Instituto Juan March
de Estudios e Investigaciones

123 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Regulation of Protein Function by Nitric Oxide

Organized by

J. S. Stamler, J. M. Mato and S. Lamas

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The lectures summarized in this publication were presented by their authors at a workshop held on the 7th through the 9th of May, 2001, at the Instituto Juan March.

Depósito legal: M-26.928/2001
Impresión: Ediciones Peninsular. Tomelloso, 27. 28026 Madrid.

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Introduction

S. Lamas, J. M. Mato and J. S. Stamler

Nitric oxide (NO) plays important roles in the function of every organ system and participates in most complex physiological responses. The history of NO biology is one replete with examples of "one more function for that molecule". But the progress in understanding the mechanism of NO action has lagged behind—until recently. The advent of new tools and reagents, and increasing support for the concept of precise regulation of protein function by NO is providing a revised picture of the biology, one defined less by physiologic response and more by core principles of signal transduction. The theme that has emerged is of NO mediating posttranslational modification of proteins containing critical thiol or transition metal centers. The aim of this workshop was to review new developments in the area of NO and related reactive nitrogen intermediate (RNI)-mediated modification of proteins (NO signaling and host defense) as well as defects in signaling pathways that underlie the basis of disease (nitrosative stress).

S-nitrosylation, or the covalent attachment of NO groups to protein sulfhydryls, has emerged as the best characterized example of RNI-induced protein modification. Its functional relevance has been shown for multiple proteins of different classes including hemoglobin, the ryanodine receptor and caspase-3. While many other intriguing examples of function-regulating protein nitrosothiols were described, their physiological relevance remains to be shown. Interesting examples of S-nitrosylation as a regulatory step were provided by the reversible inactivation of two isoforms of methionine adenosyltransferases (MATI/III), the activation of the olfactory cyclic nucleotide-gated channel, the regulation of thioredoxin and the stimulation of methyl transferase activity.

The effect of NO on proteins governing the process of gene expression was analyzed at several levels. Human iron metabolism is regulated post-transcriptionally by the IRE/IRP protein system which is exquisitively sensitive to oxidative and nitrosative stresses. In the case of IRP-1, endogenous NO is able not only to promote RNA-binding activity but also results in a reduction of IRP-1 protein levels. Models for the regulation of gene expression by oxidative and nitrosative stresses are provided by the bacterial regulator OxyR and the transcription factors AP-1 and NF-κB. Both S-nitrosylation and oxidative modifications such as S-glutathionylation may occur. NO can also interact upstream in signal transduction pathways that couple cell surface signals to gene expression, including responses that control

DNA methylation. Approaches using microarray methodology suggest that NO can control cell cycle progression by modifying critical proteins such as p53.

NO plays an important role in host defense both by eliminating infectious agents and regulating immunity. This is well exemplified by studies in Salmonella, where sustained inhibition of bacterial growth is mediated by S-nitrosoglutathione, and in the Coxackie virus model where RNI inhibit viral replication. In both of these cases critical proteins thiols may be modified through nitrosative mechanisms. A GSNO reductase that may serve a conserved function to alleviate nitrosative stress in microbes and mammals was described. NO can also interact with superoxide anion to form peroxynitrite, and the discovery of enzymes capable of metabolizing peroxynitrite and even repairing peroxynitrite-like damage point to a possible role for this species in human disease states. Nitration of tyrosine hydroxylase was described in an animal model of Parkinson disease and attributed to peroxynitrite .

Data presented at this meeting suggest that NO is a powerful regulator of cell respiration and apoptosis. Nitrosylation of mitochondrial protein targets such as cytochrome C oxidase, cytochrome C and caspases were shown to be responsible for these effects. Structural studies of model proteins such as hemoglobin and Ras provided mechanistic insights into NO-regulation of protein function. Connectivity between hemes and thiols as a means of propagating NO signals was described in studies of hemoglobin.

The atmosphere and spirit of the workshop were highly enjoyable and provided a perfect setting for scientific interaction.

S. Lamas, J. M. Mato and J. S. Stamler

Session 1: Nitrosative and oxidative stress vs redox-related signaling
Chair: Salvador Moncada

Nitrosylation and nitrosative stress

Jonathan S. Stamler

Howard Hughes Medical Institute
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Accumulating evidence indicates that the covalent attachment of NO groups to protein thiols and transition metal centers is a precisely regulated post-translational protein modification, and that these modifications underlie the specific control by NO of diverse protein functions across an expanding range of cellular mechanisms. Specificity is conferred by motifs that target NO to critical thiols, by protein-protein interactions that confine the signals in space, and by enzymes that function to regulate levels of S-nitrosylation and protect against nitrosative stress. Here I review studies in model systems that have provided the basis for these conclusions, including hemoglobin, caspase 3, OxyR , NMDA receptor, the ryanodine receptor–calcium release channel and tissue transglutaminase. Additionally, I consider themes that have emerged from our studies in microbes, nematodes and various mammals including: a) the conservation of NO control mechanisms throughout phylogeny; b) the central role of S-nitrosothiols (SNOs) in transducing NO-related bioactivity; c) the connectivity between hemes and thiols, which serves to regulate NO bioactivity and protein function; c) unanticipated roles for O_2 in modulating NO responses, e) the role of protein structure in determination of SNO structure/activity relationships; f) structural requirements and allosteric determinants for S-nitrosylation/denitrosylation reactions; and g) the existence of specific enzymes that protect cells from NO and SNO (nitrosative stress). The general importance of NO is further underscored by ongoing studies of its biology, which are redefining the functions of classical proteins (e.g. hemoglobin(s) and alcohol dehydrogenase(s)), and providing a new evolutionary perspective on what is perhaps the most ancient and conserved of signals.

Modulation of nitric oxide metabolism in macrophages by a flavonoid rich French maritime pine bark extract

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One the most efficient *in vitro* scavengers of the reactive oxygen species HO• and $O_2^{\bullet-}$ in our database of plant extracts and individual flavonoids is the procyandin-rich French maritime pine bark extract pycnogenol® (PBE). Using a model system of ascorbate/ascorbate oxidase coupled with ESR detection, PBE was remarkably effective in extending ascorbyl radical lifetime, indicating that it can play a role in the cellular antioxidant network. PBE may act in the redox antioxidant network at the interface between ascorbate and lipophilic antioxidants. Evidence for PBE in the antioxidant network is also shown by an increase in the level of antioxidants, e.g. its capacity to spare α-tocopherol and glutathione in cells cultured under oxidative stress conditions.

In elucidating the mechanism of cell regulation, the involvement of PBE redox activity and/or direct binding to enzymes or proteins and its subsequent action on activity are of importance. PBE was able to reduce cytochrome c reversibly. PBE competitively inhibited electron transport activity in isolated mitochondria and submitochondrial particles. NADH-ubiquinone, succinate-ubiquinone, and ubiquinol-cyctochrome *c* reductases were inhibited by low concentrations of PBE to a similar extent. However, at same concentration range, PBE by reducing cytochrome c acts as an electron donor for complex IV; cytochrome *c* oxidase. These studies demonstrate how the redox activity of the polyphenol structure common to PBE and flavonoids enables them to affect cell functions.

PBE dose-dependently inhibits the activities of oxidative enzymes such as xanthine oxidase (XO), xanthine dehydrogenase (XDH), horseradish peroxidase, and lipoxygenase, but does not affect the activities of glucose oxidase (GO), ascorbate oxidase or elastase. To characterize the protein binding action of PBE, studies were focused on XO and GO. Under non-denaturating conditions, PBE changed the electrophoretic mobility of XO but not of GO. Hydrophobic binding was the dominant mode of interaction between PBE and XO. The importance of PBE binding in the modulation of enzyme activity was verified by showing that PBE binds and inhibits catalase, but not superoxide dismutase.

NO production has important implications for human health. NO has key importance in the circulation regulating the vascular tone, and affects cell signalling processes involved in regulating physiological responses in many tissues. NO is produced by endogenous nitric oxide synthase (eNOS) or by an inducible enzyme (iNOS) in endothelial cells in immune cells markedly elevating NO in production, a cytotoxic response important in targeting infection, tumors, etc. PBE and other botanical extracts have been investigated with respect to modulation of NO metabolism by determining effects on: **i)** Direct quenching of NO radicals, **ii)** iNOS enzyme activity, **iii)** iNOS mRNA gene expression, and **iv)** iNOS mRNA stability. PBE has a biphasic effect at first stimulating at low concentrations and then inhibiting cell NO production; its combined effect on i, ii, iii accounts for inhibition of NO production in activated rat macrophages. PBE markedly elevates interferon INFγ induced NO production in

rat macrophages. This effect is also manifested by procyanidin trimers but not dimers or monomers. These affects are correlated and dependent on induction of TNFα production. PBE may activate signal transduction pathways involved in TNFα syntheses and secretion. Thus plant polyphenols may be considered as promising tools for the non-pharmacological control of NO during chronic inflammation or as preventive treatment against different pathologies which have been found to be associated with nitrosative stress such as arteriosclerosis, cardiovascular disease and neurodegenerative disorders and cancer.

Nitrosylation, S-adenosylmethionine and liver function

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Hepatic content of S-adenosylmethionine (AdoMet) is controlled by three methionine adenosyltransferases; MAT I, MAT II and MAT III. MAT I & III are products of the gene *MAT1A*, while MAT II is the product of the gene *MAT2A*. Fetal liver, extrahepatic tissues and hepatocarcinomas (HCC) express only *MAT2A*; while adult liver expresses large quantities of *MAT1A* and a small amount of *MAT2A*. The liver expression of *MAT2A* increases during regeneration and in response to injury. To analyze the role of MAT I/III in liver function we have disrupted *MAT1A*. *MAT1A* knockout mouse have isolated hypermethioninemia and reduced hepatic AdoMet content. Moreover, absence of MAT I/III resulted in a liver that expresses markers of an acute phase response, proliferation and de-differentiation, displays increased proliferation and is more susceptible to injury. Thus, AdoMet is a control switch that regulates liver function: at high concentrations favors the differentiated/metabolic status and at low concentrations favors the de-differentiated/proliferative status.

MAT I/III is inactivated in response to liver injury, which in its turn leads to a reduction of hepatic AdoMet content. The mechanism involves the reversible S-nitrosylation/oxidation of a cysteine residue located in position 121 (C121). NO reversibly inactivates MAT I/III by S-nitrosylation of cysteine 121 (C121). MAT II, however, has in the corresponding position a residue of glycine (G120) and, consequently, is resistant to NO inactivation. Substitution in MAT II of this glycine residue by cysteine (G120C) leads to S-nitrosylation and enzyme inactivation. Accordingly, while in wild type mouse (expressing mainly *MAT1A*) liver injury induces MAT inactivation and a decrease in AdoMet content; in knockout mouse (expressing only *MAT2A*) liver injury has no effect on MAT activity or AdoMet content. In isolated rat hepatocytes, AdoMet blocks HGF-induced DNA synthesis. NO, through inactivation of MAT I/III, reverts the blocking effect of AdoMet on HGF action. In summary, liver injury induces hepatic mass loss and cytokine production, which leads to NO and ROS generation, and MAT I/III inactivation. As a consequence of this reduction of hepatic MAT activity, AdoMet levels decrease and the liver switches from a metabolic to a proliferative status. When liver mass is recovered, MAT I/III is reactivated, AdoMet content increases and the metabolic/differentiated phenotype is restored.

References:

1. Lu SC, Alvarez L, Huang Z-Z, Chen L, An W, Corrales FJ, Avila MA, Kanel G & Mato JM (2001) *Proc Natl Acad Sci USA* In press
2. Latasa MU, Boukaba A, García-Trevijano ER, Torres L, Rodriguez JL, Caballería J, Lu SC, López-Rodas G, Franco L, Mato JM, Avila MA (2001) *FASEB J* In press
3. Avila MA, Mingorance J, Martínez-Chantar ML, Casado M, Martín.Sanz P, Boscá L, Mato JM. (1997) *Hepatology* 25:391-396
4. Ruiz F, Corrales FJ, Miqueo C, Mato JM (1998) *Hepatology* 28:1051-1057
5. Pérez-Mato I, Castro I, Ruiz FA, Corrales FJ, Mato JM (1999) *J Biol Chem* 274, 17075-17080
6. Sánchez del Pino M, Corrales FJ, Mato JM (2000) *J Biol Chem* 275: 23476-23482

Activation of the olfactory cyclic nucleotide-gated channel by S-nitrosylation

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Cyclic nucleotide-gated (CNG) channels are critical for signal transduction in the vertebrate visual and olfactory systems. These non-selective cation channels are activated by the binding of cAMP or cGMP to a particular domain of the protein (1). Three different but highly homologous subunits that are thought to assemble in a hetero-tetrameric structure have been identified for the olfactory CNG channel, the α3, the α4 and the β1b subunits. These CNG channel subunits have been found in other tissues and in the central nervous system, in particular in hippocampal neurons. Their roles in these cells have not been identified yet.

