Instituto Juan March
de Estudios e Investigaciones

43 CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Molecular Biology and Pathophysiology of Nitric Oxide

Organized by

S. Lamas and T. Michel

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INTRODUCTION

Santiago Lamas and Thomas Michel

Since the identification of endothelial-derived relaxing factor in the mid- 1980s as nitric oxide (NO), an increasingly diverse array of biological roles have been identified for this ubiquitous molecule. In different tissues, NO may function as a vasodilator, neurotransmitter, or cytotoxic agent. In a number of tissues and cell types, the physiological role of NO synthesis remains to be established. Much of the progress in this field has evolved from the identification of the molecular mechanisms responsible for NO synthesis and the catalytic process whereby L-arginine is oxidized to form L-citrulline plus NO. This reaction is catalyzed by a group of nitric oxide synthase (NOS) isoforms, of which three distinct gene products have been identified in humans. Two NOS isoforms appear to be constitutively expressed in their cells of origin: the neuronal NOS (nNOS or type I) and endothelial NOS (ecNOS or type III). By contrast, the inducible NOS (iNOS or type II) is not normally expressed in tissues, but its synthesis can be induced in a wide variety of cells by specific immune mediators. Ongoing studies in multiple cellular systems are directed towards the elucidation of the mechanisms which regulate NOS gene expression.

During this workshop, recent data on the pathophysiological consequences of iNOS gene "knockout" were presented by two different groups. Two laboratories achieved iNOS gene inactivation by homologous recombination in mouse embryo-derived stem cells, and characterized the response of homozygous iNOS knockout mice to bacterial endotoxin and to intracellular pathogens. These animals were phenotypically normal in the absence of bacteriological challenge. However, the animals were partially resistant to endotoxic shock, but were highly susceptible to certain intracellullar pathogens such as Listeria monocytogenes or Leishmania major. The physiologic and pathophysiologic roles of NO in the modulation of cardiac myocyte function were discussed, as were the roles of NO in the gastrointestinal tract under conditions of stress. The differential arginine transport pathways activated upon induction of macrophages with cytokines were presented in detail. Enzymological characterizations of the NOS isoforms focused on the role of key cofactors such as calmodulin, heme and tetrahydrobiopterin. Studies of the nNOS expressed in baculovirus were directed towards the elucidation the

protein's hydrodynamic properties, with particular emphasis on the role of tetrahydrobiopterin binding. Distinctive features of the endothelial NOS were discussed, including the roles of myristoylation, phosphorylation and palmitoylation as key mechanisms for the regulation of enzyme function and subcellular localization. Intracellular levels of Ca++, which are critical for ecNOS activation, are fine tuned by tyrosine phosphorylation and regulated by physiological processes including hemodynamic shear-stress.

New biochemical roles for NO were discussed in the context of the NO-mediated activation of transcription factors such as NF-kB, as well as the regulation of iron metabolism. The participation of NO in the regulation of alpha1-adrenergic-induced proto-oncogene expression, auto-phosphorylation of growth factor receptors and the mutual interactions of Ca++ and the NO-cGMP pathway in chromaffin cells were novel aspects explored during the workshop. It also appears that NO is also important in diverse non-mammalian systems: in the salivary glands of bloodsucking hemiptera, NO subserves functions of vasodilation and is stored in a new class of proteins, termed nitrophorins.

NO is a free radical capable of interacting with reactive oxygen species, such as the superoxide anion, to form nitrosating agents and peroxynitrites. These molecules are responsible for the NO-related DNA damage and toxicity observed in many cellular systems. The involvement of NO in apoptosis is complex: NO prevents apoptosis in B cells, but promotes programmed cell death in macrophages. The redox state of cells might represent the key switch which controls which biological role will be assumed by NO.

Regulation of NOS gene expression represents a critical regulatory pathway, and has been most extensively characterized for the iNOS. More recently, nNOS and ecNOS have been detected in diverse tissues, and these two "constitutive" isoforms appear to be subject to dynamic transcriptional regulation. Studies of potential cis-regulatory elements in the promoters of the NOS genes have led to the identification of the roles of specific transcription factors. Transcriptional control of the iNOS gene appears to be importantly determined by the rel family of transcription factors. A key member of the rel family, NF-κb, has been identified as an important determinant of the iNOS gene activation in response to cytokines as well as bacterial endotoxin. Some antioxidants and non-steroidal anti-inflammatory

drugs may inhibit the activation of this transcription factor. Upon cytokine or endotoxin stimulation, vascular smooth muscle and mesangial cells appear to share common mechanisms of transcriptional regulation of iNOS expression. In human mesangial cells, where induction of iNOS requires the concerted effects of multiple stimuli, a newly characterized lymphokine, interleukin-13, is a powerful regulator of iNOS expression and NO synthesis.

Overall, the March Foundation provided a comprehensive framework for the discussion of the recent advances of this active field of biological inquiry, in a remarkably dynamic, open and productive scientific environment.

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FIRST SESSION: PATHOPHYSIOLOGY

Chairman: Louis J. Ignarro

RELEASE OF NITRIC OXIDE BY HUMAN MONOCYTES/MACROPHAGES

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The formation of nitric oxide (NO) from L-arginine is now recognised as a ubiquitous pathway involved in a variety of physiological functions. The synthesis of NO by vascular endothelium is responsible for the vasodilator tone that is essential for the regulation of blood pressure. In the central nervous system NO is a neurotransmitter that underlies a number of functions, including the formation of memory. In the periphery, there is a widespread network of nerves, previously recognised as nonadrenergic and noncholinergic, that operate through an NO-dependent mechanism to mediate some forms of neurogenic vasodilatation and regulate various gastrointestinal, respiratory, and genitourinary tract functions. Nitric oxide also contributes to the control of platelet aggregation and the regulation of cardiac contractility. These physiological effects of NO are all mediated by the action of a constitutive NO synthase and subsequent activation by NO of the soluble guanylate cyclase (for review see 1).

In addition, NO is produced in large quantities by an inducible NO synthase (iNOS) during host defence and immunological reactions. Because it has cytotoxic properties and is generated by activated macrophages, NO is likely to have a role in nonspecific immunity. Indeed, mice in which the iNOS gene has been disrupted show increased susceptibility to parasite infection as well as reduced non-specific inflammatory responses (2). The induction of iNOS has recently been demonstrated in human monocytes/macrophages following expression and ligation of the low affinity receptor for IgE. The induction of iNOS correlates directly with the intracellular killing of Leishmania major by these cells (3).

Nitric oxide produced by iNOS is involved in the pathogenesis of conditions such as septic shock and perhaps also the hyperdynamic state of

cirrhosis and in inflammation. Recent observations have shown that the generation of NO in human breast cancer correlates positively with tumour grade (4). It has also been shown that tumours from a human tumour cell line, genetically engineered to generate NO continuously, grew faster and were more vascularised than those from wild-type cells (5). However, the NO-generating cells grew more slowly in vitro than did the wild-type cells, suggesting that NO may have a dual pro- and anti-tumour action, depending on the local concentration of the molecule.

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Physiology and Pathophysiology of Cardiac NO Signalling Pathways

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NO is formed by the oxidation of one of two equivalent guanidino nitrogens in L-arg by O_2 to form NO and L-citrulline. This reaction is catalyzed by a family of enzymes termed NO synthases. Three distinct isoforms of NOS have been identified, each the product of a separate gene. Neuronal and endothelial NOS isoforms are constitutively present in neural and endothelial cells, respectively, as well as other cell types, and a cytokine-inducible form (iNOS) has been characterized from macrophages and numerous other cell sources. Each of these three isoforms demonstrates 50-60% amino acid homology with one another.

Recent observations from this and other laboratories indicate that a constitutive form of NOS present in cardiac myocytes generates NO that appears to modulate both β-adrenergic and cholinergic signalling pathways.

We and others have also reported that induction of NOS isoforms in cardiac muscle may underlie cytokine-mediated abnormalities in myocardial contractile function. When induced and activated, iNOS is capable of generating substantial quantities of NO, resulting in the expected cascade of guanylyl cyclase activation. The specific mechanism or mechanisms by which endogenous NO production produces contractile dysfunction in the heart are under continuing study.

In this presentation, new data identifying the constitutive NOS in adult rat cardiac myocytes as the endothelial isoform (ecNOS) will be presented, as well as evidence that muscarinic cholinergic inhibition of L-type calcium channel conductance in cardiac cells involves endogenous NO production resulting in guanylyl cyclase activation and enhanced cAMP breakdown by a cGMP-activated phosphodiesterase. New evidence will also be presented suggesting that NO is capable of interfering with energy production in addition to its effects on signalling pathways in the heart.

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MODULATION BY NEURONAL NITRIC OXIDE OF STRESS-INDUCED CHANGES IN GASTROINTESTINAL FUNCTION

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There is now considerable evidence that NO plays an important role in both physiological and pathological events within the gastrointestinal tract. Thus, endogenous NO is involved in the modulation of blood flow under resting and stimulated conditions (1), and has a crucial role in the regulation of mucosal integrity in the stomach (2) and intestine as well as in intestinal secretion and ion transport. Furthermore, substantial evidence indicates that NO is implicated in the regulation of gastrointestinal motility, mediating the non adrenergic-non cholinergic relaxation of smooth muscle (3), and modulates spontaneous motility and peristalsis. NO may interact with other neurotransmitters, not only in the modulation of gastrointestinal motility, but also in the regulation of vascular tone. Thus, NO may serve as a cotransmitter released from nerves in close proximity to the gastrointestinal vasculature, may exert prejunctional actions on neuronal activity, or may be involved in the activation of neurogenic inflammatory responses. Both the constitutive and the inducible NO synthase have been widely found in gastrointestinal tissue.

Recent studies have suggested a role for NO in the alterations of gastrointestinal functions induced by stress. Thus, stress induced by low doses of endotoxin inhibited gastric acid secretion and protected the integrity of the gastric mucosa: This process required the synthesis of NO (4, 5) and the integrity of the peripheral nervous system (6). Further studies with intracerebroventricular administration of a NO synthesis inhibitor revealed that the effects of endotoxin involved the local generation of nitric oxide in the central nervous system (7). Such NO formation may be involved in intracellular communication by acting as a neuromodulator or neurotransmitter that leads to a gastric reponse to stress. A similar activation of this NO-mediated neural reflex has recently been shown with various other validated stress models (8). These findings suggest that stress activates a neural reflex involving synthesis of neuronal NO that mediates the defense of the gastrointestinal tract against injury and reduces the level of the endogenous aggression represented by gastric acid.

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SECOND SESSION: MOLECULAR APPROACHES

Chairman: Santiago Lamas

LIFE WITHOUT INDUCIBLE NITRIC OXIDE SYNTHASE: INCREASED VULNERABILITY TO INFECTION AND PARTIAL PROTECTION AGAINST ENDOTOXIC SHOCK

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Given inducible nitric oxide synthase's (iNOS) widespread cellular expression, its co-ordinate regulation by multiple agonists, its proximal and distant effects on a large number of target proteins and cell types, its paradoxically protective and pathologic consequences - can you imagine life without it? Previous attempts to induce an iNOS deficiency in mammals by pharmacologic means have been fraught with problems of non-specificity, toxicity, incomplete inhibition and ultimately, interpretation. In an attempt to bypass these problems, we generated mice defective in iNOS by homologous recombination in ES cells.

Viable, homozygous iNOS -/- mice survived weaning at the expected Mendelian ratio, developed normally as assessed by gross, histopathologic, lymphohematologic and clinical biochemical analyses, and were fertile. Their iNOS deficiency was verified by Southern blot, the absence of iNOS RNA in tissues and macrophages following induction with LPS± rMuIFN-γ, undetectable immunoreacting protein in macrophages by Western blot, and loss of enzyme activity in response to an array of LPS ± cytokine agonists. iNOS -/- mice remained globally responsive to these same eliciting agents as indicated by macrophage H_2O_2 secretion, MHC Class II upregulation and peritoneal leukocyte accumulation.

Discrepancies between iNOS -/- mice and their wild type littermates emerged when challenged with microbes or LPS. Mice defective in iNOS allowed the replication of 100-fold more *Listeria monocytogenes* in both liver and spleen compared with control animals. Similary, macrophages from iNOS -/- mice failed to restrict the *in vitro* growth of the murine lymphoma line, L1210. During endotoxic shock, however, iNOS's detrimental activities became evident. Injection with LPS resulted in a substantial fall (64% by 3.5 hr p.i.) in central arterial blood pressure followed by mortality in anesthetized wild type mice, whereas iNOS -/- mice were markedly protected from both hypotension (16% fall by 2.5 hr which thereafter stabilized) and survived the experiment. Other indices of endotoxicity, namely, elevated hepatic serum enzyme levels (ALT, ASP and LDH) and cytokines (TNF-α, IL-1β and IL-6) were unaltered by iNOS deficiency, while serum nitrate was greatly diminished. Sensitization with heat-killed *Proprionobacterium acnes* 6 days prior to injection of unanesthetized mice with LPS resulted in a rapid demise (<12 hours) of wild type and iNOS -/- mice without the Istter's increase in serum nitrate. Histopathologic examination of both genotypes showed pronounced margination of polymorphonuclear leukocytes in the lung and liver, multifocal hepatocellular necrosis and disseminated intravascular coagulation. Thus it appears that while iNOS is chiefly responsible for LPS-induced hypotension, iNOS-independent pathways can be sufficient for endotoxic death.

