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Review

The inducible nitric oxide synthase in vascular and cardiac tissue

Jean-Claude Stoclet *, Bernard Muller, Katalin György, Ramaroson Andriantsiothaina, Andrei L. Kleschyov

Laboratoire de Pharmacologie et Physico-chimie des Interactions Cellulaires et Moléculaires (UMR CNRS), Université Louis Pasteur de Strasbourg, Faculté de Pharmacie, 74, route du Rhin-F 67401, Illkirch, France

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Abstract

Expression of the inducible form of nitric oxide synthase (iNOS) has been reported in a variety of cardiovascular diseases. The resulting high output nitric oxide (NO) formation, besides the level of iNOS expression, depends also on the expression of the metabolic pathways providing the enzyme with substrate and cofactor. NO may trigger short and long term effects which are either beneficial or deleterious, depending on the molecular targets with which it interacts. These interactions are governed by local factors (like the redox state). In the cardiovascular system, the major targets involve not only guanylyl cyclase, but also other haem proteins, protein thiols, iron–non-haem complexes, and superoxide anion (forming peroxynitrite). The latter has several intracellular targets and may be cytotoxic, despite the existence of endogenous defence mechanisms. These interactions may either trigger NO effects or represent releasable NO stores, able to buffer NO and prolong its effects in blood vessels and in the heart. Besides selectively inhibiting iNOS, a number of other therapeutic strategies are conceivable to alleviate deleterious effects of excessive NO formation, including peroxynitrite ($ONOO^-$) scavenging and inhibition of metabolic pathways triggered by $ONOO^-$. When available, these approaches might have the advantage to preserve beneficial effects of iNOS induction. Counteracting vascular hyper-responsiveness to endogenous vasoconstrictor agonists in septic shock, or inducing cardiac protection against ischaemia–reperfusion injury are examples of such beneficial effects of iNOS induction.

Keywords: Nitric oxide (NO) synthase, inducible; Nitric oxide (NO); Peroxynitrite; *S*-nitrosothiol; Dinitrosyl–iron complex; Septic shock; Cardiac protection

1. Introduction

The first evidence suggesting that extra-endothelial large production of nitric oxide (NO) could be induced in blood vessels by inflammatory stimuli arose from investigations on the mechanisms of hypotension and impaired vasoconstrictor effect of catecholamines in animal models of septic shock (for review, see Stoclet et al., 1993). It was found that NO synthase (NOS) inhibitors were able to reverse the endothelium-independent hyporesponsiveness to noradrenaline (Gray et al., 1990; Julou-Schaeffer et al., 1990) and the increase in tissue level of guanosine 3′,5′-cyclic monophosphate (cGMP) (Fleming et al., 1990, 1991b) that took place several hours after administration of bacterial lipopolysaccharide in rats, or after exposure of aortic rings to lipopolysaccharide in vitro. It was soon confirmed that exposure of the rat aorta or aortic smooth muscle cells to lipopolysaccharide or cytokines, respectively, induces the activity of a NOS of the inducible type (iNOS) that was not activated by calcium (Busse and Mülsch, 1990; Fleming et al., 1991a) and was inhibited by dexamethasone (Knowles et al., 1990; Rees et al., 1990a) and by cycloheximide (Fleming et al., 1993). These early findings led to the hypothesis that pharmacological inhibition of iNOS (or NO effect) might be useful to overcome life threatening hypotension in human septic shock and perhaps in other systemic inflammatory reactions. Administration of these drugs in patients with septic shock partially restored blood pressure (Petros et al., 1991, 1994; Schneider et al., 1992a).

It is now well established that inflammatory stimuli can induce the expression of the ‘macrophage type’ or ‘inducible’ NOS (iNOS or NOS-2) in many eukaryotic cells, including animal and human vascular and cardiac cells, provided they are exposed to an appropriate cocktail of inflammatory stimuli. This may result in production of

* Corresponding author. Tel.: +33-3-88-67-69-33; Fax: +33-3-88-66-46-33; E-mail: stoclet@pharma.u-strasbg.fr

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large amounts of NO, far beyond the 'physiological' levels of NO produced by the 'constitutive' endothelial (eNOS or NOS-3) and neuronal (nNOS or NOS-1) enzymes (for reviews, see Nathan, 1992, 1997). The nomenclature adopted below (nNOS, iNOS, eNOS) for NOS isoforms and NOS inhibitors conforms to the recommendations of IUPHAR (Moncada et al., 1997).

In the cardiovascular system, induction of iNOS has been found in various pathophysiological conditions. It is associated with a variety of short term or long term beneficial or detrimental effects of NO (for reviews, see Stoclet et al., 1993, 1998a,b). The short term effects include vasodilatation, which may improve tissue perfusion but also cause life threatening drop in blood pressure if excessive. They also include inhibition of platelet adhesion and aggregation and of leucocyte adhesion, and enhanced cardiac diastolic relaxation. Long term tissue protective effects mostly result from induction of genes coding for antioxidant and anti-apoptotic mechanisms (for review, see Brüne et al., 1998). In an opposite direction, high NO tissue level can impair cell respiration and induce cytotoxic mechanisms, resulting in cardiac depression and various other deleterious effects such as endothelial injury and multi organ failure. The present review will focus on recent finding on the role of iNOS in vascular and cardiac tissues and on the molecular mechanisms governing its beneficial and detrimental effects.

2. Major biochemical mechanisms involved in the role of iNOS in the cardiovascular system

2.1. Catalytic activity of iNOS

The three NOS isoenzymes can catalyse the oxidation of L-arginine to the free radical NO and L-citrulline, using O_2 and NADPH as co-substrates. However, they produce superoxide anion ($O_2^{\cdot -}$) in the absence of L-arginine. Thus, depending on the concentrations of L-arginine, iNOS may produce either NO, or $O_2^{\cdot -}$, or both (Xia and Zweier, 1997). In the latter case, formation of peroxynitrite ($ONOO^-$) may result from the interaction between NO and $O_2^{\cdot -}$. Furthermore the co-factor tetrahydrobiopterin (BH_4) is essential for enzyme activity of the all three NOSs. Tetrahydrobiopterin is necessary to fully couple reduction of O_2 to oxidation of L-arginine by the two constitutive NOSs (Schmidt et al., 1996; Vasquez-Vivar et al., 1998). If the same is true for iNOS, it might also regulate the balance between NO and $O_2^{\cdot -}$ generation by this enzyme. Considering its marked pterin dependence, it has been proposed that antagonists like the 4-amino analog of BH_4 might selectively inhibit iNOS (Mayer et al., 1997).

