Instituto Juan March de Estudios e Investigaciones

84

CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Nitric Oxide: From Discovery to the Clinic

Organized by

S. Moncada and S. Lamas

J. S. Beckman
T. R. Billiar
L. Boscá
G. Chaudhuri
J. V. Esplugues
C. Estrada
A. García Sánchez
J. Garthwaite
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T. Michel
S. Moncada
C. Nathan
D. Pérez-Sala
P. Vallance
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Introduction

S. Lamas and S. Moncada

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Nitric oxide, discovered in biological systems a little over a decade ago, continues to arouse intense interest among the community of life scientists. Not only can this biological mediator convey crucial signals which result in a wide spectrum of effects (vasodilatation, modulation of neurotransmission, host defense in the immune response), but also it is now clear that it may act as an important regulator of general cellular processes such as gene expression and mitochondrial function.

NO is synthesized from the amino acid L-arginine through the action of a family of enzymes termed nitric oxide synthases (NOSs). Three main isoforms have been identified, two constitutive (nNOS and eNOS) and one inducible (iNOS), all sharing common functional and structural features. Two main domains may be distinguished in the NOSs: an N-terminal oxygenase domain and a reductase domain, the latter highly resembling cytochrome P-450 reductase. The three-dimensional structure of the oxygenase domain from two of these isoforms (iNOS and eNOS) has recently been determined, providing important insight into structure-function relationships of the dimeric proteins and revealing the potential interaction between substrate and allosteric modulators within the oxygenase domain. Each isoform has a particular mode of regulation: while iNOS is mainly regulated at the level of transcription, nNOS and eNOS are regulated through posttranslational modifications and protein-protein interactions. In endothelial cells as well as in cardiac myocytes, eNOS myristoylation and palmitoylation serve to target the enzyme to the particulate subcellular fraction, where eNOS is localized in plasmalemmal caveolae. These are specialized cholesterol-rich invaginations of the plasma membrane, which associate with several signalling proteins. A structural protein of caveolae, caveolin, inhibits eNOS enzyme activity, and the inhibitory eNOS-caveolin complex is reversibly disrupted by calcium-calmodulin in a regulatory cycle modulated by G protein-coupled receptors.

The so-called "constitutive" enzymes, eNOS and nNOS, can be transcriptionally or posttranscriptionally regulated by a variety of stimuli. Hypoxia, shear-stress and estrogens are among the stimuli capable of modulating eNOS mRNA and/or protein levels. Cyclosporin A (CsA), a calcineurin-inhibiting immunosuppresor, upregulates eNOS mRNA expression in endothelial cells by transcriptionally-dependent mechanisms. The protective role of NO against atherosclerosis can be improved by estradiol which, at physiological concentrations, increases eNOS expression *in vivo*.

Pharmacological approaches to the study of the functions of NO have classically used NOS inhibitors, as well as NO donors. In recent years, alternative approaches are emerging which make use of genetic tools. Knockout mice for nNOS have unveiled the participation of this enzyme in the development of neurotoxicity after cerebral ischemia and in the motility of the gastrointestinal tract. Studies in eNOS knockout mice are consistent with a protective role of eNOS against increased intimal proliferation following vessel injury. This has significant implications in atherosclerosis and makes eNOS an important target for gene therapy to restore endothelial generation of NO.

NO has long been recognized as a modulator of the immune response as it may behave as a regulator of the balance between Th1 and Th2 responses in the immune system. In iNOS-deficient mice there is a predominance of the Th1 response, as evidenced by an increased production of IL-12 and IFN-γ and decreased IL-4. In contrast, high amounts of NO down-regulate the generation of IL-12, thus reducing the Th1 response. The role of NO on gene expression in cells treated with pro-inflammatory stimuli appears to be controversial, since both positive and negative effects of NO have been encountered. Nitric oxide has been reported either to protect cells from apoptosis or to cause apoptotic

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cell death depending on the target cells, the local concentration, and the relative amounts of other reactive species.

In the nervous system, NO has been implicated in numerous phenomena ranging from the acute regulation of neural function to the long term regulation of synaptic efficacy. Clearly cGMP is an important mediator of NO actions in the nervous system. However, after prolonged NMDA receptor stimulation, NO may be produced in excess and become toxic to neurons through mechanisms that appear to be cGMP-independent. NO may play also a role in ischemia-reperfusion neurotoxicity. Nitric oxide may modulate sensory-motor processing such as eye movement and also play important roles in the regulation of gastric function by acting on the central nervous system.

When analyzing studies with NO donors several factors should be considered. Among the most critical are the diversity of the pharmacological tools employed as donors of NO and the highly variable rate of NO release by these compounds. Besides, it is possible that, in addition to NO, some of these substances favour the synthesis of other reactive intermediates. Among these is peroxynitrite ($ONOO^-$), a highly toxic and reactive radical, which interferes with signal transduction pathways and structural proteins by promoting the nitration of tyrosine residues. Genes conferring resistance to reactive nitrogen intermediates (RNI) have been recently characterized in *M.tuberculosis* and other species. One of these, encoding for alkylhydroperoxide reductase subunit C, seems to be the most widely distributed gene protecting cells directly from RNI and provides the first known enzymatic defense against an effective element of anti-tubercular immunity.

Long term exposure to NO irreversibly inhibits mitochondrial complex I under conditions of low glutathione concentration. This inhibition may result from S-nitrosylation of critical thiols in the enzyme complex since it can be reversed by exposing cells to high intensity light or by replenishment of intracellular reduced glutathione. Thus, although NO may regulate cell respiration physiologically by its action on complex IV, prolonged exposure to NO leads to persistent inhibition of complex I and potentially to cell toxicity.

Important progress has been made in the area of NO and human pathophysiology. Reduced NO-mediated dilatation has been reported in patients with cardiovascular disorders including hypertension, diabetes and hypercholesterolemia. Although levels of NO are increased in hypotensive patients with septic shock and a NOS inhibitor, L-NMMA, may restore blood pressure, the mechanisms by which infection might alter NO generation and vascular response in humans are complex and await further research. NO has been shown to inhibit cell proliferation. However, the mechanisms by which this effect takes place are still obscure and seem to be cGMP-independent, at least in part. N-hydroxyarginine , an intermediate in the NO synthesis pathway, might account for the antitumoral effects observed in a human colon carcinoma cell line. It is also possible that the inhibitory actions of NO on cell proliferation are mediated by the inhibition of ornithine decarboxylase.

NO is progressively being incorporated as a therapeutic tool. Inhaled NO is an effective therapy in neonates with pulmonary hypertension and patients with acute respiratory distress syndrome. Very recently, and through yet undetermined mechanisms, NO has been shown to improve sickling crises in patients with sickle cell anemia.

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In summary, NO continues to be a fascinating biological mediator for which new biochemical roles, physiological functions and therapeutic applications are being unravelled day by day. This workshop showed that standardization of experimental design and of the use of NO donors as well as criteria for their employment are now necessary for valid interpretation of the huge amount of literature published in this field. As usual, The Centre for International Workshops in Biology at the Fundación Juan March provided the perfect environment to foster scientific discussion, exchange of ideas and social interaction.

Santiago Lamas and Salvador Moncada

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Session 1: General Overview

Chair: Santiago Lamas

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DISCOVERY OF THE L-ARGININE-NO PATHWAY: BIOLOGICAL IMPLICATIONS

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The discovery in 1987 that endothelial cells release nitric oxide (NO) and the subsequent identification of its generation from the amino acid L-arginine revealed the existence of a ubiquitous biochemical pathway. NO is formed by a family of enzymes, the NO synthases, and plays a role in many physiological functions. Its formation in vascular endothelial cells maintains a vasodilator tone that is essential for the regulation of blood flow and pressure. NO produced by the endothelium and/or platelets also inhibits platelet aggregation and adhesion, inhibits leukocyte adhesion and modulates smooth muscle cell proliferation. Thus NO plays a role as a homeostatic regulator of vessel wall functions and a decrease in its synthesis or actions contributes to the development of some vascular pathologies (1).

NO is also synthesized in neurons of the central nervous system, where it acts as a neuromediator with several physiological functions, including the formation of memory, coordination between neuronal activity and blood flow, and modulation of pain (2). In the peripheral nervous system, NO is now known to be the mediator released by a widespread network of nerves, previously recognized as nonadrenergic and noncholinergic. These nerves mediate some forms of neurogenic vasodilation and regulate certain gastrointestinal, respiratory and genitourinary functions. All these physiological actions of NO are mediated by activation of the soluble guanylate cyclase and consequent increase in the concentration of cyclic guanosine monophosphate in target cells.

In addition, NO is generated in large quantities during host defense and immunological reactions (3). Such generation of NO was first observed in activated

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macrophages, where it contributes to their cytotoxicity against tumor cells, bacteria, viruses and other invading microorganisms. When NO is released in this way it contributes to the development of certain pathologies, including septic shock and some forms of acute and chronic inflammation. Thus NO is a physiological mediator which, when released in large quantities for long periods, acts as a defense mechanism and may be involved in pathophysiology including tissue damage.

One of the ways in which NO may be transformed from a physiological mediator to a pathophysiological entity may be through the actions it has on mitochondrial function. It has been established recently in our laboratory that at low physiological concentrations NO inhibits cytokine c oxidase in a reversible manner which is competitive with oxygen. At higher concentrations it persistently inhibits other enzymes in the respiratory cycle, either directly or through the interactions with superoxide anion leading to the generation of peroxynitrite (4). The way in which these interactions take place will be discussed in detail.

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Session 2: NO Synthases: Structure-function relationships

Chair: Foo Y. Liew

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Unique Structural Aspects of Nitric Oxide Synthases Which Determine Functionality

*Masters, B.S.S., *Martasek, P., *Roman, L.J., *Miller, R.T., *Nishimura, J.S., †Sessa, W.C., ‡Gross, S.S., §Raman, C.S., §Li, H., and §Poulos, T.J.
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The isoforms of nitric oxide synthase are known as neuronal (nNOS), inducible (iNOS), and endothelial (eNOS) and are encoded by distinct genes. Not only do they differ in molecular weight, with the nNOS being the largest, but the functions of their common product, NO•, also differ. Since all three isoforms utilize L-arginine (L-Arg) to produce L-citrulline (L-Cit) and NO•, understanding those properties which determine the localization and function of these enzymes is important. We have expressed large quantities of all three isoforms in *E. coli* in order to study their structure-function relationships. The production of modules containing only heme, flavin, or binding site(s) for tetrahydrobiopterin (BH_4) and/or other cofactors has permitted the localization of their binding, determination of spectroscopic and binding parameters, and effects of cofactors on O_2 reduction. Characterization of the cysteine331alanine (C331A) mutant of nNOS has revealed a protein with altered binding characteristics for L-Arg and BH_4. This mutant and a flavoprotein construct derived from nNOS showed that the flavoprotein domain is necessary and sufficient for superoxide anion production from this isoform. The inactive, isolated C331A nNOS mutant, after overnight incubation with high concentrations of L-Arg and BH_4, is converted to a fully active enzyme with a turnover number equal to that of the wild type enzyme. A calmodulin-minus mutant of eNOS has been expressed and both intact and heme domain constructs have been purified in large quantities. These and other mutants and modules have been examined by crystallographic methods leading to high resolution structural information regarding the binding sites for the various cofactors bound in the oxygenase domain. [Supported by NIH Grants GM52419 and HL30050 and Robert A. Welch Foundation grant AQ-1192 to BSSM.]

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Binding properties of neuronal Nitric Oxide Synthase to the dynein light chain PIN. Ignacio Rodríguez-Crespo & Paul R. Ortiz de Montellano#. Departamento de Bioquímica y Biología Molecular I. Facultad de Ciencias Químicas. Universidad Complutense de Madrid y # Dept. Pharmaceutical Chemistry. University of California. San Francisco, USA.

PIN (*P*rotein *I*nhibitor of *n*NOS) is a small polypeptide of 89 amino acids that was initially fished using the yeast two-hybrid system, and hence defined as an interacting protein of the neuronal nitric oxide synthase (Jaffrey, S. R. & Snyder, S. H. *Science* 1996, **274**, 774-778). In this first report, PIN inhibited nNOS selectively, being unable to bind or interact with neither the endothelial nor inducible isoforms. Since nNOS is only active in its dimeric form, PIN inhibition was achieved by promoting the monomerization of the enzyme. The effect of PIN on nNOS could not be reversed neither by L-Arg nor by tetrahydrobiopterin (BH_4).

Subsequently, PIN was identified as a dynein light chain (King *et al.*, 1996, JBC, **271**, 19358-19366), opening the possibility of PIN acting as a transport protein of nNOS along the axon. In order to characterize the interaction between PIN and nNOS we have synthesized the PIN cDNA by recursive PCR optimizing at the same time the codon usage, expressing it afterwards in a pET vector in *Escherichia coli*. We have also expressed in *E. coli* the nNOS fragment known to bind to PIN (residues 163-245). We have studied the interaction between PIN and full-length nNOS as well as the interaction between PIN and this fragment of nNOS that was proposed to be the binding site for PIN according to the yeast two-hybrid system.

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Genes From Bacterial Pathogens that Confer Resistance to Reactive Nitrogen and Oxygen Intermediates. Carl Nathan, M. D. Cornell University Medical College, New York, NY 10021.