REGULATION OF ARGININE TRANSPORT IN CYTOKINE-ACTIVATED MACROPHAGES

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The production of NO in cytokine-activated macrophages is dependent on extracellular arginine. Therefore, transport proteins in the plasma membrane must supply these cells with arginine for use as substrate in the synthesis of NO. We have studied three related carrier proteins (MCAT for mouse cationic amino acid transporters) that transport cationic amino acids, including arginine, across the plasma membrane. We investigated the expression of these transporters in the mouse macrophages upon induction of NOS expression and nitric oxide synthesis by γ-interferon (γINF), lipopolysaccharide (LPS), or both. Twelve hours after activation, a 7-fold increase in arginine uptake was observed in LPS and γINF-stimulated cells that correlated with expression of one of the transporters (MCAT-2B). MCAT-1 was expressed in uninduced cells, but its expression did not change upon activation. Expression studies in *Xenopus* oocytes demonstrate that the kinetic properties of MCAT-1 and MCAT-2B are consistent with the reported properties of arginine uptake in macrophages. Our findings suggest that MCAT-1 is responsible for baseline arginine transport and that induction of MCAT-2B expression accounts for the additional arginine uptake in activated macrophages during NO production.

THIRD SESSION: ENDOTHELIAL NITRIC OXIDE SYNTHASE (ecNOS)

Chairman: Salvador Moncada

BIOSYNTHESIS AND POST-TRANSLATIONAL REGULATION OF ENDOTHELIAL NITRIC OXIDE SYNTHASE

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The endothelial isoform of nitric oxide synthase (ecNOS) plays a central role in intercellular signaling in the vascular wall, and coordinates diverse extracellular stimuli to modulate blood pressure and platelet aggregation. The level of ecNOS expression may be an key determinant of its roles in vascular signaling, and ecNOS synthesis may be importantly regulated at transcriptional, post-transcriptional and post-translational levels. The promoter region of the ecNOS gene contains candidate *cis*-acting regulatory regions that may modulate gene expression in response to changes in hemodynamic shear stress or sex hormones[1], and levels of ecNOS mRNA may further be subject to dynamic regulation by cytokines [2]. The ecNOS is co-translationally modified by N-terminal myristoylation [3], and undergoes homo-oligomerization in the cell cytosol. The ecNOS protein undergoes several post-translational modifications that modulate its signaling roles, including phosphorylation[4] and palmitoylation[5].

The ecNOS is distinct among the NOS isoforms in being modified by N - terminal myristoylation, which is necessary for targeting the enzyme to the endothelial cell membrane. Membrane targeting of ecNOS is primarily stabilized by hydrophobic interactions between the enzyme's myristate moiety and membrane phospholipids[6]. In bovine aortic endothelial cells, ecNOS is phosphorylated on a serine residue(s); diverse stimuli promote phosphorylations that can be mapped to the same tryptic phosphopeptide. In response to agonists, such as bradykinin, ecNOS translocates from membrane to cytosol, accompanied by an increase in enzyme phosphorylation, but with no change in ecNOS myristoylation. Thus, myristoylation is necessary but not sufficient for ecNOS membrane association; furthermore, myristoylation is not required for ecNOS phosphorylation. The ecNOS also undergoes palmitoylation; bradykinin promotes de-palmitoylation of the enzyme, and this is the likely mechanism for agonist-promoted translocation of ecNOS. The ecNOS now joins a growing list of signaling proteins, including G protein alpha subunits, G protein-coupled receptors, and members of the Ras and Src family, that all are complexly regulated by dual acylation plus phosphorylation.

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REGULATION OF THE ENDOTHELIAL NITRIC OXIDE SYNTHASE BY SHEAR STRESS AND RECEPTOR-DEPENDENT AGONISTS

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The nitric oxide (NO) synthase isoform constitutively expressed in endothelial cells is a Ca^{2+}/calmodulin-dependent enzyme. Thus, although NO synthase activity may be modified by alterations in intracellular pH, the intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$) is the main determinant of endothelial NO production.

We have recently shown that modulating tyrosine phosphorylation in endothelial cells, using either inhibitors of tyrosine kinases or tyrosine phosphatases, can significantly influence Ca^{2+} signalling and thus endothelial NO synthase activity. Our data demonstrate that the inhibition of tyrosine kinases, and the subsequent reduction in tyrosine phosphorylation, suppresses Ca^{2+} influx whereas inhibition of tyrosine phosphatases significantly increases $[Ca^{2+}]_i$ and consequently results in the endothelium-dependent vasodilatation of isolated arteries. Agonist-induced increases in $[Ca^{2+}]_i$ also result in the tyrosine phosphorylation and activation of MAP kinases in the cytosol [1] and may provide a link between agonist stimulation and modulation of gene expression in endothelial cells.

Fluid shear stress represents the physiologically most important mechanism controlling endothelial NO production. In cultured endothelial cells, increases in shear stress are associated with relatively transient (15 min) increases in $[Ca^{2+}]_i$ and pH_i, both of which are known to enhance NO synthase activity. In a bioassay system, we could demonstrate that prolonged periods of shear stress (60 min) result in the continuous production of NO which can be separated into two components, the first being transient and Ca^{2+}-dependent while the second is sustained and Ca^{2+}-independent. This finding supports the concept that in addition to $[Ca^{2+}]_i$ and pH_i, a further mechanism is involved in the control of NO synthase activity by shear stress. This mechanism may involve a change in the orientation of the enzyme within the membrane, translocation from the membrane to the cytosol or the involvement of an, as yet unidentified, signal transduction pathway. In addition to these acute effects, shear stress also plays a role in the more chronic regulation of NOS activity. This concept is supported by the observation that expression of the NO synthase as well as the basal release of NO is significantly greater in native endothelial cells, continuously exposed to shear, than that in cultured endothelial cells maintained under static conditions [2]. Moreover, the expression of NOS in cultured endothelial cells can be upregulated by exposure to shear stress for several hours, suggesting that NOS levels may

be maintained *in vivo* as a consequence of the continuous activation of the endothelium by shear stress. Indeed, the distribution of NO synthase along the main stem of the arterial tree also appears to be determined by shear stress since higher levels of NO synthase protein were detected in porcine endothelial cells from the aortic arch, where pulsatile shear stress levels are greatest, and to decrease towards the abdominal aorta and peripheral arteries. Shear stress-induced activation of the MAP kinases may play a role in maintaining NO synthase expression since both the 42- and 44-kDa isoforms are tyrosine phosphorylated following the application of shear stress to cultured endothelial cells.

NO is also able to modulate vascular functions by altering the expression of certain endothelial proteins as well as the genes which encode them. NO mediated regulation of adhesion molecule expression appears to play a crucial role in inflammation and early atherogenesis and effects the expression of both P-selectin [3] and monocyte chemoattractant factor (MCP-1) [4]. We have observed that inhibition of NO synthesis results in the activation of the transcription factor NFκB (as assessed by gel mobility shift assay) an effect that can be reversed by exogenously applied NO [4]. An enhanced production of oxygen radical species associated with hypercholesterolemia-induced oxidative stress may be responsible for a decrease in the local concentration of NO and may, via activation of NFκB, account for the enhanced expression of other adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1). It cannot be excluded that NO also influences the activation of other transcription factors such as AP-1, which has been characterised as antioxidant responsive factor. Such observations suggest that the effects of NO on gene expression may be the consequence of a direct and/or indirect interaction with transcription factors and underline the significance of endothelium-derived NO, both in short-term and long-term vascular homeostasis.

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Expression and expressional regulation of constitutive NO synthases in various cell types

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Three isozymes of nitric oxide synthase (NOS) have been identified. While most cell types can be stimulated to express the inducible NOS II (or i-NOS), the constitutive isoforms NOS I (or nc-NOS) and NOS III (ec-NOS) are only expressed by specific cells. Nevertheless, the expression of these isoforms can be subject to regulation.

NOS I is present in specific neurons of the brain [1]. NOS I is also expressed in peripheral nitrergic nerves [2-4], in sympathetic ganglia and adrenal glands [5, 6], in certain areas of the spinal cord [7], in epithelial cells of lung, uterus and stomach [8], in pancreatic islet cells [8], in kidney macula densa cells [8], and in human, rat and guinea pig skeletal muscle [9-11]. In many neurons, NOS I is colocalized with other neurotransmitters (or neurotransmitter-synthesizing enzymes). For example, in dentate hilar neurons of the rat hippocampus, NOS I is colocalized with somatostatin [12], and in the peripheral autonomic nervous system, NOS I was found in pre- and postganglionic sympathetic neurons together with choline acetyltransferase and dopamine-β-hydroxylase, respectively [5, 6]. NOS I is considered a constitutive enzyme, but there are some indications for expressional regulation. One report suggested that estrogens can upregulate NOS I mRNA and activity in various tissues [13]. Following axotomy, a long-lasting upregulation of NOS I expression (up to 159 days) has been observed in rat brain [14]. In most brain regions investigated, enhanced NOS I expression was associated with an increased expression of the transcription factor c-jun [14]. Occlusion of the middle cerebral artery of the rat led to an upregulation of NOS I mRNA and immunoreactive protein [15]. NOS I in neuronal cells is also subject to expressional regulation during ontogeny (Dun and Förstermann, unpublished).

NOS III was first identified in endothelial cells [16, 17]. Immunohistochemical studies located the enzyme to various types of arterial and venous endothelial cells in many tissues, including human tissues [18]. NOS III-immunoreactivity has also been detected in LLC-PK_1 kidney tubular epithelial cells [19], syncytiotrophoblasts of human placenta [20], and interstitial cells of the canine colon [21]. Surprisingly, NOS III-immunoreactivity has also been described in neurons of the rat hippocampus and other brain regions [22]. Multiple compounds and conditions have been described in recent years that regulate the expression of NOS III. Shear stress produced by the flowing blood not only increases the *activity* of endothelial NOS III, it also upregulates NOS III *expression* [23]. A putative shear stress-responsive element has been described in the promoter sequence of NOS III [24]. In cultured endothelial cells, TNF-α downregulates NOS mRNA, protein and activity [23, 25, 26]. In our bovine aortic endothelial cell line, no parallel induction of NOS II was observed, indicating that the downregulation of NOS III occurs independently from NOS II induction [26]. TNF-α has recently been described to destabilize the NOS III mRNA with no effect on transcription [27]. In guinea pigs, near term pregnancy and treatment with estradiol (but not progesterone) increased calcium-dependent NOS activity and NOS III mRNA in various tissues [13]. Incubation of human endothelial cells (EA.hy 926) [28] with 17β-estradiol increased the NOS II mRNA concentration 4 fold (Kleinert and Förstermann, unpublished). These data point to a stimulatory effect of estrogens on NOS III expression. The molecular mechanisms of this regulation are currently being investigated. Also TGF-β1 produces a modest upregulation of NOS III expression in endothelial cells (Förstermann and Kleinert, unpublished). Endothelial NOS III immunoreactivity was markedly upregulated in cerebral blood vessels during cerebral ischemia [29]. NOS III mRNA and protein is also increased in growing versus resting bovine aortic endothelial cells [30]

These data indicate that the "constitutive" isoforms of NO synthase are expressed in cell types other than the ones in which they were originally detected, and that the expression of both isoforms is regulated in various ways.