Recently, we have shown that, in addition to their activation by cyclic nucleotides, nitric oxide (NO) generating compounds can directly open olfactory CNG channels through a redox reaction that results in the S-nitrosylation of a free SH group on a cysteine residue (2).

Of the 8 cysteine residues distributed throughout the rat olfactory α3 channel, biochemical evidence identified one residue located on the intracellular face of the channel as the putative target site for S-nitrosylation (2). We then focused on the cysteines located on the intracellular face of the channel and generated a series of mutant subunit constructs in which each of these cysteines was changed to a serine residue. After expression in HEK 293 cells, we were able to test each of these channels for activation by cAMP and/or NO. All mutant channels continued to be activated by cyclic nucleotides, but only one of them, the C460S mutant channel, exhibited a total loss of NO sensitivity. This result was further supported by a similar lack of NO sensitivity that we found for a natural mutant of this precise cysteine residue, the *D. melanogaster* CNG channel. The cysteine C460 is located in the C-linker region of the channel known to be important in channel gating. Kinetic analyses suggested that at least two of these cysteine C460 residues on different channel subunits were involved in the activation by NO. These results show that one single cysteine residue is responsible for NO sensitivity of the CNG channel, but that several channel subunits need to be activated for channel opening by NO (3).

Through the direct gating of NO, we have also shown that α4 CNG subunits can form functional homomeric channels in heterologous expression systems (4). We also found, *in vivo*, a conductance having the characteristics of this NO-gated α4 channel in the neurons of the vomeronasal organ which is responsible for pheromone recognition and in growing processes of hippocampal neurons in culture. With its high permeability to Ca^{2+}, the α4 subunit of the CNG channel is well suited for numerous roles in the nervous system. For example, if α4 subunits form presynaptic channels in mature hippocampal neurons, they could serve as the target of a postulated retrograde NO signal (5), coupling NO to presynaptic Ca^{2+} influx.

References:

1. Zagotta, W. N., and Siegelbaum, S. A. (1996) *Annual Review of Neuroscience* **19,** 235-63
2. Broillet, M.-C., and Firestein, S. (1996) *Neuron* **16,** 377-385
3. Broillet, M.-C. (2000) *J. Biol. Chem.* **275,** 15135-15141
4. Broillet, M.-C., and Firestein, S. (1997) *Neuron* **18,** 951-958
5. Schuman, E. M., and Madison, D. V. (1991) *Science* **254,** 1503-1506

S-Nitrosoglutathione increases expression of mature dF508del CFTR

Khalequz Zaman, Marianne McPherson, Lisa Palmer, Benjamin M. Gaston

Airway levels of S-Nitrosoglutathione (GSNO) are low in patients with cystic fibrosis (CF). The dF508 mutation of the cystic fibrosis transmembrane regulator (CFTR) gene, which has an SP1 promoter site, produces a functional protein that is rapidly degraded by the ubiquitin-proteasome system. Our data suggest that GSNO inhibits ubiquitin activation and stimulates SP1 binding We tested the effect of GSNO on dF508 CFTR expression. We incubated dF508 homozygous (BHK; CFPAC1) cells and wild type CFTR-transfected CFPAC1 cells with GSNO (0.5 - 100 μM) and performed western blots (monoclonal anti-CFTR R domain; R&D, Minneapolis, MN) following lysis in 1 % NP40 buffer (4°C). Neither BHK nor dF508 homozygous CFPAC1 cells expressed mature CFTR. Both cell lines demonstrated a time- and dose-dependent increase CFTR maturation following exposure to GSNO. Actinomycin D (15 mg/ml) had no effect on the induction of CFTR protein by GSNO; however, cycloheximide (50 mg/ml) eliminated the effect of GSNO. When untransfected CFPAC1 cells were treated with 200 mM DTT during the last 30 min of GSNO incubation, maturation was also eliminated. Further, the g-glutamyl transpeptidase (g GT) inhibitor, acivicin (100 mM), inhibited the effect of GSNO, and this inhibition was overcome by S-nitrosocysteinyl glycine. In summary, GSNO at physiological concentrations increases expression and maturation of dF508 CFTR in a post-transcriptional process that appears to 1) be g GT-dependent; and 2) involve an S-nitrosylation reaction. Taken together, these observations suggest that GSNO may increase epithelial CFTR maturation in patients with the dF508 CFTR mutation.

Session 2: Functional consequences of NO-protein interactions
Chair: Jonathan S. Stamler

Nitric oxide and the mitochondrion

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Nitric oxide (NO) is involved in a variety of physiological functions, including regulation of blood flow and the formation of memory, which are mediated via its activation of the soluble guanylate cyclase. In addition, NO is cytostatic/cytotoxic for invading microorganisms and contributes to the development of certain pathologies, such as septic shock and some forms of inflammation (1).

One potential route of NO-induced toxicity is through its interaction with other molecules, mainly superoxide anion (O_2^-). NO generated from vascular cells or from leukocytes interacts with O_2^-, leading to reduction in its vasorelaxant and platelet antiaggregatory actions (2,3). The product of the reaction between these two free radicals is peroxynitrite anion ($ONOO^-$) which is also formed in vitro by activated macrophages. This powerful oxidant, when protonated, decomposes rapidly, resulting in the formation of OH· and NO_2, both of which are tissue-damaging agents. 3-nitrotyrosine (which has been proposed to be a product of the interaction between tyrosine and $ONOO^-$) has been shown to be present in biological fluids and in a variety of inflammatory conditions including atherosclerosis (4). At present it is not clear under which conditions $ONOO^-$ is formed and whether its release leads to irreversible tissue damage through nitration of proteins. We have shown that there are very effective detoxification mechanisms for $ONOO^-$, notably its interaction with tissue thiols, which are rapidly nitrosylated (5). From these results we developed two concepts: first, the effect of $ONOO^-$ is dependent on the biological environment where it is released, and second, the protective effect of thiols is due, at least in part, to their ability to react with $ONOO^-$ and regenerate NO. We have postulated therefore that $ONOO^-$ may be damaging in conditions in which those mechanisms of detoxification are reduced.

An area of increasing interest in which NO and oxygen might be interacting is at the mitochondrion. It has been shown by our group and others that NO in physiological concentrations blocks the mitochondrial respiratory chain enzyme cytochrome c oxidase (complex IV) in a reversible manner that is competitive with oxygen (6-8). Our group went on to demonstrate that inhibition of complex IV is a physiological event and that NO generated by vascular endothelial cells under basal and stimulated conditions modulates the respiration of these cells in response to acute changes in oxygen concentration (9). It is possible that an oxygen sensor in cells is effectively complex IV, which is regulated by physiological concentrations of NO interacting with oxygen. Reports that mitochondria may have their own isoform of NO synthase (10,11) support the view that NO produced in small amounts by mitochondria themselves could regulate cellular respiration.

We have shown that prolonged exposure of murine macrophage-like cells to the NO donor DETA-NO, which releases NO for long periods in a constant manner, results in inhibition of oxygen consumption. This inhibition is reversible at low concentrations of DETA-NO and for short incubations (1 hour); however, if the concentration or the time of exposure to DETA-NO is increased then the inhibition of cellular respiration becomes

persistent, i.e. not reversible by haemoglobin (12). This inhibition happens specifically at the level of complex I, is persistent and most probably due to the generation of $O_2^{\cdot}$. If this is the case then this may be one of the early stages of NO-dependent cell pathology. The inhibition of complex I is most likely to be due to S-nitrosylation of critical thiols in the enzyme. We have repeated these studies in several cell types and have observed the same pattern of response (13). All these observations led us to hypothesise that exposure of cells to NO for long periods results in S-nitrosylation of thiols in complex I. This can be counteracted by glutathione unless the levels of this thiol are depleted, at which time irreversible inhibition of respiration begins.

We have recently investigated the effect of a constant inhibition of complex IV by DETA-NO on mitochondrial energy status and cell viability (14). We have found that blockade of complex IV by NO initiates a protective action in the mitochondrion to maintain mitochondrial membrane potential; this results in prevention of apoptosis. It is likely that during cellular stress involving increased generation of NO this compound will trigger a similar sequence of events, depending on its concentration and duration of release.

References:

1. Moncada, S., Palmer, R.M., Higgs, E.A. (1991). Pharmacol. Rev., **43** (2), 109-142.
2. Gryglewski, R.J., Palmer, R.M.J., Moncada, S. (1986). Nature, **320**, 454-456.
3. McCall, T.B., Boughton-Smith, N.K., Palmer, R.M.J., Whittle, B.J.R., Moncada, S. (1989). Biochem. J., **261**, 293-296.
4. Beckman, J.S., Koppenol, W.H. (1996). Am. J. Physiol., **271** (5 pt. 1). C1424-1437.
5. Moro, M.A., et al. (1994). Proc. Natl. Acad. Sci. USA, **91**, 6702-6706.
6. Brown, G.C., Cooper, C.E. (1994). FEBS Lett, **356**, 295-298.
7. Torres, J., Darley-Usmar, V., Wilson, M.T. (1995). Biochem. J., **312**. 169-173.
8. Lizasoain, I., Moro, M.A., Knowles, R.G., Darley-Usmar, V., Moncada, S. (1996). Biochem. J.. **314**, 877-880.
9. Clementi, E., Brown, G.C., Foxwell, N., Moncada, S. (1999). Proc. Natl. Acad. Sci. USA, **96**. 1559-1562.
10. Bates, T.E, Loesch, A., Burnstock, G., Clark, J.B. (1996). Biochem. Biophys. Res. Commun.. **218** (1), 40-44.
11. Ghafourifar, P., Richter, C. (1997). FEBS Lett., **418**, 291-296.
12. Clementi, E., Brown, G.C., Feelisch, M., Moncada, S. (1998). Proc. Natl. Acad. Sci. USA, **95**. 7631-7636.
13. Beltran, B., Orsi, A., Clementi, E., Moncada, S. (2000). Br. J. Pharmacol., **129**. 953-960.
14. Beltran, B., Mathur, A., Duchen, M.R., Erusalimsky, J.D., Moncada, S. (2000). Proc. Natl. Acad. Sci. USA, **97**, 14602-14607.

S-nitrosylation of argininosuccinate synthetase: a reversible protein modification that endows NO with the ability to limit its own synthesis

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NO-producing cells regenerate substrate L-arginine (Arg) from the NOS co-product, L-citrulline (Cit). This *Arg/Cit cycle* is ubiquitous, comprising NOS and two additional enzymes that are best known for their function in the urea cycle of liver: argininosuccinate synthase (AS) and argininosuccinate lyase (AL). Notably, AS mRNA, protein and enyme activity are coordinately induced in cells by same imunostimuli that elicit iNOS gene expression. Prior studies have shown that AS is rate-limiting to high-output NO production by vascular cells, indicating that Arg recycling can govern NO synthesis. We reveal that thiol depletion of vascular smooth muscle cells reduces the ability of Cit to serve as an indirect source of substrate for NO synthesis, without affecting the direct utilization of Arg. This suggests that AS or AL may possess one or more redox-senstive thiols. *In vitro* studies demonstrated that recombinant huAS is reversibly inactivated by NO-donors, via S-nitrosyltion of a specific Cys residue that is protected in the ATP-bound enzyme. A different site on AS is additionally found to mediate inactivation by peroxide; this latter site is protected by bound citrulline. Although AS contains 5 Cys residues in total, a single Cys residue was found to be targeted by S-nitrosylation (Cys132 in hu AS) using a novel mass spectrometric technique based on facile collision-gas induced displacement of a species with the mass of NO. Mutagenesis of the predicted NO-sensitive Cys residue to Ala resulted in neither a loss of enzyme activity, nor a change in Km for substrates, however, inactivation by NO required higher concentrations of NO-donors. In contrast, inactivation of AS by peroxide was identical in mutant and wildtype enzymes. Activation of NO synthesis in AS over-expressing cells revealed that S-nitrosylation of AS occurs *in vivo*. Sequence alignments suggest that the preferred Cys target of NO is present in all mammalian AS isoforms, but absent from ASs in bacteria and yeast, organisms which do not possess a full-length NOS homolog in their genomes. We propose that inactivation of AS by NO specifically evolved as a mechanism to prevent toxic NO overproduction in cells, by limiting the access of NOS to Arg substrate.