Mycobacterium tuberculosis (Mtb) is the single leading cause of death from infectious disease. The high-output pathway of NO production is an indispensable element of host defense against tuberculosis (TB) in mice. The catalyst of this pathway, inducible NO synthase (iNOS), is also present in alveolar macrophages from people with TB and can control mycobacterial replication in human alveolar macrophages. These findings direct our attention to genes in Mtb that may confer resistance to reactive nitrogen intermediates (RNI) alone or along with reactive oxygen intermediates (ROI). Three such genes have been cloned, *NOXR* (nitrogen oxides and oxygen intermediates resistance)-*1*, *-2* and *-3*. All confer protection against S-nitrosoglutathione (GSNO) and H_2O_2, and two confer protection against acidified nitrite, upon expression in Salmonella typhimurium (Sty), E. coli and/or Mycobacterium smegmatis (Msm). NOXR1 is a novel 15.5-kDa protein containing 4 Cys, encoded by a gene apparently confined to members of the Mtb complex. Expressed in Msm, NOXR1 confers resistance to killing by macrophages from mice that are wild type, deficient in iNOS, or deficient in the respiratory burst oxidase[1]. NOXR2 is homologous to subunit C of a two-component enzyme, alkylhydroperoxide reductase. Sty disrupted in *ahpC* become hypersusceptible to GSNO and acidified nitrite as well as alkylperoxides. *ahpC* from either Mtb or Sty fully complements the defect in resistance to RNI, but Mtb *ahpC* only partially complements the defect in resistance to ROI. Unlike protection against ROI, protection afforded by AhpC against RNI is independent of AhpF, the flavoprotein subunit of alkylhydroperoxide reductase. Expressed in human cells, Mtb *ahpC* protects against necrosis and apoptosis caused by RNI delivered exogenously or produced endogenously by transfected iNOS. Resistance to RNI appears to be a physiologic function of *ahpC*. *ahpC* appears to be the most widely distributed gene known that protects cells directly from RNI, and provides the first known enzymatic defense against an effective element of anti-tubercular immunity[2]. NOXR3 is a small, novel protein encoded by a sequence so far found only in the Mtb database. NOXR3 protects Sty from GSNO and H_2O_2 but minimally from acidified nitrite. This suggests that acidified nitrite has bactericidal actions independent of its formation of GSNO, a point reinforced by the absence of glutathione from mycobacteria, which are susceptible to acidified nitrite[3]. These findings have several implications: Mtb whose NOXRs have been inhibited may be more readily killed when exposed to RNI or ROI generated by the host's immune system or delivered as therapeutics. Divergent fates of mammalian cells exposed to RNI/ROI may reflect differential expression of endogenous genes homologous to NOXRs, such as peroxiredoxins, whose inhibition or induction may permit regulation of apoptosis and inflammation.

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Regulation and Function of eNOS

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Blood pressure homeostasis and platelet aggregation are influenced by nitric oxide (NO) generated by the endothelial isoform of NO synthase (eNOS) expressed in the vascular endothelium. eNOS is also expressed in cardiac myocytes, and importantly modulates the hormonal regulation of myocardial function. eNOS, a calcium-calmodulin dependent enzyme, is activated by a variety of G-protein coupled cell surface receptors and by hemodynamic shear stress. The eNOS protein undergoes a complex series of covalent modifications, including N-terminal myristoylation, reversible thiopalmitoylation, and phosphorylation[1]. In endothelial cells as well as in cardiac myocytes, eNOS myristoylation and palmitoylation serve to target the enzyme to the particulate subcellular fraction, where eNOS is localized in plasmalemmal caveolae. Cellular imaging studies reveal that eNOS undergoes dynamic agonist-dependent translocation from plasmalemmal caveolae to intracellular sites in response to receptor activation. Caveolae are specialized cholesterol-rich invaginations of the plasma membrane, and may serve as sites for the sequestration of diverse signaling proteins, including receptors, G-proteins, and protein kinases. The distinct lipid composition of caveolae provides a potential point for the concerted derangement of vascular signaling by alterations in cellular lipids. eNOS undergoes dynamically regulated interactions with the essential structural protein of caveolae, termed caveolin; the fact that distinct caveolin isoforms are expressed in cardiac myocytes and endothelial cells provides another potential point of regulation. Caveolin inhibits eNOS enzyme activity, and the inhibitory eNOS-caveolin complex is reversibly disrupted by calcium-calmodulin in a regulatory cycle modulated by G protein-coupled receptors[2]. The diverse biological roles subserved by eNOS expressed in different tissues likely reflect important differences in cell-specific regulation of this key signaling protein, mediated, at least in part, by cell-specific protein-protein interactions.

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Modulation of the eNOS-caveolin interaction in cardiac myocytes: implications for the autonomic regulation of heart rate.

Feron O.*, Dessy C.*, Opel D., Arstall M, Kelly RA. & Michel T. *Laboratory of Pharmacotherapy, School of Medicine, University of Louvain, Brusssels, Belgium and Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, USA.

The endothelial isoform of nitric oxide synthase (eNOS) was originally isolated and characterized in vascular endothelium but is now known to be expressed in numerous cell types including cardiac myocytes. eNOS is an enzyme acylated by the fatty acids myristate and palmitate and is thereby targeted to plasmalemmal signal-transducing microdomains termed caveolae, where the enzyme is quantitatively associated with caveolin, the structural protein of caveolae. In these studies, we have characterized myocytes isolated from gene-targeted homozygous "knock-out" mice in which the eNOS gene had been inactivated. Neonatal cardiac myocytes from these eNOS-deficient mice were transfected with cDNA constructs encoding wild-type (WT) eNOS or a myristoylation-deficient (myr$^-$) eNOS mutant. Following transfection of these cardiac myocytes, we found that the WT eNOS was targeted to plasmalemmal caveolae, but that the myr$^-$ protein was expressed exclusively in the cytosolic compartment. We next isolated the transfected myocytes from non-transfected cells using a magnetic bead selection system; these isolated cells started to beat spontaneously ~24 h after selection. In myocytes expressing the transfected WT eNOS, we found that the muscarinic cholinergic agonist carbachol evoked a marked negative chronotropic effect, accompanied by a 4-fold elevation in the myocyte cGMP level; these effects of carbachol parallel those seen in cardiac myocytes isolated from wild-type mice (which express eNOS endogenously). By contrast, after transfection of the myr$^-$ eNOS mutant into the eNOS "knock-out" cardiac myocytes, carbachol failed to exert any negative chronotropic effect whatsoever; importantly, there was also no agonist-induced increase in myocyte cGMP levels. We have next used a reversible permeabilization protocol to load intact neonatal rat myocytes with a synthetic oligopeptide corresponding to the caveolin-3 scaffolding domain (Cav3). This peptide (10 μM) completely abrogated the carbachol-evoked decrease in spontaneous beating rate of isolated myocytes; a control scrambled peptide (Cav3X) did not significantly change the beating rate. Again there was a perfect correlation with cGMP measurements: Cav3 peptide completely blocked the carbachol-induced elevation of cGMP level but the control Cav3X did not alter the agonist-evoked increase of cGMP. Our results therefore confirm the obligatory role of eNOS in coupling muscarinic receptor activation to cGMP-dependent control of cardiac contraction, and demonstrate that the caveolin-3/eNOS interaction regulates the parasympathic control of heart rate, thereby documenting for the first time the key role of eNOS caveolar location and caveolin interactions in the modulation of myocyte function.

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Modulation of eNOS expression in endothelial dysfunction.

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In recent years endothelial dysfunction has evolved as a concept related to the abnormal paracrine regulation of vascular tone. Several pathological conditions including hypertension, atherosclerosis, diabetes, vasculitides, hemolytic-microangiopathic syndromes and sickle cell disease have been associated with this general perturbation. Although it is clear that many regulatory functions of the endothelium such as the metabolism of lipoproteins, thrombogenicity and adhesion are altered in these conditions, a common cornerstone in all of them is an abnormal capacity of vessels to adequately relax in response to endothelial-mediated agonists. Central to this observation is a dysfunction of the L-arginine-nitric oxide (NO)-cGMP pathway. The complexity of this system has fostered research uncovering alterations in almost every regulatory step of the pathway. Potential modifications in the expression, function or structure of endothelial nitric oxide synthase (eNOS) may lead to severe derangements in endothelial function. Initially considered a constitutive enzyme with complex posttranslational modifications, it is now clear that regulation of eNOS expression is affected by several experimental and pathological conditions. Hypoxia, shear-stress and estrogens are among stimuli capable of modulating eNOS mRNA and/or protein levels. In our laboratory we have studied the modulation of eNOS expression in endothelial cells in culture in response to two agents implied in endothelial dysfunction in *in vivo* models: oxidized LDL (oxLDL) and Cyclosporin A (CsA). oxLDL was able to induce a dose and time-dependent decrement of eNOS mRNA and protein levels. Of interest two HMG-CoA reductase inhibitors, Atorvastatin and Simvastatin, were able to reverse this effect. These drugs also decreased the expression of another powerful vascular constrictor, pre-proendothelin 1. These observations support the hypothesis derived from clinical studies regarding the capacity of these drugs to improve endothelial function, possibly with independence of their cholesterol-lowering action.

CsA and FK-506, two calcineurin inhibiting immunosuppressors, unexpectedly increased 2-3 fold eNOS mRNA and protein levels both in bovine and human endothelial cells. This effect takes place mainly at the level of transcription as revealed by studies with inhibitors of total RNA synthesis. Transfection experiments using luciferase reporter assays showed that CsA augments eNOS promoter activity 2-fold. Using two redox-sensitive fluorescent probes, DHR123 and H_2DCFDA, we observed that CsA increased the synthesis of reactive oxygen species (ROS). ROS generating systems (xanthine/xanthine oxidase and glucose oxidase) were able to mimic CsA-mediated changes on eNOS expression and two antioxidants (catalase and superoxide dismutase) but not PDTC blunted this effect. Electrophoretic mobility shift assays revealed that both CsA and glucose oxidase were able to increase AP-1 binding activity. Thus CsA may modulate eNOS expression at least in part through the synthesis of ROS and the redox-sensitive transcription factor AP-1. Current studies are devoted to understand the role of this factor in the upregulation of eNOS expression at the promoter level, as well as to dissect the signalling pathways triggered by calcineurin-sensitive transcription factors such as NF-AT in endothelial cells. Hence, regulation of eNOS expression by drugs may serve as a model to unveil the existence of complex signalling cascades, potentially related to the mechanisms underlying endothelial dysfunction.

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Session 3: Genetics and genetic models

Chair: Salvador Moncada

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Genetic analysis of NOS isoforms in physiology and pathology using NOS mutant animals

Paul Huang

A genetic approach, using mutant animals that lack the genes for nitric oxide synthases (NOS) or those that overexpress NOS isoforms, complements the pharmacologic approach of using NOS inhibitors. This talk will review how these NOS knockout and overexpressing mice have been useful to reveal important functions of NO in physiology and pathology.

Neuronal NOS knockout mice display grossly enlarged stomachs, and parallel the human disorder infantile pyloric stenosis. This phenotype underscores the importance of neuronal NO to gastrointestinal motility. The neuroanatomy of these animals is normal, demonstrating that nNOS is not required for grossly normal development of the nervous system, including cortical whisker barrels. The nNOS mutant mice are resistant to both focal and global cerebral ischemia, providing evidence that the nNOS isoform contributes to neurotoxicity following ischemia. They also demonstrate several important examples of physiologic compensation for absence of the nNOS gene, in their cerebral blood flow response to whisker stimulation, blood flow response to hypercapnia, and nociception.

Endothelial NOS knockout mice lack endothelium-derived relaxing factor (EDRF) activity, proving that the eNOS gene is required to generate EDRF. The eNOS mutant mice are hypertensive compared to wild-type animals. In studies of cerebral ischemia, eNOS mutant mice develop larger infarcts and more severe neurologic deficits than wild-type animals, indicating that preservation of eNOS activity is important to maintain blood flow in borderline ischemic zones. The eNOS mutant mice develop increased intimal proliferation following vessel injury, suggesting that eNOS normally suppresses such responses. These findings have implications for patients at risk for atherosclerotic disease, and form the basis for attempts to restore endothelial function and NO production by dietary supplementation with arginine, hormonal manipulation, or gene therapy. eNOS mutant mice also display increased inotropic responses to adrenergic agonists, consistent with a role for NO in modulating these responses.

eNOS and nNOS mutant mice respond differently to stimulation of neurotransmitter release in microdialysis studies, indicating the separate populations of neurons may use each NOS isoform to mediate transmitter release. Combined mutant animals that lack both nNOS and eNOS display abnormalities in long-term potentiation, a model for learning and memory. The mutant animals clarify some aspects of NOS isoform substitution and localization in long-term potentiation.

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MODULATORY ROLES OF TETRAHYDROBIOPTERIN AND NITRIC OXIDE ON GENE EXPRESSION IN MESANGIAL AND ENDOTHELIAL CELLS

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Nitric oxide synthase (NOS) enzymes require several cofactors for catalytic activity. The role of the pterin cofactor, tetrahydrobiopterin (BH_4), has been elusive for some time. It seems now clear that BH_4 plays a crucial role in the interaction between NOS subunits and in the formation of the active site. The inducible isoform of NOS (iNOS) is expressed in response to pro-inflammatory stimuli in many cell types, including macrophages, hepatocytes, and smooth muscle and mesangial cells. The expression of iNOS and of BH_4 biosynthetic enzymes appears to be regulated coordinately. This ensures an adequate supply of the pterin cofactor for the newly synthesized NOS enzyme. We have observed that the biosynthesis of BH_4 is a limiting process for cytokine-induced NO generation in human mesangial cells (HMC). In addition, BH_4 availability plays a modulatory role in iNOS mRNA and protein expression. Inhibition of *de novo* synthesis of BH_4 results in reduced iNOS expression in response to cytokine stimulation, while, increasing intracellular BH_4 levels by treatment with the BH_4 donor sepiapterin, clearly potentiates iNOS induction, in part, due to iNOS mRNA stabilization. The modulatory effect of BH_4 is not restricted to iNOS induction, since it also affects the expression of other pro-inflammatory proteins, such as the inducible cyclooxygenase (COX-2), which is involved in the overproduction of prostanoids associated with many inflammatory situations.

The mechanisms by which BH_4 exerts these effects may be multiple. BH_4 supplementation leads to improved generation of NO, which is in turn a powerful modulator of gene expression. It has been shown that NO is able to influence gene transcription and mRNA stability either positively or negatively depending on the gene and the cell type under study. We have observed that endogenous generation of NO during HMC activation is important for both iNOS and COX-2 expression. However, studies with NO donors have revealed that NO can also contribute to limit iNOS and COX-2 induction in HMC by negative feedback mechanisms which involve the expression of IκBα. In addition, BH_4 may modulate mesangial cell responses to pro-inflammatory stimuli by mechanisms apparently not related to its role as a cofactor for NOS. On one hand, BH_4 can modify the tyrosine phosphorylation of several cellular proteins, as an early event in mesangial cell activation. On the other hand, BH_4 can modulate the generation of reactive oxygen species (ROS) in mesangial or endothelial cells. Supplementing these cells with BH_4 or sepiapterin leads to a decrease in the detection of ROS in response to various stimuli, including cytokines and cyclosporine A. Beyond its role in the regulation of NOS catalytic activity, BH4 may have broader implications in gene expression, specially in pro-inflammatory contexts.