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FOURTH SESSION: BIOCHEMISTRY I

Chairman: Thomas Michel

NITRIC OXIDE SYNTHASE MECHANISM AND CALMODULIN RECOGNITION

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Nitric oxide synthase (NOS) catalyzes the overall oxidation of L-arginine to citrulline and nitric oxide (•NO). Co-substrates for the reaction include O_2 and NADPH. NOS has a bound FAD and FMN, sequence homology to cytochrome P450 reductase and a P450-type heme. In addition, there is a requirement for (6*R*)-tetrahydro-L-biopterin (H_4B). Two distinct constitutive isoforms that are Ca^{2+} and calmodulin dependent and an inducible form that co-purifies with calmodulin have been isolated. The ability of NOS to utilize peroxides in place of O_2 and NADPH was investigated with both L-arginine and N^G-hydroxy-L-arginine (L-NHA) as substrates (Pufahl et al., 1995). H_2O_2 was able to support citrulline formation only with L-NHA as a substrate. Stable isotope labeling experiments with [^{18}O]-H_2O_2 established incorporation of oxygen into the ureido position of citrulline. Further product analysis was consistent with the formation of nitroxyl (NO^-). These finding lend support to a proposed NOS mechanism. The inducible isoform of nitric oxide synthase (iNOS) isolated from murine macrophages co-purifies with calmodulin (CaM) as a tightly bound subunit (Cho et al., 1992). The exact function of this tightly bound CaM, however, has not been elucidated. In order to probe the function of this unusually strong interaction between iNOS and CaM, a 30 amino acid peptide derived from the putative CaM binding site of iNOS was synthesized. Crosslinking and autoradiographic analyses demonstrated that the peptide and CaM form a 1:1 complex as well as several higher molecular weight complexes (Stevens-Truss & Marletta, 1995). When assayed in the presence of a 12-fold excess of peptide to iNOS, over 90% of the enzymatic activity was inhibited. This inhibition could be prevented with the addition of exogenous bovine CaM to the assay mixture, in a concentration-dependent manner. Native PAGE and Western blot analysis of iNOS treated with peptide revealed the formation of a peptide-CaM complex with CaM derived from iNOS. Moreover, EGTA (5 mM) caused a 30% maximal inhibition of activity that was reversed by the addition of exogenous Ca^{2+} in a concentration-dependent fashion suggesting a role for Ca^{2+} in this interaction. EGTA also changed the native PAGE mobility of iNOS and increased the intensity of a band which comigrates with CaM. These results demonstrate that the binding interaction between CaM and iNOS is tight but reversible, requires Ca^{2+}, and is atypical from other known CaM-enzyme interactions (Stevens-Truss & Marletta, 1995).

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Distinctive Enzymological Features of Brain Nitric Oxide Synthase

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Oxidation of L-arginine to L-citrulline and nitric oxide (NO) is catalyzed by a family of NO synthase (NOS) isozymes which exhibit unique complexity in their requirement for cofactors. As in other cytochromes P-450, ferro-protoporphyrin IX is involved in reductive oxygen activation which is coupled to a two-step oxidation of the substrate. The overall reaction requires input of three reducing equivalents which are shuttled from the redox-active cofactor NADPH to the heme by the flavins FAD and FMN. Thus, NOSs seem to represent self-sufficient P-450s with a flavin-containing reductase domain delivering NADPH-derived electrons to the prosthetic heme group. Tetrahydrobiopterin (H_4biopterin) represents an additional coenzyme of NOS with as yet unknown function. Current evidence suggests that the pteridine exhibits both allosteric and redox-active effects. Thus, H_4biopterin is required for assembly of the active dimeric form of inducible macrophage NOS (1), converts brain NOS into an high affinity state (2), and perhaps serves as reductant in the first step of L-arginine oxidation yielding N^G-hydroxy-L-arginine (3). The heme-site inhibitor 7-nitroindazole was found to compete with H_4biopterin binding to brain NOS (2), pointing to an interaction of the pteridine domain with the catalytic center of the enzyme. Based on sequence similarities with dihydrofolate reductase, it has been suggested that the H_4biopterin binding site may be located between the heme and calmodulin site of NOS, starting about 150 amino acids downstream from Cys_{415}, the axial ligand of the heme iron (J.C. Salerno, meeting abstracts). We have tested several synthetic peptides derived from this sequence, which has been strongly conserved within the different NOS isoforms, but none of the peptides interfered with H_4biopterin or substrate binding. Inhibition of L-citrulline formation and NADPH oxidation by a peptide corresponding to the amino acids 564-582 of brain NOS was non-competitive with L-arginine and H_4biopterin, also suggesting that this sequence is not part of the substrate or pteridine binding domains but is essentially involved in enzymatic oxygen reduction, which occurs in an L-arginine and H_4biopterin-independent manner (4). The inhibitory effect of $peptide_{564-582}$ was critically dependent on NOS concentration, with virtually no inhibition being observed at ≥50 µg NOS/ml, indicating that the peptide binds to a NOS conformation occurring at low enzyme concentrations, only. Further attempts are being made in our laboratory to identify the pteridine domain of NOS by mutational analysis and biochemical characterization of the recombinant rat brain enzyme expressed in baculovirus-infected insect cells (5).

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TRANSCRIPTIONAL REGULATION OF INFLAMMATORY NITRIC OXIDE SYNTHASE BY ANTIOXIDANTS, METAL CHELATORS, PROTEASOME INHIBITORS AND NONSTEROIDAL ANTIINFLAMMATORY DRUGS.

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Nitric oxide (NO) synthase (NOS) isoforms that are constitutively expressed, such as the neuronal (nNOS) and endothelial (eNOS) isoforms, are regulated by calcium availability, whereas inflammatory NO synthase (iNOS) is an inducible isoform that is calcium-independent and is regulated at least in part at the transcriptional level. Calcium promotes the binding of calmodulin to nNOS and eNOS to initiate catalytic activity. iNOS already has calmodulin tightly bound as a subunit and, therefore, calcium is essentially not required for catalytic activity. All three NOS isoforms can be regulated by negative feedback mechanisms mediated by NO itself. Both enzymatically generated NO and exogenous NO inhibit NOS activity by mechanisms that appear to involve heme-dependent oxidation of L-arginine to NO and L-citrulline and NOS inhibition is prevented and reversed by tetrahydrobiopterin (1-4). The iNOS appears to be more resistant than either nNOS or eNOS to the negative action of NO.

One of the transcriptional mechanisms of iNOS expression appears to involve nuclear factor kappa B (NF-kB), a nuclear transcription factor that plays an important role in the inducible expression of many genes encoding proteins engaged in immune defense and inflammation. This is consistent with the pathophysiological role of NO as an inflammatory mediator and immunomodulator. Extracellular signals including lipopolysaccharide (LPS) and certain cytokines (IL-1β and TNF-α) trigger the formation of oxygen radicals, which in turn initiate the rapid activation of NF-kB. Oxygen radicals trigger phosphorylation and proteolytic reactions that cause NF-kB to dissociate from its inhibitory IkB complex in the cytoplasm. Free NF-kB then translocates to the nucleus where it binds to the promoter of the iNOS gene to stimulate transcription. In order to further explore the role of NF-kB in the inducible expression of the iNOS gene, we examined the influence of antioxidants and protease inhibitors on NO formation and iNOS expression in LPS-activated rodent macrophages. Pyrrolidine dithiocarbamate, a weak antioxidant with metal chelating properties, inhibited NO formation (NO_X^- accumulation in cell culture medium) (5) and iNOS expression (recoverable iNOS activity from intact cells; Northern blot analysis). Other antioxidants, however, were not effective, whereas iron and copper chelating agents (desferrioxamine, bathophenanthroline, D-penicillamine, bathocuproine) were very effective in preventing or abolishing iNOS expression and NO formation. H_2O_2 and O_2^- enhanced LPS-induced expression of iNOS, and this effect was further enhanced by SOD, thereby suggesting that O_2^- serves merely as a precursor to H_2O_2, which represents the reactive oxygen species that enhances iNOS expression. Inhibitors of chymotrypsin-like cysteine proteases or chymotrypsin itself markedly inhibited iNOS expression. These data suggest that specific proteases belonging to the proteasome or multicatalytic protease complex are involved in iNOS expression. These observations support the view that NF-kB activation is intimately involved in transcription of the iNOS gene in LPS-activated rodent macrophages.

Since NO is pro-inflammatory and NOS inhibitors have been shown to possess antiinflammatory properties and the transcriptional expression of iNOS involves pathways that are also involved in the inducible expression of genes that encode proteins engaged in immune defense and inflammation, we examined the possibility that nonsteroidal antiinflammatory drugs (NSAIDs) might interfere with high-output NO production by iNOS in macrophages (6). Aspirin, sodium salicylate, ibuprofen, indomethacin, diclofenac and naproxen each inhibited NO_X^- formation by LPS-activated macrophages. NSAIDs did not directly alter iNOS catalytic activity and did not injure or kill the cells. Instead, NSAIDs decreased iNOS activity recoverable from intact cells when the drugs were added to cell cultures at or before LPS addition but not 6-hr after LPS addition. Northern blot and Western blot analyses revealed that NSAIDs interfered with transcription of DNA to iNOS mRNA, thereby preventing synthesis of iNOS protein. The molecular mechanism of action of NSAIDs appears to be inhibition of activation of NF-kB, as assessed by electrophoretic mobility shift assays. In vivo experiments using rats showed that LPS-induced accumulation of NO_X^- (approx. 10-fold) in blood at 5-hr after LPS administration (2 mg/kg; ip) is largely prevented by ip administration of NSAIDs 1-hr prior to LPS.

These studies support the view that NF-kB activation represents one important transcriptional mechanism by which LPS induces iNOS activity in rodent macrophages. The mechanism of NF-kB activation appears to involve H_2O_2 and a metal such as iron or copper, which are in turn involved in triggering a proteolytic reaction to free NF-kB from its inhibitory complex in the cytoplasm. The novel finding that NSAIDs inhibit iNOS expression by interfering with NF-kB activation represents a second mechanism of antiinflammatory action of NSAIDs that is distinct from inhibition of cyclooxygenase activity and prostaglandin production.

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FIFTH SESSION: BIOCHEMISTRY II

Chairman: Michael A. Marletta

Nitric Oxide and the Regulation of Iron Metabolism

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Regulation of iron metabolism is a fundamental process with profound medical implications. Cell growth and erythropoiesis are most critically affected by iron deficiency, while iron overload predominantly causes hepatic and degenerative disease. The underlying molecular control mechanisms are becoming increasingly well understood. Cellular iron uptake via the transferrin receptor and intracellular storage within a molecule consisting of 24 ferritin H- and L subunits is posttranscriptionally regulated through iron-responsive elements (IREs) contained in the untranslated regions of the respective mRNAs. IREs are also found in the messages encoding a rate-limiting enzyme for erythroid heme synthesis, 5-aminolevulinate synthase (eALAS), as well as mitochondrial aconitase, a Krebs' cycle enzyme of critical importance for cellular metabolic activity. These IRE-containing mRNAs are regulated by binding of a cytoplasmic protein, iron regulatory protein (IRP). IRP binding reduces the translation rates of ferritin, eALAS and aconitase mRNAs and indirectly increases the synthesis of transferrin receptors by stabilizing the mRNA against degradation.

IRP binding to IREs is regulated by multiple cellular signals: first by cellular iron levels, second by nitric oxide (NO), and third by oxidative stress (in the form of hydrogen peroxide, H_2O_2). Iron deficiency, NO and H_2O_2 independently activate IRE binding by inducing the posttranslational conversion of 4Fe-4S IRP to apoIRP. Remarkably, the induction kinetics for NO exceed those for iron starvation. The mechanism of IRP activation by NO will be discussed.

Thus, a regulatory network that controls iron homeostasis as well as heme synthesis and that affects the Krebs' cycle is operated by NO via IRP, providing a framework for understanding physiological and pathological responses that affect cell growth and metabolism as well as erythropoiesis.

Regulation of tetrahydrobiopterin biosynthesis in mammalian cells

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Tetrahydrobiopterin, a cofactor for aromatic amino acid hydroxylases, alkyl glycol ether monooxygenase and for nitric oxide synthases (NOSs), is formed from guanosine 5'-triphosphate (GTP). The first and rate-limiting step of this biosynthetic pathway is catalyzed by GTP cyclohydrolase I (EC 3.5.4.16), yielding 7,8-dihydroneopterin triphosphate. Two further enzymes, i.e. 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase, accomplish the formation of tetrahydrobiopterin (for a review see reference 1,2).

Several years ago, stimulation of GTP cyclohydrolase I activity leading to increased excretion of neopterin was observed in patients suffering from malignant disease or viral infections. It was shown that macrophages challenged with interferon-γ (IFN-γ) are the chief source of neopterin, which originates from 7,8-dihydroneopterin triphosphate, the first intermediate of tetrahydrobiopterin biosynthesis. Neopterin is found exclusively in primates but not in other mammalian species (reviewed in reference 3).

Detailed in vitro studies then showed that IFN-γ stimulates pteridine biosynthesis in various mammalian cell types including endothelial cells, fibroblasts, and tumour cell lines from different tissues. Neopterin and tetrahydrobiopterin are formed at cell- and species-specific ratios. The ratio of the two pteridines is dependent on the activity of 6-pyruvyol tetrahydropterin synthase, the second enzyme of tetrahydrobiopterin synthesis, in relation to GTP cyclohydrolase I activity (for a review see refernce 3). Besides IFN-γ, also tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1) and bacterial lipopolysaccharide (LPS) increase GTP cyclohydrolase I activity and mRNA levels either as a single stimulus or in combination with IFN-γ. A number of deactivating cytokines, i.e. IL-4, IL-10 and transforming growth factor-ß (TGF-ß) down-regulate GTP cyclohydrolase I (reviewed in reference 4).

