Nitric oxide: from basic research to clinical applications

It must have been gratifying for Boris Vargarftig (Institut Pasteur, Paris, France) to introduce a nitric oxide (NO) conference less than three days after the announcement of his Nobel Prize for the discovery of NO in biological systems. Salvador Moncada (Wolfson Institute, University College London, UK), who has inspired the NO research of most of the speakers (and audience) at this Euroconference (held on 15–16 October 1998 at the Pasteur Institute, France), had been instrumental in putting together the programme of speakers and he gave a memorable opening review lecture. The disappointment for the participants of this Euroconference was that Moncada had not shared the Nobel Prize.

Moncada's recent work examines in great detail the mechanism by which NO inhibits mitochondrial respiration. It shows that although NO inhibits complex IV in a reversible manner, long-term exposure to NO results in a persistent inhibition of complex I that is concomitant with a reduction of intracellular reduced glutathione (GSH). This does not involve peroxynitrite ($ONOO^-$); rather, it appears to involve S-nitrosylation of critical thiols in the enzyme complex because it is reversed by replenishment of intracellular GSH. These results suggest a stepwise process is induced by NO from physiology (inhibition complex IV) to pathophysiology (inhibition complex I) that is reminiscent of some features observed in neurodegenerative disorders such as Parkinson's or Alzheimer's diseases.

NO synthases

NO is formed from L-arginine by a family of enzymes called NO synthases (NOSs), of which there are three different isoforms: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). The nNOS may regulate learning and memory formation in brain and contribute to neurodegenerative disorders, while the eNOS promotes vascular relaxation and inhibits platelet aggregation. Both of these enzymes are constitutively expressed and are calcium dependent. The iNOS is inducible by endotoxins and cytokines, and overexpression of this isoform may lead to tissue injury among other harmful effects.

All NOSs are homodimeric and contain a reductase and highly conserved oxygenase protein domain within each monomer. The reductase domain binds FAD, NADPH and FMN, the oxygenase domain binds haem and tetrahydrobiopterin (BH_4), and calmodulin links the two domains.

The crystal structure of the oxygenase domain of mouse iNOS was described by Denis Stuehr (The Cleveland clinic, OH, USA). His group has carried out site-directed mutagenesis and amino acid deletions to provide structure–function information about arginine binding, BH_4 in the dimer and also the flavin-to-haem 'trans' intersubunit transfer of electrons in the heterodimer. He showed that domain swapping occurs in iNOS to properly align the reductase and oxygenase domains for NO formation.

As presented by Bernd Mayer (University of Graz, Austria), BH_4 binds with ease to the first monomer in the dimer, but slowly to the second. Depending on the condition, the physiological consequence of this is that NO, superoxide ($O_2^{-\bullet}$) and $ONOO^-$ will be formed – $O_2^{-\bullet}$ from the BH_4-free

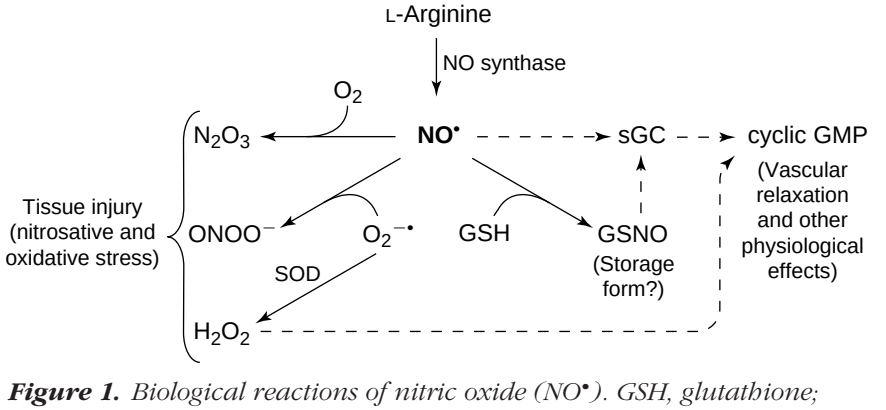

Figure 1. *Biological reactions of nitric oxide (NO•). GSH, glutathione; GSNO, S-nitrosoglutathione; N_2O_3, dinitrogen trioxide; $O_2^{-\bullet}$, superoxide anion; $ONOO^-$, peroxynitrite; sGC, soluble guanylate cyclase; SOD, superoxide dismutase.*

1359-6446/99/$ – see front matter PII: S1359-6446(98)01296-3

subunit (Fig. 1). When superoxide dismutase (SOD) is at high concentrations, NO formation is improved because the peroxynitrite reaction is reduced. In the absence of SOD but in the presence of GSH a storage form of NO, namely *S*-nitrosoglutathione (GSNO), may result that indirectly stimulates cGMP formation. If NOS is saturated with arginine, without BH_4, the product will be hydrogen peroxide, which also stimulates cGMP formation.