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Chair: Juan V. Esplugues

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Gene Therapy Using Human Inducible NO Synthase to Prevent Intimal Hyperplasia

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Nitric oxide (NO)has been shown to intimal hyperplasia in several models. In order to exploit the beneficial effects of NO to prevent restenosis, it is necessary to find a method to deliver NO to the site of vascular injury. Gene therapy using the human inducible NO synthase (iNOS) is a feasible approach. We have carried out experiments using an adenoviral vector carrying the humaniNOS cDNA (AdiNOS). AdiNOS transfer inhibited intimal hyperplasia in a rat carotid artery injury model as well as a pig artery of iliac artery injury. AdiNOS transfer also inhibited intimal hyperplasia associated with vascular allograft rejection. Mechanistic studies carried out using AdiNOS transfer into in vitro as well as in vivo indicate that NO suppresses endothelial cell apoptosis while simultaneously inhibiting smooth muscle cell proliferation. The inhibition of smooth muscle cell proliferation occurs inpart due to an upregulation of p21. Taken together, these results suggest that AdiNOS gene therapy will be an effective approach to inhibit vasoocclusive complications such as restenosis.

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GLYCOSYLATED OXYHAEMOGLOBIN CAN HAVE A ROLE IN HUMAN DIABETIC ENDOTHELIAL DYSFUNCTION

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Early glycosylation products such as glycohaemoglobin have recently arised as possible mediators of diabetic endothelial impairment. We have previously reported that human oxyhaemoglobin at nanomolar concentrations (physiological plasmatic content of free haemoglobin) inhibits nitric oxide (NO)-mediated responses in rat aortic segments only when glycosyated at pathologic range (Rodríguez-Mañas *et al.*, 1993). This effect was mediated by generation of superoxide anions (Angulo *et al.*, 1996). On the other hand, in streptozotocin-induced diabetic rats we have also demonstrated a close relationship between endothelial dysfunction and blood levels of glycosylated hemoglobin (HbA_{1c}) (Rodríguez-Mañas et al., 1998). The aim of this work is to determine whether these results may be extrapolated to humans.

In one protocol, branches of the human mesenteric arteries were obtained from abdominal fat of patients (n=19; aged 47.5±2.8 years; 46% females) suffering surgical intervention unrelated to diabetes, hypertension or vascular disease. Arterial segments were mounted in a myograph for reactivity analysis of microvessels. Cumulative vasodilatatory responses to bradykinin (BK; 0.01 to 3 μM) were tested in vessels precontracted with 35 mM K^+. Addition of 10 μM N^G-nitro-L-arginine metyl ester (L-NAME), an inhibitor of NO-synthase, markedly reduced relaxations evoked by BK (27.8±8.4% vs 92.0±1.2% of control maximal relaxation; n=4 and 5). In the same way, BK-induced responses were inhibited by 1 μM oxyhaemoglobin (43.7±6.2% vs 86.12% of control maximal relaxation; n=3 and 5). However, when physiological concentrations were employed (10 nM) non glycosylated human oxyhaemoglobin (HHb) did not modified relaxations to BK when compared to respective control curves. By contrast, 10 nM glycosylated human oxyhaemoglobin (GHHb) inhibited relaxations induced by BK when the percentage of glycosylation was 10% or higher (14%). (Table 1) Preincubation of vessels with 100 U/ml superoxide dismutase (SOD) prevented the effect induced by 10 nM 14% GHHb (Table 1).

Table 1. Effect of 10 nM non glycosylated (HHb) and glycosylated human oxyhaemoglobin (GHHb) at increasing percentages of glycosylation on pD2 values for bradykinin in human mesenteric microvessels. Effects of 100 U/ml superoxide dismutase (SOD).

	Control	10 nM Oxyhaemoglobin	14% GHHb +SOD
HHb	7.43±0.12 (6)	7.31±0.04 (7)	
GHHb 8%	7.40±0.09 (10)	7.33±0.09 (8)	
GHHb 10%	7.66±0.10 (9)	7.28±0.04* (8)	
GHHb 14%	7.48±0.07 (19)	7.02±0.08* (9)	7,39±0,16 (9)

pD2 indicates the -log M of the required concentration of bradykinin to reach the half-maximal relaxation obtained in control conditions. Number of segments is in parenthesis. * P< 0,01 vs respective control curve.

In a second protocol, vasorelaxant responses to methacholine (MCh; 1.3 to 10 $\mu g.min^{-1}$) were tested in the human forearm resistance vessels by venous occlusion plethysmography (Johnstone et al., 1993). Studies were performed in control normal subjects (n= 15), type I diabetic patients with good metabolic control (HbA_{1c} ≤ 7.5%; n= 7), and type I diabetic patients not well controlled (HbA_{1c} > 7.5%; n= 6) but without late diabetic complications. The vasoactive responses were compared by ANOVA for multiple comparisons. MCh produced a dose-dependent increase in forearm blood flow that was similar in control and well controlled diabetic patients but was significantly reduced in patients with a bad metabolic control (P=0.004 and 0.007 versus control and well contolled patients, respectively). After improvement of metabolic control with insulin during four to six weeks, lowering HbA_{1c} levels below 7.5%, maximal responses evoked by MCh significantly increased (P=0.04) and the differences versus the other groups disappeared.

The present results suggest, in human vessels, that only high glycosylated human oxyhaemoglobin impairs NO-mediated responses when tested at physiological plasmatic concentrations. This is consistent with the fact that endothelial dysfunction in diabetic patients is related to high blood levels of HbA_{1c}. In conclusion, we hypothesize that glycohaemoglobin, and perhaps other early glycosylation products, contributes to the development of vascular dysfunction in human diabetes.

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Session 4: NO in the nervous system

Chair: Louis J. Ignarro

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Role of NO in the CNS

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The CNS has a rich capacity to synthesise nitric oxide (NO), a common trigger for NO formation therein being activation of neuronal receptors for the major excitatory neurotransmitter, glutamate. Of particular significance is the NMDA class of glutamate receptor, whose associated ion channel permits substantial Ca^{2+} entry which, via binding to calmodulin, activates the neuronal NO synthase. Because of the high rate of diffusion of NO in both lipid and aqueous environment, a source is likely to influence neural function over a large tissue volume (equivalent to one containing 1-2 million synapses). Physiologically, NO has been implicated in numerous phenomena ranging from the acute regulation of neural function (e.g. by influencing neuronal excitability, neurotransmitter release, gap junction conductance) to the long-term regulation of synaptic efficacy. The best known signal transduction pathway for NO in target structures is stimulation of the soluble form of guanylyl cyclase (sGC) leading to cyclic GMP (cGMP) accumulation. The role of this pathway has now been investigated in several aspects of neural function in which NO has been implicated, including the modulation of neurotransmitter release and in different forms of synaptic plasticity. The results indicate that the sGC-cGMP pathway is likely to be a major physiological pathway for NO signal transduction. When produced in excess, for example following prolonged NMDA receptor stimulation as a result of impaired glutamate homeostasis, NO can become toxic to neurones (and possibly non-neuronal cells) through mechanisms that appear to be primarily cGMP-independent. In this way, NO is considered to be one of the mediators of the neurodegeneration taking place following cerebral ischaemia. Excess NO may also be generated in inflammatory conditions as a result of the expression of the inducible, Ca^{2+}-independent, isoform of NO synthase. This may contribute to a late phase of degeneration following cerebral ischaemia and to the functional and pathological changes occurring in disorders such as multiple sclerosis.

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MODULATION OF SENSORY-MOTOR PROCESSING BY NITRIC OXIDE: THE OCULOMOTOR SYSTEM MODEL.

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The posible participation of nitric oxide (NO) in the processing of specific sensory and/or motor signals has been tested using as a model the cat oculomotor system, due to its several advantages. First, horizontal eye movements are relatively simple, since they are performed by the conjugated action of only two muscles commanded from the abducens nucleus. Second, the abducens nucleus, as well as the related premotor areas in the brain stem are accessible to local injections and electrophysiological recordings. Third, horizontal eye movements can be accurately measured in alert animals both during spontaneous performance and during the response to vestibular stimulation (vestibulo-ocular reflex, VOR). In addition, immunocytochemical studies revealed the presence of nitrergic neurons densely grouped in the prepositus hypoglossi (PH) nucleus, a premotor nucleus involved in the control of horizontal eye movements.

To analyze the functional role of NO in the PH nucleus, adult cats were anesthetized and prepared for chronic recording of eye movements using the scleral search coil technique (Fuchs and Robinson, 1966) and for local injections and electrophysiological recording in the brain stem, according to the procedure described by Delgado-García et al. (1986). At least one week later, horizontal eye movements were recorded in the alert animal before and after injections of different drugs related with the NO-cGMP pathway in the PH nucleus.

Local inhibition of NOS by injections of either L-NAME or L-NMMA in one PH nucleus produced an immediate and long lasting nystagmus with slow phases in a direction contralateral to the injected side. The effect of L-NAME was stereospecific and reversed by local administration of L-arginine. Injections of L-arginine alone produced a mild nystagmus toward the ipsilateral side. A severe nystagmus toward the ipsilateral side was observed after injection of NO donors. When visual information was presented under light conditions, the nystagmus was considerably reduced.

The oculomotor response to vestibular stimulation was also altered when NO concentration in the PH nucleus was modified pharmacologically. A velocity imbalance was evident in all cases, directed either toward the contralateral side after NOS inhibitor injections or toward the ipsilateral side when L-Arginine or NO donors were administered.

These results indicate that a balanced NO production by PH neurons is necessary for the normal performance of spontaneous or reflex eye movements.

The PH nucleus send two kinds of signals to the abducens motoneurons: velocity signals during eye displacements and position signals during eye fixation periods. The current hypothesis is that position signals for any kind of eye movement result from the temporal integration (in the mathematical sense) of velocity signals and that this integration occurs, at least in part, in the PH

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nucleus. A failure in the processing of velocity signals result in nystagmus with linear slow phases during spontaneous eye movements and velocity imbalance during VOR. On the other hand, a failure in the velocity-to-position integrator produces an exponential decay after saccades and decreased reflex gain and increased phase lead during VOR.

Mathematical analysis of the eye movement recordings indicated that the alterations produced by NOS inhibitors or L-Arginine injections, this is, when endogenous NO concentration was changed, affected exclusively the processing of velocity signals without apparent modification of the velocity-to-position integrator function. However, administration of NO donors also altered the velocity-to-position integrator during spontaneous eye movements, although not during VOR. This finding suggest two interesting possibilities. First, the integrator mechanism may not be unique for all kinds of eye movements as it is currently considered. Second, NO donors affect targets that are not reached by the endogenous NO produced by local neurons; these targets might be part of a new velocity-to-position integrator for spontaneous eye movements.

In order to find the possible targets of NO, structures containing NO-sensitive guanylyl cyclase were identified according to the method described by Southam and Garthwaite (1993). This approach was based on the functional finding that the permeant cGMP analog 8-Br-cGMP produced the same effects as NO donors when injected in the PH nucleus, suggesting that the NO function was mediated by the activation of soluble guanylyl cyclase. Cats we perfused through the ascending aorta with SNP to maximally stimulate NO-sensitive soluble guanylyl cyclase, and then with 4 % paraformaldehyde as a fixative. Immunohistochemistry for cGMP was performed in brain stem sections containing the rostral part of the PH nucleus. Using this technique in combination with NOS immunohistochemistry, we found that, in the PH nucleus, NOS was present in a group of densely packed neurons, whereas cGMP was localized in a rich neuropil in the dorsal part of the nucleus, which is probably mediating the functional effects of endogenous NO. In addition, a cluster of NO-sensitive cGMP-producing neuronal cell bodies and neuropil were identified in an intermediate zone between the PH nucleus and the medial vestibular nucleus, far from the nitrergic neurons, but within the drug injection area. These cells could be the additional target for exogenous NO and, therefore, be responsible for the integration deficit induced by NO donors during spontaneous eye movements.

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TRANSPLANTATION OF FETAL NITRIC OXIDE SYNTHESIZING CELLS IN THE ADULT HIPPOCAMPAL FORMATION OF PRIMATES

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Fetal cells dissociated from basal forebrain tissue were stereotaxically injected into the hippocampal formation of mature Rhesus monkeys. Since the proliferation of neurons in the macaque monkey basal forebrain peaks around fetal day 45, we harvest tissue from brains at this optimal age of development. The host animals simultaneously underwent neurosurgical transection of the fornix, the major source of septohippocampal fibers, as described previously (Alonso and Amaral, 1995; Alonso et al., 1996). Following a nine month survival, grafted tissue was obvious at all eight injection locations. Although the grafted tissue was anatomically integrated within the host hippocampus, the border between the graft and the host was easy to determine since the packing density and morphology of neurons within the graft were distinctly different from the host. We observed NADPH-diaphorase positive neurons in the graft and they appeared morphologically similar to neurons observed in the basal forebrain of adult control animals. These results indicate that even though the fetal basal forebrain neurons were placed into the unusual environment of the host hippocampus, they developed into "basal forebrain" neurons rather than hippocampal neurons, i.e. their fate had apparently been determined prior to transplantation. Double labeling of the same sections with specific antibodies against neuronal nitric oxide synthase (NOS) demonstrated that these cells were NOS-immunoreactive. Stained neurons belong to type I and demonstrated dendrites with dendritic spines and varicosities. At the nine month survival period at which the transplants were evaluated, no mitotic figures were found, indicating that the grafts had already reached their maximal neuronal number. We observed examples of aberrant connections between the transplant and host tissue. The molecular layer of the dentate gyrus in the vicinity of the transplant, for example, demonstrated a dense plexus of NADPH-diaphorase positive fibers whereas the normal molecular layer receives a very meager innervation of NADPH-diaphorase positive fibers. We conclude that fetal basal forebrain tissue can be successfully transplanted to the mature monkey hippocampal formation. The dissociated fetal neurons undergo substantial proliferation and form aggregations of phenotypically heterogenous neurons reminiscent of the nuclei found in the basal forebrain The growth and successful integration of fetal tissue into the mature primate brain supports continued investigation of neural transplantation for the treatment of neurodegenerative illnesses and stroke.