Nitric oxide and the regulation of human iron metabolism

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Messenger RNAs encoding important proteins for human iron metabolism are regulated post-transcriptionally by iron-responsive elements (IREs) and iron regulatory proteins (IRPs). Binding of IRP-1 or IRP-2 to IREs controls cellular iron uptake and storage as well as iron utilization by erythroid cells, and affects the expression of mitochondrial citric acid cycle enzymes. The IRE/IRP regulatory system may also regulate systemic iron homeostasis. IRP binding to IREs either represses translation (when binding occurs to an IRE in the 5' untranslated region, e.g. ferritin) or stabilizes transferrin receptor mRNA against degradation (by binding to IREs in the 3'UTR). While IRP-2 activity is regulated at the level of protein stability/degradation, IRP-1 is a constitutively stable protein. Its IRE-binding activity is controlled by reversible incorporation/loss of a 4Fe-4S cluster, the "iron sulfur switch". Only the cluster-less apoIRP-1 binds to IREs with high affinity. Importantly, IRP-1 is activated by nitric oxide and oxidative stress in the form of H_2O_2 in cultured cells and intact organs.

One of the key issues of current research is how the IRE/IRP system and the signals that regulate it affect systemic iron homeostasis. Furthermore, regulation of iron metabolism by the IRE/IRP system in specific tissues leading to degenerative disorders represents an important field for further investigation. We have begun to investigate the regulation of iron metabolism in health and disease using microarray technology on the "iron chip", a cDNA array of some 200 human genes involved in metal metabolism as well as nitric oxide and oxidative stress pathways.

References:

Weiss, G., B. Goossen, W. Doppler, D. Fuchs, K. Pantopoulos, G. Werner-Felmayer, H. Wachter and M.W. Hentze. Translational regulation via iron-responsive elements by the nitric oxide/NO-synthase pathway. EMBO J. 12, 3651-3657, 1993.

Pantopoulos, K., G. Weiss and M.W. Hentze. Nitric oxide and the post-transcriptional control of cellular iron traffic. Trends Cell Biol. 4, 82-86, 1994.

Hentze, M.W. and L.C. Kühn. Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide and oxidative stress. Proc. Natl. Acad. Sci. USA 93,8175-8182, 1996

Muckenthaler, M., N.K. Gray and M.W. Hentze. IRP-1 binding to ferritin mRNA prevents the recruitment of the small ribosomal subunit by the cap-binding complex eIF4F. Molecular Cell 2, 383-388, 1998.

Brazzolotto, X., J. Gaillard, K. Pantopoulos, M.W. Hentze and J.-M. Moulis. Human cytoplasmic aconitase (iron regulatory protein1) is converted into its [3Fe-4S] form by

hydrogen peroxide in vitro but is not activated for iron-responsive element binding. J. Biol. Chem. 274, 21625-21630, 1999.

Pantopoulos,K. and M.W. Hentze. Nitric oxide, oxygen radicals and iron metabolism. In: Nitric Oxide: Biology and Pathobiology (L.J. Ignarro, ed.). Academic Press, 293-313, 2000.

McKie, A.T., P. Marcani, A. Rolfs, K. Brennan, K. Wehr, D. Barrow, S. Miret, A. Bomford, T.J. Peters, F. Farzaneh, M.A. Hediger, M.W. Hentze and R.J. Simpson. A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. Mol. Cell 5, 299-309, 2000.

Control of iron regulatory protein 1 expression and activities by nitric oxide

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Aconitases are a class of metalloproteins very sensitive to environmental redox signals. These monomeric proteins contain a versatile 4Fe-4S cluster of which only three of the four iron atoms are linked to cysteines of the protein backbone. The fourth iron atom is exposed and can interact with substrates, solvent or exogenous ligands. Over the last few years, there has been a growing interest in aconitases because they can also exhibit a regulatory activity. Remarkably, in mammalian cells, the cytosolic aconitase is a bifunctional protein which behaves either as an enzyme or as a RNA-binding protein (iron regulatory protein or IRP1) which binds cis-elements present on the mRNA of several proteins including ferritin and transferrin receptor. Beside iron level, environmental signals including reactive oxygen species, NO synthase 2-derived NO and peroxynitrite modulate IRP1 aconitase activity. Moreover, data obtained from biological assays and from spectroscopic experiments revealed that conversion of cytosolic aconitase into RNA-binding protein by NO occurs through direct modification of the protein. The endogenous reducing system thioredoxin is a likely partner of NO in this regard. Thus, the function of IRP1 is determined by its 4Fe-4S cluster that senses and sorts the chemical signals of its environment. Examining the post-translational modifications of IRP1 by NO and congeners is now an important objective to understand the mechanisms that underlie the regulatory switch. Moreover, the question of how the expression of this protein is regulated has remained open. It is held that IRP1 is a stable protein which is regulated post-translationally. Recently, we showed that endogenous production of NO, beside inducing IRP1 RNA-binding activity, also results in a reduction of IRP1 protein levels, as determined by Western blot analyses. No changes in IRP1 levels could be detected in stimulated macrophages from NOS2-/- mice. We also found an NO-dependent decrease in IRP1 mRNA expression in NO-producing cells. NO therefore reacts with IRP1 at two different levels: post-translationally, by boosting IRE-binding activity up to 100%, and by reducing its gene expression to approximately 50%. This control pathway may limit the expansion of active iron pool when the cell has to cope with a sustained and risky inhibition of ferritin translation. It may also represent a feed-back mechanism which tempers the consequences of a nitrosative stress.

References:

Beinert, H., and M. C. Kennedy. 1993. Aconitase, a two-faced protein: enzyme and iron regulatory factor. Faseb J 7:1442-9. Bouton, C., H. Hirling, and J. C. Drapier. 1997. Redox modulation of iron regulatory proteins by peroxynitrite. J Biol Chem 272:19969-75. Drapier, J. C., and C. Bouton. 1996. Modulation by nitric oxide of metalloprotein regulatory activities. Bioessays 18:549-56. Gardner, P. R., G. Costantino, C. Szabo, and A. L. Salzman. 1997. Nitric oxide sensitivity of the aconitases. J Biol Chem 272:25071-6. Hentze, M. W., and L. C. Kuhn. 1996. Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. Proc Natl Acad Sci U S A 93:8175-82. Oliveira, L and Drapier JC. 2000. Down-regulation of iron regulatory protein 1 gene expression by nitric oxide. Proc Natl Acad Sci U S A 97, 6550-5 Tang Y, Guest JR. 1999. Direct evidence for mRNA binding and post-transcriptional regulation by *Escherichia coli* aconitases. Microbiology. 145:3069-79.

Inhibition of the human water channel, Aquaporin-1, by nitric oxide

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The water channel aquaporin-1 (AQP1) mediates osmotically-driven water transport in capillary endothelial cells, where it colocalizes with endothelial nitric oxide synthase (eNOS). We hypothesized that NO might regulate AQP1 function through nitrosation of critical residues, e.g. the mercury-sensitive Cysteine 189. Wild-type human AQP1 (wtAQP1) and AQP1 mutated on Cysteine 189 (C189SAQP1) were expressed in *Xenopus laevis* oocytes to assess the effect of several NO donors on AQP1-mediated water permeability (P_f). In wtAQP1-expressing oocytes, 3-morpholinosydnonimine (Sin-1, 5mM) that produces NO and superoxide anions decreased P_f by 37%. This inhibition was reversed by superoxide dismutase or β-mercaptoethanol. The NO donors S-nitrosoacetylpenicillamine (SNAP, 100 μM) and DETA-NO (1mM) inhibited Pf by 29% and 24%, respectively, in the presence of a superoxide anion generator, pyrogallol. NAP and pyrogallol alone or in combination had no effect on Pf, whereas peroxinitrite (250 μM) reproduced a 39% inhibition of Pf. NO donors did not alter AQP1 localization at the oocyte plasma membrane, as revealed by immunohistochemistry. Importantly, in oocytes expressing C189SAQP1, no inhibition of Pf was observed after incubation with either Sin-1 or SNAP and pyrogallol. We conclude that under oxidizing conditions, NO inhibits AQP1-mediated water permeability through a specific interaction with Cysteine 189 on AQP1.

Modifications of tyrosine hydroxylase by peroxynitrite, effects of CO_2

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Tyrosine hydroxylase (TH) is the rate-limiting step in the biosynthesis of catecholamines and one of the proteins modified by tyrosine nitration after exposure of mice to MPTP, an animal model of Parkinson's disease. To begin investigating the mechanism of TH inactivation, purified recombinant rat TH was reacted with peroxynitrite in the presence or absence of bicarbonate. Exposure of TH to 10 fold molar excess of peroxynitrite did not result in cysteine, methionine and tryptophan oxidation. Evidence for cysteine oxidation was obtained only after partial unfolding of the protein. Stopped-flow experiments revealed a modest second order rate constant of (3.8 ± 0.9) x 103 M-1 s-1 at pH 7.4 and 25°C for the holoenzyme. Digestion and sequence analysis of the peptides indicated that nitration of Tyr 423 primarily and to a lesser extent Tyr 428 are the only modifications detected and likely responsible for the inactivation of TH by peroxynitrite. Contrary to previous reports, the addition of CO2 did not increase tyrosine nitration in TH under these experimental conditions. In order to understand this unusual effect of CO2 on tyrosine nitration, a number of proteins with various molecular masses were reacted with ONO(O)CO2-. We observed that the ability of CO2 to augment the yield of tyrosine nitration in proteins was dependent on the protein concentration and more importantly on the protein volume (Å3). The data indicates that CO2 maybe critical in promoting protein tyrosine nitration under pathophysiological environments where protein concentration is much greater than the steady state levels of nitrating agents.

Session 3: Gene regulation and NO
Chair: Carl Nathan

Transcriptional regulation by S-nitrosylation and oxidation

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Exposure to reactive oxygen and nitrogen species imposes a stress on cells by damaging essential cellular constituents. Nitrosative and oxidative stresses are ubiquitous and ancient. Accordingly, an elaborate system of antinitrosative and antioxidative defenses has evolved. Redox modifications of transcription factors are a central theme in the cellular response to changes in the redox state. These modifications ultimately lead to the activation of redox-response genes, the products of which are involved in detoxification of the reactive molecules, export, repair and other homeostatic functions. In most cases, the precise mechanisms by which these modifications occur are unknown, as multiple products can be produced (1). Thiols in particular have been identified as the regulatory sites in transcription factors. This is best exemplified by OxyR, a bacterial regulator of the oxidative and nitrosative stress response; less well characterized eucaryotic examples are NF-?B, AP-1, c-jun and c-fos (2). Importantly, both reversible and irreversible thiol modifications can be achieved during oxidative and nitrosative stress. In principle, any thiol modification may produce a functional response, and the response may be different with different modifications. For example, oxyR is activated by oxidation or nitrosylation of a critical cysteine residue (3, 4). The oxidative modification of OxyR that leads to transcriptional activation has long been thought to be a sulfenic acid (S-OH) (3), but more recently an internal disulfide (S-S) has been identified in a crystal structure (5). But these data are difficult to reconcile with the formation of a stable S-nitrosothiol (S-NO) (1, 4, 6). Here I present data showing that a disulfide is not present in naturally occurring protein, and reconcile this finding with previous results. This caveat notwithstanding, OxyR serves as an excellent model for oxidative/nitrosative regulation. More generally, a continuum of progressively oxidized forms of protein may exist, encompassing S-NO ? S-OH ? S-SR/S-S ? S-O2H ? S-O3H. The first three forms are reversible and may be used as a signal, while last two are more likely irreversible markers of injury. The existence of multiple redox-related modifications raises the possibility that proteins are able to distinguish among them.

References:

1.Stamler, J. S. & Hausladen, A. (1998) Nat. Struct. Biol. 5, 247-249.
2.Marshall, H. E., Merchant, K. & Stamler, J. S. (2000) Faseb J 14, 1889-900.
3.Storz, G., Tartaglia, L. A. & Ames, B. N. (1990) Science 248, 189-94.
4.Hausladen, A., Privalle, C. T., Keng, T., DeAngelo, J. & Stamler, J. S. (1996) Cell 86, 719-729.
5.Choi, H., Kim, S., Mukhopadhyay, P., Cho, S., Woo, J., Storz, G. & Ryu, S. (2001) Cell 105, 103-113.
6.Arnelle, D. R. & Stamler, J. S. (1995) Arch. Biochem. Biophys. 318, 279-285.

