NITRIC OXIDE AND ITS INTERACTIONS

Nitric Oxide and its Interactions

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Extracellular Diffusion is the Rate Limiting Factor in the Reaction of Nitric Oxide and Hemoglobin Encapsulated Within Phospholipid Vesicles at Very Low Hematocrits

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Nitric Oxide (NO) is a well known smooth muscle relaxation factor and as such plays a crucial role in maintaining vascular homeostasis. NO is scavenged rapidly by hemoglobin (Hb), however, under normal physiological conditions the encapsulation of Hb inside red blood cells (RBCs) significantly retards this reaction permitting NO to reach the smooth muscle. The rate limiting factors (diffusion of NO through the cell free zone vs. unstirred layer vs. RBC membrane vs. intracellular diffusion) responsible for this retardation have been the subject of much debate. An important application where it is necessary to know the role of each of these factors lies in the development of efficient hemoglobin based oxygen carriers (HBOCs) wherein the Hb would be contained within phospholipid vesicles (HbVs). In a work by Sakai et. al. [*J. Biol. Chem.* **283**:1508–1517; 2008], the authors performed stopped flow experiments of the reaction of NO with deoxygenated Hb at various concentrations inside HbVs of various radii at extremely low hematocrits. They also completed computer simulations of the experiments in which they did not account for extracellular diffusion of NO or for the lipid membrane permeability to NO. They concluded that intracellular diffusion alone is responsible for the reaction rates observed experimentally. We have performed our own computer simulations using COMSOL Multiphysics (Comsol Inc., Burlington, MA) to model the experiments of Sakai et. al. with a three dimensional model that includes extracellular diffusion and membrane permeability in addition to intracellular diffusion of NO. We find that membrane permeability does, in principle modulate NO uptake, but suggest here that this is more of an issue for protein scaffold-containing red blood cells than HbVs. Importantly, we also find that under the given experimental conditions, extracellular diffusion of NO is the main rate limiting factor.

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A Novel Role for Cytochrome c: Efficient Catalysis of S-Nitrosothiol Formation

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The physiological mechanism for the formation of S-nitrosothiols involved in NO-dependent signal transduction has not been established. In this study, we demonstrate that cytochrome c can serve as an efficient catalyst for NO-dependent S-nitrosation in vivo. Results show that the formation of GSNO by ferric cytochrome c occurs both in the presence and absence of oxygen and results in formation of reduced cytochrome c. Ferric cytochrome c reduction by NO is much faster in the presence of GSH and the rate of reduction surprisingly decreases as the concentration of NO increases. We propose that GSH initially binds to ferric cytochrome c, which is then followed by reaction of NO with this complex, yielding ferrous cytochrome c and GSNO. We further show that when submitochondrial particles or cell lysates are exposed to NO in the presence of cytochrome c, there is a robust formation of protein S-nitrosothiols and in case of submitochondrial particles, mitochondrial complex I is also inhibited. These observations suggest that cytochrome c mediates mitochondrial S-nitrosation, particularly during hypoxia, and potentially mediates the release of cytochrome c into the cytosol during apoptosis by functioning as a GSNO synthase and modulating apoptotic signaling.

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Reactions of Angeli's Salt in Whole Blood

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Angeli's salt (AS) ($Na_2N_2O_3$) decomposes into nitroxyl and nitrite, compounds of physiological interest for their impact on nitric oxide bioavailability. The potential of Angeli's salt to decrease nitric oxide scavenging by plasma hemoglobin was proposed by He et. al. [Free Radic. Biol. Med. **44**:1420–1432; 2008], when it was shown that AS preferentially reacts with cell-free Hb over red-blood-cell-encapsulated Hb to form non-nitric-oxide-scavenging ferric hemoglobin (metHb). In this study, we further examine the reactivity of AS in whole blood. Less metHb is formed when AS is reacted with red blood cells (RBCs) than when reacted with free Hb, and even less is produced when AS is reacted with whole blood, indicating that factors both inside and outside the red cell affect AS reaction with Hb in blood. Excess Hb was reacted with varying concentrations of AS; these reactions yielded a metHb concentration that was higher than predicted from the 1:2:2 (HNO to oxyHb to metHb) stoichiometry of the reaction. To account for any metHb formed by NO_2^-, we used samples of Hb + NO_2^- and subtracted the metHb formed by NO_2^-; however, this still does not account for the surplus metHb. Addition of catalase to the Hb reduced the amount of metHb formed. This may be due to direct reaction of nitroxyl with catalase or an interruption of a peroxide-driven reaction. Surprisingly, it was observed during this reaction that 1mM oxyHb containing 12.5-25 μM catalase spontaneously deoxygenates over the course of several hours; further investigation showed that deoxygenation of Hb in the presence of catalase occurs regardless of the presence of AS or NO_2^-.

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Efficient S-Nitrosation of Cellular Proteins by Cytochrome c

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S-Nitrosation of key cysteine residues has been suggested to represent a novel redox-based signaling process. However, the exact intracellular mechanisms of S-nitrosothiol formation has not yet been established. Nitric oxide (NO) is synthesized from nitric oxide synthase, but does not directly react with thiols to form S-nitrosothiols. Although S-nitrosation has been thought to be largely oxygen dependent, recent evidence points to hypoxia as being a stimulus of S-nitrosothiol synthesis. Here, we demonstrate that cytochrome c (cyt c) may represent an efficient catalyst of S-nitrosation in vivo. The ability of cyt c to generate protein S-nitrosothiols in cell lysate under anaerobic conditions was determined by incubating NO-donor, Proli/NO, (100 μM) and ferric cyt c (100 μM) with lysate from murine macrophage RAW 264.7 cells. In the absence of cyt c, a small but measureable level of S-nitrosation was observed. In the presence of added cyt c, the level of S-nitrosation was greatly enhanced approaching 1

nmol/mg protein as determined by chemiluminescence. In addition, a combination of CyDye switch labeling and two dimensional differential in-gel electrophoresis (DIGE) approach was used to analyze cell lysate samples treated with NO-donor in the presence or absence of ferric cyt c. We observed significantly increased labeling when both cyt c and NO were present as compared to NO alone. Filtration of cell lysate using a 10 kDa cut-off filter revealed about 40% of total S-nitrososthiol associated with the high molecular weight fraction and 60% associated with the low molecular weight component. Transnitrosation of model protein was also assessed in system containing protein, GSH, NO-donor and cyt c. Taken together, these observations raise the possibility that cyt c is a mediator of S-nitrosation, particularly during hypoxia, and that release of cyt c in the cytosol during apoptosis potentially releases an enzymatic activity capable of S-nitrosothiol synthesis which could in turn modulate apoptotic signaling.

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Nitrite Mediated Enhance Resuscitation From Hemorrhagic Shock

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This study determines the systemic and microvascular hemodynamic consequences of administering a low dose sodium nitrite after fluid resuscitation from hemorrhagic shock. Hemodynamic responses to hemorrhagic shock and resuscitation were studied in the hamster window chamber model. Moderated hemorrhage was induced by arterial controlled bleeding of 50% of the blood volume (BV) and the hypovolemic state was maintained for one hour. Volume restitution was performed by infusion of 25% of BV using a conventional plasma expander and hemoglobin (Hb) based oxygen carriers (HBOCs); namely: Hextend® (6% Hetastarch 670 kDa in lactated electrolyte solution), Polymerized Hb (PBH, 13g/dl, Oxyglobin™, Biopure Corp. Boston, MA) and Polyethylene Glycol Conjugated Hb (PEG-Hb, 4g/dl). Nitrite therapy was administered 10 min after fluid resuscitation, absolute nitrite doses per animal were 0 or 5 namomoles, respectively. The experimental groups were named based on the resuscitation fluid and nitrite concentration used. Systemic parameters, microvascular hemodynamics and capillary perfusion were followed during entire protocol. Exogenous nitrite maintained systemic and microhemodynamic conditions post fluid resuscitation from hemorrhagic shock, compared to no nitrite. Animals resuscitated with HBOCs had stronger response to nitrite therapy compared to Hextend. A moderated increase in plasma nitrite during the early phase of resuscitation reversed arteriolar vasoconstriction and increased capillary perfusion and venous return, improving central cardiac function. Nitrite effects on resistance vessels directly influenced intravascular pressure redistribution, sustained blood flow, and prevented tissue ischemia. In conclusion, increasing nitrite plasma bioavailability after fluid resuscitation from hemorrhagic shock can be a potential therapy to enhance microvascular perfusion and to improve overall outcome. Work support in part by grants R24-HL64395, R01-HL62354 and PPG-HL071064 to MI.

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Exogenous Nitric Oxide Induces Protection During Hemorrhagic Shock

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This study analyzed the systemic and microvascular hemodynamic changes related to increased nitric oxide (NO) availability during the early phase of hemorrhagic shock. Hemodynamic responses to hemorrhagic shock were studied in the hamster window chamber. Exogenous NO was administered in the form of nitrosothiols (nitrosylated glutathione, GSNO) and was given prior the onset of hemorrhage. Moderate hemorrhage was induced by arterial controlled bleeding of 50% of the blood volume, and the hypovolemic shock was followed over 90 min. Animals pre-treated with GSNO maintained systemic and microvascular conditions during hypovolemic hemorrhagic shock, when compared to animal treated with glutathione (GSH) or the sham group. Low concentrations of NO released during the early phase of hypovolemic shock from GSNO mitigated arteriolar vasoconstriction, increased capillary perfusion and venous return, and improved cardiac function (recovered of blood pressure and stabilized heart rate). GSNO's effect on resistance vessels influenced intravascular pressure redistribution and blood flow, preventing tissue ischemia. In conclusion, increases in NO availability during the early phase of hypovolemic shock, could preserve cardiac function and microvascular perfusion, sustaining organ function. Direct translation into a clinical scenario may be limited, although the pathophysiological importance of NO in the early phase of hypovolemia is clearly highlighted here. Work support in part by grant R01-HL62354 to Marcos Intaglietta.