By pharmacological depletion and reconstitution of tetrahydrobiopterin from intact cells it was shown that a functional role for cytokine-induced tetrahydrobiopterin biosynthesis in peripheral cells is to supply a cofactor for NOSs (3,4). Apparently, expression of GTP cyclohydrolase I and inducible NOS (iNOS) are co-ordinately regulated. However, while certain molecular mechanisms involved in iNOS expression in rodent cells have been recently elucidated (reviewed in 5), regulatory mechanisms of GTP cyclohydrolase I expression have not yet been studied in detail. Using PCR-generated probes, we studied GTP cyclohydrolase I expression in a human (THP-1) and a murine (J774A.1) monocytic cell line. Our data suggest species-specific differences in size and regulation of GTP cyclohydrolase I mRNA.

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SYNTHESIS, STORAGE AND TRANSPORT OF NO IN THE SALIVARY GLANDS OF BLOOD SUCKING HEMIPTERA - J.M.C. Ribeiro. Department of Entomology, University of Arizona, Forbes Bldg. Rm. 410, Tucson AZ, 85721, USA. FAX: (602) 621-1150. E-mail Ribeiro@ccit.arizona.edu

Blood sucking arthropods face their host's hemostatic system when attempting to feed. Accordingly, it is of selective advantage to these animals to produce anticlotting, anti-platelet and vasodilatory compounds that can neutralize the hemostatic response. A variety of such compounds are indeed injected in the form of saliva into their host's skin while the arthropod is attempting to feed or during feeding [1].

Because the blood sucking behavior is polyphyletic, a convergent evolutionary scenario allowed the development of a large diversity of such compounds. Vasodilatory prostaglandins, peptides, enzymes and even NO were "discovered" by such animals in their quest for blood. The blood sucking hemitera *Rhodnius prolixus* (reduvidae) and *Cimex lectularius* (cimicidae) both contain NO as vasodilators in their salivary glands, which is stored as a nitrosyl-Fe^{3+}hemeprotein[2-3], a new functional class of proteins named nitrophorins.

After a meal which may lead to the intake of 10 times its own weight in blood, 4th instar *Rhodnius* nymphs discharges over half of its salivary content in its host. The nitrophorins are synthesized before the molt, but they remain not charged with NO. At about molting time, an increase in diaphorase activity is observed. Concomitant with an increase in the salivary diaphorase activity, *Rhodnius* nitrophorins are charged with NO. Electron microscopic experiments indicate an intracellular/intravesicular location for the salivary diaphorase. A fine microvillar structure found in the intraluminal surface of *Rhodnius* salivary epithelium indicates the possibility that such membrane system may direct the secretion of NO preferentially to the lumen[4].

Rhodnius contain at least 4 salivary nitrophorins that probably arose by gene duplication[5]. These four nitrophorins have been sequenced and their comparison may yield information on important domains affecting heme and NO binding. Interestingly, one or the nitrophorins is also an anti factor VIII anticlotting[6]. Comparison of the evolutionary divergence between *Rhodnius* nitrophorins may also indicate the probable time that this insect had the selective pressure to increase NO secretion and thus blood-feeding.

Cimex nitrophorin has been characterized to be similar to that of *Rhodnius*, but after purification and cloning, a completely different structure was found. While *Rhodnius* nitrophorins are 19-20 kDa in size and have landmarks indicating relatedness to invertebrate hemoglobins, *Cimex* nitrophorin is a 32 kDa protein containing 30% identity and 50% similarity to human platelet inositol-tris-phosphate phosphatase (Valenzuela and Ribeiro, submitted). Because *Cimex* and *Rhodnius* evolved to blood-feeding independently, each co-opted a different molecule to become the NO carrier. Perhaps *Cimex* is indicating to us a connection between the IP and NO pathways.

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SIXTH SESSION: SIGNAL TRANSDUCTION

Chairman: Thomas W. Smith

Nitric oxide inhibits α_1 adrenergic receptor-induced proto-oncogene expression in rat aorta. BB Hoffman. Stanford University School of Medicine and VA Medical Center, Geriatrics Research, Education, and Clinical Center (182B), 3801 Miranda Ave, Palo Alto, California, USA 94304.

α_1 adrenergic receptors play an important role in regulation of blood vessel contraction. In addition, there is evidence suggesting that catecholamines promote the growth of arterial smooth muscle by activation of α_1 adrenergic receptors (1-3). In rat aorta activation of α_1 adrenergic receptors markedly induces *c-fos* gene expression (4). *c-fos* and *c-jun* are rapidly induced by phenylephrine, angiotensin II and platelet-derived growth factor and are thought to be associated with vascular hypertrophy (5).

Vasodilatation induced by acetylcholine and bradykinin are dependent on the presence of endothelium. The substance inducing these relaxation, termed endothelium-derived relaxing factor (EDRF), has been identified as nitric oxide (NO). EDRF-induced vasodilatation is mediated by cGMP although other mechanisms could be involved. cGMP-dependent vasodilators have been shown to have anti-proliferative effects in aortic smooth muscle cells. Consequently, the endothelium plays an important role in controlling smooth muscle cell growth.

Restenosis of coronary vessels after percutaneous transluminal coronary angioplasty is a frequent and major clinical problem. An important feature of restenosis after percutaneous transluminal coronary angioplasty is that injury to the endothelium apparently promotes neointima formation which contributes to narrowing of coronary arteries. It has been found that an angiotensin converting enzyme inhibitor as well as an antisense oligonucleotide against *c-myb* inhibit formation of neointima after balloon injury in animal models. In addition, the adrenergic α_1 adrenergic receptor antagonist prazosin inhibits neointimal hyperplasia after balloon injury (6, 7). These approaches presumably inhibit the effects of trophic factors, such as angiotensin II and catecholamines, on smooth muscle cell growth and proliferation. However, relatively little is known about the role of the endothelium in modulating the growth promoting effects of hormones and neurotransmitters.

The purpose of our studies was to investigate the potential expression and role of alpha$_1$ adrenergic receptor subtypes in rat aorta and cultured rat aortic smooth muscle cells in inducing the expression of *c-fos* gene and to investigate the possibility that NO might inhibit these responses. It is not known which subtypes of alpha$_1$ adrenergic receptors are involved in the growth of smooth muscle cells. Using an *in vitro* preparation of rat aorta and cultured vascular smooth muscle cells, we investigated and compared alpha$_1$ adrenergic induction of *c-fos* mRNA. Phenylephrine, a selective alpha$_1$ adrenergic receptor agonist, caused a rapid and transient increase in *c-fos* mRNA accumulation which was inhibited by prazosin, an alpha$_1$ receptor antagonist. Similarly, phenylephrine stimulated *c-jun* and *c-myc* mRNA accumulation. Chlorethylclonidine, a compound which irreversibly blocks alpha$_{1B}$ receptors, completely blocked the phenylephrine-induced increase in *c-fos* mRNA. RNase protection experiments demonstrated that rat aorta prominently expressed mRNA for alpha$_{1B}$ and alpha$_{1D}$ receptors as well as some alpha$_{1A}$ receptor mRNA. Phenylephrine-induced *c-fos* mRNA was partially inhibited by H-7, a protein kinase C inhibitor and by nifedipine, a Ca^{++} channel blocker; these two compounds together had additive effects. *In situ* hybridization showed that expression of *c-fos* mRNA induced by phenylephrine was localized to aorta's medial layer. These results suggest that alpha$_1$ receptor-induced increase in *c-fos* mRNA in aorta is mediated by a chlorethylclonidine-sensitive receptor subtype signaling via increasing intracellular Ca^{++} concentrations and activating protein kinase C.

We used expression of *c-fos* and *c-jun* mRNA in rat aorta and cultured vascular smooth muscle cells as a marker for the stimulatory effects of catecholamines on smooth muscle. The endothelium inhibits the growth of vascular smooth muscle although little is known about potential mechanisms. We have examined effects of NO on *c-fos* and *c-jun* mRNA induced by the stimulation of α_1 adrenergic receptors primarily using isolated rings of intact rat aorta *in vitro*. The muscarinic cholinergic agonist carbachol which promotes the release of nitric oxide had inhibitory effects on expression of *c-fos* and *c-jun* mRNA induced by the α_1 receptor selective agonist phenylephrine. Carbachol's action was inhibited by the muscarinic cholinergic receptor antagonist atropine. Furthermore, the nitric oxide synthase inhibitors Nω-nitro-L-arginine methyl ester and N^G-monomethyl-L-arginine blocked the inhibitory effects of carbachol on *c-fos* mRNA expression. Excess of L-arginine attenuated the effects of nitric oxide synthase inhibitors on phenylephrine-induced *c-fos* mRNA expression. A cGMP analogue mimicked the inhibitory effects of carbachol on *c-fos* and *c-jun* mRNA expression. In rat aorta, atrial natriuretic peptide also inhibited the induction of *c-fos* and *c-jun* mRNA by phenylephrine. On the other hand, in cultured vascular smooth muscle cells, neither cGMP nor atrial natriuretic peptide had inhibitory effects on the induction of *c-fos* and *c-jun* mRNA by an α adrenergic agonist. These results suggest that nitric oxide, acting through a cGMP dependent mechanism, inhibits expression of the *c-fos* and *c-jun* genes in vascular smooth muscle which may contribute to the growth-inhibiting effects of the endothelium.

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NITRIC OXIDE INHIBITS THE EPIDERMAL GROWTH FACTOR RECEPTOR TYROSINE KYNASE ACTIVITY IN TRANSFECTED FIBROBLASTS: A POSSIBLE MECHANISM FOR ITS ANTIPROLIFERATIVE EFFECT.

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Nitric oxide donors inhibit proliferation in a variety of cell types, including smooth muscle and fibroblasts. Whereas in some smooth muscle cell lines cGMP seems to be involved in the NO-mediated antiproliferative effect, this is not the case in fibroblats. We have tested the hypothesis that NO might have a direct action on the epidermal growth factor receptor (EGF-R) in these cells, based on the presence of cysteine-rich domains in the extracellular region of the EGF-R, and the observed S-nitrosylation of sulphydryl groups of certain proteins by NO.

Experiments were carried out in cultured fibroblasts transfected with the cDNA encoding sequence for the EGF-R, and overexpressing this plasma membrane receptor. The effect of different NO donors on DNA synthesis was evaluated by [methyl-^{3}H]-thymidine incorporation assays. The possible direct effect of NO on the EGF-R was tested on both the EGF-induced autophosphorylation of the receptor and its tyrosine kinase activity toward the exogenous substrate poly-L-[Glu:Tyr] using permeabilized cells.

The NO donors S-nitroso-N-acetylpenicillamine (SNAP) and 1,1-diethyl-2-hydroxy-2-nitroso-hydrazine (DEA-NO) produced a concentration-dependent inhibition of DNA synthesis in the transfected fibroblasts, without inducing cell lysis. This effect was not mediated by cGMP, since it was mimicked neither by the permeant analog 8-bromo-cGMP nor by the c-GMP phosphodiesterase inhibitor zaprinast. Both SNAP and DEA-NO also inhibited the EGF-R autophosphorylation and the phosphorylation of poly-L-[Glu:Tyr] induced by EGF in a concentration-dependent manner . However, 3-[morpholinosydnonimine hydrochloride] (SIN-1), which had no antiproliferative effect, did not affect EGF-induced phosphorylation. In preliminary experiments, reduced hemoglobin prevented the inhibitory action of DEA-NO on EGF-induced phosphorylation, verifying that the effect of this drug was due to the release of NO.

These results strongly suggest that the antiproliferative action of NO in fibroblasts is due, at least in part, to its direct effect on EGF receptors, most likely by interacting with sulphydryl groups. These findings may be relevant to the understanding of some cell growth disorders, such as those leading to vascular stenosis, and may open new perspectives for the design of therapeutic strategies.

This work was supported by grant to C.E. from the Fondo de Investigaciones Sanitarias (94/ 0388), and grants to A.V. from the Comisión Interministerial de Ciencia y Tecnología (SAF 392-93) and from the Consejería de Educación y Cultura de la Comunidad Autónoma de Madrid (AE132-93 and AE 16-94).

ACTIVATION OF NO:cGMP PATHWAY BY ACETYLCHOLINE IN BOVINE CHROMAFFIN CELLS. POSSIBLE ROLE OF Ca^{2+} IN THE DOWN-REGULATION OF cGMP SIGNALING.

