9th International Conference on Heme Oxygenase Prague 2016

BOOK OF ABSTRACT

14 – 17 September 2016

SfRBM Society for Redox Biology and Medicine

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Welcome

Dear Colleagues,
It is my great pleasure to welcome you at the 9th International Conference on Heme Oxygenase held in Prague, a charming city situated in the heart of Europe, rich in history, magnificent architecture, and unique culture.
Heme Oxygenase 2016 conference follows already long tradition of bringing together leading scientists, clinicians, young investigators and students in basic and applied research of heme oxygenases. It provides an exceptional opportunity for sharing the latest research findings, and establishing further collaboration which might accelerate progress in our exciting field.
It is my strong belief that our Conference will fulfil all your expectations!

Libor Vítek
Chairman of the 9th International Conference
on Heme Oxygenase 2016

Date & Venue

14–17 September 2016 | Purkyne Institute, Prague, Czech Republic
The conference is organized under the Patronage of Tomáš Zima, the Rector of the Charles University, Prague.

Scientific committee

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Anupam Agarwal | University of Alabama, Birmingham, USA
Asif Ahmed | Aston University, Birmingham, UK
John Belcher | University of Minnesota, Minneapolis, USA
Jozef Dulak | Jagiellonian University, Krakow, Poland
Roberta Foresti | INSERM, University Paris-Est, Paris, France
Alicja Józkowicz, Jagiellonian University, Krakow, Poland

9th International Conference on Heme Oxygenases Prague 2016

Viktor Kožich | Charles University in Prague, Czech Republic
Mahin Maines | University of Rochester Medical Center, USA
Pavel Martásek | Charles University in Prague, Czech Republic
Roberto Motterlini | INSERM, University Paris-Est, Paris, France
Jiri Neuzil | Griffith University, Gold Coast, Australia
Leo Otterbein | Harvard Medical School, Boston, USA
Prem Ponka | McGill University, Montreal, Canada
Roland Stocker | Victor Chang Cardiac Research Institute, Sydney, Australia
Young-Joon Surh | Seoul National University, Seoul, Republic of Korea
Claudio Tiribelli | Liver Research Center, Trieste, Italy
Gregory Vercelloti | University of Minnesota, Minneapolis, USA
Ron Wong | Stanford University, Stanford, USA
Tomáš Zima | Charles University in Prague, Czech Republic

Keynote speakers

Phyllis Dennery | University of Pennsylvania, Philadelphia, USA
Jiri Neuzil | Griffith University, Gold Coast, Australia
Prem Ponka | McGill University, Montreal, Canada

Professional organizer

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Programme

Day	Time	Session	Session Title, Chair	Speakers, Talk Title
14. 9. Wen	15:00-16:00	**Registration Opens**		
	16:00-16:10	**Welcome and Opening Remarks** Tomáš Zima (rector of the Charles University in Prague) / Libor Vítek		
	16:10-16:50		**Opening Lecture** Pavel Martásek, Czech Republic	**Toru Shimizu**, Tohoku University, Japan. Role of heme in nature
	16:50-17:50	**1**	**HMOX and mitochondria** Jiri Neužil, Australia	16:50-17-10. **Anupam Agarwal**, University of Alabama at Birmingham, USA. Regulation of mitochondrial function by HO-1 17:10-17:30. Rychtarcikova Z, Lettlova S, Tomkova V, Neuzil J, **Jaroslav Truksa**, Czech Acad Sci, Prague, Czech Rep; Mitochondria, iron metabolism and cancer stem cells 17:30-17:50. **Roberta Foresti**, Roberto Motterlini. INSERM, University Paris-Est, Paris, France. The interplay between CO and mitochondria
	19:30-21:30	**Welcome Reception (Carolinum)**		

15. 9. Thru	8:00-8:30		**State-of-the Art Lecture** Asif Ahmed, UK	**Phyllis Dennery,** University of Pennsylvania, USA. Role of heme oxygenase in cellular signaling
	8:30-9:30	**2**	**Role of HMOX in cellular differentiation** Roland Stocker, Australia	8:30-8:50**.** Szade K, Zukowska M, Nowak WN, Szade A, Bukowska-Strakova K, Kachamakova-Trojanowska N, Dulak J, **Alicja Jozkowicz** , Jagiellonian University, Krakow, Poland. To be still young: heme oxygenase-1 prevents hematopoietic stem cells from premature aging 8:50-9:10**. James F. George,** University of Alabama, Birmingham. HMOX1 in immune cell differentiation 9:10-9:30. **Rodriguez AM**, Mahrouf-Yorgov M, Motterlini R, Foresti R. Université Paris Est, France. Mitochondria from dying cells activate the cytoprotective function of mesenchymal stem cells through a heme oxygenase-1- dependent mechanism
	9:30-10:00	**Coffee Break**		
	10:00-11:30	**3**	**HMOX1 and cardiovascular and metabolic diseases** Jozsef Balla, Hungary	10:00-10:20**. Karl Nath**, Kang L, Juncos JP, Grande JP, Croatt AJ, Hillestad ML, Barry MA, Katusic ZS. Mayo Clinic, Rochester, MN, USA. The functional significance of the heme oxygenase system in pathologic shear stress 10:20-10:40. **Joseph F Ndisang,** University of Saskatchewan. Heme oxygenase is an important switch-box that regulates insulin signaling and glucose metabolism 10:40-11:00. **Jeremias C Kupatt**, Hinkel R, Ludwig Maximilian University, Munich, Germany. Heme oxygenase 1 gene therapy provides cardioprotection in a preclinical pig model 11:00-11:15. Zhang M, Nakamura K, Lawal A, Gong KW, Ke B, Busuttil R, Agarwal A, Kupiec-Weglinski J, **Araujo J**. UCLA School of Medicine, USA. Myeloid specific HO-1 expression protects against ischemia reperfusion injury 11:15-11:30. **Leaf DE**, Body SC, Muehlschlegel JD, McMahon GM, Lichtner P, Collard CD, Shernan SK, Fox AA, Waikar SS**.** Brigham and Women's Hospital, Boston, USA. Length polymorphisms in heme oxygenase-1 and AKI following cardiac surgery
	11:30-12:00		**State-of-the Art Lecture** Karl-Heinz Wagner	**Jiri Neužil,** Kovarova J, Bajzikova M, Dong L, Berridge MV. Griffith University, Australia. Horizontal transfer of mitochondria and its context in cancer
	12:00-13:00	**Lunch and Poster Viewing**		

	13:00-14:10	**4**	**HMOX1 and microRNAs** Barbara Wegiel	13:00-13:20. **Feng Guo**, UCLA, USA. microRNA maturation and heme oxygenase 13:20-13:40. Pietraszek-Gremplewicz K, Kozakowska M, Szade K, Ciesla M, Bronisz I, Seczynska M, Bukowska-Strakova K, Jozkowicz A, **Jozef Dulak**, Jagellonian University, Krakow, Poland. Heme oxygenase-1 and microRNAs interaction in Duchenne muscular dystrophy 13:40-13:55. **Cressatti M**, Song W, Liberman A, Galindez C, Schipper HM. MicroRNA regulation in the GFAP. HMOX1 mouse model of Parkinson disease 13:55-14:10**. Ciesla M**, Marona P, Jeż M, Seczyńska M, Kozakowska M, Loboda A, Szade A, Nowak W, Dulak J, Józkowicz A. Jagiellonian University, Krakow, Poland. Heme oxygenase-1 controls the oxidative stress – HDAC4 – miR-206 axis in rhabdomyosarcoma
	14:10-15:40	**5**	**HMOX pathway and iron metabolism** Prem Ponka, Canada	14:10-14:30. **John D Belcher**, Nathan Brinkman, Gregory M. Vercellotti. University of Minnesota, USA. HO-1: The ultimate cytoprotectant in sickle cell disease? 14:30-14:50**.** Lanceta L, Burkhardt P, Soucy P, Li C, **John W Eaton**, University of Louisville, Kentucky, USA. Heme oxygenase-1, ferritin and life in extreme environments 14:50-15:10**. Alex Sheftell**, Spartan Bioscience Inc. Ottawa, ON, Canada. Is the heme oxygenase activity of HO-1 necessary for the protection of cells from oxidative insult? 15:10-15:25**.** Ingoglia G, Sag CM, Rex N, De Franceschi L, Vinchi F, Cimino J, Petrillo S, Wagner S, Silengo L, Altruda F, Maier L, Hirsch E, Ghigo A, **Tolosano E.** University of Torino, Italy. Hemopexin counteracts systolic dysfunction induced by heme-driven oxidative stress 15:25-15:40. **Hamdi A**, Garcia dos Santos D, Sheftel A, Ponka P, McGill University, Montreal, Canada. Regulation of heme levels during erythroid cell development: a balancing act
	15:40-16:10	**Coffee Break**		

	16:10-17:40	**6**	**Biliverdin reductase** Mahin Maines, USA	16:10-16:30**.** Gibbs PE, Miralem T, **Mahin Maines**, University of Rochester, Rochester, USA. A novel hBVR-based technology to regulate insulin receptor and Akt kinase activities and treat diabetes 16:30-16:50**. Barbara Wegiel**, Harvard University, USA. Is biliverdin reductase a key regulator of inflammation? 16:50-17:10**. Terry Hinds,** University of Toledo, USA. Biliverdin reductase A and hepatic steatosis 17:10-17:25**. Barone E**, Di Domenico F, Butterfield DA, Perluigi M. Sapienza University of Rome, Italy. Impairment of biliverdin reductase-A promotes brain insulin resistance in Alzheimer disease 17:25-17:40**. Paul B.**, Vasavda C, Tokhunts R, Sbodio JI, Snowman AM, Snyder SH. Johns Hopkins University, Baltimore, USA. Neuroprotective roles of biliverdin reductase in the brain
	17:40-18:50	**7**	**Controversial role of HMOX in carcinogenesis** Alicja Józkowicz, Poland	17:40-18:00**. Elba Vazquez,** Buenos Aires, Argentina. Anti-oncogenic potential of HMOX1 18:00-18:20. Park SA, **Hye-Kyung Na,** Sungshin Women's University, Seoul, South Korea. Induction of heme oxygenase-1 by 4-hydroxyestradiol promotes mammary cell transformation and tumorigenesis 18:20-18:35**. Fest S**, Zenclussen AC. Otto-von-Guericke University, Magdeburg, Germany. HO-1 blockage is effective in stimulating the host immune system to fight against neuroblastoma 18:35-18:50. **Jaworski FM**, Gentilini LD, Gueron G, Meiss RP, Ortiz EG, Berguer PM, Ahmed A, Navone N, Rabinovich GA, Compagno D, Laderach DJ, Vazquez E, University of Buenos Aires, Argentina. In vivo hemin pre-conditioning targets the vascular and immunological compartments and restrains prostate tumor development
	19:40	**Faculty Dinner**		

16. 9. Fri	8:00-8:30		**State-of-the Art Lecture** Jozef Dulak, Poland	Garcia-Santos D, Hamdi A, Sheftel AD, **Prem Ponka,** McGill University, Canada. Iron and heme: Our friends and foes
	8:30-10:00	**8**	**HMOX and vascular system** Anupam Agarwal, USA	8:30-8:50**.** Wang K, Ahmad S, Murdoch C, **Asif Ahmed**, Aston University, Birmingham, UK. Vascular-targeted molecules to limit the risky business of pregnancy 8:50-9:10**.** Kong S, Ni J, Newington D, Dunn LL, Ayer A, Suarna C, Lam M, Maghzal G, **Roland Stocker**, Victor Chang Cardiac Research Institute, Sydney, Australia. Inhibition of vascular smooth muscle cell migration by C-terminus-truncated, enzymatically active heme oxygenase-1 9:10-9:30**. Gregory Vercelloti**, Smith A, Belcher JD. University of Minnesota, USA. Hepatic overexpression of hemopexin inhibits inflammation and vascular stasis in murine models of sickle cell disease 9:30-9:45**. Petrillo S**, Chiabrando D, Mercurio S, Merlo G, Santoro M, Silengo L, Altruda F, Tolosano E**.** University of Turin, Italy. Endothelial loss of the heme exporter Flvcr1A alters vascular integrity 9:45-10:00**. Vinchi F**, Simmelbauer A, Altamura S, Spaich S, Galy B, Hentze MW, Muckenthaler MU. University of Heidelberg, Germany. Iron causes vascular oxidation and accelerates atherosclerosis progression
	10:00-10:30	**Coffee Break**		
	10:30-12:00	**9**	**HMOX and immune system** Miguel Soares, Portugal	10:30-10:50. **Ana Zenclussen,** Otto-von-Guericke University Magdeburg, Germany. HO-1 derived carbon monoxide modulates immune cells to support pregnancy 10:50-11:10. Nakamura K, Zhang M, Kageyama S, Araujo J, **Jerzy Kupiec-Weglinski**, UCLA, Los Angeles, USA. HO-1/SIRT1/p53 axis regulates macrophage activation and attenuates liver ischemia-reperfusion injury in mice 11:10-11:30. **Brian Zuckerbraun**, University of Pittsburgh, USA, HMOX/CO and trauma/hemorrhagic shock mediated inflammatory and immune dysfunction 11:30-11:45**. Zhao H**, Kalish F, Wong RJ, Stevenson DK. Stanford University, USA. Heme oxygenase-1 affects uterine infiltration of myeloid cells and their oxidative stress in early pregnancy 11:55-12:00**. Leclerc J**, Moestrup SK, Doré S. University of Florida, USA. CD163 has distinct temporal influences on hemorrhagic stroke outcomes

	12:00-13:00	**Lunch and Poster Viewing**		
	13:00-14:30	**10**	**HMOX and inflammation** Leo Otterbein, USA	13:00-13:20. **Hun Taeg Chung,** University of Ulsan, South Korea. Carbon Monoxide Promotes Inter-Organelle Communication through the Activation of TFEB/3 and PGC-1+A24:E26 13:20-13:40. **Miguel Soares**, Instituto Gulbenkian de Ciência, Oeiras, Portugal. A central stage for heme catabolism in tissue damage control 13:40-14:00. **Marcelo T. Bozza,** Universidade Federal do Rio de Janeiro, Brasil. Heme modulates innate immune receptor signaling dependently of ROS and Syk 14:00-14:15. Dorresteijn M, Paine M, Zilian E, Fenten MG, Janciauskiene S, Figueiredo C, Eiz-Vesper B, Blasczyk R, Dekker D, Pennings B, Scharstuhl A, Smits P, Larmann J, Theilmeier G, Hoeven JG, Wagener FADT, Pickkers P, **Immennschuh S.** Hannover Medical School, Hannover, Germany. Cell-type specific down-regulation of heme oxygenase-1 by lipopolysaccharide via Bach1 in primary human mononuclear cells 14:15-14:30. **Ramos S**, Sundaram B, Tolosano E, Gozzelino R, Soares MP, Instituto Gulbenkian de Ciência, Oeiras, Portugal**.** Kidney proximal tubular epithelial cells control disease tolerance to malaria by maintaining heme/iron homeostasis
	14:30-15:45	**11**	**Novel techniques to study HMOX pathway** Claudio Tiribelli, Italy	14:30-14:50. **Gus Maghzal,** Suarna C, Ayer A, Chen YC, Peter KH, Stocker R. Victor Chang Cardiac Research Institute, Sydney, Australia- Sensitive LC-MS/MS Assay for the simultaneous detection of heme, biliverdin and bilirubin in complex biological sample 14:50-15:10**. Ichiro Morioka**, Kobe University Graduate School of Medicine, Japan. A novel measurement method for serum unconjugated bilirubin levels using a bilirubin-inducible fluorescent protein from eel muscle 15:10-15:30**.** Erapaneedi R, da Graca AP, **Friedemann Kiefer,** Max Planck Institute for Molecular Biomedicine. Approaches to image vascular development and disease in the mouse 15:30-15:45**. Jašprová J**, Teclová A, Vítek L. Charles University in Prague, Czech Republic. Novel method for quantitative determination of bilirubin photoisomers
	15:45-16:15	**Coffee Break**		

	16:15-18:00	**12**	**Young Investigators Session** Roberta Foresti, France	16:15-16:30. **Lucie Muchova,** Charles University in Prague, Czech Republic. Beneficial roles of CO in liver pathologies. 16:30-16:45**.** Tomczyk M, Szade K, Kraszewska I, Bukowska-Strakowa K, Jozkowicz A, Dulak J, **Agnieszka Jazwa**, Jagellonian University, Krakow, Poland. HMOX1 and macrophages in cardiac homeostasis and repair following myocardial infarction 16:45-17:00. **Luca Vanella**, University of Catania, Italy. Heme oxygenase-1 nuclear translocation regulates bortezomib-induced cytotoxicity in myeloma cells 17:00-17:15. **Andrew Bulmer**, Griffith University, Australia. Metabolic effects of bilirubin: key findings from animal models 17:15-17:30**. Schallner N**, Goebel U; Gallo D; Fuller P, Hanafy KA, Otterbein LE. University Medical Center Freiburg, Germany. Neuronal injury after subarachnoid hemorrhage is determined by a carbon monoxide sensing change in circadian rhythm 17:30-17:45**. Garcia dos Santos D**, Hamdi A, Zidova Z, Horvathova M, Ponka P, McGill University, Montreal, Canada. Investigations of heme oxygenase 1 and its inhibitors in β-thalassemia 17:45-18:00. **Vijayan V,** Zilian E, Saragih H, Hiller O, Figueiredo C, Aljabri A, Blasczyk R, Theilmeier G, Becker JU, Larmann J, Immenschuh S. Hannover Medical School, Hannover, Germany**.** Heme oxygenase-1 inhibits HLA class I antibody-dependent endothelial cell activation
	19:00	**GalaDinner**		

17. 9. Sat	8:00-8:45	**13**	**Natural products as modulators of HMOX system** Ron Wong, USA	8:00-8:22. Kim SH, Kim W, Zhong X, Lee HN, Kim K, Suh YG, Kim C, Cha YN, **Young-Joon Surh**, Seoul National University, South Korea. Taurine chloramine exerts anti-inflammatory and proresolving effects through induction of heme oxygenase-1 expression 8:22-8:45. **Vladimir Kren**, Vitek I. Czech Acad Sci, Prague, Czech Rep; Silymarin uncovered: Molecules or a ¨quack remedy¨
	8:45-10:15	**14**	**Gaseous transmitters** Viktor Kožich, Czech Republic	8:45-9:05. **Roberto Motterlini**, Roberta Foresti. INSERM, University Paris-Est, Paris, France. Advances in the design of pharmacological agents targeting HO-1/CO 9:05-9:25**. Andreas Papapetropoulos**, University of Athens, Greece. H2S in homeostasis and disease: paradigms from the cardiovascular system 9:25-9:45**. Csaba Szabo**, University of Texas, USA. Gaseous transmitters (NO, CO, H_2S) in cancer: pathways and interactions 9:45-10:00. **Steiger C**, Uchiyama K, Naito Y, Meinel L. University of Wuerzburg, Germany. Controlled oral delivery of therapeutic gases – local carbon monoxide for ulcerative colitis 10:00-10:15**. Doré S.** University of Florida, Gainesville, USA. Neuroprotective mechanisms of carbon monoxide and heme oxygenases on stroke outcomes
	10:15-10:45	**Coffee Break**		
	10:45-12:00	**15**	**Biological effects of bilirubin** Libor Vítek, Czech Republic	10:45-11:05**. Claudio Tiribelli,** Silvia Gazzin, Liver Research Center, Trieste, Italy. Bilirubin research overview with the focus on cellular events 11:05-11:25**. Ron Wong,** Stanford University, USA: Bilirubin neurotoxicity 11:25-11:45**. Karl-Heinz Wagner**, University of Vienna. Protective effects of bilirubin 11:45-12:00**. Zelenka J**, Dvořák A, Alán L, Zadinová M, Haluzík M, Vítek L. Charles university in Prague, Czech Republic. Hyperbilirubinemia counteracts inflammation in aging: role of redox homeostasis
	12:00-13:00	**Lunch**		
	13:00-13:45	**16**	**Heme oxygenase-2** Kanji Nakatsu, Canada	13:00-13:22**. Stephen W Ragsdale,** Fleischhacker A, Zuiderweg E, Kochert B, Engen J. University of Michigan, USA. Role of HMOX2 in oxidative stress defense 13:22-13:45**. David E. Stec**, University of Mississipi. Role of heme oxygenase-2 (HO-2) in arterial hypertension

	13:45-15:00	**17**	**Interplay between Nrf2 and HMOX pathway** Young-Joon Surh, South Korea	13:45-14:05. **Agnieszka Łoboda**, Jagiellonian University, Krakow, Poland. Nephroprotective effect of heme oxygenase-1 and Nrf2 – role of microRNAs 14:05-14:25. **Kazuhiko Igarashi**, Watanabe-Matsui M, Tohoku University, Japan. Heme regulates Bach2 protein interaction by binding to its intrinsically disordered region 14:25-14:45. **Antonio Cuadrado,** Autonomous University of Madrid. Role of Nrf2 in carcinogenesis 14:45-15:00**.** Kloska D, Kopacz A, Augustyniak A, Dulak J, Jozkowicz A, **Grochot-Przeczek A.** Jagiellonian Univeristy, Krakow, Poland. Nrf2-dependent angiogenesis is not directly related to its transcriptional activity
	15:00-16:15	**18**	**Novel approaches in chemistry and pharmacology with respect to HMOX system** Petr Klán, Czech Republic	15:00-15:20. **Hiroaki Kitagishi**, Koji, Doshisha University, Japan. Induction of HO-1 expression by selective removal of endogenous CO 15:20-15:40. **Kanji Nakatsu,** Queen's University, Kingston, Canada. Structure-activity relationship of HMOX modulation 15:40-16:00. **Kamil Sitarz,** SELVITA, Krakow, Poland. Heme oxygenase-1 as an oncology target: focus on small molecule inhibitors 16:00-16:15**. Wang B.** Georgia State University, USA. Organic carbon monoxide prodrugs that release CO under physiological conditions with tunable release rates
	16:15-16:25	**Concluding Remarks** Libor Vítek		

Time for all presentations include 3 minutes for discussion

Abstracts / Oral presentation

Roles of Heme in Nature

Shimizu T, Stranava M, Fojtíková V and Martínková M
Charles University in Prague, Czech Republic and Tohoku University, Sendai, Japan

Heme, the iron protoporphyrin IX complex, is one of the best-known and most important cofactors required for proper functioning of proteins and enzymes. In prototype heme-containing proteins, such as hemoglobin, cytochrome c and P450, heme itself is the functional center. However, there is emerging evidence that heme can also act as a primary agent in intramolecular signal transduction, i.e. heme's association with and/or dissociation from heme-responsive sensor proteins regulates various physiological functions, including transcription, protein phosphorylation and protein degradation. There is also evidence that the cofactor acts as the sensing site in heme-based gas sensor proteins. In these cases it binds gaseous molecules such as O2, NO and CO, thereby indirectly participating in the regulation of diverse physiological functions, including activities of protein kinases, guanylate cyclase and phosphodiesterase, as well as transcription, in response to changes in gas availability. We have attempted to elucidate the molecular mechanism of heme-regulated eukaryotic initiation factor 2α (eIF2α) kinase (HRI) activity by identifying the heme sensing sites, determining heme-induced global protein structural changes and characterizing the inhibition of heme binding by autophosphorylation of the proteins (1, 2). In addition, we have studied structure-function relationships of heme-based O2 sensors (EcDOS, YddV and AfGcHK) in efforts to elucidate the role of O2 binding to the heme in their intramolecular signal transduction processes (3-6). We will discuss these and other aspects of emergent roles of heme in physiological functions

References: (1) Miksanova M et al. (2006) Biochemistry 45, 9894; (2) Igarashi J et al. (2008) J. Biol. Chem. 283, 18782; (3) Martínková M et al. (2013) J. Biol. Chem. 288, 27702; (4) Stranava M et al. (2014) J. Inorg. Biochem. 140, 29; (5) Fojtíková V et al. (2015) Biochemistry 54, 5017; (6) Shimizu T et al. (2015) Chem. Rev. 115, 6491.

Regulation of mitochondrial function by HO–1

Anupam Agarwal, MD
University of Alabama at Birmingham, Birmingham, AL 35294

Mitochondria are key organelles that are abundant in heme proteins and considered energy "powerhouses" for all cells. They serve as a source and target for reactive oxygen species and possess inherent redox signaling mechanisms to protect against oxidative stress. Mitochondrial dysfunction underlies the pathogenesis of several pathological conditions and is also associated with normal processes such as aging. Recent studies have highlighted the role of heme oxygenase-1 (HO-1) in mediating its protective effects through restoring mitochondrial function, effects that are mediated through one or more of the by products of the HO enzyme system, namely biliverdin/bilirubin and carbon monoxide. HO-1 expression regulates mitochondrial biogenesis in the heart by inhibiting mitochondrial fission and promoting fusion. HO-1 expression also modulates mitophagy through the proteins, PINK1 and Parkin. This presentation will review recent work regarding the key role of HO-1 in mediating its protective effects via restoration of mitochondrial function.

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Mitochondria, iron metabolism and cancer stem cells

Zuzana Rychtarcikova[1,2], Sandra Lettlova[1,3], Veronika Tomkova[1,3], Jiri Neuzil[1,4] and Jaroslav Truksa[1]
[1]Institute of Biotechnology, Czech Academy of Sciences, Prague, Czech Republic, [2]Charles University in Prague, Faculty of Pharmacy in Hradec Kralove, Hradec Kralove, Czech Republic, [3]Charles University in Prague, Faculty of Sciences, Prague, Czech Republic, [4]School of Medical Science, Griffith University, Southport, Qld, Australia

The role of iron metabolism in the phenotype and maintenance of cancer stem cells (CSCs) has not received much attention so far. We have studied iron metabolism using the *in vitro* model of cancer stem cells derived from cell lines of breast and prostate origin and have found a marked alterations in the iron uptake, export and handling. Our data suggest that CSCs generated by our model are more sensitive to death induced by the iron chelators and exhibit activation of the IRP machinery leading to activation of iron uptake and a decrease in iron export. Importantly, CSC salso show marked deregulation of the Fe-S cluster biogenesis pathway and higher production of ROS including mitochondrial ROS.

One of the interesting candidate genes whose expression has been altered was HMOX1. In order to delineate the role of HMOX1 in cancer progression and CSC phenotype, we generated doxycycline inducible MCF-7 cell line, expressing high amount of the HMOX1 at the mRNA as well as protein level. Cell over-expressing HMOX1 show only slight deregulation of cellular proliferation *in vitro* but exhibit moderately higher tumor growth in the *in vivo* model of xenotransplanted human MCF7 cells in the BALB/c nude mice. Our data suggest that overexpression of HMOX1 modulates carcinogenesis process and its role in the biology of CSC and cancer resistance is under investigation.

The interplay between CO and mitochondria

Roberta Foresti[1,2] and Roberto Motterlini[1,2]
[1]INSERM U955, Equipe 12, Créteil, 94000, France, [2]University Paris Est, Faculty of Medicine, Créteil, 94000, France

Until few years ago scientists believed that the interaction of carbon monoxide (CO) with mitochondria led exclusively to inhibition of cytochrome c oxidase, the last enzyme in the respiratory chain of mitochondria, thus poisoning the cell and preventing energy production. However, more recent evidence indicates that small quantities of CO can regulate mitochondrial function by alternative mechanisms. We showed in isolated mitochondria that CO-releasing molecules (CO-RMs) exert an uncoupling activity, a phenomenon which consists in dissipating the mitochondrial proton gradient to generate heat instead of using it for energy production. We have now started to study the effect of CO-RMs in different cell types using an analyzer that monitors in real time the metabolic profile of living cells. Our results confirm that small concentrations of CO-RMs (5-50 μM) gradually increase cellular respiration and higher CO levels inhibit mitochondrial function. ATP content is not diminished by concentrations of CO that cause uncoupling, suggesting that the mitochondrial targets responding to low levels of CO are different from cytochrome c oxidase. By measuring complex activities in permeabilized cells we also observed that the uncoupling effect involves primarily complex I and complex III. Interestingly, a gradual and sustained increase in respiration is observed in cells after treatment with hemin (1-5 μM), the substrate for heme oxygenase activity. This effect is abolished by a heme oxygenase inhibitor, suggesting that endogenous CO also modulates mitochondrial function. In addition, the ability of CO to increase oxygen consumption prevented the depression of respiration and the decrease in ATP levels elicited by lipopolysaccharide in BV2 microglia cells. An uncoupling activity may be one of the mechanisms by which this gas exerts anti-inflammatory actions. This peculiar effect of CO may also have important implications in the context of obesity and metabolic imbalances.

Role of heme oxygenase in cellular signaling

Phyllis A. Dennery, M.D
University of Pennsylvania, USA

Many reports have highlighted the importance of heme oxygenase (HO), the rate-limiting enzyme in heme degradation in various physiologic processes. It is well known that the reaction releases bile pigments and carbon monoxide (CO), which are important antioxidant and signaling molecules. However, the importance of this protein goes beyond its enzymatic action since its cytoprotective and modulatory effects occur even when the protein is devoid of enzymatic activity. The HO protein plays a role in cellular signaling, including transcription factor activation, binding to proteins, phosphorylation, modulation of protein function and alteration of mitochondrial metabolism amongst others. The signaling functions of HO-1 may have particular relevance in clinical circumstances, including cancer, as migration of HO-1 into the nucleus is observed with cancer progression and metastasis. In addition, along with oxidative stress, the pleiotropic functions of HO-1 modulate antioxidant defense. This talk will review the cell signaling roles of HO-1 and describe recent advances in understanding how the nuclear form of HO-1 in particular alters cellular respiration and glycolysis. This will provide insights into potential therapeutic strategies using HO in cellular injury and repair.

To be still young: heme oxygenase-1 prevents hematopoietic stem cells from premature aging

Krzysztof Szade, Monika Zukowska, Witold N. Nowak, Agata Szade, Karolina Bukowska-Strakova, Neli Kachamakova-Trojanowska, Jozef Dulak, Alicja Jozkowicz
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Heme oxygenase-1 (HO-1) regulates the response of hematopoietic stem cells (HSC) to acute stress. Our aim was to investigate whether HO-1 influences the HSC function in a steady state conditions.
We found that HO-1 in HSC (defined as $Lin^-cKit^+Sca1^+CD48^-CD150^+CD34^-$ cells) is localized mainly in the nucleus where it forms foci, especially in aged animals. HSC from young HO-1 deficient ($HO\text{-}1^{-/-}$) mice lose quiescence, extensively proliferate and contain a higher fraction of γH2AX-high cells, that may indicate the enhanced DNA damage. Young $HO\text{-}1^{-/-}$ individuals have also more myeloid cells in peripheral blood, and their single sorted HSC show increased myeloid differentiation *in vitro*. All these features are typical for aged hematopoietic system. Accordingly, RNA-seq analysis revealed that transcriptome of young $HO\text{-}1^{-/-}$ HSC closely resembles the transcriptome of old $HO\text{-}1^{+/+}$ HSC, with deregulated cell cycle checkpoints, symptoms of replication stress, enhanced translesion DNA synthesis, and activated DNA damage response accompanied by DNA repair based on Fanconi anemia and homologous recombination pathways. Finally, when we transplanted purified HSC from $HO\text{-}1^{-/-}$ donors to $HO\text{-}1^{+/+}$ recipients they showed clearly impaired repopulation capacity in all tested lineages.
The expression of HO-1 in HSC was relatively low. Much higher level we found in the HSC niche, especially in reticular cells and $CD31^+Sca1^+$ endothelial cells localized to the bone metaphysis, suggesting that HO-1 may be even more important in the niche than in HSC themselves. Indeed the GO enrichment analysis showed that lack of HO-1 affects genes involved in integrin-mediated cell adhesion and genes regulating hematopoietic stem cell proliferation and differentiation both in $CD31^+Sca1^+$ endothelial cells and reticular cells. Importantly, $HO\text{-}1^{+/+}$ HSC transplanted to $HO\text{-}1^{-/-}$ animals gave worse reconstitution of peripheral blood after 32 weeks and were unable to reconstitute any $HO\text{-}1^{+/+}$ secondary recipient. On the other hand, transplantation of $HO\text{-}1^{-/-}$ HSC to the $HO\text{-}1^{+/+}$ niche preserved their repopulation capacity in $HO\text{-}1^{+/+}$ secondary recipient. Concluding, HO-1 prevents HSC from premature aging and HO-1 expression in the niche is necessary for proper reconstitution capacities of HSC.

HMOX1 in immune cell differentiation

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Cellular differentiation is a tightly controlled process central to immune cell function and immune system control. Immune responses are typically initiated when multiple signals are integrated by mononuclear phagocytes, which are comprised of macrophages, monocytes, and dendritic cells. These cells differentiate into regulatory and effector cells in innate immune responses, and they also differentiate into antigen presenting cells that drive differentiation and expansion of lymphocytes in specific immune responses. Mononuclear phagocytes are of particular interest in the context of HO-1 because they tend to handle larger quantities of heme. They are phagocytic (e.g. disposal of senescent erythrocytes) and are recruited to areas of tissue damage and cellular stress. This presentation focuses on how HO-1 modulates phenotypic and functional differentiation of mononuclear phagocytes. Data are reviewed that show involvement of HO-1 or heme degradation products in multiple mononuclear phagocyte functions known to be driven by distinct signaling pathways. How deficiencies or overexpression of HO-1 in specific cellular compartments affects immune cell differentiation and function will also be discussed.

Mitochondria from dying cells activate the cytoprotective function of mesenchymal stem cells through a heme oxygenase-1- dependent mechanism

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The clinical use of mesenchymal stem cells (MSC) holds great promise for treating a broad range of organ injury and in particular that caused by ischemic insults, which remain a major cause of mortality worldwide. Nevertheless, the outcome of clinical trials revealed that efficacy of MSC therapy is modest and must be improved to achieve significant functional recovery in diseased organs. For this reason, intensive efforts are currently devoted to dissect the molecular mechanisms underlying the regenerative capacity of MSC in order to exploit this knowledge for maximizing their therapeutic usage. It is believed that the surrounding environment, in where MSC are located or engrafted control their regenerative function through the release of danger cues which remain to be identified. In line with this idea, recent studies relate that MSC communicate with their microenvironment through bidirectional exchanges of mitochondria and that transfer of mitochondria from MSC to somatic cells renders tissues more resistant to injury. However, the role of somatic mitochondria conveyed to MSC is still unknown. Here, we identified that mitochondria released by damaged somatic cells act as danger-signaling organelles that trigger the anti-apoptotic function of MSC through a cascade of events that lead to the induction of the strong cytoptotective enzyme heme oxygenase-1 (HO-1). More precisely, by using a co-culture system consisting of MSC and H_2O_2-insulted cardiomyocytes or endothelial cells, we demonstrate that somatic mitochondria are engulfed and subsequently degraded by MSC, leading to induction of HO-1. These phenomenons promote stimulation of mitochondrial biogenesis in MSC allowing the transfer of healthy mitochondria to damaged somatic cells which exhibit enhanced survival. Blockade of mitophagy or heme oxygenase activity abolishes the rescuing properties of MSC. Similar mechanisms occur after exposure of MSC to exogenous mitochondria isolated from somatic cells and are recapitulated in a model of myocardial infarction *in vivo*. Specifically, MSC engrafted into mouse infarcted hearts reduce damage, up-regulate HO-1 and increase mitochondrial biogenesis while inhibition of mitophagy or HO-1 fails to protect against cardiac apoptosis. Thus, our studies reveal that mitochondria are a critical environmental cue that controls the regenerative properties of MSC and open novel therapeutic avenues in regard to the obligatory role of HO-1 in sensing mitochondrial signals which may be instrumental for maximizing cell therapy efficacy.

The Functional Significance of the Heme Oxygenase System in Pathologic Shear Stress.

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Endstage kidney disease is most commonly treated by hemodialysis, and the most favored form of hemodialysis vascular access is the arteriovenous fistula (AVF). This form of vascular access, however, has a relatively high rate of failure to adequately mature (approximately 50% of created AVFs) such that the created AVF can effectively sustain dialysis; the average lifespan of AVFs that adequately mature is relatively limited at 5 years. The AVF subjects the venous segment to high blood flow rates and intraluminal pressure, and to pathologic shear stress. We questioned whether HO-1 is induced in the AVF and the functional significance of any such induction. In a murine AVF model (end-artery to side-vein), HO-1 mRNA and protein are induced in the arterial and venous segments, and the creation of this model in HO-1$^{-/-}$ mice is attended by decreased AVF blood flow, increased neointimal hyperplasia, premature loss of vascular patency, and exaggerated vasculopathic gene expression (Kidney Int 2008, AJP 2011). Subsequent studies in another murine model of pathologic shear stress, the partial carotid artery ligation model, confirmed that the induction of HO-1 is a vasoprotective response in vivo (AJP 2015). In this model, as in the AVF model, HO-2 also confers vasoprotective effects (AJP 2013, AJP 2015). Finally, we created a murine AVF model that more closely mirrors what occurs clinically, namely, creating the AVF in the setting of chronic kidney disease and by anastomosing end-vein to side--artery in fashioning the AVF (AJP 2016). In this setting, the presence of chronic kidney disease impairs AVF blood flow, increases venous wall thickening, and augments the induction of HO-1 in the AVF. In this model, the prior upregulation of HO-1 by adeno-associated viral delivery of HO-1 substantially improves AVF blood flow and decreases venous wall thickening, while the acute administration of CORM-3 increases AVF blood flow. We conclude that induction of HO-1 is a protective response in pathologic shear stress, in general, and in the AVF, in particular, and opens up therapeutic avenues to improve maturation and longevity of the AVF.