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A Molecular Model for the Susceptibility of Protein Thiols to S-Nitrosation

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S-Nitrosation, the addition of an –NO moiety in the thiol group of cysteine residue of target proteins, is regard as a reversible nitric oxide (NO)-mediated post-translational modification. An increasing number of publications have shown the importance of S-nitrosation in the modulation of protein function and the alteration of cell phenotype. Furthermore, the developing field of S-nitrosoproteomics, the global identification and characterization of S-nitrosated proteins in cells, tissues, and organs, emphasizes the importance of S-nitrosation in biological systems. However, the mechanism of S-nitrosation is still obscure. Several factors are suggested to be involved in the S-nitrosation of protein thiols, such as the accessibility of the protein thiol to NO and S-nitrosating agents, the low pKa of the protein thiol, the availability of metal ions, the abundance of oxygen, and the proximity of hydrophobic microenvironments. In order to understand the effects of the environmental factors that control S-nitrosation, we have established a molecular model for assessing the propensities of protein thiols to S-nitrosation. Green fluorescent protein (GFP) has been chosen because of its well-characterized three-dimensional structure which allows us to introduce cysteine residue in the desired protein microenvironments. The GFP from *A. vinelandii* has 238 amino acids and a barrel shape structure with a hydrophobic core. It has two cysteine residues at amino acid 48 and 68, and both of them are located in a limited solvent-accessible region. To test the susceptibility of S-nitrosation, the wild-type GFP was transient expressed in Chinese hamster ovary cells (CHO) and the S-nitrosation of the cysteines was detected in both intact cells and cell free system after S-nitrosocysteine treatment by biotin-switch method. We found the cysteines of wild-type GFP are not easily S-nitrosated making GFP a potential molecular test system for assessing environmental factors in the control of protein S-nitrosation. We are currently introducing cysteine residues in several protein locations to examine the microenvironmental effects on S-nitrosation in the intracellular environment. This model could help us uncover the fundamental rules of S-nitrosation.

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Isoflavone Induced eNOS Activation is Associated with Alterations of the F-Actin Cytoskeleton and Mitochondrial ROS Production

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Menopause is associated with an increased risk of cardiovascular disease and growing concerns over the use of hormone replacement therapy have prompted a search for alternative therapies. Recently, we reported that physiological concentrations of equol (100nM) rapidly (2min) activate eNOS via phosphorylation of Akt and ERK1/2 (Joy *et al.,* 2006). In the present study, we have investigated whether equol (100nM) induced eNOS activation is associated with (i) increased production of reactive oxygen species (ROS), (ii) alterations of the F-actin cytoskeleton and (iii) the effect of actin depolymerisation on mitochondrial ROS formation. Human umbilical vein endothelial cells (HUVEC) were incubated in Krebs Henseleit buffer containing lucigenin (5µM), vehicle (DMSO) or equol (100nM) ± inhibitors of flavoproteins/NADPH oxidase (Nox) (apocynin, 10µM; diphenylionium, 1µM) or the mitochondrial complex-I inhibitor rotenone (5µM). Mitochondrial ROS were also measured in HUVEC loaded with Mitosox red for 30min (± the actin depolymerising agent cytochalasin-D, 2.5-5µM). Cells were then treated for 20min with vehicle (DMSO) or equol (100nM, ± cytochalasin-D). Similarly, F-actin was visualized using Phalloidin by confocal fluorescence microscopy. Data denote means ± S.E.M. of n=3-4 different cultures, ERK1/2, Akt and eNOS phosphorylation was abrogated by inhibiton of Nox and mitochondrial complex I. Equol stimulated ROS production was attenuated by inhibitors of flavoproteins/Nox and rotenone. The appearance of cytosolic F-actin stress fibers in response to equol was paralleled by increased mitochondrial ROS production, with depolymerisation of F-actin dose-dependently attenuating Mitosox red fluorescence. In summary, equol induced alterations in the F-actin cytoskeleton modulate mitochondrial ROS production and subsequent eNOS activation.

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Site-specific CPB1 Tyrosine Nitration and Patho-Physiological Implications Following its Physical Association with NOS-3 in Experimental Sepsis

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Lipopolysaccharide (LPS)-induced sepsis results in oxidative modification and inactivation of carboxypeptidase B1 (CPB1). Here, immunoprecipitated CPB1 was probed for tyrosine nitration using monoclonal nitrotyrosine-specific antibodies in a murine model of LPS-induced sepsis. Tyrosine nitration of CPB1 was significantly reduced in the presence of NOS inhibitors, and xanthine oxidase (XO) inhibitor allopurinol and NOS-3 KO mice. CPB1 tyrosine nitration and loss of activity by the concerted action of NOS-3 and XO were also confirmed in vitro using both the nitric oxide donor 3-morpholinosydnonimine (SIN-1) and peroxynitrite. LC/MS/MS data indicated five sites of tyrosine nitration in vitro including Tyr-248, the tyrosine at the catalytic site. The site and protein specific nitration of CPB1 and the possible high nitration yield to inactivate it were elucidated by confocal microscopy. The studies indicated that CPB1 co-localized with NOS-3 in the cytosol of sinus lining cells in the red pulp of the spleen. Further analysis of CPB1-immunoprecipitated samples indicated immunoreactivity to a monoclonal NOS-3 antibody, suggesting protein complex formation with CPB1. XO, NOS inhibitors and NOS-3 KO mice injected LPS had decreased levels of C5a in spleens of septic mice, indicating peroxynitrite as a possible cause for CPB1 functional alteration. Thus, CPB1 co-localization, coupling, and proximity to NOS-3 in the sinus lining cells of spleen red pulp could explain the site-specific tyrosine nitration and inactivation of CPB1. These results open up new avenues for investigation of several enzymes involved in inflammation and their site-specific oxidative modifications by protein-protein interactions as well as their role in sepsis.

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Repression of Classical Nuclear Export by S-Nitrosylation of CRM1

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We report that cellular chromosomal region maintenance 1 (CRM1) became *S*-nitrosylated upon extensive exposure to endogenous or exogenous nitric oxide (NO). This abrogated the interaction of CRM1 with nuclear export signals (NESs) and repressed classical protein export. Analysis by mass spectrometry and involving the use of *S*-nitrosylation mimetic mutations indicated that modification at either of two specific cysteines of CRM1 was sufficient to abolish the CRM1-NES association. Moreover, ectopic overexpression of the corresponding *S*-nitrosylation-resistant CRM1 mutants rescued NO-induced repression of nuclear export. The inactivation of CRM1 by NO facilitated the nuclear accumulation of the antioxidant response transcription factor Nrf2. We speculate that this is important for promoting a cytoprotective transcriptional response to nitrosative stress.

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Determination of Nitric Oxide Donor Targets in Vivo with Electron Spin Resonance

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Besides its activity on vascular tone, nitric oxide (NO) activity can as also regulate oxygen consumption in tissues and prevent pathologies such as atherosclerosis and fibrosis through adaptation to hypoxia. For therapeutic strategy, it appears crucial to better understand which organs lack NO bioavailability and to which extent current and future NO-donor therapies can locally elevate its content. We therefore adapted to rat an in vivo spin trapping approach based on the affinity of colloidal $Fe(DETC)_2$ for NO. Adult Sprague Dawley rats were orally given nitroglycerin (10 µmol/kg, NTG) or its solvent for a total of 2 hours. During the last 30 minutes, DETC was injected intraperitoneally and an iron/citrate solution was injected subcutaneously. $Fe(DETC)_2$ is very lipophilic when it carries NO and is therefore trapped into tissue plasma membranes. Upon sacrifice, blood was collected to measure nitrosylated haemoglobin (HbNO) and $Fe(DETC)_2$-NO was measured in frozen tissues. We observed a higher increase in NO in the liver and the kidneys than in aorta, heart and lungs. The level of HbNO also increased importantly and appeared to be a reliable marker of NTG metabolism. Ex vivo study of each type of organ in the presence of 10 µM NTG confirmed that the liver and the kidneys were more likely to enzymatically metabolize NTG since they formed approximately ten times more NO over a 1h period. An elevated NO production could potentially have

deleterious consequences in the cardiovascular system through formation of peroxynitrite and hydroxyl radicals. However, systemic oxidation of the spin probe CMH in NTG-infused rats revealed only a moderate increase of oxidative stress. We therefore speculate that, in vivo, nitrate–type NO donors could be converted to HbNO in the liver and the kidneys and then slowly deliver NO to other organs with minimal secondary ROS production, supporting an endocrine role of NO.