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REGULATION OF THE NITRIC OXIDE/CYCLIC GMP SYSTEM IN ASTROGLIAL CELLS

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In the central nervous system (CNS) nitric oxide (NO) has been involved in brain development, synaptic plasticity, neuroendocrine secretion, sensory processing and cerebral blood flow. These actions appear to be mediated to a large extend by cyclic GMP (cGMP) increases resulting from NO stimulation of soluble guanylyl cyclase (sGC) in target cells (1). In the normal brain, NO is predominantly synthesised by the Ca^{2+}/calmodulin-dependent neuronal NO synthase or NO synthase type I (NOS-I), that has been localised in discrete populations of neurones of all brain regions. Although initial histochemical studies failed to demonstrate the presence of NOS-I in glial cells, using primary cultures enriched in astrocytes from different brain regions, we and others demonstrated that these cells can generate NO in response to agonists that increase intracellular Ca^{2+} (reviewed in 2). In cultures of cerebellar astrocytes, where the largest NO-dependent cyclic GMP accumulations are measured, we have observed this response upon stimulation of α_1-adrenergic receptors (3), glutamate Ca^{2+}-permeable AMPA receptors (4) and endothelin ET_A receptors. The NOS isoform constitutively expressed in these cells is recognised by specific anti-NOS-I antibodies and has biochemical characteristics similar to the cerebellar granule cell enzyme (5). However, differences in sensitivity to the aldehydes used as tissue fixatives and in the degree of association to membranes, suggest that the astroglial and neuronal enzymes may be different NOS-I variants. In agreement with our results, immunostaining for NOS-I has been demonstrated in hippocampal and cerebellar astrocytes and Bergmann glia in lightly fixed rat brain sections (6).

More recently, we have observed that Ca^{2+} has a double regulatory role on intracellular cGMP in CNS cells. Both in cerebellar astrocytes and granule cells in culture, the same agonists that stimulate NO-dependent cGMP formation decrease cGMP generation in response to direct activation of sGC by NO donors (7). This effect

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requires extracellular Ca^{2+} and is prevented by a calmodulin inhibitor. Decreases in cGMP are less pronounced if a phosphodiesterase (PDE) inhibitor is present indicating that agonists are activating cGMP degradation by a Ca^{2+}/calmodulin-dependent PDE. In fact, in cytosolic fractions from both cell types we found that the predominant hydrolysing activity at μM cGMP has the characteristics of a type I PDE, the Ca^{2+}/calmodulin-dependent family (8). This activity and NOS-I show the same EC_{50} for Ca^{2+} (5,8). These results indicate that cGMP rises will mainly occur in cell compartments different from those where NO˙ is generated in a Ca^{2+}-dependent manner. This may explain reports from immunohistochemical studies in cerebellar slices showing that stimulation of neuronal NO production by N-methyl-D-aspartate increases cGMP in astroglial cells more than in neurones, whereas NO donors produce more generalised increases in cGMP (9).

Astrocytes as well as microglia can express Ca^{2+}-independent inducible NOS or NOS type II (NOS-II) when treated with bacterial endotoxin or combinations of cytokines *in vitro* and under inflammatory conditions *in vivo* (2). Also in this case we have observed an inverse regulation of NO and cGMP formation by the same stimulus. Treatment of astroglial cells for more than three hours with bacterial lipopolysacharide (LPS) induces NOS-II and simultaneously down-regulates sGC with similar concentration-dependence. Both LPS effects require transcription and protein synthesis but appear to occur by independent mechanisms. Treatment of cells with the NOS inhibitor N^{w}-monomethyl-L-arginine or with agents that prevent NOS-II induction such as dexamethasone or the tyrosine kinase inhibitor genistein do not block the LPS-induced down-regulation of sGC, indicating that NO is not implicated. The pro-inflammatory cytokine interleukin-1ß (Il-1ß) also decreases sGC activity in astroglial cultures. Down-regulation of sGC could be a mechanism to prevent excess cGMP formation in astrocytes activated under inflammatory conditions when NO would be overproduced as a result of NOS-II induction.

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Postnatal modification of cerebral expression of nitric oxide synthase in rats subjected to intrauterine hypoxia before delivery

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The role of nitric oxide (NO) in hypoxic-ischaemic brain damage is controversial, as it may exert a dual role as a powerful dilator of cerebral blood vessels and as a mediator of tissue injury. Pregnant female Wistar rats were killed by decapitation just before delivery and kept at 37°C for 35 min to produce intrauterine hypoxia. The pups were then delivered surgically, revived by thoracic massage and placed with a wet-nurse rat during the postnatal period. Control pups were born naturally and kept with their own mothers. Control and hypoxic rats were anaesthetized and perfused transcardially with saline and fixative on postnatal days 0, 1, 2, 3, 4, 5, 7, 10, 15, 20 and 25 for immunocytochemical examination of their brains with polyclonal primary antibodies against neuronal (nNOS) and inducible (iNOS) isoforms of nitric oxide synthase and nitrotyrosine. With all three antibodies the experimental rats showed an increase in immunoreactive neurons in all cortical areas as compared with control rats on post natal days 0-4. At day 5 the number of immunoreactive neurons was similar in both groups, and thereafter declined in the experimental group, immunoreactive neurons being more numerous in the control animals. At day 7 immunoreactivity to iNOS and nitrotyrosine was absent from all cortical areas. The immunocytochemical data for nNOS immunoreactivity corresponded with parallel biochemical determinations of calcium-dependent NOS activity in brain tissue from non-perfused animals. The results suggest that hypoxia immediately before delivery is a potent determinant of increased neuronal expression of nNOS and iNOS during the early postnatal period, increasing the formation of nitrotyrosine in tissue proteins. At day 5 the expression of nNOS was similar in both groups because of an increased expression in the controls, which may be related to a role of NO in cortical lamination.

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Session 5: NO in the liver and gastrointestinal tract

Chair: John Garthwaite

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Nitric Oxide is a Potent Inhibitor of Apoptosis in the Liver

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We have previously shown that the inhibition of the inducible NO synthase (iNOS) results in an increase in apoptosis in the liver during endotoxemia. Furthermore, administration of a liver specific NO donor was found to almost completely inhibit the fulminant hepatic failure and apoptosis following TNF and galactosamine administration to rats. Experiments were carried out to determine the mechanism by which NO inhibits hepatocyte apoptosis. We initially focused our attention on the caspase cascade. Caspase-3-like activity is dramatically upregulated in hepatocyte stimulated to undergo apoptosis by exposure to TNF + actinomycin D. The increase in caspase-3-like activity was almost completely suppressed by exposure to NO donors, overexpression of iNOS, or induction of iNOS by cytokines. The inhibition of caspase activity could be partially reversed with DTT indicating that the suppression was due in part to S-nitrosylation of activated caspase. We also found that NO-induced cGMP elevations suppressed caspase-3-activation. Therefore, NO inhibits apoptosis in hepatocytes by two distinct mechanisms, 1) a cGMP-dependent, and 2) cGMP independent mechanism. It may be possible to take advantage of the antiapoptotic activity of NO to suppress liver damage seen as part of fulminant hepatic failure.

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NITRIC OXIDE IN THE GASTROINTESTINAL TRACT

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The widespread distribution of the different isoforms of nitric oxide synthase (NOS)) in the stomach indicates the important role that nitric oxide has in the physiology and pathophysiology of gastric function. This role has been evaluated by selective blockade of endogenous NO synthesis or by exogenous administration of NO donors. Different experimental studies support a crucial role for NO in the regulation of the resting mucosal microcirculation and an important interaction between endogenous NO, sensory neuropeptides and prostanoids in preserving the gastric mucosal integrity (1,2). Constitutive release of NO does not mediate basal and pentagastrin-stimulated gastric acid secretion; however, exogenous administration of low doses of NO donors decreases acid output induced by neuronal stimulus while does not modify acid responses induced by direct secretagogues (3).

Synthesis of NO plays also an important role in changes in gastric functions associated with pathophysiological circumstances. Thus, this mediator contributes to the gastric vasodilatation observed in cirrhosis, portal hypertension, and renal failure. For the last years, we have been studying the role of NO in the pathophysiology of gastric function under stress situations. Moderate somatic stress, induced by the acute administration of low doses of endotoxin, increases gastrointestinal transit and gastric mucosa resistance to damage (4, 5) and inhibits gastric acid secretion, through endogenous synthesis of NO (6). The doses of endotoxin used in these studies do not produce any significant fall in blood pressure, suggesting an action of endotoxin independent of any vascular changes. Inducible NOS does not seem to be involved in the acute inhibitory effects of endotoxin since they take place in less than 60 min and

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are not influenced by pretreatment with dexamethasone.

These acute inhibitory effects of endotoxin required the integrity of the nervous system and they seem to be mediated by a neuronal NOS isoform. Recent functional studies by specific administration of NOS inhibitors in the cisterna magna support that the activation of a common L-arginine:NO pathway in the central nervous system mediates changes in gastric functions induced by stress. Immunohistochemcial and functional studies attribute to the dorsal motor nucleus of the vagus the specific location in the brain where NO synthesis regulates gastric function (7).

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INDUCIBLE NITRIC OXIDE SYNTHASE EXPRESSION IN CHRONIC VIRAL HEPATITIS: EVIDENCE FOR A VIRUS-INDUCED GENE UP-REGULATION.

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Increased nitric oxide (NO) production may contribute to the pathological changes featuring some inflammatory diseases, but the role of NO in chronic viral hepatitis is still unknown. We compared the inducible nitric oxide synthase expression (NOS2) in the liver of patients with chronic viral hepatitis with that of both non-viral liver disease and histologically normal liver. NOS2 expression was assessed by immunohistochemical and in situ hybridization studies of liver biopsy sections. An intense hepatocellular NOS2 reactivity was detected in chronic viral hepatitis, whereas it was weakly or not observed in non-viral liver disease or normal liver, respectively.

In addition, we determined whether the hepatitis B virus (HBV) might regulate the synthesis of this enzyme. NOS2 mRNA and protein levels as well as enzyme activity were assessed in cytokine-stimulated HBV-transfected and untransfected hepatoma cells. Transfection with either HBV genome or HBV X gene resulted in induction of NOS2 mRNA expression, being the maximal induction of this transcript and NO production observed in cytokine-stimulated HBV-transfected cells. These results indicate that hepatotropic viral infections are able to up-regulate the NOS2 gene expression in human hepatocytes, suggesting that NO may mediate important pathogenic events in the course of chronic viral hepatitis.

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Session 6: NO and inflammation

Chair: Thomas Michel

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NO in immunology: regulatory role

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High concentrations of NO produced by inducible NO synthase (iNOS) has long been known to have cytotoxic effect. Thus NO donors (such as SNAP) have anti-T cell proliferative effect, whereas NOS inhibitors (such as L-NMMA) enhance T cell responses. Using a strain of iNOS-deficient mice, we have demonstrated that NO may have selective effect on Th1 subset of $CD4^+$ T cells. Mutant mice infected with virus, bacteria or parasites produced elevated concentrations of IFNγ but decreased levels of IL-4 compared with the control wild-type mice. This strongly suggests that NO may be required to regulate Th1 cell expansion which has been implicated in a range of autoimmue diseases. The mechanism for such selective effect of NO is unclear, but could be due to the preferential inhibition of the transcription of IL-12 gene in macrophages. IL-12 is a major driving cytokine for the differentiation of Th1 cells. We have recently found that while high doses of NO can block IL-12-driven Th1 cell differentiation, low doses of NO enhance Th1 cell development in vitro. The homeostatic effect of NO in immune responses and its implications in immunopathology will be discussed.

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MODULATION OF COX-2 EXPRESSION BY NO DONORS IN HUMAN MESANGIAL CELLS

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Nitric oxide (NO) can regulate cyclooxygenase (COX) activity and/or expression. It has been reported that NO has either positive or negative effects on prostaglandins formation and COX-2 expression depending on the experimental system under study.[1 2 3]

We have observed that NO exerts a dual effect on COX-2 expression in cytokine-stimulated human mesangial cells (HMC). NO amplifies IL-1β/TNF-α-induced COX-2 expression at early time-points (up to 4-8 h for mRNA and up to 16-24 h for protein expression), but displays inhibitory effects on COX-2 expression at later times of induction.

The study of the effect of sodium nitroprusside (SNP), one NO donor, on COX-2 protein expression induced by various stimuli shows that the amplifying effect of NO results greater when HMC are stimulated with IL-1β alone or with the combination of IL-1β/TNF-α. However, when COX-2 expression is fully induced with a combination of three stimulants (IL-1β/TNF-α/LPS) the potentiating effect of NO is no longer apparent.

Electrophoretic mobility shift assays show that at early times SNP increases IL-1β/TNF-α- induced activation of NF-κB while at later times, a moderate decrease in NF-κB activation is observed. These effects correlate well with opposite changes in IκBα protein levels. In cells supplemented with SNP, IκBα levels are lower at early times of induction and higher at later times compared to cells treated with cytokines alone. These changes in NF-κB activation might be implicated in the dual effect of SNP on COX-2 expression.

In addition, recent results suggest that in HMC, NO donors could modulate COX-2 expression through a cGMP-dependent pathway. The potential roles of guanylate cyclase activation, cGMP generation and AP-1 activation as mediators of the effect of NO donors are currently under study.

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Inducing Constitutive NO Synthases: Biological Significance

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There are various agents that can induce the constitutive NO synthases and the first evidence that this occurs came from studies related to estradiol. We had demonstrated that basal release of NO was greater with endothelium-intact aortic rings from female rabbits than those from males. Oophorectomy diminished both circulating estradiol concentration and basal NO release to levels seen in male rabbits. We also observed that estradiol replacement in oophorectomized animals increased NO release compared to placebo treated ovariectomized animals (1). We speculated that the increased release of NO by estradiol may offer an explanation for the protective effect of estradiol against the development of atherosclerosis. We however did not demonstrate the mechanism by which estradiol increased NO release (1).