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Chromaffin cells from adrenal medulla provide a good model for investigation of the molecular modulatory mechanism of neurosecretion. Stimulation of ionotropic nicotinic acetylcholine receptors in chromaffin cells triggers secretion of catecholamines through a mechanism which implies membrane depolarization and activation of voltage-dependent Ca^{2+}-channels. Acetylcholine also elevates cyclic GMP (cGMP) in bovine chromaffin cells although the link between the agonist and the intracellular increase in the second messenger had not yet been completely understood (1,2). In this study, we show that acetylcholine increased cGMP in a transitory (peak at 1 min) and concentration-dependent manner (estimated half maximal increase, $EC_{50}=61\pm5\mu M$) and such a increase was inhibited by the arginine analogue, N-nitro-L-arginine methyl ester (half maximal inhibitory concentration, $IC_{50}=259\pm54\mu M$) The acetylcholine-induced increase in cGMP was also inhibited by a calmodulin antagonist (calmidazolium, $30\mu M$) and by the absence of extracellular calcium. Other agents which increased cytosolic calcium concentration ($[Ca^{2+}]_i$) as did acetylcholine, such as the nicotinic-agonist, 1,1-dimethyl-4-phenylpiperazonium (DMPP), high-KCl (50mM) and ionomycin, also caused a rise in cGMP. Chromaffin cells also possess metabotropic acetylcholine receptors: muscarinic receptors, which were traditionally thought to be responsible for the increase in cGMP induced by acetylcholine in these cells (3). However, under our experimental conditions, the muscarinic-agonist, muscarine, failed to increased both the $[Ca^{2+}]_i$ and the cGMP. These results indicate that the cGMP produced by acetylcholine acting on nicotinic receptors was mediated by NO (nitric oxide) release, which can be generated by the enzyme NO synthase (NOS). This enzyme is L-arginine and Ca^{2+}/CaM-dependent and synthesizes NO in response to a variety of $[Ca^{2+}]_i$-increasing stimuli.

The production of cGMP in bovine chromaffin cells is also stimulated in a manner independent of calcium signaling by sodium nitroprusside (SNP) and C-type natriuretic peptide (CNP), which act spontaneously generating NO and stimulating the particulate guanylate cyclase, respectively. The calcium ionophore, ionomycin, was able to partially inhibit the cGMP production induced by SNP and CNP. This inhibition was abolished by calmidazolium indicating a role for Ca^{2+}/CaM signaling in the degradation of the cyclic nucleotide.

In conclusion, acetylcholine, through nicotinic receptor stimulation, and other stimuli which increase $[Ca^{2+}]_i$, activate the NOS and the released NO activates the soluble guanylate cyclase. Moreover, the increase in $[Ca^{2+}]_i$ also stimulates a Ca^{2+}/CaM phosphodiesterase that could down-regulate the levels of cGMP. Thus, Ca^{2+} ions through calmodulin signaling play a dual role in cGMP metabolism, on the one hand stimulating NOS to synthesize NO and on the other activating a phosphodiesterase to tightly regulate the levels of the cyclic nucleotide in these cells.

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SEVENTH SESSION: NO TOXICITY

Chairman: Steven R. Tannenbaum

WORKSHOP ON

MOLECULAR BIOLOGY AND PATHOPHYSIOLOGY

OF NITRIC OXIDE

Genotoxicity of NO·

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The focus of our research has been on the nature and extent of DNA damage in generator cells such as macrophages producing NO· and $O_2^{\cdot-}$ and in their target cells. The mutagenicity of NO· has been established in bacteria (1) and human cells (2). In tissues undergoing an inflammatory reaction, both the infiltrating and resident cell populations produce a time-dependent mixture of nitrogen oxide radicals and oxygen radicals. These different radical species interact forming new reactive intermediates which may contribute to DNA damage. Although the flux of radicals per unit time is low, an inflammatory condition that continues for years becomes a significant risk factor for carcinogenic cell transformation. The relative contributions of various radical species to the carcinogenic process must be assessed through both chemical analysis of DNA and through genetic and molecular biological analysis of mutations in surviving exposed cells.

The key chemical reactions for DNA damage are as follows(3):

1. Autoxidation of NO· to NO_2 leads to N_2O_3, a powerful nitrosating agent. Nitrosation of DNA leads to deamination of nucleic acid bases, and formation of DNA and protein cross-links.

2. Free-radical termination of NO· and O_2·⁻ occurs at diffusion controlled rates to yield peroxynitrite. The latter species is capable of causing both one- and two-electron oxidations at physiological pH. In DNA these reactions lead to oxidized bases, cross-links, and DNA single-strand breaks.

The complexity of NO·-induced damage has led us to take a combined approach of assessing chemical damage by chromatographic and spectroscopic methods, and the biological effects of this damage by measuring cytotoxicity, mutagenesis, and the role of DNA repair pathways. In stimulated macrophages and mammalian target cells (CHO) NO· potentiated both deamination and oxidative damage to DNA. Both types of damage were inhibited by NMA, an NOS inhibitor (4). In *E. coli*, NO· activated an oxidative-stress response (soxRS) previously thought to be responsive only to O2·- (5). This multigene system was shown to be essential not only for protection of cells against NO·, but also from killing by peritoneal macrophages. On the other hand, NMA not only protected cells against killing, but also prevented induction of the soxRS regulon.

In order to mimic the chemical and biological effects of NO· delivered by macrophages, we have developed a membrane permeation system for exposure of cells to controlled fluxes of NO· (6). Toxicity and mutagenicity of NO· delivered in this manner to bacterial and mammalian cells is much greater than when an equivalent dose is delivered by syringe as a gas or as a saturated NO· solution. The efficacy of NO· as a nitrosating agent and as a biological signaling agent is also enhanced when the delivery

rate is low and comparable to that produced by macrophages. Analysis of the chemistry and biochemistry of NO· production by macrophages has demonstrated that the events and biological effects are kinetically controlled (3). Failure to take this into consideration can lead to serious errors.

In summary, the chemistry of DNA damage by NO·, although potentially complex with respect to the overall mechanisms and the actual alterations of the nucleic acid, might arise from only two fundamental processes: reaction with oxygen to form nitrosating species and reaction with superoxide to form peroxynitrite. Chemical analysis of DNA, analysis of mutational spectra in key genes, and elucidation of the role of DNA repair enzymes will enable us to better understand the complexity of this system.

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INVOLVEMENT OF NITRIC OXIDE IN THE CONTROL OF PROGRAMMED CELL DEATH IN DISTINCT CELL TYPES.

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Programmed cell death (PCD), also known as apoptosis, is a process through which individual cells are deleted in the course of development or cell turnover in many tissues (1,2). Negative selection of the B and T cell lineages in the immune system is mediated by apoptosis, and the current view implicates growth factors, a wide array of cytokines and hormones as well as the level of expression of bcl-2 and related proto-oncogenes as critical factors controlling PCD (3).

In contrast to T cells, *ex vivo* isolated purified splenic B-cells, when kept unstimulated, suffer a progressive time-dependent degradation of DNA that can be followed by the appearance of a DNA fragmentation observed by agarose gel electrophoresis, by flow-cell cytometry analysis of the DNA staining and through the appearance of nucleosomes in the cytosol. However, PCD is not observed when the B cells are stimulated either with LPS, membrane surface immunoglobulin mAb plus IL-4 or in the presence of substances that produce a continuous and sustained release of nitric oxide, such as 3-morpholinosydnonimine (SIN-1). This prevention appears to be mediated by an increase in cyclic GMP and leads to the maintenance of significant levels of bcl-2 transcription (4).

A similar protective role of nitric oxide against apoptosis in human B cells has been recently reported, suggesting that this is a general effect of nitric oxide on mammalian B cells (5).

In contrast to this prevention of PCD by nitric oxide in B cells, macrophages confronted to nitric oxide donor substances or in response to cytokines that promote nitric oxide synthase expression display characteristic apoptotic features in agreement with their physiological function. However, this nitric oxide-

triggered apoptosis of macrophages is also observed when these cells are stimulated with bacterial products and in the absence of nitric oxide synthesis, suggesting that nitric oxide in these cells contributes to provides a coordinate response to other cytokines and extracellular factors to commit activated macrophages to PCD.

In conclusion, the protective role of nitric oxide against PCD in B-cells contrasts with the nitric oxide-dependent induction of apoptosis observed in other cell types such as macrophages, suggesting the existence of cell-specific pathways in the response to nitric oxide. This opposite response to nitric oxide in B-cells and macrophages is not unique since neuroprotective and neurodestructive effects of nitric oxide have been reported in neurons and this complex behaviour has been explained on the basis of the different redox states of nitric oxide once released in the cytoplasm of the cell.

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Bcl-2 PREVENTS DEVELOPMENT OF NITRIC OXIDE-MEDIATED APOPTOSIS.

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Nitric oxide (NO) is known as an effector agent in cell-mediated cytotoxicity and apoptosis (1). The biochemical changes in the cells undergoing apoptosis can be correlated with the modification of several cellular enzymes and structures by NO. These include inhibition of enzymes involved in energy generation [respiratory chain enzymes, cis-aconitase in Krebs cycle, and glyceraldehyde-3-phosphate dehydrogenase in glycolytic pathway], inhibition of ribonucleotide reductase [key enzyme in synthesis of DNA precursors], DNA damage with subsequent activation of poly(ADP-ribose) polymerase and activation of Ca^{2+}/Mg^{2+}-dependent endonuclease (2). NO also reacts with superoxide, yielding peroxynitrite which decomposes to form highly reactive hydroxyl radical causing lipid peroxidation (3). Morphological changes in apoptosis are characterized by cytoplasmic membrane blebbing with release of membrane-bounded apoptotic bodies, cell shrinking, and DNA fragmentation (4).

Despite of extensive biochemical and morphological studies revealing several apparently different ways of apoptosis induction, key events underlying development of apoptosis remain largely unknown. The bcl-2 proto-oncogene has been shown to prevent apoptosis in multiple contexts and to function in the antioxidant pathway [i.e. in protection against reactive oxygen species], (5). Because of the interconnection of reactive nitrogen and oxygen species [RNS, ROS] metabolism, it could be that expression of bcl-2 gene prevents NO-mediated cellular damage.

To test this hypothesis, an inducible vaccinia virus [VV] expression system was used. We have generated two recombinant viruses with murine inducible nitric oxide synthase [WRNOS] or human bcl-2 [WRbcl2] genes, which are expressed upon induction by IPTG. As control, identical construct expressing chloramphenicol acetyltransferase [WRCAT] was used. Simultaneous infection by WRNOS and WRCAT caused cell death of HeLa G cell line, as characterized by cellular morphology and DNA fragmentation. In contrast, when WRNOS and WRbcl2 were used, development of apoptosis was prevented. bcl-2 overexpression did not, however, affect NO formation [determined in medium as nitrites, NO oxidation products] nor rescue vaccinia virus growth [NO inhibits VV DNA formation, see accompanying abstract], suggesting that bcl-2 does not interact directly with NOS nor functions as an exclusive terminal acceptor of NO. It is rather likely that NO dammaging effects are largely due to its interaction with ROS and these are consequently neutralized by bcl-2.

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EIGHTH SESSION: GENE REGULATION

Chairman: Charles Lowenstein

Structure and function of the inducible nitric oxide synthase

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The inducible nitric oxide synthase (i-NOS) is a 135 kDa protein related to the family of cytochrome P450 enzymes, and it is about 50% identical to the other NOS isoforms. Much more is known about the regulation of i-NOS than is known about its structure. All NOS isoforms contain consensus binding regions for NADPH, FAD, and FMN in the carboxy-terminal portions of the deduced amino acid sequences, and a calmodulin binding region in the middle. Presumably the basic, hydrophilic calmodulin binding site has a higher affinity for calmodulin in the i-NOS isoform than the constitutive isoforms, accounting for the physiological independence of i-NOS from calcium. The amino-terminal half of i-NOS shares extensive regions of homology with the other NOS isoforms, and presumably contains the sites for heme binding, arginine binding, tetrahydrobiopterin binding, and dimerization.

Since i-NOS is always active once expressed, the primary mechanism of regulation of i-NOS is transcriptional. The regulation of i-NOS is best characterized in murine macrophages. Multiple stimuli, including LPS, IL-1ß, and TNF-α, activate the degradation of inhibitory factor kappa B and the activation of nuclear factor kappa B (NF-κB), a transcription factor. NF-κB induces transcription of a set of genes including i-NOS by binding to an NF-κB binding site upstream of the TATA box of the i-NOS gene. Interferon-gamma alone cannot induce i-NOS, but it increases the transcription of previously induced i-NOS by stimulating phosphorylation of the interferon response factor 1 which binds to an interferon stimulated response element further upstream of the TATA box. Inhibitors of i-NOS transcription include transforming growth factor beta (TGF-ß) and iron. The stability of the i-NOS mRNA is increased by TGF-ß and decreased by an unknown protein binding to destabilizing sequences downstream of the stop codon. The regulation of the human i-NOS gene has not been characterized in as much detail, but its induction is more tightly controlled, since LPS alone is insufficient to stimulate transcription of i-NOS in human cells.

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Molecular Mechanisms regulating INOS gene activation by cytokines in vascular smooth muscle cells.