Heme oxygenase is an important switch-box that regulates insulin signaling and glucose metabolism

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Abstract

Impaired insulin signaling and deregulated glucose metabolism are associated with the progressive alterations in structure and function of vital organs like the heart and kidneys in diabetic patients. With the escalation of cardio-renal and cardio-metabolic complications novel strategies are needed. Our recent studies indicate that upregulating the heme oxygenase (HO) system with HO-inducers potentiates insulin signaling and improve glucose metabolism in animal models of type-1 and type-2 diabetes including (i) streptozotocin-induced diabetic rats, (ii) Zucker diabetic fatty rats (ZDF), (iii) obese Zucker rats, (iv) Goto-Kakizaki rats as well as other models that display glucose intolerance like spontaneously hypertensive rats and uninephrectomized DOCA-salt hypertensive rats, suggesting a universal role of the HO-system in regulating insulin signaling and glucose metabolism. Interestingly, treatment with HO-inducers like hemin or heme arginate (i) abated inflammation, (ii) suppressed oxidative stress, (iii) enhanced fundamental proteins implicated in the insulin signal transduction pathway like IRS-1, PI3K and PKB, (iv) reduced insulin-tolerance (IPITT), (v) increased insulin sensitivity and the inability of insulin to enhance GLUT4 was overturned. Corresponding, diabetic complications including cardiomyopathy and nephropathy were markedly improved. Collectively, these studies suggest that the HO-system could be considered and important switch box that when potentiated adequately can rescue organ damage in diabetes.

Heme oxygenase 1 gene therapy provides cardioprotection in a preclinical pig model

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Heme oxygenase-1 (HO-1) is an inducible stress-responsive enzyme reducing heme to bilirubin, carbon monoxide (CO) and free iron (Fe^{2}), thereby exerting anti-inflammatory and pro-survival effects. Although efficient cardioprotection of HO-1 overexpression has been reported in rodents, its role in attenuating postischemic inflammation is unclear. In a large animal model, we assessed the efficacy of regional application of a cardiomyotropic adeno-associated virus (AAV2.9) encoding heme oxygenase-1 in attenuating postischemic inflammation and ischemia-reperfusion injury. In a porcine model of ischemia (60min LAD occlusion, 24 hours reperfusion), postischemic influx of MPO^{+} neutrophils and $CD14^{+}$ monocytes was attenuated by forced human HO-1 expression via recombinant adeno-associated virus (rAAV), similar to HO-1 transgenic (HO-1 tg) pigs. Concomitantly, we also found a decrease in infarct size from 58±4% of the area at risk (controls) to 43±3% (rAAV.hHO-1) or 36±2% (hHO-1 tg). Functionally, rAAV.hHO-1 and hHO-1 tg left ventricles displayed a smaller loss of ejection fraction (ΔEF 26±3% in controls, 15±3% in rAAV.hHO-1 and 17±2% in hHO-1 tg hearts). In conclusion, we have shown that hHO-1 gene therapy attenuates postischemic inflammation and myocardial dysfunction in porcine hearts. The efficacy of rAAV.hHO-1 was similar to that of ubiquitous hHO-1-overexpression, pointing to the transduced cardiomyocyte compartment as the main target of hHO-1-enhancing therapies.

Myeloid Specific HO-1 Expression Protects Against Ischemia Reperfusion Injury

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Heme oxygenase-1 (HO-1) is a highly inducible enzyme with cytoprotective, anti-apoptotic, anti-inflammatory and immunodulatory properties. We have previously shown that global expression of HO-1 inhibits damage induced by liver ischemia reperfusion (IRI). This protection was not only dependent on the level of HO-1 expression in response to the ischemia but it appeared more closely related to the level of basal expression prior to the ischemia. It is not known, however, whether such protection was due to HO-1 expression in hepatocytes or in infiltrating inflammatory cells. To dissect the role of HO-1 expression in macrophages (MØ) and neutrophils, we have generated myeloid specific HO-1 knockout (KO) mice and myeloid specific HO-1 transgenic (Tg) mice. KO mice were generated by crossing floxed HO-1 KO mice with lysM Cre Tg mice in the C57BL/6 background, resulting in HO-1 KO fl/fl, lysM cre (+/+) mice. HO-1 deletion was confirmed in alveolar MØs (AMs) and peritoneal MØs (PMs) by qPCR and western blotting analysis. Tg mice were generated by crossing floxed human HO-1 Tg mice with lysM Cre Tg mice aforementioned in the C57BL/6 background as well, resulting in HO-1 Tg fl/fl, lysM cre (+/+). Overall overexpression of HO-1 was confirmed in AMs by qPCR . We subjected these mice vs. corresponding littermate Cre-/- controls to partial (75%) warm hepatic IRI (n=6/group). Myeloid specific expression of HO-1 played a protective role against hepatic IRI. Thus, KO mice exhibited increased plasma levels of AST and ALT, higher Suzuki score and marked neutrophil and macrophage infiltration as compared with controls, indicating that the myeloid deletion of HO-1 exacerbated the ischemic injury. On the other hand, Tg mice showed decreased release of AST and ALT, lower Suzuki score and reduced neutrophil and macrophage infiltrates as compared with controls, indicating that HO-1 overexpression ameliorated the ischemic injury. Furthermore, modulation of HO-1 expression levels altered pro-inflammatory M1 (IL 1 β, CCL2, TNF-α) and anti-inflammatory M2 (Arg1 and CD163-M) gene expression profiles in KO and Tg mice as compared with their controls, in synthony with similar findings obtained with LPS-treated bone marrow-derived MØs from both KO and Tg mice, suggesting that HO-1

expression plays an important role in MØ polarization. In conclusion, myeloid expression of HO-1 diminished hepatic IRI, likely by modulating macrophage polarization.

Length Polymorphisms in Heme Oxygenase-1 and AKI following Cardiac Surgery

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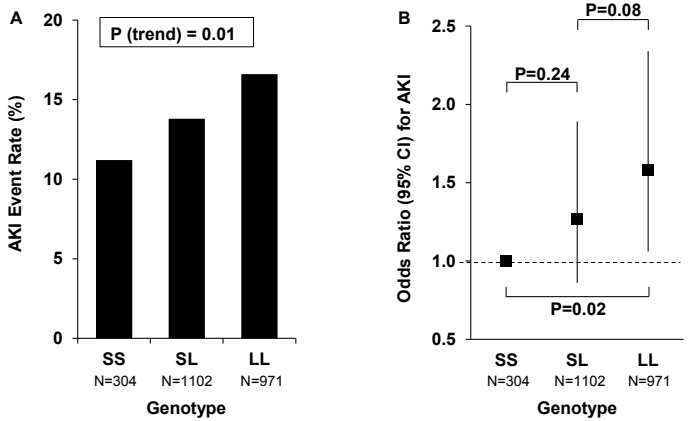
BACKGROUND: Heme oxygenase-1 (HO-1), the rate-limiting enzyme in the degradation of heme, has a central role in the pathophysiology of acute kidney injury (AKI) in animal models, but data on HO-1 in human AKI are sparse. Since length polymorphisms in the number of GT dinucleotide repeats in the HO-1 gene promoter are inversely associated with HMOX1 mRNA expression, we hypothesized that these polymorphisms might also be associated with risk of AKI following cardiac surgery.
METHODS: We used DNA fragment analysis to determine the number of (GT)n repeats in the HMOX1 promoter in 2377 Caucasian patients who underwent cardiac surgery with cardiopulmonary bypass. We categorized patients as having the short (S) or long (L) allele if they had <27 or ≥27 repeats, respectively, consistent with prior studies. Accordingly, patients were categorized into three mutually exclusive genotypes: SS, SL, or LL. AKI following cardiac surgery was defined as an increase in serum creatinine ≥0.3 mg/dl within 48 hours, ≥50% within 5 days, or need for renal replacement therapy.
RESULTS: Rates of AKI in patients with the SS, SL, and LL genotypes were 11.2, 13.8, and 16.6%, respectively (P for trend, 0.01; Figure 1A). Patients with the LL versus SS genotype had 1.58-fold (95% CI, 1.06 to 2.34, P=0.02) higher odds of AKI (Figure 1B). When analyzed using an additive genetic model, the odds ratio for AKI per L allele was 1.25 (95% CI, 1.05 to 1.49, P=0.01). After adjusting for baseline and operative characteristics, the odds ratio was unchanged. Further, these findings were confirmed in sensitivity analyses using alternative definitions of AKI and alternative cutpoints for the S and L alleles.
CONCLUSIONS: In conclusion, longer GT repeats in the HMOX1 gene promoter are associated with an increased risk of AKI following cardiac surgery. These results are consistent with heme toxicity as a pathogenic feature of cardiac surgery-associated AKI, and HO-1 as a potential therapeutic target.



Horizontal Transfer of Mitochondria and Its Context in Cancer

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Besides being the cellular powerhouse, mitochondria are organelles deciding the fate of a cell, and are also sites of convergence and divergence of multiple signalling pathways. What makes mitochondria different from other organelles is that they have their own, mitochondrial (mt) DNA coding for 13 proteins, all being subunits of respiratory complexes. We initially observed that cancer cells depleted of their mtDNA (r^0 cells) form tumours in mice with a delay, and discovered that the tumour cells, again, comprised mtDNA. Next generation sequencing, later confirmed by digital droplet single cell PCR, identified the host origin of mtDNA, pointing to horizontal transfer of mitochondrial genome (1). Our subsequent experiments revealed that mtDNA is required by cancer cells to recover their respiration (r^0 cells completely rely on glycolysis for their energetic needs), in order to form and propagate tumours. Analysis of ATP generation points to the role of respiration in metabolic re-modelling rather than in the cell's bioenergetics. Using transgenic mice with red fluorescence mitochondria in somatic cells, we documented that mtDNA moves between cells in whole mitochondria. Our research clearly documents a novel, unexpected paradigm, i.e. horizontal transfer of (mitochondrial) genes that has a profound consequence for cancer biology and treatment. A number of additional questions concerning horizontal transfer of mitochondria and its molecular regulation need to be resolved. They include the initial signal that triggers the process of mitochondrial transfer, the direction of movement of the organelles and its mode. It is likely that the process of horizontal transfer of mitochondria is related to pathologies other than cancer, as well as to normal cell physiology.

(1) Tan A et al (2015) Mitochondrial genome acquisition restores respiratory function and tumorigenic potential in cancer cells without mitochondrial DNA. Cell Metabolism 21, 81-94.

microRNA maturation and heme oxygenase

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Abstract

microRNAs (miRNAs) regulate about 40% of all protein-coding genes and serve critical functions in development, cell physiology and stress responses. Canonical miRNAs are transcribed as long primary transcripts (pri-miRNAs), which must be processed via sequential cleavages by the Drosha and Dicer ribonucleases in nucleus and cytoplasm respectively before mature miRNAs (~22 nt) are produced. Previous studies have shown that heme is required for the Drosha cleavage step by serving as a cofactor of Drosha's RNA-binding partner protein DGCR8. In cultured human cells, heme supply from the media and heme biosynthesis both modulate pri-miRNA processing. Here I will present evidence demonstrating that heme degradation by heme oxygenases also regulate miRNA maturation. I will also discuss how heme acquisition, biosynthesis and degradation jointly control miRNA-mediated gene regulation network in important biological processes.

Heme oxygenase-1 and microRNAs interaction in Duchenne muscular dystrophY

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Differentiation of myoblasts is dependent on miR-206,-1 and -133a/b. Recently, we showed that expression of myoD and myomiRs and consequently, differentiation of murine myoblasts was inhibited by heme oxygenase-1 (*Hmox1*), an anti-inflammatory enzyme degrading heme to CO, iron ions and biliverdin. Inversely, differentiation of *Hmox1*-deficient murine primary myoblasts, showing enhanced expression of myomiRs was accelerated (Kozakowska et al, Antioxid Redox Signal 16:113-27; 2012).
In muscles of dystrophin-deficient *mdx* mice the expression of *Hmox1* is consistently increased from week 8 up to 12 months. Importantly, elevated expression of *Hmox1* is observed in myeloid cells, while the expression of miR-1,-133a and -133b was decreased in muscles of *mdx* mice. However, expression of miR-206 was upregulated in gastrocnemius and diaphragm of *mdx* mice starting from 2nd week of age till at least one year. Interestingly, single cell analysis demonstrated that in SCs (α7integrin+ CD34+), the expression of *Hmox1* was decreased while miR-206 was increased, indicating for SC-specific regulation of *Hmox1* and miR-206.
The double knockout mice, lacking *Hmox1* and dystrophin, showed significant impairment of exercise capacity on treadmill in comparison to *mdx* mice, aggravated muscle injury as evidenced by higher level of creatine kinase (CK) and lactate dehydrogenase (LDH), increased infiltration with inflammatory cells and increased expression of miR-146a.
Similar effects were observed in *mdx* mice treated with tin protoporphyrin, a pharmacological inhibitor of *Hmox1* activity. Global RNA sequencing demonstrated significant effect of dystrophin deficiency on SCs transcriptome, while lack of *Hmox1* was weakly pronounced. Transcriptome of activated satellite cells (α7i+CD34-) also differed significantly from the α7i+CD34+ cells. SCs isolated from *mdx* mice showed disturbed and accelerated differentiation.
The results indicate for the relationship between *Hmox1* and myomirs in muscle damage. *Hmox1* exerts both satellite cells-specific effect and influences inflammation in dystrophic muscles. *Hmox1* is an important modulator of DMD progression.

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MicroRNA Regulation in the GFAP.HMOX1 Mouse Model of Parkinson Disease

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Given an aging population that is both growing larger and living longer, neurodegenerative disorders are becoming increasingly prevalent in the developed world. Idiopathic Parkinson disease (PD) is a movement disorder of uncertain etiology that afflicts 1-2% of the population over 65 years of age. PD is characterized pathologically by progressive degeneration of dopaminergic (DA) neurons in the substantia nigra pars compacta (SN) and striatum (STM), formation of fibrillar inclusions (Lewy bodies) in these cell populations and depletion of DA and other bioaminergic neurotransmitters. This results in a slew of motor, autonomic and cognitive disabilities. Our laboratory has recently engineered a novel transgenic (TG) mouse, GFAP.HMOX1, in which the stress-inducible protein, heme oxygenase-1 (HO-1), is overexpressed in astrocytes between 8.5 and 19 months of age. This model recapitulates many neuropathological, neurochemical and behavioural features of PD. Recent evidence suggests that dysregulated microRNA (miRNA) expression

may play a pivotal role in aging and neurodegenerative conditions, like PD. MiRNA are noncoding RNA species that negatively regulate target genes via mRNA cleavage, mRNA degradation or protein translation repression. GFAP.HMOX1 neural tissues exhibited altered patterns of miRNA and target mRNA expression akin to those observed in human PD subjects. Several genes involved in the DA system were significantly downregulated at the mRNA and/or protein level in TG STM compared to wild-type (WT) controls, while select miRNAs targeting these genes were significantly upregulated. Alpha-synuclein, involved in the formation of Lewy bodies in PD subjects, was significantly upregulated at both the mRNA and protein level in both SN and STM, while several miRNAs targeting alpha-synuclein were significantly downregulated. Genes involved in other pathways known or suspected to play a role in PD pathology, such as oxidative stress, apoptosis, autophagy and mitophagy, mitochondrial biogenesis and reelin expression, were significantly elevated at the mRNA and/or protein level in the experimental samples compared to WT preparations. Furthermore, several miRNAs were negatively correlated with target genes involved in the aforementioned systems. Many of these whole--brain findings were recapitulated in neurons co-cultured with TG astrocytes compared to WT preparations, suggesting that HMOX1 overexpression in the astrocytic compartment significantly compromises neuronal integrity. Alterations in key miRNA expression levels could be used as a suitable preclinical biomarker for PD, considering the stable nature of miRNAs and detectability in human plasma, serum or total blood, as well as urine and saliva.

Heme oxygenase-1 controls the oxidative stress – HDAC4 – miR-206 axis in rhabdomyosarcoma

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Rhabdomyosarcoma (RMS) is the tumor characterized by disturbed myogenic differentiation. In myoblasts, myogenesis is impeded upon overexpression of heme oxygenase-1 (HO-1), the effect mediated by inhibition of cEBPδ-dependent myoD transcription and downregulation of myomirs, including miR-206. Herein we analyzed a role of HO-1 in progression of RMS.
Experiments were performed using in vitro model of six human RMS cell lines of different clinical aggressiveness. Histological staining and gene expression analyses were also done in 31 clinical primary tumor samples. Additionally, to evaluate role of HO-1 in tumor stroma cells, SMS-CTR cells of eRMS origin were co-cultured with primary mesenchymal stem cells (MSCs) expressing (HO-1 WT) or not (HO-1 KO) HO-1. Finally, effect of HO-1 inhibition was evaluated in vivo using aRMS xenograft spheroid assay in the mice.
We found that expression of HO-1 is elevated in cell lines and in clinical primary tumors of aRMS phenotype. Moreover, upregulation of HO-1 can be induced in the mild eRMS form by forced expression of Pax3-FoxO1 fusion gene, the hallmark oncogene typical for aRMS. Accordingly, incubation of RMS cells with HO-1 inhibitor (tin protoporphyrin, SnPP) led to upregulation of markers of myogenic differentiation, like myogenin or myosin, the effect more pronounced in the eRMS cells. Differently from eRMS, in the aRMS cell line no effect of SnPP on myoD was observed. SnPP significantly decreased Pax3-FoxO1- induced SDF-1/CXCR4 and cMET/HGF gene expressions. However, differently from eRMS, in the aRMS cell line no effect of SnPP on myoD was observed. Instead, SnPP - mediated inhibition of HO-1 strongly upregulated the production of miRNA206 both in eRMS and aRMS, what was accompanied by translocation of histone deacetylase-4 (HDAC4) from nucleus to cytoplasm. HDAC4, when present in the nucleus, is known to repress miRNA206 expression. Thus, it appears that the antioxidative HO-1, by affecting HDAC-4-miRNA206 pathway, may abrogate the myogenic differentiation in RMS. Interestingly, co-culturing of eRMS cells with MSCs isolated from HO-1 KO mice resulted in a more pronounced myogenic differentiation in comparison to HO-1 WT co-culture, indicating a plausible role of HO-1 activity not only in tumor cells but also in stroma cells. Finally, inhibition of HO-1 in aRMS in vivo setting

by systemic treatment with SnPP led to reduction of tumor growth, decrease in tumor vascularization and promotion of myogenic differentiation.
Thus, inhibition of HO-1 induces miR-206-dependent myogenic program in RMS and can be considered as therapeutic strategy for treatment of this malignancy.

HO-1: The Ultimate Cytoprotectant in Sickle Cell Disease?

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Patients with sickle cell disease (SCD) have unrelenting hemolysis that continually releases hemoglobin and heme into the vasculature. Heme, a damage-associated molecular pattern, can activate the pattern recognition receptor toll-like receptor 4 (TLR4), leading to oxidative stress, inflammation and vaso-occlusion. Intravenous infusion of the hemoglobin-binding protein haptoglobin or the heme-binding protein hemopexin into humanized mouse models of SCD inhibits vaso-occlusion in steady-state or after hemoglobin challenge. Moreover, induction of the cytoprotective heme metabolizing enzyme heme oxygenase-1 (HO-1) or overexpression of HO-1 by gene therapy induces cellular cytoprotective responses that inhibit oxidative stress, inflammation and vaso-occlusion in SCD mice. The nuclear factor erythroid-2-related factor-2 (Nrf2) transcriptional pathway is the principal cellular defense system responding to pro--oxidative and pro-inflammatory stress. Dimethyl fumarate (DMF), a drug approved for treatment of multiple sclerosis, provides cytoprotection by activating Nrf2-responsive genes including HO-1, haptoglobin, hemopexin, and ferritin. Inhibition of HO activity with tin protoporphyrin abrogates the cytoprotection in SCD afforded by supplementation with haptoglobin/hemopexin or treatment with DMF suggesting that HO-1 is the ultimate cytoprotectant in SCD.

Heme Oxygenase-1, Ferritin and Life in Extreme Environments

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Heme oxygenase-1 (HO-1) is well known to protect against a number of cytotoxic stresses including oxidants. We earlier found that the protective effects of HO-1 over-expression against oxidant challenge were actually due to the linked induction of ferritin. Indeed, over-expression of ferritin – in the absence of HO-1 over-expression – was sufficient to confer protection against oxidants such as hydrogen peroxide. Long ago, the idea was floated that the cytotoxic effects of ionizing radiation and hydrogen peroxide were similar. Because we previously found evidence that increases in intracellular 'loose' (redox-active) iron sensitized cells to radiation, we wondered whether the protective effects of HO-1/ferritin might also extend to ionizing radiation.

siRNA knockdown of HO-1 in NIH 3T3 fibroblasts concomitantly reduced ferritin synthesis and sensitized to killing by ionizing radiation (Co^{60}). Furthermore, overexpression of ferritin heavy chain (Fth) in 3T3 cells greatly increased clonogenic survival following radiation exposure (from 30% in controls to 60% in Fth overexpressing cells). This is supported by results of comet assays performed after radiation exposure with Fth overexpressing cells showing no increase in tail length. The involvement of DNA double strand breaks is further indicated by greatly increased expression of gamma H2AX following radiation exposure in wild type 3T3 cells compared to cells overexpressing Fth. These results suggested

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that iron-mediated DNA damage might be responsible for the cytotoxic effects of radiation as has been previously suggested. In support of this idea, and as expected from our earlier work, intracellular levels of 'loose' iron were substantially lower in Fth overexpressing cells.

We sought to determine whether pharmacologic induction of ferritin might have similar radio-protective effects. 1,2-dithiole-3-thione (D3T) was earlier reported to upregulate Fth expression mediated by activation of Nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Indeed, pre-incubation of 3T3 cells with D3T increased the expression of Fth by >5-fold. Concomitant with the increased expression of Fth, we found that cells pre-treated with D3T were resistant to killing by 3Gy of ionizing radiation (~70% survival after D3T vs. ~2% in controls) and 6Gy (20% survival vs. 0% in controls) as assessed by clonogenic survival. Because D3T increases expression of a variety electrophile/antioxidant responsive genes (including HO-1), we selectively knocked down Fth with siRNA prior to the exposure of 3T3 cells to D3T. In this case, there was no increase in Fth and no significant radioprotection indicating that Fth expression, but not that of other antioxidant enzymes, was most important in promoting cell survival. We conclude that, at least in this experimental system, the protective effects of HO-1 against oxidants and radiation are mediated by the linked induction of ferritin and that the suppression of redox active intracellular iron is most important in such protection.

Is the heme oxygenase activity of HO-1 necessary for the protection of cells from oxidative insult?

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The products of heme catabolism by HO-1 are ferrous iron, biliveridin, and carbon monoxide. In addition to its primary function, the recycling of erythrocyte iron, this enzyme has been implicated in numerous cytoprotective mechanisms mainly in the context of oxidant insult. Implicit in most reports of HO-1 cytoprotection are effects on the cellular handling of heme and/or iron. While biliverdin and its metabolite, bilirubin, are antioxidants, ferrous iron catalyzes the production of hydroxyl radicals through Fenton chemistry. Thus, the release of iron from heme may counterproductive in protecting the cell from oxidative stress. Furthermore, there are a number of non-heme stimulators of HO-1 induction, bringing to question the source of substrate for HO-1 in these paradigms.
Induction of HO-1 by sodium arsenite induces HO-1, but has no effect on iron metabolism in a macrophage-like cell line. The latter requires a supply of exogenous iron. Stable overexpression of HO-1 in the same cell line afforded cells protection against oxidative stress, but also did not affect intracellular iron metabolism. The protection of cells by HO-1 without an apparent change in intracellular heme catabolism raises the question of whether the degradation of heme is indeed the mechanism through which HO-1 exerts its protective effects. Other work showing the localization of HO-1 in the nucleus, among other organelles, is consistent with a function of HO-1 beyond its ability to catabolize heme. Congruent with this idea, HO-1 mutants unable to degrade heme nonetheless confer cytoprotection and/or carcinogenicity to tissues. Taken together, our results as well as those of other groups, suggest that the protection of cells by HO-1 from oxidative insult is not due to the catabolism of considerable amounts of cellular heme.

Hemopexin Counteracts Systolic Dysfunction Induced by Heme-driven Oxidative Stress

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Heart failure is a leading cause of morbidity and mortality in patients affected by different disorders associated to intravascular hemolysis. The leading factor is the presence of pathologic amount of pro-oxidant free heme in the bloodstream, due to the exhaustion of the natural heme scavenger Hemopexin (Hx). Here, we evaluated whether free heme directly affects cardiac function, and tested the therapeutic potential of replenishing serum Hx for increasing serum heme buffering capacity.
In mice, Hx loss/depletion resulted in heme accumulation and enhanced ROS production in the heart, which ultimately led to severe systolic dysfunction. Similarly, high ROS, reduced systolic Ca2+ transient amplitudes and reduced fractional shortening were observed in primary cardiomyocytes exposed to free heme. In keeping with these Ca2+ handling alterations, oxidation and CamKII-dependent phosphorylation of Ryanodine Receptor 2 were higher in Hx-/- hearts than in controls. Intriguingly, ROS production, Ca2+ mishandling and systolic failure were prevented in cells exposed to heme in the presence of Hx. Consistently, administration of Hx to hemolytic mice preserved cardiac function.
We show that heme-mediated oxidative stress perturbs cardiac Ca2+ homeostasis and promotes contractile dysfunction. Heart Free Heme (HFH) toxicity contributes to left ventricular dysfunction in β-thalassemic mouse model.

Conclusion. Scavenging heme, Hx counteracts cardiac heme toxicity and preserves left ventricular function. Our data generate the rationale to consider the therapeutic use of Hx to limit the cardiotoxicity of free heme in hemolytic disorders.

Regulation of Heme Levels during Erythroid Cell Development: A Balancing Act

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Heme is a complex of iron with protoporphyrin IX, which is essential for most forms of life. However, heme can cause severe oxidative damage in its "free" non-protein bound form. Hence, cells are equipped with mechanisms that maintain a fine balance between heme synthesis and catabolism.
The synthesis of heme in vertebrates involves eight enzymes, four of which are cytoplasmic and four of which are mitochondrial; all are encoded by nuclear genes. The first step occurs in the mitochondria and involves the condensation of succinyl CoA and glycine to form ALA, a reaction catalyzed by ALA synthase (ALA-S). The next four biosynthetic steps, which take place in the cytosol, eventually lead to the formation of coproporphyrinogen III. The final three steps of the biosynthetic pathway, including the insertion of Fe2+ into protoporphyrin IX by ferrochelatase, occur in the mitochondria. Differences in iron metabolism and genes for ALA-S account for the variation in the regulation of heme synthesis rate in erythroid cells as compared to other cells in mammals (Ponka & Sheftel, In: *Iron Physiology and Pathophysiology*, Chapter 10, Springer, p. 191, 2012). The intracellular path of iron from transferrin-endosomes to ferrochelatase is still obscure or, at best, controversial. The prevailing opinion is that iron, after its export from endosomes, spreads into the cytosol, from where the metal mysteriously finds its way into mitochondria. An alternative view, conceived in our laboratory, is that the highly efficient transport of iron toward ferrochelatase in erythroid cells requires direct interaction

between transferrin-endosomes and mitochondria ("kiss-and-run" hypothesis). Despite the longevity of the prevailing opinion, experimental evidence (Richardson et al. *Blood* 87:3477, 1996; Zhang et al. *Blood* 105:368, 2005; Sheftel et al. *Blood* 110: 125, 2007) only supports the latter hypothesis, which sees favorable reception among Cell Biologists (McBride, *BMC Biology* 13:8, 2015).
The only physiological mechanism of heme degradation is by heme oxygenases (HO). The heme-inducible isoform, HO-1, has been extensively studied in numerous nonerythroid cells but, until recently, virtually nothing was known about the expression and potential significance of HO-1 in developing red blood cells. We have demonstrated that HO-1 is present in erythroid progenitors and that its expression is upregulated during erythroid differentiation. Overexpression of HO-1 in erythroid cells impairs hemoglobin synthesis, whereas HO-1 absence enhances hemoglobinization in cultured erythroid cells. Based on these results, we conclude that HO-1 controls the regulatory heme pool at appropriate levels for any given stage of erythroid differentiation. Hence, our results reveal the importance of HO-1 expression for erythroid development and expand our knowledge about the fine regulation of hemoglobin synthesis in erythroid cells that involves not only the synthesis of heme, but also its degradation.

A Novel hBVR-Based Technology to Regulate Insulin Receptor and Akt kinase Activities and Treat Diabetes.

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Biliverdin reductase A (hBVR) reduces the heme oxygenase activity product, biliverdin, to bilirubin; it is directly activated by insulin receptor kinase (IRK) and is a key regulator of glucose metabolism. hBVR is the upstream activator of the PI3K/PDK1/MAPK /Akt signaling pathway .An hBVR –based peptide KYCCSRK, corresponding to the C-terminal segment of hBVR, has an unprecedented ability to activate glucose uptake through direct interaction with the intracellular kinase domain of insulin receptor and inducing conformational change similar to that induced by insulin binding to extracellular domain of IRK. We developed a peptidase-resistant formulation of the peptide that was efficient in both mice and cell culture systems. The peptide was constructed of D-amino acids, in reverse order, and blocked at both termini The peptide improved glucose clearance in both wild-type and Ob/Ob mice; it lowered blood glucose levels and suppressed glucose-stimulated insulin secretion. IRK activity was stimulated in the liver of treated mice and in cultured cells. The peptide potentiated function of IRK's downstream effector, Akt. hBVR and Akt isozymes have overlapping pleiotropic functions in the signaling pathway. The isozymes (Akt1-3) are activated by sequential phosphorylation at T308 by PDK1 and autophosphorylation at S473. Phosphorylation of glycogen synthase kinase 3 (GSK3) isoforms α/β by Akts inhibits their activity; unphosphorylated GSK3β inhibits activation of various genes. Akt(RxRxxSF) and PDK1 (RFxFPxFS) binding motifs are present in hBVR . hBVR and Akt1 co- immunoprecipitated, and in-cell Förster resonance energy transfer (FRET) and glutathione S-transferase pulldown analyses identified Akt1 pleckstrin homology domain as the interactive domain. Immunoprecipitation analysis showed that PDK1 and hBVR interact through hBVR's PDK1 binding motif, RFGFPAFS and formation of the PDK1/hBVR/Akt1 complex. sihBVR blocked complex formation. Findings identify: hBVR and its fragment as previously unknown activator of Akt: a key mediator of Akt1/GSK3 pathway ;a novel effector of Akt activation; and, regulator of glucose metabolism (supported by NIH ES-R01-004066).
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Is biliverdin reductase a key regulator of inflammation?

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Biliverdin reductase, a key signaling molecule and enzyme that converts biliverdin to bilirubin, has been recognize to have strong immunomodulatory effects. Our and other laboratories have recently demonstrated a critical role of BVR in the membrane as a mediator of anti-inflammatory IL-10 via activation of PI3K-Akt signaling. Furthermore, lack of BVR amplified enodotoxin-mediated injury to the liver in part via increase in TLR4-TNF signaling. BVR is a transcriptional regulatory and can bind nucleic acids to regulate gene expression in addition to its kinase and signaling function. We have recently asked whether membrane BVR can recognize DNA fragments released from necrotic cells and which are immunogenic and recognized as danger associated molecular patterns (DAMPs). Indeed, we found that biliverdin reductase (BVR, BLVRA) binds extracellular mtDNA. The interaction between BVR and CG-rich DNA then activates PI3K and IRF3 signaling, leading to TNF production. Conditional deletion of the BVR gene in macrophages abrogated liver damage in several DAMPs-driven models of sterile inflammation. This is in contrast to the elevated responses to the endotoxin-induced organ injury in mice lacking BVR in macrophages. The protective effects of BVR deletion were independent of the antioxidant properties of BVR enzymatic activity on biliverdin, which competes with mtDNA for binding to BVR. We also found that BVR is upregulated in monocytes from trauma patients, which have high levels of circulating DNA. In summary, BVR as an immune sensor, which modulates responses based on the cause of inflammation.

Biliverdin reductase A and hepatic steatosis

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Non-alcoholic fatty liver disease (NAFLD) is the most rapidly growing form of liver disease, and if left untreated can result in non-alcoholic steatohepatitis (NASH) ultimately resulting in liver cirrhosis and failure. Biliverdin reductase-A (BVRA) is a multi-functioning protein primarily responsible for the reduction of biliverdin to bilirubin. Also, BVRA functions as a kinase and transcription factor, regulating several cellular functions. We report here that liver BVRA protects against hepatic steatosis by inhibiting glycogen synthase kinase-3β (GSK3β) by enhancing serine 9 phosphorylation, which inhibits its activity. We show that GSK3β phosphorylates serine 73 (pS73) of the peroxisome proliferator-activated receptor alpha (PPARα), which in turn, increased ubiquitination and protein turnover as well as decreased activity. Interestingly, liver-specific BVRA knockout (LBVRA-KO) mice had increased GSK3β activity and pS73 of PPARα, which resulted in decreased PPARα protein and activity. Furthermore, the LBVRA-KO mice exhibited increased plasma glucose and insulin levels, and decreased glycogen storage, which may be due to the manifestation of hepatic steatosis observed in the mice. These findings reveal a novel BVRA-GSKβ-PPARα-axis that regulates hepatic lipid metabolism and may provide unique targets for the treatment of NAFLD.

Impairment of Biliverdin Reductase-A promotes brain insulin resistance in Alzheimer Disease

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Clinical studies suggest a link between peripheral insulin resistance and cognitive dysfunction. Interestingly, post--mortem analyses of Alzheimer disease (AD) subjects demonstrated insulin resistance in the brain proposing a role for cognitive deficits observed in AD. However, the mechanisms responsible for the onset of brain insulin resistance (BIR) need further elucidations. Biliverdin reductase-A (BVR-A) emerged as a unique Ser/Thr/Tyr kinase directly involved in the regulation of insulin signaling. Indeed, once activated via Tyr phosphorylation by insulin receptor (IR), BVR-A is able to phosphorylate the insulin receptor substrate (IRS)-1 on inhibitory domains, representing an upstream regulator in the insulin signaling cascade. Because we previously demonstrated the oxidative/nitrosative stress (OS/NS)-induced impairment of BVR-A in human Alzheimer disease (AD) brain, here we hypothesize that BVR-A dysregulation could be associated with the onset of BIR in AD.
To this aim, we performed a longitudinal analysis to evaluate BVR-A protein levels and activation in the hippocampus of 3xTg-AD and WT mice at 3, 6, 12 and 18 months of age (n=6/group). Changes of BVR-A have been then correlated with changes about (i) IR/IRS1 protein levels and activation state (ii) total OS/NS markers levels (PC, HNE, 3-NT), (iii) changes of Aβ and tau pathology, (iv) TNF-α levels and (v) mTOR activation. Subsequently, ad hoc experiments have been performed in SH-SY5Y cells treated with (i) insulin, (ii) hydrogen peroxide/peroxynitrite or (iii) a specific silencing RNA (siRNA) for BVR-A, to clarify the molecular mechanism(s) underlying changes observed in mice.
Our results highlighted two distinct phases in the hippocampus of 3xTg-AD mice: a first insulin signaling hyper-activation (3-6 months) followed by a persistent IRS1 inihibition and thus insulin resistance at both 12 and 18 months. In this picture, we found that BVR-A levels and activation start to decline early, at 6 months of age, prior the accumulation of Aβ and tau pathology, and remain persistently reduced until 18 months, possibly because the increased OS/NS levels in the same time-frame. In addition, because TNF-α is known to inhibit BVR-A promoter activity and TNF-α has been also demonstrated to be a conceivable mediator of BIR in AD, we wondered to check whether reduced BVR-A levels were associated with changes of TNF-α. Interestingly, an increase of TNF-α levels is evident only at 18 months, thus highlighting the impairment of BVR-A as an early event in the onset of BIR. Similar changes have been found during the normal ageing process in WT mice, but later in life. Experiments on SH-SY5Y cells further confirmed that either lack of BVR-A or OS/NS-induced impairment of BVR-A promotes BIR. Finally, we identified the sustained activation of mTOR, as one of the feedback mechanisms leading to insulin resistance following BVR-A impairment both in mice and cells.
In conclusion, we propose a novel mechanism for which: OS/NS-induced impairment of BVR-A is firstly responsible for a sustained activation of IRS1, which then causes the stimulation of negative feedback mechanisms (i.e. mTOR) aimed to turn-off IRS1 hyper-activity and thus insulin resistance. Similar alterations characterize the normal ageing process in mice, positing BVR-A impairment as a possible bridge in the transition from normal ageing to AD.