It was work from Dr. Moncada's group (2) that provided the first evidence that estradiol increases both endothelial and neuronal nitric oxide synthase (NOS) in guinea-pigs due to an increase in the transcription of the endothelial and neuronal NO synthase gene. This work challenged the concept of classifying the NOS as "constitutive" and "inducible" as the so-called "constitutive" NOS like endothelial NOS (eNOS) and neuronal NOS (nNOS) dould actually be induced. Similarly, the "constitutive" enzyme could be down regulated in the absence of the stimulating agent.

The biological implications of the induction of the constitutive NO are numerous and are thought to be important in various systems. In the cardiovascular system, the shear stress leads to increase in eNOS which in turn allows the cardiovascular system to adjust to increased blood flow. It is now felt that nitric oxide produced by endothelial cells may protect against atherogenesis. Lipid peroxides upregulate endothelial nitric oxide synthase (3) and this may buffer the atherogenic action of lipid peroxides and thus upregulation of eNOS may provide some attenuation against the development of atherosclerosis. Similarly, the estradiol induced increase in eNOS expression may be partially responsible for the protective effect of estradiol against atherosclerosis. Recently, it has been demonstrated that in rabbits fed a cholesterol enriched diet, there was a significant reduction in the extent of atherosclerosis development if an estrogen was simultaneously administered and this protective effect of estrogen was significantly attenuated if ^{G}N-nitro-l-arginine methyl ester (L-NAME), an inhibitor of NOS, was also administered along with estradiol (4).

Estradiol has been shown to increase nNOS in the cerebellum and work from our laboratory recently indicated increased nNOS in the gastrointestinal tract of pregnant rats compared to non-pregnant controls. The increase in nNOS observed was due to an increase in gene expression modulated by estradiol. There was an increase in immunostaining of nNOS in the nerve cells and nerves of the myenteric plexus during pregnancy and following administration of estradiol (5). The increase in nNOS in nerves innervating the gastrointestinal tract during pregnancy suggests that many of the gastrointestinal effects in pregnancy like delayed gastric emptying and slowing of the gastrointestinal motility may be mediated by release of NO from the non-adrenergic non-cholinergic neurones innervating the gastrointestinal tract.

The effects of estradiol on NOS gene expression seen in various cell types may be mediated by increased gene expression via an action on estrogen receptors (2) as well as by its antioxidant action as antioxidants can also increase eNOS gene expression (6).

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Mechanisms of nitric oxide-dependent apoptosis: role of mitochondrial mediators

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Apoptosis or programmed cell death constitutes a general process of tissue remodelling, cell replacement and organ size control. Elucidation of the signals that turn on the apoptotic pathway constitutes an area of intense research and several mechanisms underlying in its activation have been identified (1,2).

In understanding such mechanisms of apoptosis, it has recently been established that mitochondrial dysfunction might initiate this process (2). Proteins such as Bcl-2 that inhibit apoptosis are localized in the mitochondria, suggesting a role for this organelle in the induction of apoptotic death. Indeed, the release of mitochondrial pro-apoptotic factors, for example cytochrome c, is blocked by Bcl-2 (3). In this context of regulation of apoptosis by mitochondrial mediators, species highly reactive with prosthetic groups or aminoacid residues of mitochondrial proteins are likely candidates to control apoptosis. One of these is NO, a pleiotropic molecule that either protects cells from apoptosis or causes apoptotic cell death depending on the local concentration, the nature of the target cells and the presence of other reactive molecules such as superoxide, which produces peroxinitrite, a strong oxidant species (4). Low doses of NO protect B-lymphocytes from receptor-induced, or viral infection-dependent apoptosis (5). High levels of NO induce apoptosis in macrophages, heaptocytes, glial cells, neurons and distinct cells of the immune system.

Several pathways have been found to modulate NO-dependent induction of apoptosis (6,7). One of these involves the release of mitochondrial pro-apoptotic mediators that induce DNA degradation identified using reconstituted systems of isolated nuclei and mitochondrial supernatants (2,7). Regarding its mechanism of action, NO induced a large mitochondrial permeability transition, as determined by colloidosmotic swelling. This process was inhibited by bongkrekic acid, caspase inhibitors and cyclosporine A or its related non-immunosuppressive derivatives (7). In line with this, NO produced by chemical NO-donors is sufficient to stimulate isolated purified mitochondria to release apoptogenic factors which induce DNA fragmentation as well as other apoptotic alterations in the nuclear morphology. This induction of apoptosis is accompanied by an alteration of the mitochondrial inner transmembrane potential, as measured by the shift in the potential sensitive probe dihexyloxacarbocyanine (DiOC6). This change has been identified as an early event in the process of NO-dependent apoptosis by the use of several inhibitors. However, analysis of NO-dependent mitochondrial changes in various cell types indicates a cell-specific response in terms of the changes in the transmembrane potential.

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Post-translational regulation of human inducible nitric oxide synthase by tyrosine phosphorylation

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Human inducible nitric oxide synthase (iNOS) is regulated at the transcriptional level by cell stimulation with a mixture of several cytokines. Not much is known yet on its post-translational regulation but few recent data suggest that the protein might be controlled by phosphorylation. In order to better characterize the function of human iNOS we used an in vitro model of colon carcinoma (DLD-1 cells). We induced iNOS by exposure of the cells to a mixture of interferon-γ, interleukin-1β and IL-6. INOS induction was assessed by Western blot analysis and activity measured by conversion of 3H-arginine to 3H-citrulline. In our conditions iNOS protein and the activity were present in both membranes and cytosol fraction, determined following subcellular markers. Analysis using phosphotyrosine antibodies revealed that iNOS was phosphorylated on tyrosine residues. When the activity, reached a steady state we briefly exposed activated cells to vanadate, a tyrosine phosphatase inhibitor and found a significant increase in enzyme activity. Taken together, these results demonstrate that tyrosine kinase and phosphatase are involved in the post-translational modification of iNOS and may potentially play a role in modulating the functional activity of the enzyme in human colon cancer cells.

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Chair: Lisardo Boscá

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TRANSDERMAL NITROGLYCERIN PREVENTS INDOMETHACIN INDUCED-LEUKOCYTE ACTIVATION AND GASTRIC BLOOD FLOW REDUCTION.

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Transdermal nitroglycerin protects the rat gastric mucosa against damage induced by indomethacin at doses clinically used (1). Gastrolesivity induced by NSAIDs results in a reduction in blood flow and the activation of inflammatory responses in the gastric mucosa (2). In the present study we investigate the effects of this mode of administration of an NO-donor on these mechanisms of gastric injury. Conscious male rats received indomethacin (20 mg/kg, s.c.) 30 min after application of transdermal patches releasing nitroglycerin (NGC, 166 ng kg^{-1} min^{-1}) or placebo (PP). Three hours later, animals were killed and gastric lesions were measured. Two further groups of experiments were performed in anaesthetized (pentobarbital, 50 mg/kg , i.p.) rats. In the first group, the change (%) of indomethacin (20 mg/kg, s.c.) -induced gastric mucosal blood flow (GMBF) was evaluated by laser-Doppler flowmetry using an ex vivo gastric chamber preparation. In the second one, intravital microscopy was used to analyze the effect of transdermal nitroglycerin on indomethacin-induced leukocyte-endothelial cells interaction within the mesenteric microcirculation. Indomethacin was administered by superfusion (50 µg/ml) and the absolute number of rolling (NR), rolling velocity (RV), adherence (A) and emigration (E) of leukocytes quantified. In both cases, indomethacin was administered thirty minutes after patch application and GMBF, inflammatory events and blood pressure registered for 60 min. Animals treated with transdermal NGC exhibited a 70±13% (n=7, $p<0.05$) diminution in the level of gastric damage induced by indomethacin (30±5 mm, n=7). This dose or NGC did not modify blood pressure.

min	0		30		60	
	PP	NGC	PP	NGC	PP	NGC
GMBF (%)	100	100	89±2⁺	96±7	84±6⁺	96±8
NR	16±3	11±1	48±13⁺	10±2*	34±6	11±5*
A	1.5±0.2	2.7±0.7	8.8±1.6⁺	3.6±0.8*	9.8±2.3	2.5±1.5*
RV	75±17	52±13	44±6	39±4	44±8	47±15
E	2.5±0.5	1.0±0.5	3.8±0.6	2.6±0.3	4.5±0.9	3.0±1.0

($n \geq 5$; mean ± s.e.m). $^{+}p<0.05$ vs respective basal values and *$p<0.05$ vs respective PP values.

In conclusion, gastroprotective doses of transdermal nitroglycerin prevent the reduction of mucosal blood flow and the leukocyte-endothelial cells interaction induced by indomethacin without modifying systemic blood pressure.

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Glucocorticoid induced proteolytic cleavage of inducible nitric oxide synthase by calpain I in IFN-γ - stimulated RAW 264. 7 cells: evidence for the involvement of the calmodulin binding site

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Proteolytic degradation of inducible nitric oxide synthase (iNOS) is one of the key steps by which the synthetic glucocorticoid dexamethasone controlles the amount of iNOS protein and thus the production of nitric oxide (NO) in IFN-γ - stimulated RAW 264.7 cells (Walker et al., 1997). We show here that iNOS is a molecular substrate for cleavage by the calcium-dependent neutral protease calpain I using cytoplasmic extracts from dexamethasone - treated RAW 264.7 cells and ^{35}S - radioactively labeled iNOS synthesized *in vitro* with a transcription / translation coupled reticulocyte lysate system in a cell - free degradation assay. Besides a conformational determinant located between amino acids 737 and 324, a preferential cleavage site for calpain I exists in the calmodulin (CaM) - binding domain present at amino acids 501 to 532 in iNOS. The access of the CaM - binding region is critical for substrate cleavage as incubation of *in vitro* synthesized iNOS with CaM reduces the kinetic of iNOS degradation by calpain I. Incubation of RAW 264.7 cells with IFN - γ in the presence of CaM - inhbitors such as W - 7 or Calmidazolium blocks the formation of iNOS protein. These data suggest that CaM binding is an essential prerequisite for the formation and stability of iNOS. Furthermore, by immunoprecipitaion we demonstrate that treatment of RAW 264.7 cells with dexamethasone reduces cytosolic CaM levels. We suggest that part of the regulation of calpain I - dependent iNOS degradation by dexamethasone may be achieved by decreasing cytosolic CaM levels and thus potentially affecting CaM - binding to iNOS.

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Nitric oxide-mediated apoptosis in human malignant lymphoid cells involves the CD95 pathway and strictly requires caspase activity

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Nitric oxide (NO), an important effector molecule involved in immune regulation and host defense, was previously shown to induce apoptosis in mouse lymphoma cells. Here we report that the NO donor glycerol trinitrate (GTN) induced apoptosis in Jurkat cells that are sensitive to CD95-mediated kill. In contrast, the CD95-resistant Jurkat subclone, showed substantial protection from apoptosis after exposure to NO. NO induced the mRNA expression of CD95 (Fas/APO-1) and TRAIL/APO-2 ligands. Moreover, NO triggered apoptosis in freshly isolated human leukemic lymphocytes which were also sensitive to anti-CD95 treatment. The ability of NO to induce apoptosis was completely blocked by a broad spectrum ICE-protease/caspase inhibitor. Moreover, NO-mediated cell death was found to correlate with FLICE/caspase-8 activation which was abrogated by the inhibitor of protein synthesis cycloheximide. Similar results were obtained in an *in vitro* experimental model of co-culture of human lymphoid target cells with activated bovine endothelial cells generating NO as effectors. Furthermore, the inhibition of endogenous NO production with the inducible NO synthase inhibitor N^G-monomethyl-L-arginine caused a complete abrogation of the apoptotic effect. Our data provide evidence that production of endogenous NO by endothelial cells or exposure to exogenous NO triggers apoptosis in human neoplastic lymphoid cells through the CD95 signaling pathway. The observed apoptotic effect strictly requires caspase activity including FLICE, the most CD95 receptor-proximal caspase.

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Session 7: NO: Pharmacology and toxicology

Chair: Patrick Vallance

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NO and Peroxynitrite: Interactions and Biological Significance

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Nitric oxide has a reputation as being a highly reactive and toxic molecule, but it is relatively inert at biologically relevant concentrations. However, nitric oxide competes with superoxide dismutase for superoxide to form peroxynitrite ($ONOO^-$). When the concentration of nitric oxide approaches that of intracellular superoxide dismutase, peroxynitrite becomes a major toxic product of nitric oxide. The toxicity of peroxynitrite is much greater than hydroxyl radical because peroxynitrite is far more selective and attacks key chemical moieties such as iron/sulfur centers, zinc fingers, and thiolate anions in proteins like tyrosine phosphatases. Consequently, peroxynitrite readily damages signal transduction pathways, interrupts electron transport and affects the structural integrity of cells by modifying structural proteins. Peroxynitrite can readily transverse cellular membranes both as a acid through the lipid bilayer and as an anion through bicarbonate exchange proteins. Peroxynitrite reacts rapidly with carbon dioxide to form a relatively short lived but highly reactive intermediate that increases nitration of tyrosines. In addition, peroxynitrite reacts directly with many metalloproteins including superoxide dismutase to catalyze tyrosine nitration. Nitrotyrosine has become an important marker of peroxynitrite formation in many disease states. Although many other reactions can form nitrotyrosine *in vitro*, peroxynitrite is still the most effective agent to form nitrotyrosine in biological tissues. Peroxynitrite-injured cells become more susceptible to apoptosis when also treated with certain growth factors such as acidic fibroblast growth factor. Oxidative damage to signal transduction pathways may help influence which cells proliferate or die as part of wound healing.