The events leading to NO formation in cells in which iNOS expression is induced are illustrated in Fig. 1. In general, the L-arginine tissue content seems sufficient to saturate iNOS. However, in some conditions like sepsis or exposure to endotoxin, NO production by iNOS is critically dependent on extracellular L-arginine supply in vascular smooth muscle cells (Beasley et al., 1991) and in blood vessels (Schneider et al., 1992b, 1994; Schott et al., 1993). Interestingly, pro-inflammatory cytokines and lipopolysaccharide enhance the transport on L-arginine in vascular smooth muscle cells, in conditions in which they induce iNOS expression (Wileman et al., 1995), allowing sustained NO production. Several cationic amino-acids transporters have been characterized in animal cells (for review, see Devés and Boyd, 1998). Recent studies have shown that inflammatory mediators enhance the gene expression of an inducible cationic amino-acids transporter in vascular smooth muscle cells (Gill et al., 1996), in cardiac myocytes (Simmons et al., 1996) and in vascular endothe-

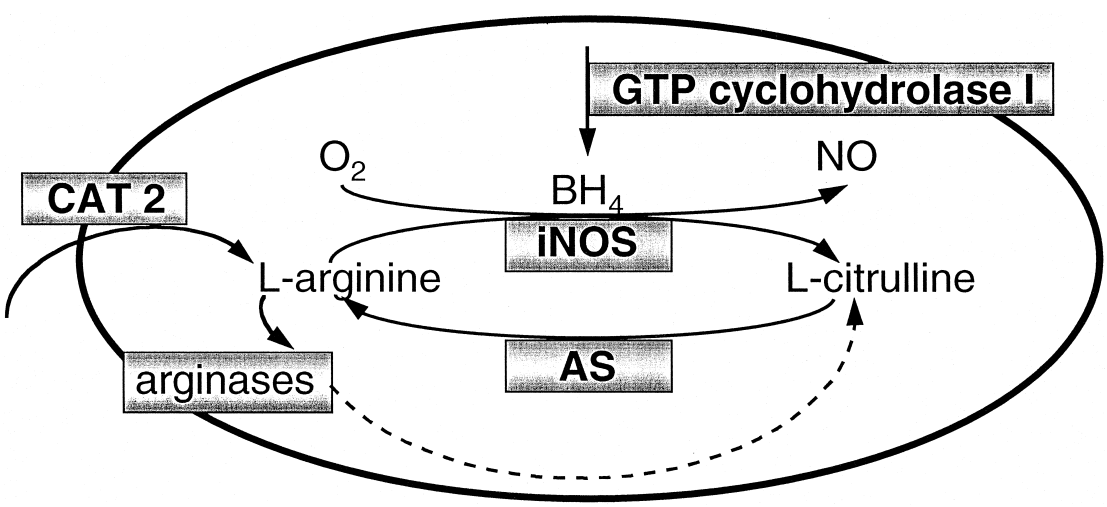

Fig. 1. Metabolic pathways induced by inflammatory stimuli, leading to NO formation in vascular smooth muscle cells. AS: arginosuccinate synthase, CAT 2: cationic amino acid transporter 2, iNOS: inducible NO synthase, BH_4: tetrahydrobiopterin.

lial cells (Irie et al., 1997). In addition, L-citrulline recycling to L-arginine may contribute to provide substrate in support of NO synthesis in some tissues. In vascular smooth muscle, inflammatory stimuli induce the expression of both iNOS and arginosuccinate synthase, which provides iNOS with a continuous flux of L-arginine (Xie and Gross, 1997). In addition, the activities of the arginase isoenzymes may regulate the synthesis of NO (for review, see Wu and Morris, 1998). Relationships between the pathways of L-arginine metabolism are complex owing to compartimentalized expression of involved enzymes and transporters.

De novo BH_4 synthesis in also required for lipopolysaccharide- or cytokine-induced NO generation. GTP cyclohydrolase I, the rate-limiting enzyme in the synthesis of BH_4, is co-induced with iNOS by lipopolysaccharide in vascular smooth muscle cells (Hattori and Gross, 1993) and cardiac myocytes (Kasai et al., 1997). Expression of the two genes appears regulated by activation of the nuclear factor κB(NFκB), allowing cellular NO production by iNOS (Hattori et al., 1996).

N^G-OH-L-arginine (L-OHarg), a stable intermediate in the oxidation of L-arginine to NO and L-citrulline, can be liberated from the active site of NOS and it is released from cells in which a high iNOS activity is expressed, including cytokine stimulated vascular smooth muscle cells (Hecker et al., 1995a). It is also released in the circulation in lipopolysaccharide treated rats (Hecker et al., 1995c). Due to the high specificity of NOS catalysed oxidation of L-arginine to L-OHArg, it might represent a marker of NOS activity. The findings that it can enter cells via a cationic amino acid carrier (Chenais et al., 1993; Schott et al., 1994) led to the suggestion that L-OHArg may produce paracrine effects in tissues. In vascular smooth muscle cells, L-OHArg may be oxidized into nitrogen oxides (Schott et al., 1994), and produce relaxation (Hecker et al., 1995a). The mechanism of oxidation of L-OHArg into nitrogen oxides in vascular smooth muscle cells may involve cytochrome P-450 and $O_2^{\cdot -}$ (Renaud et al., 1993; Schott et al., 1994; Vetrovsky et al., 1996). In addition, L-OHArg can serve as substrate for iNOS in cells exposed to inflammatory agents (Vetrovsky et al., 1997). It is also a potent inhibitor of arginases (Boucher et al., 1994; Hecker et al., 1995b). Thus, there are multiple pathways by which L-OHArg may directly or indirectly cause NO formation in tissues.

2.2. Molecular mechanisms of the effects of NO in the heart and blood vessels

The nature of tissue effects of the free radical NO produced by iNOS is governed by its interaction with molecular targets, which may either trigger effects, or contribute to formation of releasable stores or transport forms, or represent a sink for elimination of NO. The current evidence supports the view that NO is directed towards one or another molecular target by multiple factors, including not only the concentration of NO, but also the nature of local targets, O_2, local pH and presence of transition metals (for reviews, see Nathan, 1992; Henry et al., 1993; Stamler, 1994; Stoclet et al., 1998b).

2.2.1. cGMP-dependent and -independent effects of NO

It is generally assumed that activation of the soluble guanylyl cyclase, which is an haemoprotein, accounts for a large part of the effects of 'physiological' concentrations of NO produced by eNOS and nNOS, whereas higher NO levels produced by iNOS can activate other less sensitive targets which would trigger toxic effects. This is certainly an oversimplified view. Some effects of NO derived from eNOS or nNOS are cGMP-independent. This is the case for instance for activation of some K^+ channels which contribute to NO-induced relaxation in some vessels (Bolotina et al., 1994). Reversible inhibition of mitochondrial complex IV (cytochrome c oxidase), resulting in reduced O_2 consumption in neighbour cells by endothelium-derived NO (Shen et al., 1995), interactions with many proteins like cyclo-oxygenases (Salvemini et al., 1993), inhibition of the angiotensin converting enzyme (Ackermann et al., 1998), are other examples of cGMP-independent effects of NO. Vice versa, cGMP is involved in many effects of iNOS-derived NO, including not only haemodynamic effects, but also inhibition of smooth muscle migration and proliferation, inhibition of platelet aggregation and leucocyte adhesion (for reviews, see Moncada et al., 1991; Nathan, 1992; Sarkar and Webb, 1998). Whatever the origin of NO, cGMP is implicated not only in short but also in long term effects of NO; the cascade of events triggered by activation of cGMP-dependent protein kinase regulates vascular smooth muscle phenotype (Boerth et al., 1997).