Flavin cofactor binding-site inhibitors are less common than active-site inhibitors, but such pharmacological tools (e.g. anti-pterins) that displace BH_4 from NOS unexpectedly do not lower basal NOS activity. The view of Harald Schmidt (University of Würzburg, Germany) is that basal nNOS activity may be independent of BH_4, which may bind to the dimerization domain thereby preventing monomerization and increasing stability. Other pterin-based activators and inhibitors of NOS should prove to be useful tools to understand BH_4 binding and to modulate enzyme activity.

William Sessa (Yale University, New Haven, CT, USA) showed that eNOS is compartmentalized by myristoylation and palmitoylation to the Golgi area and to the cholesterol- and glycolipid-rich regions of the plasma membrane called caveolae. This subcellular localization is critical for optimal NO production. The process of eNOS docking to these membranes is regulated by interaction with caveolin and by heat shock protein 90 (HSP-90), inhibition of which lowers agonist-stimulated NO production.

The activation of eNOS in response to receptor-agonist calcium elevation is transient compared with the activation by shear stress. Tyrosine kinase inhibitors lower stress-activated NOS, which is insensitive to calcium removal. Shear stress studies on eNOS showed that although tyrosine and serine residue phosphorylation occurs it does not account entirely for the calcium independency. According to Rudi Busse (JWG Universität, Frankfurt, Germany), eNOS is part of a 'multimolecular complex' consisting of proteins which co-precipitate with eNOS and are sensitive to tyrosine phosphorylation.

NO and cell death

There are several mechanisms by which NO can induce DNA damage. NO from NO-donors or from cytokine-induced iNOS is sufficient to cause DNA strand breaks directly. But single strand breaks can also be caused if either $ONOO^-$ is formed, which oxidizes guanine and produces abasic sites, or if the nitrosating agent N_2O_3 is formed, which cross links DNA and deaminates bases. Steven Tannenbaum (Massachusetts Institute of Technology, Cambridge, MA, USA) hypothesized that the DNA damage arising from NO is dependent on the dose, cell type and cellular environment, and that it will cause cells to either undergo apoptosis or experience enhanced mutation. His lecture concentrated on NO chemistry and how the formation and action of damaging species (i.e. $ONOO^-$, N_2O_3, Fenton reaction ferryl species and hydroxyl radicals) may differ in cells located at different distances from the site of NO production.

A high concentration of NO is potentially cytotoxic and cell death induced by NO may be necrotic or apoptotic, but NO may also signal cell protection. Apoptosis caused by cytokine- and lipopolysaccharide-induced iNOS and protection by low-dose NO was exquisitely demonstrated and discussed by Bernhard Brüne (University of Erlangen-Numberg, Erlangen, Germany). He described how treatment of RAW 264.7 macrophages with GSNO or cytokines led to p53 tumour-suppressor gene expression, caspase activation, poly ADP ribose polymerase (PARP) cleavage and Bcl2 expression. Protection by low-dose NO results when the intracellular level of nuclear factor κ-binding (NF-κB) is increased. It is suggested that protective proteins such as HSP-70, haemoxygenase-1 and COX-2, as well $NO/O_2^{-\bullet}$ interactions and thiol modification of caspases, could favour protection over cell death.

Targets of NO

An important receptor for NO is soluble guanylate cyclase (sGC), which mediates many physiological actions of NO such as vasorelaxation and inhibition of platelet aggregation or synaptic transmission (Fig. 1). The stimulatory effect of NO is mediated by interaction with the prosthetic haem group of sGC. Doris Koesling (Free University of Berlin, Germany) presented a novel mechanism of stimulating sGC by using YC-1 – a benzylindazole derivative. This compound activates sGC independently of NO or carbon monoxide. By binding to an allosteric site on sGC, YC-1 sensitizes sGC to its natural agonist and also to carbon monoxide. Also by exerting inhibitory effects on cGMP phosphodiesterases, this compound increases cGMP several hundred fold.

Other targets for NO are metalloenzymes that contain Fe–S clusters and/or redox sensitive sites. One such example is aconitase and was described by Jean-Claude Drapier (ICSN-CNRS, Gif-sur-Yvette, France). Another presentation by Rafael Radi (University of Montevideo, Uruguay) highlighted the biological reactions of $ONOO^-$ – the reaction product of NO and superoxide $O_2^{-\bullet}$. He showed how this highly reactive and cytotoxic molecule accounts for both O_2- and NO-dependent toxicity.

It should be noted that signalling for NO is diverse. For example, Ted M. Dawson's (Johns Hopkins University, Baltimore, MD, USA) investigations of neuronal cell development and survival suggest that NO activation of the GTP-binding protein p21 Ras – specifically its GTPase activity by NO-redox sensitive modification – causes activation of extracellular-regulated kinase (ERK).