Neuroprotective roles of Biliverdin Reductase in the Brain

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Biliverdin reductase (BVR) is a highly conserved enzyme involved in the conversion of biliverdin, which is derived from heme, to bilirubin. BVR exists as two isoforms, BVR-A and BVR-B, of which BVR-A is enriched in adults. In addition to its role in bilirubin generation, BVR-A is a kinase and transcription factor, and participates in several signaling pathways. Besides, these activities, the BVR pathway plays significant roles in maintenance of redox balance in cells. Cell culture studies have shown that depletion of BVR-A results in elevated oxidative stress and associated cellular damage. Oxidative stress is a hallmark of several neurodegenerative diseases such as Alzheimer's disease and Huntington's disease, which mediates neurotoxicity. Using BVR-A knockout mice, we show that depletion of BVR-A is associated with elevated oxidative stress in the brain and altered cytoprotective response to stress stimuli. Mouse embryonic fibroblasts and primary neurons derived from BVR knockout mice exhibit increased susceptibility to oxidants such as hydrogen peroxide and homocysteic acid. Treatment of mice with NMDA, an excitotoxin, results in increased cell death and larger lesions in BVR-A knockout mice. NMDA receptor activation is known to elevate superoxide production via the NADPH

oxidases, enzymes which are known to be inhibited by the BVR-bilirubin pathway. Thus deletion of BVR would elevate oxidative stress via this pathway. A histochemical analysis of the brains of the BVR knockout mice reveal enlarged ventricles and cortical thinning, features frequently observed in neurodegeneration. Taken together, our findings uncover important roles for BVR in neuroprotective processes. Modulating the BVR/bilirubin pathway may offer therapeutic benefits in neurodegenerative diseases involving redox imbalance.

ANTI-ONCOGENIC POTENTIAL OF HMOX1

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Currently our view of cancer has evolved to include, in addition to the transformed cells that have deregulated homeostatic mechanisms, a wide spectrum of cells of the tumor microenvironment. The dialogue established between the tumor cells and its microenvironment, is an essential determinant of the characteristics of the tumor progression.
The completion of the human genome sequence has generated great expectations in the development of new cancer therapies. However, considering the heterogeneity of most tumors, a single biomarker does not seem sufficient to predict the disease outcome. Furthermore, the problem widens when analyzing and grading the architectural complexity of the epithelial structures. In this regard, Innovative high-throughput "omics" platforms are now identifying and quantifying new specific and sensitive biomarkers for detection, stratification and treatment. Although some progress has been made, there is an urgent need to chart a coherent road map with clearly define milestones to guide therapeutics efforts.
Heme-oxygenase 1 (HMOX1), has been shown to govern a plethora of biological processes and molecular functions associated with anti-tumoral effects in several cancers, ranging from cell proliferation, invasion and migration impairment, to exerting co-regulatory functions at the transcriptional level and preventing DNA damage. This presentation is intended to describe the most pressing issues of HO-1 in cancer, also emphasizing its role in modifying the tumoral microenvironment, favoring a less aggressive phenotype.

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Induction of Heme Oxygenase-1 by 4-Hydroxyestradiol Promotes Mammary Cell Transformation and Tumorigenesis

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Estrogen (17β-estradiol, E2) undergoes oxidative metabolism by CYP1B1 to form 4-hydroxyestradiol (4-OHE2), a putative carcinogenic metabolite of estrogen. Our previous study showed that 4-OHE2-induced production of reactive oxygen species (ROS) contributed to neoplastic transformation of human breast epithelial (MCF-10A) cells. In

this study, 4-OHE2, not E2, increased the expression of heme oxygenase-1 (HO-1), a sensor and regulator of oxidative stress, in MCF-10A cells. Silencing the HO-1 gene in MCF-10A cells suppressed 4-OHE2-induced cell proliferation and transformation. In addition, subcutaneous administration of 4-OHE2 markedly enhanced the growth of the MDA--MB-231 human breast cancer xenografts, which was retarded by zinc protoporphyrin (ZnPP), a pharmacological inhibitor of HO-1. 4-OHE2-induced HO-1 expression was mediated by NF-E2-related factor (Nrf2). We speculate that an electrophilic quinone formed as a consequence of oxidation of 4-OHE2 binds directly to Kelch-like ECH-associated protein 1 (Keap1), an inhibitory protein that sequesters Nrf2 in the cytoplasm. This will diminish association between Nrf2 and Keap1. 4-OHE2 failed to interrupt the interaction between Keap1 and Nrf2 and to induce HO-1 expression in Keap1-C273S or C288S mutant cells. Lano-LC-ESIMS/MS analysis in MCF-10A-Keap1-WT cells which were treated with 4-OHE2 revealed that the peptide fragment containing Cys288 gained a molecular mass of 287.15 Da, equivalent to the addition of a single molecule of 4-OHE2-derived ortho-quinones. In conclusion, induction of HO-1 expression by 4-OHE2 may led to transformation of normal breast cells and tumor promotion of breast cancer cells, respectively. Therefore, HO-1 represents a novel target for chemoprevention and treatment of breast cancer.

HO-1 blockage is effective in stimulating the host immune system to fight against neuroblastoma

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Heme oxygenase (HO)-1 has been proposed to have immunomodulatory effects. This could be tumor-protective, allowing the tumor to escape the immune response of the host. Indeed, several tumor cells express HO-1 to a great extent. So does neuroblastoma (NB), the most common extracranial solid childhood tumor. Here, we employed a syngeneic mouse model of NB to target HO-1 by systemic as well as tumor-specific Zinc protoporphyrin-mediated HO-1 blockage. Systemic ZnPPIX treatment inhibited HO-1 activity albeit not its expression, provoking a maturation of T cells and the stimulation CD4 and CD8 T-effector cell responses. This resulted in 50% reduction of primary tumor growth as well as to a diminution in the number of spontaneous liver metastases. The same could be achieved when pre-treating tumor cells with ZnPPIX. In summary, HO-1 emerges as a novel immune regulator in NB and emerges as a promising target for the development of therapeutic approaches.

In vivo hemin pre-conditioning targets the vascular and immunological compartments and restrains prostate tumor development

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Prostate cancer (PCa) is the second leading cause of cancer incidence in men worldwide. Most current therapies against this disease are designed to target the tumor cell. However, the surrounding microenvironment plays a leading role in enabling tumor growth and dissemination. "Pre-conditioning" strategies constitute a relatively unexplored and exciting opportunity to shape tumor fate. In the present study we used hemin, a well-known inducer of the homeostatic enzyme Heme Oxygenase-1 (HO-1), in an in vivo pre-conditioning model to assess PCa development. The stroma of fully immunocompetent mice (n=5) was conditioned by subcutaneous administration of hemin (200µl, 10µM) or its vehicle PBS prior to T-C1 tumor challenge. Hemin pre-conditioned animals showed a significant increase in tumor latency (57 days in hemin-treated mice vs. 47 days in control mice; $P<0.01$) and a significant decrease in the initial tumor growth rate ($P<0.05$). Histological analysis of the tumors revealed impaired vascularization, as determined by Masson's trichrome staining. In vitro experiments indicated that hemin-treated HUVEC exhibit decreased tubulogenesis only in the presence of T-C1-derived conditioned media ($P<0.001$). We also studied whether hemin pre-conditioning could influence the crosstalk between tumor and endothelial cells. In fact, T-C1 cell motility was significantly impaired when a wound-healing assay was performed in the presence of hemin pre-treated HUVEC conditioned media ($P<0.05$). Moreover, tumor cell adhesion to the endothelium was severely reduced when HUVEC were pre-treated with hemin ($P<0.01$). An in vivo Matrigel plug assay confirmed that s.c. hemin preconditioning hinders tumor-associated neo-vascularization in C57Bl/6 mice (n=5; $P<0.01$). Hemin treatment boosted the CD8+ immune response by inducing their proliferation and degranulation in vitro, both in a normal and in a tumor microenvironment. To verify whether these findings were relevant in vivo, we tested specific cytotoxicity following hemin pre-conditioning by performing a cytotoxic-Tlymphocyte (CTL) assay. Hemin pre-conditioning resulted in enhanced antigen-specific cytotoxicity in vivo (n=5; $P<0.01$). When effector lymphocytes were treated ex vivo prior to adoptive transfer into C57Bl/6 mice, it also led to an augmented antigen-specific cytotoxicity (n=5; $P<0.01$). Interestingly, a significant systemic increase in the frequency of CD8+ T lymphocytes was observed in hemin preconditioned tumor-bearing mice. Tumors from hemin-conditioned mice showed reduced expression of Galectin-1 ($P<0.01$), key modulator of tumor angiogenesis and immunity, evidencing a clear and persistent remodeling of the microenvironment. Data obtained at the mRNA and protein levels revealed a subset of PCa patients and PCa patient-derived xenografts, respectively, with mild HO-1 and low Gal-1 expression. Taken altogether, these data showcase a novel function of an already human-used drug as a novel means of boosting the endogenous anti-tumor response.

Iron and heme: Our friends and foes

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Iron is a vitally important element in virtually all organisms, because of its unsurpassed versatility as a biological catalyst. It serves as metal cofactor for many proteins and enzymes, either non-heme or hemoproteins. In the latter, iron is inserted like a gem in the center of protoporphyrin IX. Under normal conditions both intracellular iron trafficking and heme levels are impeccably regulated, preventing the accumulation of noxious free iron and highly toxic intermediates of heme biosynthesis. In mammals, there is an iron cycle that entails the movement from plasma transferrin to hemoglobin in developing red blood cells, and the release of iron back to plasma transferrin from macrophages that recycle the hemoglobin iron of senescent erythrocytes. Under normal circumstances, hemoglobin-processing macrophages simultaneously convey iron to plasma at the same rate as the metal is delivered from transferrin to developing erythroid cells. The crucial role in this loop is played by heme oxygenase 1 (HO1) that catabolizes heme to biliverdin upon the release of Fe^{2+} and carbon monoxide. Importantly, we have recently reported that HO1 is expressed also in erythroid progenitors as well as more mature hemoglobin-synthesizing cells. These and other results have allowed us to conclude (*Blood* 123:2269, 2014) that HO1 controls the "regulatory" heme pool at appropriate levels and thus plays an important role as a co-regulator of erythroid differentiation. Since erythroid cells can degrade "uncommitted" ("regulatory") heme, they probably do not need to export "toxic heme", an idea that has become remarkably popular in the past ten years.

Vascular-targeted molecules to limit the risky business of pregnancy

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Preeclampsia is a life-threatening vascular disorder of pregnancy due to a failing stressed placenta. Millions of women risk death to give birth each year and globally each year, almost 300,000 lose their life in this process and over 500,000 babies die as a consequence of preeclampsia. Despite decades of research, we lack pharmacological agents to treat it. Maternal endothelial oxidative stress is a central phenomenon responsible for the preeclampsia phenotype of high maternal blood pressure and proteinuria. In 1997, we discovered that vascular endothelial growth factor (VEGF) stimulated nitric oxide release. This led us to suggest that preeclampsia arises due to the loss of VEGF activity. Researchers showed that high soluble Flt-1 (sFlt-1) and soluble endoglin (sEng) elicit the severe preeclampsia phenotype in pregnant rodents. We demonstrated that heme oxygenase-1 (Hmox1) / carbon monoxide (CO) pathway prevents placental stress and suppresses sFlt-1 and soluble endoglin (sEng) release. Hydrogen sulfide generating cystathionine-γ-lyase (CSE) system also inhibits these anti-angiogenic factors. These two enzymes suppress sFlt-1 and sEng and protect against the preeclampsia phenotype in mice. Importantly, hydrogen sulfide restores placental vasculature, and in doing so improves lagging fetal growth. These molecules act as the inhibitor systems in pregnancy and when they fail, this triggers preeclampsia. Discovering that statins induce these enzymes led us to a double-blind randomized control trial to develop a low-cost therapy (StAmP Trial) for preeclampsia. In this talk, we discuss Bradford-Hill causation criteria for disease causation and how the protection against cellular stress hypothesis that states that the protective pathways mitigate cellular stress by limiting elevation of anti-angiogenic factors and oxidative stress and the subsequent clinical signs of preeclampsia appear to fulfill the Bradford-Hill causation criteria, making the roadmap to this pathway a good candidate for developing diagnostics and therapeutics to target the pathogenesis of preeclampsia.
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Inhibition of Vascular Smooth Muscle Cell Migration By C-Terminus-Truncated, Enzymatically Active Heme Oxygenase-1

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Heme oxygenase-1 (Hmox1) is an ~32 kDa enzyme that degrades heme to carbon monoxide, Fe^{2+} and biliverdin, and that plays a primary role in iron homeostasis. Previous studies utilising a pharmacological approach showed that chemical inducers of Hmox1 attenuate cardiovascular disease, including angioplasty- and stent-induced neointimal hyperplasia and vascular disease. We observed that treatment of rat aortic vascular smooth muscle cells (RASMC) with stressors such as hypoxia or hemin increased the formation of a truncated form of Hmox1. Mass spectrometric analysis confirmed truncated Hmox1 (hereafter referred to as $Hmox1_{\Delta 23}$) to lack the 23 C-terminal amino acids. Increased expression of $Hmox1_{\Delta 23}$ induced by hypoxia was associated with increased heme oxygenase activity, as assessed by the accumulation of biliverdin at 4-fold higher concentration in the culture media compared with normoxia. Consistent with this observation, purified $Hmox1_{\Delta 23}$ retained ~20% of Hmox1 full length enzymatic activity *in vitro*, as assessed by conversion of heme to biliverdin, also determined by liquid chromatography-mass spectrometry. Fractionation of RASMC subjected to hypoxia indicated at least part of the $Hmox1_{\Delta 23}$ expression to be present in the nucleus. This conclusion was confirmed by confocal microscopy using FLAG-Hmox1-6HIS constructs. Over-expression of $Hmox1_{\Delta 23}$ in RASMC also significantly decreased cell migration. This was attenuated partially by mutation of either the His25 required for heme binding or a putative nuclear localisation signal at Ser229 of Hmox1. Mutating both, His25 and Ser229 completely abolished the ability of $Hmox1_{\Delta 23}$ to inhibit RASMC migration. We also identified an amino acid sequence within the C-terminus possibly required for stress-induced 'truncation' of Hmox1. We are currently using adenoviral delivery of different Hmox1 isoforms and mutants to assess their ability to inhibit intimal hyperplasia *in vivo* using a rat carotid balloon injury model.

Hepatic Overexpression of Hemopexin Inhibits Inflammation and Vascular Stasis in Murine Models of Sickle Cell Disease

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Sickle cell disease (SCD) patients have low serum hemopexin (Hpx) levels due to chronic hemolysis. We hypothesize that in SCD mice, hepatic overexpression of hemopexin will scavenge the proximal mediator of vascular activation, heme, and will inhibit inflammation and microvascular stasis. To examine the protective role of Hpx in SCD, we transplanted bone marrow from NY1DD SCD mice into $Hpx^{-/-}$ or $Hpx^{+/+}$ C57BL/6 mice. Dorsal skin fold chambers were implanted in week 13 post-transplant and microvascular stasis (% non-flowing venules) evaluated in response to heme infusion. $Hpx^{-/-}$ sickle mice had significantly greater microvascular stasis in response to heme infusion than $Hpx^{+/+}$ sickle mice ($p<0.05$), demonstrating the protective effect of Hpx in SCD. We utilized *Sleeping Beauty (SB)* transposon-mediated gene transfer to overexpress wild-type rat Hpx (wt-Hpx) in NY1DD and Townes-SS SCD mice. Control SCD mice were treated with lactated Ringer's solution (LRS) or a luciferase (Luc) plasmid. Plasma and hepatic Hpx were significantly increased compared to LRS and Luc controls. Microvascular stasis in response to heme infusion in NY1DD and Townes-SS mice overexpressing wt-Hpx had significantly less stasis than controls ($p<0.05$). Wt-Hpx overexpression markedly increased hepatic nuclear Nrf2 expression, HO-1 activity and protein, the heme-Hpx binding protein and scavenger receptor, CD91/LRP1 and decreased NF-κB activation. Two missense (ms)-Hpx *SB*-constructs that bound neither heme nor the Hpx receptor, CD91/LRP1, did not prevent heme-induced stasis. In conclusion, increasing Hpx levels in transgenic sickle mice via gene transfer activates the Nrf2/HO-1 anti-oxidant axis and ameliorates inflammation and vaso-occlusion.

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Endothelial Loss of the Heme Exporter Flvcr1A alters Vascular Integrity

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Objective: The Feline Leukemia Virus Subgroup C Receptor 1 (Flvcr1) is an highly conserved heme export protein existing as two different isoforms, Flvcr1A and 1B. Mice lacking the 1A isoform die midgestionally due to extended haemorrhages, thus suggesting an involvement of Flvcr1A in the maintenance of vessels' integrity (Chiabrando et al., JCI 2012). The purpose of this work is to assess the role of Flvcr1A in the vasculature.
Matherials and Methods: We generated an endothelial knockout model for Flvcr1A (Flvcr1Afl/fl;Tie2-cre) and we isolated endothelial cells (ECs) from the embryo through magnetic separation or cell sorting. We also generated a zebrafish model of Flvcr1A deficiency. Finally, we downregulated Flvcr1A in primary human umbilical endothelial cells (HUVECs) and primary human microvascular endothelial cells (HMECs).
Results: Flvcr1Afl/fl;Tie2-cre embryos died around embryonic day 14.5 (E14.5) due to severe haemorrhages. At E11.5 embryos' capillaries appeared disrupted and dilated. Similar vascular alterations were also visible in zebrafish morphants. Electron microscopy analysis on ECs from Flvcr1Afl/fl;Tie2-cre embryos highlighted a strong cytoplasmic vacuolation and an altered swollen morphology of mitochondria and endoplasmic reticulum (ER), thus resembling paraptotic cell death. Notably, HUVECs and HMECs without Flvcr1A showed high levels of intracellular heme and increased reactive oxygen species (ROS) production, despite the up-regulation of Heme oxygenase 1 (Ho-1) and the inhibition of Aminolevulinic acid synthase 1 (Alas1). This suffering condition of knockout ECs finally leads to lower viability, diminished cell adhesion and impaired tubulogenesis in vitro.
Conclusions: Flvcr1A loss in endothelial cells compromises the integrity of vessels thus leading to haemorrhages and embryonic death. Electron microscopy analysis shows that knockout ECs display features of paraptosis, a cytoplasmic vacuolation-mediated form of programmed cell death. Hence, the absence of Flvcr1A triggers endothelial cellular damage and loss of adhesive properties. All these data definitively demonstrate that Flvcr1A has an essential role in the maintenance of vascular integrity.

Iron Causes Vascular Oxidation and Accelerates Atherosclerosis Progression

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Iron accumulates in atherosclerotic lesions but its role in atherogenesis is still debated. In the "iron hypothesis" (1981), Sullivan proposed that iron is detrimental for the cardiovascular system, promoting atherosclerosis progression.
So far, epidemiological data and studies in animal models have provided conflicting evidence regarding a role of excess iron in atherogenesis and cardiovascular disease

In this study we aimed to investigate the role of iron overload in the development of atherosclerosis. To this purpose, a mouse model of type IV Hereditary Hemochromatosis, in which the hepcidin/ferroportin regulatory circuitry is disrupted due to a point mutation in the iron exporter ferroportin, was interbred with ApoE-null mice (ApoE-/-), that show increased susceptibility to atherosclerosis.
Plaque formation was analyzed in ApoE-/-FPNwt/C326S mice at 6 and 12 months of age.
ApoE-/-FPNwt/C326S mice show high serum iron and cholesterol levels, as expected.
Importantly, these mice show strongly increased lesion size and number at both 6 and 12 months of age compared to age-matched Apo-/- mice. The atherosclerotic phenotype positively correlates with higher levels of circulating iron and oxidized LDLs.
Iron is deposited in the artery media layer, which correlates with vascular oxidative stress, and increased expression of the iron storae proteins Ferritins and the anti-oxidant enzymes HO-1, SOD-1 and catalase.
We observed increased vascular permeability, reduced nitric oxide availability and sustained activation and inflammation of the vascular endothelium. Within the atherosclerotic plaques, collagen deposition is reduced and lipid content is increased, indicating enhanced plaque instability and faster disease progression. Plaque macrophages are significantly elevated and correlate with increased iron-induced CCL2 levels, contributing to increased lesion vulnerability. The increased expression of vascular smooth muscle cell actin and the presence of calcifications potentially play a key role in arterial stiffness in ApoE-/-FPNwt/C326S mice. Indeed, the hyperthophic remodeling of the cardiac left ventricle in these mice occurs as a compensation for the enhanced arterial stiffness.
Prolonged administration of a low-iron diet rescues the severe atherosclerotic phenotype of ApoE-/-FPNwt/C326S mice, proving that iron is detrimental for this disease. Experiments are ongoing to test the effect of iron chelation therapy.
Our data suggest that high circulating iron levels strongly enhance the severity and promote the progression of atherosclerosis, indicating that systemic iron overload is a risk factor for atherosclerosis and predisposes to cardiovascular disease.
Our findings have potential implications for those pathological conditions with elevated systemic iron levels, ranging from patients with hemochromatosis to anemic patients dependent on chronic blood transfusions or intravenous iron administration.

HO-1-derived carbon monoxide modulates immune cells to support pregnancy.

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Immune cells are highly relevant for pregnancy success. At early pregnancy stages, cells of the innate immune system promote the remodeling of uterine spiral arteries so that they can adapt to the demands of the fetus. Later, cells of the adaptive immune system orchestrate the specific tolerance towards the semi allogeneic fetus. Beside our previously reported findings showing that Heme Oxygenase-1 (HO-1) and its metabolite carbon monoxide (CO) are indispensable for implantation and placentation, we now describe the importance of HO-1 and CO for immune cell modulation. Hmox1(+/-) or Hmox1(-/-) implantations presented fewer uNK cell numbers compared with Hmox1(+/+) sites. Accordingly, Hmox1(+/-) and Hmox1(-/-) implantations had shallow SA development that was accompanied by intrauterine growth restriction and gestational hypertension. Application of CO at low dose during early to midgestation prevented intrauterine growth restriction in Hmox1(+/-) mothers, this being associated with enhanced in situ proliferation of uNK cells and normalization of angiogenic parameters. Most importantly, CO improved SA remodeling and normalized blood pressure, ensuring a proper fetal growth. Using an allogeneic mouse model for miscarriages, we found that HO-1 is able to maintain maternal dendritic cells (DCs) in an immature state, which contributes to the expansion of the peripheral Treg population. This brings to light one essential pathway through which Treg mediates the semi-allogeneic fetus tolerance.
In conclusion, HO-1-derived CO emerges as a key molecular player in pregnancy; it positively influences uNK cells that results in SA remodeling and adequate fetal growth while modulating maternal Treg cells to ensure immunological tolerance towards the growing fetus.

HO-1/SIRT1/p53 Axis Regulates Macrophage Activation and Attenuates Liver Ischemia-Reperfusion Injury in Mice

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Background/Aim: The mechanism by which macrophage heme oxygenase-1 (HO-1) - Sirtuin1 (SIRT1) signaling promotes resistance against sterile inflammation in tissue damage remains elusive. As tumor suppressor protein p53 may be critical in macrophage activation, we have analyzed how macrophage HO-1/SIRT1/p53 axis affects inflammation response in the liver subjected to ischemia-reperfusion injury (IRI). Methods/Results: Livers in groups of wild type (WT) and myeloid-specific HO-1 transgenic (HO-1TG) mice (C57/BL6) were subjected to partial warm ischemia (90 min) followed by reperfusion (6 hr). Upregulation of myeloid HO-1 attenuated the severity of hepatic IRI, as compared to WT controls (sAST: 5,074±2,986 vs. 13,927±2,704 IU/L; $p<0.05$, n=4-7). Western analysis showed increased levels of HO-1, SIRT1, p19, p53, MDM2 and decreased expression of p-Stat1, p-IkBa and iNOS in IR-stressed HO-1TG livers. This correlated with enhanced RT-PCR expression of SIRT1, Noxa, and p21 and lower levels of IRI cytokine signature (MCP1, TNFα, IL-1β, CXCL10) in HO-1TG livers ($p<0.05$). Higher levels of SIRT1, p19, p53, MDM2, PUMA, and lower level of p-Stat1 in HO-1TG bone marrow-derived macrophage (BMM) cultures were abolished after transfection of HO-1TG macrophages with SIRT1-siRNA. Then, we asked how pretreatment with SIRT-1 activator, resveratrol (Res) may affect the severity of liver IRI in myeloid-specific HO-1 knockout (HO-1KO) vs. FLOX-control (Con) mice. While myeloid-specific HO-1 deletion worsened IRI, Res treatment has rescued HO-1 deficient livers from IR-damage (sAST: Con – 10,764±3,238; HO-1KO – 16,379±3,239; HO-1 KO + Res – 6,946±999 IU/L; $p<0.05$, n=4-6). Adjunctive Res treatment restored otherwise suppressed cytoprotective signaling pathway (SIRT1, p19, p53, and MDM2) in HO-1 deficient livers, data supported by RT-PCR analysis of IRI cytokine signature ($p<0.05$). Addition of Res to HO-1-deficient BMM cultures restored SIRT1, p19, p53, MDM2, PUMA, Noxa, p21 expression while depressing p-Stat1, TNFα, MCP1, iNOS, IL-1β and IL-12. Res upregulated p19/p53/MDM2 expression in WT BMM cultures while p19-siRNA transfection abolished the ability of Res to induce p53. Conclusion: Myeloid cell-specific HO-1/SIRT1/p53 axis regulates inflammation and promotes hepatoprotection in IR-stressed livers. Macrophage SIRT1 upregulates p53 via p19 signaling. These studies describe a new class of HO-1/SIRT1/p53 regulatory mechanism, with broad implications for sterile inflammation and tissue damage conditions.

HMOX/CO and trauma/hemorrhagic shock mediated inflammatory and immune dysfunction

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Trauma is a leading cause of morbidity and mortality worldwide. Although neurological injury and/or hemorrhage account for early mortality, in those patients that survive the initial traumatic injury, the development of multiple organ dysfunction with or without nosocomial infection leads to later mortalities and prolonged morbidities. A combination of genetic, epigenetic, and environmental factors predispose patients to the development of this later phenotype of injury-induced critical illness. Patients predisposed to immune dysfunction may be able to be detected early by measuring points of convergence such as cytokines and chemokines.
Heme, heme-related signaling molecules, and heme oxygenases are one such pathway that likely influences immune responses to trauma and hemorrhage. This includes heme scavenging responses, such as hemopexin levels, as well as heme oxygenases responses, including promoter polymorphisms. The role of heme and heme oxygenases in trauma and immune dysfunction in trauma will be highlighted. This includes the influence of heme oxygenase enzyme signaling on mitochondrial dynamics, oxidative phosphorylation, and signaling, and how these responses influence immune cell phenotypes in trauma models and in humans.

HEME OXYGENASE-1 AFFECTS UTERINE INFILTRATION OF MYELOID CELLS AND THEIR OXIDATIVE STRESS IN EARLY PREGNANCY

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Background: Infiltrating myeloid cells in pregnant uteri can affect multiple processes during gestation, including embryo implantation, placental development, fetal-maternal tolerance, pathogen defense, and parturition. Heme oxygenase-1 (HO-1), a stress-response protein, has anti-inflammatory, antioxidant, and pro-angiogenic properties. We have previously reported that a partial maternal deficiency of HO-1 results in a smaller litter size, associated with
a reduction in uterine natural killer (NK) cells and significant abnormal placental vascular development.
Objective: Here, we investigated how HO-1 affects myeloid cell infiltration into pregnant uteri and their oxidative status.
Method: Uteri were collected at E8.5-10.5 from timed pregnancies of wild-type (WT) FVB or HO-1-deficient (HO-1+/-, Het) breeding pairs. Single cell suspensions were prepared from bone marrow, blood, and uteri from pregnant dams of both genotypes. Using flow cytometry (FC), myeloid populations were identified and HO-1 expression levels were measured using specific antibodies. Cell proliferation rates were evaluated by in vivo BrdU pulse-labelling. Gene expression profiles of cytokines in WT and Het uteri were measured by PCR arrays and compared. Oxidative stress in individual cells was quantitated by FC measurements of total reactive oxidative species (ROS) and glutathione (GSH).
Results: Using FC, HO-1 was found predominantly expressed in circulating and local uterine myeloid cells, specifically neutrophils and monocytes/macrophages. Neutrophils and monocytes/macrophages were significantly reduced in pregnant Het uteri compared to pregnant WT uteri. In vivo BrdU assays showed that HO-1 deficiency did not affect cell proliferation or blood cell populations. Using PCR arrays, gene expression of cytokines (Csf1, Csf3), chemokines (Ccl1, Ccl2, Ccl6, Ccl8, Ccl11, Ccl12, Cxcl4, Cxcl9, Cxcl12), and their receptors (Ccr1, Ccr2, Ccr3, Ccr5) were significantly reduced in pregnant Het uteri compared to WT uteri. Moreover, after CSF1R and CCR2 receptor expression on myeloid cells of blood and uteri from pregnant and non-pregnant mice were characterized, we found that a deficiency in HO-1 significantly reduced CCR2 expression in infiltrating uterine monocytes/macrophages and dendritic cells. In addition, myeloid ROS and GSH levels were significantly higher in Het uteri.

Conclusion: These data reveal that HO-1 regulates not only cytokine/chemokine production in pregnant uteri, but also myeloid receptor number and cellular oxidative stress levels, suggesting a role of HO-1 in the recruitment of myeloid cells during pregnancy.

CD163 has Distinct Temporal Influences on Hemorrhagic Stroke Outcomes

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Background and Purpose—Extracorpuscular hemoglobin (Hb)-induced toxicity following intracerebral hemorrhage (ICH) is an important contributor to secondary brain damage and poor outcomes. Knowing that CD163 is a Hb scavenger receptor that has potent anti-inflammatory effects and CD163-positive macrophages/microglia accumulate in the brain with time post-ICH, it is of interest that there are no studies investigating the role of CD163 after ICH.
Methods—ICH was experimentally induced in wildtype and CD163-/- mice and various anatomical and functional outcomes were temporally assessed by histology and neurobehavioral testing.
Results—Acutely (72h), CD163-/- mice have 33.2±4.5% ($p<0.0001$), 43.4±5.0% ($p=0.0002$), and 34.8±3.4% ($p=0.0003$) less lesion volumes, hematoma volumes, and tissue injury, respectively. Whereas, at 10d, CD163-/- mice have 49.2±15.0% larger lesion volumes ($p=0.0385$). Temporal data examination revealed an inflection point at 4d post-ICH, where CD163-/- mice perform significantly better on neurobehavioral testing and have less mortality before 4d, but increased mortality and worse functional outcomes after 4d ($p<0.05$). Immunohistochemical staining at 72h shows that CD163-/- mice have significantly less Hb, iron, and blood brain barrier dysfunction, increased astrogliosis and cortical neovascularization, and no change in heme oxygenase 1 expression. At 10d, CD163-/- mice have increased iron and hematomal VEGF immunoreactivity, no change in heme oxygenase 1 expression, and decreased astrogliosis.
Conclusion—These novel findings reveal that CD163 has distinct temporal influences on ICH outcomes, with early injurious properties but delayed beneficial effects. The results are consistent with a primary anti-inflammatory role for CD163 after ICH, rather than Hb clearance per se, although the two are uniquely intertwined. CD163 may represent a key targetable immunomodulatory receptor after ICH.

Carbon Monoxide Promotes Inter-Organelle Communication through the Activation of TFEB/3 and PGC-1a

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Membrane-bound organelles are specialized biological and biochemical functional units within highly compartmentalized eukaryotic cell. Theses organelles are often further partitioned into subdomains, thus providing a mechanism to segregate specific processes into different regions within the same organelle. Although this segregation is necessary for separating potentially incompatible functionalities, integration of cellular function depends upon efficient cross--talk between multiple of organelles. Such inter-organelle communication (IOC) can be performed most frequently by a bioactive molecule such as carbon monoxide (CO) formed by one organelle and effective to the other organelle. But the strongest IOC might be the direct physical contact between organellar membranes such as mitochondria-associated membranes (MAMs) between mitochondria (Mt) and endoplasmic reticulum (ER). Recently we observed that CO could be formed by PERK-induced heme oxygenase (HO-1) by the stimulation of the PERK with exogenous CO-donor, CORM. More recently, we found that HO-1/CO system mediates ER stress (ERS)-induced promotion of adaptive mitochondrial responses (increase on ATP production and mitochondrial biogenesis). In this study, we present several IOCs

which are dependent on various major transcription factors. In addition to the IOC between ER and Mt, we observed another IOC between ER and lysosome, which is mediated by CO-PERK-TFEB/3 pathway. We also found that PGC-1α, FGF21 and sestrin-2 could be activated by exogenous and endogenous CO. Some other examples of IOC in addition to CO-PERK-NRF1 and CO-PERK-TFEB/3 pathways will be presented in the posters.
In conclusion, cross-talks between ER and other organelles including Mt and lysosome emerge as crucial IOC for the system HO-1/CO, through which cellular homeostasis and survival are modulated.

A central stage for heme catabolism in tissue damage control

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Host protection from infection relies on a variety of immune-driven resistance mechanisms that sense and target pathogens for containment, destruction or expulsion. As a trade off however, this evolutionary conserved defense strategy can cause dysfunction and damage to host parenchyma tissues, i.e. immunopathology. Therefore, immune--driven resistance mechanisms must be coupled to tissue damage control mechanisms that limit the extent of dysfunction and damage imposed to host parenchyma tissues during infection. These tissue damage control mechanisms are regulated by a number of evolutionary conserved stress and damage responses. These drive the expression of effector genes protecting parenchyma tissues against different forms of stress and damage associated with infection. Induction of heme oxygenase-1 (HO-1) and ferritin expression, by these stress responses, is central to confer tissue damage control, limiting the severity of infectious diseases. This evolutionary conserved defense strategy, which does not carry a direct negative impact on pathogens, is referred to as disease tolerance. Here I will discuss our recent body of work towards revealing the mechanisms via which heme detoxification by the HO-1/ferritin pathway contributes to the establishment of disease tolerance to infection.

Heme modulates innate immune receptor signaling dependently of ROS and Syk

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In the past years our group made the original discovery that heme activates innate immune receptors, including TLR4 and NLRP3, and potentiates the cytokine production induced by microbial molecules dependently of Syk. Interestingly, the mechanisms and consequences of heme-induced activation differ from the prototypic activators of these receptors but the reasons are not well understood. Our current study presents evidences implicating the generation of reactive oxygen species (ROS) by heme, including mitochondrial ROS, as an essential step to macrophage activation and TLR4 signaling. Mitochondrial ROS contribute to heme-induced TNF, selectively inducing phosphorylation of ERK1/2. Although ROS affect TLR4 signaling, generation of ROS, including mtROS, by heme-stimulated macrophages occurs independently of TLR4. This finding is in contrast to the requirement of TLR4 to LPS-induced ROS generation. Lack of TLR4 and TNFR1 or the use of a mitochondrial-target antioxidant are protective in a model of hemolysis. We also observed that Syk is essential to the activation of TLR4 and NLRP3 signaling pathways triggered by heme through modulation of ROS production. These observations support the contention that targeting these pathways might be beneficial in the treatment of hemolytic disorders.

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Cell-Type Specific Down-Regulation of Heme Oxygenase-1 by Lipopolysaccharide via Bach1 in Primary Human Mononuclear Cells

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Heme oxygenase (HO)-1 is the inducible isoform of heme degradation, which is up-regulated by multiple stress stimuli. HO-1 has major immunomodulatory and anti-inflammatory effects via its functions in mononuclear cells. Contradictory findings have previously been reported on HO-1 gene regulation by the toll-like receptor (TLR)4 ligand lipopolysaccharide (LPS) in mononuclear cells from different species. Therefore, we re-investigated the effect of LPS on HO-1 gene expression in various types of murine and human mononuclear cells in vitro and in vivo. Remarkably, LPS up-regulated HO-1 mRNA levels in primary murine macrophages and human monocytic leukemia cell lines, but down-regulated HO-1 mRNA levels in primary human peripheral blood mononuclear cells (PBMCs), CD14+ monocytes, macrophages, dendritic cells and granulocytes. Furthermore, experiments with human CD14+ monocytes revealed that activation of other TLRs including TLR1, -2, -5, -6, -8 and -9 down-regulated HO-1 mRNA expression. LPS-dependent down--regulation of HO-1 expression was specific, because other typical LPS-inducible genes such as NADP(H)--quinone-oxidoreductase-1, peroxiredoxin-1 and cyclooxygenase-2 were up-regulated under the same experimental conditions. Notably, LPS induced expression levels of Bach1, a critical transcriptional repressor of HO-1. Moreover, knockdown of this nuclear factor enhanced basal and LPS-dependent HO-1 expression in mononuclear cells. Finally, down-regulation of HO-1 in response to LPS was confirmed in PBMCs obtained from human individuals subjected to experimental endotoxemia. In summary, LPS down-regulates HO-1 expression in primary human mononuclear cells via a Bach1-mediated pathway. As LPS-dependent HO-1 gene expression in human and mouse macrophages exhibits an opposing regulatory pattern, the findings may suggest an important species-specific difference of myeloid HO-1 function in inflammatory responses.