2.2.2. Formation of protein S-nitrosothiols

Although the free radical NO itself do not nitrosate thiols, formation of *S*-nitrosothiols is a major pathway in NO signalling (Stamler, 1994). The mechanisms of formation of *S*-nitrosothiols are not entirely elucidated. *S*-nitrosation of critical thiol groups can modify structure and function of various proteins. Well known examples of the role of such mechanisms are *S*-nitrosation of K^+ channels which may explain the abovementioned cGMP-independent hyperpolarization of vascular smooth muscle cells (Bolotina et al., 1994), and activation of the cardiac ryanodine-sensitive calcium release channel (Xu et al., 1998). *S*-nitrosation of proteins also triggers longer term effects of NO resulting from modulation of transcription factors function (Henderson et al., 1994), protein kinase C (Gopalakrishna et al., 1993) and phosphotyrosine phosphatase (Caselli et al., 1994) activities. It also probably accounts for irreversible inhibition of mitochondrial complex I caused by long term exposure to NO (Clementi et al., 1998).

In general, protein *S*-nitrosothiols are more stable than low molecular weight *S*-nitrosothiols. Low molecular weight thiols appear to play a crucial role in *trans*-*S*-nitrosative reactions allowing transfer of NO moiety from one thiol to the other (Scharfstein et al., 1994). Once *S*-nitrosated, they can either transfer NO to protein cysteine residues or release free NO. Thus, trans *S*-nitrosation reactions may transfer NO moiety to targets or contribute to release of NO from *S*-nitrosated proteins (Williams, 1996; Hogg et al., 1997).

An example suggesting the involvement of the displacement of NO from stores by a low molecular weight thiol is provided by studies performed in lipopolysaccharide-exposed rat aorta. In this tissue, *N*-acetylcysteine produced vasorelaxation in the presence of a NOS inhibitor, whereas it had no relaxing effect in control vessels or in vessels incubated with lipopolysaccharide but without L-arginine (Muller et al., 1996). Furthermore, in these conditions, the relaxing effect of *N*-acetylcysteine was cGMP-independent, and it was inhibited by a non-specific K^+-channel blocker, tetrabutyl ammonium, and by KCl depolarization (Muller et al., 1998). One possible interpretation is that, in these conditions, *N*-acetylcysteine-induced relaxation resulted from *trans*-*S*-nitrosation of thiol groups of K^+ channels.

2.2.3. Formation of dinitrosyl–iron complexes with thiols

Paramagnetic dinitrosyl–iron complexes are present in NO overproducing cells and tissues (for reviews, see Henry et al., 1993; Vanin and Kleschyov, 1998). In the literature, there are contradictory views on the role of these NO-derived species. Their general formula is $(NO)_2Fe(RS)_2$, where RS is either a low molecular weight thiol or a thiol protein. Low molecular weight dinitrosyl–iron complexes are labile compounds, which can serve both as NO donors activating soluble guanylyl cyclase (Mülsch et al., 1991) and as powerful nitrosative agents (Boese et al., 1995). Conversely, protein-bound dinitrosyl–iron complexes are much less mobile, more stable and it is very unlikely that they can efficiently serve as NO donors or nitrosating agent themselves. In intracellular environment, the equilibrium between low molecular weight dinitrosyl–iron complexes and protein-bound dinitrosyl–iron complexes strongly favours formation of the protein-bound form. However, increase in concentration of low molecular weight thiols shifts the equilibrium toward low molecular weight dinitrosyl–iron complexes. Thus, at high local concentration of low molecular weight thiols, protein-bound dinitrosyl–iron complexes can represent a slow releasable reservoir of biologically active NO, while at low concentration of low molecular weight thiols (which can occurs in some intracellular compartments or near hydrophobic parts of proteins), protein-bound dinitrosyl–iron complexes can be very stable.

Dinitrosyl–iron complexes were found in endothelial cells stimulated with bradykinin (Mülsch et al., 1993) and in rat aorta exposed to lipopolysaccharide (Muller et al., 1996; Kleschyov et al., 1997). In rat aorta, lipopolysaccharide-induced iNOS activity resulted in accumulation of large amounts of protein-bound dinitrosyl–iron complexes in the media layer. This accumulation was dependent on exogenous L-arginine and on the presence of adventitia. As discussed below (Section 4.1.), NO released from adventitial cells can diffuse in the media, and produce both immediate relaxation via a cGMP-dependent mechanism and contribute to generation of *N*-acetylcysteine-releasable NO store. Protein bound dinitrosyl–iron complexes might be a good candidate for participating in this NO storage, as *N*-acetylcysteine facilitates the formation of low molecular weight dinitrosyl–iron complexes. Interestingly, dinitrosyl–iron complexes possess antioxidant properties which might contribute to tissue protection (Gorbunov et al., 1997).

2.2.4. Peroxynitrite

The reaction of NO with $O_2^{\cdot-}$ is so fast that it occurs practically at diffusion controlled rate, resulting in formation of $ONOO^-$. This is most important not only because iNOS may be able to catalyze the formation of both $O_2^{\cdot-}$ and NO (as discussed above), but also because NO may react with $O_2^{\cdot-}$ locally produced by other enzymes, such as NAD(P)H oxidase.

Formation of $ONOO^-$ is generally associated with cytotoxicity. For example, it may account for endothelial injury (Bouloumie et al., 1997) and for vascular failure in endotoxic shock (Szabo and Dawson, 1998). However, it seems that $ONOO^-$ can also exert some beneficial effects. A recent study showed that physiologically relevant concentrations of $ONOO^-$ decreased the severity of ischaemic insult and preserved coronary endothelial function in a feline model of ischaemia–reperfusion (Nossuli et al., 1998).

The molecular mechanisms by which $ONOO^-$ exert its effects are diverse. $ONOO^-$ can produce DNA strand breakage, initiate peroxidation of lipids and oxidize, nitrate or nitrosate a variety of proteins, thus modifying their function (Beckman and Koppenol, 1996). In each particular case it is unknown whether $ONOO^-$ acts itself or via the formation of secondary $ONOO^-$-derived reactive radicals. From chemical perspective, $ONOO^-$ is relatively stable at high physiological pH. A slight acidic pH will favour generation of highly reactive species ($OH^{\cdot}$, $NO_2^{\cdot}$). At neutral pH, nitration reactions catalyzed by transition metals will take place. In slightly alkaline medium (pH 8), $ONOO^-$ will isomerise directly in NO_3^- (Beckman and Koppenol, 1996; Muijsers et al., 1997). Carbon dioxide, due to its high concentration and high reaction rate with $ONOO^-$ (5.8×10^4 M^{-1} s^{-1}) is thought to limit the reactions of $ONOO^-$ in vivo. It seems that only a few number of biotargets can react directly with $ONOO^-$ under physiological conditions. However, the reaction of CO_2 with $ONOO^-$ produces a range of the secondary

reactive species (nitrating, nitrosating, oxidizing), among which $NO_2^{\cdot}$ and $CO_3^{\cdot}$ (Squadrito and Pryor, 1998). It has been demonstrated recently that nitration of tyrosine residues of proteins is indeed mediated by $NO_2^{\cdot}$ rather than $ONOO^-$ itself (Pfeiffer and Mayer, 1998).