NO modulators: therapeutic agents

The actions and reactions of NO suggest that it may either exacerbate disease states – such as stroke, Alzheimer's and Parkinson's disease, Huntingdon's chorea, septic shock, asthma and other inflammatory airway diseases – or be therapeutic – as for pulmonary hypertension, vascular disorders and male impotence. NO synthesized by eNOS seems to have exclusively physiological actions whereas NO produced by iNOS and nNOS may be pathologic. Thus, selectivity among NOS isoforms is highly desirable for therapeutic purposes.

NO acute toxicity in brain appears to require $O_2^{-\bullet}$ with which it reacts to form $ONOO^-$. Such neurotoxicity by $ONOO^-$ could result from the inhibition of respiratory chain enzymes and from its DNA-damaging ability. Also, activation of the DNA repair enzyme PARP by $NO/ONOO^-$ results in loss of cellular NAD leading to a fatal depletion in energy stores. Dawson showed that inhibition of PARP *in vitro* attenuates toxicity in cortical cultures, whereas PARP-knockout mice are resistant to focal ischaemia and to the neurotoxin MPTP, which reproduces features of Parkinson's disease.

Evaluation of the neuroprotective properties of NOS inhibitors and antioxidants has been examined by researchers at Beaufour-Ipsen (les Ulis, France). Interestingly, they have found that in combination, these two types of drug exert a synergistic neuroprotective effect. Thus, they have developed novel compounds possessing such dual activity, and Pierre-Etienne Chabrier described **1** – a selective nNOS inhibitor and potent antioxidant (Fig. 2). This compound shows remarkable efficacy in delayed post treatment in models of transient ischaemia to reduce infarct volume and to improve behavioural recovery.

Richard Knowles from Glaxo Wellcome (Stevenage, UK) described the pathologies associated with iNOS (septic shock and tumour growth) and the role of NO in autoimmune diseases and resistance to infection. He reported expression of iNOS in human gastric, CNS, breast and other tumours, and described how in mouse, the growth of tumours *in vivo* is inhibited by compound **2** – a selective iNOS inhibitor. According to Knowles, resolution of conditions like septic shock may require precisely timed use of a selective iNOS inhibitor because nonselective NOS inhibitors such as L-NMMA exacerbate early microvascular leakage by affecting vascular eNOS.

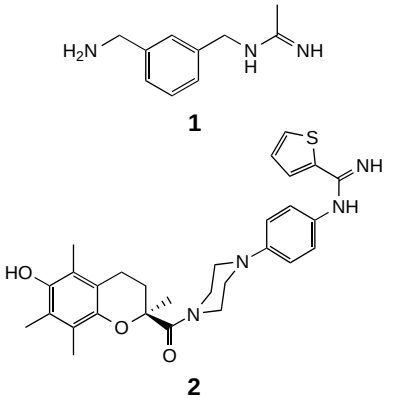

Figure 2. *Selective NOS inhibitors:* ***1****, BN80933 (Beaufour-Ipsen), is a selective nNOS inhibitor with potent antioxidant activity, and* ***2****, 1400W (Glaxo Wellcome), is a selective iNOS inhibitor.*

Peter J. Barnes (National Heart & Lung Institute, London, UK) presented data of exhaled NO by patients with different lung diseases. He found greatly increased levels of NO in airway samples from patients with asthma, but not from patients with other lung diseases. However, NO cannot be used as a diagnostic marker for asthma, because increased levels also occur in other inflammatory diseases and if nasal air contaminates the airways' sample. Barnes described how inhaled NOS inhibitors and steroids can reduce exhaled NO. However, because of the multifactorial nature of inflammatory disease, NO can also tip the balance between cytotoxic and cytoprotective cytokine-producing lymphocytes. More advanced clinical studies were presented by Claes Frostel (Karolinska Hospital, Stockholm, Sweden) who described the use of inhaling NO at 0.1–100 parts per billion to improve oxygenation in both hypoxic newborns and acute lung injury in adults.

In a more prospective way, Victor Dzau (Harvard University, Cambridge, MA, USA) introduced gene therapy strategies using either eNOS or iNOS and their potential benefit to treat vascular proliferative disorders. Gordon Letts (NitroMed, Bedford, MA, USA) explained how Viagra, a phosphodiesterase V inhibitor, modulates the NO–cGMP system, but is ineffective in males not producing enough basal NO. Thus, developing compounds containing 'parent drug-inert linker-NO producer' components is relevant for the male impotence market, and may also have a promising future in other therapies.

Conclusion

This Euroconference showed how dynamic NO basic research is and emphasized clearly its therapeutic potential. To complete the experience for registered participants, invited speakers had contributed to a conference proceedings book reminiscent of a Paris telephone directory, although much more interesting.

Irene C. Green and*
Pierre-Etienne Chabrier‡
**University of Brighton*
Brighton, East Sussex
UK BN2 4GJ
tel: +44 1273 678404
fax:+44 1273 678433
e-mail: i.c.green@sussex.ac.uk
‡at Les Ulis, France