NITROGEN(II) OXIDE (NITRIC OXIDE, NO): ITS ORIGIN, FATE AND PHYSIOLOGICAL SIGNIFICANCE. A REVIEW

Petr VETROVSKY[1] and Gustav ENTLICHER

Department of Biochemistry, Faculty of Science, Charles University, 128 40 Prague 2, Czech Republic; e-mail: [1] p.vetrovsky@usa.net

Received December 31, 1996
Accepted June 26, 1997

1. Nitrogen Oxide (NO) 1356
1.1. A View Back 1356
1.2. Chemical Properties of NO in Solutions 1357
1.3. Molecular Targets of NO 1358
2. NO-Synthase 1359
2.1. Catalytic Mechanism of NOS 1359
2.2. General Structure and Properties of NOS 1360
2.3. NOS Isoforms and Their Tissue Distribution 1362
2.4. NOS Activity Regulation 1364
2.5. NOS Inhibitors 1364
3. N^G-Hydroxy-L-arginine 1365
3.1. N^G-Hydroxy-L-arginine and Its Metabolism to NO by NOS 1365
3.2. N^G-Hydroxy-L-arginine and Its Metabolism to NO by Other Enzymes than NOS 1366
3.3. The Mechanism of NO Formation upon Oxidative Cleavage of C=N(OH) Bond by NOS and Cytochromes P450 1366
3.4. Other Alternative Pathways for NO Biosynthesis from N^G-Hydroxy-L-arginine 1367
3.5. Physiological Properties of N^G-Hydroxy-L-arginine 1370
4. Superoxide Ion 1371
4.1. Formation of Superoxide and Hydrogen Peroxide by Cellular Systems 1371
4.2. Leukocyte-Derived Superoxide and Hydrogen Peroxide 1372
4.3. Superoxide Generation by Non-Phagocyte NAD(P)H-Oxidase 1373
4.4. Reaction Between NO and Superoxide Ion 1373
5. Roles of NO in Mammalian Physiology 1375
5.1. Cardiovascular System 1375
5.2. Immune System 1375
5.3. NO in Central Nervous System 1376
5.4. NO in Peripheral Nervous System 1376
5.5. NO in the Modulation of Uterine Contractility 1377
References 1377

The nitrogen oxide (NO), a free radical molecule, plays a key role in the regulation of mammalian physiology and pathophysiology, *e.g.*, in cardiovascular, nervous or immune systems. This molecule is produced from guanidino moiety of amino acid L-arginine with N^G-hydroxy-L-arginine as intermediate and L-citrulline as a co-product of this reaction. This conversion is catalyzed by an ezyme called NO-synthase. The NO-synthase belongs to the cytochrome P450 superfamily and four its isoenzymes are known so far. Two (denoted NOS-I and III) are constitutive, generate lower amounts (pmol) of NO and are regulated by Ca^{++}/calmodulin system. The others (NOS-II and IV) are induc-

ible, produce a larger quantity of NO (nmol) and are regulated at a transcriptional level. The constitutive form is present, for example, in endothelial and neuronal cells whereas the inducible form is *de novo* synthesized as a consequence of certain stimuli (including cytokines) in macrophages, vascular smooth muscle cells and other cells. There are several target molecules for NO depending on cells. The most frequent target is supposed to be the soluble guanylate cyclase. However, superoxide anion is a very important target for NO, too. The reaction between these two molecules leads to the production of peroxynitrite, the fate of which depends on environmental conditions. Therefore, the importance of this reaction is still debated. This review deals with the nature of NO, the mechanisms of its production, the role of intermediate N^G-hydroxy-L-arginine and summarizes the biology of superoxide anion with respect to its reaction with NO. A review with 218 references.

Key words: L-Arginine; N^G-Hydroxy-L-arginine; Nitric oxide; Nitrogen(II) oxide; NO-Synthase; Superoxide ion.

1. NITROGEN OXIDE (NO)

1.1. A View Back

Nitrogen oxide (NO) was considered to be only an irritant atmospheric pollutant even not many years ago. From 1981 to 1986, 10 publications appeared on mammalian biological effects[1] of NO. This changed dramatically in 1986–1987 when several separate directions of biomedical investigation unexpectedly converged on NO. In 1986, during the fourth symposium devoted to vasodilatation, both Furchgott and Ignarro[2,3] simultaneously proposed that endothelium-derived relaxing factor (EDRF)* may be NO. The initial evidence for this proposal was obtained in Furchgott's studies of the characteristics of the transient relaxation of rabbit aorta produced by a factor generated in $NaNO_2$ solutions on acidification. It was found that the relaxing effect of NO was blocked by haemoglobin and by generators of superoxide ion and was markedly potentiated by superoxide dismutase. These characteristics strongly resembled those of EDRF, known for several years. Ignarro at the same symposium also proposed that EDRF might be NO or some closely related unstable radical species because EDRF and NO exhibited identical properties (see Table I). Shortly afterwards, Palmer *et al.*[4], using