Kidney proximal tubular epithelial cells control disease tolerance to malaria by maintaining heme/iron homeostasis

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Disease tolerance is an evolutionarily conserved defense strategy against infection that preserves the functional output of host parenchyma tissues without interfering directly with pathogens. Host genes controlling iron-heme metabolism are essential to the establishment of disease tolerance to malaria, the disease caused by Plasmodium infection. This protective strategy relies on several mechanisms limiting the cytotoxic effects of labile iron-heme. Here we addressed further the mechanism via which adaptive responses regulating iron-heme metabolism preserve the functional output of parenchyma tissues and confer disease tolerance to malaria. We found that, as it accumulates in plasma during Plasmodium infection, labile heme is excreted through the kidneys via a mechanism that is not regulated by the plasma

hemoglobin or heme scavengers, haptoglobin (HP) and/or hemopexin (HX), respectively. As it accumulates in urine, labile heme is probably taken up by kidney proximal tubular epithelial cells, where it is catabolized by heme oxygenase 1 (HO-1, encoded by the Hmox1 gene). Deletion of the Hmox1 allele in kidney proximal tubular epithelial cells results in overt kidney damage and compromises survival to Plasmodium infection, without interfering with the host pathogen load. In a similar manner, deletion of the ferrtin H chain (FtH) allele in kidney proximal tubular epithelial cells also results in overt kidney damage and compromises survival to Plasmodium infection without interfering with the host pathogen load. This suggests that heme catabolism by HO-1 and storage of the resulting labile iron in kidney proximal tubular epithelial cells is required to prevent the development of acute kidney injury during Plasmodium infection, a hallmark of severe malaria. In conclusion, we reveal that disease tolerance to malaria relies on a tissue damage control mechanism that operates specifically in kidney proximal tubular epithelial cells and that maintains heme/iron homeostasis during Plasmodium infection.

Sensitive LC-MS/MS Assay for the Simultaneous Detection of Heme, Biliverdin and Bilirubin in Complex Biological Samples

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A current limitation to the heme oxygenase (Hmox) field is the low sensitivity and specificity of methods used to determine both Hmox activity as well as endogenous concentrations of heme, biliverdin and bilirubin in complex biological systems. Because Hmox activity does not necessarily correlate with Hmox mRNA or protein content, a reliable assay is needed to determine Hmox activity. Spectrophotometric measurement of the bile pigments has limited specificity and requires a relatively large amount of sample. Here, we describe the development and application of a sensitive and specific method to simultaneously detect heme, biliverdin and bilirubin by liquid chromatography with tandem mass spectrometry (LC-MS/MS). The method detects these metabolites with detection limits of 250 attomoles on column for heme and biliverdin, and 500 attomoles for bilirubin. n-Methyl protoporphyrin IX, meso-biliverdin and meso-bilirubin are used as internal standards for heme, biliverdin and bilirubin, respectively. Applying the method to purified Hmox1 protein and mutants, yeast cells, murine blastocysts (~2,000-3,000 cells per sample), blood plasma, and arterial lesions from mouse models of atherosclerosis (with 1 mg of tissue) afforded the specific detection of heme, biliverdin and bilirubin. We also successfully determined Hmox activity in lysates, nuclear or cytoplasmic fractions from hypoxia--treated vascular smooth muscle cells. Our results confirm the applicability of the LC-MS/MS method to quantify endogenous heme and bile pigments, and to assess Hmox activity in biological samples.

A Novel Measurement Method For Serum Unconjugated Bilirubin Using A Bilirubin-Inducible Fluorescent Protein From Eel Muscle

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Background: An increase in serum unconjugated bilirubin (indirect bilirubin) is associated with the development of brain injury in neonates. Current serum bilirubin measurements using the diazo method, bilirubin-oxidase method, or spectrophotometry do not directly measure unconjugated bilirubin, and have not been updated for several decades. Recently, a unique fluorescent protein from eel muscle, UnaG, was cloned and is characterized as having high affinity and specific binding to unconjugated bilirubin, and not conjugated bilirubin (direct bilirubin) (Cell, 2013).
Objective: To establish unconjugated bilirubin measurement using UnaG (UnaG method) in newborn sera.
Methods: For the UnaG method, we used a 200-µL reaction mixture solution containing 2 µM of UnaG (150 µL) and diluted artificial unconjugated bilirubin solutions or sera (50 µL). Fluorescence intensities were measure at 527 nm using a micro plate reader (SH-9000, Corona Electric, Ibaraki, Japan) and reached maximum levels at 10 min after mixing. First, various bilirubin concentrations, ranging from 0.0 to 66.8 mg/dL, using artificial bilirubin standard solutions (supplied by Arrows Co Ltd, Osaka, Japan) were measured and a standard curve created. Sera were then diluted 200-fold using phosphate buffer saline and fluorescence intensities were measured. Serum concentrations of unconjugated bilirubin were then extrapolated from the standard curve. Linear regression analysis was performed to compare unconjugated bilirubin concentrations using by UnaG method and by the conventional bilirubin-oxidase method (Unitika Co., Okazaki, Japan). Serum unconjugated bilirubin concentration by the bilirubin-oxidase method was calculated with the following formula: [unconjugated bilirubin] = [total bilirubin] – [conjugated bilirubin].
Results: A total of 140 serum samples obtained from 93 newborns with serum conjugated bilirubin concentrations < 1.0 mg/dl were analyzed. Unconjugated bilirubin concentrations measured by the UnaG and bilirubin oxidase methods were strongly correlated ($y = 1.01x + 0.17$, $r = 0.943$, $P < 0.001$). Unconjugated bilirubin concentrations were similar across 14 serum samples with conjugated bilirubin concentration ≥ 1.0 mg/dl as determined by the UnaG and bilirubin-oxidase methods ($P = 0.31$).
Conclusion: The UnaG method yielded results that were at least comparable to those obtained by the conventional bilirubin-oxidase method in newborn sera. Most importantly, this method can measure unconjugated bilirubin directly regardless of conjugated bilirubin concentrations.

Approaches to image vascular development and disease in the mouse

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Our laboratory aims to understand the morphogenetic mechanisms that shape the vasculature, in particular the lymphatic vessel system. In addition, we investigate pathological alterations that are caused by or result in malfunction of the blood and lymphatic vascular systems. However, a comprehensive understanding of the development, function and pathofunction of the vasculature requires imaging of its 3-dimensional structure with cellular resolution over time. In the recent past, the introduction of light sheet microscopy to reconstruct both the blood and lymphatic vessel systems in wholemount-stained mouse embryos and fetuses has greatly enhanced our understanding of the underlying developmental processes. In addition, there is a strong necessity for the development of chronic intravital imaging technologies that allow high resolution optical imaging in a living model organism over time. Towards this goal, we are applying optical windows for use in multiphoton laser scanning microscopy in conjunction with transgenic model systems that allow label-free visualization of vessels and their functional environment.
One aspect that recently occupied the centre of our attention is the function of hypoxia during development or as an integral component of disease. Hypoxia is an intensively investigated condition with profound effects on cell metabolism, migration and angiogenesis during development and disease. Physiologically, hypoxia is linked to tissue homeostasis and maintenance of pluripotency. Hypoxia also contributes to pathologies including cardiovascular diseases and cancer. Despite its importance, microscopic visualisation of hypoxia is largely restricted to the detection of reductively activated probes by immunostaining. Therefore, we developed a novel family of genetically encoded fluorescent sensors. These sensors detect activation of HIF transcription factors reported by the oxygen-independent fluorescent protein UnaG, isolated from Japanese eel. They comprise sensors with different switching and memory behavior and

combination sensors that allow distinction of hypoxic and reoxygenated cells. We tested these sensors on orthotopically transplanted glioma cell lines. Using a cranial window, we visualized hypoxia intravitally at cellular resolution. In tissue samples, sensor activity was detected in regions, which were largely devoid of blood vessels, correlated with HIF-1α stabilization and were highly heterogeneous at a cellular level. Frequently, we detected recently reoxygenated cells outside hypoxic areas in the proximity of blood vessels, suggestive of hypoxia-promoted cell migration (Erapaneedi *et al.* (2016) EMBO J 35:102-113).

A NOVEL METHOD FOR QUANTITATIVE DETERMINATION OF BILIRUBIN PHOTOISOMERS

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Neonatal hyperbilirubinemia is a common condition affecting more than 60 % of newborn babies. To prevent potential bilirubin neurotoxicity (which is generally associated with serum bilirubin concentrations above 340 µmol/L), the phototherapy is being used worldwide as a gold standard treatment. During phototherapy, bilirubin is transformed into more polar derivatives called bilirubin photoisomers (PI); EZ/ZE-bilirubins and Z-lumirubin being the major ones. Although phototherapy as a treatment option has been used for decades, the method for accurate and quantitative determination of bilirubin PI in the circulation is still lacking. The aim of our study was to optimize conditions for lumirubin preparation, and to establish a reliable analytical method for determination of bilirubin PI in the circulation.
Bilirubin PI were prepared from the stock solution of bilirubin using phototherapeutic device (TSE, Ostrava, Czech Republic). To optimize the yield of produced bilirubin PI, different conditions were tested (initial bilirubin concentration, type of albumin used to dissolve bilirubin, light intensity and the length of light exposure). Lumirubin produced was isolated using TLC, to be used as a calibration standard for HPLC analysis, which was based on a modified method by McDonagh (1982). Mobile phase consisted of di-n-octylamine acetate in methanol with 10% of H2O (0.1 mol/L), the stationary phase was Poroshell 120, SB-C18 column (4.6 x 100 mm, 2.7 µm; Agilent, CA, USA). The signal was recorded at 453 nm, bilirubin dimethylester was used as an internal standard. The method was tested on sera of hyperbilirubinemic neonates treated with phototherapy.
The highest yield of lumirubin without interference with other products was reached when bilirubin was dissolved in rabbit serum albumin to get the final concentration of 500 µmol/L, and photo-irradiated by the light of intensity 70 µW/cm2/nm for 30 minutes. Prepared lumirubin was isolated by TLC and used for calibration of the HPLC method. The method enabled to separate EZ/ZE-bilirubin, Z-lumirubin, as well as unconjugated bilirubin, and was linear in the concentration range 5-150 µmol/l. Testing of samples of human neonates treated with phototherapy revealed that major bilirubin PI represented not more than 15 % of initial bilirubin concentration after discharged for phototherapy, predominantly being represented by ZE/EZ-bilirubin, while Z-lumirubin concentrations were less than 5 µmol/l.
In conclusion, we have optimized the method for quantitative determination of bilirubin PI in human serum samples. It is expected that use of this method might help to improve the care of neonates suffering from severe neonatal jaundice.
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Beneficial role of CO in liver pathologies

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Carbon monoxide (CO) is a small gas molecule with a strong affinity to a heme moiety of various heme proteins. Its bond to hemoglobin leads to a formation of carbonylhemoglobin (COHb) which could result in a disturbance of oxygen delivery, relative functional anemia and tissue toxicity. On the other hand, in trace amounts, CO can modulate biological activity of some heme proteins and trigger signaling cascades exerting anti-inflammatory, anti-apoptotic and anti-proliferative effects in multiple organ systems including the liver.
The liver is an organ rich in heme proteins and with a relatively high heme oxygenase activity, an endogenous source of CO. CO appears to be important in regulating hepatic perfusion, bile flow and immune response. Its hepatoprotective effects have been described in liver transplantation and I/R injury after perfusion with exogenous CO. Furthermore, CO has an important role in modulating intrahepatic vascular resistance and thus the severity of portal hypertension and may be closely related to the hyperdynamic circulatory state in cirrhosis. Its anti-inflammatory properties participate in a protection against LPS or GalN/LPS induced liver injury.
CO may have a beneficial effect on different types of cholestatic diseases through limiting the contractility of bile canaliculi, stimulating bile flow and modulating the expression of liver transporters. We have demonstrated that administration of heme, the source of CO in organism, increased bile flow by the induction of hepatocyte transporters expression in the model of ethinylestradiol-induced cholestasis in rats. Moreover, it stimulated liver Multidrug resistance protein 3 expression *via* a Nrf2-dependent mechanism transporting bile acids from cholestatic liver into plasma and supporting their clearance into the urine.
Based on the persuasive experimetal data, CO is now in a spotlight as a potential therapeutic agent. CO can be delivered into organism by inhalation or in the form of various CO-releasing molecules. However, in designing the experiments, tissue specific distribution and elimination of CO as well as a balance between beneficial and toxic effects should be taken in account. We have shown that CO half-life in the rat liver after CO inhalation was 0.6±0.3h affecting early phase inflammatory response in the model of endotoxin-induced liver injury.
In conclusion, CO is a molecule with a great hepatoprotective potential, however, research on CO kinetics, studies on CO signaling and development of new CO releasing molecules remain major challenges for further successful clinical development.

HMOX1 and Macrophages in Cardiac Homeostasis and Repair Following Myocardial Infarction

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Upon myocardial infarction (MI) immune system becomes activated by extensive necrosis of cardiomyocytes. Overactive and prolonged immune responses can be responsible for heart failure in patients surviving the ischemic episode. Cardioprotective effects of heme oxygenase-1 (HMOX1) were previously demonstrated in experimental models of MI. Nevertheless, its importance in suppression of post-ischemic inflammation remains incompletely understood.
The aim of this study was to investigate the role of HMOX1 in the monocyte/macrophage-mediated late acute and subacute phases of inflammation in a mouse model of MI.
HMOX1 knockout (Hmox1-/-) and wild type (Hmox1+/+) mice were subjected to a permanent ligation of the left anterior descending (LAD) coronary artery. Shortly after MI WT mice responded with a strong up-regulation of cardiac

Hmox1 expression, which decreased with time. During the 3-week follow-up a potent deterioration of heart function and impaired post-MI recovery in Hmox1-/- mice was accompanied by higher numbers of classical inflammatory Ly6C(hi) monocytes in the peripheral blood and upregulation of monocyte chemoattractant protein-1 (MCP-1) in the heart. These post-MI changes in the absence of HMOX1 were associated with increased abundance of pro-inflammatory MHCII+Ly6C+CD11c+ and MHCII++Ly6C+CD11c+ cardiac macrophages. Moreover, Hmox1-/- bone marrow (BM) transplantation to Hmox1+/+ recipients revealed that HMOX1 deprivation restricted to BM-derived blood cells does not, per se, impair post-ischemic recovery.
HMOX1 provides a timely resolution of inflammation after MI by restricting monocytopoiesis, inflammatory monocytes recruitment to the heart and macrophage-mediated cardiac remodeling. This has a direct impact on infarct expansion and heart function.

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"Heme oxygenase-1 nuclear translocation regulates bortezomib induced cytotoxicity in myeloma cells"

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Multiple myeloma (MM) is a clonal B-cell malignancy characterized by an accumulation of clonal plasma cells in the bone marrow leading to bone destruction and bone marrow failure. Several molecular mechanisms underlie chemoresistance among which heme oxygenase-1 (HO-1) could play a major role. We evaluated the impact of HO-1 in MM following bortezomib (BTZ) treatment, which represents the first-line therapy in the treatment of patients with MM. We measured cell viability, reactive oxygen species (ROS) formation, endoplasmic reticulum (ER) stress, HO-1 expression and compartmentalization and cellular genetic instability. Results showed that BTZ significantly reduced cell viability in different MM cell lines and induced ER-stress and ROS formation. Concomitantly, we observed a significant overexpression of both HO-1 gene and protein levels. Surprisingly, inhibition of HO activity with SnMP failed to increase BTZ sensitivity in MM cells whereas inhibition of HO-1 nuclear translocation by E64d, a cysteine protease inhibitor, increased sensitivity to BTZ and decreased genetic instability as measured by cytokinesis-block micronucleus assay. In conclusion, although the molecular mechanism and the pathophysiological significance of HO-1 nuclear localization during cancer progression remain elusive, our data on MM cells suggest that BTZ sensitivity depends on HO-1 nuclear compartmentalization and not on its enzymatic activity.

Metabolic Effects of Bilirubin: Key Findings from Animal Models

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Circulating concentrations of bilirubin, a terminal product of haem catabolism and potent scavenger of reactive oxygen and nitrogen species, is clearly associated with protection from cardiovascular and metabolic disease. Our current understanding of the protective mechanisms induced by bilirubin, however, remains limited and requires further interrogation. This presentation aims to demonstrate that bilirubin (or its altered metabolism) profoundly influences hepatic and whole body metabolism, lowering circulating cholesterol concentrations and reducing fat mass in hyperbilirubinaemic rodents. Bilirubin may induce these effects by influencing transcriptional regulation/gene expression,

evidenced in the heart and liver of Gunn rats. Considering bilirubin's potent radical scavenging ability, recent evidence suggests that it reduces mitochondrial ROS production and potentially influences cellular red-ox signalling and age associated metabolic deterioration. Furthermore, new molecular targets of bilirubin have recently been identified, including PPARs, indicating that bilirubin may also influence cellular function independent of the Aryl Hydrocarbon Receptor. These data provide new insight into the possible physiological importance of bilirubin, which could be harnessed to resist age associated deterioration of cell function and potentially extend the life-span.

Neuronal Injury After Subarachnoid Hemorrhage Is Determined by a Carbon Monoxide Sensing Change in Circadian Rhythm

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Abstract
Background: Subarachnoid hemorrhage (SAH) is associated with a temporal pattern of stroke incidence. We have shown that HO-1 and CO protect against SAH-induced brain injury. We hypothesized that natural oscillations in gene expression controlling circadian rhythm dictate the severity of neuronal injury. Moreover, that the protective gene heme oxygenase-1 (HO-1/*Hmox1*) and its product carbon monoxide (CO) contribute to resolution and restoration of rhythm.
Methods: Murine SAH model where homologous blood was injected at various time points of the circadian cycle. Readouts included 'clock gene' expression in the brain by qPCR, locomotor activity, vasospasm, neuroinflammatory markers and apoptosis. Additionally, cerebrospinal fluid (CSF) and peripheral blood leukocytes from human SAH patients and controls were analyzed for 'clock gene' expression.
Results: Significant elevations in the 'clock genes', *Per-1*, *Per-2* and *NPAS-2* were observed in the hippocampus (HC) and suprachiasmatic nucleus (SCN) in animals subjected to SAH at "zeitgeber" time ZT12 compared to ZT2. ZT12 animals showed a significant reduction in cerebral vasospasm, neuronal apoptosis and microglia activation. In animals with microglia-specific HO-1 deficiency (*Lyz-Cre-Hmox1$^{fl/fl}$*) the ZT12 elevations in *Per-1*, *Per-2* and *NPAS-2* expression were suppressed in the SCN, which correlated with increased injury severity. Treatment with a low dose of CO rescued the *Lyz-Cre-Hmox1$^{fl/fl}$* mice, restored the ZT12 increase in *Per-1*, *Per-2 and NPAS-2* expression and reduced neuronal apoptosis.
Conclusions: We demonstrate that 'clock gene' expression regulates the severity of SAH and in part requires microglial HO-1 activity likely due to clearance of erythrocytes. Exposure to CO can rescue the loss of HO-1 and warrants further clinical investigation in patients with SAH.

Investigations of Heme Oxygenase 1 and its Inhibitors in β-Thalassemia

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Thalassemias are a heterogeneous group of red blood cell disorders ranging from a clinically severe phenotype requiring lifesaving transfusions (thalassemia major) to a relatively moderate symptomatic disorder, sometimes requiring

transfusions (thalassemia intermedia). Though considered a major cause of morbidity and mortality worldwide, there is still no universally available cure for thalassemia major. The reason for this is due to, at least in part, the lack of full understanding of pathophysiology of thalassemia. The underlying basis of thalassemia pathology is the premature apoptotic destruction of erythroblasts causing ineffective erythropoeisis. In β-thalassemia, β-globin synthesis is diminished causing α-globin accumulation. Unpaired globin chains that accumulate in thalassemic erythroblasts are bound to heme. In addition, in β-thalassemia an erythroid specific protease destroys excess α globin chains, likely leading to the generation of a pool of "free" heme in erythroblasts.

"Unshielded" heme is toxic, but this toxicity will likely be augmented, if heme oxygenase 1 (HO1) can release iron from heme. Until recently, virtually no information about the expression of HO1 in erythroblasts has been produced. However, we have lately provided unequivocal evidence that this enzyme is present in erythroid cells[1]. Based on this novel and important finding, we hypothesize that in β-thalassemic erythroblasts HO-mediated release of iron from heme is the major culprit responsible for the damage of developing erythroid cells.

To test this hypothesis, we exploited the mouse model of β-thalassemia known as th3/th3. Our data indicates that HO1 expression is increased in the liver of β-thalassemic mice compared to wild type mice. Importantly, we observed that erythropoietin-mediated induction of erythroid differentiation of fetal liver (FL) cells isolated from β-thalassemic fetuses causes an increase in HO1 mRNA and protein levels. Our finding of increased ferritin levels in β-thalassemic FL cells indicates enhanced heme catabolism and consequent iron release from heme. To investigate the contribution of HO 1 to the pathology associated with β-thalassemia, wild type and thalassemic (th3/+) mice were injected with 40 µmole/kg/d of tin-protoporphyrin IX (SnPP, HO1 inhibitor) during a 4 week period, 3 times a week. We found that when compared to PBS injected β-thalassemic mice, β-thalassemic mice injected with SnPP display an increase in hemoglobin levels, red blood cell counts, reticulocyte counts and a decrease in liver iron content.

Our results indicate that β-thalassemic erythroblasts have high levels of HO1, which predictably degrades "free" heme. Further research is needed to determine whether iron liberated from heme by HO1 is directly responsible for the damage of β-thalassemic erythroblasts.

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Heme Oxygenase-1 Inhibits HLA class I Antibody-Dependent Endothelial Cell Activation

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Antibody-mediated rejection is a key limiting factor for long-term graft survival in solid organ transplantation. HLA class I (HLA I) antibodies (Abs) play a major role in the pathogenesis of antibody-mediated rejection via their interactions with HLA molecules on vascular endothelial cells (ECs). The antioxidant enzyme heme oxygenase (HO)-1 has anti-inflammatory functions in the endothelium. As complement-independent effects of HLA I Abs can activate ECs, it was the goal of the current study to investigate the role of HO-1 on activation of human ECs by HLA I Abs. In cell cultures of various primary human macro- and microvascular ECs treatment with monoclonal pan- and allele-specific HLA I Abs up-regulated the expression of inducible proinflammatory adhesion molecules and chemokines (VCAM-1, ICAM-1, interleukin-8 and MCP-1). Pharmacological induction of HO-1 with cobalt-protoporphyrin IX reduced, whereas inhibition of HO-1 with either zinc-protoporphyrin IX or siRNA-mediated knockdown increased HLA I Ab-dependent

up-regulation of VCAM-1. Treatment with two carbon monoxide (CO)-releasing molecules, which liberate the gaseous HO product CO, blocked HLA I Ab-dependent EC activation. Finally, in an in vitro adhesion assay exposure of ECs to HLA I Abs led to increased monocyte binding, which was counteracted by up-regulation of HO 1. In conclusion, HLA I Ab-dependent EC activation is modulated by endothelial HO-1 and targeted induction of this enzyme may be a novel therapeutic approach for the treatment of antibody-mediated rejection in solid organ transplantation.

Taurine chloramine exerts anti-inflammatory and proresolving effects through induction of heme oxygenase-1 expression

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Resolution of inflammation is an important process in the host defence against infection and subsequent tissue damage. Effective clearance of apoptotic neutrophils by macrophages, termed efferocytosis, is an essential process in the resolution of inflammation. Taurine is one of the most abundant free amino acids in the cell. It is abundant in some foods, such as fish, meat, egg, cheese, milk, etc. The stored taurine reacts stoichiometrically with hypochlorous acid (HOCl), a highly toxic antibacterial oxidant produced from H_2O_2 by the myeloperoxidase activity of the activated neutrophils in the presence of chloride ion. This results in the generation of taurine chloramine (TauCl). The concentration of TauCl is markedly elevated in neutrophils when subjected to oxidative burst. Once the activated neutrophils undergo apoptosis, TauCl is then released and acts as a local autacoid in the inflamed tissues. We have previously reported that TauCl, when injected into the peritoneum of mice, stimulates the resolution of zymosan A-induced peritonitis. Furthermore, when the macrophages obtained from peritoneal exudates were treated with TauCl, their efferocytic ability was elevated. In the murine macrophage-like RAW264.7 cells exposed to TauCl, the proportion of macrophages engulfing the apoptotic neutrophils was also increased. In these macrophages treated with TauCl, expression of heme oxygenase-1 (HO-1) was elevated. TauCl, when treated to peritoneal macrophages isolated from HO-1 knockout mice, failed to induce efferocytosis. In a subsequent study, intraperitoneal administration of TauCl protected against inflammatory tissue damage in mice infected with *Candida albicans* by stimulating the fungal phagocytic activity of macrophages. We found that the enhanced phagocytic activity of macrophages in TauCl-treated mice was mediated by carbon monoxide (CO), a byproduct of the HO-1-catalyzed reaction. Dectin-1 plays a significant role in cellular signaling involved in phagocytosis and inflammatory response through interaction with a ligand, β-glucan fungal surface carbohydrate. The CO-releasing molecule potentiated the phagocytic activity of macrophages by inducing dectin-1 expression in macrophages. In conclusion, TauCl potentiates phagocytic activity of pathogen-engulfing macrophages through upregulation of HO-1 expression and subsequent CO production.

Silymarin Uncovered: Molecules or a "Quack Remedy".

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Silymarin (flavonoid extract from milk thistle fruits - *Silybum marianum*) has been traditionally used in various medicinal application since ancient times [1]. Long-term application proved this extract to be efficient and completely safe having no or very low toxicity even at the high and prolonged dosage. Number of controversies often arises mostly due to non-standard composition of this phytopreparation, use of various undefined mixtures, mismatching silymarin *vs.* silybin, and also due to ignoring the chemistry of respective components of silymarin [2].
Major components of silymarin are flavonolignans silybin (**1**), isosilybin, silychristin, silydianin, and 2,3-dehydrosilybin. All these compounds (besides silydianin) exist in the nature in the form of two diastereomers. Both the extract (silymarin) and respective isolated flavonoids have broad spectrum of biological activities operating at various cell levels [1]. Silybin is mainly used in the prevention and treatment of various liver diseases and as a protectant against a number of hepatotoxins and mycotoxins. Moreover, both silybin and 2,3-dehydrosilybin have been identified as rather effective natural compounds in the prevention and treatment of some types of cancer (e.g. prostate cancer). Natural silybin is an equimolar mixture of two stereomers silybin A (**1**) and silybin B (**2**), which are inseparable at the preparatory scale. We have developed an enzymatic separation of both diastereomers at the preparatory scale [3]. Biological activity of both diastereomers differs substantially.
We will demonstrate in this presentation that single components of silymarin have profoundly different biological activities and that it is absolutely crucial to work with "molecules" to disclose respective "molecular" effects. We shall demonstrate these effects e.g. on the interactions with AhR receptors and also in modulation of serum concentrations of bilirubin *via* modulation of heme oxygenase (HMOX) and bilirubin UDP-glucuronosyl transferase (UGT1A1).

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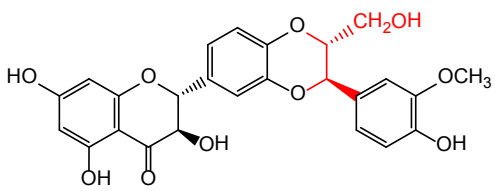
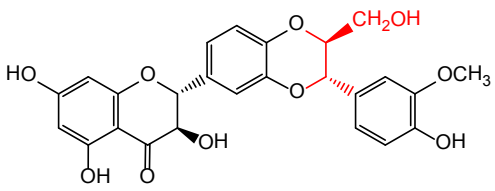


1 Silybin A (2*R*, 3*R*, 10R, 11*R*)



2 Silybin B (2*R*, 3*R*, 10*S*, 11*S*)

Advances in the design of pharmacological agents targeting HO-1/CO

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The transcription factor Nrf2 and its downstream target heme oxygenase-1 (HO-1) are essential protective systems against oxidative stress and inflammation. The products of HO-1 enzymatic activity, biliverdin and carbon monoxide (CO), actively contribute to this protection suggesting that activation of the Nrf2/HO-1 axis can be exploited for therapeutic applications in a variety of diseases. We have recently developed a new class of hybrid molecules, termed HYCOs, that activate Nrf2/HO-1 and simultaneously release CO (1,2). Based on these recent published findings, we present here data on HYCO-3, a compound obtained by coordinating the Nrf2 activator ethyl fumaric ester to a newly characterized CO-releasing molecule (CORM-401). In BV2 microglia cells, HYCO-3 (1-10 μM) increased Nrf2/HO-1 expression, delivered detectable amounts of CO intracellularly and inhibited nitrite production induced by lipopolysaccharide (LPS). Similarly, using a model of LPS-induced inflammation in mice, we found that oral administration of HYCO-3 (40 mg/kg) increases the expression of HO-1 in various organs as well as the amount of CO-hemoglobin in the blood circulation while markedly attenuating the pro-inflammatory response. We conclude that molecules with dual activity that simultaneously target Nrf2 and release CO are pharmacologically active and represent a feasible strategic approach to maximize the therapeutic actions of HO-1 against inflammatory disorders.

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H_2S in homeostasis and disease: paradigms from the cardiovascular system

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Following the discovery that mammalian cells are capable of producing H_2S, this molecule has undergone a dramatic metamorphosis from a dangerous pollutant to a biologically relevant molecule. H_2S is now considered to be the newest member of the gasotransmitter family that also includes nitric oxide and cardon monoxide, and is recognized as an endogenous mediator with important roles in homeostasis, physiology and disease. H_2S is generated through the action of three enzymes, namely cystathionine-β synthase (CBS), cystathionine-γlyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST). All three enzymes have been shown to be present in the vessel wall and in the heart. H_2S controls fundamental mammalian cellular responses, including growth, differentiation, migration and cell death. Reduced H_2S levels have been documented in several conditions associated with endothelial dysfunction including hypertension, atherosclerosis, preeclampsia, diabetic vascular complications and heart failure. Administration of H_2S ameliorates all of these diseases and conditions. We will review evidence regarding the signaling pathways mediating the biological activities of H_2S and present examples of therapeutic applications of H_2S donors.

Gaseous transmitters (NO, CO, H_2S) in cancer: pathways and interactions

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The gasotransmitters hydrogen sulfide (H_2S), nitric oxide (NO), and carbon monoxide (CO) exhibit different pharmacological effects at low and high concentrations. The role of the three gasotransmitters on cancer cell proliferation has not yet been studied simultaneously. First, we quantified the expression of NO, CO and H_2S-generating enzymes in primary colon cancer tissues and in HCT116 colon cancer cells. There was an increased expression of the NO synthases eNOS, iNOS, and nNOS in colon cancer tissue (compared to surrounding normal tissue) and in HCT116 tumor cells (compared to the non-transformed cell line NCM356). Moreover, there was an increase in the H_2S-producing enzymes CBS and CSE as well as an upregulation of HO-1. Next, we evaluated the effect of donation or inhibition of the biosynthesis of each of the three gasotransmitters on the proliferation of HCT116 cells. DETA and SNAP were used for NO donation, CORM3 for CO donation and AP39 for H_2S donation; L-NMA was used to inhibit NO biosynthesis, zinc protoporphyrin to inhibit CO biosynthesis and aminooxyacetic acid to inhibit H_2S biosynthesis. All donors produced a bell-shaped response: enhancement of HCT116 proliferation at low concentrations and inhibition at higher concentrations. Inhibition of NO, CO or H_2S biosynthesis all suppressed cell proliferation. Combination of different donors or combination of different inhibitors did not produce marked additive or synergistic effects. We conclude that each of the three gasotransmitters exert bell-shaped pharmacological effects in the control of tumor cell proliferation and their effects converge on similar or overlapping effector pathways.

Controlled Oral Delivery of Therapeutic Gases – Local Carbon Monoxide for Ulcerative Colitis

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Carbon monoxide (CO) is an endogenous signal transmitter involved in numerous physiological systems including the gastrointestinal (GI) homeostasis. A therapeutic approach using CO however is challenged by inappropriate delivery modes. Here, we describe a micro scale Oral Carbon Monoxide Release System (M-OCORS) functional for local and controlled GI delivery of CO (**Figure 1**). With the overall identical set-up CO release from the M-OCORS was tailorable from several minutes to almost one day. Likewise, after the oral application in mice the *in-vivo* carboxyhemoglobin formation over time was tailorable as a function of the *in-vitro* release. Designed for intestinal delivery an extended release formulation of M-OCORS proved efficacy in 2,4,6-trinitrobenzenesulfonic acid (TNBS) induced colitis in mice. Addressing the safety profile of the ruthenium based OCORS concept we propose a modification by switching to the iron based Esterase-Triggered OCORS (E-OCORS).

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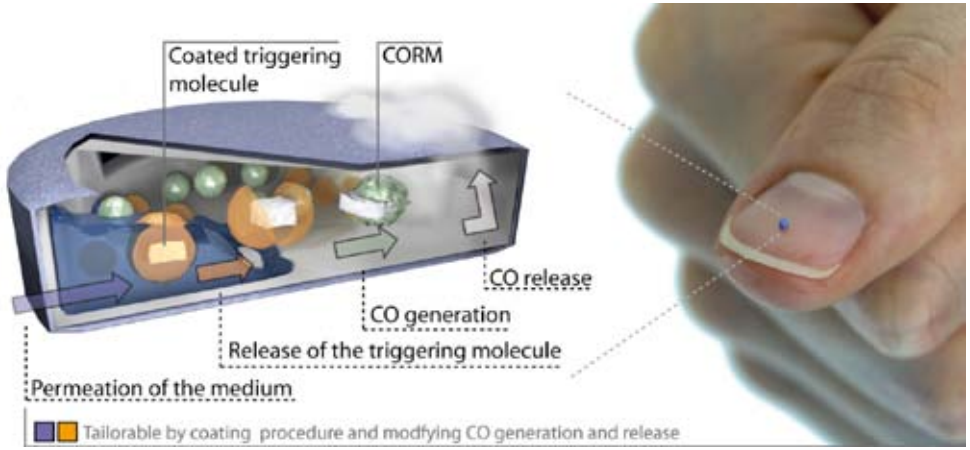

Figure 1: Functional principle of the Oral Carbon Monoxide Release System (OCORS) and the micro-scale OCORS (M-OCORS) in comparison to a thumbnail. The CO release rate of OCORS is tailorable as a function of the hydrophobicity and nature of coatings controlling two consecutive rate controlling steps ultimately contacting the Carbon Monoxide Releasing Molecule (CORM) and the triggering molecule thereby generating CO. In a first step the permeation of the medium (e.g. intestinal fluid) into M-OCORS is controlled by the type and hydrophobicity of the cellulose acetate shell (purple). In a second step a coating around the triggering molecule (orange) controls the reaction between CORM and the triggering molecule. CORM-2 and sodium sulfite were used as CORM and release trigger for M-OCORS, accordingly.

Neuroprotective Mechanisms of Carbon Monoxide and Heme Oxygenases on Stroke Outcomes

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Carbon monoxide (CO) is a gaseous second messenger produced endogenously when heme oxygenase (HO) enzymes catabolize heme. We have previously documented that the constitutive HO2 would be consistently neuroprotective in ischemic stroke paradigms while HO1 would dictate various ischemic and hemorrhagic outcomes. We have demonstrated that CO can be therapeutic in ischemia-reperfusion brain injury and showed that 250ppm CO exposure provided better neuroprotection than 125 or 500ppm. However, it was unclear whether CO can also offer protection in permanent ischemic stroke or what mechanism underlies the effect. The HO1 neuroprotection is shown to be regulated by Nrf2; therefore, we investigated whether CO might partially exert neuroprotection by modulating the Nrf2 pathway.
To evaluate potential protective effects of CO, we exposed C57BL/6 wildtype and Nrf2-/- mice to 250ppm CO or control air immediately after permanent middle cerebral artery occlusion. Infarct volume and neurological deficits were assessed on day 7. Molecular mechanisms of Nrf2 pathway activation by CO were also investigated. Mice exposed to CO after permanent ischemia had significantly reduced infarct size from 25.2±4.8% (air) to 16.4±3.7% (CO) at 7d. Additionally, CO treatment led to Nrf2 dissociation from Keap1, nuclear translocation, increased binding activity of Nrf2 to HO1 antioxidant response elements, and elevated HO1 expression 6-48h after CO exposure. We also investigated the expression of HO1 and HO2 after CO exposure and found that HO1 expression gradually increased from 6 to 48 hours after CO exposure; in contrast, CO had no effect on HO2 expression. The overall CO neuroprotection was essentially completely abolished in Nrf2-/- mice. Low-concentration of exogenous CO represents a neuroprotective agent for stroke combination treatment and its beneficial effect would be at least partially mediated by activation of the endogenous Nrf2 pathway.