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Lipid Membrane Solubility and Permeability of Nitrogen Dioxide

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Nitrogen dioxide ($^{\bullet}NO_2$) is an important oxidant species in biology. Its reactivity to cellular targets may be regulated by compartmentalization, but the permeability of membranes to this species is not known. The high reactivity of $^{\bullet}NO_2$ with organic compounds and water prevents a direct measurement of this property, but indirect studies suggest values between $4x10^{-4}$ and 10 $cm.s^{-1}$, a range too wide to make useful predictions. Herein we estimate membrane permeability to $^{\bullet}NO_2$ based on $^{\bullet}NO_2$ solubility in membranes (both properties are related) using "in silico" and empirical relationships approaches. Quantum calculations combining Density Functional Theory (DFT) and Tomasi´s polarizable continuum model (PCM) were performed to obtain the solvation free energy for $^{\bullet}NO_2$ and reference gases in various solvents, from which the theoretical solubilities were derived. In parallel, experimental solubility data for several diatomic and triatomic gases in different solvents was used to set empirical relationships (experimental vs. experimental and experimental vs. theoretical) in order to estimate $^{\bullet}NO_2$ solubility in different solvents. The best correlation was obtained between experimental and theoretical data for different gases in methanol, ethanol and octanol. $^{\bullet}NO_2$ solubility in octanol was determined to be 3.4 times higher than in water, being thus 1.7 times higher in membranes than in water. This result let us predict that membrane permeability to $^{\bullet}NO_2$ should be higher than that of CO_2 (0.35 $cm.s^{-1}$). Considering the permeability of an equally thick layer of water is 50 $cm.s^{-1}$, it can be concluded that membranes are moderately low barriers to $^{\bullet}NO_2$ transport.

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Protein Glutathionylation of RAW 264.7 Cells after S-nitrosocyteine Exposure

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Protein glutathionylation is the reversible formation of a mixed disulfide between a protein thiol and low molecular weight glutathione (GSH). Reactive oxygen species (ROS)/reactive nitrogen species (RNS) can cause protein glutathionylation and it is an important cellular response to oxidative stress. Protein glutathionylation has also emerged as a potential posttranslational regulation mechanism of a variety of proteins. We have previously shown that exposure of cells to S-Nitrosocysteine (CysNO) results in extensive protein S-nitrosation and loss of cellular GSH. It is often thought that S-nitrosation may lead to S-glutathionylation of protein thiols through the reaction of GSH with protein S-nitrosothiols. In this study we examined if exposure of cells to CysNO leads to protein glutathionylation. To investigate the global protein glutathionylation induced by CysNO-dependent thiol oxidation, mouse leukemic monocyte macrophage cells (RAW 264.7) were exposed to CysNO and the global cellular glutathionylation is analyzed by western blot using an antibody specific for protein glutathionylation. No increase in protein glutathionylation was observed upon treatment of cells with CysNO, whereas the positive control, diamide, caused extensive protein glutathionylation in these cells. This indicating the same treatment that can cause protein thiol nitrosation is not inducing extensive protein glutathionylation despite extensive loss of cellular GSH. It is possible that extensive S-nitrosation prevents S-glutathionylation by blocking available free thiols.

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Chronic Exposure to Nitric Oxide Decreases Mitochondrial Reserve Capacity in Endothelial Cells and Increases the Sensitivity to Oxidative Stress

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The regulation of bioenergetic responses to nitric oxide (NO) in endothelial cells remains poorly understood since it is often assumed these cells have minimal requirements for oxidative phosphorylation. In this study we hypothesized that the mitochondrial reserve respiratory capacity in endothelial cells is depressed by NO, resulting in increased vulnerability to oxidative stress. In the first series of experiments, Bovine Aortic Endothelial Cells (BAEC) were exposed to the NO donor Deta NONOate (DetaNO; 100-500µM; 16h), and bioenergetic function assessed. DetaNO resulted in a 40-70% inhibition of mitochondrial respiration that was not reversible on removal of NO. These data also showed that BAEC utilize just 35% of their maximal mitochondrial function under normal conditions. Thus, we next determined the effects of NO on this reserve capacity and found a dose-dependent decrease with complete inhibition by 500µM DetaNO. To determine the effects of oxidative stress on these cells, we followed the treatments above with exposure to the redox cycling agent DMNQ, which generates both hydrogen peroxide and superoxide. While none of the concentrations of DetaNO tested above were toxic to the cells alone, subsequent exposure to 7.5µM DMNQ increased apoptotic cell death within 6h. Mitochondrial function was assessed at 2h and was found to be completely inhibited. These data have important implications for our understanding of the impact of NO on endothelial cell function and highlight the importance of mitochondria even in cells with low bioenergetic demand.

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Regulation of Nitric Oxide Bioavailability by Cytoglobin, a Globin Expressed in Fibroblasts and Smooth Muscle

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Disposition of the second messenger nitric oxide (NO) in mammalian tissues occurs through multiple pathways including reaction with erythrocyte hemoglobin, red muscle myoglobin, and metabolism by NO dioxygenase activities in non-striated tissues. Although the newly discovered globin cytoglobin binds molecular oxygen and dioxygenates NO *in vitro*, the lack of an associated reductase activity has raised doubts about the ability of cytoglobin to dioxygenate NO *in vivo*. ***We hypothesize that non-striated***

cells can dioxygenate NO and that cytoglobin contributes to this activity. For this purpose, we stably expressed short hairpin RNA targeting cytoglobin in mouse fibroblasts. This resulted in an 80 percent reduction in cytoglobin protein expression. Wild type cells demonstrated intracellular nitrate generation upon addition of exogenous NO that was oxygen-dependent and cyanide-inhibitable. The sustained dioxygenation of NO observed over a 30 min exposure to NO was diminished in cells with low cytoglobin expression. These cells were also more sensitive to NO-induced inhibition of cell respiration and proliferation. Normal response to NO could be re-established through expression of human cytoglobin in the knock down cells. We have demonstrated cytoglobin message and protein expression in the intact rat aorta as well as primary cultured vascular smooth muscle (VSM) and adventitial fibroblasts from various species. Cultured VSM cells generate nitrate upon addition of exogenous NO, consistent with the NO dioxygenation observed in the immortalized fibroblasts. Exposure of VSM cells to the pro-inflammatory cytokine IL-1β resulted in upregulation of cytoglobin message and protein and was associated with an increase in NO consumption as compared to untreated cells. IL-1β induced changes in cytoglobin expression and rates of NO consumption were completely inhibited with pretreatment of VSM cells with the IL-1β receptor antagonist. These results are consistent with our observation that cytoglobin is upregulated in response to vascular injury in vivo. In summary, our study reveals a pivotal role for cytoglobin in cell-mediated NO dioxygenation to regulate nitrosative stress and cell respiration during conditions of chronic NO exposure. These results indicate cytoglobin may represent an important NO sink in the vessel wall in response to vascular injury and inflammation.

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Physiological Parameters of DNIC Formation and Degradation

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Although dinitrosyl iron complexes (DNIC) were first observed in the 1960s and are appreciated to be a biologically relevant product of endogenously and exogenously produced nitric oxide (NO), many fundamental, physiological properties of these species remain to be explored. Our studies examined the concentration of NO as it relates to formation of DNIC complexes in RAW 264.7 macrophages by measuring the EPR detectable g = 2.04 signal of this large paramagnetic species. Macrophages were chosen to facilitate the comparison of endogenous and exogenous NO treatments. The DNIC concentrations achieved from endogenous stimulation of RAW 264.7 cells was far lower than could be obtained with super physiological doses of NO, yet DNICs were detected long after NO exposure in both cases. LPS-stimulated, NO-producing macrophages revealed that DNIC formation paralleled the onset of NO production. Maximal intercellular DNIC concentrations were observed at 12 hours and gradually decayed, independent of NO production, out to 36 hours. The kinetics of DNIC decomposition (biological half-lives) at both 21% and 1% oxygen were measured after treatment with NO; no difference in the rate of decay at either oxygen concentration was detected. RAW cells treated with LPS at different oxygen concentration, on the other hand, showed different magnitudes and stabilities of DNICs. In general, these experiments define many of the physiological parameters of DNIC formation and stability induced by exogenous and endogenous NO sources at differing oxygen concentrations. Thus they provide invaluable data on the kinetics of the NO iron interaction and demonstrate differences in the fate of exogenous and endogenous NO.

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Thrombospondin-1, via CD47, Blocks Endothelial-Dependent Arterial Relaxation by Limiting Endothelial Nitric Oxide Synthase Activation

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The secreted matricellular protein thrombospondin-1 (TSP1) is an important negative regulator of nitric oxide/cGMP signaling in vascular cells under conditions of ischemic stress and in the acute physiological control of blood pressure. Signaling through its receptor CD47 limits activation of soluble guanylate cyclase and cGMP-dependent protein kinase, but we now show that TSP1 simultaneously acts upstream to inhibit activation of eNOS. Acetylcholine activates eNOS via increasing cytoplasmic calcium, and both acetylcholine- and calcium-mediated activation of eNOS in endothelial cells is inhibited by TSP1. This suggested that endogenous TSP1 could regulate arterial tone by limiting NO production in vascular endothelium. Ex vivo, the dilation of arteries from TSP1 and CD47 null mice in response to physiologic activation of eNOS by acetylcholine was significantly greater than in wild type vessels. Treating the luminal endothelial compartment of wild type and TSP1 null but not CD47 null arteries with TSP1 inhibited vessel relaxation to acetylcholine and potentiated vasoconstriction to phenylephrine. *In vivo*, acetylcholine-stimulated hypotension in TSP1 null mice was blocked by intravenous replacement of TSP1. Also a CD47 antibody altered arterial and blood pressure responses to vasoactive agents. Thus circulating TSP1, through CD47, limits endothelial-dependent arterial relaxation. This demonstrates a role for circulating TSP1 as a hypertensive agent supporting blood pressure through continuous modulation of both NO production and signaling.

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Does Erythrocyte Membrane PDI Interacts with Band-3 and Glut 1 in Transfer of NO-Equivalents in Red Blood Cells?