In cells, the dismutation of $O_2^{\cdot -}$ by superoxide dismutase probably limits the formation of $ONOO^-$. The $ONOO^-$-induced effects may be also counteracted by endogenous anti-oxidant activity. In such cellular defence, the important role belongs to glutathione. Indeed, in various cell types, including endothelial and vascular smooth muscle cells, glutathione depletion enhanced cytotoxic effects of $ONOO^-$ (Castro et al., 1998; Cuzzocrea et al., 1998). In isolated blood vessels, it has been reported that depletion of intracellular glutathione reduces the relaxing effect of $ONOO^-$, and that $ONOO^-$ reacts with glutathione to form an intermediate product that promotes time- and thiol-dependent release of NO and vasodilatation (Mayer et al., 1995; Balazy et al., 1998). This reaction product was identified as *S*-nitrosoglutathione in one study (van der Vliet et al., 1998) and as *S*-nitroglutathione in another study (Balazy et al., 1998). These data emphasize the importance of the interaction between $ONOO^-$ and glutathione that may provide both cytoprotection and generation of a releasable storage form of NO. Reaction of $ONOO^-$ with sugars or other compounds containing an alcohol functional group also generate compounds that behave as NO donors (Moro et al., 1995).

Uric acid, the product of purine metabolism, is another endogenous compound that avidly reacts with $ONOO^-$. Recently, the reaction product of uric acid and $ONOO^-$ has been identified as a nitrated uric acid derivative. Interestingly, this product possesses potent vasorelaxing properties related to protracted and thiol-independent release of NO (Skinner et al., 1998).

Recent studies have discovered several new endogenous defence mechanisms against $ONOO^-$-mediated injury. Glutathione peroxidase can function as a $ONOO^-$ reductase, producing nitrite (Roussyn et al., 1996; Briviba et al., 1998). Selenoprotein P (Arteel et al., 1998), melatonin (Gilad et al., 1997) and bilirubin (Minetti et al., 1998) also efficiently protect against $ONOO^-$-mediated oxidation and nitration reactions. Moreover, a 'nitrotyrosine denitrase'-like activity was found in some tissues (Kamisaki et al., 1998).

2.2.5. Poly(ADP-ribose) polymerase activation

Some well established effects of $ONOO^-$ in vascular tissue include DNA strand breakage and inhibition of mitochondrial respiration (Muijsers et al., 1997; Szabo and Dawson, 1998). Single strand breaks leads to the activation of a nuclear enzyme, poly(ADP-ribose) polymerase activation. This has been reported in vascular smooth muscle cells expressing iNOS (Cuzzocrea et al., 1998). The physiological function of poly(ADP-ribose) polymerase is not fully understood but it plays an important role in maintaining genomic stability in the base excision repair pathway. Poly(ADP-ribose) polymerase is involved in different cellular responses to genotoxic damage, including cellular survival, cellular division and DNA replication, DNA repair, cellular transformation and differentiation and cell death, as well as apoptosis (Muijsers et al., 1997; Le Rhun et al., 1998; Szabo and Dawson, 1998).

Overactivation of poly(ADP-ribose) polymerase leads to a rapid cellular depletion of its substrate, nicotine adenine dinucleotide (NAD^+), and subsequent inhibition of cellular ATP-generating pathways (Le Rhun et al., 1998). The ultimate consequences are acute cellular dysfunction and cellular death. Indeed, poly(ADP-ribose) polymerase activation contributes to the development of vascular hyporeactivity, endothelial dysfunction and recruitment of pro-inflammatory cells such as neutrophils during systemic inflammation induced by many inflammatory agents including zymosan or lipopolysaccharide. Recent studies in poly(ADP-ribose) polymerase knock out mice indicate that poly(ADP-ribose) polymerase is involved in organ injury caused by inflammation and ischaemia–reperfusion. However it should be noted that at very high concentration of $ONOO^-$, poly(ADP-ribose) polymerase-independent cytotoxic mechanisms become predominant (Szabo et al., 1997a).

In addition to the direct cytotoxic pathway regulated by DNA injury and poly(ADP-ribose) polymerase activation, it appears that poly(ADP-ribose) polymerase plays an important role in regulating the expression of a variety of genes including the iNOS. Indeed, poly(ADP-ribose) polymerase seems to be required in the pathway leading to the activation of NFκB-mediated gene transcription of iNOS in macrophages (Le Page et al., 1998). The latter pathway may be crucial for the anti-inflammatory effect of either pharmacological inhibition of poly(ADP-ribose) polymerase or deletion of its gene, since NFκB-pathway is a key element for the production of many other inflammatory products such as tumor necrosis factor-α, interleukin-6, cyclo-oxygenase metabolites and other oxidant species and adhesion molecules.

3. Pharmacological modulation of iNOS and its products

3.1. Inhibition of iNOS activity

3.1.1. Inhibition of human NOS isoenzymes

Comparisons of the potency and selectivity of inhibitors on NOS activities are difficult, mainly because of variations in the source and level of purity of NOS isoenzymes. The present review focuses on data obtained on highly purified or recombinant NOS from human origin (Garvey et al., 1994, 1996, 1997; Faraci et al., 1996; Hagen et al., 1998), with particular reference to inhibitors of eNOS and iNOS.

N^G-substituted analogs of L-arginine represent the first class of drugs described to inhibit NO synthesis. Although these compounds inhibit NOS activity by competition with L-Arginine at the active site, their precise mechanism of action varies with the NOS isoform and the analog studied (Marletta, 1994). Data recently obtained on purified human NOSs (Table 1) largely confirm those previously reported on NOS from various sources in that none of these analogs of L-arginine displays selectivity for iNOS.

The first drugs characterized as selective inhibitors of human iNOS were aminoguanidine and L-N^6-(1-iminoethyl)-lysine. In comparison to data previously obtained on partially purified inducible and constitutive NOS (Misko et al., 1993; Moore et al., 1994), their selectivity for iNOS is less on recombinant enzymes from human origin, especially in the case of aminoguanidine.

As compared to the abovementioned inhibitors, some *S*-substituted isothioureas are much more potent inhibitors of human iNOS (Garvey et al., 1994). However, they demonstrate modest selectivity for iNOS. *S*-methylisothiourea, *S*-ethylisothiourea, *S*-isopropylisothiourea and aminoethylisothiourea are only 1.5- to 4-fold selective for iNOS vs. constitutive human enzymes.

A higher degree of selectivity for iNOS vs. eNOS is obtained with some bis-isothiourea derivatives (*S,S′*-(1,3-phenylenebis(1,2-ethanediyl))bisisothiourea), substituted 2-iminohomo piperidinium salts and 2-iminopiperidines. However, their selectivity for iNOS vs. nNOS inhibition appears narrow (Garvey et al., 1994, Hansen et al., 1998; Webber et al., 1998). By contrast, 1400W (*N*-(3-(aminomethyl)benzyl) acetamidine) and (+)-*cis*-4-methyl-5-pentylpyrrolidin-2-imine display large selectivity for iNOS vs. both eNOS and nNOS (Garvey et al., 1997; Hagen et al., 1998). Compound 1400W behaves as a slow and tight binding inhibitor of iNOS (Garvey et al., 1997) and is the most potent and selective inhibitor of the inducible enzyme described up to date.

3.1.2. Effects of NOS inhibitors in isolated blood vessels and intact animals

Many approaches have been used to address the efficacy and selectivity of NOS inhibitors in biological systems. The consequences of eNOS inhibition in blood vessels have been currently assessed by studying the ability of inhibitors to increase tone or to inhibit the effects of endothelium-dependent vasodilators in isolated vascular preparations and to increase systemic arterial blood pressure in intact animals. Those of iNOS inhibition have been evaluated by the ability of inhibitors either to reverse the vascular hyporeactivity to contractile agonists in vascular preparations exposed to lipopolysaccharide (in vitro or ex vivo) or to attenuate lipopolysaccharide-induced delayed hypotension or rise in plasma concentration of nitrate in intact animals.