Bilirubin research overview with focus on cellular events

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Unconjugated bilirubin is recognized as the most potent endogenous antioxidant substance. While hyperbilirubinemia has been long recognized an ominous sign of liver dysfunction, more recent data strongly indicate that mildly elevated bilirubin levels protect from a wide array of diseases associated with increased oxidative stress. Convincing evidence have now been obtained to support the notion that bilirubin and all the machinery involved in its production and metabolism are deeply involved in several crucial steps of cellular pathways and homeostasis. This occurs by a complex, intricate network involving several genes and pathways, pointing to the fact that bilirubin and related enzymes have more important functions than merely representing the waste products described in the 80´s. Recent discoveries pointed to a major role of bilirubin in immunosuppression and inhibition of protein phosphorylation resulting in modulation of intracellular signaling pathways involved in vascular, autoimmune diseases and cancer. As we proceed in understanding of the multiple roles of bilirubin and related compounds in modulating of various cellular pathways, the term "bilirubinomics" to describe this growing field of study is coming to the horizon.

Bilirubin Neurotoxicity

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Bilirubin is a bioactive product formed in the heme degradative pathway. It is a potent anti-oxidant known to exhibit potent antioxidant properties preventing the oxidative damage triggered by a wide range of oxidative stressors. However, at high levels, bilirubin can be neurotoxic and if left untreated, this hyperbilirubinemia can progress to silent or long-term symptomatic neurodevelopmental impairments and manifest as the syndrome of bilirubin-induced neurologic dysfunction (BIND), especially in the preterm infant. The impact has long-lasting functional and structural neurological injury that leads to alterations in the processing of afferent inputs, which can then lead to disordered efferent function, presenting as abnormalities in clinical extrapyramidal function, sensory processing of hearing, visual responses, and learning. The mechanisms underlying or contributing to the sequelae of bilirubin neurotoxicity are believed to be multifactorial, but are yet to be fully elucidated. Although in vitro studies have shown that bilirubin accumulates within neurons, neuronal processes, and microglia, cell-dependent sensitivity to bilirubin toxicity and the role of each cell type still requires further study. In this talk, current experimental models to study bilirubin toxicity will be presented.

Protective effects of bilirubin

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Bilirubin, the principal tetrapyrrole, bile pigment and catabolite of haem, is an emerging biomarker of disease resistance, which may be related to several recently documented biological functions.

Initially believed to be only toxic in infants, the perception of bilirubin has undergone a transformation; it is now also considered to be a molecule that may promote health in adults. Data from the last decade demonstrate that mildly elevated serum bilirubin levels, which are found in the condition of Gilbert´s Syndrome (GS; relatively common condition in the Caucasian population affecting 5-10 of the adult population), are strongly associated with reduced prevalence of chronic diseases, particularly cardiovascular diseases (CVDs), as well as CVD-related mortality and risk factors. Recent data also link bilirubin to other chronic diseases, including cancer and Type 2 diabetes mellitus, and to all-cause mortality. Therefore, there is evidence to suggest that bilirubin is a biomarker for reduced chronic disease prevalence and a potential predictor of all-cause mortality, which is of important clinical significance.
In the presentation, detailed information on the association between bilirubin and all-cause mortality, as well as the pathological conditions of CVD, cancer, diabetes and neurodegenerative diseases will be provided.
The mechanistic background concerning how bilirubin and particularly its metabolism may influence disease prevention and its clinical relevance will also be discussed.
Given that the search for novel biomarkers of chronic diseases, as well as for novel therapeutic modalities, is a key research objective for the near future, bilirubin (and its metabolism) represents a promising candidate, meeting the criteria of a biomarker, and should be considered more carefully in clinical practice as a molecule that might provide insights into disease resistance.
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Hyperbilirubinemia Counteracts Inflammation in Aging: Role of Redox Homeostasis

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Mild constitutive hyperbilirubinemia is associated with a reduced risk of cardiovascular diseases, diabetes, and cancer. Since these pathologies are associated with aging, inflammation and oxidative stress, we investigated whether hyperbilirubinemia interferes with ROS homeostasis in cell cultures, and with inflammation, senescence and mitochondrial dysfunction in aged rats.
Human embryonic kidney cells and rat primary fibroblasts showed a dose-dependent decrease in the ratio of oxidized/reduced glutathione, intracellular H2O2 levels, and mitochondrial ROS production, with increasing bilirubin concentrations in the culture media.
Compared to their normobilirubinemic siblings, aged hyperbilirubinemic Gunn rats showed significantly smaller amounts of visceral fat, better glucose tolerance, and decreased serum levels of proinflammatory cytokines TNFα, IL-1β, and IL-18. Simultaneously, livers from Gunn rats showed decreased expression of senescence markers, cell cycle inhibitors p21 and p16. Mitochondria from aged Gunn rats showed higher respiration and lower H2O2 production compared to controls.

In conclusion, we demonstrated that mildly elevated serum bilirubin is generally associated with attenuation of oxidative stress, and with better anthropometric parameters, decreased inflammatory status, increased glucose tolerance, fewer signs of cellular senescence, as well as enhanced mitochondrial function in aged rats.

Role of Heme Oxygenase-2 in Oxidative Stress Defense

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We have been studying the regulation of heme oxygenase-2 (HO2) by a heme responsive motif (HRM) domain, which contains a heme binding site made up of a conserved Cys-Pro core sequence flanked on one side by basic amino acids, at the other by a hydrophobic residue, with a distal His residue often completing the heme coordination sphere. Our results indicate that the HRMs can act as a switch allowing proteins to be regulated by redox and heme. In this switch, heme binds to the Cys residue in the HRM inducing a conformation change in HO2 that modulates its activity, (ii) oxidative stress conditions cause reaction of the HRM's Cys thiolate with a proximal Cys to form a disulfide bond, which releases the Cys ligand and thus affinity for heme diminishes. We will describe structural and functional results indicating that the HRMs of HO2 form a redox-regulated heme shuttle from solution to the catalytic core.

Role of Heme Oxygenase-2 (HO-2) in Arterial Hypertension

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Heme oxygenase-2 (HO-2) is the constitutively expressed isoform of heme oxygenase. Previous studies have demonstrated the important role for HO-2 as a major vasodilator of blood vessels especially in the brain and kidney. Given its preeminent role as a major vasodilator in the kidney, we wanted to examine the effect of loss of HO-2 on blood pressure and renal blood flow in response to angiotensin II, blockade of nitric oxide (NO), and renovascular hypertension using HO-2 specific knockout mice. Administration of angiotensin II and the NO synthesis inhibitor, L-NAME, resulted in identical increases in blood pressure in both male and female HO-2 knockout as compared to wild-type (WT) mice. Interestingly, loss of HO-2 did not enhance renal vasoconstriction to L-NAME and resulted in an attenuation in renal vasoconstriction in response to ANG II. While a similar increase in blood pressure in response to L-NAME treatment was observed between WT and KO mice, KO mice exhibited significantly enhanced cardiac hypertrophy as compared to WT mice. In response to 2 kidney, 1 clip, renovascular hypertension, HO-2 KO male mice exhibited a greater increase in blood pressure as compared to WT mice an effect that was not observed in female mice. However, female HO-2 KO mice did exhibit greater cardiac hypertrophy as compared to WT mice under basal conditions and in response to renovascular hypertension despite no difference in blood pressure. In conclusion, HO-2 appears to act in a sex-specific fashion to enhance the blood pressure response to some forms of hypertension. It also appears to play a role in protecting the heart especially in conditions of NO deficiency and may play a role in protecting female hearts from pressure-induced hypertrophy.

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Nephroprotective effect of heme oxygenase-1 and Nrf2 – role of microRNAs.

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Kidney fibrosis, the formation of excess fibrous connective tissue is a key determinant of the progression of renal diseases and it contributes to progressive decrease in glomerular filtration and tubular function. One of the possible cause of the renal fibrosis is chronic dietary exposure to ochratoxin A (OTA), a toxic secondary metabolite produced by several molds of the Aspergillus and Penicillium genera.
We have previously shown that in porcine tubular epithelial cells, OTA disrupts antioxidant/cytoprotective system and increases ROS production by down-regulating NF-E2-related factor-2 (Nrf2)/heme oxygenase-1 (HO-1) pathway [1, 2]. We also found that the changes in the level of microRNAs processing enzymes and microRNAs content is important mechanism of OTA toxicity. To further evaluate the mechanisms of OTA toxicity we analyzed the direct consequence of both HO-1 and Nrf2 deficiency (in a model of HO-1 or Nrf2 knock-out mice) on OTA-induced changes.
We have shown that OTA up-regulates the number of pro-fibrotic, pro-inflammatory and pro-apoptotic factors including microRNAs, whereas it down-regulates VEGF as well as anti-fibrotic BMP-7 level in HO-1/Nrf2-dependent way, and additionally such effects might be also sex-dependent. Moreover, the induction of Nrf2/HO-1 axis with sulforaphane (SFN) and cobalt protoporphyrin (CoPPIX) was able to halt nephrotoxic effects of OTA. Importantly, we found the regulation of microRNAs expression to be affected by OTA treatment. Accordingly, p53-regulated miR-34a and pro-fibrotic miR-21 were potently induced, whereas anti-fibrotic microRNAs were down-regulated by OTA administration. Modulation of Nrf2/HO-1 pathway as well as microRNAs expression may provide a therapeutic approach to OTA-triggered renal diseases.

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Heme regulates Bach2 protein interaction by binding to its intrinsically disordered region

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The transcription factors Bach1 and Bach2 repress the expression of heme oxygenase-1 gene (*Hmox1*) by binding to its upstream enhancer regions. They are both heme receptors as well, and their DNA binding activity, nuclear loclalization, and stability are inhibited by direct binding of heme, resulting in derepression of *Hmox1*. The heme-mediated regulation of Bach1 and Bach2 underlie the substrate induction of heme oxygenase-1 in diverse cells. Bach1 and Bach2 possess multiple cysteine-proline (CP) motifs each of which is involved in formation of 5-coordinate heme complex. In

addition, Bach1 and Bach2 also bind heme in 6-coordinated mode. However, little has been known for the molecular mechanisms of heme-Bach interaction. Using Bach2 as a model, we found that an intrinsically disordered region (IDR) of Bach2 mediates both 5- and 6-coordinated heme binding. Heme did not induce any secondary structure formation upon binding to this region. However, we found that heme promoted binding of several proteins to the Bach2 IDR, suggesting a conformational alteration of IDR upon heme binding. These observations suggest that the IDR of Bach2 is important for heme binding, and consequently for its functional regulation.

Role of NRF2 in Carcinogenesis

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Transcription factor Nrf2 controls the expression of more than 1% of human genes related to biotransformation reactions, redox homeostasis, energetic metabolism and proteostasis. One of the best characterized Nrf2-genes is Hmox1 coding heme oxygenase -1 (HO1). The Nrf2/HO-1 axis plays crucial roles in cancer initiation an d progression and therefore, a great effort is being made to elucidate the mechanisms that regulate this axis.
The main mechanism of Nrf2 regulation is at the level of protein stability by the KEAP1 the ubiquitin E3 ligase adapter Keap1, which is a redox and electrophile sensor. However, we have been characterizing a different regulation of Nrf2 stability that connects this protein with cell signaling at the level of glycogen synthase kinse-3 (GSK-3). This kinase phosphorylates specific serine residues in the Neh6 domain of Nrf2 to create a degradation domain that is then recognized by the ubiquitin ligase adapter β-TrCP and tagged for proteasome degradation by a Cullin1/Rbx1 complex. These pathways include those activated by ligands of tyrosine kinase, G protein-coupled, metabotropic, and ionotropic receptors that activate phosphatidyl inositol 3-kinase (PI3K)/ATK and by the canonical WNT signaling pathway, where a fraction of Nrf2 interacts with Axin1/GSK-3.
Constitutive activation of these signaling pathways during tumor development eliminates the GSK-3 control and lead to persistent activation of Nrf2/HO1 up to levels that permit cancer cells to become tolerant drugs and to oxidant environment. We will show results indicating that oncogenic activation of NRF2 by loss of its negative regulation by the tumor suppressor PTEN may be relevant to a large number of tumors, including endometrioid carcinomas.

Nrf2-dependent Angiogenesis Is Not Directly Related To Its Transcriptional Activity

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Nrf2, a master regulator of oxidative stress response in cells, is a transcription factor. Our data demonstrate that angiogenesis driven by SDF-1 and GDF-15, but not VEGF, depends on Nrf2 presence in all-or-none manner and is not directly related to its transcriptional activity. We showed that SDF-1 and GDF-15 dose-dependently induce angiogenic response, measured by tube formation, migration and vessel lumen formation, in human endothelial cells, what was blocked in the absence of Nrf2. Angiogenesis induced by SDF-1 and GDF-15 was evidenced by reporter assay and ChIP assay to be independent of a direct Nrf2 transcriptional activity, what is in line with poor phosphorylation, lack of acetylation and delayed nuclear translocation of Nrf2 driven by these two factors. We also demonstrated that it is not related to oxidative stress, as silencing of Nrf2 does not cause increase of reactive oxygen species in endothelial cells and treatment of cells with N-acetyl cysteine does not reverses detrimental angiogenesis-related effect of Nrf2

deficiency. A role of Nrf2 in angiogenic response of endothelial cells is linked to its involvement in actin cytoskeleton rearrangements instead, at least via mechanisms preventing cellular senescence.

Induction of HO-1 Expression by Selective Removal of Endogenous CO

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The physiological roles of endogenous carbon monoxide (CO) have not been fully understood because of the difficulty in preparing a loss-of-function phenotype of this molecule. Here, we have utilized synthetic CO-depleting agents, hemoCDs, which are the 1:1 supramolecular inclusion complexes of Fe(II)porphyrin with per-*O*-methylated β-cyclodextrin dimers. We have previously reported that hemoCD is capable of capturing endogenous CO in the blood of rats [Ref 1]. In this study, we used hemoCDs showing different CO affinities to investigate the depleting effect of endogenous CO in the blood of mice. Intraperitoneal injection of the hemoCD solution to mice induced the pseudo-knockdown state for endogenous CO in the blood, in which the mRNA level of heme oxygenase-1 (HO-1) in the mice liver was temporarily but strongly enhanced. The amount of CO in the blood of mice was temporarily disappeared, and then quickly returned to the normal revel after the renal clearance of hemoCD. The result suggests that the biological feedback for the CO homeostasis was activated by the HO-1 induction in the liver. The plausible pathway for the CO/HO-1 system is assumed as follows: HemoCD in blood primarily removes CO from cell-free CO-Hb released from senescent red blood cells. The removal of CO causes the formation of cell-free oxy-Hb, which is quickly oxidized to met-Hb by the reaction with reactive oxygen species such as H_2O_2 in blood. Cell-free met-Hb readily releases free hemin in blood that triggers the induction of HO-1. It is assumed that endogenous CO plays a crucial role in suppressing the autoxidation reaction of ferrous Hb to met-Hb through its ligation to the heme iron. These results suggest that the major role of endogenous CO in blood is to adjust the free hemin concentrations in the bloodstream [Ref 2].
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Structure Activity Relationship of HMOX Modulation

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Elucidation of the role of heme oxygenase (HMOX, HO) in various biological systems can be approached pharmacologically, which implies exploiting drugs to activate or inhibit the HO system(s). The first generation of HO inhibitors (metaloporphyrins) were criticized for their effects on other enzyme systems. Thus, the focus of our laboratory has been the design of HO inhibitor drugs that have a non-porphyrin basis. Our in vitro assay for HO activity measured CO production, and used rat spleen microsomes for HO-1 and rat brain microsomes for HO-2, respectively. A series of compounds based on azalanstat (4-[[(2S,4S)-2-[2-(4-chlorophenyl)ethyl]-2-(imidazol-1-ylmethyl)-1,3-dioxolan-4-yl] methylsulfanyl]aniline) yielded a number of good HO inhibitors, some of which were selective for HO-1. The effects of alterations of major moieties on enzyme selectivity and potency will be presented. X-ray crystallography revealed that the inhibitors bound in the HO catalytic site and binding involved the substrate, heme. Substitution of the imidazole group of the lead compound with triazoles or tetrazoles decreased inhibitory activity on cytochromes P450. Screening of a chemical library yielded a series of inhibitors based on clemizole that possessed selectivity for HO-2. Structure

activity relationships of this series will also be discussed. HO inhibitors were also tested against enzymes obtained from nude mice. Several drugs were significantly more effective against rat spleen and brain microsomal HO than mouse sourced enzymes.
Supported by the Canadian Institutes of Health Research

Heme oxygenase-1 as an oncology target: focuse on small molecule inhibitors
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Heme oxygenase-1 (HO-1) is highly induced in a variety of cancer types and its increased expression is correlated with more advanced tumor development stage as well as poorer survival of patients. Multiple lines of evidence connected genetic and pharmacological HO-1 inhibition with enhanced sensitivity of cancer cells to standard-of-care chemotherapy, as well as suppression of cancer metastasis in experimental *in vitro* and *in vivo* models. Public domain data on specific HO-1 inhibitors is however limited. Recently developed imidazole-based derivatives are so far the only non-porphyrin based, isozyme selective HO-1 inhibitors. To address the need for a new generation of HO-1 inhibitors, we initiated a drug discovery program aimed at identification of novel, potent and selective small molecule inhibitors of HO-1. The main hit finding strategy was virtual HTS (vHTS) followed by structure-based rational design. The screening led to the identification of several chemically diverse molecules which actively inhibit the catalytic function of HO-1 at submicromolar concentrations. Detailed characterization of the mechanism of action of identified compounds, based on numerous biochemical, biophysical and analytical methods, as well as research work on experimental models aimed at defining the most effective therapeutic indications for HO-1 inhibitors in oncology will be presented.

Organic Carbon Monoxide Prodrugs that Release CO under Physiological Conditions with Tunable Release Rates
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Carbon monoxide (CO) has been recognized as a critical signaling molecule in mammals with therapeutic effects in treating cancer, inflammatory diseases, and sickle cell disease among others. The past decade has seen a tremendous amount of work in understanding CO pharmacology and its mechanism of actions. All indications are that the time has come to develop CO-based therapeutics for various indications. One key issue, however, is the difficulty in controllable delivery of CO in a pharmaceutically acceptable form. Ideally, one would like to see CO in a pill or an ampule for oral and/or injection/infusion-based deliveries.

Along this line, the past decade has seen many metal-based CO-releasing molecules (CO-RMs) and photo-sensitive organic CO-RMs. These compounds have played critical roles in helping the understanding of CO pharmacology and its mechanism of actions. Some of these compounds also have the potential to be developed further as therapeutics in human with select indications.

Taking the view of pharmaceutical sciences, it is our belief that small organic molecules capable of caging and releasing CO under desired conditions can be very valuable research tools. In addition, the pharmaceutical industry has extensive experience in developing small organic molecules as therapeutics with well understood criteria in the area of absorption, distribution, metabolism, excretion, and toxicity. As a consequence, organic CO-prodrugs should be well-

positioned for future development as therapeutics for various indications. For these reasons, we have undertaken an effort to develop small organic molecule CO-prodrugs, which can release CO under physiological conditions without the need for light activation. In addition, it is almost certain that the desirable CO-release rate would be different depending on the specific indication. Thus having tunable release rates and/or triggered release using biological triggers will be very much desirable.

For our design, we cage carbon monoxide as a carbonyl group in a ketone. By taking advantage of Inverse Electron Demand Diels-Elder reactions, we have synthesized a series of more than 70 organic CO-prodrugs with half-lives ranging from 2 min to weeks under physiological conditions without the need for light. In addition, these prodrugs also allow for tuning of water solubility, membrane permeability as well as conjugation with targeting molecules. These properties and features are critical to their future pharmaceutical development.

This presentation will discuss the chemistry of these CO-prodrugs and associate pharmaceutical issues.

Abstracts / Posters

1.
Heme Oxygenase-1 (HO-1) Mitigates Ferroptotic Cell Death in Proximal Tubule Cells
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Introduction: Ferroptosis is an iron-dependent form of regulated cell death that is triggered under conditions of glutathione depletion. Recent studies have highlighted the role of ferroptosis in mediating cell death in models of acute kidney injury (AKI). HO-1 is a cytoprotective enzyme that is robustly induced in renal proximal tubules in AKI and is a source of intracellular iron (required for ferroptosis) due to its ability to catabolize the breakdown of heme into iron, biliverdin, and carbon monoxide. Therefore, the purpose of this study was to elucidate the role of HO-1 in regulating ferroptotic cell death in renal proximal tubular epithelial cells.

Methods: Immortalized proximal tubule epithelial cells (PTEC) obtained from HO-$1^{+/+}$ and HO-$1^{-/-}$ mice were treated with erastin, an inducer of ferroptosis, and analyzed for morphological changes and cellular metabolic activity using the Alamar blue assay. Induction of HO-1 in response to erastin treatment, and following co-treatment with anti-oxidants or iron chelators were determined using real time PCR and western blotting.

Results: Treatment of HO-$1^{+/+}$ PTECs with erastin resulted in a dose-dependent increase in HO-1 expression, as well as significant inhibition of cellular metabolic activity compared to vehicle-treated controls (mean ± SEM, control: 100±1.8; erastin 0.1μM: 91.6±2.5; erastin 1μM: 58.3±0.9, and erastin 10μM: 62.5±1.8%, $p<0.0001$, n=3/group). Iron supplementation using ferric ammonium citrate in cells treated with 1μM erastin further reduced cell viability from 54.7±1.5% to 38.4±1.5%, $p=0.0012$. Interestingly, co-treatment with 1μM hemin (HO-1 inducer), 0.1mM deferroxamine (iron chelator), or 0.1M N-acetyl-L-cysteine (glutathione replenisher) significantly increased cell viability. To test the dependency on HO-1 in mediating ferroptosis, HO-$1^{-/-}$ PTECs were treated with erastin. Such treatment resulted in a dose-dependent increased sensitivity to ferroptosis (mean ± SEM, control: 100±3.1; erastin 0.1 μM: 68.7±2.2; 1 μM: 44.0±0.7, and 10 μM: 53.8±1.9 %, $p<0.0001$, n=3/group).

Conclusion: HO-1 induction appears to attenuate erastin-induced ferroptotic cell death in renal epithelial cells; therefore, it may serve as a viable therapeutic target for intervention in AKI.

2.

Heme induced contractile dysfunction in human cardiomyocytes by myofilament protein oxidation

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Introduction: Under pathological conditions free intracellular heme predisposes for oxidant-mediated tissue damage. Previous results in our laboratory showed alterations in contractility machinery and structure of the sarcomere function. The aim of this project was to elucidate the mechanism beside the dysfunction of the contractile protein machinery of human cardiomyocytes.
Methods: Force measurements were performed in skinned human cardiomyocytes isolated from left ventricle. Ca2+--activated active force (Factive), Ca2+-independent passive force (Fpassive) were monitored before and after 20-minutes-long incubations in the presence of increasing heme concentrations (1µM-100µM) with or without added H2O2 and/or the antioxidant DTT. A protein biotinylation and sulfenylation assays were performed to determine the relative levels of sulfhydryl (SH) group oxidation and sulfenic acid formation for individual proteins in the presence of increasing heme concentrations (1µM-300µM).
Results: Following 100 µM heme exposures, Factive decreased to 11±2% and Fpassive increased to 568±61% (mean±SEM, P<0.05, n=8) with structural disturb. The half maximal inhibitory concentration of heme dependency (IC50) on Factive was 19 µM. SH group decreased after 30 µM of heme concentration to almost complete oxidation of all myofilament proteins at 300 µM. Partial restoration in the SH content was observed in a protein running at 140 kDa after exposure with DTT. However Titin and the other proteins did not show changes in the SH content. Moreover sulfenic acid formation was increased in MHC, cMyBPC and alpha actinin, after exposure to 300 µM heme. Hemopexin and alpha 1 microglobulin blocked the effects on the mechanical parameters.
Discussion: Our observations suggest that free heme modifies SH content and sulfenic acid formation in cardiac contractile machinery proteins, specifically on thick filaments; leading to important mechanical limitations in their physiological functions. The heme induced antiparallel changes in Factive and Fpassive that may potentially explain part of the systolic and diastolic cardiac dysfunctions not only in haemolytic diseases, but also during heart failure and myocardial ischemia-reperfusion injury.

3.

An Additional Heme Oxigenase-1 Knockout Increases Liver Inflammation and Fibrosis in Mdr2 Knockout Mice

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Background: Deletion of the multi drug resistance protein 2 (Mdr2) in mice causes hepatic inflammation and fibrosis with progression to hepatocellular carcinoma (HCC) at about 12 month of age (Mdr2 knockout mouse; Mdr2ko; FVB.129P2-Abcb4tm1Bor). We have recently shown that induction of heme oxygenase-1 (HO-1) in Mdr2ko mice interferes with liver inflammation, fibrosis formation and proliferation. We established a double knockout mouse for Mdr2 and HO-1 (Mdr2/HO-1ko) to further investigate effects of HO-1 on chronic liver inflammation and its consequences.
Methods: Liver damage was monitored by alanine aminotransferase (ALT) levels. Leukocyte infiltration and neoductuli formation was visualized by H&E staining of liver slices. Spleen weight was measured as a sign of inflammation. TNF--alpha and its receptors were analyzed as mediators of inflammation. Fibrotic remodeling was analyzed by measuring the hepatic hydroxyproline content, as well as mRNA expression of Collagens, Matrix-Metallo-ases (MMPs), and tissue--inhibitors of MMPs (TIMPs). Flow cytometry (FACS) was used to analyze liver immune cell populations. Bone marrow derived dendritic cells (BM-DCs) were analyzed for cytokine production and expression of maturation markers by flow cytometry. Female and male (F/M) FVB/N background control (wt), Mdr2ko, and Mdr2/HO-1ko mice were analyzed at the age of 12 weeks.
Results: Mdr2/HO-1ko survived better than HO-1single knockout mice (4.2% vs. 0.2% of offspring). ALT levels were significantly up-regulated in Mdr2ko (F/M), and Mdr2/HO-1ko (M) mice compared to wt. Female Mdr2/HO-1ko displayed higher ALT levels than Mdr2ko mice. Liver hydroxyproline levels were significantly higher in Mdr2/HO-1ko mice compared to Mdr2ko mice (F/M). Fibrosis Markers Colagen 1, MMP-13 and TIMP-1 are highly expressed in female Mdr2ko and Mdr2/HO-1ko compared to the male counterparts. MMP-9 was significantly induced in Mdr2/HO-1ko compared to wt and Mdr2ko mice, while male Mdr2ko and Mdr2/HO-1ko only show a slight induction. Spleen weight was doubled in Mdr2ko (F/M) mice compared to wt, while Mdr2/HO-1ko (F/M) mice showed a 5 fold elevated spleen weight. H&E staining revealed similar levels of leukocyte infiltration and neoductuli formation in Mdr2ko and Mdr2/HO-1ko mice (F/M). FACS analysis showed increased frequencies of T-cells and NKT-cells as well as CD11c+ dendritic cells (DCs) in Mdr2/HO-1ko mice compared to Mdr2ko mice. Mdr2/HO-1ko mice showed highly activated BM-DCs (indicated by frequencies of MHCII+CD86+ cells), as well as a 5 fold increase in IL-12 production even without re-stimulation compared to Mdr2ko mice.
Conclusion: Mdr2/HO-1ko mice have a stronger fibrotic and inflammatory phenotype compared to the single Mdr2ko. The stronger inflammation can be explained by higher frequencies of T-cells, NKT-cells and mature DCs. Increased production of IL-12 in BM-DCs indicate a stronger Th1 response in Mdr2/HO-1ko mice compared to Mdr2ko.

4.

Pharmacologic induction of ferritin prevents osteoblastic transformation of smooth muscle cells

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Vascular calcification is a frequent complication of atherosclerosis, diabetes, and chronic kidney disease. In the latter group of patients, calcification is commonly seen in tunica media where smooth muscle cells (SMC) undergo osteoblastic transformation. Risk factors such as elevated phosphorus levels and vitamin D3 analogs have been identified. In light of earlier observations by our group and others, we sought to inhibit SMC calcification via induction of ferritin. Human aortic SMC were cultured using β-glycerophosphate with activated vitamin D3, or inorganic phosphate with calcium, and induction of alkaline phosphatase and osteocalcin as well as
accumulation of calcium were used to monitor osteoblastic transformation. Additionally, to
examine the role of vitamin D3 analogs, plasma samples from patients on hemodialysis who had
received calcitriol or paricalcitol were tested for their tendency to induce calcification of SMC. Addition of exogenous ferritin mitigates the transformation of SMC into osteoblast-like cells. Importantly, pharmacologic induction of heavy chain ferritin by 3H-1,2-Dithiole-3-thione was able to inhibit the SMC transition into osteoblast-like cells and calci-

fication of extracellular matrix. Plasma samples collected from patients after administration of activated vitamin D3 caused significantly increased alkaline phosphatase activity in SMC compared to samples drawn prior to activated vitamin D3 and here, again induction of ferritin diminished the osteoblastic transformation. Our data suggests that pharmacologic induction of ferritin prevents osteoblastic transformation of SMC. Hence, utilization of such agents that will cause enhanced ferritin synthesis may have important clinical application in prevention of vascular calcification.

5.

Proximal tubule-specific HO-1 modulates the progression of acute kidney injury to chronic kidney disease

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Acute kidney (AKI) is a growing epidemiological issue, associated with high morbidity and mortality. AKI has the propensity to lead to chronic kidney disease (CKD), though the mechanism is unclear. AKI has been shown to be ameliorated by the induction of heme oxygenase-1 (HO-1), a cytoprotective enzyme that catalyzes the breakdown of pro--oxidant heme, into by-products (carbon monoxide, biliverdin and iron), which have pro-survival properties that have proven to be beneficial in AKI. HO-1 is predominately expressed in the proximal tubules (PT) of the nephron, which are also most susceptible to injury.
To evaluate the role of PT-specific HO-1 in the transition from AKI to CKD, we used transgenic mice, generated using the cre-lox system, to manipulate HO-1 expression specifically in the PT. We examined the progression to CKD using unilateral kidney ischemia injury (25 minutes) followed by reperfusion.
We demonstrate that selective PT-specific HO-1 deletion lessens the severity of fibrotic remodeling in the injured kidney, as evident by decreased expression of fibronectin, α-smooth muscle actin, and reduced collagen deposition. PT deletion of HO-1 also led to significantly reduced expression of inflammatory markers, such as *TNF-α,* in the injured kidneys compared to their wild-type littermates. *Interestingly,* HO-1 led to decreased levels of urinary neutrophil gelatinase-associated lipocalin (NGAL), a biomarker of AKI, at 24h post-injury and reduced NGAL gene expression in the injured kidneys at day 21. These results suggest a role of PT-specific HO-1 in the fibrotic remodeling post-ischemic injury. Macrophages play a vital role in modulating fibrosis and therefore, our current efforts are aimed at determining macrophage accumulation and phenotype and its association with fibrosis in the absence of PT-HO-1. These results suggest a role of PT-specific HO-1 in modulating fibrotic remodeling post-ischemic injury and may provide insight into the generation of therapies for preventing progression from AKI to CKD.

6.

Sex and Age Dependent HO-1 Expression in Cisplatin-Mediated Acute Kidney Injury

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Background: Acute kidney injury (AKI) is a clinical condition associated with high morbidity and mortality. In animal models of AKI, heme oxygenase-1 (HO-1), a 32 kDa microsomal enzyme, is robustly induced and is cytoprotective through its anti-oxidant, anti-apoptotic, and anti-inflammatory effects.

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Methods: To study the influence of age and gender on HO-1 expression in response to AKI, 4 months (young) and 16-17 months (aging) old male and female C57BL/6 mice, respectively, were subjected to AKI with a single intra-peritoneal cisplatin (Cp) injection (20 mg/kg body weight) or normal saline as vehicle control. 1 and 3 days post cisplatin/vehicle injections, the mice were sacrificed and kidneys were processed for western blot analysis. Serum was collected for creatinine measurement, a marker of renal function, and performed using LC-MS/MS. Data values indicate mean±SEM using one way ANOVA with Tukey's post-test.

Results: Serum creatinine values (marker for renal injury) in aging males (3.64±0.40 mg/dL) and females (3.40±0.44 mg/dL) showed worsening of kidney function as compared to the young males (1.69±0.66 mg/dL) and females (0.61±0.12 mg/dL)($p<0.01$ and $p<0.0001$, respectively), after 3 days post Cp injection. Western blot data obtained from kidney lysates indicate that young males had significantly higher HO-1 induction as compared to young females 1 day post Cp-induced AKI. Young females and males had significantly higher HO-1 induction as compared to aged females and males, respectively, 1 day post Cp AKI. 3 days post Cp, in contrast to aging females, HO-1 protein expression in young females returned to values comparable to the baseline vehicle control treated values. The increased HO-1 protein levels persisted in young and aging males 3 days post Cp as compared to saline/vehicle-treated control mice. Markers of autophagy like ATG5 were significantly decreased in aging females compared to young females 3 days post Cp. Levels of p62 were significantly higher in all the aging mice as compared to their younger counterparts after 1 and 3 days post Cp injection.

Conclusions: The results of this study indicate that age and sex influence renal HO-1 expression and alter the autophagy pathway following Cp-induced AKI which may contribute to worse outcomes. Further studies are ongoing to examine the putative mechanisms involved so that they can be exploited as a potential therapeutic target in AKI.

7.

Heme Oxygenase-1 Modulates Renal Inflammation Associated Lymphangiogenesis

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Inflammation associated lymphangiogenesis (IAL) is characteristic of many pathological conditions such as tumorigenesis, wound healing, inflammatory bowel disease, and transplant rejection. Despite its prominent role in many conditions, relatively little is known about IAL. Heme oxygenase-1 (HO-1) is a well characterized stress inducible enzyme with potent anti-inflammatory properties. HO-1 is a key regulator of angiogenesis, but its role in IAL remains to be fully elucidated. Stimulation of lymphangiogenesis is modulated by vascular endothelial growth factors (VEGFs), specifically VEGF-C, VEGF-D, and their receptor VEGF-R3. To study IAL in acute kidney injury and determine the role of HO-1 in this process, we induced injury in HO-1+/+ and HO-1-/- mice via unilateral ureteral obstruction (UUO), a model of progressive renal inflammation and fibrosis. Protein and mRNA levels of the lymphangiogenic growth factors were analyzed at baseline and on days 1 and 7 after surgery via western blot and qPCR. Additionally, to determine the roles of individual cell types in this process, primary proximal tubule cells, and bone marrow derived monocytes were cultured and exposed to hypoxia to mimic ischemic conditions and the levels of markers were analyzed. Higher levels of VEGF-R3 expression were observed in the kidneys of HO-1-/- mice at baseline and seven days after UUO compared to their wild--type littermates. Also, higher levels of VEGF-D were seen in the kidneys of HO-1-/- mice 7 days after UUO compared to HO-1+/+ kidneys. A similar pattern of expression was found in the macrophages: in the HO-1-/- cells, VEGF-R3 expression was more pronounced at baseline and this increased further after hypoxia. The expression of markers associated

with lymphangiogenesis was reversed in the proximal tubule cells with significant decrease in VEGF-R3 expression after hypoxia in the HO-1-/- cells. These results suggest that HO-1 plays a major role in the cross-talk between tubular and inflammatory cells following injury to modulate IAL and hence may serve as a major regulator of the inflammatory process and resolution following injury.

8.

Antiangiogenic Effects of Blue-green Cyanobacteria Spirulina sp. on Pancreatic Cancer

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Background: Spirulina sp, blue-green cyanobacteria, is a popular nutraceutical having potent antioxidant properties and likely to affect also carcinogenesis. Among other substances, it is rich in phycocyanobilin (PCB), a tetrapyrrolic molecule closely related to bilirubin, an endogenous antioxidant. The aim of our study was to assess possible inhibitory effect on human pancreatic cancer cells of S. sp. and also its effect on angiogenesis.
Methods: The effect of S. sp. extract was tested on human pancreatic cancers grown in athymic mice, human pancreatic cancer cells (PA-TU-8902) and immortalized endothelial cells (Ea.hy926) were used for our studies. Markers of vascularization (CD31, VEGF) were analyzed immunohistochemically in tumors, VEGF production as well as ERK signaling pathway was assessed in pancreatic cancer cells. In vitro migration and invasiveness assays were performed on pancreatic cancer and endothelial cells.
Results: Compared to untreated cells, experimental therapeutics significantly decreased proliferation of human pancreatic cancer cell in vitro in a dose-dependent manner. The anti-proliferative effects of S. sp. extract were also shown in vivo, where inhibition of pancreatic cancer growth was evidenced since the third day of treatment ($p<0.05$). Tumors of mice treated with S. sp. extract exhibited much lesser degree of vascularization as measured by CD31 immunostaining ($p=0.004$), but surprisingly, expression of VEGF tended to be substantially higher (by 45%, $p=0.08$). S. sp. extract as well as PCB significantly increased production of VEGF under both normoxic and hypoxic conditions. S. sp. extract significantly decreased phosphorylation of ERK signaling protein. Inhibitory effects of S. sp. extract on pancreatic cancer and endothelial cell in migration and invasion were also demonstrated.
Conclusion: Treatment of pancreatic cancer with S. sp. extract is associated with decreased proliferation with various anti-angiogenic features which might account for reported anticancer effects of this blue-green cyanobacteria.