Nitric oxide (NO) is a potent vasorelaxant [1], but also plays a role in physiological processes as diverse as neurotransmission [2] and host defence [3]. There are three types of enzyme which synthesize NO, the NO synthases [4]. Of these two are constitutive, but the third is normally only produced following transcriptional activation of its gene [5,6]. This inducible NO synthase (iNOS) is produced in a variety of cell types following stimulation with a number of different factors, the most important of which are proinflammatory cytokines and lipopolysaccharide [4,7]. Production of NO by this enzyme is important in the pathophysiology of a number of conditions. For example, normal vascular smooth muscle produces no NO in the resting state. However, following stimulation with cytokines such as interleukin-1 (IL-1), interferon-gamma (IFN-γ) and tumor necrosis factor-α (TNF-α), iNOS is synthesised [8-10], leading to NO production, vasorelaxation and hypotension [11]. This is thought to be the principal cause of the hypotension so characteristic of septic shock [12]. Hence, an understanding of the mechanisms underlying the control of iNOS production not only is important in understanding the mechanisms of cytokine action, but also in illuminating the pathophysiological processes in conditions such as septic shock.

The recently cloned promoter of the murine gene for iNOS contains numerous potential sites for the binding of a number of different transcription factors [13]. This approximately 1.7 kb DNA element confers inducibility by IFN-γ and lipopolysaccharide (LPS) in macrophages. A key region of the promoter in mediating the response to LPS is a downstream NF-κB site, which extends from position -85 to -76 [14]. The synergic effect of IFN-γ, however, requires the presence of the 5' region of the promoter [13,15]. Moreover, the importance of the 5' region of the promoter is shown by recent work using transgenic mice with targeted disruptions in the gene for the transcription factor interferon regulatory factor-1 (IRF-1). These animals fail to induce iNOS following a variety of stimuli [16]. Of note, there are two potential binding sites for IRF-1 within the 5' portion of the promoter extending from position -900 to -924 [16,17].

The area of the promoter which mediates the synergistic effects of IFN-γ has not been mapped in any detail. Moreover, these studies on the promoter of iNOS have so far been performed in macrophages, and have only studied the effect of LPS and IFN-γ. However, a number of different cell types can produce iNOS after stimulation by LPS and IFN-γ, as well as pro-inflammatory cytokines such as IL-1, TNF-α, and IL-2 [10]. Cytokines are rarely produced in isolation, and their ability to interact with one another increases the range of biological effects which they mediate. Given the complexity of the iNOS promoter and the necessity to integrate the stimulatory effects of a number of pro-inflammatory cytokines, different mechanisms might apply in cell types other than macrophages and following different stimuli. In addition, while many studies have been performed on the effects of a single cytokine on a given promoter's function, there are relatively few studies which have analysed the way in which a number of cytokines interact to produce increased transcriptional activation of a gene.

For these reasons, we decided to study the function of the promoter of iNOS within vascular smooth muscle cells, where the production of NO by iNOS plays an important part in the pathophysiology of septic shock. We chose to analyse the effects of three pro-inflammatory cytokines, TNF-α, IFN-γ and IL-1 on iNOS promoter function, since these cytokines are all produced during septic shock from a variety of different causes [18], and are known to synergise in the production of iNOS from vascular smooth muscle cells [10].

To understand the molecular mechanisms of iNOS gene activation, we have defined the cytokine responsive element of the murine iNOS promoter transfected into a VSM cell line, and the role of NF-κB/Rel family proteins in gene activation. The combination of IL-1, IFN-γ and TNF-α stimulate promoter activity by a factor of 8.4 fold; single cytokines show little activity, while pairs of cytokines produce an

intermediate effect. Using a series of promoter deletion mutants, we have defined the cytokine responsive element from position -890 to -1002; this region contains an NF-κB site as well as a number of interferon response elements. Nuclear proteins from cytokine-stimulated VSM cells which bind to an oligonucleotide containing this NF-κB site are composed of p65 together with an unidentified protein of 50 kDa, which is not a known rel family member. Promoter mutants with a 2 bp change within this NF-κB site have an activity of only approximately 40% of the wild type promoter. In addition, protein binding to this site is abolished by a specific inhibitor of NF-κB activation, which also abrogates iNOS activity. The important role of an upstream NF-κB site which binds an unusual p65-containing NF-κB complex in cytokine activation of iNOS in VSM cells differs markedly from the LPS activation of iNOS in macrophages.

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REGULATION OF INDUCIBLE NITRIC OXIDE SYNTHASE (iNOS) EXPRESSION IN MESANGIAL CELLS

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Mesangial cells are modified vascular smooth muscle cells located in the renal glomerulus which control macromolecular traffic and glomerular filtration. When exposed to proinflammatory cytokines (TNF-α, IL-1-β) or lipopolysaccharide (LPS) they synthesize nitric oxide through de novo induction of an iNOS enzyme which is 88 % similar to maciNOS. In rat mesangial cells (RMC) we have been able to show that both TNF-α and LPS, independently, induce iNOS expression, which is enhanced in the presence of both agents and is dependent on protein and RNA synthesis . Both dexamethasone (Dx) and pirrolidin dithiocarbamate (PDTC), an NF-κB inhibitor with antioxidant properties, suppress iNOS mRNA expression in a dose-dependent fashion. Whereas Dx is effective only when added before or simultaneously with the inductive stimuli (IS), PDTC inhibited iNOS mRNA expression after the addition of the IS as well. Electrophoretic mobility shift assays (EMSAS) performed in nuclear extracts of RMC showed that oligomers containing a consensus binding sequence for the transcription factor NF-κB retarded the migration of a protein-DNA complex which was clearly visible after 4-6 h of exposure to LPS + TNF-α and less abundant with pre-treatment and co-treatment of RMC with PDTC but not affected by Dx. EMSAS in similar nuclear extracts performed with an oligomer with the consensus binding sequence for the transcription factor AP-1showed retardation of a protein-DNA complex after 2 h of LPS/TNF-α treatment , an effect which was suppressed by pretreatment with Dx and PDTC. However in nuclear extracts of RMC treated for 6h there was a slight enhancement of the retarded AP-1 sequence-protein complex in the presence of PDTC. In order to better define the participation of these transcription factors in the activation of iNOS transcription, ongoing efforts are directed towards the molecular characterization of the iNOS promoter in RMC and the analysis of the promoter activity in transfected RMC.

Studies in human mesangial cells (HMC) have demonstrated that they require the presence of multiple IS in order to express iNOS. Interleukin-13, an antiinflammatory lymphokine, is able to abrogate NO synthesis and iNOS mRNA expression over a wide temporal window. Present studies are devoted to clarify the molecular mechanisms of this effect, including the role of tetrahydrobiopterin on inducible NO synthesis and the potential regions of interaction between IL-13 and the iNOS promoter in HMC.

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POSTERS

INTERSPECIES DIFFERENCES IN THE NADPH-DIAPHORASE LABELING OF THE OLFACTORY BULB IN INSECTIVORES, RODENTS AND PRIMATES

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The distribution and the morphology of NADPH-diaphorase (ND) active neurons and fibers were studied in the olfactory bulb of an insectivorous (hedgehog, *Erinaceus europaeus*), a rodent (rat, *Rattus norvegicus*), and three species of primates, i.e., the cynomolgus macaque monkey (*Macaca fascicularis*), the pig-tail macaque monkey (*Macaca nemestrina*) and the man. The technique was carried out by means of a direct histochemical method using ß-NADPH as co-substrate and nitroblue tetrazolium as chromogen. A relatively common pattern was observed in all three primates, whereas the ND-staining pattern showed substantial differences between macrosmatic and microsmatic mammals. In primates, all olfactory fibers demonstrated ND-positive labeling, whereas in the rat and hedgehog, an insectivorous with an extraordinary development of the olfactory structures, only a subpopulation with a topographically distinct distribution was stained. In rodents and insectivores, periglomerular cells were ND-labeled, whereas they were ND-negative in primates. Darkly stained cells were identified as superficial and deep short-axon cells, with spiny dendrites. Deep short-axon cells were present in all species, whereas superficial short-axon cells were observed in all of them with the exception of the hedgehog. In the hedgehog, we observed a more complex ND staining pattern than those previously observed in rodents, including the presence of specific chemically and morphologically defined new neuronal types. In primates, by contrast, the ND-staining pattern was more simplified. These data suggest that the complexity of the ND-staining in the olfactory bulb of one species correlates with the importance of olfaction in the biology of such species.

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DIFFERENT SUSCEPTIBILITY OF ASTROCYTES AND NEURONES TO PEROXYNITRITE-MEDIATED MITOCHONDRIAL DAMAGE

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Nitric oxide has been implicated in the neurotoxicity associated with glutamate receptor stimulation [1], suggesting that nitric oxide may be involved in the pathogenesis of certain neurodegenerative diseases [2]. While a relationship between brain mitochondrial dysfunction and nitric oxide in the pathogenesis of neurodegenerative diseases is still not known, we have presented evidence showing that the induction of nitric oxide synthase in astrocytes causes inhibition of the mitochondrial respiratory chain, with cytochrome c oxidase being the most susceptible component [3]. The nitric oxide derivative, peroxynitrite anion ($ONOO^-$), seems to be the agent responsible for the nitric oxide-mediated neurotoxicity [4]. Since astrocytes possess higher concentrations of antioxidants/antioxidant enzymes [5], we hypothesized that production and release of $ONOO^-$ by astrocytes might be more deleterious to neurones than to astrocytes themselves. Accordingly, the aim of the present study was to compare the susceptibility of neurones and astrocytes in primary culture to $ONOO^-$, with particular reference to the activity of the mitochondrial respiratory chain components. A single exposure of the neurones to $ONOO^-$ (initial concentrations of 0.01-2.0 mM) caused, after a subsequent 24 h incubation, a dose-dependent decrease in succinate-cytochrome c reductase (60% at 0.5 mM) and in cytochrome c oxidase (52% at 0.5 mM) activities. NADH-ubiquinone-1 reductase was unaffected. In astrocytes, the activity of the mitochondrial complexes was not affected up to 2 mM $ONOO^-$. Citrate synthase was unaffected in both cell types under all conditions studied. However, lactate dehydrogenase activity released to the culture medium was increased by $ONOO^-$ in a dose-dependent manner (40% at 0.5 mM) from the neurones, but not from the astrocytes. Neuronal glutathione concentration decreased by 39% at 0.1 mM $ONOO^-$ but astrocytic glutathione was not affected up to 2 mM $ONOO^-$. In isolated brain mitochondria, only succinate-cytochrome c reductase activity was affected (22% decrease at 1 mM $ONOO^-$). We conclude that the acute exposure of $ONOO^-$ selectively damages neurones whilst astrocytes remain unaffected. Intracellular glutathione appears to be an important factor for ameliorating $ONOO^-$-mediated mitochondrial damage. This work supports the hypothesis that the neurotoxicity of nitric oxide is mediated through mitochondrial dysfunction.

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THE ROLE OF NITRIC OXIDE (NO) IN OVARIAN FUNCTION

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The ovary is a highly vascular organ and many of its activities are related to changes in rates of blood flow. Nitric oxide (NO) has attracted considerable attention as an important mediator of vasoactivity (1,2). We carried out three studies to examine the potential role of NO as a vasoactive factor in the ovary. Experiment 1:The first study focussed on the role of NO in the ovulatory process. In the rabbit, follicular rupture involves vasodilatation and increased vascular permeability within the wall of the preovulatory follicle. Thus the objective of this study was to determine whether inhibiting NO synthesis by blocking nitric oxide synthase (NOS) activity with N^G-nitro-L-arginine methyl ester (L-NAME), would affect hCG-induced ovulation. Sexually mature New Zealand White rabbits were used. In the first part of the experiment, L-NAME (40 mg/kg; n=11) was administered subcutaneously 1 h prior to intravenous administration of hCG (100 IU). Additional doses of L-NAME were given every three hours. Control rabbits (n=6) received saline vehicle and hCG. Twelve hours after the first dose of L-NAME or saline vehicle, rabbits were anesthetized and ovaries were removed and examined for ovulation points. Ovulatory efficiency (percent of large follicles ovulated) was determined. L-NAME significantly ($p<0.01$) reduced hCG-induced ovulation *in vivo* (control 68%; L-NAME 25%). To further establish the role of NO in the ovulatory process and to avoid systemic effects of L-NAME, an isolated *in vitro* perfusion system was used. Ovarian arteries were cannulated *in situ* and the ovaries excised and perfused. The medium for one ovary contained L-NAME (n=4), while the contralateral ovary served as control (n=4). hCG was administered to both ovaries (100 IU/150 ml) and perfusion continued for 10 hours thereafter. Ovulation efficiency was 51% in the controls but reduced to 8% in the L-NAME treated animals, confirming the results of the *in vivo* experiment. Experiment 2: A second study used immunolocalisation techniques with specific anti NOS antibodies to establish the presence of inducible and constituitive (endothelial) NOS in the ovary of rats and rabbits. Pseudopregnant rabbits were examined on days 4, 7, 11 and 18 after injection of hCG. NOS was localized in luteal cells and vascular endothelium throughout pseudopregnancy. Rats were examined during the estrus cycle and at six stages during pregnancy, days 3, 8, 13, 16, 22, and 23. During the estrus cycle, thecal vessels showed abundant NOS stain. During pregnancy, the corpora lutea stained richly with the stages of luteal formation and early luteal regression appearing to exhibit more intense endothelial NOS staining than the period of peak progesterone secretion. Experiment 3:To establish whether the NOS is important for CL function, pseudopregnant rabbits were treated with L-NAME (120 mg/kg/day) or saline from day 11 (maximum progesterone production) to day 18 (functional luteolysis). On day 18, ovaries were removed and perfused with 0.01mM L-NAME or medium alone for 6 h. A 53 % decrease in progesterone secretion was seen in ovaries perfused with L-NAME. Ovaries from saline treated rabbits perfused with L-NAME secreted 36% less progesterone than ovaries perfused with medium alone. To elucidate the mechanism underlying reduced progesterone secretion observed with L-NAME treatment, the integrity of luteal DNA was analyzed for evidence of apoptosis (physiological cell death, 3). Increased levels of DNA breakdown were observed in CL from ovaries perfused with L-NAME compared to CL perfused with medium alone.