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NO a novel type of neurotransmitter in the autonomic nervous system

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Nitric oxide (NO) has been implicated as an important neuromediator in both the central and peripheral nervous systems (Garthwaite et al., 1988; 1989). The mechanisms behind NO release from neurons remain one of the fundamental gaps in our knowledge of nitrergic neurotransmission in the peripheral nervous system. NO has been postulated as a putative neurotransmitter on the grounds that it is synthesized by neurons and released from nerves upon nerve stimulation (Wiklund et al., 1993). Furthermore, NO is quickly inactivated by oxidation and the pharmacological profile of authentic NO has been shown to be identical to the profile for the nitrergic neurotransmitter (Boeckxstaens et al., 1995, Wiklund et al 1993). However, NO shows differences from the classical neurotransmitters. NO is a gaseous molecule acting on the heme moiety of the soluble guanylyl cyclase, without preceding interaction with cell-surface receptors. Furthermore, the synthesis, storage and release of NO have been suggested to differ from these of the classical neurotransmitters. Studies of the subcellular localization of nNOS in neuronal tissue have shown a predominantly cytosolic distribution (Förstermann et al., 1991). In agreement with this, the ultrastructural localization of NOS in enteric neurons has been shown to be patchy and the enzyme is non-homogeneously distributed throughout the nerve (Llewellyn-Smith et al., 1992). In contrast to this, NO has been suggested to be stored as a chemically more stable NO-containing molecule, such as S-nitrosothiols (Rand and Li 1995). There is discrepancy between the pharmacological profiles of free NO and the S-nitrosothiols (Iversen et al., 1994), but it has been suggested that S-nitrosothiols could still be the precursors by releasing free NO at the cell membrane of the nerve terminal (Rand and Li 1995). Hitherto, however, there is no fundamental evidence that discriminates between NO release by enzymatic activity and NO release from vesicular stores. An observation that speaks in support of a non-vesicular release of NO is the enduring stable release of NO during hours of nerve activation (Kasakov et al., 1995). In contrast, vesicularly stored classical transmitters are exhausted by long-term stimulation of cholinergic and adrenergic nerves.

Our studies have shown that NO formation may occur along the whole nerve cell including the terminal, axon and soma as demonstrated by visualization of nerve-evoked NO formation (Wiklund et al., 1997), suggesting a non-vesicular release. The clostridial neurotoxins have provided possibilies to study the calcium-dependent exocytotic release of neurotransmitters from neuronal tissue (Montecucco and Schiavo, 1995). BTX B has been shown to inhibit vesicular neurotransmitter release by cleavage of the synaptic vesicle protein VAMP, thereby blocking neuronal exocytosis (Montecucco and Schiavo, 1995). The clostridial neurotoxins have been shown to affect membrane fusion itself, without interacting with other elements of nerve terminal function such as membrane potential, voltage-gated Ca^{2+} currents, and the morphology of intracellular structures (Montecucco and Schiavo, 1995). We have found that BTX B blocked the nerve-induced cholinergic and tachykininergic contractile responses and markedly

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inhibited the nerve stimulation-evoked release of [^{3}H]-choline, but had no effect on nerve-induced relaxations in the guinea pig intestines. The relaxations were equally inhibited by an NO-synthase inhibitor and a selective inhibitor of soluble guanylyl cyclase, both in BTX B-treated and in control preparations. Nerve stimulation-evoked NO synthase-dependent outflow of NO/NO_2^- was unaffected by BTX B. Based on the present findings we suggest that the nerve-dependent formation of NO is independent of intact VAMP in the enteric nervous system. This view implies that vesicle-membrane fusion is not necessary for NO formation in nerve tissue, and favors the hypothesis of a non-vesicular release of NO following nerve activation.

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Ignacio Lizasoain. NO AND BRAIN ISCHAEMIA.

Nitric oxide (NO) overproduction has been postulated to significantly contribute to ischaemia-reperfusion neurotoxicity. Inducible NO synthase (iNOS) synthesizes NO in large quantities for long periods of time. Therefore we investigated the expression and localization of iNOS after oxygen and glucose deprivation (OGD) in rat forebrain slices. In this experimental model, calcium-independent NOS activity reached a maximum 180 min after the end of a 20 min OGD period. During the same period of time, the calcium-independent activity was absent in control forebrain slices. To test whether this calcium-independent NOS activity was due to the expression of iNOS, the effects of the addition of dexamethasone, cycloheximide and pyrrolidine dithiocarbamate were determined. All of them inhibited the induction of the calcium-independent NOS activity measured in the rat forebrain slices after OGD. Furthermore, OGD caused the expression of the gene encoding iNOS in rat forebrain slices, as assessed by the detection of iNOS message and protein in these samples. A 6-fold increase in the iNOS mRNA levels was observed at 180 min and the time course of the expression of iNOS mRNA was in agreement with the temporal profile of iNOS enzymatic activity. Immunohistochemistry analysis revealed that iNOS was highly expressed in neurones, astrocytes and microglial cells. These results demonstrate for the first time that iNOS is expressed in neurones after OGD, and that this expression occurs in short periods of time.

We have also used this experimental model to study the effect of OGD on neuronal NOS (nNOS). In OGD-exposed rat forebrain slices, a decrease in the calcium-dependent NOS activity was found 180 min after the OGD period, which was parallel to the increase during this period in calcium-independent NOS activity. Both dexamethasone and cycloheximide, which completely inhibited the induction of the calcium-independent NOS activity, caused a 40-70% recovery in calcium-dependent NOS activity when compared with slices collected immediately after OGD. The NO scavenger oxyhaemoglobin completely recovered calcium-dependent NOS activity, suggesting that NO formed after OGD is responsible of this down-regulation. Consistently, the exposure to the NO donor DETA-NONOate during 20 min caused a decrease in the calcium-dependent NOS activity present in control rat forebrain slices. Furthermore, OGD and DETA-NONOate caused a decrease in both nNOS mRNA and protein. In summary, our results indicate that iNOS expression down-regulates nNOS activity in rat brain slices exposed to oxygen-glucose deprivation.

These findings suggest that 1) NO can play an important pathogenic role in the tissue damage that occurs after cerebral ischaemia and that 2) there are important and complex interactions between NOS isoforms which may help to gain further insight into the physiological and pathophysiological events that occur during and after cerebral ischaemia.

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Session 8: NO in health and disease

Chair: Bettie Sue S. Masters

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Nitric oxide in human vasculature

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All three isoforms of NOS have been implicated in cardiovascular control in humans. Inhibition of NO generation with L-NMMA causes vasoconstriction and this is most evident in resistance vessels (small arteries and arterioles) [1]. The maximum decrease in blood flow in response to L-NMMA is about 40-50% at constant perfusion pressure, indicating a near doubling of vascular resistance. This effect has been seen in the forearm arterial bed, the renal bed, and coronary and cerebral circulations [2]. When L-NMMA is given systemically, arterial blood pressure rises and the increase in pressure is due to vasoconstriction. Conduit vessels appear to show less vasoconstriction to L-NMMA suggesting that these vessels may have reduced basal generation of NO. Most veins do not constrict to L-NMMA, indicating lack of basal NO-mediated dilatation in this vessel type in humans. Reduced NO-mediated dilatation has been reported in patients with cardiovascular disorders including hypertension, diabetes and hypercholesterolaemia, and in smokers. Recently it has been shown that the conversion of ^{15}N arginine to $^{15}NO_3$ is decreased in patients with hypertension, suggesting that the decreased NO-mediated dilatation is due to a decrease in NO generation [3].

Mechanisms underlying changes in NO generation in disease states are not fully understood and are likely to differ between diseases. One possibility is that endogenous inhibitors of NOS are important under certain conditions. We have identified such an endogenous inhibitor [4] and found that it accumulates in patients with renal failure. Recently several groups have reported that the inhibitor also accumulates in patients with hypercholesterolaemia and have suggested that this might contribute to inhibition of NOS activity. This area will be reviewed in the lecture and the mechanisms of synthesis and degradation of the inhibitor will be discussed.

Over production of NO generation in the vasculature has also been implicated in disease. Septic shock is characterised by vasodilatation and vascular collapse. Nitrate levels are increased in patients with sepsis and the NOS inhibitor L-NMMA restores BP in patients with septic shock [5]. Although it is clear from animal models that induction of iNOS underlies these effects, the precise mechanisms by which infection might alter NO generation in humans has not been established. Recently we have undertaken experiments attempting to induce NO generation in human blood vessels in vivo. Bacterial endotoxin produced a rapid vasodilatation that was not mediated by NO. In contrast, the pro-inflammatory cytokine interleukin-1 (IL-1) induced a slowly developing vasodilatation that was mediated by NO and reversed by L-NMMA. These experiments will be described together with results of studies exploring the molecular basis of the changes seen.

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Novel Antitumor Actions of the Arginine-Nitric Oxide Pathway

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The objective of this study was to elucidate the role of nitric oxide (NO) synthase (NOS) in the growth of the Caco-2 human colon carcinoma cell line. Activation of NOS and consequent increased production of NO are believed to elicit anti-proliferative effects attributed to NO itself (1). Additional studies have suggested that increased NOS activity or added NO causes cytostasis (2). These studies are important, but inconclusive in that cyclic GMP did not account for the cytostatic effect of NO in many cell systems. Moreover, studies addressing the role of NOS activation on cell proliferation were based on the assumption that NO is the only potentially active species generated from arginine by NOS, and the effect of the NOS intermediate, N-hydroxyarginine (NOHA), was not addressed. The latter possibility takes on special significance in light of our recent finding that cytokine-activated cultured cells generate and accumulate relatively large quantities of NOHA in addition to NO or its oxidized metabolites (3). Further, the accumulation of NOHA by other cell types and in plasma has been reported (4). These observations indicated that NOHA may serve not only as an intermediate in the production of NO but may also play a role as a distinct biological mediator in its own right. One such role for NOHA conceivably might be to function as an endogenous inhibitor of arginase activity, a view that is consistent with the observations that NOHA inhibits arginase activity (3,5,6). Arginase, which catalyzes the conversion of arginine to ornithine plus urea, is important not only in the urea cycle but also in biochemical pathways essential to cell proliferation and wound healing. For example, ornithine is a precursor to polyamines and proline, and in mammalian cells is derived from arginine via the catalytic action of arginase. Ornithine is converted to putrescine by ornithine decarboxylase, and putrescine is then converted to the polyamines spermidine and spermine, which function to stabilize DNA. Accordingly, inhibition of arginase activity by NOHA could result in inhibition of cell proliferation, and this action could account for at least part of the mechanism by which activation of the NOS pathway leads to cytostasis.

The two novel observations reported here are first that NOHA inhibits Caco-2 tumor cell proliferation, likely by inhibiting arginase activity, and second that NO causes cytostasis by inhibiting ornithine decarboxylase activity. Both arginase and ornithine decarboxylase are enzymes involved in the conversion of arginine to polyamines required for cell proliferation. Cell growth was monitored by cell count, cell protein analysis and DNA synthesis. NOHA (1-30 μM) and NO in the form of DETA/NO (1-30 μM) inhibited cell proliferation by 20-80%. The cytostatic effect of NOHA was prevented by addition of reaction sequence products that are distal to arginase such as ornithine, putrescine, spermidine or spermine to cell cultures. The cytostatic effect of the ornithine decarboxylase inhibitor, a-difluoromethylornithine (DFMO), was prevented by addition of reaction products distal to ornithine decarboxylase such as putrescine, spermidine or spermine but not ornithine. Interestingly, the cytostatic effect of NO (DETA/NO) was also unaffected by ornithine, but was prevented by putrescine, spermidine or spermine. Thus, the cytostatic action of NO closely resembled that of DFMO, and this suggested that NO might be a potent inhibitor of ornithine decarboxylase. The cytostatic effect of NOHA was not attributed to conversion to NO, and the effect of NO was independent

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of cyclic GMP. NOHA inhibited ornithine and urea production by Caco-2 cells and inhibited arginase catalytic activity (85% at 3 μM) by competitive mechanisms with respect to arginine substrate, whereas NO (DEA/NO and SNAP) inhibited ornithine decarboxylase activity (60% at 10 μM) without affecting arginase activity. The mechanism of inhibition by NO of ornithine decarboxylase appears to be via S-nitrosylation of the critical cysteine-360 sulfhydryl at the catalytic site of ornithine decarboxylase. S-Nitrosothiols such as SNAP and GSNO and CYSNO were particularly potent inhibitors of ornithine decarboxylase activity present in Caco-2 tumor cells and also of purified recombinant mammalian ornithine decarboxylase.

Co-culture of Caco-2 cells with LPS/cytokine-activated murine macrophages or rat aortic endothelial cells markedly slowed Caco-2 cell proliferation, and this was blocked by NOS inhibitors such as S-ethylisothiourea and N-methylarginine. These observations that two products of the NOS pathway, NOHA and NO, inhibit sequential steps in the arginine-polyamine pathway implicate a novel biological role for NOS in the inhibition of cell proliferation. This complementarity of anti-proliferative mechanisms for NOHA and NO suggests that NOS activation represents an important physiological and/or pathophysiological process to modulate the growth of tumor cells and perhaps other cell types.

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Aerocrine functions of nitric oxide

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NO gas, when inhaled at 5-80 parts per million (ppm), has been shown to dilate the pulmonary circulation of lambs in which pulmonary hypertension had been induced pharmacologically [1], and in anesthetized pigs in endotoxin shock [2]. Inhalation of NO by patients with adult respiratory distress syndrome (ARDS) alleviates the pulmonary hypertension and hypoxia associated with this condition [3]. This is thought to be achieved by NO being distributed selectively to ventilated pulmonary areas, thus increasing blood flow preferentially in well-ventilated alveoli and improving the ventilation-perfusion ratio. Recent studies have shown that NO inhalation therapy may be effective in ARDS also in the sub-ppm concentration range [4]. Interestingly, large amounts of endogenous NO is excreted in the nasal airways of healthy subjects [5, 6]. A very large NO source is situated in the paranasal sinuses, where an "inducible-like" NO synthase is constitutively expressed in the epithelium [7]. NO released into the sinuses enters the nasal cavity via the sinus ostia and contributes largely to the levels of NO found in the nasal cavity [8]. This NO will follow the airstream to the lower airways and the lungs with every inhalation. Thus, a continuous NO flushing of the lower airways takes place, with inhaled NO concentrations of about 0.1 ppm. Since most of the NO excretion takes place in the nasal airways, a particularly high amount of NO will be inhaled if a subject is inhaling through the nose.