9.

Heme Oxygenase as a Key Enzyme to Demonstrate Oxidative Stress in a Mouse Model of Acute Intermittent Porphyria

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Heme oxygenase (HO), a known oxidative stress-inducible protein, plays a key role in heme catabolism, where heme, a prooxidant, is converted to bilirubin, an antioxidant. HO increases after heat shock or glutathione depletion. GSH and Catalase are of vital importance in protecting tissue from oxidative damage. Oxidative stress could be one of the reasons for the neuropsychiatric syndrome of acute Porphyrias. We have reported that porphyrinogenic drugs altered HO causing

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oxidative and nitrosative stress in brain of *CF1* mice. The aim was to investigate if the anaesthesia with Isoflurane and Sevoflurane developed oxidative stress in a mouse model of Acute Intermittent Porphyria (AIP). Allylisopropilacetamide (AIA), Veronal and ethanol, known porphyrinogenic drugs were also evaluated. HO, Catalase and GSH levels were measured in liver, kidney and brain of T1 (PBG-D activity 50% reduced) and AIP (PBG-D activity 70% diminished) mice. In T1 mice, Isoflurane caused no variations in liver and encephalon HO activity, while kidney enzyme was 48% ($p<0.05$) induced; in females, Isoflurane produced only an increase in encephalon (100%, $p<0.05$) without any alteration in liver and kidney. Sevoflurane augmented 75% ($p<0.01$) in male kidney enzyme activity, being hepatic and encephalon enzyme unaltered; no variations were detected in female tissues. AIA induced 110% ($p<0.01$) and 200% ($p<0.01$) liver and kidney HO activity respectively of male T1 mice; in female group, the enzyme remains in its basal levels. When Veronal was administered to T1 males, the activity was only increased in kidney (200%, $p<0.05$); on the contrary, HO activity showed a reduction (80%, $p<0.01$) in encephalon of females, being unaltered in the other tissues. Ethanol caused an induction in liver HO activity (85% $p<0.05$) of male mice without no variation in kidney and encephalon; while kidney female T1 mice enzyme activity was 60% ($p<0.05$) reduced. Catalase activity and GSH levels varied depending on the drug and the sex. When Isoflurane was administered to T1 male mice, the activity of Catalase increased in liver (155%, $p<0.01$), kidney (275%, $p<0.01$) and encephalon (70%, $p<0.05$) without any effect in T1 females or AIP groups. In AIP female mice, Isoflurane affected the kidney increasing (50%, $p<0.05$) GSH levels. Sevoflurane only altered Catalase in encephalon T1 male mice, reducing 65% ($p<0.05$) its activity. On the contrary, in AIP mice Catalase activity increased in male liver (30%, $p<0.05$) and in male (50%, $p<0.05$) and female encephalon (238%, $p<0.05$). Clinical symptoms typical of acute attacks were observed by effect of AIA. In conclusion, the instauration of oxidative stress as a consequence of the drugs assayed was produced being more pronounced in males. This fact is different to that observed in humans where females are more sensitive to develop AIP.

10.

The GFAP.HMOX1 Mouse Model of Parkinson Disease – microRNA, mRNA and Protein Expression Profiles

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Idiopathic Parkinson's disease (PD) is a movement disorder of unknown etiology characterized by the accelerated loss of dopaminergic (DA) neurons and accumulation of alpha-synuclein in the pars compacta of the substantia nigra (SN) and striatum (STM) brain regions. In recent years, Dr. Hyman Schipper's laboratory has engineered a novel parkinsonian GFAP.HMOX1 mouse model, in which glial heme oxygenase-1 (HO-1), a stress protein induced in the PD SN, is overexpressed in the astroglial compartment. This model recapitulates many neuropathological, neurochemical and behavioural features of the disease. The objective of the current study was to further characterize the GFAP.HMOX1 mice through a broad genetic screen, evaluating key genes involved in various pathways that have been implicated in PD pathogenesis. MicroRNA (miRNA), mRNA and protein expression levels were analyzed both in vivo and in vitro. GFAP.HMOX1 neural tissues exhibited altered patterns of miRNA and target mRNA expression akin to those observed in human PD subjects. Several genes involved in the dopaminergic system were significantly downregulated, including dopamine transporter (DAT), tyrosine hydroxylase (TH), Nurr1, Pitx3 and LMX1B, at the mRNA and/or protein level in transgenic (TG) SN and STM compared to wild-type (WT) controls, while select miRNAs targeting these genes were significantly upregulated. Alpha-synuclein, involved in the formation of Lewy bodies in PD subjects, was significantly upregulated at both the mRNA and protein level in both brain regions, while several miRNAs targeting alpha-synuclein were significantly downregulated. Genes involved in other pathways known or suspected to play a role in PD pathology, such as oxidative stress, apoptosis, autophagy and mitophagy, mitochondrial biogenesis and reelin regulation were

significantly elevated at the mRNA and/or protein level in the experimental samples compared to WT preparations. Many of these whole-brain findings were recapitulated in neurons co-cultured with TG astrocytes compared to WT controls. Overexpression of astrocytic HMOX1 in mice between 8.5 and 19 months of age promotes several neuropathological, neurochemical, behavioural and regulatory features of idiopathic PD. Curtailment of glial HO-1 hyperactivity by pharmacological or other means may afford neuroprotection in PD and other aging-related neurodegenerative disorders.

11.

The Nuclear Form of HO-1 Preferentially Promotes Glycolysis Under Hyperoxic Stress

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Heme oxygenase-1 (HO-1) is a stress-inducible enzyme that catalyzes the degradation of heme, generating the products CO and biliverdin/bilirubin which exhibit antioxidant properties. Cytoplasmic HO-1 is tethered to the ER via a C-terminal transmembrane segment. Cleavage of this anchor allows localization of a truncated, catalytically inactive form of HO-1 to the nucleus, as has been observed in fetal lung cells in hyperoxia and in several cancer tissues. While cytoplasmic HO-1 is associated with cytoprotection directly via its antioxidant activities, nuclear HO-1 also plays a role in protection against oxidative injury via its interaction with nuclear proteins and transcription factors, resulting in altered gene expression of several rate-limiting enzymes in key metabolic pathways including glycolysis. Interestingly, nuclear HO-1 is also associated with a proliferative phenotype. Although HO-1 has been shown to play a role in glucose metabolism and altered mitochondrial dynamics, the mechanism is currently unknown. In an effort to investigate this we used a Seahorse Bioanalyzer to measure glycolysis and oxidative phosphorylation (oxphos) in MEF cells expressing only the nuclear, truncated HO (HO-TR). We find that HO-TR has increased glycolytic activity under hyperoxic stress compared to HO+/+ or HO-/- cells. Separately, oxphos levels were unaffected between air and hyperoxia; however, the presence of heme inhibited oxphos activity of cells lacking HO-1 catalytic activity, that is, HO-TR and HO-/-. Our results suggest a role of nuclear HO-1 in upregulating glycolysis during hyperoxic stress. They also suggest that catalytically active HO-1 is required for homeostasis of mitochondial function. Taken together our results suggest that nuclear HO-1 is cytoprotective under hyperoxic stress via a metabolic shift toward glycolysis and highlight a putative role of HO-1 in mitochondrial dynamics and metabolic regulation.

12.

CO Modulation of Ca2+ Channels in Cardiovascular Disease

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Heme oxygenase-1 (HO-1) is induced in response to numerous cell stresses associated with cardiovascular disease, including oxidation and inflammation, and subsequently HO-1 derived CO mediates a plethora of favourable effects in vascular cells (1). CO is now established as a vital endogenous signalling molecule and is emerging as a modulator of numerous ion channels.

Cardioprotective effects of CO could be mediated via inhibition of the L-type Ca2+ channel. We found that CO inhibited Ca2+ currents in rat cardiomyocytes and in HEK Cav1.2 cells. Inhibition was mediated by CO-induced increased mitochondrial reactive oxygen species acting at identified cysteine residues on the C-terminal cytoplasmic region of the channel (2). CO also has beneficial effects on vascular cells. Both HO-1 induction (via CoPPIX) and CO application (applied via CO-releasing molecule, CORM-3) inhibited proliferation of rat aortic vascular smooth muscle cells (VSMC), (A7r5 cells), human saphenous vein smooth muscle cells, and HEK Cav3.2 cells (3). We propose that CO limits proliferation in these cells via inhibition of the T-type Ca2+ channel. T-type Ca2+ currents were significantly reduced in A7r5 cells and HEK Cav3.2 cells in the presence of CORM-2 or HO-1 induction via CoPPIX (3, 4). Our data suggest that CO inhibits Cav3.2 T-type Ca2+ channels by disrupting the extracellular redox modulation of the channel by thioredoxin (4).

HO-1 derived CO is an important gasotransmitter with clinical implications. Indeed, there are clinical trials underway involving CO gas for several disorders (5). Its ability to modulate Ca2+ channels of vascular importance may provide cardioprotection and also be beneficial in vascular diseases. In the latter case, by limiting pathological VSMC proliferation central to cardiovascular disorders, the burden of the associated complications, such as vessel stiffening, occlusion and myocardial infarction, could be reduced.

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13.

Concentration-Dependent Effects of Thioredoxin Reductase-1 Inhibition on Heme Oxygenase-1 Expression by Lung Epithelial Cells

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Bronchopulmonary dysplasia (BPD), characterized by disrupted lung development, it is the most common respiratory morbidity in premature infants. Recently published in vivo data from our lab revealed that thioredoxin reductase-1 (TrxR1) inhibition in a newborn mouse model of BPD activated nuclear E2-related factor (Nrf2), induced heme-oxygenase-1 (HO-1), and improved lung development. We have consistently observed disproportionate increases in HO-1 induction by TrxR1 inhibitors in vivo and in vitro when compared to other Nrf2-regulated genes. The present studies were designed to test the hypothesis that TrxR1 inhibition induces HO-1 in lung epithelial cells in a concentration--dependent manner. Murine transformed club cells (mtCC) were treated with the increasing concentrations (0.1 to 1 μM) of the TrxR1 inhibitor auranofin (AFN) or vehicle control for 4 hours. Cell lysates were collected at the conclusion of treatment and HO-1 protein expression was determined by western blot. Data (mean+-SEM) were log transformed and were analyzed by one-way ANOVA with Tukey's multiple comparison post hoc (n=3). Significance was accepted at $p < 0.05$. Our data indicated concentration-dependent increases in HO-1 expression in AFN-treated cells. Specifically, HO-1 expression was significantly greater with cells treated with 0.25 μM AFN than in control-treated cells (2.56+-0.13 vs 1.03+-0.11; $p<0.0001$). We observed an additional increase in HO-1 expression in cells treated with 0.75 μM AFN when compared to cells treated with 0.25 μM AFN (3.43+-0.2 vs 2.56+-0.13; $p=0.0148$). HO-1 expression was not

significantly different between cells treated with 0.75 μM or 1 μM AFN. Consistent with our hypothesis, our data revealed a concentration-dependent effect of AFN on HO-1 protein expression in lung epithelial cells. Additional studies are currently underway to assess the time-dependent effects of AFN on HO-1 induction by AFN. Future mechanistic studies will determine the contribution of Nrf2 toward HO-1 induction by TrxR1 inhibitors and will determine the specific role of HO-1 in pulmonary protection by TrxR1 inhibitors in vitro and in vivo. Collectively, our data support the potential clinical utility of therapeutic TrxR1 inhibition to protect against lung injury and to improve lung development in prematurely born infants.

14.

Heme oxygenase-1 downregulation is associated with endothelial dysfunction in scleroderma

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Systemic Sclerosis (SSc) or scleroderma is a complex, autoimmune connective tissue disease that leads to fibrosis of the skin and internal organs. Although tissue fibrosis is the hallmark of SSc, endothelial cell (EC) dysfunction is a very early pathological manifestation of this disease (1,2).
The most important profibrotic growth factor involved in SSc is transforming growth factor (TGF)-β, however the putative link between aberrant TGF-β signalling and EC dysfunction is still not clear. Recent studies have shown that TGF-β suppresses the expression of heme oxygenase (HO)-1 by displacing the Nrf-2 transcription factor away from the antioxidant response element, the promoter of HO-1 and other cytoprotective genes (3).
Here we explored whether the inhibitory effect of TGF-β on HO-1 expression could be the link between aberrant TGF-β signalling and the endothelial dysfunction associated with the pathogenesis of SSc.
Using immunohistochemistry we examined the expression of HO-1 in both skin and lung biopsies from healthy controls and patients with SSc. HO-1 expression was less prominent in the skin of SSc patients. By contrast, HO-1 was only detected in the areas of SSc lung biopsies infiltrated by immune cells. Expression of HO-1 was assessed by western blotting (n=4) and quantitative PCR (n=3) in skin fibroblasts isolated from SSc patients and healthy subjects under normoxia (control), chronic hypoxia (CH, 1% oxygen for 24h to induce HO-1 expression), TGF-β (5ng/mL), and the combination of CH+TGF-β. Basal HO-1 expression was downregulated in SSc fibroblasts compared with healthy controls. HO-1 expression was upregulated by CH and not significantly affected by TGF-β. By contrast, TGF-β reversed the CH-dependent upregulation of HO-1 expression under CH.
Importantly our results show that aberrant TGF-β signalling within SSc is responsible for the downregulation of HO-1, and might be responsible for the endothelial dysfunction associated with this condition.

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15.

Effects of H2S on the Heme Coordination Structure and Catalytic Activity of the Heme-based Gas Sensor Proteins

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Heme-based gas sensor proteins participate in important bacterial functions such as antibiotic resistance, sporulation, biofilm formation or virulence. Since these bacterial properties affect human\'s life their regulation pathways have become an important target for studying. Heme-based gas sensor proteins are always composed of at least two domains: N-terminal heme containing domain which bounds the signal molecule and C-terminal functional domain with catalytic or transcriptional activity. According to the heme iron ligand and/or redox state in the N-terminal domain the C-terminal domain is either active or inactive. It was described how the presence of gases such as O2, CO or NO influence activity of these proteins. As H2S has been reported as an important biological signal in some systems, we have decided to examine the effect of H2S on heme coordination structure and catalytic activity of several heme-based gas sensor proteins. For this purpose we have chosen three model heme-based gas sensor proteins to study, namely a globin-coupled histidine kinase from Anaeromyxobacter sp (AfGcHK), a globin-coupled diguanylate cyclase from Escherichia coli (YddV) and a direct oxygen sensor from Escherichia coli (EcDOS). As two of these proteins have globin structure of sensor domain and one has the PAS fold, it is interesting to compare their interactions with H2S. For this part of our research we used optical absorption spectroscopy and mass spectrometry. The second part of the research was to determine how the presence of H2S influenced the catalytic reactions of each protein. It will be also discussed how our findings can contribute to explanation of molecular mechanism of signal transduction between the N-terminal and C-terminal domain of heme-based gas sensor proteins.
References: Du Y et al. (2013) BioMetals 26, 839; Yan F. et al.(2015) BioMetals 28, 637; Fojtikova V et al. BioMetals submitted
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16.

Anti-inflammatory and Pro-healing Activities of HYCOs, Novel Nrf2/HO-1 Activators that Simultaneously Release Carbon Monoxide

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Oxidative stress and inflammation are constant features of many chronic disorders including skin diseases and diabetes. Despite many advances in the treatment of these pathologies, patients' morbidity and mortality remains high indicating the need to develop more efficient therapeutic approaches. Our group is currently working on drug discovery strategies that target endogenous antioxidant and anti-inflammatory pathways crucial for the cellular reparative response. Specifically, we are focusing on the nuclear factor-erythroid 2-related-factor 2 (Nrf2), a transcription factor controlling the expression of several detoxifying and cytoprotective genes, including heme oxygenase-1 (HO-1). HO-1 converts heme to biliverdin, iron and carbon monoxide (CO), bioactive products that contribute to its antioxidant and anti-inflammatory effects. Small molecules activators of the Nrf2/HO-1 axis have been shown to ameliorate the function of cells and

tissues subjected to damaging stressful stimuli. Likewise, delivery of CO *via* CO-releasing molecules (CO-RMs) prevents or diminishes tissue injury caused by inflammation and oxidative stress. We are currently working on the synthesis and characterization of a new class of molecules that activate Nrf2/HO-1 and simultaneously release CO. These compounds were termed hybrid CO-releasing molecules (HYCOs), and their dual activity was obtained by coordinating a CO-RM moiety to known Nrf2/HO-1 inducers (1,2). Fourteen HYCOs were characterized *in vitro* by measuring their ability to release CO to cells, their capacity to induce the Nrf2/HO-1 axis, cell toxicity and their anti-inflammatory effect using human THP-1 monocytes and HaCaT keratinocytes. Our data show that five among the 14 compounds tested were able to significantly induce Nrf2 and HO-1 protein expression and release CO in both cell types. In addition, HYCO-6, HYCO-7 and HYCO-13 decreased the production of pro-inflammatory markers such as TNF-α, IL-8, IL-6 and IL-1β in THP-1 cells challenged with lipopolysaccharide. When a linear wound was created in keratinocytes in culture, HYCO-6 and HYCO-13 accelerated wound closure in a concentration-dependent manner. In addition, preliminary data show that oral administration of HYCO-6 was able to induce Nrf2 and HO-1 protein expression in the skin and accelerated the closure of wounds in a murine model of cutaneous wound healing. Our results suggest that HYCOs are pharmacologically active and possess anti-inflammatory and protective properties both *in vitro* and *in vivo*.

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17.

THE ROLE OF HEME OXYGENASE-1 IN NEONATAL SEPSIS

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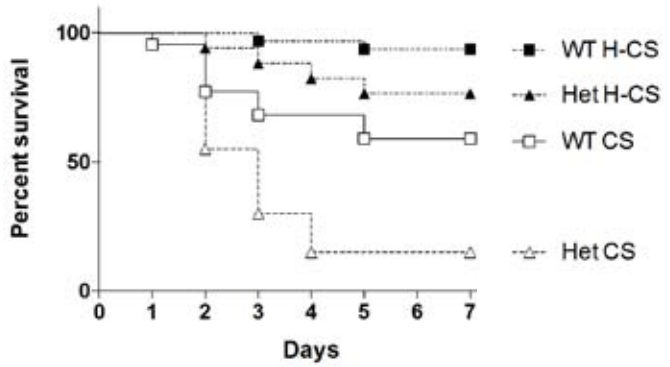
Sepsis in preterm infants is characterized by an initial bacterial invasion followed by a systemic inflammatory response. Its pathophysiology has not been well elucidated due to the lack of appropriate animal models. Heme oxygenase-1 (HO-1), a stress response protein, can protect against many physiologic and pathologic conditions during the newborn period through its anti-inflammatory, antioxidative, and anti-apoptotic properties. Therefore, we investigated the role of HO-1 in neonatal sepsis using a preterm sepsis mouse model. To induce sepsis, the non-surgical cecal slurry (CS) model was applied to 4d-old mouse pups, whose age is equivalent to that of human preterm infants. In brief, adult cecums were first harvested, and then their contents were diluted with PBS-glycerol to 100 mg/mL for CS stock preparations. HO-1 heterozygote (Het, HO-1+/-) and wild-type (WT) mice were administered CS IP at a dose of 2.0 mg/g (LD40 for WT mice based on preliminary studies), and then monitored for survival for up to 7d. To study the protective role of HO-1, 30-μmol heme/kg was given subcutaneously to 3d-old pups of both genotypes 24h prior to sepsis induction and monitored for survival. Liver HO activity of both genotypes was determined via gas chromatography, and gene expression profiles were measured using PCR arrays and then compared between all groups for both genotypes. Treatment with 2.0-mg CS/g caused a significantly higher mortality in Het (85.0%, n=20) than in WT (40.9%, n=22, $p<0.01$) pups. 24h after heme administration, liver HO activity increased 64% and 55% over age-matched WT and Het control pups (496±85, n=10, and 328±36, n=13, $p<0.01$, respectively). Most importantly, pre-treatment with heme significantly reduced mortality in both WT and Het pups (6.3%, n=32 and 23.5%, n=17, respectively). In addition, expression profiles measured 6h post-sepsis induction showed significant increases in cytokines, pattern recognition receptors, and other immune-related genes in both genotypes. These increases were attenuated in pups of both genotypes pre-treated with heme. Because HO-1 deficiency is associated with an increase in mortality following sepsis induction and HO induction significantly reduces mortality, we conclude that HO-1 may confer protection against sepsis in preterm infants.

Figure. Survival curves of 4d-old WT and Het pre-treated with or without heme (30 μmol/kg BW) prior to sepsis induction.



18.

Transcriptional Regulation of Transferrin Receptor by Heme in Erytroid Cells

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The function of cell surface transferrin receptor (TfR) is to mediate cellular iron uptake from plasma glycoprotein, transferrin (Tf). In proliferating nonerythroid cells, TfR expression is negatively post-transcriptionally regulated by intracellular "free" iron (also referred to as "labile iron pool", LIP) through iron-responsive elements (IREs) localized in the 3' untranslated region (UTR) of TfR mRNA. IREs are recognized by iron regulatory proteins (IRP-1 and IRP-2), which are specific cytoplasmic RNA-binding proteins that respond to cellular iron levels. At low iron levels IRPs bind to IREs in the 3' UTR of TfR mRNA preventing degradation of the transcript. On the other hand, the expansion of the LIP inactivates the binding of IRP-1 to IREs and leads to a degradation of IRP-2, resulting in a rapid disintegration of TfR mRNA.
Erythroid cells are the largest consumers of iron which is delivered to them exclusively by Tf *via* TfR. Developing red blood cells regulate TfR expression not only at the level of mRNA stability (please see above), but also by transcription[1]. Recently, we have provided unequivocal evidence that heme oxygenase 1 (HO1) is present in erythroid cells where it controls intracellular regulatory-heme levels[2]. Modulation of HO1 expression caused changes in intracellular heme levels and, consenquently, alterations in TfR expression in erythroid cells[2]. For example, HO1-deficient erythroid cells had significantly increased TfR mRNA and protein levels[2].

Here we provide evidence that TfR expression and cellular uptake of iron from Tf is stimulated by enhanced heme synthesis. Incubation of erythroid cells with 5-aminolevulinic acid (ALA) increased TfR expression as well as iron incorporation into heme. This effect of ALA can be completely prevented by the inhibitors of heme synthesis (succinylacetone [inhibits ALA dehydratase] or N-methylprotoporphyrin [inhibits ferrochelatase]), indicating that the effect of ALA requires its metabolism to heme. The induction of TfR mRNA expression by ALA is a result of increased mRNA synthesis since the effect of ALA can be abolished by actinomycin D. Additionally, TfR promoter was activated *in vitro* by addition of ALA and hemin in differentiated (DMSO) murine erythroleukemia cells. Importantly, site-directed mutagenesis of erythroid active element[1] in TfR promoter abolished the heme mediated effects of ALA and hemin. These results suggest that heme may directly or indirectly interact with TfR promoter activating gene expression. Hence, our results indicate that in erythroid cells heme serves as a positive feedback regulator that maintains high TfR levels thus ensuring adequate iron availability for hemoglobin synthesis.

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[2]Garcia-Santos D, et al. (2014) Heme oxygenase 1 is expressed in murine erythroid cells where it controls the level of regulatory heme. *Blood* 123: 2269.

19.

Inflammation-driven Heme/Iron Cytotoxicity In Parkinson's Disease

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Heme is a metallo-compound, essential for the survival of most organisms. However, the ability of the central iron (Fe) atom, contained within its protoporphyrin ring, to participate in the Fenton reaction and generate highly reactive hydroxyl radicals renders heme potentially toxic. Under inflammatory conditions, the release of heme from hemoproteins leads to an exacerbated oxidative stress, a deleterious effect associated with tissue damage and disease severity. As immune-mediated inflammatory diseases often cause a certain degree of hemolysis and vascular leakage, it is reasonable to assume that the cytotoxicity of heme/Fe may also affect less accessible organs, such as the brain, thus increasing the risk and severity of neurodegenerative diseases. This hypothesis has been investigated in mice, in which the exogenous administration of heme or the release of this molecule upon inflammation/infection was shown to enhance the severity of Parkinson's disease (PD). An increased susceptibility to pharmacologic PD was observed when mice are exposed to heme and subsequently treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin precursor that, once converted into the active metabolite, irreversibly damages the dopaminergic neurons (DNs). Our results demonstrate that from circulation, heme is capable to enter the brain and trigger neuroinflammation. While this is associated with the recruitment of immune cells, which breach the BBB and enter the brain, an increased level of circulating heme was shown to contribute to the occurrence of brain Fe overload, causing an exacerbated locomotor dysfunction in response to PD induction. Therefore, our findings suggest that the release of heme in circulation upon immune-mediated inflammatory diseases is capable to prime the brain and contribute to the development of neurodegenerative diseases, such as PD.

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20.

HO-1 GOVERNS THE CYTOSKELETON AT FILOPODIA: PULLING THE BRAKES ON THE MIGRATORY CAPACITY OF PROSTATE TUMORAL CELLS

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Prostate Cancer (PCa) is the second leading cause of cancer in men. The misregulation of the expression of cytoskeletal proteins plays an important role in cancer, augmenting the capacity to colonize distant organs and metastasize, as well as conferring resistance to chemotherapy. We have previously showed that heme-oxygenase 1 (HO-1) has a strong anti-tumoral effect in PCa. High-throughput proteomic platforms are now identifying and quantifying new specific biomarkers for PCa detection, stratification and treatment. In an effort to identify HO-1 molecular partners associated to the integrity of the cellular architecture and assess actin dynamics of PCa cells under HO-1 modulation, we undertook an in-depth mass spectrometry-based proteomics study to build the HO-1 interactome in PCa,
The proteomics analysis of HO-1 interacting factors revealed several cytoskeletal-associated proteins involved in the regulation of actin filament dynamics. We also performed a bioinformatic screening across the *Oncomine* platform, which showed that the RNA expression profiles of the cytoskeletal HO-1 interacting proteins lie within the 22 percent of the most consistently low or high-expressed genes in prostate adenocarcinoma compared to normal prostate tissue. We took advantage of 2D migration assays to assess motility changes. As a result, we observed a reduced frequency in migration events, trajectory and cell velocity under hemin exposure. Moreover, a significant higher proportion of filopodia-like protrusions among neighboring cells and cell-cell contacts were observed under HO-1 modulation. Forced-expression of HO-1 resulted in an alteration of cell protrusions in transwell co-culture systems of PC3 cells with MC3T3 cells (pre-osteoblastic cell line). Altogether, we demonstrate for the first time that HO-1 interacts with cytoskeletal proteins highly misregulated in PCa, alters cell migratory patterns, induces the remodeling of the actin filament architecture at filopodia towards a less invasive phenotype, showcasing its relevance as a key homeostatic factor against the aggressive and metastatic disease.

21.

Possible Connection Between Heme Oxygenase-1/CO System and Autophagy in Cardiomyocytes

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Diseases affecting the cardiovascular system are still a serious public health problem, which is most often caused by the ischemic of the myocardium. Several group have also demonstrated that the increased activity of heme oxygenase-1/CO system and autophagy could protect the myocardium against ischemic events. However, it is still not clear whether a connection between HO-1/CO system and autophagy processes exists in the myocardium.
In the current project we aimed to examine the possible connection between the two systems. H9c2 cardiomyoblast cells were treated with 100μM hem, or vehicle (20mM NaOH solution) for 24-h, and an untreated group was used as the control group. After the treatment of the cells, cell viability/cytotoxicity was measured by MTT assay. Furthermore,

FITC-conjugated phalloidin staining was carried out to determine the alterations in cell size after the treatments. To study the autophagic process CytoID staining was carried out and cells were studied by fluorescence microscope and by flow cytometry. Moreover, Western blot analysis was performed to study the level of HO-1, and certain autophagy related proteins.
We have detected a slight decrement in cell viability in the hem treated group, which may indicate the toxic effect of high concentration of hem. The cell size did not alter after the treatment. As it was expected a robust induction of HO-1 were detected in the hem treated group. An enhanced number of autophagosome were detected by CytoID staining, and elevated level of Lc3-II was found in the hem treated group.
Taken together, our results show that, there is a connection between HO-1/CO system and autophagy process, but further experiments (e.g, lower hem concentration or different HO-1 inducer) needs to be carried out to precisely understand the nature of the connection.
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22.

Heme Oxygenase-1 Expressed in Bone Marrow-derived Myeloid Cells Determines Cellular Responses to DNA Damage

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Radiation and chemotherapy used as treatment for cancer patients cause severe side effects due to injury to the healthy tissues. DNA damage in the intestinal epithelial cells may be irreversible and long-lasting even after the end of cancer therapy. Heme oxygenase-1 (HO-1) and heme degradation products are known to be protective homeostatic molecules. We have previously reported that HO-1/CO activates DNA repair via ATM-H2AX signaling pathway.

In current studies, we hypothesized that myeloid-derived HO-1 is critical for restoration of epithelial function in response to radio- or chemo- toxicity. We applied bone marrow transplant (BM Tx) using recipient mice lethally irradiated to eliminate myeloid cell. This dose of irradiation also induced DNA damage in multiple other proliferative tissues. Mice lacking HO-1 in myeloid cells (LysM-Cre:Hmox1fl/fl), or controls (Hmox1fl/fl) mice were used as donors of BM.

We have showed a higher levels of H2AXγ in colon and kidney in the recipients receiving transplant from LysM--Cre:Hmox1fl/fl compared to control Hmox1fl/fl mice. Apart from more DNA damage, the mice receiving LysM--Cre:Hmox1fl/fl bone marrow also had increased expression of p16INK4 and less Ki67 staining, suggesting lower proliferation of epithelium. Further, we demonstrated higher H2AXγ and p16INK4 in the macrophages from LysM--Cre:Hmox1fl/fl compared to Hmox1fl/fl mice in response to chemotoxin treatment. Lower macrophage function in LysM-Cre:Hmox1fl/l mice is a likely contributor of the decreased repair of epithelium, as BM Tx from control Hmox1fl/fl mice readily restored a repair.

The combination of impaired DNA repair and cell cycle inhibition in the organs of mice receiving bone marrow lacking HO-1 expression specifically in myeloid cells, suggests an important role for myeloid cells-derived HO-1 in recovery of gut epithelium following genotoxic treatments.

23.

Hemin Inhibits Acetylation of High Mobility Group Box 1 (HMGB1) through Upregulation of Histone Deacetylase 4 (HDAC4) in LPS-activated RAW 264.7 cells

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High mobility group box1 (HMGB1) plays an important role as a late mediated inflammatory cytokine of septic shock. We previously reported that heme oxygenase-1 (HO-1) inducers inhibit HMGB1 release in LPS-activated macrophage and CLP-induced septic mice. Although HMGB1 secretion modified by histone deacetylase (HDAC)s has been reported, precise role of HO-1 in this event is not clear. This study was designed to investigate whether hemin, HO-1 activator, inhibits the acetylation of HMGB1 through upregulation of HDACs in LPS-activated RAW 264.7 cells. Hemin significantly inhibited HMGB1 acetylation and secretion in LPS-activated RAW264.7 cells, whereas gene silencing of HO-1 reversed this result. HO-1 overexpression reduced in LPS-stimulated HMGB1 release along with increasing of HDAC4 expression. Hemin and HO-1 overexpression significantly increased HDAC4 mRNA and protein expression in a time- and dose-dependent manner but not HDAC1, 2 and 3 mRNA levels. Importantly, gene silencing of HO-1 abrogated hemin-induced HDAC4 expression, but knockdown of HDAC4 did not affect HO-1 expression, suggesting that HO-1 upregulates HDAC4 expression in RAW 264.7 cells. In addition, HDAC4 overexpression prevented LPS-stimulated HMGB1 secretion, concomitant with inhibiting the acetylation of HMGB1. LPS treatment enhanced HDAC4 phosphorylation and its translocation to cytoplasm which promoted ubiquitination and degradation of HDAC4 (but not mRNA), resulting in the release of HMGB1. Interestingly, hemin reversed in LPS-induced the phosphorylation, ubiquitination and degradation of HDAC4. Taken together, these findings indicate a novel mechanism by which HO-1 prevents the acetylation of HMGB1 by regulating HDAC4 in LPS-activated RAW264.7 cells.

Keywords: Heme oxygease-1, High mobility group box1, Histone deacetylase

24.

Carbon Monoxide Attenuates Alzheimer-related Phenotypes via the Inhibition of NF-κB-mediated BACE1 Expression

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The abnormal secretion and accumulation of amyloid peptides (Aβ), the major constituent of plaques, which is generated by sequential proteolytic cleavage of the amyloid precursor protein (APP) via β-secreatse (BACE1) and the γ-secretase complex, are thought to be the initial causative events in the development of Alzheimer's disease (AD). Previous studies have indicated that carbon monoxide (CO), a reaction product of heme oxygenase (HO)-1 activity, protects against Amyloid-β-induced toxicity and promotes neuroprotection. However, the signaling cascades involved in the carbon monoxide-induced mitigation in Aβ levels and BACE1 expression levels have not been elucidated. In this study, we show that carbon monoxide significantly reduced BACE1-mediated cleavage of APP and Aβ production by decreasing BACE1 gene expression *in vivo* and *in vitro*. CO markedly improved memory deficits in AD transgenic mice.

The regulation of BACE1 gene expression by CO was dependent on nuclear factor–kappa B (NF-κB) signaling. NF-κB activity is tightly regulated by the mammalian sirtuin SIRT1. In the presence of the SIRT1 inhibitor sirtinol, carbon monoxide releasing molecule (CORM) failed to elicit any significant decrease in BACE1 protein levels. We also found that CO inhalation attenuates elevation of BACE1 level in mouse brains after feeding a high-fat, high-cholesterol diet. CO decreased the cholesterol oxidation product 27-hydroxycholesterol (27-OHC)-induced NF-κB activation and BACE1 expression. These data suggest that CO reduced the NF-κB-mediated transcription of BACE1 and consequently decreased amyloid-β genesis.

25.

Carbon Monoxide Enhances Mitophagy and Mitochondrial Biogenesis via TFEB/3 and PGC-1α Induction

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During last decade, several groups showed that heme oxygenase-1 (HO-1)/carbon monoxide (CO) system mediates anti-diabetic, anti-obestogenic and anti-hypertensive effects as well as anti-septic, anti-inflammatory, anti-oxidative and mitochondrial biogenic properties. Mechanistically, HO-1/CO system increases adiponectin, pAKT, pAMPK and PPARγ in adipocytes among metabolic tissues.
The author's group reported that CO stimulates only PERK branch among three of unfolded protein response (UPR) branches, and induces HO-1 expression via activation of Nrf2. Activated PERK phosphorylates eukaryotic translation initiation factor 2 alpha (eIF2α), which attenuates global translation except specific UPR-dependent genes, such as activating transcription factor 4 (ATF4). ATF4 promotes cell survival and metabolic homeostasis by inducing transcriptional upregulation of a number of genes required for autophagy, redox homeostasis and metabolic hormones such as FGF21 and Sestrin 2. Under starvation condition, inactivation of mTORC1 allows nuclear translocation of TFEB and TFE3, two transcription factors that mediate cellular adaptation to stress by promoting autophagy and mitochondrial biogenesis. In this study, we addressed whether HO-1/CO system promotes nuclear translocation of TFEB and TFE3 and this leads to increases of mitophagy and mitochondrial biogenesis. We found that CO induced PERK phosphorylation. And PERK-mediated activation of calcineurin promoted nuclear translocation of TFEB and TFE3. This leads to increased mitophagy and mitochondrial biogenesis via induction of PGC-1α.

26.

Carbon Monoxide-induced Sestrin 2 Improves Hepatic Steatosis Through Activation of Autophagy

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Nonalcoholic fatty liver disease (NAFLD), the hepatic manifestation of the metabolic syndrome, has become one of the most common causes of chronic liver disease over the last decade in developed countries. NAFLD includes a spectrum

of pathological hepatic changes, such as steatosis, steatohepatitis, advanced fibrosis, and cirrhosis. Autophagic function is decreased in the liver during the development of NAFLD. Although our previous reports showed that carbon monoxide (CO) has a benefical effect on NAFLD, its molecular mechanisms remain unclear. In the present study, we aimed to exam the effect of CO on methionine/choline-deficient (MCD) diet or medium-induced hepatic steatosis, to explore the possible mechanism. In the present study, we found CO, a reaction product of heme oxygenase (HO) activity, induces sestrin 2 and protects against methionine choline deficient (MCD)-induced NAFLD progression through activation of autophagy. Our results showed that levels of sestrin 2 expression were increased in PERK/eIF2α/ATF4 dependent manner after treatment with CO-releasing molecule (CORM). CO-induced sestrin 2 upregulation contributed to autophagy induction through AMPK activation and mTORC1 inhibition in hepatocyte cell lines. Furthermore, we investigated that CO significantly induces expression of sestrin 2 and enhances autophagy in the livers of mice fed the MCD diet or MCD medium. Conversely, knockdown of sestrin 2 abrogated the autophagy activation and mTOR inhibition by CO. These results indicate that CO improves hepatic steatosis through autophagy pathway induced by sestrin 2 upregulation.