Oxidative and nitrosative stresses as regulators of the activity of transcription factors

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Both oxidative and nitrosative stresses may result in the inactivation of the binding to DNA of redox-sensitive transcription factors. The underlying biochemical mechanisms may involve oxidation or nitrosylation of critical thiols within the DNA binding domains of these proteins. However, S-glutathionylation, the formation of a mixed disulfide between glutathione and the redox-sensitive cysteine residues, has been shown to occur under NO exposure (1) and pro-oxidative conditions (2) in c-Jun, one of the AP-1 constituents. This modification may be functionally important as it is reversible (3) and has been detected in other transcription factors, such as NF-κB, by using covalent chromatography with a modified S-nitrosoglutathione sepharose (4). The response of the transcription factor NF-κB to perturbations in the redox homeostasis of cells may play an important role in modulating functions in which NF-κB is involved. We pursued the identification of S-glutathionylation and other oxidative modifications within the redox sensitive Cys 62 of the NF-κB subunit, p50 (5). Under pro-oxidative conditions, mimicked by physiological changes in the GSH/GSSG ratio, we detected the formation of a mixed disulfide between glutathione and Cys 62, within the DNA binding domain of p50. This S-glutathionylation was associated with an inhibition of DNA-binding activity and was reversible. Mass spectrometry studies (MALDI-TOF and nano ES QIT MS) confirmed glutathionylation at Cys 62 and revealed the potential formation of a sulfenate moiety within the same residue. Structural simulations based on molecular modeling suggested the existence of specific electrostatic interactions between glutathione and p50 that could favor glutathionylation. This modification may represent a mechanism by which oxidative stress signals are transduced into gene expression changes.

References:

1. Nitric oxide inhibits cJun DNA binding by specifically targeted S-glutathionylation.
P.Klatt, E.Pineda-Molina, S.Lamas
Journal of Biological Chemistry 274: 15857-15864, 1999.

2. Redox regulation of cJun DNA binding by reversible S-glutathiolation.
P. Klatt, E.Pineda-Molina, M. García de Lacoba, C. A. Padilla, E. Martínez-Galisteo, J.A. Bárcena, S.Lamas.
FASEB Journal 13: 1481-1490, 1999.

3. Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress.
P. Klatt and S.Lamas
European Journal of Biochemistry 267: 1-18, 2000.

4. Novel application of S-nitrosoglutathione-Sepharose to identify proteins which are potential targets for S-nitrosoglutathione-induced mixed-disulphide formation.
P. Klatt, E. Pineda-Molina, D. Pérez-Sala, S.Lamas
Biochemical Journal 349: 567-578, 2000.

5. S-glutathionylation of the p50 subunit of NF-kB: a mechanism for redox-induced inhibition of DNA-binding.
E. Pineda-Molina, P. Klatt, J. Vázquez, A. Marina, M. García de Lacoba, D. Pérez-Sala, S. Lamas.
(Submitted)

Activation of defense responses by nitric oxide

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Cells may encounter low nitric oxide levels due to intercellular signaling processes, or higher fluxes as a result of inflammation and the output of phagocytic activity of the immune system. The latter can be cytostatic or cytotoxic, and cells may need to counteract these effects. We have explored the defensive genetic responses against NO in bacterial and mammalian cells. In *Escherichia coli*, we found that the *soxRS* system, which was discovered as a response to superoxide, is also involved in the cellular response to NO. The *soxRS* system is triggered in two stages: pre-existing SoxR protein is activated and stimulates transcription of the *soxS* gene; the resulting SoxS protein then induces the expression of a large number of defense genes. We found that this system can be activated by exposure of *E. coli* to pure NO diffused into the growth medium. Bacteria phagocytosed by murine peritoneal macrophages also activated *soxRS* in an NO-dependent manner. Moreover, *soxRS* activation contributed to the survival of *E. coli* after phagocytosis by macrophages, and individual *soxRS*-regulated genes have been shown to contribute to this survival. We have shown that the same two-stage regulatory mechanism operates in the *Salmonella typhimurium* (*enterica*) the *soxRS* system, although the set of regulated genes differs. The *S. typhimurium soxRS* system does not contribute detectably to survival in isolated macrophages or during mixed infections of mice. Evidence has accrued, however, that the *soxRS* system plays a role in the development of clinical antibiotic resistance in both *E. coli* and *S. typhimurium*.

The signaling mechanism employed by SoxR to respond to NO demonstrates a novel gene-activation pathway. The SoxR protein is a homodimer of 17-kDa subunits, each containing a [2Fe-2S] center. In resting SoxR, these iron-sulfur centers are maintained in the reduced state, and upon cellular exposure to superoxide-generating drugs (e.g., paraquat), they are oxidized (E_0' –285 mV) to activate transcriptional activity. The response of SoxR to NO occurs in the absence of oxygen, and so is not due to the adventitious generation of superoxide in NO-treated cells. Instead, physical analysis shows that a new form is generated by NO, in which the iron-sulfur centers of SoxR are modified to form mixed dinitrosyl-iron-dithiol complexes anchored to the protein. This destructive reaction activates SoxR as a transcription activator nearly as potent as the oxidized form. Intriguingly, the nitrosylated iron-sulfur centers are removed rapidly in cells following NO exposure, which points to efficient cellular pathways to process this type of protein lesion.

Because NO cannot be contained by cells, its production during inflammatory responses will affect surrounding cells indiscriminately. We therefore tested whether specific sets of genes are induced in mammalian cells by fluxes of NO that are just below the toxic level. These studies showed that exposure to sublethal NO activates expression of various gene products, including the enzyme heme oxygenase 1(HO1), in many cell types. In rodent motor neurons, this activation contributes to a complex inducible resistance to higher levels of NO. The expression of HO1 mRNA is strongly activated in normal human fibroblasts in response to NO, but this induction is not primarily transcriptional. Instead, there is a pronounced increase in the stability of the HO1 message independent of active protein

synthesis (the half-life is increased from <2 h to >10 h). This change occurs rapidly after NO exposure and seems to be triggered by NO rather than oxygen-dependent by-products. Fibroblasts also exhibit an inducible resistance to NO, with increased survival not only of toxic NO treatment, but also of challenges with the DNA-damaging agents bleomycin and methyl methane sulfonate. In parallel with this resistance, NO-dependent DNA damage occurs at a lower level in cells expressing inducible NO resistance. Such cells also show diminished levels of protein nitrotyrosines. We are exploring whether these differences arise from diminished formation of molecular damage or more effective removal or repair of the damaged molecules. The diverse genes activated in response to NO provide cells with a dynamic ability to cope with changing levels of this biological free radical.

References:

1. A. Koutsolioutsou, E.A. Martins, D.G. White, S.B. Levy & B. Demple (2001) A *soxRS*-constitutive mutation contributing to antibiotic resistance in a clinical isolate of *Salmonella enterica* (serovar Typhimurium). *Antimicrob Agents Chemother* **45**: 38-45.
2. H. Ding & B. Demple (2000) Direct nitric oxide signal transduction via nitrosylation of iron- sulfur centers in the SoxR transcription activator. *Proc Natl Acad Sci U S A* **97**: 5146-50.
3. J.C. Marquis & B. Demple (1998) Complex genetic response of human cells to sublethal levels of pure nitric oxide. *Cancer Res* **58**: 3435-40.
4. A. Bishop, J.C. Marquis, N.R. Cashman, & B. Demple (1999) Adaptive resistance to nitric oxide in motor neurons. *Free Radic Biol Med* **26**: 978-86.
5. C. Bouton & B. Demple (2000) Nitric oxide-inducible expression of heme oxygenase-1 in human cells: Translation-independent stabilization of the mRNA and evidence for direct action of NO. *J Biol Chem* **275**: 32688-93.

Mechanisms of gene regulation by nitric oxide

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Nitric oxide (NO) modulates transcription factors that bind specific cis-regulatory DNA responsible for coordinating spatial and temporal patterns of gene expression initiated by a changing microenvironment. In this way NO helps to orchestrate gene transcription and forms the basis of functional cell responses to accommodate metabolic requirements and to coordinate endogenous defense mechanisms against a variety of stress and disease conditions. There is a marked overlap between signalling pathways triggered by NO, superoxide, and hypoxia. The chemokine MIP-2 (macrophage inflammatory protein 2) [1] and the matrix metalloproteinase-9 [2] will be discussed as paradigms of NO- and superoxide-regulated genes. Understanding of the redox-based regulation of signal transduction and gene expression will provide insights into how cell activities are constantly coordinated and how promising new therapies may be developed [3].

References:

[1]. Walpen S, Beck KF, Schaefer L, Raslik I, Eberhardt W, Schaefer RM, Pfeilschifter J:
Nitric oxide induces MIP-2 transcription in rat renal mesangial cells and in a rat model of glomerulonephritis. FASEB J. 15, 571-573 (2001).

[2]. Eberhardt W, Huwiler A, Beck KF, Walpen S, Pfeilschifter J: Amplification of interleukin-1 beta-induced MMP-9 expression by superoxide in rat glomerular mesangial cells is mediated by increased activities of NF-kappaB and AP-1 and involves activation of the MAPK-pathways. J. Immunol. 165, 5788-5797 (2000).

[3]. Pfeilschifter J, Eberhardt W, Beck KF: Regulation of gene expression by nitric oxide. Pflügers Arch. Eur. J. Physiol. (2001) in press.

Regulation of gene expression and cell proliferation by nitric oxide

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Nitric oxide (NO) induces long lasting changes in cell physiology; however, the gene targets of NO remain uncharacterized and the signaling pathways that lead to gene activation by NO are still obscure. We have used a cDNA microarray hybridization approach to obtain transcript profiles and identify genes affected by NO. Our results demonstrate that NO is a potent regulator of gene expression in mammalian cells. A subset of genes affected by NO is involved in cell cycle progression. Another, partially overlapping subset of genes is dependent on tumor suppressor p53 for activation by NO, as revealed by using cells from knock-out mice deficient in p53. Other subsets of genes affected by NO depend on guanylate cyclase, protein kinase C, and PI3 kinase activity, as revealed by using selective inhibitors of these enzymes. Since phosphorylation of particular residues of the p53 molecule has been correlated with its functional activity, we determined the phosphorylation pattern of the p53 molecule after exposure to NO and compared it with the phosphorylation patterns that develop upon treatment with gamma-irradiation, UV light, and adriamycin. We found that NO induced a specific signature pattern of p53 phosphorylation, distinct from the patterns evoked by other inducers. Our results suggest that NO uses multiple signaling pathways to regulate gene activity and control cell proliferation in mammalian cells.

Session 4: NO and modulation of proteins involved in infection and immunity
Chair: Santiago Lamas

Enzymes that prevent and repair peroxynitrite-mediated injury: studies with microbial pathogens

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Knock-out mice have helped identify infections against which NOS2 or phox constitute essential, nonredundant host defenses[1]. Mice deficient in both NOS2 and phox reveal that these enzymes also constitute mutually redundant defenses, to the extent that without them, the species could not survive in the wild[2]. Thus, the antimicrobial effects of NOS2 are more widespread than revealed by the $NOS2^{-/-}$ mouse. Much of the antimicrobial action of NOS2 may be mediated by formation of peroxynitrite ($OONO^-$). If we can identify enzymes that prevent or repair $OONO^-$-mediated damage, we can use the phenotype of bacteria deficient in these enzymes as a probe to gauge the importance of $OONO^-$-mediated antibacterial effects.

We and others have identified three levels of enzymatic defense against $OONO^-$: (i) Prevention. SOD and NO dioxygenase can draw off the precursors of $OONO^-$, preventing its formation.

(ii) Catabolism. S. typhimurium deficient in the peroxiredoxin AhpC were sensitive to killing by NO_2^- and S-nitrosoglutathione (GSNO)[3]. The peroxiredoxin from M. tuberculosis protected transfected human cells from autotoxicity caused by NOS2[3]. Yet AhpC did not detectably react with NO or GSNO[4]. Instead, we found that peroxiredoxins from S. typhimurium, M. tuberculosis and H. pylori acted as peroxynitrite reductases. They break down $OONO^-$ fast enough to protect bystander molecules from oxidation. The mechanism is the reversible oxidation of active-site Cys to the sulfenic acid, followed by intramolecular disulfide bond formation and reduction either by a dedicated flavoprotein (AhpF) or by thioredoxin (Trx). Trx in turn is reduced by its own dedicated flavoprotein, Trx reductase. Tyr nitration is a much less sensitive indicator of the reaction of AhpC with $OONO^-$ than is oxidation of the acidic active site Cys[4]. These findings imply that NO_2^-, GSNO and NOS2 can cause cytotoxicity via $OONO^-$.

(iii) Repair. Killing of E. coli by NO_2^- required O_2 and thus may have involved formation of $OONO^-$. Killing by NO_2^-, GSNO and H_2O_2, but not by acid, ethanol or urea, was dependent on oxidation of Met to Met-SO, as revealed by the phenotype of mutants deficient in peptidyl methionine sulfoxide reductase (msrA) or in which msrA's active site Cys was mutated to Ser[5]. NO_2^- and GSNO do not oxidize Met, while $OONO^-$ does. Thus, some reactive nitrogen intermediates (RNI) may kill bacteria by giving rise in the cell to $OONO^-$, which oxidizes critical Met residues. MsrA may be the first example of an enzyme that repairs $OONO^-$-mediated injury.

Agents that inhibit pathogens' RNI resistance mechanisms may improve immunity in those diseases for which RNI represent an important but imperfect element of host control. Mammalian homologs of these enzymes may be important determinants of inflammatory tissue damage.