As expected from data obtained on isolated enzymes, N^G-substituted analogs of L-arginine do not display selectivity for iNOS inhibition in isolated vessels and intact animals. N^G-nitro-L-arginine methylester (L-NAME) for instance at a dose which elevated mean arterial blood pressure in untreated rats (Rees et al., 1990b), also prevented delayed hypotension and rise in circulating levels of nitrates caused by lipopolysaccharide (Paya et al., 1995). In vascular tissue exposed to lipopolysaccharide, these analogs restored contractile responses to agonists (Julou-Schaeffer et al., 1990; Fleming et al., 1991b) and in the same range of concentrations, also inhibited the influence of basal and agonist-stimulated eNOS activity (Rees et al., 1990b).

L-N^6-(1-iminoethyl)-lysine has been reported to attenuate lipopolysaccharide-induced delayed hypotension and rise in circulating nitrate in rats (Faraci et al., 1996; Wray et al., 1998) at about 5 times lower doses than those required to elevate arterial blood pressure in control animals. Few information exist on its potential selectivity in isolated vascular preparations.

Table 1
Effects of some inhibitors on human NOS isoenzymes

Inhibitor	iNOS	eNOS	nNOS
L-NMMA	0.86[a]	0.41[a]	0.84[a]
L-NA	0.67[a]	0.039[b]	0.015[b]
L-NIO	0.34[a]	0.81[a]	0.23[a]
L-N^6-(1-iminoethyl)-lysine	0.9[d]	10.4[d]	4.1[d]
Aminoguanidine	6.4[d]	22.1[d]	30.3[d]
S-methylisothiourea	0.12[a]	0.20[a]	0.16[a]
S-ethylisothiourea	0.019[a]	0.039[a]	0.029[a]
S-isopropylisothiourea	0.0098[a]	0.022[a]	0.037[a]
S-aminoethylisothiourea	0.59[a]	2.1[a]	1.8[a]
S,S′-(1,3-phenylenebis(1,2-ethanediyl))bisisothiourea	0.047[a]	9[a]	0.25[a]
(+)-*cis*-4-methyl-5-pentylpyrrolidin-2-imine	0.25[d]	226[d]	3.2[d]
N-(3-(aminomethyl)benzyl) acetamidine (1400W)	0.007[c]	50[a]	2[a]

L-NMMA: N^G-monomethyl-L-arginine; L-NA: N^G-nitro-L-arginine; L-NIO: *N*-iminoethyl-L-ornithine.
K_I values (μM) on human purified ([a]) NOS (Garvey et al., 1994, 1996, 1997)
K_D values (μM) on human purified ([b]) or recombinant ([c]) NOS (Garvey et al., 1996, 1997)
IC_{50} values (μM) on human recombinant ([d]) NOS (Faraci et al., 1996; Hagen et al., 1998)

Most *S*-substituted isothioureas increased mean arterial blood pressure when injected to anaesthetized rats (Southan et al., 1995). *S*-methylisothiourea reversed lipopolysaccharide-induced hypotension in the rat, the amplitude of blood pressure elevation being larger than that observed in control animals administered with the same range of doses (Szabo et al., 1994). This finding distinguishes *S*-methylisothiourea from N^G-monomethyl-L-arginine (L-NMMA) and L-NAME which increased blood pressure to a similar extend in both control and lipopolysaccharide-treated rats. It has been interpreted as indicating a selective inhibitory effect of *S*-methylisothiourea on iNOS in vivo (Szabo et al., 1994).

Aminoethylisothiourea also produced sustained arterial blood pressure elevation in lipopolysaccharide-treated rats, whereas no such effect was observed in control animals (Southan et al., 1996), suggesting a selective inhibition of iNOS in vivo. However aminoethylisothiourea undergoes chemical modifications at physiological pH, rearranging into mercaptoethylguanidine as well as small amounts of 2-aminothiazoline (Southan et al., 1996). The latter behaves as a non-selective NOS inhibitor, whereas the former scavenges peroxynitrite $ONOO^-$ (Szabo et al., 1997b) and exerts a dual inhibitory effect on iNOS and cyclooxygenases activity (Southan et al., 1996; Zingarelli et al., 1997). In addition, mercaptoethylguanidine may dimerise into guanidinoethyldisulphide, which in addition to inhibiting iNOS, also induces relaxation by other mechanism(s) (Szabo et al., 1996). These multiple properties may contribute to the apparent selectivity of aminoethylisothiourea for iNOS in vivo. Aminoethylisothiourea also decreases the expression of iNOS protein in vitro and in vivo (Ruetten and Thiemermann, 1996) by inhibiting the translation of iNOS mRNA into functional protein as well as accelerating degradation of already translated protein (Wei et al., 1998). Although potentially relevant for anti-inflammatory therapy, all these properties represent important limitations for the use of isothioureas as pharmacological agents to evaluate specifically the role of iNOS activity in the cardiovascular system.

Aminoguanidine is one of the selective iNOS inhibitors that has been largely evaluated, both in isolated blood vessels and in vivo. In vascular preparations removed from lipopolysaccharide-treated rats, aminoguanidine enhanced the response to vasoconstrictors (Griffiths et al., 1993; Joly et al., 1994) at concentrations at which it did not affect the response related to eNOS activity (Hasan et al., 1993; Misko et al., 1993). Accordingly, in lipopolysaccharide-treated rats, aminoguanidine increased blood pressure and restored responses to vasopressor agents at doses that did not produce hypertension in controls (Wu et al., 1995). Also in rats, aminoguanidine attenuated lipopolysaccharide-induced rise in circulating nitrate at about 9 times lower doses than those elevating arterial blood pressure in untreated animals (Faraci et al., 1996). Such data indicate a selective inhibitory effect of aminoguanidine on iNOS in vascular tissue as well as in vivo. However, other data do not support such selectivity, especially in intact animals. For instance aminoguanidine inhibited both constitutive and inducible NOS in rat intestinal microvasculature (Laszlo et al., 1995). Administered subcutaneously to anaesthetized rats, it substantially increased blood pressure, the response reaching its maximal effect at a much slower rate (within 1 h) than the one produced by L-NMMA at a similar dose (Laszlo et al., 1995). Moreover, besides the inhibition of iNOS activity, aminoguanidine prevents the induction of iNOS (Ruetten and Thiemermann, 1996), possesses direct scavenging properties against free radicals and $ONOO^-$ (Yildiz et al., 1998) and inhibits the activity of various enzymes (Southan and Szabo, 1996).

The effects of more recently described selective iNOS inhibitors are not fully characterized at the present time. The use of *S,S′*-(1,3-phenylenebis(1,2-ethanediyl)) bisisothiourea in biological system is limited by its poor cellular penetration and acute toxicity (Garvey et al., 1994). (+)-*cis*-4-methyl-5-pentylpyrrolidin-2-imine elevated blood pressure in mice, but the effective doses were much larger than those inhibiting lipopolysaccharide-induced elevation of plasma nitrate levels (Hagen et al., 1998). In animals, compound 1400W attenuated lipopolysaccharide-induced delayed hypotension and rise in circulating nitrate (Wray et al., 1998, Hamilton and Warner, 1998). The selectivity of compound 1400W for iNOS was confirmed in isolated arteries (in which it appeared at least 1000-fold selective for iNOS vs. eNOS) and in intact rats, where it diminished delayed vascular injury induced by lipopolysaccharide without exacerbating acute vascular leakage attributed to eNOS inhibition, even at 50 times larger doses (Garvey et al., 1997).