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Red Blood Cell (RBC) NOx-metabolism has been focus of much attention as it is implicated in hypoxic vasodilatation. RBCs scavenge nitrite that is generated from oxidation of NO in blood stream. However, the mechanisms by which NO-equivalents leave red blood cells and induce vasodilatation are unclear. In preliminary studies we have observed that RBC membrane protein disulfide isomerase (PDI) can be *S*-nitrosated. Other studies have shown that PDI is secreted from many cells including red blood cells, platelets, and endothelial cells. Little is known concerning the identity of proteins that are closely associated with PDI in the plasma membrane of erythrocytes that could potentially transfer NO-equivalents onto PDI. Role of anion exchanger (AE1 or band 3), which is the most abundant integral membrane protein of erythrocytes, has previously been studied with respect to NO-efflux. GLUT1 is a member of a family of glucose transporters (GLUTS), which is expressed at high levels in erythrocyte membrane. GLUT1 contains free thiols that can serve as a sink for NO^+. Here we explored the possibility of PDI acting as a protein that can transfer NO-equivalents from *S*-nitrosated membrane proteins such as GLUT1 and band 3 to the outside of RBCs. Our results indicate that when RBCs are exposed to nitrite under normoxic conditions, PDI is *S*-nitrosated. Band 3 and GLUT1 co-immunoprecipitated with PDI in nitrite treated red blood cell membrane, indicating specific interaction of

band 3 and GLUT1 with PDI. However, neither band 3 nor GLUT1 co-immunoprecipitated with each other. The proposed postulated role of PDI on RBC membrane is PDI getting *S*-nitrosated in lungs and loses NO group in tissues under hypoxic conditions.

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Identification of Nitrotryptophan in the Nitrated Proteins from Peroxynitrite-Treated PC12 Cells by Using LC-ESI-MS/MS Analysis

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We have shown that the formation of 6-nitrotryptophan ($6NO_2Trp$), which is a new nitrated amino acid, by the reaction of reactive nitrogen (RNS) species with Cu/Zn SOD and lysozyme. Production of RNS and 3-nitrotyrosine has been reported in some neurodegenerative diseases, such as Parkinson's syndrome or Alzheimer disease. In this study, we tried to detect $6NO_2Trp$-containing proteins by using newly developed antibody for $6NO_2Trp$ residues in peroxynitrite-treated lysate from neuron-like PC12 cells. Western blot analysis for 2D-PAGE showed seven intensively stained immuno-reactive spots in the lysate, which were then subjected to trypsin digestion and LC–ESI-MS/MS analyses. We identified several peptides containing nitrotryptophan in those amino acid sequences and identified to be L-lactate dehydrogenase A, malate dehydrogenase 1, and others by MASCOT search with significant ions scores ($p>0.05$). In addition, we found that the molar ratio of 3-nitortyrosine to $6NO_2Trp$ was about 6:1 in the proteinase K-digested PC12 cell lysate by using HPLC-coularray system. We conclude that $6NO_2Trp$ could be a useful new marker of nitrative stress in neurodegenerative disease.

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Oxygen Dependence of XOR Nitrite Reductase Activity

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Under severe hypoxia or anoxia, XOR catalyzes the one e^- reduction of NO_2^- to NO at the Mo-cofactor. However, the conditions that favor e^- transfer to NO_2^- at the Mo-cofactor over e^- transfer to O_2 at the FAD site have not been fully described. Using both NO probes and chemiluminsence detection methods, we analyzed NO_2^- reduction from purified XOR in an O_2 controlled environment. At 0% O_2, XOR reduced NO_2^- generating 17.0 ± 3.2 nM/min NO from xanthine while similar rates (22.9 ± 4.8) were observed with NADH as reducing substrate. At O_2 tensions up to 2%, there were O_2-dependent delays in the onset of NO production (8 min-0.5%, 12 min-1% and 30 min-2%). NO generation did not occur until all O_2 was consumed, suggesting that O_2 decreases NO_2^- reduction by indirect oxidation of the Mo via e^- withdrawal at the FAD. This was confirmed with experiments in which the FAD poison, DPI abrogated delay times. Superoxide generation did not affect NO detection, as the presence of SOD (100 U/ml) did not alter rates of NO formation or lag times, indicating that divalent reduction of O_2 predominates at low O_2 tensions. Experiments with XDH, the dehydrogenase form of XOR which has lower affinity for O_2, did not demonstrate improved rates of NO formation or reduced lag times. Combined these data indicate tissue O_2 tensions must approach zero before XO-dependent NO_2^- reduction occurs and NO formation realized.

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The Reaction of S-Nitrosothiols with a Water-soluble Phosphine gives S-Alkyl Phosphonium Products as Potential Bio-markers

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S-Nitrosothiols (RSNOs) represent an important class of post translational thiol modifications that may control many cGMP-independent signaling processes. S-Nitrosation of regulatory proteins mediates thrombosis, vascular inflammation and various cardiac actions. S-Nitrosated proteins may also act as relatively stable metabolites and/or sources of nitric oxide (NO) mediating cGMP-dependent processes. Given the relative chemical reactivity and instability of the S-nitrosothiol group in proteins, accurate direct detection and labeling of this post translational modification has been difficult. Previous work indicates that a reaction between S-nitrosothiols and triaryl phosphines gives covalent products and we now report the reaction of S-nitrosated cysteine and glutathione with a water-soluble phosphine, tris(4,6-dimethyl-3-sulfonatophenyl) phosphine trisodium salt hydrate (TXPTS). A combination of LC/MS spectrometry and one and two-dimensional proton, carbon and phosphorus nuclear magnetic resonance (NMR) experiments show the products of this reaction include a unique S-alkyl phosphonium ion ($RS\text{-}^+PR_3$), phosphine oxide ($O{=}PR_3$) and a phosphorus-based aza-ylide ($HN{=}PR_3$). Chemiluminescence and gas chromatographic studies suggest the absence of NO or nitroxyl (HNO) formation from these reactions and reduction of the reaction mixture generates starting phosphine and a target-site thiol. Incubation of the S-nitrostated bacterial peroxiredoxin mutant, C165S alkyl hydroperoxide reductase (AhpC) with TXPTS, generates a protein-derived S-alkyl phosphonium ion as judged by mass spectrometry giving evidence of direct in vitro labeling of a S-nitrosated protein. Control experiments show the phosphine does not react with free thiols or disulfides, suggesting a promising future for this reagent and labeling strategy for further in vivo S-nitrosothiol investigation.

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Nitrite Bioactivation in Cardiomyocytes

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Apart from regulation of vascular tonus, nitric oxide (NO) plays an important role in maintaining cardiac function. Circulating nitrite has recently been shown to induce vasodilation. It has been shown by others that nitrite-mediated vasodilation is based on the reduction of nitrite in red blood cells (RBC) to a bioactive intermediate which diffuses to smooth muscle cells (mechanism 1). However, we have previously shown that, in heart tissue homogenates, the reduction of nitrite to NO is predominantly mediated by mitochondria. Nitrite reduction was nearly fully inhibited by myxothiazol, a specific inhibitor of the mitochondrial respiratory chain (mechanism 2). The objective of this study was to clarify the predominant mechanism(s) (1 or/and 2) of exogenous nitrite bioactivation in an in vitro model of cardiomyocytes (HL-1) co-cultured with RBC. Isolated RBC and HL-1 were incubated in various proportions with or without nitrite. Nitrosyl complexes of hemoglobin (NO-Hb), NO, and cGMP were determined by electron spin resonance spectroscopy, confocal microscopy (DAF), and ELISA. RBC or HL-1 cells alone as well

as in combination, reduce nitrite to NO, yielding NO-Hb complexes in an oxygen-dependent manner. Free NO was detected in HL-1 cells but not in RBC. Nitrite reduction by HL-1 cells was accompanied by increased cGMP levels, while in co-culture with RBC this increase was significantly reduced. NO-Hb and cGMP levels were diminished by the specific mitochondrial inhibitors myxothiazol and potassium cyanide. Our data suggest that the bioactivation of nitrite in cardiomyocytes under hypoxic conditions is catalysed predominantly by mitochondria. Our data also suggest that cardiac and vasodilatory effects of nitrite may be controlled by different mechanisms of nitrite bioactivation in different types of cells and intracellular compartments.

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Interacting Profiles of Oxygen and Nitric Oxide Concentration Dynamics in the Hippocampus

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The ubiquitous messenger molecule nitric oxide (•NO) is a known regulator of a myriad of cellular processes in organ systems ranging from the cardiovascular to nervous or immune systems. While soluble guanylate cyclase is classically viewed as the main mediator of •NO actions in mammalian cells, •NO can also regulate cell metabolism/respiration by inhibiting cytochrome c oxidase, the terminal complex of the mitochondrial respiratory chain. In view of this current understanding we performed simultaneous recordings of •NO and O_2 in the CA1 subregion of the rat hippocampal slice.We had previously shown that NMDA-evoked •NO production is dramatically different among the principal cell layers of the hippocampal slices, with the following gradient CA1>>CA3=DG. Considering the role played by •NO in LTP on the CA1 subregion and this regions higher sensitivity in several degenerative disorders associated with oxidative stress, we questioned whether NMDA evoked •NO production in CA1 could effect tissue oxygen consumption.Using 2 microelectodes inserted into the CA1 subregion of the hippocampal slice we succeed in simultaneously recording •NO concentration dynamics and the evolution of pO_2 upon NMDA receptor stimulation. The main findings were: 1) endogenously produced •NO inhibited tissue oxygen consumption; 2) the extent in inhibition of O_2 consumption is directly related to maximal •NO concentration (r= 0.62); 3) the direct relationship between produced •NO and variation in O_2 consumption rate was confirmed by pre-treating slices with 3-Br-7-NI, a potent inhibitor of nNOS; 4) decrease in O_2 consumption was observed only after •NO concentration passed the average threshold value of 271.8 ± 89.3 nM (n=14). We have also found that the profile of variation in pO_2 varies with the shape of the •NO response: while very low •NO concentrations (typically below 50 nM) lead to reversible inhibition of O_2 consumption, for higher concentrations, O_2 consumption is inhibited irreversibly, with a new steady state pO2 being reached; also, decreasing the mid-width of •NO signals resulted in partial or total (rarely) reversion of O_2 consumption to values closer to initial steady state (prior to NMDA application).These results obtained in a system close to the in vivo strongly support the current paradigm obtained in simple systems for O_2 and •NO interplay in the regulation of cellular respiration.