Conclusion: These data suggest that NO plays a significant role in the mechanism of hCG-induced ovulation in the rabbit. Furthermore the data suggest that NO plays an important role in CL function in the rabbit and rat and inhibition of NO may lead to vessel constriction, loss of trophic support, and subsequent apoptosis.

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EXPRESSION OF THE CITRULLINE-NITRIC OXIDE CYCLE IN RODENT AND HUMAN PANCREATIC β-CELLS.

INDUCTION OF ARGININOSUCCINATE SYNTHETASE BY CYTOKINES

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Nitric oxide (NO) may be a mediator of pancreatic β-cell damage in insulin-dependent diabetes mellitus. We have previously shown that both rodent (Eizirik et al. FEBS Lett 308: 249-252, 1992) and human (Eizirik et al. J Clin Invest 93: 1698-1974, 1994) β-cells express mRNA for the inducible form of nitric oxide synthase (iNOS), and produce large amounts of NO upon exposure to cytokines. iNOS requires the amino acid arginine for NO formation. It has been shown in other cell types that IFN-γ and bacterial lipopolysaccharide induce the enzyme argininosuccinate synthetase (AS), enhancing the capacity of these cells to regenerate arginine from citrulline and maintain NO production in the presence of low arginine concentrations. To characterize the expression of AS in insulin-producing cells, RINm5F cells (RIN cells) were exposed to IL-1β or to TNF-α + IFN-γ. After 4-6 h there was a significant and parallel induction of AS and iNOS mRNA. IL-1β-induced AS and iNOS mRNA expression was prevented both by an inhibitor of the activation factor NF-κB (PDTC), an inhibitor of gene transcription (actinomycin D) and a blocker of protein synthesis (cycloheximide), suggesting coregulation of AS and iNOS by cytokines. The fact that RIN cells exposed to IL-1β in the presence of citrulline, but absence of arginine, were able to produce NO demonstrate the expression of AS enzymatic activity. Similar expression of AS activity was present in primary culture of adult rat islets exposed to IL-1β and in human pancreatic islets cultured in the presence of IL-1β + TNF-α + IFN-γ. To our knowledge, these observations with human islets are the first demonstration of a functioning citrulline-NO cycle in differentiated human tissue. Taken as a whole, the present data suggest that regulation of AS activity may play a role in modulation of NO production in insulin-producing cells.

Scavenging of Nitric Oxide by Imidazolineoxyl N-oxides in Vascular Endothelium. EPR measurement of NO Synthase Activity

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Nitric oxide is known to be a mediator in a number of important biological processes. NO synthases constitute an expanding family of unique enzymes responsible for the biosynthesis of nitric oxide in mammalian cells. The disadvantages of the methods applied for NO measurements in present time stimulate the development of a new approaches. Overproduction and cytotoxic action of nitric oxide in some diseases and disorders, including septic shock and multiorgan failure, determine the importance of searches of new scavengers of NO. Recently we propose a new series of Imidazolineoxyl N-oxides as spin traps for nitric oxide [1]. A high efficiency of NO trapping by imidazolineoxyl N-oxides could be useful for a therapeutic regulation of nitric oxide. It was found these radicals react with NO forming stable iminonitroxides with dramatic changes in EPR spectra. Spectrophotometric stop-flow studies give the bimolecular rate constants of the imidazolineoxyl N-oxides with NO equal to about 10^4 $M^{-1}c^{-1}$. The sensitivity of this EPR method for the measurement of nitric oxide production is down to 0.03 uM NO per min in a flat quartz cell of 0.2 ml. However the fast reduction of the imidazolineoxyl N-oxides biological samples (for example, it's a few sec in rat cerebella cytosol) does not allow quantitative estimation of NO production. To overcome this limitation we have used the imidazolineoxyl N-oxide with charged trimethylammoniophenyl group (INTA) incorporated into the inner volume of large unilamellar phosphatidylcholine liposomes. In this case the liposome barrier is easily penetrated by small hydrophobic molecular, NO, but the reduction of the radical, INTA, in rat cerebella cytosol is slow (ca. 1% per min). The rate of NO production by NO synthase from rat cerebellum measured by INTA is in a reasonable agreement with that obtained by spectrophotometric method.

We have found that imidazolineoxyl N-oxide antagonize NO produced in vascular endothelium. The physiological effect of imidazolineoxyl N-oxide studied on the Wistar rats and rats with inherited hypertension [2] has been effectively prolonged using charged radical, INTA, incorporated in liposomes.

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LARGE SCALE PURIFICATION OF RAT BRAIN NITRIC OXIDE SYNTHASE FROM A BACULOVIRUS OVEREXPRESSION SYSTEM

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Nitric oxide synthase (NOS) isozymes catalyze the conversion of L-arginine to L-citrulline and NO. These enzymes are highly complex proteins, consisting of a heme-containing oxygenase domain coupled to a FAD- and FMN-containing cytochrome P-450 reductase. For unknown reasons NOSs require tetrahydrobiopterin (H_4B) as cofactor for the coupling of oxygen activation to substrate metabolism. For expression of neuronal NOS with the baculovirus/insect cell system, the cDNA encoding rat brain NOS (Bredt et al., Nature 351, 714-718, 1991) was cloned in the expression vector pVL1393, which was used for infection of Sf9 cells (4.5×10^9 cells/3,000 ml) at a ratio of 5 pfu/cell. NOS was expressed to a high level (~5 % of total soluble protein), but the purified enzyme was heme-deficient and had only poor specific activity. Supplementing the culture medium with 4 µg/ml of hemin resulted in a pronounced increase in the heme content of NOS and allowed a convenient large scale purification of the highly active enzyme from the cell supernatants by chromatography over 2'5'-ADP-sepharose. From 3,000 ml cell suspension, we obtained about 100 mg of homogenous NOS with a specific activity of approximately 1 µmol citrulline $\times$ mg^{-1} $\times$ min^{-1}. Further chromatography of the 2´5´-ADP-sepharose eluate over calmodulin-sepharose did not significantly increase purity (>99 %) and specific activity of the enzyme. Purified NOS contained 0.93 mol of heme per subunit and exhibited a native light absorbance spectrum typical for hemeproteins with a Soret band at 396 nm (ε = 100 mM^{-1} cm^{-1}). HPLC analysis of purified NOS preparations revealed presence of FAD, FMN, and H_4B at molar ratios of 0.18, 0.23, and 0.45, respectively. Catalytic activity of the enzyme was increased 4.8- and 2.7-fold by addition of exogenous FAD/FMN and H_4B, respectively, demonstrating a good correlation between the amount of endogenously bound cofactors and respective basal NOS activities. Our data suggest that baculovirus-mediated expression of brain NOS in insect cells may be useful to obtain large quantities of the functionally intact enzyme for further biochemical, biophysical, and mutational analysis.

Dexamethasone differently affects interleukin 1β- and cyclic AMP - induced nitric oxide synthase mRNA expression in renal mesangial cells

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Inducible nitric oxide synthase (iNOS) is expressed in renal mesangial cells in response to two principal classes of activating signals that interact in a synergistic fashion. These two groups of activators comprise inflammatory cytokines such as interleukin (IL-)1 β or tumour necrosis factor α and agents that elevate cellular levels of cyclic AMP (cAMP). We examined whether dexamethasone differently affects iNOS induction in response to IL-1β and a membrane-permeable analogue, N^6,O-2'-dibutyryladenosine 3',5'-phosphate (Bt_2cAMP). Nanomolar concentrations of dexamethasone suppress IL-1β- as well as Bt_2cAMP-induced iNOS protein expression and production of nitrite, the stable endproduct of nitric oxide (NO) formation. In contrast, dexamethasone prevents induction of iNOS mRNA in response to Bt_2cAMP without affecting IL-1β-triggered increase in iNOS mRNA levels, suggesting that dexamethasone acts at different levels, depending on the stimulus used to suppress iNOS induction in mesangial cells. We currently perform further experiments to unravel the underlying mechanism(s). Preliminary data suggest that the IL-1β - dependent induction of iNOS is suppressed at the translational level, whereas the cAMP-dependent induction of iNOS mRNA is blocked at the transcriptional level.

INHIBITION OF VACCINIA VIRUS DNA REPLICATION BY INDUCIBLE EXPRESSION OF NITRIC OXIDE SYNTHASE.

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Phagocytic cells of the monocyte/macrophage lineage play a central role in non-specific defense of the living organism against all sorts of infectious agents and other pathogens. They exert inflammatory, tumoricidal, antimicrobial, antiparasitic and antifungal activities, most of which include reactive oxygen and nitrogen species (ROS, RNS) formation. Inhibition of DNA synthesis through its effect on ribonucleotide reductase (RR) and inhibition of enzymes involved in energy generation are key events in the cytostasis and killing of intracellular parasites by NO (1).

Macrophage inhibition of virus growth and the antiviral properties of NO have been intensively studied only recently. NO was shown to inhibit growth of three DNA viruses: herpes simplex, ectromelia and vaccinia (VV), as determined by reduction of virus titers. The site of NO action was not, however, characterized (2, 3). We have previously found that interferon γ (IFNγ)-activated macrophages inhibit VV growth at the level of DNA synthesis, and this inhibition correlated with increased NO production (4).

In view of multiple effects exerted by IFNγ on macrophages, it was important to establish if NO was directly responsible for inhibition of VV DNA replication. Thus, we have generated a recombinant VV expressing the mouse inducible nitric oxide synthase under an inducible promoter (WRNOS). Upon induction by IPTG, NO is generated and virus growth is inhibited in a dose dependent manner. VV growth is inhibited at the level of DNA synthesis, and this inhibition is similar or identical to the inhibition caused by IFNγ-activated macrophages: the block in VV DNA synthesis results in the inhibition of viral late protein synthesis. Virus particles assembled before the onset of NO-mediated inhibition remain infectious, indicating a specific effect of NO on VV replication, probably on RR.

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Nitric oxide modulated activation induces cell death in monocytes.

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Apoptosis or programmed cell death concerns immune cells exposed to specific activation signals that are delivered via the antigen receptor, alternative activation pathways, endogenous immunosuppressive molecules (e.g., glucocorticoids, transforming growth factor), cytotoxic effector molecules (e.g., granzymes, TNF), or a deficiency in obligate trophic factors. Cross-linking of the activation marker CD69, which belongs to the natural killer complex, causes apoptosis of LPS-preactivated monocytes in a medium containing L-arginine (nitric oxide precursor). Nevertheless, when cells were incubated in the same conditions but in a medium lacking L-arginine or with a L-NAME (analogous of L-arginine which inhibits NO synthase) apoptosis did not occur. The effect of L-NAME was time-dependent, showing that addition of this analogous after 4 hours or more of antiCD69/LPS activation failed to inhibit apoptosis. These results suggest that nitric oxide pathway could be involved in the control mechanisms triggering apoptosis.

NITRIC OXIDE PARTICIPATES IN THE GENERATION OF A MOTOR SIGNAL IN PHYSIOLOGICAL CONDITIONS: A STUDY IN THE OCULOMOTOR SYSTEM.

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This study attempts to evaluate the possible role of nitric oxide (NO) as a physiological modulator in the central nervous system. The oculomotor system was selected as a model because its circuits are well known, its output can be measured in alert animals, and the nuclei involved are accessible for neuronal activity recording and for focal injections. Horizontal eye movements are generated by activation of neurons present in the abducens (ABD) nucleus, which are activated in turn by premotor neurons localized in the reticular formation (RF), prepositus hypoglossi (PH) and medial vestibular (MV) nuclei. The objectives of the work were: i) to reveal the possible existence of NO synthase (NOS) containing neurons within the nuclei involved in the control of eye movements, and ii) to evaluate possible changes in eye-movement behavior upon injection of NO donors or NOS blockers in these nuclei.