We have shown that arterial oxygenation is improved in healthy awake subjects during nasal breathing as compared to breathing through the mouth [9]. Furthermore, in intubated patients, who are deprived of self-inhaled NO, we have shown that pulmonary function is improved by adding air derived from the patient's own nose to the inspiratory flow of the ventilator [10]. These effects were probably due to NO, since exogenous NO in the same concentration range had similar effects in these patients.

The involvement of autogenous NO in regulation of pulmonary function may represent a novel physiological principle, namely that of an enzymatically produced airborne messenger. The term "aerocrine" may be appropriate for this action of NO in the airways. In fact, these finding may help to explain one biological role of the enigmatic human paranasal sinuses, the major sources of NO in the upper airways.

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INHALED NITRIC OXIDE

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INTRODUCTION

The major objective of this lecture is to describe the effects of inhaling low levels of nitric oxide (NO) on the hemodynamic and gas exchange function of both the normal and diseased lung. Considerable attention will be paid to safety and hazards of inhaled NO therapy. During the past few years remarkable progress has been made in understanding the NO-guanylate cyclase signal transduction system. NO has been given considerable clinical investigation in pulmonary artery hypertension and adult respiratory distress syndrome (ARDS) patients. This lecture concentrates on this area of clinical research.

Pulmonary hypertension with severe hypoxemia may complicate the care of patients with diseases such as chronic pulmonary hypertension, ARDS, chronic respiratory failure, congenital heart disease, and after cardiopulmonary bypass.

Numerous vasodilator therapies aimed at reducing pulmonary hypertension have been tested in these patients. Systemic vasodilation and hypotension occur with all the currently available intravenous vasodilators tested in dosages sufficient to reduce the pulmonary artery pressure. In addition, intravenous infusions of systemic vasodilators such as nitroprusside or prostacyclin (PGI_2) markedly increase the venous admixture (1,2).

NITRIC OXIDE

In 1987, the gaseous molecule NO was identified as an endothelium-derived relaxing factor (EDRF) (3,4). NO is an ideal local transcellular messenger because of its small size, lipophilic nature, and short duration of action (5) and its numerous functions in various tissues have been reviewed (6). In vascular endothelial cells, NO is synthesized from the terminal guanidine nitrogen of L-arginine and diffuses rapidly into subjacent vascular smooth muscle (7). There, NO binds to the heme iron complex of soluble guanylate cyclase. The resulting nitrosylheme activates guanylate cyclase, stimulating the production of cyclic guanosine 3',5'-monophosphate (cGMP) and subsequently relaxing vascular smooth muscle (7,8). When NO diffuses into the intravascular space, its biologic activity is limited by avid binding to hemoglobin. Interestingly, the nitroso-vasodilators we have used for decades, such as nitroglycerin and nitroprusside, act by releasing NO (9).

Endothelium-dependent relaxation in pulmonary arteries occurs in response to a variety of physical and pharmacologic stimuli (10). Endogenous NO can be measured in the exhalation of rabbits, guinea pigs, and humans (11). In normal lungs, however, baseline pulmonary vascular tone is very low and the administration of acetylcholine or the addition of exogenous NO has little effect on pulmonary vascular resistance (12 - 14). In patients with pulmonary hypertension, on the other hand, acetylcholine infusion or NO inhalation can reduce pulmonary vascular resistance (12,13). It is possible that in some acute and chronic pulmonary hypertensive states, such as ARDS, or chronic pulmonary hypertension, the production of endogenous NO is impaired (15,16). This might produce further vasoconstriction and foster platelet aggregation (17). Evidence supporting this hypothesis is indirect at this time. Such patients may have an intact response to inhaled NO even though their response to intravenous acetylcholine is impaired (18).

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NO Inhalation in ARDS

We hypothesized that inhaled NO should diffuse into the pulmonary vasculature of ventilated lung regions and cause relaxation of pulmonary vascular smooth muscle, thereby decreasing pulmonary hypertension in ARDS (19,20). Since the NO is inhaled, the gas should be distributed predominantly to well-ventilated alveoli and not to collapsed or fluid-filled areas of the lung. In the presence of increased vasomotor tone, selective vasodilation of well-ventilated lung regions should cause a "steal" or diversion of pulmonary artery blood flow towards well-ventilated alveoli, improving the matching of ventilation to perfusion and improving arterial oxygenation during ARDS. Such an effect would be in marked contrast to the effects of intravenously administered conventional vasodilators (such as nitroprusside, nitroglycerin, or prostacyclin). These intravenous agents also decrease PA pressure, but by nonselectively dilating the pulmonary vasculature, they augment blood flow to nonventilated areas, thereby increasing right-to-left shunting and reducing the PaO_2. Also unlike available intravenous vasodilators, inhaled NO, because it is avidly bound to hemoglobin and rapidly inactivated, should not produce systemic vasodilation.

Rossaint and coworkers compared the effects of NO inhalation (18 and 36 parts per million (ppm)) to intravenously infused prostacyclin in nine patients with ARDS (21). NO selectively reduced mean pulmonary artery pressure from 37 ± 3 to 30 ± 2 mmHg (mean $\pm$ SE). Oxygenation improved due to a decreased venous admixture ($Q\bar{V}_A/Qt$). During NO breathing, the PaO_2/FIO_2 ratio increased from 152 ± 15 mmHg to 199 ± 23 mmHg. While the intravenous infusion of prostacyclin also reduced pulmonary artery pressure, mean arterial pressure and PaO_2 decreased as QV_A/Qt increased. Subsequent reports documented that inhalation of lower concentrations of NO (< 20 ppm) effectively reduced pulmonary artery pressure and improved PaO_2 (22 - 25). Even very small inhaled concentrations (as low as 250 parts per billion NO) may be effective in some patients (26). Right ventricular ejection fraction may increase in some patients responding to inhaled NO, suggesting that the observed decreases of pulmonary artery pressure may be hemodynamically important (24,25).

A marked variation has been reported for the hemodynamic and respiratory effects of NO inhalation, both among patients and within the same patient at different times in their illness (22,27,28). It is possible that preexisting pulmonary disease as well as the concomitant administration of other vasoactive drugs may contribute to the observed variability. In general, the baseline level of pulmonary vascular resistance appears to predict the degree of pulmonary vasoconstriction reversible by NO inhalation. Those with the greatest degree of pulmonary hypertension appear to respond best to NO inhalation (22,28). Dellinger recently reported a dose-response analysis of a randomized trial of NO in 177 ARDS patients (29), a trial which was too small to obtain significant outcome data.

Tachyphylaxis has not been observed even when NO inhalation was continued for up to 53 days (21). Pulmonary artery pressure and PaO_2 quickly return to baseline values, however, after discontinuation of the gas. Occasionally, sudden discontinuation of inhaled NO can produce problematic pulmonary vasoconstriction and possibly bronchoconstriction (22,30,31). The reason for this is unclear. Possibly, the addition of exogenous NO may decrease NO synthase activity (32) or increase tissue cGMP phosphodiesterase activity.

The vasoconstrictor almitrine besylate has been given intravenously to enhance pulmonary vasoconstriction during NO breathing. This agent has further reduced Qs/Qt in ARDS in combination with NO inhalation (33).

NO Inhalation in Neonatal Respiratory Failure

At birth, there is a sustained decrease of pulmonary vascular resistance and an increase of pulmonary blood flow, in part due to increasing oxygen tensions. If this does not occur, persistent pulmonary hypertension of the newborn (PPHN) may result. Persistent pulmonary hypertension of the newborn is a syndrome characterized by an increased pulmonary vascular resistance, increased right-to-left shunting across the ductus arteriosus and foramen ovale, and severe hypoxemia. Extracorporeal membrane

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oxygenation (ECMO) is often used to support these infants, because conventional vasodilator therapy is limited by severe systemic hypotension and may reduce PaO_2 by increasing right-to-left shunting. It has been hypothesized that endogenous production of NO by the pulmonary vasculature might be decreased in PPHN. If so, then inhaled NO might provide an effective therapy for these severely ill infants (34, 35). Multiple small clinical studies of NO inhalation have been performed in neonates, infants, and children with various types of acute respiratory failure. In general, pulmonary hypertension is reduced and systemic arterial oxygenation is improved with inhalation of less than 20 ppm NO. Nitric oxide inhalation in babies with PPHN and hypoxic respiratory failure has been studied in randomized multicenter trials(36,37). As in adults, however, the response is variable. In the neonatal lung, the degree of improvement with NO appears to depend upon the presence of mature surfactant.

Laboratory studies of the neonatal pulmonary circulation have also documented that inhaled nitric oxide is an effective pulmonary vasodilator (38). Additionally, important experimental evidence is accumulating that the inhalation of nitric oxide attenuates chronic hypoxic pulmonary vascular remodeling of the pulmonary circulation (39,40). Conceivably, inhaled nitric oxide therapy might be used to limit the chronic pulmonary vascular changes which accompany neonatal acute respiratory failure.

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POSTERS

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VASCULAR EUTROPHIC REMODELLING INDUCED BY CHRONIC NITRIC OXIDE INHIBITION IN CEREBRAL AND MESENTERIC RESISTANCE ARTERIES. A CONFOCAL MICROSCOPY STUDY.

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Studies in smooth muscle cells (SMC) in culture suggest that nitric oxide (NO) has antiproliferative properties. It has been hypothesised that a reduction of NO production in some vascular diseases (e.g.. hypertension) could play a role in the associated changes in vascular architecture (remodelling). Laser Scanning Confocal Microscopy (LSCM) offers a new approach allowing determination of the vascular morphology of intact vessels at the cellular level (Arribas et al., Hypertension 30:1455-1464, 1997).
The **objective** of this study was to determine the effects of chronic NO inhibition on the remodelling process in intact arteries, where the interaction between different cell types is maintained.
Methods. 8 week old WKY rats were treated for three weeks with L-NAME (10 mg/kg/day), administered in the drinking water. Basilar and third order mesenteric resistance arteries were dissected, mounted on a perfusion myograph and stained intraluminally with the nuclear dye Hoechst 33342 for 5 min. The arteries were pressurised at half of the systolic pressure of the rat and fixed under pressure. Serial optical sections of the wall and lumen were obtained with a LSCM (364nm EX; x40 oil and x10 air objectives). Metamorph Image Analysis Software was used to analyse the images obtained and for 3-dimensional reconstructions of the vessel wall.
Results. L-NAME treated rats developed hypertension (L-NAME, 200 ± 2.8 mm Hg, control 134.2 ± 2.1 mm Hg; $p<0.01$). Both basilar and mesenteric arteries from L-NAME treated rats showed: (1) a significant reduction in lumen but no change in wall thickness, (2) a decrease in SMC and endothelial cell number and (3) a reduction in endothelial nuclei area and shape factor. In addition, there was a significant increase in adventitial cell number in the basilar but not in mesenteric resistance arteries.
Conclusions. These results suggest that:
(1) Hypertension induced in the rat by chronic NO inhibition is associated with eutrophic inward remodelling of basilar and mesenteric arteries.
(2) Remodelling induced by NO inhibition involves significant cellular changes, despite no changes in overall wall dimensions.
(3) The increase in adventitial cell number in cerebral, but not in mesenteric arteries, might be related to the existence of nitrergic innervation in the basilar artery.
(4) The contribution to these vascular alterations of NO inhibition and hypertension itself needs to be determined.
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Effect of NOS2 induction on glutathione metabolism in rat cultured astrocytes

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Reduced glutathione (GSH) is considered to be a major antioxidant compound protecting central nervous system cells against excess nitric oxide (•NO) biosynthesis. We have previously described that exposure of exogenous, authentic peroxynitrite (ONOO—) causes depletion of GSH stores in neurones but not in astrocytes. It appears that GSH depletion is involved in the specific neuronal susceptibility to excess •NO-mediated damage to the mitochondrial respiratory chain leading to neurotoxicity. Whilst GSH depletion may account for the different vulnerability to •NO/ONOO— found in different cell types, the mechanism for the specific resistance of astrocytes to the damaging effects of •NO/ONOO— is not understood. In order to address this question, we have studied the possible role of nitric oxide synthase (NOS2) induction on GSH metabolism. Lipopolysaccharide-mediated NOS2 induction was accompanied by a time-dependent increase in glucose-6-phosphate dehydrogenase (G6PD) mRNA levels in rat cultured astrocytes. The mechanism responsible for G6PD induction and other pathways related with GSH biosynthesis were also studied. Since NADPH is required for GSH regeneration from GSSG, NOS2-mediated G6PD induction may represent a possible protective mechanism responsible for the resistance of glial cells against excess •NO exposure.

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ROLE OF NO AND ANGIOTENSIN IN ARTERIAL PRESSURE CHANGES DURING ACUTE NECROTIZING PANCREATITIS

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In severe acute pancreatitis dramatic decreases in arterial blood pressure are often observed. In these circumstances, the response to different vasoconstrictors is strongly depresssed, but the causes of this lack of response are not yet known. We have used the experimental model of retrograde taurocholate infusion through the pancreatic duct to induce necrotizing acute pancreatitits in rats. Male Wistar rats (300-350g of bw) were anesthethized with uretane (20%, 1ml/100 g bw) and the right carotid artery was cannulated. Arterial pressure values were recorded by means of a Mac Lab informatic system (ADInstruments) and vasoactive substances were infused through the femoral vein. The decrease (21±3mm Hg) in mean arterial pressure (MAP) in pancreatitis was gradually attained after a meantime of 59.5±7min. After the hypotension, the effect of several vasoactive substances was checked, in order to know which mechanisms could be affected by pancreatitis. The effects of L-NAME infusion (50mg/Kg/h for 30 min), followed by a bolus of angiotensin II (225ng/Kg) and the effects of a bolus injection of angiotensin II (225ng/Kg) on (MAP), systolic (SP), diastolic and pulse pressures and heart frequency were analyzed in control and pancreatitic animals. In terms of absolute values (mm Hg), the increase of MAP was significantly higher after the L-NAME infusion (52±6 in controls, 34±3 in pancreatitics) compared with the effect of angiotensin (23±3 in controls, 10±2 in pancreatitics). Similar effects were observed on SP. Therefore, nitric oxide (NO) plays an important role to control arterial blood pressure, not only in normal but also in pancreatitic rats and though the increase of MAP and SP were smaller in pancreatitic animals compared with control ones, the response to vasoconstrictors is yet very important in pancreatitis. Moreover, when angiotensin was injected after the L-NAME infusion, the MAP was significantly increased (28±3 in controls, 19±3 in pancreatitics). Therefore L-NAME and angiotensin could restore normal tension values in acute pancreatitis. On the other hand, the effect of perindopril (2.3mg/Kg), an inhibitor of the angiotensin converting enzyme (ACEI) was also studied in order to know to what extent angiotensin is effective in pancreatitic animals. ACEI produces a decrease in MAP of 24±4 mm Hg in control compared with 12±2 in pancreatitic animals. Our results demonstrate that angiotensin operates even in pancreatitis Therefore, the combined effect of L-NAME and angiotensin could be useful to preserve the arterial pressure after hypotension derived from pancreatitis.