3.2. NO and reactive nitrogen oxides scavenging

The principal natural scavenger of NO, oxyhaemoglobin, has been widely used in experimental systems to investigate the effects of NO. Some other scavengers of NO are also known, including hydroxocobalamin (Rochelle et al., 1995), spin traps like Fe-diethyldithiocarbamate (Vanin and Kleschyov, 1998), imidazolineoxyl *N*-oxides like carboxy-PTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide] (Akaike et al., 1993), iron chelates (Kazmierski et al., 1996) and ruthenium complexes (Fricker et al., 1997). Superoxide anion may also be considered as an NO scavenger, which is able to reduce the direct effects of NO. Thus, $O_2^{\cdot-}$ generators like hydroquinone, pyrogallol and LY83583 (6-anilino-5,8-quinolinedione) inhibit NO-induced vasorelaxations (Mülsch et al., 1989; Cherry et al., 1990). All these drugs do not discriminate between endothelial-derived NO, NO released from nerve endings, and NO from another cell origin subsequent to the induction of iNOS activity. As a consequence, they should produce arterial hypertension when administered in vivo. Furthermore, they are not specific

and they may inhibit vasorelaxation via various mechanisms.

Since under pathophysiological conditions NO can be readily converted into an array of reactive forms, including potentially toxic ones such as $ONOO^-$ and $NO_2^{\cdot}$, the use of appropriate and selective scavengers might have some advantages over NOS inhibitors. In this regard, plenty of both natural and synthetic compounds which exert $ONOO^-$ scavenging activities have been discovered over the last few years. It is necessary to point out that most of them have been evaluated in strictly defined systems in vitro. The physiological and pharmacological relevance of these findings remain still largely unknown.

As mentioned above (Section 2.2.4), some naturally occurring compounds, such as glutathione, uric acid, melatonin and bilirubin can react with $ONOO^-$ and prevent its toxic effects. Some of them have proven to be of pharmacological interest. For instance, uric acid minimized tissue damage and improved both survival and clinical symptoms in experimental allergic encephalomyelitis and multiple sclerosis (Hooper et al., 1997, 1998).

Synthetic mimics of glutathione peroxidase such as selenomethionine, selenocystine and especially ebselen, very rapidly react with $ONOO^-$ to produce nitrite and protect against $ONOO^-$-mediated oxidation and nitration (Roussyn et al., 1996; Briviba et al., 1998).

Lazaroids (Fici et al., 1997) and flavonoids have been described as scavengers of $ONOO^-$. Some of the latter are about 10 times more effective than the well known $ONOO^-$ scavenger ebselen (Haenen et al., 1997). A dietary polyphenol, clorogenic acid, very efficiently scavenges $ONOO^-$ and provides DNA protection in the presence of horseradish peroxidase (an analog of human myeloperoxidase). The high rate of the reaction suggests its physiological and pharmacological importance (Grace et al., 1998).

Among the most promising compounds which can efficiently interfere with $ONOO^-$ are certain water-soluble iron (III) porphyrins, such as 5,10,15,20-tetrakis(2,4,6-trimethyl-3,3-disulfonatophenyl)porphyrinato iron (III) and 5,10,15,20-tetrakis(*N*-methyl-4′-pyridyl)porphyrinato iron (III) which catalyze the isomerization of $ONOO^-$ to nitrate. In animal experiments, these compounds inhibited carrageenan-induced inflammation while they did not elevate blood pressure, suggesting a lack of interaction with NO derived from eNOS (Salvemini et al., 1998).

3.3. Other pharmacological approaches

Other conceivable pharmacological approaches to protect tissues against the deleterious effects of excessive NO production include inhibition of L-arginine availability, inhibition of the expression of the iNOS gene and inhibition of the cytotoxic mechanisms triggered by NO.

With respect to L-arginine availability, inhibition of cellular uptake may reduce selectively large NO formation in conditions of iNOS activation, if the intracellular pool of L-arginine remains sufficient to meet with the lower NO formation catalyzed by the constitutive eNOS and nNOS. Indeed, it has been reported that administration of L-lysine, a competitor of L-arginine at cationic aminoacids transporter, reduces haemodynamic alterations and NO production in rats after lipopolysaccharide (Liaudet et al., 1997). However, L-lysine interacts not only with cationic amino acids transporter 2, whose expression is induced by lipopolysaccharide (see Section 2.1 and Fig. 1), but also with other L-arginine carriers. One of these carriers (cationic amino acids transporter 1) is coupled with eNOS in caveolae, allowing direct delivery of L-arginine to the enzyme (McDonald et al., 1997). As a consequence, endothelial NO formation may depend on extracellular L-arginine supply. Selective blockade of cationic amino acids transporter 2 might be a means to reduce NO production by iNOS without impairing eNOS activity. As discussed above (Section 2.1), there are other potential targets to achieve the same goal by modulating L-arginine metabolic pathways (Fig. 1). Pharmacology of these pathways warrants further investigation.

Inhibition of the iNOS gene expression might be of therapeutic value for the treatment of inflammatory reactions such as those triggered by sepsis. It is well known that glucocorticoids are able to inhibit the induction of iNOS by lipopolysaccharide in rats (Knowles et al., 1990) and in human vessels (Tsuneyoshi et al., 1996a). However, dexamethasone prevented but was unable to reverse lipopolysaccharide-induced vascular hyporeactivity in rats (Paya et al., 1993). This might explain negative results obtained with high dose glucocorticoid therapy in human septic shock and in lipopolysaccharide-exposed human arteries (Tsuneyoshi et al., 1996a). The same might be true for many other agents that can prevent induction of iNOS by cytokines in cultured vascular smooth muscle cells, including growth factors (Scott-Burden et al., 1992), and inhibitors of NFκB activation, including antioxidants like dithiocarbamates (Schini-Kerth et al., 1994) and protease inhibitors like *N*-α-tosyl-L-lysine chloromethyl ketone (Schini-Kerth et al., 1997). It is worth mentioning that endothelium might inhibit iNOS expression in blood vessels via the release of angiotensin II activating on angiotensin AT_1 receptors (Monton et al., 1997; Nakayama et al., 1994). Other drugs such as *N*-acetyl-5-hydroxytryptamine that inhibit the release of inflammatory cytokines may also reduce both iNOS induction in vascular smooth muscle cells and hypotension caused by lipopolysaccharide (Klemm et al., 1995).

Another conceivable approach is to alleviate some toxic effects of excessive NO production without altering NO production. $ONOO^-$ scavenging is discussed above. Poly(ADP-ribose) polymerase inhibition has also been proposed (for review, see Szabo and Dawson, 1998). Indeed, the poly(ADP-ribose) polymerase inhibitors such as 3-aminobenzamide attenuate NAD and ATP depletion. They

reduce systemic and local inflammatory response and associated tissue injury caused by endotoxin and ischaemia–reperfusion, and they improve the survival rate of mice injected with a high dose of lipopolysaccharide. A variety of compounds are able to inhibit poly(ADP-ribose) polymerase. The available information concerning these compounds have been discussed recently by Szabo and Dawson (1998). Poly(ADP-ribose) polymerase inhibition might prove a valuable strategy in pathological conditions associated with overproduction of nitrogen oxides, subject to further evaluation of the physiological role of poly(ADP-ribose) polymerase and its importance in maintaining genomic stability.