References:

1. Nathan, C. F. and M. U. Shiloh. Proc. Natl. Acad. Sci. 97: 8841-8848, 2000.

2. Shiloh, M. U., MacMicking, J., Nicholson, S., Brause, J., Potter, S., Fang, F., Marino, M., Dinauer, M. and Nathan, C. Immunity 10: 29-38, 1999.

3. Chen, L., Q.-w. Xie, and C. Nathan. Molecular Cell 1: 795-805, 1998.

4. Bryk, R., P. Griffin and C. Nathan. Nature 407, 211-215, 2000.

5. St. John, G., J. Ruan, N. Brot, H. Weissbach, and C. Nathan. In preparation.

Inhibition of bacterial DNA replication by nitrogen oxides

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Nitrogen oxides produced by phagocytic cells have been strongly implicated as antimicrobial mediators against parasites, fungi, bacteria and viruses. Both humans and experimental animals produce dramatically increased quantities of nitric oxide (NO) during infection, and expression of inducible NO synthase can be directly demonstrated in infected tissues. Inhibition of NO synthase exacerbates microbial proliferation during experimental infections or phagocyte killing assays, and a variety of chemical NO-donors have been shown to inhibit or kill diverse microbial species *in vitro*. Although NO can exert synergistic antimicrobial activity in concert with reactive oxygen species, observations in a murine systemic *Salmonella* infection model have shown that initial bacterial killing by phagocytes is primarily dependent on the NADPH phagocyte oxidase, with subsequent sustained inhibition of bacterial growth mediated by NO. Analysis of repair-deficient bacterial strains indicates that bacterial DNA replication is an important target of NO. In the absence of the RecBC repair proteins, replication arrest can result in lethal double-strand breaks mediated by the RuvC resolvase. Inhibition of bacterial growth correlates with zinc release, suggesting that nitrogen oxides interfere with DNA replication by targeting DNA-binding zinc metalloproteins.

References:

DeGroote, M.A., D. Granger, Y. Xu, G. Campbell, R. Prince and F.C. Fang. "Genetic and redox determinants of nitric oxide cytotoxicity in a *Salmonella typhimurium* model," *Proc Natl Acad Sci U.S.A. 92*:6399-403, 1995.

Fang, F.C., ed. *Nitric Oxide and Infection*. New York: Plenum, 1999.

Vazquez-Torres, A., J. Jones-Carson, P. Mastroeni, H. Ischiropoulos and F.C. Fang. "Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages *in vitro*." *J. Exp. Med. 192*:227-36, 2000.

Mastroeni, P., A. Vazquez-Torres, F.C. Fang, Y. Xu, S. Khan, C.E. Hormaeche and G. Dougan. "Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival *in vivo*." *J. Exp. Med. 192*:247-47, 2000.

Vazquez-Torres, A. and F.C. Fang. "Oxygen-dependent anti-*Salmonella* activity of macrophages." *Trends Microbiol. 9*:29-33, 2001.

Anti-viral mechanisms of peroxynitrite

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In response to infections, phagocytes synthesize superoxide, which can combine with other molecules to generate microbicidal oxidants, including hypochlorous acid, hydroxyl radical, and peroxynitrite.[1-4] Peroxynitrite, the product of nitric oxide and superoxide, is used by the host to fight infections, and bacteria have developed mechanisms to detoxify peroxynitrite.[5] However, the precise mechanisms by which peroxynitrite and its derivatives kill pathogens are not well understood. We have examined the effect of peroxynitrite upon poliovirus replication. By adding peroxynitrite to infected cells, we have found that peroxynitrite inhibits viral replication by several distinct mechanisms.

Peroxynitrite inhibits poliovirus replication by directly modifying the virion prior to infection. Peroxynitrite modifies viral RNA, reducing its infectivity. Peroxynitrite modifies the viral capsid proteins, reducing viral attachment to the host cell. And peroxynitrite modifies the structure of the virion as well.

Peroxynitrite also inhibits poliovirus replication after the virion has entered the cell. Addition of peroxynitrite to cells infected with poliovirus reduces viral replication, blocking viral protein synthesis and viral RNA replication. Peroxynitrite inactivates specific viral proteins during replication.

Thus peroxynitrite inhibits viral replication by acting both inside and outside of infected cells. Peroxynitrite alters properties of the virion outside of the host cell, and peroxynitrite also interferes with the intracellular process of viral replication. These mechanisms by which peroxynitrite inhibits poliovirus infection may prove to be general mechanisms by which reactive nitrogen and oxygen intermediates kill other pathogens as well.

References:

1.Babior BM. Phagocytes and oxidative stress. Am J Med 2000; 109:33-44.
2.Bogdan C, Rollinghoff M, Diefenbach A. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. Curr Opin Immunol 2000; 12:64-76.
3.Fang FC. Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. J Clin Invest 1997; 99:2818-25.
4.Nathan C, Shiloh MU. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. Proc Natl Acad Sci U S A 2000; 97:8841-8.
5.Bryk R, Griffin P, Nathan C. Peroxynitrite reductase activity of bacterial peroxiredoxins. Nature 2000; 407:211-5.

Cytokine regulation and antimicrobial activity of inducible nitric oxide synthase: fresh air to established concepts?

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Two central immunological features of type 2 nitric oxide synthase (iNOS2 or NOS2) have been its strong transcriptional and posttranscriptional regulation by cytokines and its high out-put production of NO exerting a strong antimicrobial activity. This view is challenged by two recent studies.

Rutschman et al. (*J. Immunol.166, 2173, 2001*) provided evidence that the suppression of macrophage NO production (nitrite accumulation) by IL-4 or IL-13 results from the induction of arginase and the subsequent depletion of arginine from the culture medium, because (a) the addition of exogenous L-arginine fully restored the production of NO in macrophages pretreated with IL-4 or IL-13, and (b) IL-4 and IL-13 failed to inhibit the production of NO in STAT6-/- macrophages lacking the expression of arginase. These results, which essentially confirm previous data by Modolell et al. (*Eur. J. Immunol.* 25: 1101, 1995), contrast which several other reports in which IL-4 and IL-13 were shown to downregulate the expression of iNOS mRNA and/or protein in mouse macrophages (e.g., Bogdan et al., *J. Leukoc. Biol.* 55: 227, 1994; Sands et al., *Eur. J. Immunol.* 24: 2345, 1994; Takeda et al., *J. Immunol.* 157: 3220, 1996; Bogdan et al., *J. Immunol.* 159: 4506, 1997).

Iniesta et al. (*J. Exp. Med.* 193: 777, 2001) argue that the antileishmanial activity of bone marrow-derived macrophages activated by IFN-γ plus LPS is not due to the production of NO, but at least in part results from the generation of the iNOS-intermediate N^{ω}-hydroxy-L-arginine (LOHA) and its inhibitory effect on the arginase of the parasite. This idea is based on the observation that (a) LOHA alone can mediate death of *Leishmania* within macrophages and that (b) exogenously added ornithine (the product of the arginase reaction) can revert the killing of *Leishmania* by cytokine-activated macrophages expressing iNOS.

Here, we demonstrate that the previous concepts still hold true.

- Pretreatment of mouse peritoneal macrophages with IL-4 or IL-13 clearly suppressed the expression of iNOS protein in response to IFN-γ plus LPS. However, in the past we had already observed that the downregulation of iNOS by IL-13 was more prominent in Western blot analyses than in ^{35}S labeling experiments that *per se* required the replacement of the culture medium after the pretreatment phase in order to achieve sufficient incorporation of ^{35}S-methionine (Bogdan et al., *J. Immunol.* 159: 4506, 1997). This led us to hypothesize that IL-13 might act via depletion of a medium component that is required for the synthesis of iNOS. We now show that the suppression of iNOS protein by IL-13 is completely prevented by the addition of arginase inhibitors, the exchange of medium or the supplementation of arginine after the pretreatment phase. Furthermore, the effect of IL-13 can be mimicked by the addition of arginase or the reduction of the arginine concentration in the medium. These results not only provide an explanation for the seemingly discrepant previous data, but also suggest a novel posttranscriptional mechanism of iNOS regulation.

- Stimulation of peritoneal exudate macrophages with IFN-γ plus TNF (or LPS) led to the induction of iNOS and the subsequent killing of intracellular *Leishmania (L.) major* amastigotes. This effect could not be overcome by the addition of exogenous L-ornithine (up to 1 mM). Furthermore, in unstimulated but infected macrophages lacking iNOS, the addition of arginase inhibitors (up to 1 mM) did not lead to a decrease of the number of viable intracellular parasites. When iNOS+/+ macrophages were cocultured with *L. major*-infected iNOS-/- macrophages, stimulation with IFN-γ/TNF induced parasite killing provided the two macrophage populations were in close vicinity. Killing was not observed in transwell-chambers, where the infected iNOS-/- and iNOS +/+ macrophages were separated by a membrane and ca. 0.4 cm away from each other. These results suggest that killing of *L. major* by cytokine-activated macrophages is due to the iNOS-dependent generation of a labile antimicrobial product and is not mediated by the inhibition of arginase.

Session 5: Modulation of hemoproteins by NO
Chair: Steven S. Gross

New regulatory and molecular aspects of NO-sensitive guanylyl cyclase

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The signalling molecule nitric oxide (NO) has an important function in the cardiovascular and nervous systems. Most of the effects of NO are mediated by the activation of soluble guanylyl cyclase (sGC), the enzyme which catalyses the conversion of GTP to cGMP. sGC which contains a prosthetic heme group required for NO stimulation is a heterodimer consisting of an α and β subunit. Although two α (α_1, α_2) and two β subunits (β_1, β_2) have been identified on cDNA level, only two heterodimers have been shown to occur on the protein level ($\alpha_1\beta_1$, $\alpha_2\beta_1$) with the $\alpha_1\beta_1$ heterodimer representing the most abundant isoform.

Recent data suggested an interaction of the C-terminus of the α_2 subunit with PDZ domains. PDZ domains are adaptor domains mediating interactions of proteins, thereby leading to the formation of functional complexes of signaling proteins. As the neuronal NO synthase has been shown to interact with a PDZ domain of PSD-95, we considered a possible $\alpha_2\beta_1$/PSD-95 interaction and showed that the immobilized C-terminal peptide of the α_2 subunit precipitates PSD-95 and related proteins (PSD-93, SAP97, SAP 102) from brain homogenates. Using immunopurified antibodies against the α_1, α_2 and β_1 subunits, we were able to detect all guanylyl cyclase subunits in brain homogenates. Further experiments have to show whether $\alpha_2\beta_1$/PSD-95 complexes exist *in vivo* and whether the interaction with PSD-95 leads to the membrane association of the $\alpha_2\beta_1$ isoform.

Accumulating evidence suggests that a lack of NO caused by either endothelium removal, gene-disruption or inhibition of the endothelial NO synthase leads to increased NO sensitivity of aortic vessels paralleled by an augmentation of NO-induced cGMP levels. Here, we show that after long-term L-NAME treatment of rats this increase in NO sensitivity is not due to augmented expression of sGC in rat aortae. In aortic tissue and platelets, NO leads to a tremendous increase in cGMP within less than 30 seconds (50- to 100-fold) and a rapid decline reflecting the tightly controlled balance of guanylyl cyclase and phosphodiesterase activities. Inverse to the increase in sensitivity caused by relative NO shortage, NO by itself concentration-dependently led to desensitisation of the cGMP response. Further experiments have to show whether this desensitization occurs on the level of guanylyl cyclase or phosphodiesterases.

Reductive nitrosylation of human methemoglobin occurs preferentially within beta-subunits and leads to the formation of SNO-hemoglobin

David J. Singel

Previous studies of the interactions of NO with hemoglobin have implicated the predominance of reaction channels that alternatively eliminate NO as nitrate, or tightly complex it on alpha-subunit ferrous hemes. Both channels could effectively quench NO bioactivity. More recent work has provided support for the idea that NO groups can rapidly transfer from the hemes to cysteine thiols within the beta-subunit to form bioactive nitrosothiols that maintain NO bioactivity. In order to probe for a nitrosothiol-forming reaction channel between heme and cys(beta-93), we have studied the reaction of NO with human methemoglobin in which reductive nitrosylation of oxidized hemes could support the oxidative requirements of heme-to-thiol NO group transfer chemistry. We find that exposure of methemoglobin to NO at added-NO/heme ratios less than ~0.1, which embrace physiological conditions, quantitatively yields hemoglobin-nitrosothiol and ferrous nitrosylated hemes within the beta-subunits of the hemoglobin tetramer. This selective processing within the beta-subunits reveals a means by which the NO-group can be dislodged from a heme-Fe(II)NO complex to make the nitrosothiol hemoglobin derivative, and establishes the connectivity between beta-subunit hemes and thiols that preserves NO bioactivity.