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A Rapid Reaction of Nitric Oxide with Hemin-GSH Complex: a Mechanism for S-nitrosothiols Formation

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Nitrosation of thiol groups by nitric oxide (NO) to form S-nitrosothiols (SNOs) is involved in the regulation of various cellular processes. However, the mechanism for SNOs formation *in vivo* is not understood. One route for SNOs formation that has been proposed in presence of oxygen involves the oxidation of NO to N_2O_3, which can nitrosylate the thiols. This mechanism does not explain the formation of SNOs in blood milieu where the life-span of NO is less than 2ms. The other alternate mechanism is the oxidation of NO by metal compounds and metalloproteins to NO^+, a powerful nitrosation agent if the thiol is in close contact. Along these lines we have been studying the formation of SNOs by hemin. Hemin, the oxidized form of iron protoporphyrin, is a prosthetic group of several important hemoproteins that is also involved in signal transduction pathways. Hemin forms a complex with glutathione coordinating with iron without reduction under anaerobic conditions. NO alone does not reduce hemin to heme. However, we observed that NO readily reduces the hemin to heme when NO is added to the hemin-GSH complex. This reductive nitrosylation simultaneously resulted in the formation of GSNO. The finding that sodium azide had no effect on the GSNO formation rules out the involvement of N_2O_3, thought to be formed during reductive nitrosylation. When NO was added to the membrane associated hemin-GSH complex, the hemin was reduced and SNOs were detected on the membrane and supernatant. Hemin and glutathione, ubiquitous molecules in cells that can exist as complexes under hypoxic conditions, have, thus, been shown to form SNOs when they come in contact with NO. These results provide a potential mechanism for the formation of SNOs *in vivo* and perhaps the mechanism for hypoxic vasodilation.

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Hypoxic Nitrite Reduction to Nitric Oxide Varies by Cell Type

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Nitrite anion acts as a reservoir for bioavailable NO due to its reduction back to nitric oxide in permissive tissue environments such as hypoxia. A majority of studies examining nitrite reduction to NO have focused on the red blood cell due to the nitrite reductase activity of deoxyhemoglobin. However, several other nitrite reduction mechanisms could be invoked during hypoxia in other cells of the cardiovascular system. Here we examined the hypothesis that nitrite reduction to NO readily occurs in both endothelial and vascular smooth muscle cells under hypoxic culture conditions. Human primary endothelial or vascular smooth muscle cells from various vessel types were cultured under normoxic or 1% hypoxia conditions for 18 hours and then incubated with various doses of sodium nitrite to evaluate NO production using DAF fluorescence and chemiluminescence detection. We discovered that nitrite reduction to NO was dose dependent and vascular cell type specific such that venous endothelial (EC) and vascular smooth muscle cell (VSMC) nitrite reduction occurred at lower doses of sodium nitrite (1-100 µM) compared to arterial EC and VSMC's. Importantly, nitrite treatment of all cell types under normoxia did not result in NO production. Control experiments under hypoxia were performed using sodium nitrite plus DAF without cells or cell experiments containing the NO scavenger cPTIO demonstrating that nitrite

was unable to mediate direct nitrosylation confirming the generation of bonafide NO. Nitrite reduction to NO could be completely blocked by the addition of xanthine oxidase inhibitors allopurinol or Febuxostat. However, inhibition of NOS enzyme activity with L-NAME did not affect nitrite reduction to NO. Lastly, human primary aortic adventitial fibroblasts were much less effective at reducing nitrite to NO under hypoxic conditions. This study demonstrates that chronic cellular hypoxia significantly increases reduction of nitrite to NO in various cardiovascular cell types through a mechanism involving xanthine oxidase. Our data also indicate that nitrite dependent NO generation can directly occur in end-target tissues thus bypassing potential biological sinks of NO such as hemoglobin.

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A Novel Method to Measure Nitrite and Nitrosyl Hemoglobin in Whole Blood using Electron Paramagnetic Resonance Spectroscopy

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Reported levels of nitrogen oxides in the blood have varied considerably, from the nanomolar to the micromolar range. In the case of nitrosyl hemoglobin (HbNO), the total amount has been given as 2.5 µM in venous blood (Nat Med. 2002; 8:711) to approximately 200 nM (J Biol Chem. 2005; 280:40583). Basal levels of HbNO have also been specified as 0.18 % of total hemoglobin (PNAS. 2007; 104:17593), approximately 18 µM. Nitrite is considered an important signaling molecule (Med. Res. Rev. 2009; 29:683-741) so determining whole blood nitrite levels accurately is also important, and nitrite concentrations have been reported in the range of 200 nM to nearly 500 nM (Blood. 2005; 106:734). In our study, we have used electron paramagnetic resonance (EPR) as a tool to measure basal nitrosyl hemoglobin and nitrite in blood. Nitrite reacts with deoxyhemoglobin to form HbNO. Sodium dithionite effectively deoxygenates whole hemoglobin; yet, it does not react with nitrite efficiently. Whole blood was treated with dithionite and known concentrations of nitrite for HbNO formation. All of the added nitrite is converted to HbNO. Thus, the measured HbNO signal corresponds to the amount of basal nitrite and HbNO plus the added nitrite. Based on this method, we have found that the total amount of basal HbNO and nitrite in blood is less than one micromolar, which suggests some high reported levels of basal HbNO are erroneous. Additional work is being conducted to adapt this method to also measure nitrosothiols.

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Effect of Hypertonic Resuscitation in Pancreatitis and Nitric Oxide Interactions

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It has been shown a hepatic injury following acute pancreatitis (AP) and a positive correlation with severity of the disease. Hypertonic Solution (HS) limited local and end-organ injury in pancreatitis, reducing mortality. To test whether HS may exert tissue protection by modulating hepatic oxidative stress in liver during AP, Wistar rats were divided in: C- control animals not suffered insult nor treatment; NT- animals submitted to AP that did not received treatment; NS- animals submitted to AP and had received normal saline (0,9% NaCl 34 ml/Kg); HS- animals submitted to AP and had received hypertonic solution(7,5% NaCl 4 ml/kg). AP was induced by retrograde infusion of 2,5% sodium taurocholate into the pancreatic duct transduodenally. Assays were performed in liver tissue after 4, 12 and 24 hours of pancreatitis induction to analyze Nytrotirosine expression (immunohistochemistry), Apoptosis (Western Blot of Capases 2 and 7, APAF-1 and Apoptosis Inducing Factor – AIF), Nitrite/nitrate (Griess Reaction), Lipid peroxidation (TBARs), ALT production (automatic analyzer). Data (mean ± SEM) were analyzed by ANOVA. Values of $p<0,05$ were considered significant. Correlations were determined by linear regression. HS significantly reduces nitrite/nitrate levels ($p<0,01$ vs. NS at 12h) and lipid peroxidation ($p<0,05$ vs. NT at 12h), consequently reducing nitrotyrosine formation ($p<0,05$ vs. NS at 24h). Lipid peroxidation and nitration showed a positive correlation of $R=0.52$ ($p<0.001$). The proteins involved in mitochondrial pathway of apoptosis (Casp2, APAF-1, AIF) have not been activated in pancreatitis. The Casp7 expression increased in NT group ($p<0,01$ vs. C at 4h), remained similar in control and treated groups. Hepatic damage (ALT release) was reduced in HS group ($p<0,01$ vs. NS at 12h). In conclusion, hypertonic resuscitation prevents the dangerous effects of peroxynitrite in the time critical resulting in diminished liver damage.

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Nitroarachidonic Acid: The First Peroxidase Inhibitor of Prostaglandin Endoperoxide H Synthase 1 and 2

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Prostaglandin endoperoxide H synthase (PGHS) is a bifunctional heme-peroxidase that catalyzes the oxidation of arachidonate (AA) to prostaglandin G2 (cyclooxygenase activity, COX) following reduction to prostaglandin H2 (peroxidase activity, POX). We have previously synthesized and chemically characterized nitroarachidonate ($AANO_2$), a novel anti-inflammatory signaling mediator. Herein, the interaction of $AANO_2$ with PGHS was analyzed. $AANO_2$ inhibited POX of both ovine-PGHS-1 and murine-PGHS-2 via a slow tight binding two-step-mechanism. In contrast to the reported PGHS-2 inhibitors which selectively affect COX activity, $AANO_2$ exhibited time- and concentration-dependent inhibition of POX in PGHS-2 without affectation of COX; k_{obs} plotted against $AANO_2$ concentrations showed hyperbolic functions with $k_{inact}=0.045s^{-1}$ and $K_{inact}=0.019$ µM for PGHS-1 and $k_{inact}=0.057\ s^{-1}$ and $K_{inact}=0.020$ µM for PGHS-2. The obtained plots intersect the y axis at zero suggesting an isomerization step follow $AANO_2$ reaction with the enzyme, leading to an irreversible inactive state. Mass spectrometry analysis showed that $AANO_2$ did not exert covalent modifications of the protein. Instead, we observed a disturbance in the interaction of the heme moiety at the holo enzyme when $AANO_2$ was present. This suggests that the limiting step might correspond to a conformational transition of the protein due to a reaction with $AANO_2$ leading to the displacement of heme. PGHS inhibition by $AANO_2$ was also demonstrated in activated platelets, where the nitro-fatty acid significantly decreased tromboxane-B2 formation. Current studies are focusing to determine the pharmacological potential of $AANO_2$ as first selective inhibitor of POX in PGHS-2.