27.

FGF21 Mediates the Metabolic Effects of Carbon Monoxide on Insulin Sensitivity and Energy Expenditure in Mice

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Carbon monoxide (CO), a diatomic low molecular weight gas, shows anti-inflammatory, anti-proliferative, and anti-apoptotic effects on a variety of cellular injury models. Also, it recently emerged as a regulator of glucose and lipid homeostasis and energy utilization. However, the mechanisms underlying the metabolic actions of CO remain unknown. Here, we demonstrate that the pluripotent metabolic hormone FGF21 is a downstream effector of CO. Treatments with CO enhanced both expression and secretion of FGF21 in hepatocytes, thereby increasing serum levels of FGF21 in mice. Importantly, FGF21-knockout mice were refractory to several beneficial effects of CO, including attenuation of ER stress-mediated hepatic steatosis and diet-induced obesity (DIO) associated hyperglycemia, hypertriglyceridemia, insulin resistance, and hepatic steatosis. Moreover, CO lowers blood glucose levels, enhances insulin sensitivity, increases mitochondrial fatty acid oxidation and promotes energy expenditure by stimulating beiging. Collectively, these data suggest that CO is a potent inducer of FGF21 expression and that CO critically depends on FGF21 to exert its energy consuming and insulin sensitizing effects.

28.

Organic Carbon Monoxide Prodrugs that Release CO under Physiological Conditions with Tunable Release Rates

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Acetaminophen (APAP) is a commonly used over-the-counter analgesic drug with an excellent safety profile when ad-

ministered in proper therapeutic doses. However, APAP overdose can cause potentially acute liver damage. The toxicity of APAP begins with a reactive metabolite that binds to cysteine sulfhydryl groups of proteins. This leads mainly mitochondrial oxidant stress and dysfunction. Carbon monoxide (CO) has anti-oxidant, anti-inflammatory, anti-apoptotic and cyto-protective properties. In this study, we hypothesized that carbon monoxide ameliorates APAP-induced liver damage and ER stress. Our results indicated that CO reduced CHOP that mediates cell death induced by APAP overdose in AML12 cells and in liver tissues. Moreover, increased level of ALT in serum after challenge of APAP was dramatically declined by CO inhalation or CORM3 injection. Finally, ER stress induces CHOP mediated cell death by PERK/eIF2a/ATF4 signal of ER stress brunch in vitro. In conclusion, carbon monoxide protects against acetaminophen overdose-induced hepatic cell death.

29.

Pterostilbene 4′-glucoside Protects Mice from LPS-induced Acute Lung Injury via Induction of Heme Oxygenase-1

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Demethylether analog of resveratrol, pterostilbene, is well known to exhibit effects of anti-inflammation, anti-oxidation and cell proliferation. Heme oxygenase-1 (HO-1), a stress-inducible protein, has potential anti-inflammatory effect. Acute lung injury is characterized by an acute inflammatory process in the airspaces and lung parenchyma. In this study, we suggest that pterostilbene 4′-glucoside (4-PG), as a compound of pterostilbene, could benefit LPS-induced acute lung injury attenuation through induction of HO-1. HO-1 expression was increased with 4-PG in murine macrophage cells. 4-PG decreased levels of pro-inflammatory cytokines such as TNF-α and IL-1β in vitro. To investigate the role of HO-1 in the anti-inflammatory effects of 4-PG, we utilized LPS-induced acute lung injury models in HO-1 WT and KO mice. The expression of HO-1 and pro-inflammatory cytokines were determined in bronchoalveolar lavage fluids (BALF) and lung tissues. We found that 4-PG increased the expression of HO-1 while inhibited pro-inflammatory cytokines. Taken together, these findings suggest that 4-PG mediates anti-inflammatory effects on LPS-induced acute lung injury through induction of HO-1.

30.

Ferryl hemoglobin triggers inflammatory response upon intraventricular hemorrhage in premature infants

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Background: Intraventricular hemorrhage (IVH) that is bleeding inside or around the ventricles is most common in preterm infants and is the leading cause of neurodevelopmental impairment in these children. The molecular mechanism of post-IVH neuroinflammation and subsequent brain damage is largely unknown therefore we lack any therapeutic intervention to prevent the development of neurological disability following IVH. We hypothesized that hemolysis and hemoglobin (Hb) oxidation play critical roles in the pathomechanism of IVH-associated neuroinflammation. Hb oxidation leads to the formation of various Hb oxidation products including metHb (Fe^{3+}), ferrylHb (Fe^{4+}) and oxoferryl Hb (Fe^{4+}=O) species. Ferryl- and oxoferryl-species are unstable and return to the Fe^{3+}state by reacting with specific amino acids of the globin chains. In these reactions globin radicals are produced followed by termination reactions leading to the formation covalently crosslinked Hb multimers, referred here as ferrylHb. MetHb and ferrylHb can release heme moiety, that exhibit pro-oxidant and pro-inflammatory activities.
Methods: Cerebrospinal fluid (CSF) were collected by spinal trap or ventricular reservoir puncture on day 1-3 (early) or day 5-7 (late) post-IVH from preterm infants (n=30, mean gestational age at birth: 28.1 week). Concentrations of Hb oxidation products were determined spectrophotometrically, covalently crosslinked Hb forms were detected by Western blot. Levels of soluble adhesion molecules (ICAM-1, VCAM-1, E-selectin) were measured by ELISA.
Results: We detected more Hb in late CSF compared to early CSF (27.7 ± 4.5 vs. 8.9 ± 1.1 µmol/L). MetHb concentration was below 1 µmol/L in early CSF, but was markedly elevated in late CSF (57.2 ± 10.0 µmol/L). Covalently crosslinked Hb was undetectable in early CSF, but were present in high amounts in late CSF samples. Non Hb-bound heme concentration was 3.2 ± 0.9 µmol/L in early CSF, and was markedly elevated in late CSF (106.3 ± 24.7 µmol/L). FerrylHb content of CSF samples strongly correlated with the levels of sVCAM-1, sICAM-1 and sE-selectin. In endothelial cells, late, but not early CSF samples induced the expression of VCAM-1, ICAM-1 and E-selectin and triggered intercellular gap formation and monocyte adhesion.
Conclusion:
Following IVH, hemolysis and oxidation of Hb occurs. We identified ferrylHb, as the ultimate oxidized Hb form driving the post-IVH inflammatory response.

31.

Valproic Acid Downregulates Heme Oxygenase-1 Independently of Nrf2 by Increasing Ubiquitination and Proteasomal Degradation

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Aims. Heme oxygenase-1 (HO-1; HMOX1 in human, Hmox1 in mice) is an antioxidative enzyme affecting wide range of sub-cellular processes. It was shown to modulate tumor growth or vascular-related diseases, thus being putative molecular target for tailored therapies. Therefore it is of importance to elucidate novel compounds regulating HO-1 activity/expression and to delineate mechanisms of their action. In the present study we aimed to understand mode of action of valproic acid (VA), an antiepileptic drug, on HO-1 expression.
Results. We demonstrated that HO-1 expression is decreased by valproic acid at protein but not mRNA level in human rhabdomyosarcoma cell lines: SMS-CTR (embryonic) and CW9019 (alveolar). Nrf2 transcription factor,the activator of HO-1 expression, was not inhibitedalthough its repressor Bach1 was upregulated in response to VA. Further analyses showed that miRNAs predicted to target HMOX1 were downregulated excluding miRNA-dependent inhibition as a mechanism of HMOX1 regulation. However, co-immunoprecipitation assay showed higher level of ubiquitinated HO-1 after VA treatment. Accordingly, MG132, an inhibitor of proteasomal degradation, reversed the effect of VA on HO-1 suggesting that decrease in HO-1 expression by VA is through protein stability.
The inhibitory effect of VA on HO-1 was also observed in murine cells including embryonic fibroblasts isolated from Nrf2-deficient mice, what confirmsNrf2-independent effect of the compound. Importantly, VA decreased also HO-1 expression and activity in murine skeletal muscles in vivo.
Innovation. The study describes the mechanism of VA action on heme oxygenase-1 expression and indicates its potential application for treatment of diseases associated with overexpression of HO-1.

Conclusion. Our data indicate that VA downregulates HO-1 independently of Nrf-2 transcription factor by acting through ubiquitin-proteasomal pathway leading to decrease in protein level.

32.

Carbon Monoxide Regulates Endothelial Cells Bioenergetics

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Carbon monoxide (CO), a ubiquitous and versatile signaling molecule produced in mammalian cells by heme oxygenase enzymes, can affect cell metabolism. We have previously demonstrated that CO induced a two-component metabolic response in endothelial cells: uncoupling of mitochondrial respiration and inhibition of glycolysis [1], but the mechanisms involved are not completely understood. Using a CO-releasing agent (CORM-401), we characterized the acute effects of CO on bioenergetics and metabolism in intact EA.hy926 endothelial cells by live cell imaging techniques. Treatment of cells with CORM-401 did not affect the production of reactive oxygen species but induced a mild mitochondrial depolarization. In addition, CORM-401 increased mitochondrial calcium content and accelerated NADH consumption as well as FAD production, confirming increased activities of complexes I and II. The observed effects were accompanied by enhanced ATP production through oxidative phosphorylation and reduced ATP production from glycolysis. An inactive CORM-401 depleted of CO induced neither of these effects, thus supporting a direct role of CO in the observed mitochondrial and metabolic activities. In conclusion, we demonstrated that quiescent endothelial cells rely primarily on glycolysis, maintaining their mitochondrial membrane potential by glycolysis-derived ATP. In the presence of CO, mitochondrial calcium increases leading to activation of mitochondrial respiration and a shift in ATP production from glycolysis to oxidative phosphorylation.

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33.

Colonic microbial ecology contributes to suppression of intestinal inflammation in Bach1-deficient mice.

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Background Inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease, is a chronic and recurrent inflammatory disorder of the intestinal tract. Although, the precise pathogenesis of IBD remains unclear, the colonic microbial ecology potentially affect onset of IBD. BTB and CNC homolog 1 (Bach1) is a transcriptional repressor of heme oxygenase-1 (HO-1), meaning that HO-1 expression is constitutively high in Bach1-deficient mice. Our previous report show that deficiency of Bach1 inhibited intestinal inflammation through the regulation of macrophage function. More recently, it has been reported that carbon monoxide, which is one of the byproduct of heme degradation, regulates microbial clearance in colonic lumen. In this study, we investigate that whether the microbial ecology of Bach1-deficeint mice contributes to the inhibition of intestinal inflammation by using the co-housing experiments.

Methods Mice were treated with 2,4,6-trinitrobenzene sulfonic acid (TNBS) to induce intestinal inflammation, and the respective degrees of mucosal injury were evaluated macroscopically, histologically, and biochemistry. For co-housing experiments, age- and gender-matched WT and Bach1-deficient mice were co-housed at 1:1 ratios for 4 weeks, followed by obtaining the fresh stool samples from each mouse.

Results TNBS-induced colonic damage was suppressed significantly in single-housed Bach1-deficient mice. However, co-housing of Bach1-deficient mice with WT mice resulted in development of comparably severe colitis in single-housed Bach1-deficient mice. Regarding the microbial composition, we analyse currently under intense investigation.

Conclusion Co-housing experiments revealed that the colonic microbial ecology of Bach1-deficient mice may contribute to inhibition of intestinal inflammation.

34.

Effects of Sirtuin-activating compounds on Heme Oxygenase 1 in D-Galactosamine/Lipopolysaccharide--induced hepatotoxicity in rats

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Our previous studies have shown that STACs (Sirtuin-activating compounds) attenuate D-Galactosamine/Lipopolysaccharide (D-GalN/LPS)-induced hepatotoxicity through a mechanism that has not yet been fully elucidated. The aim of the present study was to investigate the role of Heme Oxygenase 1 (HMOX1) in the cytoprotective effects of Quercetin (dietary polyphenol) and SRT1720 (synthetic SIRT1 activator). Male Wistar rats were randomly assigned into 6 groups: (1) Control, (2) Quercetin, (3) SRT1720, (4) D-GalN/LPS, (5) Quercetin + D-GalN/LPS and (6) SRT1720 + D-GalN/LPS. After twenty-four hours, the effects of these treatments were evaluated by biochemical studies, real-time PCR and Western blot. D-GalN/LPS treatment downregulated SIRT1 expression and drastically upregulated HMOX1. Bilirubin and other conventional markers of liver injury (such as aminotransferases and conjugated dienes) were also markedly increased. Pretreatment of D-GalN/LPS rats with Quercetin and SRT1720 increased SIRT1 expression and significantly decreased HMOX1 expression and the aforementioned markers. Collectively, these findings show that dramatic upregulation of HMOX1 and concomitant bilirubin upsurge may be involved in the liver-toxic effects of D-GalN/LPS. STACs act in part through HMOX1 downregulation and drugs that modulate HMOX1 could provide a pharmacological means against experimental liver intoxication. This study was supported by the research program PRVOUK-P25/LF1/2 and grant GAUK-916314.

35.

Crocin Inhibits Nitric Oxide Production by Upregulation of Heme Oxygenase-1 via Calcium/Calmodulin--Dependent Protein Kinase 4

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Crocin is a water-soluble carotenoid pigment that is primarily used in various cuisines as a seasoning and coloring agent, as well as in traditional medicines for the treatment of edema, fever, and hepatic disorder. In this study, we demonstrated that crocin markedly induces the expression of heme oxygenase-1 (HO-1) which leads to an anti-inflammatory response. Crocin inhibited inducible nitric oxide synthase (iNOS) expression and nitric oxide production via

downregulation of nuclear factor kappa B activity in lipopolysaccharide-(LPS-)stimulated RAW 264.7 macrophages. These effects were abrogated by blocking of HO-1 expression or activity. Crocin also induced Ca2+ mobilization from intracellular pools and phosphorylation of Ca2+/calmodulin-dependent protein kinase 4 (CAMK4). CAMK4 knockdown and kinase-dead mutant inhibited crocin-mediated HO-1 expression,Nrf2 activation, and phosphorylation of Akt, indicating that HO-1 expression is mediated by CAMK4 and that Akt is a downstream mediator of CAMK4 in crocin signaling. Moreover, crocin-mediated suppression of iNOS expression was blocked by CAMK4 inhibition. Overall, these results suggest that crocin suppresses LPS-stimulated expression of iNOS by inducing HO-1 expression via Ca2+/calmodulin-CAMK4-PI3K/Akt-Nrf2 signaling cascades. Our findings provide a novel molecular mechanism for the inhibitory effects of crocin against endotoxin-mediated inflammation.

36.

The Role of HO-1 in Regeneration of Skeletal Muscle after Cardiotoxin-Induced Injury

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Heme oxygenase-1 (HO-1) is a heme-degrading enzyme of well-known anti-inflammatory and cytoprotective properties. We have found that it also strongly influences murine skeletal myoblast differentiation in vitro, inhibiting the expression and activity of MyoD (muscle regulator transcription factor) and expression of myomirs (the group of muscle specific miRNA: miR-1, miR-133a, miR-133b, miR-206), while increasing mir-146a (*Kozakowska et al, Antioxid Redox Signal 2012*). The aim of the present study is to investigate the role of HO-1 in skeletal muscle regeneration.
Gastrocnemius muscles of HO-1+/+ and HO-1-/- 3-month-old mice were injected with cardiotoxin (CTX, 25 µl of 20 µM) to induce sterile muscle injury. In HO-1+/+ mice the expression of HO-1 was strongly induced at day 1 after injection, followed by steady decrease to the normal level at 28 day after injury. Lack of HO-1 was associated with higher muscle degeneration (evidenced by higher levels of lactate dehydrogenase and creatine kinase in the plasma) and inflammation (assessed by increased level of WBC, monocytes and granulocytes in peripheral blood, semi-quantitative histopathological analysis of gastrocnemius muscle sections and associated with elevated protein level of IL-6, IL-1b and MCP-1 in skeletal muscle tissue on the 1st and 3rd day after injury). Interestingly, lack of HO-1 decreases the ratio of M1/M2 macrophages on the 1st day after injury. Flow-cytometry analysis revealed no changes in the number of muscle satellite cells (mSC; CD45-CD31-Sca1-a7i+CD34+) on the 1st, 3rd and 28th day after injury, whereas population of activated mSC (CD45-CD31-Sca1-a7i+CD34-) was decreased on the 3rd day in HO-1-/- mice. At those stages HO-1-/- mSC proliferated better what may be associated with the changed proportion of M1/M2 macrophages observed in skeletal muscle upon regeneration. This effect can be also mimicked in vitro, by the stimulation of cells with mitogenic IL-1b, IL-6, MCP-1 - the group of cytokines that were found to be elevated in vivo. Precocious rounds of activation of HO-1-/- mSC throughout the lifetime can lead to the premature exhaustion of their population, as evidenced by decreased number of mSC in 2-year-old animals lacking HO-1. Lack of HO-1 affects inflammatory reaction in skeletal muscle leading to increased proliferation of mSC. In turn, precocious activation of mSC may lead to exhaustion of their pool.

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37.

Antioxidant Potential of CORM-401 in Murine Intestinal Epithelial MODE-K Cells under Oxidative Stress

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Abstract

NADPH oxidase (NOX) and mitochondrial complexes (I and II) are the major sources of reactive oxygen species (ROS) production contributing to tumor necrosis factor (TNF)-α/cycloheximide (CHX)-induced apoptosis in mouse MODE-K intestinal epithelial cells (IECs). Carbon monoxide (CO)-releasing molecules (CO-RMs) are developed to liberate controlled amounts of CO in biological systems, to mimic the antioxidant and cytoprotective effects of CO. These effects of CO may be linked to its binding to oxidative hemoproteins such as NOX and mitochondrial complexes. At mitochondrial level, CO can induce a transient burst of superoxide anion ($O_2^{\bullet-}$) production thought to promote a preconditioning state, allowing to counteract subsequent oxidative stress. We previously reported that the water-soluble CORM-A1 reduces both TNF-α/CHX-induced ROS production and apoptosis in MODE-K IECs; in cytoprotective concentration, it only inhibits NOX-induced ROS production by TNF-α without induction of mitochondrial $O_2^{\bullet-}$ *per se*. CORM-401 is a recently developed water-soluble compound, which can release up to three CO per mole of compound and in vitro can release CO faster under oxidative conditions. The influence of CORM-401 was now assessed in MODE-K cells using TNF-α/CHX-, hydrogen peroxide (H_2O_2)-, rotenone- and antimycin-A-induced ROS generating systems. Intracellular total ROS and mitochondrial $O_2^{\bullet-}$ production levels together with cell death were assessed by flow cytometry. Additionally, the influence on TNF-α/CHX-induced changes in mitochondrial membrane potential (Ψ_m) and mitochondrial function was studied. CORM-401 (50 µM) did not increase basal mitochondrial $O_2^{\bullet-}$ or intracellular total ROS production during 3 h of incubation. CORM-401 partially reduced both TNF-α/CHX-induced total cellular ROS production and cell death, without influencing TNF-α/CHX-induced mitochondrial $O_2^{\bullet-}$ production. CORM-401 decreased antimycin-A-induced but not rotenone-induced mitochondrial $O_2^{\bullet-}$ production; it also did not influence TNF-α/CHX-induced mitochondrial depolarization and mitochondrial dysfunction. When tested versus a cytotoxic concentration of H_2O_2 (7.5 mM incubated for 1 h), co-treatment of MODE-K cells with CORM-401 during the 1 h exposure to H_2O_2 clearly reduced H_2O_2-induced ROS production and cell death while CORM-A1 (100 µM) had no influence. Surprisingly, only pre-treating the MODE-K cells with CORM-401 during 1 h before exposure to H_2O_2 also was effective but to a lesser extent than co-treatment. Combined pre- and co-treatment showed the highest effectiveness. CORM-401 acts thus solely on NOX-derived ROS to protect MODE-K cells from TNF-α/CHX-induced cell death. It showed potential to release CO faster during severe oxidative stress conditions in MODE-K cells, which could be of therapeutic benefit for cytoprotection of IECs.

38.

Myeloid Cell Heme Oxygenase-1 Regulates the Transition of Acute Kidney Injury to Chronic Kidney Disease

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Acute kidney injury (AKI) is associated with a high risk of morbidity and mortality and an increased risk of progression to chronic kidney disease (CKD). CKD is characterized by deterioration of vital kidney function, inflammation, and fibrosis. We have previously demonstrated that expression of heme oxygenase-1 (HO-1) in myeloid cells mitigates damage and regulates inflammatory cell trafficking following AKI. For this study, we utilized a model of the AKI to CKD transition in mice by inflicting unilateral ischemia-reperfusion injury (for 30 minutes), while leaving the contralateral kidney intact and followed up for 3 weeks. This model is characterized by fibrosis with macrophage and lymphocytic infiltration, indicating a role for inflammation. Given the importance of macrophages in regulating kidney damage after AKI, we hypothesized that HO-1 deficiency in myeloid cells would lead to worse outcomes in the murine model of the AKI to CKD transition. We used cre-lox recombinant mice in which HO-1 is selectively deleted in myeloid cell populations (LysM-HO-1). Surprisingly, we found LysM-HO-1- /- mice were protected, exhibiting less proteinuria and renal fibrosis, when compared with floxed control mice (LysM-HO-1+/+) (urinary albumin-creatinine ratio 0.131 ± 0.019 and 0.188 ± 0.017 in LysM-HO-1-/- and floxed control mice, respectively, $n \geq 8$, $p = 0.04$). In addition, greater absolute numbers of bone marrow-derived macrophages (F4/80lowCD11bhi, $7.22x106 \pm 6x105$ versus $4.90x106 \pm 6.2x105$, $n \geq 5$, $p = 0.03$) and NK cells (NK1.1+, $4.51x106 \pm 6.2x105$ versus $2.44x106 \pm 4.5x105$, $n \geq 5$, $p = 0.02$) were observed in injured kidneys from LysM-HO-1-/- mice, indicating these cell types may be responsible for protection in this model. Further, myeloid cell HO-1 deficiency resulted in a trend toward lower proportions of pro-fibrotic tissue-resident macrophages (F4/80hiCD11blow, $12.14 \pm 1.3\%$ versus $16.56 \pm 1.6\%$, $n \geq 5$, $p = 0.07$). These studies demonstrate that HO-1 expression by myeloid cells regulates progressive kidney disease in the AKI to CKD model. These results have potential implications for developing cell- based therapy

39.

HMOX and hematophagy

Lima G
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Hematophagous insects ingest several times their body weight with each blood meal. However, they are able to survive the high toxicity imposed by free heme. HOs are present in the genomes of mosquitoes and tsetse flies that transmit a wide range of tropical diseases including malaria, dengue, zika and sleeping sickness. However, their role in blood feeding is unknown. Here, we have cloned and expressed HOs from the malaria vector Anopheles gambiae and Glossina morsitans morsitans, the vector for Human African Trypanosomiasis. HPLC analysis of midgut contents isolated from G. morsitans morsitans provided evidence for the degradation of heme and subsequent increase of biliverdin in the midgut of this insect during the first 3 days following a single blood meal. Comparison of recombinant recombinant HOs suggests that the heme oxygenase from G. morsitans morsitans degrades heme slower and binds heme differently from other hematophagous insects and humans but similarly to observed in D. melanogaster. Finally, antibodies raised against recombinant enzymes have been used to track the tissue distribution of HO in An. gambiae and G. morsitans morsitans. HO appears to be highly expressed in the midgut as well as reproductive organs and oenocytes. This work is shedding new light on role of HO in hematophagy.

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40.

Cyclically expressed heme oxygenase protects the fruit fly's retina against light-induced damage

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Circadian rhythms have been detected almost in all organisms. In animals, daily changes are observed in physiology, in biochemical processes and in behavior. Most of these, called circadian, are maintained in constant darkness (DD), have a period of about 24 h and are entrained by light/dark (LD) cycles.
Drosophila melanogaster is a model organism very useful in chronobiology research. In contrast to two isoforms of heme oxygenase found in mammals, in Drosophila there is only one gene encoding HO that plays an important role in development and in controlling the signaling pathway of DNA damage. Since HO may play a cytoprotective function and the retina is a site of intense physiological processes of phototransduction that generates high level of reactive oxygen species (ROS) we examined if this protein is cyclically expressed in the retina.
We have found that in the retina of Drosophila that holds an autonomous peripheral circadian clock, ho gene is rhythmically expressed, with two peaks at the beginning of the day and during the night. We concentrated on the elucidation of the role of HO in the retina in the morning, during the first peak of ho mRNA rhythm. This peak is maintained in light/dark (LD12:12) and in DD conditions. We found that 1h light pulse in DD enhances the level of ho expression. This effect was noted only at CT1 but not at other time points. Interestingly, the canonical phototransduction pathway is not involved in this process, since in norpA mutants (mutant of retina-specific phospholipase C leading to almost complete blindness) the level of ho mRNA was still increased after light exposure at CT1. On the other hand, this effect was clock-dependent because in per0 (mutant of main clock gene, completely arrhythmic) the expression of ho was activated by light not only at CT1 but also at other time points.
To conclude, ho gene expression is clock-controlled and the rhythm of ho mRNA is bimodal in the retina of wild-type flies. Moreover, the expression of ho is enhanced by light in the morning and this process is PER-dependent. The role of HO in the retina is unknown but there are evidences that it may protect photoreceptors from the oxidative stress caused by daylight in the morning.

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41.

Knockdown by shRNA or knockout by CRISPR/Cas9 – the comparison of both systems in heme oxygenase-1 targeting efficiency

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Genes knock-down (KD) through RNA interference for many years served as invaluable tool to study gene functions. However, with development of CRISPR/Cas system for genome editing the possibility to efficiently create gene knock-out (KO) cell lines was opened. In our work we compared shRNA and CRISPR/Cas9 systems to inhibit heme oxygenase-1 (HO-1) expression in terms of targeting efficiency and labor intensity. HO-1 is an inducible enzyme catabolizing heme to CO, Fe^{2+} and biliverdin which is subsequently converted to bilirubin by biliverdin reductase. Thus, in addition to the assessment of HO-1 on mRNA and protein level, the measurement of bilirubin concentration can be used as an indicator of HO-1 activity.
shRNA KD HEK 293 cell lines were developed using different shRNA sequences against HO-1 delivered by lentiviral vectors at various MOI. Transduction efficiency was checked by the assessment of GFP+ cells while basal and hemin-induced HO-1 inhibition was confirmed on mRNA, protein and activity level. For CRISPR/Cas9 three sgRNA sequences were first tested for gene targeting efficiency in puromycin-selected cell population. Cel-I nuclease assay showed targeting efficiency around 30% for each sgRNA. Two sgRNAs were used to establish clonal cell lines and out of 31 tested clones 22 were mutated (71%). Most of the clones did not express HO-1 as showed by western blot and failed to increase bilirubin concentration after hemin treatment.
In conclusion, we have shown that both technologies can be used for inhibition of HO-1 *in vitro*, however, CRISPR/Cas9 system exerts more prominent effect than shRNA.

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42.

Heme-associated Properties of BLVRB/FLR

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In the course of proteomic analysis of developing erythroid cells for studying the metabolism of heme and iron, we have observed dramatic up-regulation and high-level expression of biliverdin-IXbeta reductase or biliverdin reductase B (BLVRB), also known as NADPH-flavin reductase (FLR), during erythroid differentiation. It is intriguing that an enzyme apparently involved in the heme degradation pathway is highly expressed in cells with a high level of heme synthesis and content. BLVRB/FLR possesses both pharmacological importance for the treatment of certain forms of methemoglobinemia (via reduction of methemoglobin in the presence of administered methylene blue) and multifaceted biochemical properties (as reductases of flavins, non-alpha isomers of biliverdin and redox cycling agents, and heme binding). We have focused on the heme-associated properties of BLVRB. Recombinant mouse BLVRB could be readily isolated as a reddish brown heme binding protein in *E. coli*. It has a submicromolar specific binding affinity to ferric heme but very weak interaction with protoporphyrin IX. BLVRB in the erythroid cell extracts could be isolated by hemin-agarose and showed peroxidase activity when resolved on a native gel. From bioinformatic expression analyses, BLVRB was found to be specifically highly expressed in those cells/tissues having high levels of heme synthesis (e.g. developing and mature erythroid cells, fetal and adult liver), high heme-degradative activity (macrophages) or potential exposure to heme-associated molecules (endothelial cells). The BLVRB protein expression was modestly increased upon treatment of K562 erythroid and J774 macrophage cells with hemin, and markedly up-regulated upon induction of erythroid differentiation in mouse erythroleukemia MEL cells. Depletion of BLVRB by shRNA in MEL cells, however, did not exhibit marked effects on the hemoglobinization. The biological functions of BLVRB/FLR remain to be elucidated but may be related to its heme-binding properties in addition to its NADPH-dependent reductase activities, as well as its specific expressions in cells with high heme content and turnover.

43.

Reduced T Cell Response In Chlamydia-Induced Dysplasia Is Associated With Elevated HO-1 Expression

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Introduction: We employed a mouse model in which cervical dysplasia was induced by the vaginal application of Chlamydia muridarum to Hmox1 sufficient and deficient mice. As it is known that HO-1 is upregulated in different conditions of cell stress (e.g. infection), we aimed to investigate whether HO-1 is involved in dysplasia formation and furthermore to analyze the resulting immune response.
Materials and Methods: In a mouse model for Chlamydia-induced cervical dysplasia Hmox1 wild type, heterozygous and knock-out mice were vaginally infected with Chlamydia muridarum and sacrificed on day 7 post infection. The dissected uterus was histologically assessed regarding the grade of dysplasia formation. Lymphocytes were isolated from cervix and draining lymph nodes and stained for different cell surface markers to characterize T cell subsets by flow cytometry. Relative mRNA expression of HO-1 in the cervix was analyzed by qRT-PCR.
Results: Infected mice developed dysplasia compared to mock-infected animals regardless of their genotype, even though a slight increase of the dysplasia score could be observed in heterozygous and knock-out mice compared to wild type controls. The analysis of T cell subsets in the paraaortic lymph nodes of infected animals showed a significant decrease in the frequency of total CD4+ and CD8+ cells, their activated subtypes as well as in Foxp3+ regulatory T cells and Th17 cells. This could be confirmed in the cervix for activated and IFNγ producing CD4+ T cells. These results correlated with an increase of mRNA expression of HO-1 in the cervix in heterozygous and wildtype animals after infection.
Conclusion: Our results suggest an association between increased HO-1 expression and Chlamydia-induced dysplasia in mice that goes in hand with a reduced T cell response. Mechanisms responsible for this impaired immune response will be further investigated.

44.

Signal Transduction in Heme-Containing Oxygen Sensor Proteins

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In heme-based gas sensor proteins, heme acts as the sensing site for binding of gaseous molecules, including O2, NO, CO and H2S, and indirectly regulates many physiological functions, including the activities of protein kinases, guanylate cyclase, phosphodiesterase, and transcriptional regulatory factors, in response to gas availability. Conceptually, these proteins are always composed of at least two domains: one is a sensor domain (heme-based gas sensing) and the other is a functional domain. However, the structure-function relationship and mechanisms of communication between these domains have not been fully understood. Therefore, we selected three model systems, namely (i) a globin-coupled histidine kinase, AfGcHK, from Anaeromyxobacter sp. strain Fw109-5, (ii) a globin-coupled heme-based oxygen sensor diguanylate cyclase, YddV, from Escherichia coli and (iii) a direct oxygen sensor from Escherichia coli (EcDOS) in order to study the signal transduction in heme-containing oxygen sensor proteins.
The research contributed to expand our understanding of heme-containing sensor proteins´ function. Nevertheless, although the proteins are involved in various important physiological functions, the molecular mechanism of their sensing functions and signal transduction remains still to be elucidated. Especially the interaction between two domains, the heme-containing sensor and its functional domains, is therefore currently studied by various techniques (spectroscopy, hydrogen-deuterium exchange, enzyme kinetic analysis, crystallography etc.). Moreover, our work shed more light on protein-protein and inter-domain interactions in general.

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45.

Optimization of HO Activity Assay and Searching for Novel HO-1 Inhibitors

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Heme oxygenase-1 (HO-1), an inducible form of the enzyme converting heme to carbon monoxide (CO), ferrous ion (Fe2+) and biliverdin, can be overexpressed in some pathological conditions, including certain tumors. Specific inhibition of this enzyme may be considered as an anti-tumor treatment, that could increase sensitivity of the cancer cells to chemotherapy or radiotherapy. Unfortunately, so far described inhibitors are of limited application in clinics. Widely used in in vitro studies, metalloporphyrins are unselective and exert many sides effects including increase in HO-1 mRNA level. On the other hand, detailed information about pharmacokinetics and biodistribution of the recently developed, new compounds (imidazole-dioxolane derivatives) is lacking.
The aim of a present study was to obtain new compounds able to inhibit HO activity. Moreover, we focused at the optimization of the HO activity assay based on the extraction of bilirubin with chloroform. The optimization of the protocol described previously1 includes the application of purified rat biliverdin reductase (BVR) protein instead of rat liver cytosol as a source of biliverdin reductase. We standardized the assay and routinely use 25 µg/mL concentration of BVR. We applied HO activity assay to compare the effectiveness of known HO inhibitors with newly synthesized compounds. Both tin (SnPPIX) and zinc (ZnPPIX) protoporphyrins very potently diminished HO activity in pancreatic cancer cell line, PANC-1. Screening of the library of new compounds led to the selection of several inhibitors with submicromolar EC50s. These rate of inhibition was very similar to the results obtained for SnPPIX.
The obtained results indicate that our novel HO-1 inhibitors are of potential interest, therefore further tests should be performed to fully establish their potential applications as anti-cancer drugs.
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This work was supported by the National Centre for Research and Development (NCBR) (grant no PBS2/B7/15/2014). The Faculty of Biochemistry, Biophysics and Biotechnology of the Jagiellonian University is a partner of the Leading National Research Center (KNOW) supported by the Ministry of Science and Higher Education.

46.

Still alive: mesenchymal stromal cells are resistant to oxidative stress despite the low level of heme oxygenases

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Stem and progenitor cells are considered promising tools in regenerative medicine. However, the prerequisite for success of such therapies is good cell survival upon the administration. Mesenchymal stromal cells (MSC), which are multipotent cells derived from adult tissues, are commonly used in many pre-clinical and clinical trials. Heme oxygenase-1, enzyme that degrades heme to billiverdin, carbon monoxide and Fe2+, is important cytoprotective and antioxidant factor, which can affect stem cell performance, survival and differentiation. Therefore, the aim of our study was to characterize murine bone marrow-derived mesenchymal stromal cells lacking functional Hmox1 gene.
We used bone marrow-derived mesenchymal stromal cells (BM-MSC) isolated from Hmox1+/+ or Hmox1-/- mice. Using flow cytometry we characterized BM-MSC phenotype. Hmox1+/+ and Hmox1-/- cells were differentiated to adipocytes, osteoblasts and myofibroblasts what was confirmed with analysis of marker expression. Then, we analyzed the MSC response to the stressors such as H2O2, hemin or increased glucose concentration.
Both MSC Hmox1+/+ and Hmox1-/- showed similar phenotype, differentiation capacities and production of cytokines or growth factors. Interestingly, Hmox1+/+ and Hmox1-/- cells showed similar survival in response to 50 µmol/L hemin even in the presence of increased glucose concentration, conditions that were unfavorable for Hmox1-/- bone marrow-derived proangiogenic cells (BDMC). Surprisingly, Hmox1+/+ and Hmox1-/- MSC stood firm against up to 100 µmol/L hemin for 6 hours. What is more, Hmox1+/+ MSC but not fibroblasts retained low ROS levels even after prolonged incubation with 50 µmol/L hemin. However, both cell types have comparable level of Hmox1 expression and similarly increase its levels in response to hemin. Furthermore, MSC Hmox1-/- stronger than fibroblasts Hmox1-/- induced expression of heme exporter Flvcr1, antioxidant genes such as Fth1, Nqo1, Cat, Prdx6, and enzymes of glutathione pathway: Gclc, Gclm, Gss, Gsr and Gstp1. Changes in the expression of glutathione pathway genes in response to hemin treatment were functionally confirmed with higher levels of reduced glutathione and better GSH/GSSG ratio in MSC Hmox1-/- in comparison to fibroblasts Hmox1-/-.

Concluding, lack of Hmox1 does not influence MSC phenotype and tested functions. MSC Hmox1-/- showed higher resistance to the hemin treatment than BDMC. MSC Hmox1+/+ better withstand hemin than fibroblasts regardless of the similar Hmox1 expression level. The mechanism of MSC resistance to oxidative stress and hemin might be related to their efficient antioxidant gene response and activation of glutathione pathway.

47.

Interplay of gasotransmitter and superoxide signaling in Amyotrophic lateral sclerosis.