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MODULATION OF ANGIOTENSIN-CONVERTING ENZYME BY NITRIC OXIDE.

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The aim of the present study was to determine the effect of nitric oxide (NO) on angiotensin-converting enzyme (ACE) activity.

A biochemical study was performed in order to analyze the effect of the NO-donors, SIN-1 and diethylamine/NO (DEA/NO), and of an aqueous solution of nitric oxide on the ACE activity in plasma from 3-month old male Sprague-Dawley rats and on ACE purified from rabbit lung. SIN-1 significantly inhibited the activity of both enzymes in a concentration-dependent way between 1 and 100 µM. DEA/NO inhibited the activity of purified ACE from 0.1 µM to 10 µM and plasma ACE, with a lower potency, between 1 and 100 µM. An aqueous solution of NO (100 and 150 µM) also inhibited significantly the activity of both enzymes. Lineweaver-Burk plots indicated an apparent competitive inhibition of Hip-His-Leu hydrolisis by NO-donors.

Modulation of ACE activity by NO was also assessed in the rat carotid artery by comparing contractions elicited by Ang I and Ang II. Concentration-response curves to both peptides were performed in arteries with endothelium in the presence of the guanylyl cyclase inhibitor, ODQ (10 µM), and the inhibitor of NO formation, L-NAME (0.1 mM). NO, which is still being released from endothelium in the presence of 1 µM ODQ, elicited a significant inhibition of Ang I contractions at low concentrations (1 and 5 nM). In the absence of endothelium, 1 µM SIN-1 plus 10 µM ODQ, as well as 10 µM DEA/NO plus 10 µM ODQ induced a significant inhibition on Ang I-induced contractions at 1 and 5 nM and at 1 - 100 nM, respectively.

	Ang I, log (M)	Ang II, log (M)
control (with endothelium)	-7.46 (-7.7, -7.23)	-7.84 (-8.16, -7.52)
+ 10 µM ODQ	-7.43 (-7.81, -7.1)	-8.56 (-8.93, -8.18)*
+ 0.1 mM L-NAME	-8.21 (-8.52, -7.91)*	-8.61 (-8.88, -8.36)*
E- (without endothelium)	-8.17 (-8.45, -7.89)*	
E- + 10 µM ODQ plus 1µM SIN-1	-7.47 (-7.75, -7.15)#	
E- + 10 µM ODQ plus 10µM DEA/NO	-6.56(-7.02, -6.11)#	

Table: EC_{50} values for Ang I and Ang II-induced concentration-response curves in the rat carotid artery in the absence and presence of ODQ and L-NAME. Data are presented as geometric means and confidence intervals. *p < 0,05 with respect to control. #p < 0,05 with respect to arteries without endothelium .

In conclusion, the present resuls demonstrate that: i) NO and NO-releasing compounds inhibit ACE activity in a concentration-dependent and competitive way and ii) NO release from endothelium physiologically reduces conversion of Ang I to Ang II.

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INCREASE IN NEURONAL NITRIC OXIDE SYNTHASE EXPRESSION DURING MORPHINE DEPENDENCE AND WITHDRAWAL IN MICE.

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Opiate dependence and withdrawal has been correlated with increase in calcium-dependent nitric oxide synthase (NOS) activity throughout the CNS (1,2). To elucidate whether morphine dependence and abstinence modifies the expression of neuronal NOS and to study the topographic pattern of these changes, morphine dependence was induced by s.c. implantation of a 75 mg morphine pellet in adult male CD1 mice. At days 4 to 7 post-implantation, withdrawal was induced by s.c. administration of 1 mg/kg naloxone. All the animals underwent behavioural changes consistent with opiate withdrawal (micturition, diarrhoea, stereotyped movement – grooming-, tremor, shaking and jumping). Fifteen min after naloxone administration, brains were perfused by cardiac punction with 4% *p*-formaldehyde in PBS and postfixed in the same solution for 3 h at room temperature. After fixation, slices of brain tissues (50 μm) were incubated with a specific policlonal antibody anti nNOS at a 1:3000 dilution. Immunohistochemical analysis revealed an increase in nNOS expression in olfactory bulb, anterior and posterior olfactory nucleus, locus coeruleus (*LC*), nucleus prepositus hipoglosii (one of the main aminoacidergic inputs to *LC*), nucleus tractus solitarius and cerebellum (mainly in Purkinje cells). The present data provide evidence for the increase in nNOS expression in select populations of NO synthesising neurones during morphine dependence and withdrawal in areas related with motor and autonomic changes which take place in opiate withdrawal.

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Triggering of peritoneal macrophages with Interferon alpha/beta attenuates the expression of inducible Nitric Oxide Synthase through a decrease in NF-kappaB activation.

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Summary

Triggering of peritoneal macrophages with IFN-γ and a low concentration of LPS induced the expression of the inducible nitric oxide synthase (iNOS). However, when stimulation was accomplished in presence of IFN-α/β the synthesis of NO was significantly inhibited. At the mRNA level, IFN-α/β decreased the content of iNOS and addition of type I INFs during the initial two hours upon IFN-γ/LPS activation, was required for the inhibitory effect. Evaluation of the transcriptional activity using run-on assays of nascent RNA indicated that INF-α/β inhibited the transcription of iNOS. Transfection experiments using a 1.7 kb promoter sequence corresponding to the 5' flanking region of the murine iNOS gene showed a decreased promoter activity when type I IFNs were included in the synergistic stimulation with IFN-γ and LPS. Analysis of the transcription factors which participate in iNOS expression revealed a marked decrease of NFκB activation, a nuclear factor required for the transcription of this gene. The degradation of IκBα and IκBβ which is required for the translocation of NFκB to the nucleus was inhibited in the presence of IFN-α/β. However the activity of other transcription factors such as IRF-1, which is involved in the expression of iNOS in response to IFN-γ, was not affected by IFN-α/β stimulation. These results suggest that in the presence of IFN-α/β the activity of the iNOS promoter is impaired, and this attenuated NOS expression could be important in pathophysiological situations in which secretion of type I INFs occurs.

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MECHANISM OF NITRIC OXIDE MEDIATED INHIBITION OF VACCINIA VIRUS DNA SYNTHESIS

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Nitric oxide (NO) and peroxynitrite play a major role in the inhibition of growth of tumor cells and different kinds of intracellular pathogens. These inhibitory properties are mediated by inhibition of DNA synthesis and by the effect on host cell metabolism – especially by disturbing of mitochondrial function, changes in redox potential, modification of proteins (1).

It was previously shown that NO produced by activated macrophages inhibited growth of DNA viruses characterized by virus titers (2, 3). Using vaccinia virus (VV), we have demonstrated that this NO-mediated inhibition of virus growth occurred at the level of DNA synthesis (4). The inhibition of virus growth and DNA synthesis could be, however, caused by two different mechanisms: first, it could be due to a specific, NO-mediated inhibition of the individual enzymes involved in the synthesis of VV DNA or DNA precursors as in case of tumor cells; additionally, it could be due to the inhibition of the energy metabolism by NO.

Using VV recombinant expressing murine macrophage inducible NO-synthase (iNOS), we have determined activity of three VV encoded enzymes involved in VV DNA synthesis – ribonucleotide reductase (RR), DNA polymerase, and thymidine kinase (TK). Among the three enzymes, only activity of VV RR was inhibited by NO. The inhibition of RR activity by NO was detected at 4 hours post infection (h.p.i.), VV DNA synthesis characterized by thymidine incorporation was decreased at 6 h.p.i., and virus titers were decreased since 8 h.p.i.

We have also characterized the effect of NO production on host cell energy metabolism by measuring the levels of total cellular ATP and ADP. In time of inhibition of VV RR activity and VV DNA synthesis, there was, however, no decrease in total ATP levels observed. Thus, energy depletion does not appear to be the direct cause of inhibition of VV DNA synthesis. In conclusion, inhibition of VV RR seems to be the first and specific cause of inhibition of virus DNA synthesis by NO.

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EXCITATORY AMINO ACIDS ARE INVOLVED IN THE EXPRESSION OF INDUCIBLE NITRIC OXIDE SYNTHASE IN AN *IN VITRO* MODEL OF BRAIN ISCHAEMIA

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Oxygen and glucose deprivation (OGD) causes the expression of a calcium-independent nitric oxide synthase (NOS) activity corresponding to inducible NOS (iNOS) in different CNS cell types including neurones in rat forebrain slices (1). We have now investigated whether glutamate might be involved in the mechanisms by which OGD leads to the expression of iNOS in our model. A calcium-independent NOS activity appeared in slices exposed to OGD but not in control slices. The NMDA receptor antagonist dizocilpine (100 nM) blocked this expression. OGD also caused the release of glutamate to the bathing solution. Incubation of control slices with glutamate (100 μM) for 20 min also caused the expression of a calcium-independent NOS activity, which was inhibited by the inhibitor of the activation of NF-κB pyrrolidine dithiocarbamate. These data suggest that activation of NMDA receptors by glutamate released after OGD is involved in the expression of a calcium-independent NOS activity in rat forebrain slices *via* activation of the transcription factor NF-κB.

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SEGREGATION OF NOS EXPRESSION AND Ca^{2+} RESPONSE TO NITRIC OXIDE IN ADRENERGIC AND NORADRENERGIC BOVINE CHROMAFFIN CELLS

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Previous work has demonstrated that Nitric Oxide (NO) can be an important intracellular messenger in the regulation of neurosecretion in chromaffin cells. Since standard chromaffin cell cultures are mixed populations of noradrenaline (NA) and adrenaline (A) producing cells, it would seem important to understand the functional differences between these individual components. The use of fluorescence imaging techniques for the recording of cytosolic calcium from single chromaffin cells together with the immunoidentification of individual cells with specific antibodies against tyrosine hidroxylase, N-phenyl ethanolamine N-methyl transferase and nitric oxide synthase (NOS), has allowed us to measure single cell calcium responses in identified adrenergic, noradrenergic and nitrergic chromaffin cells, thus helping us to clarify the differential role of NO in the function of these chromaffin cell types.

53±2% of chromaffin cells were able to synthesize NO (NOS^+ cells) being these cells mainly noradrenergic (82±2%). Results indicate that NO donors such as sodium nitroprusside, molsidomine and isosorbide dinitrate evoke $[Ca^{2+}]_i$ increases in a 62±4% of chromaffin cells, the response to NO donors being between 30 and 50% of that of 20 μM nicotine. Cells responding to NO donors were mainly adrenergic (68±5%) although also 45±9% of NA cells gave $[Ca^{2+}]_i$ increasing responses. The distribution of NO responding cells between NOS^+ and NOS^- was very similar in the whole population (63±5 and 60±7%, respectively), but these differences were more prominent when considering the distribution of NO response between noradrenergic and adrenergic NOS^+ cells; while 73±6% of adrenergic NOS^+ cells evokes $[Ca^{2+}]_i$ increases by NO stimulation, only 35±11% of noradrenergic NOS^+ cells respond.

Taken together these results seem to indicate that 1) NO could act within adrenal medulla as both an intracellular and intercellular messenger and 2). Noradrenergic cells seem to be specialized in NO synthesis while adrenergic cells with an endocrine function could mainly act as a target of neurosecretory action of this second messenger

Key words: Nitric Oxide, chromaffin cells, catecholamines, calcium, immunocytochemistry

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NITRIC OXIDE MODULATES THE SOMATOSTATIN RECEPTORS, SOMATOSTATIN-LIKE IMMUNOREACTIVE CONTENT AND PROTEIN KINASE C IN THE RAT HIPPOCAMPUS

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Nitric oxide (NO), a simple gas with free radical chemical properties, has been demonstrated to serve as a neurotransmitter and neuromodulator in the central nervous system, modulating the wakefulness and circadian rythms, learning and memory, feeding, drinking, and the release of other neurotransmitters such as norepinephrine and dopamine. Anatomical, physiological and functional studies suggest an interaction between the somatostatinergic system and NO. Thus, the aim of the present study was to determine whether the somatostatinergic system is modulated by NO. Administration of the NO synthase inhibitor Nω–Nitro-L-Arginine Methyl Ester (L-NAME) (50 mg/kg, i.p.) during eight days resulted in a significant increase of somatostatin-like immunoreactivity (SSLI) content in the rat hippocampus, no changes being observed after four days of treatment. Administration of the NOS substrate L-arginine (150 mg/kg, i.p.) had no effect on this parameter. The specific binding of ^{125}I-Tyr^{11}-SS to SS receptors in hippocampal membranes from rats treated with L-NAME during eight days was markedly increased as compared with control rats. This increase was due to an increase in the maximal number of SS receptors. Coadministration of L-arginine and L-NAME during eight days reverted the number of SS receptors to control values whereas administration of L-arginine alone had no effect on the hippocampal SS receptors. In addition, L-NAME administered for four or eight days inhibited translocation of the protein kinase C isoform PKCα whereas a stimulation of PKCε translocation was detected after eight days of L-NAME treatment.Taken together, the present data suggest that NO may play an important role in many of the functions modulated by the somatostatinergic system in the hippocampus.

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