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NO Cytoprotection: DNICs Mediated Antioxidant Effects

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The labile iron pool (LIP) is a small but chemically significant fraction of total cellular iron. Due to its redox properties, the LIP is capable of generating highly oxidizing intermediates through its interaction with reactive oxygen species (ROS). These can lead to oxidative damage via DNA strand breaks, base modifications, protein oxidations, and lipid peroxidations. The LIP is known to form dinitrosyl iron complexes (DNICs) when exposed to nitric oxide (NO). Chelation of cellular iron via DNIC formation should serve an antioxidant function by preventing deleterious redox reactions. We observed that HCC breast cancer cells treated with NO (spermine nonoates, 10-50μM) were partially protected against ROS mediated cytotoxicity (H_2O_2 exposure). Furthermore, pretreatment with NO markedly decreases the oxidative stress induced by peroxide treatment as measured by DCFDA fluorescence. These results could be replicated by substituting iron chelators (dipyridal, desf) for NO. NO concentrations correlated with concentration-dependent increases in DNIC formation. These results support the hypothesis that NO is an antioxidant by its ability to chelate cellular iron. This could play a significant role in variety of diseases involving ROS mediated toxicity like cancer and neurodegenerative disorders.

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Membrane Facilitated Release of NO from Nitrite Reacted Deoxyhemoglobin

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Nitric oxide (NO) plays a crucial role in human physiology by regulating vascular tone and blood flow. However, NO's high reactivity in the circulation requires a mechanism that can retain NO bioactivity and allow for more distal and sustained effects within the microcirculation. A proposed mechanism involves the reduction of nitrite back to NO in red blood cells (RBCs) by its interaction with deoxygenated hemoglobin via the formation of stable intermediates that conserve NO bioactivity and can potentially release NO to the vasculature when needed. It is, however, necessary to explain how the NO is released from these intermediate species and furthermore, how can the released NO in the RBC bypass the scavenging role of hemoglobin. It has been shown that both oxygenated and deoxygenated hemoglobin bind to the cytoplasmic domain of band 3, the RBC trans-membrane anion exchange protein. NO released from nitrite reacted hemoglobin bound to the membrane can diffuse out of the cell before being quenched by the large pool of intracellular hemoglobin. To determine whether the RBC membrane may be involved in releasing NO, we studied two reaction systems by NO chemiluminescence: (1) the reduction of nitrite in the presence of membrane-bound deoxyHb and (2) the interaction of nitrite reacted deoxyHb with the membrane. Our studies suggest that prior binding of deoxyHb to the membrane inhibits the reaction of nitrite by a significant, reduced, concentration of the initial nitrite bound intermediate and negligible concentrations of the delocalized intermediate and Hb(II)NO. On the other hand, binding of nitrite reacted deoxyHb results in a significant decrease in the concentration of all of the nitrite reacted species present. In both cases about 10% of the added nitrite is released as NO into the gas phase, a level that is 2 orders of magnitude greater than the level detected in the absence of membranes. These results indicate a role for the membrane in releasing NO from nitrite reacted deoxyHb, and potentially into the vasculature.

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Nitric Oxide-Mediated Inhibition of Taurocholate Uptake in HuH-NTCP Cells

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The sodium-dependent bile salt cotransporter (NTCP) plays a crucial role in the formation of bile by mediating cotransport of sinusoidal Na^+ and taurocholate (TC). During sepsis-induced cholestasis, there is a decrease in NTCP dependent uptake of bile acids and a concomitant increase in nitric oxide (NO) levels in hepatocytes. It has been shown in isolated rat hepatocytes that NO can inhibit Na^+-dependent uptake of taurocholate. The purpose of the current study was to further investigate the mechanism of NO-mediated inhibition of TC uptake. The present studies were performed in the human hepatoma cell line, HuH-NTCP, which stably expresses NTCP. We find that NO inhibits TC uptake in HuH-NTCP cells as it does in isolated rat hepatocytes. Using two NO donors, sodium nitrosoprusside (SNP) and S-nitrosocysteine (SNOC), we demonstrate that NO can inhibit TC uptake in HuH-NTCP cells. HuH-NTCP cells were incubated with increasing concentrations of either SNOC or SNP. The effect of NO on the initial rate of TC uptake was determined. Both donors inhibit TC uptake, albeit with different potencies. We have also examined the timing by which NO donors inhibit TC uptake. Both SNOC and SNP inhibit TC uptake with SNOC showing substantial inhibition as early as 15 minutes. We have performed kinetic analysis with SNOC to further investigate the mechanism by which NO inhibits TC uptake. Over a range of doses of SNOC (0.5 – 5 mM) and range of concentrations of TC (0 – 100 μM) these analyses reveal that NO lowers the V_{max} of TC uptake but does not alter the K_m of TC for NTCP indicating non-competitive inhibition. One way that NO may inhibit NTCP function is by direct binding of the protein via S-nitrosylation. In order to determine whether NO modifies NTCP via S-nitrosylation we have performed experiments using the biotin switch technique. We find that NTCP is S-nitrosylated under identical conditions where NO inhibits TC uptake. Moreover, we find that dithiothreitol (DTT) can reverse NO-mediated inhibition of TC uptake. This provides further evidence that NO inhibits TC uptake via modification of cysteine thiols. Our results indicate that NO inhibits TC uptake through thiol modification and this inhibition may be due to S-nitrosylation of NTCP.

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Nitrite Stimulates Mitochondrial Biogenesis in Hypoxic Myocytes

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Nitrite, once considered to be merely an inert byproduct of nitric oxide (NO) oxidation, is now accepted as an endocrine storage form of NO that mediates a number of physiological responses including hypoxic vasodilation, angiogenesis, and cytoprotection from ischemia/reperfusion (I/R) injury. On a subcellular level, nitrite is emerging as an important regulator of mitochondrial function that inhibits respiration during hypoxia and attenuates reactive oxygen species formation after I/R. Here we show evidence that nitrite also regulates mitochondrial number during hypoxia. Cultured myocytes (H9C2) treated with nitrite (20-100 μM) and subjected to hypoxia (1% O_2) for 6 days showed a significant increase in mitochondrial number in comparison to

cells treated with nitrate (as a control) or left at normoxia. The mechanism of this increased mitochondrial number appears to occur through the traditional mitochondrial biogenesis pathway as it was associated with an increase in the expression of PPAR gamma co-activator 1 alpha (PGC1-α) and nuclear respiratory factor 2 (NRF2). Functionally, cells with increased mitochondrial number showed an increase in the rate of uncoupled respiration, while basal respiration rate was unchanged. This was accompanied by increased ATP generation. These data suggest a novel role for nitrite as a regulator of mitochondrial number and expand the role of nitrite as a regulator of hypoxic mitochondrial function. This nitrite-dependent modulation of mitochondrial number may present a fundamental mechanism by which myocytes adapt to chronic ischemia to prevent cell death.

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Oxidation of Coumarin Boronate to Hydroxy-coumarin by Different Fluxes of Nitric Oxide and Superoxide: Quantitative Measurements of Peroxynitrite under Various Nitric Oxide/ Superoxide Fluxes

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Even though peroxynitrite ($ONOO^-$) has been recognized as one of the most important reactive nitrogen species formed under a variety of inflammatory conditions, methodologies for direct detection and quantification of this species have been lacking. We report here the use of a novel boronate-based fluorogenic probe to quantitate $ONOO^-$ formed in xanthine/xanthine oxidase/NONOate system. Under various fluxes of nitric oxide ($^{\bullet}NO$) and superoxide ($O_2^{\bullet-}$), the coumarin boronate (CB) was oxidized to a fluorescent product, 7-hydroxycoumarin (7-OH-C) that was detected and quantitated by spectrofluorimetry as well as HPLC with fluorescence detection. Superoxide or $^{\bullet}NO$ alone failed to oxidize CB to 7-OH-C. Formation of 7-OH-C was unaffected by catalase and partially inhibited by SOD. This product was also formed during oxidation of CB by authentic $ONOO^-$ (≈ 85% yield). The rate constant between CB and $ONOO^-$ was determined to be ca. 1×10^6 $M^{-1}s^{-1}$. The yield of 7-OH-C increased linearly up to 1:1 flux ratio of $^{\bullet}NO$:$O_2^{\bullet-}$ and reached plateau at higher fluxes of superoxide. No bell-shaped response was observed. We conclude that $ONOO^-$ is formed as a major intermediate at all fluxes of $^{\bullet}NO$ and $O_2^{\bullet-}$ in xanthine/xanthine oxidase/NONOate system.