4. Pathophysiological implications of iNOS

The systemic inflammatory syndrome triggered by sepsis is the most striking pathological state in which iNOS is involved. However, local induction of iNOS in blood vessels and in the heart has also been implicated in a variety of diseases. These include proliferative diseases like restenosis after angioplasty and atherosclerosis, in which anti-migration, anti-proliferative and pro- and anti-apoptotic effects of NO may be involved (for review, see Stoclet et al., 1998a). The involvement of iNOS has also been reported in human cardiac allograft rejection (Lewis et al., 1996; Ravalli et al., 1998; Szabolcs et al., 1998). However, there is also evidence for a beneficial role of iNOS induction in cardiac protection against ischaemia/reperfusion injury, in animal models (see below). The present review will focus on septic shock and cardiac effects of iNOS.

4.1. Sepsis and endotoxaemia

Induction of iNOS by bacterial products may be considered as a part of the defence mechanisms against infection (for review, see Wong and Billiar, 1995). However, it has been suggested that the resulting overproduction of NO (and other reactive nitrogen oxides) may be involved in life threatening drop in blood pressure and in multiorgan failure that may occur in septic shock (Stoclet et al., 1993, 1998a).

In blood vessels and in vascular smooth muscle cells, induction of iNOS can be caused not only by exposure to lipopolysaccharide from gram-negative bacteria, but also by exposure to cell wall composites of gram-positive bacteria (Auguet et al., 1992; Martin et al., 1997). In endotoxaemic animals, induction of iNOS occurs not only in conduit arteries, but also in resistance arteries (Schneider et al., 1992b, 1994; Mitchell et al., 1993) and in veins (Vallance et al., 1992). Overproduction of NO or NO-mediated vascular hyporeactivity have been reported not only in animal models, but also in human arteries exposed to lipopolysaccharide (Thorin-Trescases et al., 1995; Tsuneyoshi et al., 1996a) and in patients with sepsis (Ochoa et al., 1991; Lorente et al., 1993; Tsuneyoshi et al., 1996b).

Both endothelial cells (Radomski et al., 1990) and smooth muscle cells express iNOS in response to proinflammatory stimuli (Busse and Mülsch, 1990; Beasley et al., 1991; Fleming et al., 1991a). Because smooth muscle cells are the major cell type of the vascular wall, it has been generally assumed that, in blood vessel, iNOS is mostly located in medial smooth muscle cells and in endothelium. Recently, it has been reported that in isolated rat aorta exposed to lipopolysaccharide, the adventitia is a more powerful source of NO than the media (Kleschyov et al., 1998). Furthermore, NO released by the adventitia can reach soluble guanylyl cyclase in the medial layer and contribute greatly to vascular hyporeactivity to noradrenaline caused by lipopolysaccharide. It also greatly participates in the abovementioned generation of releasable NO store in the aorta (Muller et al., 1996, 1998). Induction of iNOS immunoreactivity has also been found predominantly in the intima and in the adventitia of human small omental arteries from patients in the hyperdynamic phase of septic shock (Stoclet et al., 1997).

Despite the induction of iNOS, resistance arteries from endotoxaemic rats (Schneider et al., 1992b, 1994; Mitchell et al., 1993; Martinez et al., 1996) and from human patients with septic shock (Stoclet et al., 1997) are not hyporeactive to noradrenaline or other vasoconstrictor agonists ex vivo (contrary to conduit arteries from endotoxaemic rats). Pharmacological inhibition of NOS activity unmasks hyper-responsiveness to vasoconstrictor agonists in these vessels (Mitchell et al., 1993; Stoclet et al., 1997) and in septic patients in vivo (Avontuur et al., 1999). These findings are consistent with the view that iNOS derived NO counteracts arterial hyper-reactivity induced by sepsis, both in animal models and in patients. In small arteries from endotoxaemic rats, an increase in intracellular Ca^{2+} concentration is involved in the increase in reactivity to noradrenaline (Martinez et al., 1996).

Much has been learned on the role of iNOS from studies in iNOS knock out mice. The acute inflammatory responses are decreased in these animals (Wei et al., 1995), who display resistance to the hypotension induced by injection of lipopolysaccharide (MacMiking et al., 1995). However, eradication of iNOS does not consistently reduce lipopolysaccharide-induced mortality in iNOS $(-/-)$ mice compared with genetically matched controls (Laubach et al., 1995; MacMiking et al., 1995). A recent study of vascular effects of lipopolysaccharide in iNOS deficient mice provide direct evidence that iNOS accounts for impaired contraction of the carotid artery in wild type animals (Gunnett et al., 1998), but reduces cardiac oedema (Laubach et al., 1998) and leucocyte–endothelium interactions (Hickey et al., 1997) in endotoxaemia. Thus, detrimental and beneficial effects of iNOS derived NO may contend each other after lipopolysaccharide administration.

Interestingly, the lack of iNOS gene did not fully abolish lipopolysaccharide-induced tyrosine nitration (an index of $ONOO^-$ formation) in aorta and lung (Zingarelli et al., 1998). As this effect of lipopolysaccharide was abolished by L-NMMA, these findings suggest that lipopolysaccharide can induce iNOS-independent enhanced NO generation and subsequent $ONOO^-$ formation in mice.

4.2. Beneficial and deleterious effects of iNOS induction in heart disease

Various stimuli (cytokines, lipopolysaccharide, ischaemia) can induce iNOS in different cellular constituents of cardiac tissue, including cardiac myocytes, endocardial endothelium, tissue macrophages, fibroblasts, endothelium and smooth muscle of the microvasculature (Kelly et al., 1996). The beneficial or deleterious consequences following induction of iNOS in these cells are less clear.

There is increasing evidence for a role of iNOS in the pathophysiology of chronic heart failure. An increased iNOS expression in patients with dilated cardiomyopathy was first reported by De Belder et al. (1993), suggesting that this may contribute to pathophysiological changes underlying this disease. Subsequent studies report both the presence (Haywood et al., 1996; Satoh et al., 1997; Adams et al., 1998) and absence (Thoenes et al., 1996) of iNOS in hearts from patients with dilated cardiomyopathy. The data on iNOS expression in other types of heart failure (ischaemic heart disease, inflammatory and peripartum cardiomyopathy) are based on measurements of NO metabolites (Winslaw et al., 1994; De Belder et al., 1995) or iNOS quantification by histochemistry (Habib et al., 1996) and are also diverging (Haywood et al., 1996; Thoenes et al., 1996). Recently, Vejlstrup et al. (1998) identified iNOS mRNA and iNOS protein in myocardial biopsies from patients with both dilated cardiomyopathy and ischaemic heart disease. iNOS was located primarily and invariably in the endothelium and vascular smooth muscle cells of the myocardial vasculature and its expression appeared to be associated with the condition of heart failure per se rather than related to the heart failure etiology. The importance of those observations remains to be proven, and it is currently not clear whether iNOS induction is beneficial or deleterious to the compromised heart.