Session 6: NO-mediated regulation of proteins involved in apoptosis and cancer
Chair: José M. Mato

Regulation of apoptosis by NO

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Apoptosis plays an important role in the development of the organism but also under various pathological conditions. Nitric oxide exhibits contradictory effects in the regulation of apoptosis. Both pro- and antiapoptotic effects have been demonstrated. The proapoptotic effects seem to be linked to pathophysiological conditions, where high amounts of NO are produced by the inducible nitric oxide synthase. In contrast, the continuous release of endothelial NO inhibits apoptosis and may contribute to the antiatherosclerotic function of NO.

In endothelial cells low concentration of exogenous NO-donors or shear stress-induced stimulation of endogenous endothelial NO-synthesis inhibits apoptosis induced by various pro-atherosclerotic factors such as oxLDL or angiotensin II. The mechanism by which NO inhibits apoptosis includes several transcriptional and posttranscriptional events. Importantly, NO was shown to directly inhibit caspases, a family of cysteine proteases, which execute apoptosis. The inhibition of caspases by NO is mediated via S-nitrosylation of the essential cysteine residue located in the active site of the enzyme as shown by experiments with the isolated enzymes or intact cells. Moreover, recent studies further provide evidence that NO S-nitrosylates thioredoxin, a redox-regulatory protein and, thereby, additionally modulates apoptosis signalling.

Since increasing age is one of the major risk factors for the development of atherosclerosis, we further assessed the regulation of apoptosis in aged endothelial cells. Compared to young endothelial cells, aged endothelial cells revealed a higher sensitivity to pro-apoptotic stimuli such as TNFα or oxLDL. Whereas stimulus-induced apoptosis and caspase activation was elevated up to 4-fold in aged endothelial cells, basal apoptosis remained unaffected. The increase in apoptosis sensitivity in aged endothelial cells was associated with reduced eNOS protein content and S-NO levels. Moreover, addition of exogenous NO or overexpression of the endothelial NO-synthase prevented apoptosis induction, suggesting an important role for down-regulation of NO-bioavailability in age-associated increase in apoptosis sensitivity. Taken together, the apoptosis-suppressive effect of NO in endothelial cells may contribute to the profound anti-inflammatory and anti-atherosclerotic effects of endothelial-derived NO. Furthermore, the support of cell survival may play a critical role for the pro-angiogenic effects of NO.

Regulation of caspase activity and processing by nitric oxide

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It is appreciated that the production of nitric oxide (NO) may serve as a cytotoxic molecule to produce cell death along an apoptotic or necrotic pathway but also gained attention as an inhibitor of cell destruction. Diverse molecular targets such as caspases emerged as important regulators that are directly or indirectly affected by NO and in turn modulate the outcome of cell demise.

Apoptosis as a result of substantial NO-formation is characterized by caspase activation as determined by the cleavage of various substrates such as PARP (poly ADPribose polymaerase) or U1-70kDa small ribonucleoprotein. In addition, a pan caspase inhibitor (Z-Asp-CH_2-DCB) blocked apoptosis as well as caspase-3 activity while accumulation of the tumor suppressor p53 remained unaltered. Apparently, activation of caspases occurs in close association with initiation of NO-elicited cell death, i.e. apoptosis.

To suppress apoptosis by NO several signaling pathways are envisioned:

I) Upregulation of cell protective proteins by NO may interfere with apoptosis at a point upstream to caspase activation/processing. In macrophages we delineated expression of cyclooxygenase-2 (Cox-2) as a protective principle. Prestimulation of macrophages with a subtoxic dose of NO-donors activated the transcription factor NF-κB as well as AP-1 and promoted immediate early gene expression of Cox-2. NF-κB supershift analysis implied an active p50/p65-heterodimer following NO addition. Degradation of IκB and activation of a luciferase reporter construct, containing four copies of the NF-κB-site derived from the murine Cox-2 promoter confirmed NF-κB activation. A NF-κB decoy approach (oligonucleotides directed against NF-κB) or transfection of a dominant-negative c-Jun mutant (TAM-67) abrogated not only Cox-2 expression but also inducible protection. Thus, blocking NO-mediated inducible protection at the level of NF-κB and/or AP-1 restored the occurrence of apoptotic features.

II) A second model proposes S-nitrosation of active caspases to interfere with progression of apoptosis. Although, *in vitro* S-nitrosation and inactivation of active caspases occurs, we question this posttranslational modification to account for apoptosis inhibition. In Jurkat cells we blocked Fas- as well as etoposide-induced apoptosis by NO downstream of mitochondria. Interestingly, caspase activity was not restored by the reducing agent dithiothreitol, as predicted for S-nitrosations. Instead, processing of procaspase-9, -3, and -8 was attenuated. In line, formation of the apoptosome was attenuated under the impact of NO. Using an *in vitro* binding assay we demonstrate that NO attenuates a CARD/CARD interaction between procaspase-9 and Apaf-1. NO may hinder apoptosome assembly, which is a prerequisite for apoptosis execution in type II cells.

Evidently, the impact of NO on multiple pathways and targets determines the role of NO in affecting apoptotic cell death versus cell survival with the notion that cellular susceptibility varies considerably.

References:

B. Brüne, A. von Knethen and K.B. Sandau:
Nitric Oxide (NO): An Effector of Apoptosis.
Cell Death and Differentiation 6:969-975 1999

B. Zech, M. Wilm, R. van Eldik and B. Brüne:
Mass spectrometric analysis of nitric oxide modified caspase-3.
J. Biol. Chem. 274: 20931-20936 1999

A. von Knethen, D. Callsen and B. Brüne:
Superoxide attenuates macrophage apoptosis by NF-κB and AP-1 activation that promotes cyclooxygenase-2 expression.
J. Immunology 163:2858-2866 1999

Nitric oxide mediates guanine nucleotide exchange on the Ras onco-protein

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Ras proteins cycle between active GTP-bound and inactive GDP-bound states to mediate signal transduction pathways that promote cell growth and differentiation. The primary mechanism for Ras activation is believed to occur by interaction with guanine-nucleotide exchange factors (GEFs). Ras-GEF interactions are highly regulated and result in elevated levels of Ras-GTP by facilitating GDP dissociation. Recently, a novel mechanism of Ras activation has been proposed, whereby nitric oxide (NO) modification of ^{118}Cys, like GEF interaction, populates Ras in its biologically active form by stimulating GDP release (1,2). We have previously characterized a Ras C118S variant that is insensitive to NO modification. We found the GTPase activity, GDP dissociation rate and structure of the C118S mutant to be similar to wild-type Ras (2). We have also investigated the structural features and biochemical properties of Ras protein stably modified with nitric oxide, and found that stable nitrosylation of ^{118}Cys has little effect on the nucleotide exchange, effector recognition, or structure of Ras. Our results, however, do suggest that transient nitrosylation of ^{118}Cys increases guanine nucleotide dissociation of Ras and therefore should populate Ras in its biologically active GTP-bound state *in vivo*. We propose a mechanism that describes how nitric oxide labeling of Ras effects the guanine nucleotide bound state and the subsequent downstream signaling of Ras. We believe that the basis of Ras activation by NO, is destabilization of a crucial interaction between residues in the GDP-binding pocket and the nucleotide. In particular, the ^{119}Asp carboxylate side-chain is involved in multiple contacts with the guanine nucleotide and is important for high affinity binding. The ^{119}Asp carboxylate side-chain is also a necessary nucleophile in facilitating nitrosylation and denitrosylation of ^{118}Cys. Hence, transient nitrosylation or denitrosylation competes with guanine nucleotide substrate interactions thereby facilitating guanine nucleotide dissociation and GTP-loading of Ras.

References:

1. Lander HM, Hajjar DP, Hempstead BL, Mirza UA, Chait BT, Campbell S, Quilliam LA. A molecular redox switch on p21(ras). Structural basis for the nitric oxide-p21(ras) interaction. J Biol Chem. 1997 Feb 14;272(7):4323-6.

2. Mott HR, Carpenter JW, Campbell SL. Structural and functional analysis of a mutant Ras protein that is insensitive to nitric oxide activation. Biochemistry. 1997 Mar 25;36(12):3640-4.

Regulation of apoptotic signal transduction by nitrosylation of cytochrome

Joan Mannick

Release of cytochrome c from mitochondria is a central event regulating apoptosis. When mitochondria recieve an apoptotic signal, cytochrome c is released from the mitochondrial intermembrane space into the cytoplasm where it binds to Apaf1 and caspase-9, leading to the activation of effector caspases and apoptotic cell death. The mechanisms that regulate cytochrome c function during apoptosis are unkown. During Fas-induced apoptosis of human lymphocyte cell lines, we find that cytochrome c is S-nitrosylated in mitochondria and then released into the cytoplasm. The Soret band of S-nitrosylated cytochrome c is not shifted suggesting that NO is not bound to the heme. Our data suggest that one hour after Fas crosslinking, 80% of cytochrome c released into the cytoplasm is S-nitrosylated. Since cytochrome c has only two cysteine residues which covalently bind heme via thioether linkages, our data also suggest that one of these bonds is not present in the subpopulation of cytochrome c released into the cytoplasm, allowing a free cysteine to be S-nitrosylated. In Bcl-2 and Bcl-XL overexpressing cells, S-nitrosylated cytochrome c remains in mitochondria after Fas crosslinking. S-Nitrosylated cytochrome c increases caspase activation as compared to equal concentrations of control cytochrome c. In summary our data suggest that 1) cytochrome c is S-nitrosylated during Fas-induced apoptosis; 2) S-nitrosylated cytochrome c is the subpopulation of cytochrome c that is released from mitochondria; and 3) the function of S-nitrosylated cytochrome c is to increase caspase activation during apoptosis.

POSTERS

Regulation of heme oxygenase-1 mRNA turnover by nitric oxide

Bouton C and Demple B

Heme oxygenase-1 (HO-1) is a stress inducible protein that plays important cellular defense roles against oxidant injury. This protein catalyses the rate limiting step in heme catabolism in mammalian cells by degrading the heme molecule to yield equimolar amounts of biliverdin, carbon monoxide (CO), and iron (Fe). These three by-products of heme catabolism by HO-1 are now suspected to be biologically active. Biliverdin and bilirubin which is formed when biliverdin is reduced by biliverdin reductase, are both potent peroxyl radical scavengers. Fe released by heme degradation regulates genes, especially ferritin that would limit free iron from participation in the Fenton reaction. Finally and very recently, several groups have shown that the signal molecule CO mediates potent anti-inflammatory and anti-apoptotic effects through a selective inhibition of pro-inflammatory cytokines. The ability of cells to modulate HO-1 expression must certainly contibute to the effectiveness of HO-1 in antioxidant and anti-inflammatory defenses. The mechanism of HO-1 induction is therefore of great interest. Nitric oxide (NO) biosynthesis through expression of the inducible NO synthase occurs in the inflammatory process and strongly induces the expression of HO-1 mRNA and protein level in many cell types. However, the specific contribution of transcriptional or post-transcriptional effects to this induction has remained unresolved. Here we elucidate further the role of mRNA stabilization in the induction of HO-1 by NO. We have used two diazeniumdiolates as NO donors because of their controlled and predictable ability to release NO under physiological conditions. This approach allowed us to demonstrate a direct correlation between the NO flux rate in the range 1-30 nM/s and the induction of HO-1 mRNA in human fibroblasts. We then used a specific NO scavenger (carboxy-PTIO) that completely prevented the inducible expression of HO-1 by NO. Interestingly, carboxy-PTIO by scavenging NO also released NO2., the closest oxygen-dependent product of NO which failed to induce HO-1mRNA. These last results point to direct signaling action of NO in this induction. By using three different rates of NO release we also found a gradual increase in the HO-1 mRNA half-life up to 120 nM/s. Morover, the change in message stability occurs immediately upon NO exposure and in the absence of active transcription. We also showed here that stabilization of the HO-1 message did not require de novo protein synthesis. These observations suggest that cells are able to sense and respond directly to various levels of NO by adjusting the stability for HO-1 mRNA. Fine-tuning mRNA stability for HO-1 and perhaps for other transcripts may constitute an important cellular response to NO stress environment.