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The Relationship Between Arginase, NOS, and ADMA to Acute Lung Injury in Sepsis

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More than 750,000 patients in the United States develop sepsis annually. Previously we have demonstrated that the pathophysiological responses of bacteria are dependent on the nature of the causative agent such as *Pseudomonas aeruginosa* (P. aer.) or Methicillin-resistant *Staphylococcus aureus* (MRSA). We hypothesize that asymmetrical dimethyl-arginine (ADMA) causes a greater decrease in nitric oxide (NO) production and a larger increase in arginase activity in *P.aer*.-induced sepsis compared to MRSA-induced sepsis. METHODS: Ewes were operatively prepared and randomized, after a 7-day recovery period, into control, MRSA, and PA groups (n=6). Injury consisted of instillation of 2-5 x 10^{11} CFU of live MRSA or PA into the airway, and the animals were sacrificed after 24 hours. In addition, C57Bl/6J and iNOS knockout mice (n=6) were nasally inoculated with 2-5 x 10^5 CFU of live MRSA or PA and were sacrificed after 8 hours. RESULTS: PA induced a more severe lung injury compared to MRSA (PaO_2/FiO_2: 319 ± 82 vs 205 ± 72, $p<0.05$). PA-treated sheep had a significantly higher ADMA compared to MRSA-treated animals (1.79 μM ± 0.14 vs 1.16 ± 0.24, $p<0.05$) and had significantly higher arginase activity (1.55 ± 0.16 μM urea/μG protein vs 1.07 ± 0.11, $p<0.05$). PA-treated sheep had significantly lower plasma NOx compared to MRSA-treated ewes. C57Bl/6J mice treated with PA vs MRSA had significantly higher arginase activity (0.16 μM urea/μG protein ± 0.0038 vs 0.60 ± 0.12, $p<0.05$) and significantly higher protein oxidation (1124 ± 140.1 vs 3223 ± 440.7, $p<0.05$). iNOS knockout mice treated with MRSA had significantly lower arginase activity than wild-type mice (0.36 ± 0.04 μM urea/μG protein vs 0.21 ± 0.01, $p<0.05$). CONCLUSION: The results strongly suggest that the severity of acute lung injury in PA sepsis is due to the increased activity of ADMA and arginase. Treatment strategies for PA and MRSA should consider their different host responses. For possible therapeutic intervention, effects of arginase inhibitors should be tested.

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Upregulation of Nox4 by TGF-β1 Oxidizes SERCA and Induces Vascular Dysfunction in the Prediabetic Zucker Rat

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Migration of smooth muscle cells (SMCs) is an important pathological process in several vascular occlusive diseases, including atherosclerosis and restenosis, both of which are accelerated by diabetes mellitus. To determine the mechanisms of abnormal SMC migration in type-2 diabetes, the obese Zucker rat (ZO), a pre-diabetic leptin receptor-deficient model of obesity and insulin resistance, was studied. In culture, ZO aortic SMCs showed a significant increase in Nox4 mRNA and protein levels compared with ZL. SERCA nitrotyrosine-294,295 and cysteine-674 (C674)-SO_3H were increased in ZO SMCs by immunoblotting, indicating oxidant stress. Unlike ZL, nitric oxide (NO) failed to inhibit serum-induced SMC migration in ZO, while transfection of Nox4 siRNA or overexpression of SERCA2b WT, but not SERCA C674S mutant in which C674 was mutated to serine, could restore the response to NO. In addition, knockdown of Nox4 by Nox4 siRNA but not by control siRNA decreased SERCA oxidation in ZO SMCs. In ZO SMCs, the protein level of transforming growth factor beta 1 (TGF-β1) was increased. Blockade of TGF-β1 by anti-TGF-β1 antibody restored inhibition of SMC migration by NO in ZO SMCs. In ZL SMCs, TGF-β1 blocked the effect of NO by a mechanism involving upregulation of Nox4-based NADPH oxidase. Corresponding to the results in aortic SMCs, immunochemistry confirmed that TGF-β1, Nox4, and SERCA C674-SO_3H were significantly increased *in vivo* in ZO aorta compared with ZL. These studies indicate that the upregulation of Nox4 by TGF-β1 in ZO SMCs is responsible for the abnormal response to NO by a mechanism involving the redox regulation of SERCA C674.

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NO-Mediated S-nitrosation of Protein Tyrosine Phosphatase in Endothelial Cells under Flow

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It is known that laminar shear flow (LF) exerts atheroprotection to endothelial cells (ECs) due to the increased NO production. NO can react with the Cys residue (S-nitrosation) in proteins. S-nitrosation in ECs and its physiological significance remain unclear. Protein tyrosine phosphatase (PTP) contains catalytic Cys that is susceptible for oxidative modification. ECs exposed to TNFα or H_2O_2 increased oxidative modification in PTP. However, ECs under flow lead to a sustained S-nitrosation in PTPs including SHP2. ECs expressing catalytic inactive mutant of SHP2 abolished this S-nitrosation. ECs treated with siRNA to eNOS or an eNOS inhibitor (L-NAME) suppressed this S-nitrosation. Similarly, ECs exposed to TNFα followed by LF showed a reduced S-nitrosation. In contrast, ECs pretreated with an inhibitor to NADPH oxidase (NOX) or siRNA to NOX2/4 increased flow-induced S-nitrosation. Consistently, S-nitrosation in PTPs remains at elevated level in ECs under flow followed by TNFα treatment indicating that flow protects PTP from oxidative modification. In conclusion, LF to ECs enhances the S-nitrosation of proteins and this highly reversible S-nitrosation protect proteins against oxidative inactivation and thus essential for maintaining vessel integrity.

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Vascular (NO) Availability from LDLr-/- mice: Age and Diet

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Endothelial function is impaired in aging because of a decrease in nitric oxide (NO) bioavailability. We assessed the potential mechanisms and end-points underlying the NO's availability from aging model of dyslipidemic mice. LDLr-/- mice (3 months-old) were submitted to 15, 30 and 60 days treatment Chow diet (C15, C30 and C60) or High fat diet 1.25% cholesterol-enriched (H15, H30 and H60) the aortas were analyzed by: i) Biotin-switch technique for *S*-nitrosylation; ii) Expression of nitrotyrosine, nNOS, iNOS eNOS and eNOS phosphorylated at 1179 serine residue (p-eNOSser1179); iii) Nitrite and nitrate generations by Sievers chemiluminescence. Results:i) S-NO content decreased in relation to age on LDLr-/- mice C60 compared to C15 and C30 mice (0.23±0.06 *vs* 0.56±0.06, p<0.01and 0.67±0.4, p<0.001 respectively) and this decreased was potentiated by diet on LDLr-/- mice H60 compared to H15, H30 mice (0.20±0.05 *vs* 0.74±0.11, p<0.001 and 0.53±0.03, p<0.05, respectively); ii) expression levels of the nitrotyrosine, nNOS, iNOS, eNOS were not altered by age or high fat diet; Moreover, p-eNOSser1179 expression was increased in acute phase by diet on LDLr-/- mice H15 compared to C15 (1.2±0.1 *vs* 0.53±0.08, p<0.01) and H30 compared to C30 (1.74±0.07 *vs* 1.16±0.08, p<0.05), but on chronic phase this "emergential activation" was blunted . The aging on LDLr-/- mice with both diets induced marked decrease on p- eNOSser1179 (C60 *vs* C30 mice 0.60±0.1,and 1.16±0.08, p<0.05); iii) Nitrite and nitrate generation were marked increase in relation to age, but the high fat diet blunted this profile. We demonstrate that the content *S*-nitrosylated proteins in aorta from LDLr-/- mice was impaired by age and it can be releated to reduced eNOS phosphorilated status which mediated decreases eNOS enzymatic activity and NO production. Thus suggesting that p- eNOSser1179 downregulation may be involved in age-dependent impaired NO bioavailability and can be modulated by environmental factors, such the diet. [Financial Support: FAPESP and CAPES]

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Role of Glutaredoxin 1 in the Defense Against Nitrosative Stress in Rat 3Y1 Cells

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The mechanism of S-nitrosylation of cellular proteins is one of enigmatic processes in the cell biology. Emerging evidence suggests that thioredoxin plays a role in both S-nitrosylation and denitrosylation of selective proteins such as caspases. Glutaredoxin 1 (Grx 1), a low molecular weight cytosolic oxidoreductase of the thioredoxin superfamily, is responsible for removal of glutathione moiety from glutathionylated proteins. However involvement of Grx 1 in the process of S-nitrosylation and denitrosylation remains unclear. Therefore we assessed a role for Grx 1 under nitrosative stress conditions using murine fibroblast 3Y1 cells. Transfection of 3Y1 cells with a siRNA against Grx 1 down regulated mRNA for Grx 1 and decreased disulfide reductase activity of the cells. The Grx 1 down regulation sensitized cells to the NO/O_2^- donor SIN-1 (3-morpholinosydnonimine) but not to other NO donors or oxidative stressors. Interestingly, however, while SIN-1 treatment increased the level of S-nitrosylated proteins in control cells, that in Grx 1 knock-down cells was unchanged by SIN-1 treatment. These results suggest that Grx 1 may mediate S-nitrosylation of cellular proteins under nitrosative stress conditions, which may paradoxically be a cellular counter response to nitrosative stress.