Acute heart failure occurs when a coronary artery is occluded. A sustained occlusion leads to myocardial cell death (infarction). Early reperfusion of ischaemic myocardium can prevent the extent of the infarcted zone, but can also generate life-threatening arrhythmias or render cardiac contractile function reversibly impaired for a period (myocardial stunning). The role of NO in ischaemia–reperfusion injuries has been recently reviewed (Curtis and Pabla, 1997). The presence of iNOS in myocardium was not identified in hearts shortly reperfused (30–60 min) (Wang et al., 1997), indicating that there is no overproduction of NO due to iNOS during the early phase of reperfusion. Simultaneously, ischaemia–reperfusion almost totally abolished eNOS activity. Administration of NOS substrate or NO donors before reperfusion, which are expected to supplement NO deficit (Siegfried et al., 1992; Weyrich et al., 1992) markedly reduced reperfusion-induced injuries. This may also be achieved by previous iNOS induction (see preconditioning), which in turn can produce NO or induce other protective mechanisms (Richard et al., 1996).

Interestingly, in infarcted rabbit myocardium, 24 h after coronary artery ligation, iNOS activity was significantly increased with a peak activity on postoperative day 3 (Suzuki et al., 1996). The effect persisted for 14 days and immunohistochemical localization revealed macrophages as a major source for iNOS expression. Most importantly, *S*-methylisothiourea, an inhibitor of iNOS, significantly improved left ventricular performance and increased regional myocardial blood flow. This argues for a deleterious role of iNOS in these experimental conditions.

However, in an opposite direction, induction of iNOS has also been involved in myocardial preconditioning (Bolli et al., 1997). The preconditioning of myocardium is an adaptive response in which brief exposure to a series of transient sublethal ischaemic periods markedly enhances the ability of the heart to withstand a subsequent, potentially injurious ischaemic insult. The cardioprotective effects of preconditioning occur in two phases: one early, which develops immediately after the preconditioning challenge and lasts a few hours ('classical preconditioning'); the second delayed, which appears 24 h later and may last for several days ('second window of protection'). There is increasing evidence that NO derived from iNOS is involved in the late phase of preconditioning. Administration of different iNOS inhibitors (aminoguanidine, *S*-methylisothiourea) abolished the cardioprotective effects of preconditioning on myocardial stunning (Bolli et al., 1997) and on infarct size (Takano et al., 1998; Imagawa et al., 1999). Aminoguanidine reduced also the delayed protection afforded by preconditioning with rapid cardiac pacing on the number of ventricular premature beats, the incidence of life-threatening ventricular arrhythmias and the survivals in dogs (Kis et al., 1998). In addition, the results obtained with non-selective NOS inhibitors suggest that eNOS activity might contribute to the classical preconditioning.

Both protective phases of preconditioning can be mimicked with pharmacological agents. It has been known for several years that lipopolysaccharide induces delayed cardioprotection against ischaemia, comparable to late phase of preconditioning (Brown et al., 1989; Song et al., 1996). It was hypothesized that iNOS induction was involved in the cardioprotective effects of lipopolysaccharide. Recently, a relatively non-toxic endotoxin derivative, monophosphoryl lipid A has been developed. Administration of monophosphoryl lipid A in dog, rabbit and rat 24 h before coronary occlusion significantly reduced the myocardial infarct size (Yao et al., 1993; Elliott, 1996), the incidence

of ventricular arrhythmias (Végh et al., 1996; Tosaki et al., 1998), and it attenuated the coronarian endothelial dysfunction (Richard et al., 1998). These effects were accompanied by an increased iNOS activity (Zhao et al., 1997) or iNOS mRNA expression (Tosaki et al., 1998). In addition they could be blocked by aminoguanidine or L-NAME. Although the mechanisms responsible for the beneficial effects of monophosphoryl lipid A are still unknown, these results suggest that iNOS induction is involved. Treatment with agents such as monophosphoryl lipid A, which is able to pharmacologically mimic the preconditioning without modifying blood pressure or heart rate (Song et al., 1998), could be used in various clinical situations or in patients at high risk of myocardial infarction.

Although these data suggest a protective role of iNOS, simultaneous production of high levels of NO and reactive oxygen species can initiate apoptosis. The mechanisms which will determine whether the role of NO is beneficial or detrimental have been recently reviewed (Brüne et al., 1998). Depending on the redox state of the tissue, on the balance between NO and $O_2^{\cdot-}$ and the initiation of adaptive mechanisms, the consequences of iNOS induction may be very different, as discussed above.

5. Conclusion

Despite accumulation of a large amount of information, it is still difficult to discriminate in which situations the high output NO production resulting from iNOS induction is beneficial or detrimental in cardiac and vascular tissue.

The current evidence suggests that induction of iNOS in vascular and cardiac tissue may be a part of systemic or local adaptation to injury, counteracting other tissue alterations. This may be the case in the hyperdynamic phase of septic shock and in cardiac preconditioning, for instance. In these circumstances, iNOS-derived NO produces short term effects (for instance haemodynamic effects, inhibition of leukocyte adhesion and migration, of platelet aggregation, etc...) and long term effects via the induction of expression of genes (for instance, anti-oxidant and anti-apoptotic genes).

It is noteworthy that the expression of iNOS has also been reported in various physiological conditions in some tissues: for instance, it plays a role in fetal circulation in rats (Bustamante et al., 1996) and is expressed during cardiomyogenesis (Ji et al., 1999). Thus, the physiological role(s) of iNOS is not excluded, although not well documented.

However, there is no doubt that high NO level may become deleterious, especially via formation of $ONOO^-$ and other reactive nitrogen species. This formation and the resulting activation of cytotoxic pathways largely depends on local redox state and pH, and on the presence of appropriate targets. The activation of poly(ADP-ribose) polymerase is an example of such cytotoxic pathway.

The exact nature of iNOS derived damaging species is largely unknown. Cytotoxicity may result from nitrosation, nitration or oxidation of various proteins (in mitochondrial complexes, for instance), from protein bound dinitrosyl–iron complexes formation (with aconitase, for example), or from direct DNA damage. However, it seems that *S*-nitrosation, some nitration, and dinitrosyl–iron complex generating reactions, mediated by reactive nitrogen oxide species, may also be involved in beneficial effects of NO. These include the generation of releasable NO stores which can buffer high NO level and provide long term effects of NO in vascular and cardiac tissues. Furthermore, NO-induced gene transcription may cause long term tissue protection against nitrosative and oxidative stress. Considering all these reactions and the influence of local factors such as the redox state and the pH, one realizes the extreme complexity of the signaling pathways triggered by iNOS products.

Elucidation of the abovementioned mechanisms may provide a number of conceivable future therapeutic strategies to alleviate NO-induced tissue injury, bearing in mind that a physiological level of NO activity is required to maintain normal vascular and cardiac functions. Specific inhibition of iNOS is one of these strategies. In this respect, the effect of iNOS inhibitors on $O_2^{\cdot-}$ production by the enzyme remains an unanswered question. It might be more appropriate, in certain circumstances, either to inhibit cytotoxic pathways (like poly(ADP-ribose) polymerase) triggered by nitrogen oxides, or to enhance defence mechanisms against $ONOO^-$. Partially reducing NO production by acting upstream on substrate or co-substrate availability is another possibility. Acting on the mechanisms regulating the expression of the iNOS gene has not been considered in detail here, but this is obviously another topic which warrants investigation.

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