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The gaseous signaling molecule hydrogen sulfide has been shown to impact almost all cellular processes ranging from neuronal signaling to vasorelaxation. H2S is generated from cysteine, which is also a component of the antioxidant glutathione. Thus, there exists an intersection between antioxidant functions mediated by glutathione and H2S metabolism. H2S levels in the cell are regulated within a narrow physiologic range. Too much H2S production as well as paucity of the gasotransmitter can prove deleterious to the cell. We have shown earlier that aberrant H2S metabolism occurs in neurodegenerative states such as Huntington's disease and Parkinson's disease, where diminished sulfhydration was observed. We now show that in certain neurodegenerative diseases such as familial ALS, increased production of H2S occurs, leading to neurotoxicity. Mutations in superoxide dismutase 1 (SOD1) have been associated with several familial forms of ALS, with the G93A mutation being the most widely studied. Elevated H2S production has been demonstrated in the G93A mouse model of ALS. We now show that the G93A SOD1 mutant binds strongly to cystathionine gamma lyase (CSE), the biosynthetic enzyme for cysteine and H2S, and stimulates its activity. G93A SOD1 binds more tightly to CSE as compared to the wild type enzyme. Depleting the diet of G93A mice of cysteine ameliorates disease symptoms and enhances survival. Our results support the requirement for maintaining optimal H2S homeostasis in vivo.

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48.

THE INFLUENCE OF SIMVASTATIN ON HO-1 AND NRF2 IN ABDOMINAL AORTIC ANEURYSM PATIENTS

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Background: An abdominal aortic aneurysm (AAA) is a dilatation of the aorta frequently associated with an atherosclerotic background where excessive inflammation and oxidative stress is observed. Studies indicated that increased HO-1 activity may influence arthrosclerosis and HO-1 polymorphism in patients influences AAA episodes. HO-1 expression is under control of different transcription factors like Nrf2 or NF-kB and its activity may be modulated by drugs including statins.

Aim: This study was undertaken to verify the effect of simvastatin treatment on HO-1 and Nrf2-regulated genes in AAA patients.
Material and methods: The study was designed to analyse gene expression of HMOX-1 and Nrf2- targeted genes (NQO1 and GCLM) in circulating leucocytes and AAA wall of patients on simvastatin (n=10) or without statins (n=10). Patients were on simvastatin (20 mg or 40 mg per day) for at least 6 months. The patients were matched by age-, sex-, and AAA diameter. Patients demographic data, blood morphology and gene expression of HMOX-1, NQO1 and GCLM (by real time-PCR) was analysed.
Results: The results indicated that simvastatin treated patients had significantly higher expression of HMOX-1 in AAA wall comparing to the control ($p<0.05$). Moreover, simvastatin group had upregulated GCLM but not NQO1 in AAA wall ($p<0.05$).
Regarding blood morphology patients had comparable number of neutrophiles, lymphocytes and monocytes. The expression of HMOX-1 in leukocytes did not vary. However, significantly higher NQO1 but not GCLM was detected in the simvastatin group ($p<0.05$).
Conclusion: Simvastatin treatment may influence HO-1 in aortic wall but not in circulating leucocytes. Moreover, simvastatin may influence Nrf2 signaling pathway via GCLM in AAA wall and via NQO1 in circulating leucocytes. However, further studies are necessary to verify this effect.

49.

Different Approaches for HO-1 Inhibition in Cancer Cells – Are Novel Chemical Inhibitors as Effective as shRNA-mediated Gene Silencing?

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Heme oxygenase-1 (HO-1) through degradation of pro-oxidant heme into carbon monoxide (CO), ferrous ions (Fe2+) and biliverdin exhibits cytoprotective, anti-apoptotic and anti-inflammatory properties. All of these potentially beneficial functions of HO-1 can be, unfortunately, translated into pathophysiological processes, such as tumorigenesis. Indeed, elevated level of HO-1 was shown to contribute to literally all hallmarks of cancer. Therefore, HO-1 can be proposed as novel, therapeutic target for anticancer treatment in many types of tumors. Nonetheless, possibilities of specific inhibition of HO-1 are strongly limited. Providing a great alternative to current strategies of HO-1 inhibition, novel class of HO activity inhibitors with promising, anticancer effectiveness was studied.
In current study we aimed to validate the effect of genetic and pharmacological inhibition of HO-1 in vitro in two types of cancer: pancreatic cancer and hereditary leiomyomatosis and renal cell cancer (HLRCC) associated kidney cancer, as a model of synthetic lethality of HMOX1 and fumarate hydratase (FH) genes1.
We demonstrated that high HO-1 mRNA and protein levels in pancreatic cancer cell line, PANC-1, significantly affects susceptibility to anticancer treatment. Genetic inhibition of HMOX1 using shRNA approach resulted in pronounced inhibition of HMOX1 mRNA level and decrease in viability and proliferation of cancer cells. Additionally, increase in susceptibility to gemcitabine was observed. Pharmacological inhibition of HO activity using well-known inhibitor, ZnPPIX, diminished viability and clonogenic potential of Panc-1 cell line, but at the same time strongly induced HO-1 mRNA level. Treatment with novel small molecule HO inhibitors showed promising, anticancer effectiveness by decreasing cancer cells viability and clonogenic potential. Furthermore, genetic inhibition of HMOX1 in FH-deficient cell line, UOK

262, resulted in significantly diminished viability and proliferation properties of cancer cells. Chemical inhibition of HO activity using ZnPPIX resulted in similar effects as in PANC-1 cell line. Noteworthy, treatment with novel inhibitors of HO activity resulted in a decrease of UOK 262 cell viability and clonogenic activity.
In conclusion, current study points out the possible relevance of novel inhibitors of HO activity as potential anticancer treatment in different types of tumors. However, their further investigation is still needed.
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50.

Hydrogen sulfide rescues preeclampsia-like phenotype aggravated by high sFlt-1 in placenta growth factor (PlGF) deficiency.

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Introduction
Low circulating levels of placenta growth factor (PlGF) is strongly associated with the onset of preeclampsia, a maternal hypertensive disorder characterized by high blood pressure and proteinuria after 20 weeks of gestation. Although, PlGF-deficient mice are born healthy and fertile at a Mendelian ratio, the physiological importance of PlGF in the pathogenesis of preeclampsia is unclear. We hypothesised that decreased levels of PlGF in pregnancy exacerbates the fetal growth restriction associated with preeclampsia in the presence of high soluble Flt-1 (sFlt-1). Earlier studies showed that heme oxygenase-1 (HO-1) pathway inhibits sFlt-1 and as hydrogen sulfide (H2S) stimulates HO-1, we argued H2S donors will recuse the defects.
Methods
Pregnant PlGF-/- mice were injected with adenovirus encoding sFlt-1 (Ad-sFlt-1) at high (i) 1.5x109 pfu/ml and low (ii) 0.5x109 pfu/ml doses. Mean arterial blood pressure (MBP), biochemical and histological assessments of maternal kidney, placenta and embryos were performed.
Results
Ad-sFlt-1 significantly increased MBP and induced severe glomerular endotheliosis in PlGF-/- mice at E10.5 gestation compared to wild-type animals. High sFlt-1 also significantly elevated albumin-creatinine ratio and increased levels of urinary kidney injury molecule-1, a marker for proximal tubule injury. At a high dose of sFlt-1, there was complete fetal resorption in the pregnant PlGF-/- mice, and even the lower dose of sFlt-1 induced severe fetal resorption and abnormal placental vascularization. H2S-releasing agent, GYY4137, significantly reduced resorption, hypertension and proteinuria in Ad-sFlt-1 treated pregnant PlGF-/- mice. To determine if placental PlGF is critical for preventing fetal growth restriction associated with preeclampsia, we generated haploinsufficient PlGF+/- placentas and embryos were generated in wild-time dams and exposed to high sFlt-1 environment. This resulted in reduced fetal resorption, gestational hypertension and proteinuria when compared to pregnant PlGF-/- mice.
Conclusions
Placental PlGF is a critical protective factor against the damaging effects of high sFlt-1 associated with preeclampsia and activation of the H2S pathway appears to rescue preeclampsia phenotype even under low PlGF environment possibly by upregulating other protective pathways such as the HO-1/CO system.

51.

Novel Caffeic Acid Phenethyl Ester (CAPE) Analogues as Inducers of Heme Oxygenase-1

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Heme Oxygenase-1 (HO-1) is a metabolic enzyme strongly involved in relevant biological processes including cytoprotection, modulation of inflammatory response, anti-oxidative functions, regulation of cellular proliferation, angiogenesis, cardiovascular homeostasis, and immuno-modulation. HO-1 induction and/or activation is able to counterbalance, at least in part, oxidative stress and inflammation. For this reason, HO-1 can be regarded as an attractive target to ameliorate different stress-related pathologies, among which diabetes may be considered one of the most representative [1, 2]. Caffeic acid phenethyl ester (CAPE) – a natural polyphenolic compound – behaves as HO-1 inducer and possesses a plethora of beneficial effects under oxidative stress conditions [3]. In this work we present a small focused series of caffeic acid phenethyl ester analogues designed and synthesized with the aim of obtaining more potent HO-1 inducers. The capacity of these new compounds to modify the levels of HO-1 was evaluated in human mesenchymal stem cells (hMSCs) derived from bone marrow. Some of the tested compounds were found to be good HO-1 inducers and 3-(3,4-dihydroxyphenyl)-(2E)-2-propenoic acid 2-(3,4-dimethoxyphenyl)ethyl ester (VP961) was the most potent. VP961, tested to measure HO-1 protein expression and HO activity in in vitro system, resulted more potent than the parent compound CAPE, both as inducer and as direct activator of the enzyme. VP961 showed antioxidant properties in a model of H2O2-mediated ROS production and cytoprotective effects in a model of H2O2 cells viability impairment. To the best of our knowledge, VP961 is the first known compound able to activate directly HO-1 enzyme and to induce at the same time its protein expression. Further studies to asses the potential application of these properties in diabetes are ongoing.
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52.

Increased Heme oxygenase 1 Expression Observed in Tissues with High Bacterial Load During Acute Salmonella enterica Infection in Mice.

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Heme oxygenase 1 (HO-1) has been strongly associated with intestinal immune homeostasis, functioning as an anti-inflammatory and cytoprotective agent. This anti-inflammatory effect has leaded to consider HO-1 as a potential therapeutic target in several chronic and acute inflammatory diseases of the gut. It has been demonstrated that HO-1 induction results in a reduction of the colitis score in an IBD murine model, and its enzymatic product, carbon monoxide (CO), has been proposed as a potential treatment for IBD. However, the role of HO-1 induction during acute intestinal infection has not been tested yet. Salmonella enterica is the leading cause of infectious gastroenteritis in

humans, and the infection consists in acute inflammation of the intestine, colitis and, in severe cases, systemic bacterial spreading. Recent in vitro studies have demonstrated a role of HO-1 in the host immune response to Salmonella infection. The purpose of this work was to evaluate the expression of HO-1 in acute intestinal inflammation caused by Salmonella enterica serovar Typhimurium in mice. C57BL/6 mice were orally infected with Salmonella enterica serovar Typhimurium (S. Typhimurium) and, after 5 days post infection, ho-1 mRNA was evaluated by qPCR in spleen, liver, intestine and mesenteric lymph nodes. The results show higher expression of ho-1 in ileum and colon of infected mice, as compared to uninfected mice. Further, histological analyses showed increased inflammatory infiltrate in the tissues showing increased ho-1 expression in infected mice. In addition, a correlation analyses between bacterial load v/s ho-1 expression was performed for each tissue analyzed. Interestingly, the results show a positive correlation between bacterial load in feces and ho-1 expression in ileum and ascendant colon of infected mice. These results indicate that the presence of higher bacterial loads in the intestinal lumen is inducing ho-1 expression in the intestinal tissue. Taken together, these results suggest the potential role of HO-1 and its enzymatic products in Salmonella infection in the intestine. Further analyses need to be performed to evaluate whether the interaction between HO-1 and Salmonella is beneficial or detrimental for the infected host.

53.

TARGETING H-FERRITIN TO MITIGATE VALVULAR MINERALIZATION

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Objective – Calcification of heart valves is a frequent pathologic finding in atherosclerosis and chronic kidney disease. During calcification valvular interstitial cells transdifferentiate into osteoblast-like cells.
Approach and Results - Cultured cells of calcified valve from patients undergoing complete valve replacement for stenosis exhibited significant susceptibility to mineralization and transdifferentiation into osteoblast-like cells in response to phosphate and calcium compared to those harvested from valves without calcification. This was supported by higher level of extracellular calcium accumulation and expression of osteocalcin in extracellular matrix as compared to healthy valves. Importantly, mineralization/transdifferentiation process of valvular interstitial cells (VIC) was shown to be inhibited with iron and apo-ferritin, and the beneficial effect was more pronounced in healthy compared to diseased valves. The protection was mediated by ferroxidase activity of H-ferritin. Induction of ferritin expression by 3H-1, 2-dithiole-3-thione (D3T) mimicked such a benefit in interstitial cells preventing the mineralization/transdifferentiation processes. Expression of H-ferritin was higher in diseased valves as compared to healthy valves. However, such expression in calcified valve tissues was not homogenous as evidenced by alkaline phosphatase (ALP) and H-ferritin double immunostaining. In regions where H-ferritin positive cells were present lack of calcium deposition and ALP staining was observed. Conversely, in mineralized and ALP positive tissue, H-ferritin positive cells were not detected.
Conclusions – Ferritin in valvular interstitial cells is a stratagem in controlling valvular mineralization and osteoblastic differentiation. Utilization of D3T to induce ferritin expression may be a potential candidate in prevention of valvular calcification.

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54.

ANTIPLATELET ACTIVITIES OF CARBON MONOXIDE-RELEASING MOLECULES (CO-RMs): COMPARISON OF CO--RMs WITH DIFFERENT KINETICS OF CO RELEASE

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Carbon monoxide (CO) plays important regulatory roles in the cardiovascular system such as prevention of excessive adhesion and aggregation of platelets. Therefore, CO-releasing molecules (CO-RMs) represent a promising class of potential novel therapeutics, which may be used in antiplatelet therapy. Although previous studies demonstrated that CO-RMs inhibit platelet aggregation in in vivo and in vitro models, the mechanism of the antiplatelet activity of CO-RMs is not clear. The anti-aggregatory effect of CO-RMs appear to depend, among other factors, on the kinetics of CO-release (Chlopicki et al. Naunyn Schmiedebergs Arch Pharmacol. 2012;385(6):641-50) and the presence of a strong CO-acceptor seems to be a pre-requisite for the pharmacological action and the effectiveness of these compounds to modulate endothelial phenotype (Fayad-Kobeissi et al. Biochem Pharmacol 2016;102:64-77).

In this study we investigated the comparative effect of CO-RMs that possess different characteristics: 1) CORM-401, a manganese-based carbonyl complex that releases 3 moles of CO/mole of compound; 2) CORM-A1, a non-metal boron--containing carbonate that spontaneously generate CO; and 3) a group of rhenium-CO-RMs that are coordinate to a vitamin B12 scaffold. For each compound, we characterized the kinetic of CO release using a myoglobin assay and the anti-platelet effect by optical aggregometry as well as adhesion measurement performed by Quart Crystal Microbalance with Dissipation (Q-Sense E4).

All tested CO-RMs displayed a concentration-dependent CO release with various degrees of acceptor-driven release of CO, that was rather independent from the kinetic of CO-release. All examined CO-RMs displayed a similar potency as antiplatelet agents and their half-life of CO release were in a similar range. Interestingly, DiBr (c-(α,α-Dibromo)-lactone-cyanocobalamin-ReCORM), which displayed an acceptor-driven CO release, was slightly stronger than B12-CORM (Vitamin B12-ReCORM), and others B12-analogues.
In conclusion, acceptor-driven CO release might also contribute to the antiplatelet activities of CO-RMs, but further studies are needed to elucidate the relative importance of this phenomenon in the anti-platelet effects of CO-RMs.

55.

Transition-Metal-Free CO-Releasing BODIPY Derivatives Activatable by Visible to NIR Light as Promising Bioactive Molecules

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Carbon monoxide is well-known for its lethal effects in mammals because it binds to hemoglobin more strongly than oxygen.[1] However, it has been recognized as an important cell-signalling molecule with substantial therapeutic potential protecting from vascular, inflammatory or even cancer diseases.[2] Carbon monoxide-releasing molecules (CORMs) have been developed to deliver CO into the cell.[3] However, these molecules often suffer from toxicity, low water solubility, extremely short half-lives and an uncontrolled CO release limiting thus their therapeutic potential. Light-triggered CO liberation from a photochemically active CORMs (photoCORMs)[4] is an alternative activation strategy that allows for a precise spatial and temporal control over the CO release. PhotoCORMs based on organic compounds, such as BODIPY dyes (COR-BDPs), address this challenge because, unlike some photoCORMs containing transition metals, are nontoxic and thermally stable. Their high molar absorption coefficients, solubility in water and simple synthesis make them perfectly suitable for biological applications. Here, a BODIPY photoCORMs that release CO upon irradiation with visible-to-NIR (up to 730 nm) light are presented.[5] We demonstrate their performance in both *in vitro* and *in vivo* experiments.

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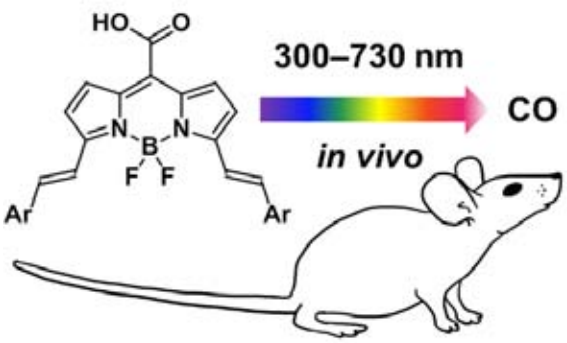
Attachment graphics:



56.

HO-1 And Nrf-2 Deficiency Affects Generation And Differentiation Of Induced Pluripotent Stem Cells

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Activation of p53, a major roadblock for successful iPSCs generation, can be triggered by increased oxidative stress. Therefore, here we evaluated the role of heme oxygenase-1 (HO-1, encoded by Hmox1 gene) and Nrf2 (encoded by Nfe2l2 gene), two major anti-oxidative and cytoprotective factors, in reprogramming process. Additionally, their effect on iPSCs differentiation was investigated.
Hmox1-/- fibroblasts demonstrated elevated level of p53 and p53-regulated miR-34a and 14-3-3σ protein, which inhibit cells in the G2/M phase of cell cycle, what was also observed in these cells. Additionally, lack of HO-1 resulted

in increased expression of miR-29a, let-7d as well as Hdac5 and Hdac7. Importantly, Hmox1-/- cells generated lower number of iPSCs colonies upon reprogramming than their control counterparts. The role of HO-1 in this process was further confirmed by its increased efficiency after stimulation of fibroblasts with cobalt protoporphyrin (CoPPIX) (HO-1 activator) which acts in HO-1-dependent manner as similar effect was not observed in Hmox1-/- cells. Interestingly, valproic acid (VA) shown previously to enhance reprogramming, in our hands decreased expression of HO-1 in murine fibroblasts, what was followed by less efficient iPSCs generation. Similarly, impaired reprogramming was observed in Nrf2-lacking fibroblasts, which demonstrated lower level of HO-1 in comparison to their control counterparts. Additionally, sulforaphan, an Nrf2 activator, increased the number of iPSCs generated from murine fibroblasts.
Hmox1+/+ and Hmox1-/- iPSCs, as well as Nfe2l2+/+and Nfe2l2-/- cells demonstrated similar expression of pluripotency markers such as Oct-4, Sox2 and miRNAs belonging to miR-290 family. Additionally, Hmox1+/+ and Hmox1-/- iPSCs were able to spontaneously differentiate via embryoid bodies to cells originating from three germ layers. However, lower number of contracting clusters was observed in HO-1-lacking cells. Interestingly, no teratomas were formed by Hmox1-/- iPSCs upon subcutaneous injection into immunocompromised mice (0 of 8 injections) in contrast to their control counterparts (5 of 8). Similar effects were not observed in case of Nfe2l2-/- iPSCs since their pluripotency was confirmed both in vitro and in vivo.
These results indicate that HO-1 and Nrf2 deficiency attenuates reprogramming efficacy whereas the lack of HO-1 may additionally affect differentiation potential of iPSCs.
Supported by National Science Center (NCN) grants MAESTRO (2012/06/A/NZ1/00004), HARMONIA (2014/14/M/NZ1/00010) and PRELUDIUM (2013/11/N/NZ1.02399) and VENTURES programme funded by the Foundation for Polish Science.

57.

Crystal Structure of Biliverdin and NADP bound Biliverdin IXα Reductase

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Biliverdin IXα reductase (BVR-A) is a classical enzyme that is responsible for the last step in heme degradation and produces bilirubin (BR), a lipophilic major antioxidant in the cells. BVR-A reduces the C10 methine bridge of the biliverdin IXα (BV) using the NAD(P)H as a cofactor. Mammalian BVR-A structures, including the cofactor bound form, has been already determined [1,2], but the molecular mechanism of BVR-A reaction, in particular the BV binding site and the catalytic residue, yet remains undetermined.
In cyanobacteria, BV is normally reduced by ferredoxin-dependent bilin reductases, which are not homologous to BVR, to produce phycobilins, photosynthetic pigments, but cyanobacteria also have mammalian BVR-A homolog, which is possible to reduce BV to BR using NAD(P)H [3]. Here, we determined the crystal structure of the cyanobacterial BVR-A (Syn BVR) in complex with BV and NADP at 2.6 Å resolution [4]. The overall folding of Syn BVR was similar to those of mammalian BVR-A. Syn BVR consists of two structural domains (N-terminal and C-terminal domains) and an obvious cleft between them constitutes the active sites. The N-terminal domain has a typical Rossmann fold, in which a parallel β-sheet is surrounded by five α-helices. The C-terminal domain contains eight β-strands and six α-helices.

BV binding manner in Syn BVR was surprising; two BV molecules (termed proximal and distal BV relative to the bound NADP) bind with the stacked geometry in the active site. Titration assay also suggested that two BV molecules bound to Syn BVR. The nicotinamide moiety of the NADP, an origin of the hydride, located close to the C10 methine bridge of the proximal BV, which means that the hydride is directly transferred from NADPH to the C10 position of the proximal BV. The distal BV would not be reduced to BR because the distal BV was located far away from the NADP. The distal BV would fix the proximal BV at the appropriate position and may function as the proton donor for the catalysis.

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58.

GT-repeat Polymorphism in the Heme Oxygenase-1 Gene Promoter Shows Association with Severe Carotid Atherosclerosis

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Background: Cardiovascular diseases are still a leading cause of mortality. Several factors influence their development, from which the oxidative stress harming the cells of the cardiovascular system seems to be an important element. Because of this the protective, anti-oxidant mechanisms can inhibit atherosclerosis and plaque formation. Heme oxygenase 1 is an important component of this cyto- and cardio-protective system, the amount/activity of which is regulated by different polymorphisms located in the promoter of its gene. The most important of these genetic variants is the GT(n) repeat polymorphism.
Methods: In order to evaluate the association of the GT(n) polymorphism and severe atherosclerosis DNA sample were isolated from peripheral blood of 101 patients undergoing surgical removal of atherosclerotic lesions located in their arteria carotis externa/interna. 368 individuals representing the Hungarian population served as a control group. The repeat number of the GT region was determined by a PCR applying fluorescently labeled primer, followed by fragment length analysis using capillary electrophoresis (ABI 310 analyzer).
Results: The length of the GT-repeats showed a typical trimodal distribution both in controls and patients, with maximal frequencies around 23, 30 and 37 repeats. Based on these, 3 allele types were defined: short (S; repeat number <29), medium (M; r.n. 30-33) and long (L; r.n. >34). The allele frequencies were significantly different between the controls and patients (p=0.003), showing a higher rate of the L (4.9 vs. 10.4%) and a lower rate of the S (43.0 vs. 33.7%) allele in the patients. Significant difference (p=0.008) was observed in the genotype distribution, too. The rate of the genotypes containing the L allele (LL, LM, LS) was significantly higher in the patients (20.8 vs. 9.6%; p=0.002) while percent of the S genotypes (SS, SM, LS) was lower in the patients compared to the controls (68.8 vs. 58.4%; p= 0.048).
Conclusions: The longer GT-repeat number in the promoter of the HMOX-1 gene is associated with reduced expression

of the protein. Knowing the protective role of HMOX-1 activity against the development of atherosclerosis the elevated rate of the L genotypes of the GT(n) polymorphism, associated with a probable reduced HMOX-1 activity, might contribute to the development of severe carotid atherosclerosis in our patient cohort.

59.

Natural hepatoprotective polyphenols increase intracellular concentration of bilirubin in the liver by inhibition of UGT1A1

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Introduction: Bilirubin is a powerful antioxidant, which is responsible for 10% of blood antioxidant capacity. Its mildly elevated concentrations in blood protect organism from diseases caused by oxidative stress. Serum concentrations of bilirubin are affected by enzymes, *i.e.* heme oxygenase (HMOX) responsible for production of bilirubin and bilirubin UDP-glucuronosyl transferase (UGT1A1) catalyzing its biotransformation in the liver. The aim of our study was to analyze potential intracellular bilirubin-modulating effects of polyphenols contained in the milk thistle (*Silybum marianum*) extract silymarin, in particular on both key enzymes of bilirubin metabolism.
Methods: The human hepatoblastoma cell line (HepG2) was exposed to different concentrations of individual polyphenolic compounds (flavonolignans) isolated from silymarin. Based on *in vitro* studies the most efficient compounds were selected and used for *in vivo* mice studies. Selected polyphenols were applied intraperitoneally for seven days to C57BL/6 mice. Markers of liver damage, expression of *UGT1A1* mRNA and intracellular concentration of bilirubin in the liver were then analyzed.
Results: All used natural *S. marianum* polyphenols increased intracellular concentration of bilirubin in HepG2 cells in a similar extent as a positive control atazanavir, a known inhibitor of UGT1A1. *I.p.* application of 50 mg/kg of the flavonolignan mixture to experimental animals led to significant down-regulation of *UGT1A1* mRNA expression (46 ± 3 % of control, $p < 0.005$) in the liver and also to a significant increase of intracellular bilirubin concentrations (0.98 ± 0.03 vs. 1.21 ± 0.02 nmol/mg, $p < 0.05$) in the liver tissue.
Conclusion: Polyphenolic compounds contained in the milk thistle affect the heme catabolic pathway with important modulation of intracellular metabolism of bilirubin. This phenomenon might contribute to hepatoprotective effects of silymarin.
Supported by grants AZV ČR (16-27317A), GAČR 16-06008S, and GAUK 337815.

60.

Carbon monoxide promotes intestinal mucosal healing in rats colitis model

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Background An abundance of recent data on the cytoprotective and anti-inflammatory effect of carbon monoxide (CO) supports that CO is a promising therapeutic gas for various inflammatory diseases. However, it remains unclear whether CO promotes the intestinal mucosal healing. Therefore, we evaluated the effect of CO in the intestinal mucosal healing using rat colitis model.

Methods Acute colitis was induced with trinitrobenzene sulfonic acid (TNBS) in male Wistar rats. CO-saturated solution was made by bubbled 50% CO gas into saline. In healing phase experiment, CO solution was intrarectally administrated twice a day from day 3 after the induction of colitis. The distal colon was removed at day 7 after the induction of colitis, and the ulcer lesions were measured and evaluated chemically. In addition, the wound healing assays were performed to determine the enhanced restitution of rat intestinal epithelial (RIE) cells treated with CO-saturated medium.
Results The intracolonic administration of CO solution ameliorated TNBS-induced colonic ulceration with accelerated the healing of the colonic ulceration. In addition, the wound assay revealed that CO-saturated medium enhanced the migration of RIE cells through the activation of Rho kinase.
Conclusions The rectal administration of CO-saturated solution accelerated the colonic ulcer healing through the promoted epithelial restitution. Based on these data, CO may thus represent a novel therapeutic approach for the treatment of inflammatory bowel disease.

61.
The role of bilirubin in inflammation
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Introduction: Bilirubin has recently attracted the attention of scientists because of its potential cytoprotective properties. Mildly elevated bilirubin levels exert antioxidant, anti-inflammatory and immunomodulatory effects in patients with diseases associated with increased oxidative stress and chronic inflammation. However, the exact mechanisms of protective actions of bilirubin have not been fully elucidated so far. The aim of the study was to assess the pathophysiological role of bilirubin in inflammation in vitro and in vivo using hyperbilirubinemic Gunn rats.

Methods: Intracellular bilirubin levels and mRNA expression of selected cytokines (IL-6, IL-10, TNF-α) were determined in primary hepatocytes isolated from Gunn rats and corresponding normobilirubinemic controls, and in HepG2 cells before and after exposure to 20 μM and 100 μM bilirubin. Viability of primary hepatocytes was measured after incubation of cells with 20 μM and 100 μM bilirubin, with or without adding of pro-inflammatory cytokine TNF-α (100 ng/ml). The level of phosphorylation of the p65 subunit of the transcription factor NF-κB was determined using Western blot. Gunn rats were treated with lipopolysaccharide (LPS, 6 mg/kg, intraperitoneally), control animals received saline. After 1 and 12 h, rats were anesthetized and sacrificed. Blood and organs were collected for analysis of inflammatory and hepatic injury markers including liver mRNA expression of selected cytokines (IL-6, IL-10, TNF-α).
Results: Intracellular bilirubin levels were similar in both primary hepatocytes before and after bilirubin exposure. After exposure to 100 μM bilirubin, mRNA expression of pro-inflammatory cytokines IL-6 and TNF-α increased significantly ($p<0.01$, and $p<0.05$, respectively), while anti-inflammatory IL-10 decreased ($p<0.01$) in primary hepatocytes from both Gunn and control animals. Cell viability of Gunn primary hepatocytes was significantly higher after incubation with bilirubin ($p<0.05$) and also in the presence of TNF-α ($p<0.001$) compared to control primary hepatocytes. The exposure of HepG2 cells to TNF-α in the presence of 20-40 μM bilirubin led to a decrease in phosphorylation of the NF-κB p65 subunit compared to cells without bilirubin ($p<0.001$). Similar results were observed using primary hepatocytes. In in vivo experiments with 12 h exposure to LPS in Gunn rats, the different kinetics of liver cytokines was observed between hyperbilirubinemic Gunn rats and respective controls with slightly decreased IL-6 after 1 h and increased IL-10. ALT activity significantly decreased in hyperbilirubinemic Gunn rats after 12 h exposure to LPS compared to control animals ($p<0.05$).
Conclusion: Bilirubin seems to protect the liver cells against LPS-induced inflammatory injury by mechanism including modulation of NF-κB pathway activation through the decreasing level of its p65 subunit phosphorylation.
The study was supported by the Charles University in Prague, 1st Faculty of Medicine, project GA UK No. 168216.

62.
Heme metabolism in mitochondrial DNA-depleted breast cancer cells
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Mitochondrial alterations are linked to several pathological conditions including cancer. To study such defects, ρ0 cells lacking mitochondrial DNA (mtDNA) and thus respiratory function, were used. Heme metabolism is tightly connected to mitochondrial function. It has been reported that heme oxygenase (HMOX), the key enzyme in the heme degradation, is involved in anti-oxidant activities in ρ0 cells and can be translocated into mitochondria under conditions of oxidative stress. While the anti-oxidant effects of HMOX are established, its role in cancer cell proliferation is still controversial. After grafting ρ0 cells in syngeneic mice, tumor growth was observed after a long lag period of 20 days, concomitant with gradual acquisition of mtDNA and recovery of respiration. We used this model to study possible changes in heme metabolism during this process.
Mouse breast carcinoma cells 4T1, 4T1ρ0 (D0) and cell lines isolated after 5, 10, 15 and 20 days (D5, D10, D15, D20) post grafting of ρ0 cells in mice were used for in vitro experiments. HMOX activity, mRNA and protein, as well as cellular heme content and ROS production were evaluated. Hemin (MHA), CoPP and SnMP treatments of parental, D0, D15 and D20 cells were used to characterize HMOX activation potential and the effect on cellular viability.
While HMOX1 mRNA was increased already from D0 and on all following days compared to parental cells, HMOX activity was gradually increased from D5. HMOX2 mRNA was not significantly changed except for mild increase in D5. Interestingly, cellular heme content was elevated in all D0-D20 cell lines. Furthermore, ROS production was significantly augmented from D0-D20. Treatment with MHA increased HMOX activity in parental 4T1 and D0 cells, but not D15 and D20 cells over their basal activity. CoPP treatment increased activity in all cell lines, whereas SnMP had the opposite effect. Although MHA and both porphyrins rapidly decreased ATP production in all cell lines, none of the short-term treatments significantly influenced the cell viability.
HMOX expression was elevated in all ρ0 cell-derived lines with delayed effect on HMOX activity. We conclude that HMOX activation may be a response to elevated ROS production and heme content in the cells gradually acquiring mtDNA, and may account for accelerated tumor growth.

63.
Role of heme-Bach2 pathway in bone marrow macrophage
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Heme is an essential molecule for living organism. Heme plays a major role in gas sensing, antioxidant defense, and signal transduction. Recently, several repots have revealed that heme-iron metabolism is linked to macrophage polarization. Bach2 is a transcriptional factor, which is involved in the regulation of development and functions of B- and

T-cells. We revealed a new function for heme as a ligand of Bach2 and as a modulatory signal involved in plasma cell differentiation. In addition, we showed that Bach2 repressed the M2 polarization of alveolar and peritoneal macrophages. However, it remains unknown whether Bach2 regulates heme metabolism and macrophage polarization in the bone marrow. Firstly, we measured the expression of Bach2 in bone-marrow macrophage using Bach2-td RFP reporter mice. We found the Bach2-negative and -positive populations in the bone marrow macrophage. After isolating these two populations, we carried out a DNA microarray analysis. The gene ontology (GO) analysis of genes with differential expression revealed molecular distinctions between RFP-negative and -positive macrophage populations. RFP-negative macrophages showed enrichment of GO terms such as "immune system process" whereas RFP-positive population showed enrichment of GO terms "tetrapyrrole metabolic process". In RFP-positive population, we found that hrg-1 gene, which encodes heme transporter, was directly repressed by Bach2. These results suggest that Bach2 may coordinate heme metabolism and macrophage polarization in a subset of bone marrow macrophages.

64.

Labile Heme Impairs Hepatic Microcirculation and Promotes Hepatic Injury in Septic Rats

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Labile heme inflicts hepatic damage when released into the circulation during systemic inflammation and sepsis. Accordingly, hemolysis and decreased concentrations of heme-scavenging proteins coincide with an unfavorable outcome in critically ill patients. We investigated the impact of labile heme on hepatic sinusoidal microcirculation and hepatocellular integrity as the liver is a key player in heme metabolism and host response to infection. Adult male rats were used for in vivo and ex vivo studies. Isolated livers from septic or control animals were perfused with carbogen saturated Krebs-Henseleit buffer containing heme, protoporphyrin IX or heme and albumin. In vitro studies were performed using non-parenchymal (immortalized human hepatic stellate (LX-2)) or parenchymal cells (Hepa 1-6). We provide evidence that labile heme significantly increases portal pressure via a mechanism that involves hepatic stellate cell-mediated sinusoidal constriction. Moreover, labile heme exerts direct cytotoxicity to hepatocytes in vitro. Heme binding by albumin attenuates heme-mediated vasoconstriction in vivo and prevents heme-mediated hepatocyte cytotoxicity in vitro. Heme perfusion potentiated tissue damage during polymicrobial sepsis, but not after bacterial lipopolysaccharide (LPS) administration, despite increased concentrations of plasma heme scavangers.

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29	Chung	Hun Taeg	Pterostilbene 4′-glucoside Protects Mice from LPS-induced Acute
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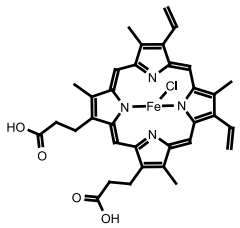

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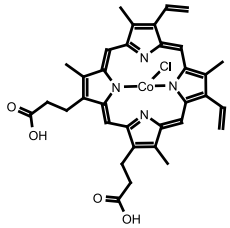

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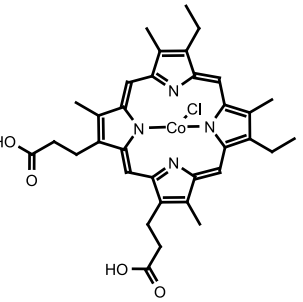

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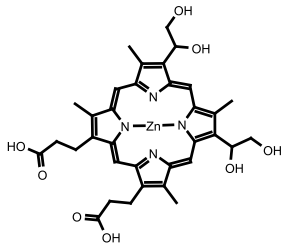

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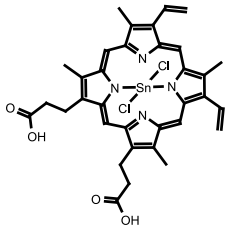

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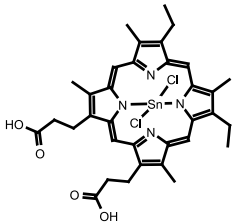

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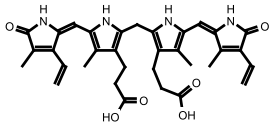

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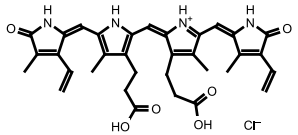

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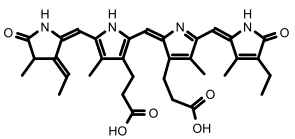

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