IMPLICATION OF TNF-α CONVERTASE (TACE /ADAM 17) IN iNOS EXPRESSION AFTER EXPERIMENTAL BRAIN ISCHAEMIA

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Summary

Tumour necrosis factor-α (TNF-α) is a major immunomodulatory and proinflammatory cytokine which is shed in its soluble form by a membrane-anchored zinc protease, identified as a disintegrin and metalloproteinase (ADAM) called TNF-α convertase (TACE; ADAM17). The role of this protease in the adult nervous system remains poorly understood. During cerebral ischemia and subsequent reperfusion, expression and release of TNF-α have been shown. We have investigated the expression and activity of TACE in an *in vitro* model of brain ischemia consisting of rat forebrain slices exposed to oxygen-glucose deprivation (OGD). OGD caused the release of TNF-α, an effect which was inhibited by a hydroxamate-based metalloprotease inhibitor, BB-3103, with an IC_{50} of 0.1 μM, suggesting that TNF-α release results selectively from TACE activity. Assay of TACE enzymatic activity on a fluorescein-labelled peptide spanning the cleavage site in pro-TNF-α, as well as Western blot and RT-PCR analyses showed that TACE is present in control forebrain and, more interestingly, that TACE expression is increased in OGD-exposed tissue. TACE enzymatic activity from OGD-exposed slices was significantly inhibited by cycloheximide, suggesting that *de novo* synthesis of TACE contributes to TNF-α release after ischaemia. Moreover, it was also inhibited by bisindolylmaleimide I, indicating that TACE activity is regulated by PKC. These findings posed the question of what was its function therein. Among other actions, TNF-α has been described to be involved in the expression of inducible nitric oxide synthase (iNOS), a high-output NOS isoform associated to cellular damage, but the link between TNF-α release after brain ischaemia and iNOS expression in this condition has not been shown. We have now found that iNOS expression in OGD-subjected brain slices is inhibited by BB-3103 at concentrations below 1 μM, indicating that shedding of TNF-α by TACE plays a necessary part in the induction of this NOS isoenzyme after OGD. Taken together, these data demonstrate that 1) TACE/ADAM17 activity accounts for the majority of TNF-α shedding after OGD in rat forebrain slices, 2) an increase in TACE expression contributes, at least in part, to the rise in TNF-α after OGD and 3) iNOS expression in OGD-subjected brain slices results from TACE activity and subsequent increase in TNF-α levels.

Inhibition of cytokine-induced expression of MMP-9 expression by PPARα agonists is indirect, and is related to inhibitory effects exerted by NO

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High output levels of nitric oxide (NO) are produced in rat cytokine-stimulated mesangial cells (MC) by the inducible nitric oxide synthase (iNOS). We recently have shown that NO inhibits the cytokine-induced expression and activity of matrix metalloproteinase-9 (MMP-9). We now tested whether ligands of the PPAR family could influence the cytokine-induced expression of MMP-9. PPAR agonists have been shown to regulate genes coding for proinflammatory proteins, including iNOS, COX-2 and MMP-9. We found that PPARα-agonists dose-dependently inhibit the IL-1ß evoked gelatinolytic activity in cellular supernatants, which is paralleled by a decrease in steady-state MMP-9 mRNA level. PPAR agonists on their own had no effect. The inhibition on MMP-9 correlates with increased levels of nitrite in the conditioned media. Coincubation of MC with IL-1ß, PPARα agonists and L-NMMA most surprisingly resulted in a strong amplification of IL-1ß-mediated increase in MMP-mRNA level. The inhibitory effects of PPARα agonists on MMP-9 steady state mRNA levels were not due to an inhibition of the ranscriptional activity since MMP-9-promoter reporter constructs were superinduced by IL-1ß and PPARα agonists. By experiments with actinomycin D, an inhibitor of *de novo* transcription, we found that exogenously given NO exerts a negative effect on MMP-9 mRNA stability whereas PPARα agonists were without effect. Our data suggest that inhibitory effects of PPARα agonists on cytokine-induced expression of MMP-9 are indirect and primarily due to a superinduction of iNOS.

Molecular mechanisms involved in soluble guanylyl cyclase regulation

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Although soluble guanylyl cyclase (sGC) was discovered in 19691,2, the progress in understanding the physiological significance of sGC-cGMP signalling has been slow until the discovery of nitric oxide (NO)3,4. Purification of mammalian sGC has yield an enzyme heterodimeric in nature formed by two subunits, termed a and b of which four types exist (a1, a2, b1, b2)5-7. Since this enzyme plays a pivotal role in the transduction of inter- and intracellular signals conveyed by NO and due to the ubiquitous nature of NO-sGC-cGMP, the knowledge of the molecular aspects of sGC regulation will be critical to understand the physiological and pathophysiological significance of this signal transduction pathway. Several recent papers have actually described different modulatory pathways of sGC expression and functionality8. We have been studying different molecular aspects of sGC regulation on chromaffin cells. First, we have found that changes in phosphorylation levels of the b subunit of sGC are accompanied by differences in NO-elicited cGMP accumulations9. Second, long-term treatment with DETA/NO caused a decrease in subsequent NO-stimulated cGMP levels, wich was accompanied by a reduction in the protein levels. Although the levels of both a and b subunits were lower in DETA/NO-treated cells than in control after 24 hours treatment, the amount of b subunit started to diminish at shorter times. This effect could be explained by a down-regulation of sGC synthesis (transcriptional or translational level) or by an increase of the degradation rate. Incubation with the protein synthesis inhibitor (cychloheximide (CHx) 10 mg/mL) during different periods of time caused a time-dependent reduction of NO-stimulated cGMP, and the estimated half-life for this activity was 41.5 ± 0.9 hours. The half-life estimated when cells were treated with 50 mM DETA/NO was 36.5 ±2 hours and with the two drugs together the half-life was shorter, being 25.7 ±3.7 hours. When the same estimations for subunit b level were performed the values were very similar to that obtained for the activity. The effect of DETA/NO was not mediated by cGMP production because the same long-term treatment with CNP, which increased the cGMP levels in the same way as DETA/NO, did not cause any reduction on NO-stimulated cGMP increases nor in sGC protein levels. On the other hand co-treatment with DETA/NO and the cGMP permeant analogue Sp-8-Br-PET-cGMP counteracted the effect of DETA. Surprisingly, treatment with the sGC inhibitor ODQ for 48 hours produced a reduction on sGC levels and enhanced the effect caused by DETA/NO.

References:

1.-Hardman J.G and Sutherland E.W (1969) J. Biol. Chem. 244: 6363-6367. 2.-White A.A and Aurbach G.D (1969) Biochim. Biophys. Acta. 191: 191-196. 3.-Palmer R.M., Ferrige A.G and Moncada S (1987) Nature 327: 524-526. 4.-Ignarro L.J., Buga G.M., Wood et al. (1987) Proc. Natl. Acad. Sci. 84: 9265-9269. 5.-Koesling D, Schultz G and Bohme E (1991) FEBS Lett. 280: 310-316. 6.-Koesling D, Bohme E and Schultz G (1991) FASEB J. 5: 2785-2791. 7.-Koesling D and Friebe A (1999) Rev. Physiol. Biochem. Pharmacol. 135: 41-65. 8.-Andreopoulos S and Papapetropoulos A (2000) Gen. Pharmacol. 34: 147-157. 9.-Ferrero R, Miras-Portugal M.T and Torres M (2000) J. Neurochem. 75: 2029-2039.

Title: Cytoplasmic location of hepatitis B virus X protein is essential for the transcriptional activation of the inducible nitric oxide synthase gene. Role of nuclear factor κB family proteins.

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Nitric oxide appears to play a central role in the pathogenesis of many inflammatory disorders. An enhanced intrahepatic expression of the inducible nitric oxide synthase (iNOS) gene exists during chronic hepatitis B virus infection, and we have shown that viral X protein transcriptionally activates this cellular gene, but the basis for this activity remains to be defined. We aimed to explore the involvement of different nuclear factor κB proteins in the X-mediated iNOS promoter upregulation as well as the effect of the intracellular distribution of the X protein on its transactivational capability. Cotransfecting human hepatocyte-derived cells with wild-type or mutated iNOS promoter and with the X expression vector, we found that the functional inactivation of the proximal nuclear factor κB-binding site at the iNOS promoter markedly reduced the X-mediated transcriptional activity. Using mobility shift assays, we found an increased amount of κB proteins in the nuclear extracts from X-transfected cells, forming DNA-protein complexes comprised mainly by p50 and p65. Indirect immunofluorescence experiments on X-transfected liver cells showed that cytoplasmic location of viral protein stimulates the iNOS promoter, but when X protein is relocated exclusively to the nucleus, it no longer activates the mentioned promoter. In conclusion, cytoplasmic location of X protein is essential for the transcriptional activation of the iNOS gene through the nuclear translocation of p50-p65 heterodimers.

Post-transcriptional regulation of heme oxygenase-1 by nitric oxide

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Heme-oxygenase-1 (HO-1) catalyzes heme degradation, and is considered one of the most sensitive indicators of cellular stress. HO-1 expression is induced by NO and its activity is required for the adaptive resistance of neurons to NO (Bishop and Demple, 1999). HO-1 mRNA levels are also increased in response to NO in human fibroblasts, and transcriptional induction is only partially responsible. Instead, the HO-1 mRNA half-life is substantially increased (Marquis and Demple, 1998; Bouton and Demple, 2000). We assessed HO-1 mRNA induction by chronic and burst NO exposures in various cell lines, and also observed the post-transcriptional regulation of HO-1 mRNA in NIH3T3 murine fibroblasts, in which chronic NO exposure increases the half-life of the HO-1 transcript from 3.5 h to >10 h. In characterizing the mechanism of HO-1 mRNA stabilization by NO, we found that HO-1 mRNA stabilization is not due to the use of an alternative transcriptional start site. Also, since deadenylation can be the rate-limiting step in mRNA degradation, we have set up an assay to assess the rate and extent of deadenylation of HO-1 mRNA. We have found that deadenylation precedes decay of HO-1 mRNA in both control and NO-treated cells. Treatment with NO results in *de novo* synthesis of fully polyadenylated HO-1 mRNA and a longer half-life of this population of mRNAs. Future work will be aimed at determining whether NO stabilizes HO-1 mRNA by decreasing its deadenylation rate and identifying the cis-regulatory elements within the HO-1 transcript that mediate its stabilization in response to NO.

References:

Bishop, A., Marquis, J.C., Cashman, N, R. and Demple, B. (1999). Adaptive resistance to nitric oxide in motor neurons. Free Radic. Biol. Med. 26: 978-986.

Bouton C. and Demple B. (2000). Nitric oxide-inducible expression of heme oxygenase-1 in human cells. J. Biol. Chem. 275: 32688-32693.

Marquis, J.C. and Demple, B. (1998). Complex genetic response of human cells to sublethal levels of pure nitric oxide. Cancer Res. 58:3435-3440.

Nitric oxide-induced apoptosis in chromaffin cells from adrenal medulla: Protective effects of NGF and insuline

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Nitric oxide (NO) donors and endogenous activators of nitric oxide synthase (NOS) (citokines and glutamatergic agonists) are able to induce apoptotic cellular death in bovine chromaffin cells. However, oxidative NO species (peroxynitrites) induce both a mixte necrotic and apoptotic cell death.

Apoptotic cell death-induced by NO and peroxynitrites is characterised by DNA fragmentation, formation of apoptotic bodies and activation of caspases 3 and 9. Moreover it is accompanied by a cell arrest in G_0G_1 phase and a decrease in cell number in G_2M and S phases of cell cycle.

During NO-induced apoptosis, an activation of NF-κB translocation into nucleus occurs, with a parallel increase in Iκ-Bα degradation. This fact seems to be a defence mechanism against cell death induction by NO since cell treatment with SN50, an inhibitor of NF-κB translocation, increases apoptosis induced by NO.

The growth factors NGF and insuline are able to rescue chromaffin cells from NO-induced apoptosis and to reverse cell cycle alterations induced by it. These effects seem to be carried out through NGF and insuline binding to specific receptors with intrinsic tyrosine-kinase activity and subsequent activation of signal transduction pathways of MAPK's and PI3K.

These results, taken together, indicate that bovine chromaffin cells constitute a good model to study the molecular mechanisms involved in the apoptotic cell death induced by NO and glutamate, both mediators in neurodegenerative processes in the brain, and to study the molecular mechanisms of neuroprotection.

Regulation of hepatic methionine adenosyltransferase by S-nitrosylation

Isabel Pérez-Mato

In the liver S-adenosylmethionine (AdoMet) serves as the methyl donor for many biological methylation reactions (such as DNA, proteins, phospholipids, adrenergic, dopaminergic and serotoninergic molecules) and provides the propylamine group for the synthesis of polyamines (1-3). AdoMet is synthesized from methionine and ATP by the enzyme methionine adenosyltransferase (MAT). There are two MAT genes; one is expressed only in the liver, and the other is expressed in extrahepatic tissues and fetal liver (2-4). Up to 85% of all methylation reactions and as much as 50% of methionine metabolism occurs in the liver (5), which agrees with this tissue having the highest specific activity of MAT (6). Moreover, in the liver the half-life of AdoMet is of only about 5 minutes (6).