NADPH-diaphorase staining and NOS immunohistochemistry of cat brain stem sections were used for the morphological study. Functional experiments were carried out in 5 cats prepared for chronic recording of eye movements by means of the magnetic search-coil technique. Air-pressure driven local injections (0.1-0.5 μl) were performed with 6-8 μm tip diameter glass micropipets, containing the different drugs at a concentration of 80 mM. The ABD nucleus, located by the antidromic field potential induced by electrical stimulation of the ipsilateral VIth nerve, was used as a reference for injections.

A large number of NO-producing neurons were visualized in PH, distributed in patches all along the nucleus. Scattered labelled neurons were also observed in the other nuclei of the horizontal oculomotor system. Unilateral injections of the NOS inhibitors L-nitoarginine methyl ester (L-NAME) and L-N-monomethylarginine (L-NMMA) in the anterior third of the PH produced conjugated eye movements with slow phases directed to the contralateral side. The effect was dose-dependent, appeared within 1-2 minutes after injection, lasted for 30-60 minutes and was more evident in complete darkness. The effect of L-NAME was stereo specific, because no effect was observed upon D-NAME injections. Administration of L-arginine together with L-NMMA abolished or even reversed the effect of the NOS inhibitor. Unilateral injections of the NO donor sodium nitroprusside (SNP) in the PH nucleus produced the opposite effect, this is, conjugated eye movements with slow phases directed to the ipsilateral side.

These results indicate that NO produced by PH neurons modulates the generation of the motor signal that control horizontal eye movements.

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INDUCTION OF CALCIUM-INDEPENDENT NITRIC OXIDE SYNTHASE ACTIVITY IN HUMAN NEUROBLASTOMA CELLS BY TNF-α.

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Cytokines are soluble molecules involved in regulatory functions. As well as having a key role in inflammatory and immune responses they also have important regulatory effects on the growth and differentiation of cells from the nervous system. Among those, tumor necrosis factor-α (TNF) which has been demonstrated at the site of neural injury has a differential effect on several cell types of the central nervous system (CNS). Thus, we have shown previously that TNF induces astrocyte and glial proliferation but induced the differentiation and growth arrest of neuroblastoma cells. Intercellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule-1 (VCAM-1) are major adhesion receptors, widely expressed on cells of hematopoietic and non-hematopoietic origin. Their cell surface expression is regulated by various proinflammatory cytokines. Unstimulated cells express ICAM-1 or VCAM-1 at low or undetectable levels, while their expression is markedly augmented by TNF, IFN-γ or IL-1. During neuropathological disorders of the CNS, a communication and interaction network, based on enhanced expression of adhesion molecules and enhanced production of cytokines, between infiltrating leukocytes, brain microvessel endothelial cells, macroglia, microglial cells and neurons has been clearly documented.

Nitric oxide (NO), is derived from L-arginine by the NADPH-dependent enzyme NO synthase, of which there are there types encoded for by different genes. Isoform I or nNOS is Ca2+-dependent and it is constitutively present in neuronal and certain epithelial cells. The isoform II or iNOS is Ca2+-independent and it is found mainly in macrophages where it can be induced by lipopolysaccharide and cytokines. Isoform III or eNOS, which is expressed in endothelial cells, is also Ca2+-dependent. Its expression is mostly constitutive although it can be enhanced in some situations.

The purpose of our work was to study the effect of TNF on cultured cells from neural origin (SK-N-SH neuroblastoma cell line). Unstimulated SK-N-SH cells express ICAM-1 and VCAM at low levels. Their expression is markedly increased after exposure to inflammatory cytokines, such as TNF in a dose-dependent manner. Induction of adhesion molecules of SK-N-SH was accompanied by the arrest of cell growth. Induction of VCAM-1 by TNF reached a plateau 4 hr after induction, whereas the kinetics of induction of ICAM-1 was somewhat slower. The induction of neuroblastoma cell adhesion by TNF can be inhibited by a nitric oxide synthase inhibitor, L-N-monomethyl arginine (L-NMMA). Moreover, the adhesion of SK-N-SH can be induced directly in a dose dependent manner by the addition of the NO generators, nitroprusside or S-nitro-N-acetyl-penicylamine, into the culture medium. These results indicate that TNF induce the adhesion of neuroblastoma cells via NO production. This is in contrast with the results found in other cell types, such us endothelial cells and leukocytes, where NO generation by NO donors has been shown to down regulate adhesion. The kinetics and substrate requirements of NOS activated in neuroblastoma cells by TNF treatment were determined by measuring the conversion of L-(14C)arginine to L-(14C)citruline by a dialyzed cytosolic fraction indicating that is a Ca2+-independent enzyme. Thus, TNF seems to induce a Ca2+-independent inducible form of NOS comparable to the isoform characteristic of activated macrophages.

We propose that leukocyte-derived cytokines, through the NO generation, induce the expression of adhesion molecules on the surface of neural cells that facilitate the subsequent attachment of leukocytes. Leukocyte adherence may contribute to some of the neural cell injury seen with various inflammatory diseases of the nervous system.

NITRIC OXIDE IMPLICATION IN THE CONTROL OF NEUROSECRETION BY CHROMAFFIN CELLS

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In this work we have studied the effects of pure nitric oxide (NO) on the regulation of catecholamine (CA) secretion by chromaffin cells, as so well the possible presence of its synthesizing enzyme L-arginine: NO synthase (NOS) in these cells.
Our results show that NO produces a large stimulation of basal CA secretion. This effect was calcium and concentration-dependent (EC_{50} = 64±8 μM) and was not due to non-specific damage of the tissue by NO.
NO also modulates the CA secretion evoked by nicotine in a dose- dependent manner. While it has a stimulatory effect on the CA secretion evoked by low doses of nicotine (< 3 μM) (EC_{50} = 16±3 μM), it produces a dose-dependent inhibition of the CA secretion induced by high doses of nicotine (≥ 30 μM) (IC_{50} = 52±6 μM).
The mechanism by which NO modulates CA secretion seems to be through the increase in the cGMP levels since there was a close correlation between the CA secretion and the cGMP levels.
The presence of a specific activity of L-arginine: NO synthase (NOS) in chromaffin cells has been demonstrated by two independent methods: release of [^{14}C]citrulline from [^{14}C]arginine and formation of a NO-hemoglobin complex. NOS activity was about 0.5 pmol per min per mg protein. It was calcium and mainly calmodulin dependent and could be specifically blocked by the NOS inhibitor N-methyl-L-arginine.
These results suggest that NO could be an important intracellular messenger in the regulation of neurosecretion in chromaffin cells.

Running title: Role of nitric oxide in neurosecretion

Key words: nitric oxide, L-arginine: NO synthetase, catecholamine secretion, chromaffin cells, cyclic GMP

LIVER ENDOTHELIAL CELLS PARTICIPATE IN T CELL DEPENDENT HOST RESISTANCE TO LYMPHOMA METASTASIS BY PRODUCTION OF NITRIC OXIDE IN VIVO.

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Tumor growth and metastasis of *lacZ* transduced murine lymphoma ESbL cells inoculated to syngeneic DBA/2 mice are characterized by a transient plateau phase with a constant tumor diameter and low metastatic load, indicating a host response against the tumor. Here we show that endothelial cells participate in T-cell dependent anti-metastatic response by producing NO *in situ*. Liver endothelial cells were isolated and examined directly *ex vivo* without further manipulation. NO production in liver endothelial cells reached the highest level during the plateau phase but declined towards the end of it, followed by an overall breakdown of host response leading to progressive tumor growth and high load of liver metastasis. Mice subjected to antitumor immunization and subsequent challenge with a tumorigenic dose of ESbL-*lacZ* cells showed, in comparison to non-immunized challenged controls, reduced liver metastasis and increased endothelial NO production. Adoptive transfer of antitumor immune spleen cells from semi-allogeneic B10.D2 mice into tumor bearing animals during the plateau phase caused a regression of primary tumor and metastases, together with a preservation of the high level of NO synthesis in endothelial cells. In immunoincompetent mice (SCID) tumor growth and metastasis were progressive and there was no endothelial NO response. Preimmunization of immunocompetent mice with both live and irradiated tumor cells at different sites of the body led to an induction of NO production by liver endothelial cells. These results reveal a novel role of endothelial cells in the suppression of lymphoma metastasis in the liver. The inducible endothelial cell NO response is apparently dependent and induced by mature T lymphocytes.

REGULATORY INTERACTIONS BETWEEN IRON METABOLISM AND THE NITRIC OXIDE PATHWAY

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We have previously shown that nitric oxide (NO), a short-lived radical formed upon cleavage of L-arginine to L-citrulline and NO catalysed by the enzyme NO-synthase (NO), modulates intracellular iron regulation via iron-responsive-elements (IRE) in macrophages. NO activates the IRE-binding function of iron-regulatory-protein (IRP), the central factor being responsible for posttranscriptional control of iron metabolism, thus resulting in repression of the synthesis of the iron storage protein ferritin. Based on these observations we investigated if on the opposite iron may influence NO biology. Taking the murine macrophage cell line J774 we could demonstrate that addition of ferric iron nitrate (50μM) to IFN-γ/LPS stimulated cells, the latter procedure strongly induces iNOS in this cell line, drastically reduced NOS activity as compared to stimulation with IFN-γ/LPS alone. Controversely, iron chelation by addition of desferrioxamine increased cytokine/LPS induced NOS activity up to 2.5-fold. We demonstrated that these observations are neither due to a direct effect of iron towards the hemoprotein NOS, nor due to alteration of the intracellular availability of the NOS cofactor tetrahydrobiopterin. Since these effects could not be attributed to a general alteration of the IFN-γ/LPS by the metal, as shown by determination of MHC class II antigen expression, we demonstrated that cytoplasmatic NOS mRNA concentration was reduced upon iron supplementation and increased by desferrioxamine. Determination of mRNA half life did not show significant differences with either treatment. By means of nuclear run off experiments we could then clearly demonstrate that nuclear transcription for NOS mRNA is reduced by iron and enhanced by desferrioxamine treatment. The alterations of transcription appear to quantitatively account for the changes of NOS activity. In summary, our results suggest the existance of an autoregulatory loop for iron metabolismus and the NO/NOS-pathway: iron deprivation of cells results in increased NOS gene transcription leading to enhanced NO formation. NO stimulates IRE-binding by IRP. High affinity binding to the IREs in the 5'-untranslated region of ferritin represses ferritin translation and thus iron storage, which in turn would lead to an increase in "free" cellular iron via enhancement of transferrin receptor expression and consecutive iron uptake. The opposing response is then induced by iron mediated inhibition of NOS transcription and NO formation as indicated by the data presented here. This would consecutively result in reduction of IRE-binding acitivity of IRP and therefore lead to increased ferritin translation and iron storage. This mechanism could provide a tool for activated macropahges to link maintenance of iron metabolism with optimal formation of NO for host defense. The functional interaction of iron metabolism with the NO pathway may further account for iron distrubances causing anemia of chronic disease, which frequently occurs in patients with chronic inflamatory disorders.

Correlation of pteridine and nitric oxide synthesis in humans *in vivo*

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In vitro experiments demonstrated that cytokines induce GTP-cyclohydrolase I in parallel to nitric oxide synthase. This leads to increased intracellular tetrahydrobiopterin concentrations, which are required for full activity of nitric oxide synthase in intact cells. In this study we were interested to assess the significance of these findings for clinical situations with endogenous cytokine formation in humans *in vivo*. We chose two diseases with high cytokine-mediated effects on the metabolism, graft versus host disease and human immunodeficiency virus type-1 (HIV-1) infection. For the investigation of graft versus host disease, we investigated the follow-up of 22 consecutive patients (1056 samples) undergoing bone-marrow transplantation. For HIV-1 infection, we investigated samples of 110 patients with all stages of disease. We assessed plasma or serum concentrations of nitrite plus nitrate, the products of NO endogenously formed, neopterin, a measure for cytokine-mediated induction of GTP-cyclohydrolase I in humans, and soluble tumour necrosis factor receptor 75 (sTNFr75), a measure for activity of the TNF-axis in vivo.

In the bone marrow transplantation group, we found a significant increase of nitrite plus nitrate levels preceeding the onset of clinical symptoms of graft-versus-host disease by up to three days and being paralleled by an increase in neopterin and sTNFr75. In infectious complications, neopterin and sTNFr75 were also elevated, but nitrite plus nitrate concentrations did not differ significantly from controls. In HIV-1 infection, nitrite plus nitrate levels were slightly but significantly higher in patients with advanced disease. In all stages, nitrite plus nitrate significantly correlated to neopterin and sTNFr75.

These results suggest that in graft-versus-host disease and in HIV-1 infection GTP-cyclohydrolase I and nitric oxide synthase are induced in parallel in humans *in vivo* under the control of cytokines.

List of Invited Speakers

MOLECULAR BIOLOGY AND PATHOPHYSIOLOGY OF NITRIC OXIDE

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