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Endothelial and Blood eNOS: Shared Roles in Nitrite Homeostasis and Blood Pressure Regulation

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Mice genetically deficient in constitutive nitric oxide synthase ($eNOS^{-/-}$) are hypertensive compared to C57Bl/6 wild type (WT) mice, indicating the importance of constitutively produced nitric oxide ($NO^{\bullet}$) to blood pressure (BP) regulation and vascular homeostasis. In addition to vascular endothelium, blood cells (platelets, leukocytes and even erythrocytes) express eNOS protein. **Objective:** The objective was to determine the contribution of eNOS in blood cells to intravascular nitrite and regulation of BP under physiological conditions. **Methods:** We cross-transplanted WT and $eNOS^{-/-}$ mice to produce chimeras expressing eNOS only in blood (BC+/EC-) or vascular endothelium (BC-/EC+), confirmed by flow cytometry, western blot, RT-PCR and immunohistochemistry. Arterial BP was assessed in anesthetized and conscious (radiotelemetry) chimeras in the absence/presence of pharmacologically induced thrombocytopenia. Whole blood and plasma nitrite were quantified using reductive chemiluminescence. NOS activity was assessed in vivo (oral NOS inhibitor L-NAME or NOS substrate L-

arginine effects on BP). **Results:** BPs and nitrite concentrations inversely correlated across all chimeric groups (plasma: r^2=.9967, p=.0017; whole blood: r^2= .77, p=.1218). BP for chimeras expressing only blood eNOS (BC+/EC-) was lower (MAP: -18.80 mmHg mean difference; p < .0001) than BP of chimeras globally deficient for eNOS (BC-/EC-), a result not changed by platelet depletion (-18.45 mmHg mean difference). Moreover, limited expression of eNOS to either endothelium or blood was sufficient for NOS inhibition or NOS stimulation to produce classical BP responses (L-NAME: 6.75±4.27 mmHg for BC+/EC+, 3.16±4.77 mmHg for BC+/EC- and 2.92±4.27 mmHg for BC-/EC+; L-arginine: -8.96±3.97 mmHg for BC+/EC+, -7.43±4.43 mmHg for BC+/EC- and -6.90±3.97 mmHg for BC-/EC+), whereas global deficiency of eNOS expression elicited paradoxical BP responses (L-NAME: -12.03±5.52; L-arginine: 0.59±5.12 mmHg). **Conclusion:** These findings indicate that physiological BP regulation is the purview of eNOS in blood as well as in vascular endothelium. Additional experiments are underway to establish leukocyte- versus erythrocyte-specific eNOS-associated effects on BP regulation.

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Ultraviolet B Light-Induced Nitric Oxide /Peroxynitrite Imbalance in Keratinocytes – Implication in Apoptosis, Necrosis and Carcinogenesis

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Ultraviolet B light (UVB) induces production of nitric oxide ($NO^{\bullet}$), which can either promote or inhibit UV-induced cell death. The mechanism for regulation of UVB-induced and $NO^{\bullet}$-mediated pro- or anti-apoptosis is not clear. In this study, we determined real-time concentration of $NO^{\bullet}$ and peroxynitrite ($ONOO^{-}$) and their role in regulation of membrane integrity and apoptosis. Nanosensors (diameter 300-500 nm) were used for direct in situ simultaneous measurements of $NO^{\bullet}$ and $ONOO^{-}$ generated by UVB in cultured keratinocytes and mice epidermis. An exposure of keratinocytes to UVB immediately generated $ONOO^{-}$ at maximal concentration of 190 nM followed by $NO^{\bullet}$ release with a maximal concentration of 91 nM. The kinetics of UVB-induced $NO^{\bullet}/ONOO^{-}$ was in contrast to cNOS agonist stimulated $NO^{\bullet}/ONOO^{-}$ from keratinocytes. After stimulating cNOS by calcium ionophore, $NO^{\bullet}$ release from keratinocytes was followed by $ONOO^{-}$ production. The [$NO^{\bullet}$] to [$ONOO^{-}$] ratio generated by UVB decreased below 0.5 indicating a serious imbalance between cytoprotective $NO^{\bullet}$ and cytotoxic $ONOO^{-}$ - a main component nitroxidative stress. The $NO^{\bullet}/ONOO^{-}$ imbalance increased membrane damage and cell apoptosis was partially reversed in the presence of free radical scavenger. Based on these results, we propose a novel mechanism through which UVB may induce membrane damage and cell apoptosis: UVB activates cNOS and production of $NO^{\bullet}$, which is rapidly scavenged by photolytically and enzymatically generated superoxide ($O_2^{\bullet-}$) to produce high levels of $ONOO^{-}$, which enhances oxidative injury and apoptosis of the irradiated cells.

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Energy Metabolism, Nitric Oxide and Superoxide Anion Production During Rabbit Heart ex vivo Ischemia and Reperfusion

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Left ventricle mitochondrial function of rabbit hearts exposed to ex vivo ischemia-reperfusion was studied, with special attention to NO and $O_2^{\bullet-}$ metabolism. Isolated hearts were perfused according to Langendorff technique. After 15 min of stabilization (0/0), ischemia was induced for 15 min (15/0), followed by 5 or 30 min of reperfusion (15/5 and 15/30). Tissue slice O_2 consumption rates were 5% lower after ischemia, and 15 and 40% lower after 5 and 30 min reperfusion, respectively. Ischemia and 5 min of reperfusion decreased state 3 and state 4 mitochondrial respiration with malate/glutamate. These effects were not observed with succinate, suggesting that ischemia-reperfusion damages complex I. For complex I activity, a decrease of about 30% was observed for 15/0, and the decline was irreversible with 5 or 30 min of reperfusion. Complex II and IV activities were 15% lower after 15 min of ischemia, but they were restored by 5 and 30 min of reperfusion. $O_2^{\bullet-}$ production was enhanced by 100% during ischemia and reperfusion. Mitochondrial NO production was decreased by about 35% in 15/0 and 15/5 hearts. After 30 min of reperfusion mtNOS activity was enhanced, reaching values 20% lower than 0/0 hearts. These results were in accordance with mtNOS functional activity measured through O_2 consumption, in the presence of L-arginine or L-NMMA: 58% (0/0), 37% (15/0), 23% (15/5) and 34% (15/30). Endogenous mitochondrial NO and NO-derived species are involved in the bioenergetic regulation and the impairment of mitochondrial function observed during rabbit heart ischemia-reperfusion.

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Methicillin-resistant *Staphylococcus aureus*-induced Vascular Hyperpermeability is Mediated by Nitric Oxide and Reactive Nitrogen Species

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Previously we have demonstrated that Gram-positive bacteria (methicillin-resistant *Staphylococcus aureus* [MRSA]) caused more pronounced vascular hyperpermeability compared to Gram-negative bacteria *Pseudomonas aeruginosa* (PS) using our well-established ovine model of sepsis. In the present study, we have tested our hypothesis that the excessive NO and resultant reactive nitrogen species (RNS) are primarily responsible for the MRSA-induced severe vascular leakage, using *in vitro* cell culture techniques.Methods: Human microvascular endothelial cells (HMVEC) were challenged with 10^5 CFU of live MRSA and co-incubated with or without non-specific NO synthase (NOS) inhibitor L-NAME, and peroxynitrite decomposition catalyst FeTMPyP. HMVECs were treated with 1mM L-NAME and 5μM FeTMPy for 30 minutes prior to challenge with 10^5 CFU of MRSA. Cells infected with MRSA were also co-incubated with or without angiopoietin-2 (5μg/mL) and Tie-2 (5μg/mL) receptor antibodies. HMVEC cell permeability was measured by quantifying the amount FITC-Dextran that passed through the confluent HMVEC cell monolayer (n=4). Results: MRSA significantly increased endothelial cells permeability starting 4 hrs after incubation. Co-incubation of the infected cells with L-NAME or FeTMPyP

reversed these permeability changes. Treatment of the cells with angiopoietin-2 or Tie-2 receptor antibody prevented the MRSA-induced hyperpermeability as well. Discussion and Conclusion: Excessive NO and resultant RNS formation mediate MRSA-induced vascular hyperpermeability. Our results indicate also the interaction of potent permeability factor angiopoeitin-2 and Tie-2 receptor interaction is responsible for these MRSA-induced hyperpermeability. Future studies are warranted to clarify the link between NO, RNS and angiopoeition-2/Tie-2 pathway.

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Monitoring the Peroxynitrite Generation by Activated Macrophages: The Use of Novel Boronate-Based Fluorogenic Probes

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Boronates (boronic acids and their esters) have been recently shown to react a million times faster with peroxynitrite ($ONOO^-$) than with hydrogen peroxide (H_2O_2) and over a hundred times faster than with hypochlorite (HOCl) at pH 7.4 (A. Sikora et al. Free Radic. Biol. Med., in press). Here we demonstrate the application of boronate-based fluorogenic probes for the detection and quantitation of peroxynitrite generated by activated macrophages (RAW 264.7 cells). The incubation of interferon γ (IFNγ) and lipopolysaccharide (LPS) and/or phorbol 12-myristate 13-acetate (PMA)-activated macrophages with the boronate fluorogenic probe causes the L-NAME-sensitive, time-dependent accumulation of the fluorescent product with the boronate moiety replaced by hydroxyl group. We have tested 3 different fluorogenic probes with the fluorescent products emitting in different optical regions: coumarin-7-boronate (blue fluorescence), N,N-dimethylxanthamide-3'-boronate (green fluorescence) and resorufin-7-boronate (red fluorescence). In case of all three probes the activation of macrophages causes an increase in the fluorescence intensity observed in the medium. The HPLC analyses confirmed that in case of all probes tested, the corresponding hydroxylated fluorescent derivatives (umbelliferone, N,N-dimethylxanthamide or resorufin) are formed. Also HPLC analyses of the cell lysates confirmed that the probes can enter the cells and thus, can trap both intra and extracellularily generated oxidants. The quantitative analysis of the production of peroxynitrite by activated macrophages is presented.