Reduced levels of AdoMet and/or MAT activity, resulting in the abnormal metabolism of methionine, have been found in human cirrhosis, and in a variety of experimental models including liver injury induced by ethanol, CCl4 and galactosamine (3,7). The importance of this alteration in AdoMet synthesis in the pathogenesis of a variety of liver disorders is suggested by the finding that exogenous AdoMet administration protects from the hepatotoxic effect induced by a variety of agents, such as ethanol, CCl4, paracetamol, TNF and galactosamine (3,7). The cellular factors regulating hepatic AdoMet levels are beginning to be defined. One such factor is nitric oxide (NO). In previous studies we demonstrated that conditions that induce NO synthesis, such as septic shock and hypoxia, induce the inactivation of hepatic MAT without affecting the expression of the liver-specific MAT gene (8,9). Further, we have reported previously that incubation of rat hepatocytes with S-nitrosoglutathione monoethyl ester (EGSNO), a cell permeable NO donor, induces MAT inactivation (10). We have also shown that purified rat liver MAT is inactivated by incubation with NO donors (3-morpholinosydnonimine, S-nitroso-N-acetyl-penicillamine and S-nitrosoglutathione) (8,10). In addition, we have recently demonstrated that liver MAT is S-nitrosylated both in vitro and in vivo (10) and further, we have identified cysteine 121 as the site of molecular interaction of NO and liver MAT (8). Because these results indicate that NO, or related molecules, is a critical regulator of liver MAT activity, we were interested in whether AdoMet content in hepatocytes is regulated by NO.

There are few studies trying to identify the active site features that control protein S-nitrosylation. It has been recently proposed (11), that protein S-nitrosylation involves an acid-base catalyzed SNO/SH exchange reaction where the target cysteine residue is localized next to basic and acidic amino acids. We were interested in determining the structural factors that govern liver MAT S-nitrosylation and inactivation using liver recombinant enzyme and mutants of MAT where the acidic and basic amino acids in the vicinity of cysteine 121 were replaced by serine by site-directed mutagenesis. Replacement of the acidic (aspartate 355) or basic (arginine 357 and arginine 363) amino acids located in the vicinity of cysteine 121 by serine leads to a marked reduction in the ability of nitric oxide to S-nitrosylate and inactivate hepatic methionine adenosyltransferase. These results indicate that protein S-nitrosylation is regulated by the basic and acidic amino acids surrounding the target cysteine. Recognition of the topology involved in protein S-nitrosylation is likely to prove useful in identifying new targets of protein S-nitrosylation.

References:
1. Cantoni, G.L. (1975). Annu. Rev. Biochem. 44, 435-451. 2. Kotb, M. and Geller, A.M. (1993). Pharmacol. Ther. 59, 125-143 3. Mato, J.M., Alvarez, L., Ortiz, P. and Pajares, M.A. (1997). Pharmacol. Ther. 73, 1-16. 4. Kotb, M. Mudd, S.H., Mato, J.M., Geller. A.M., Kredich, N.M., Chou, J.Y. and Cantoni, G.L. (1997) Trends in Genetics. 13(2), 51-52. 5. Mudd, S.H. and Poole, J.R. (1975) Metabolism. 24, 721-735. 6. Finkelstein, J.D. (1990) J. Nutr. Biochem. 1, 228-237. 7. Mato. J.M., Alvarez, L., Corrales, F. and Pajares, M.A. (1994) in The Liver Biology and Pathobiology (Arias. I.M., Boyer, J.L., Fausto, N. and Schaachter, D.A., eds.), pp.461-470, Raven Press, New York. 8. Avila, M.A., Mingorance, J., Martínez-Chantar, M.L., Casado, M., Martín-Sanz, P., Boscá, L. and Mato, J.M. (1997) Hepatology. 25, 391-396. 9. Avila, M.A., Carretero, M.V., Rodríguez, E.N. and Mato, J.M. (1998) Gastroenterology. 114, 364-371. 10. Ruiz, F., Corrales, F.J., Miqueo, C. and Mato, J.M. (1998) Hepatology. 28, 1051-1057. 11. Stamler. J.S., Toone, E.J., Lipton, S.A. and Sucher, N.J. (1997) Neuron. 18, 691-696

Nitric oxide directly activates DNA methyltransferase and induces the silencing of CpG island-containing genes

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Methylation status of control regions in the genome plays a critical role in the regulation of gene expression. In susceptible genes containing 5´ CpG islands, cytosine methylation favors a repressive chromatin structure that prevents the binding of transcriptional activators of the promoter. DNA Methyltransferase (DNA MeTase) is the major DNA methylating enzyme that produces 5´methylcytosine. DNA MeTase is a large protein with an NH2-terminal putative regulatory domain comprising two thirds of the protein with eight cysteine residues, and a COOH-terminal catalytic domain with the adenosylmethionine binding region and a proline-cysteine catalytic center. The signals and mechanisms involved in the regulation of DNA MeTase activity are poorly known. Nitric oxide (NO) has been implicated in a wide range of biological functions, many of them are not readily explained by the well established effects of NO on soluble guanylyl cyclase. We have observed that in RIN cells IL-1β activates DNA MeTase and induces the silencing of several CpG island-containing genes (FMR1 and HPRT). These effects are due to the production of NO after the induction of iNOS by the interleukine, although they are not mediated by the activation of guanylyl cyclase since incubation with 2 mM dybutiryl cGMP did not modify the enzymatic activity neither gene expression. In Jurkat cells, NO-induced FMR-1 silencing and methylation of FMR-1 CpG island of the promoter. In adition, the effect of NO donors is directly exerted on a nuclear extract suggesting that S-nitrosylation of some of the cysteine residues present in the DNA MeTase may be involved in the activation by NO. The greater prevalence and reactivity of thiols over biological nucleophiles explain the propensity for S-nitrosothiol (RS-NO) formation. The activation of DNA MeTase by NO is strongly dependent on the concentration of dithiothreitol (DTT) in the reaction medium. The maximun activation of DNA MeTase by NO is observed at 0.2 mM DTT, although the basal activity of the enzyme increases with higher DTT concentrations. These results suggest that the integrity of the SH groups at the catalytic center, maintained by DTT, is necessary for the activity of the enzyme. However, activation by NO could be due to the reaction with SH groups of cysteines at the regulatory domain. Nitrosothiols are exceptionally labile and can be reduced by high concentration of DTT. We have also observed that the specific protease V8 activates the DNA MeTase and that this effect is lightly potentiated by NO. Besides the effect of IL-1β on the expression of FMR1 and HPRT genes in RIN cells, we have observed that NO donors induce the silencing of other CpG island-containing genes in different cells type. For instance, in Caco cells we have notice the silencing of E-cadherin after the exposure to NO donors. Thus, our data suggest that nitrosylation of DNA MeTase may be a general phenomenon to regulate gene expression.

Nitric oxide and dopamine differentiation

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The nitric oxide (NO) donor, S-nitroso-N-acetyl-D,L-penicillamine (SNAP), produced differentiation of human neuroblastoma NB69 cells to a dopamine phenotype, as shown by phase-contrast microscopy and tyrosine hydroxylase (TH) immunocytochemistry. NB69 cells were treated with 50 to 750 μM of SNAP in serum-free-defined medium for 24 h. SNAP treatment did not increase the number of necrotic or apoptotic cells. However, a decrease in the number of viable cells at 750 μM of SNAP was observed. In addition, a decrease in 3H-thymidine uptake was detected at the highest dose of SNAP. An increase in the antiapoptotic Bcl-2 and Bcl-xL protein levels and a decrease in the proapoptotic Bax and Bcl-xS protein levels were also detected by Western blot analysis after SNAP treatment. At low doses (50-125 μM), SNAP induced an increase in catecholamine levels, 3H-dopamine uptake, TH activity and monoamine metabolism, while a decrease in all these parameters was observed at high doses (250-750 μM). The TH protein content, analyzed by Western blot, remained unchanged in SNAP-treated cells throughout the range of doses studied, when compared with the control group. In addition, cGMP levels and nitrite concentration, measured in the supernatant of SNAP-treated cells, increased in a dose-dependent manner, as compared to control levels. The guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3a]quinoxaline-1-one (ODQ) did not revert the SNAP-induced effect on 3H-dopamine uptake to control values. These results suggest that NO, released from SNAP, induces differentiation of NB69 cells and regulates TH protein at the post-transcriptional level through a cGMP-independent mechanism.

S- and N-nitrosylation of nitric oxide synthases

Robin J. Rosenfeld

Nitric oxide (NO) is known to regulate blood pressure homeostasis, long-term memory, and the immune response via multiple mechanisms including binding to metal centers and S-nitrosylation of cysteine residues. The duality of cytotoxic and cytoprotective roles requires tight regulation of NO formation and release from nitric oxide synthase (NOS). Tetrahydrobiopterin (H4B), which is a redox active-cofactor and allosteric regulator of NOS, binds at the dimer interface adjacent to the active site and forms a radical during the NOS-catalyzed conversion of L-arginine to citrulline and NO. Here we report the atomic resolution structure of inducible NOS with N-nitrosylated H4B. N-NitrosoH4B was generated during the process of cocrystallization of the iNOS oxygenase domain with RSNOs suggesting a NOS-dependent reaction occured during crystallization and data collection. N-nitrosylation of H4B is an unprecedented biochemical reaction that may be fundamental to NO-release and H4B regeneration in the catalytic cycle of NOS. We have also studied the interaction of RSNO's with NOSs spectroscopically and measured the formation of S- and N-nitrosylated NOS during catalytic turnover.

A p38 mitogen-activated protein kinase-regulated phosphotyrosine-protein phosphatase acting on the epidermal growth factor (EGF) receptor and on NOIPP-58, a nitric oxide-induced 58 kDa phosphoprotein activated by EGF

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We have shown in previous works that the intrinsic tyrosine kinase activity of the human epidermal growth factor receptor (EGFR) overexpressed in murine EGFR-T17 fibroblasts is reversibly inhibited by nitric oxide in permeabilized and intact cells [1, 2]. Moreover, similar results were obtained using the receptor isolated from rat liver plasma membrane [3]. This inhibitory process is mediated by the S-nitrosylation of the receptor, and correlates well with the inhibition exerted by nitric oxide on EGF-mediated cell proliferation [1, 2]. Thereafter, we demonstrated in intact cells that the tyrosine kinase activity of the EGFR overexpressed in A431 tumor cells from a human epidermoide carcinoma is also inhibited by nitric oxide. Moreover, and in contrast with the results obtained in EGFR-T17 fibroblasts, the treatment of these tumor cells with nitric oxide also induces the phosphorylation in tyrosine residues of a 58 kDa protein when the cells were treated with EGF but not in its absence. We denoted this protein NOIPP-58 for Nitric Oxide-Induced 58 kDa Phospho-Protein [4].

Now we demonstrate in A431 tumor cells that in addition to the effects of nitric oxide on the EGFR tyrosine kinase activity and on the phosphorylation of NOIPP-58, this agent also activates the p38 mitogen-activated protein kinase (p38MAPK) without affecting the p42/p44MAPKs pathway. The nitric oxide-induced activation of the p38MAPK was also observed in different fibroblast cell lines such as EGFR-T17, N7xHERc, Swiss-3T3, NIH-3T3 and clone 2.2. Most interesting, the p38MAPK appears to regulate a phosphotyrosine-protein phosphatase (PY-PP) that dephosphorylates both the EGFR and NOIPP-58. Thus, we have found that the shutdown of the p38MAPK pathway by selective inhibitors such as SB202190 and SB203580 results in the dephosphorylation of the EGFR and NOIPP-58, processes that are prevented by PY-PP inhibitors, such as vanadate and most efficiently by peroxovanadate, a readily cell permeant agent. These dephosphorylations supposedly result in the inactivation of the receptor and its putative substrate NOIPP-58. Therefore, we conclude that the activation of the p38MAPK pathway induced by nitric oxide could be a defense mechanism to maintain, among other functions, the EGFR and its signaling capacity partially active under nitrosative stress.

References:

1. Villalobo, A., Gómez, C., and Estrada, C. (1996) In: The Biology of Nitric Oxide part 5. (Moncada, S., Stamler, J., Gross, S., and Higgs, E. A. Eds.) p. 130. Portland Press Ltd., London.
2. Estrada, C., Gómez, C., Martín-Nieto, J., De Frutos, T., Jiménez, A., and Villalobo, A. (1997) Biochem. J. 326, 369-376.
3. Villalobo, A., Gómez, C., De Frutos, T., Siedenberg, M., Martín-Nieto, J., and Estrada, C. (1997) FEBS Special Meeting on Cell Signalling Mechanisms. Amsterdam, Abstract P4-092.
4. Hernández-Hernando, S., Ruano, M. J., Estrada, C., and Villalobo, A. (2000) In: The biology of nitric oxide, Part 7 (Moncada, S., Gustafsson, L., Wiklund, P., and Higgs, E. A. Eds.) p. 134. Portland Press Ltd., London.

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The lectures summarized in this publication were presented by their authors at a workshop held on the 7th through the 9th of May, 2001, at the Instituto Juan March.

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