* Abbreviations used: cGMP, cyclic guanosine 3′,5′-monophosphate; EDRF, endothelium-derived relaxing factor; FAD, flavine adenine dinucleotide; FMN, flavine mononucleotide; L-NAME, N^G-nitro-L-arginine methyl ester; L-NMMA, N^G-monomethyl-L-arginine; LPS, lipopolysaccharide; LTD, long-term depression; LTP, long-term potentiation; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; NANC, nonadrenergic noncholinergic; NMDA, *N*-methyl-D-aspartate; NOS, NO-synthase (E.C. 1.14.13.39.); NOS-I, neuronal NOS; NOS-II, macrophage NOS; NOS-III, endothelial NOS; NOS-IV, hepatic NOS; cNOS, constitutive NOS; iNOS, inducible NOS; NO_x, nitrite and nitrate; $^{\bullet}O_2^-$, $O_2^{\bullet-}$, O_2^-, superoxide ion; OH-L-Arg, N^G-hydroxy-L-arginine; ROI, reactive oxygen intermediates; SOD, superoxide dismutase; TGF, transforming growth factor; THB, tetrahydrobiopterine; IP_3, inositol 1,4,5,-trisphosphate; PKC, protein kinase C.

simultaneous chemical assays and bioassays showed that NO produced by endothelial cells accounted for most if not all of the biological activity of EDRF. Furthermore, following these experiments, the same group demonstrated[5] that ^{15}NO produced by endothelial cells was produced from the ^{15}N-guanidine residue of L-arginine. Hibbs *et al.*[6] had previously shown that the cytotoxic activity of activated macrophages was associated with a biochemical pathway synthesizing L-citrulline and nitrate from the amino acid L-arginine.

An interdisciplinary torrent of research was triggered which now bears NO on its crest as a major secretory product of mammalian cells, with critical functions in homeostasis and host defense. It is surprising that such simple, fleeting and indiscriminate reactant can convey sufficient information in a regulated manner to help control vital servomechanisms like systemic blood pressure, respiration, digestion, penis erection, uterine contractility, platelet aggregation, cerebral blood flow, hormone synthesis and neuronal synaptic plasticity. It is equally surprising that a molecule with such delicate assignments should also be empowered to destroy microbes and tumor cells.

1.2. Chemical Properties of NO in Solutions

Nitrogen oxide (NO), a radical molecule with unpaired electron, is an uncharged, relatively hydrophobic gas. Under physiological conditions, NO may be interconverted among different redox forms (nitrosonium, NO^+, and nitroxyl anion, NO^-) with distinc-

TABLE I
Comparison of characteristics of EDRF and nitric oxide (modified from ref.[3])

Characteristics	EDRF	Nitrogen oxide (NO)
Liberated by endothelial cells	yes	yes
Relaxes smooth muscle cells	yes	yes
Inhibits platelet aggregation	yes	yes
Induces platelet disaggregation	yes	yes
Inhibits platelet adhesion	yes	yes
Stability (half-life in seconds) in bioassay	3.6 ± 0.1	4.1 ± 0.2
Receptor	guanylate cyclase	guanylate cyclase
Second messenger	cGMP	cGMP
Inhibition by haemoglobin	yes	yes
Effect potentiated by SOD	yes	yes
Not affected by methemoglobin	yes	yes
Reacts with superoxide	yes	yes
oxygen	yes	yes

tive chemistries. This is physiologically very important and contributes to the diversity of biological actions of NO (ref.[7]). Nitrogen oxide (NO) is well known for its reaction in gas phase with O_2 to give nitrogen dioxide (NO_2). The hydrophobicity of NO results in a high diffusion ability in biological systems: NO readily penetrates cell membranes. Studies of NO actions in bioassay systems that utilize physiological salt solutions have suggested[8] that NO is highly unstable with an apparent half-life of 6 to 60 s, owing to reactions with O_2 and superoxide anion ($O_2^{\bullet-}$). The reaction with O_2 to form NO_2 involves two molecules of NO and thus the reaction rate falls exponentially as NO concentration is decreased linearly. At physiologically relevant levels of NO (1–50 nmol/l) it is unlikely that this reaction proceeds at a significant rate compared to other processes, in particular the reaction with superoxide ion[9].

The addition of NO to an aqueous saline environment, under physiological conditions of temperature, oxygen pressure, and pH, results in accumulation of nitrite and lower amounts of nitrate[8]. Higher production of nitrate from NO requires presence of another oxidizing agent[10].

Nitrogen oxide (NO) also readily reacts with superoxide providing peroxynitrite[11], with heme and nonheme iron[12,13], thiols[14], amines[15] as well as with various chemical species, such as hydroxylamine[16] or N^G-hydroxy-L-arginine[17]. Furthermore, NO is considered as a compound which is easily oxidized to nitrosonium[18] or reduced yielding nitroxyl[19]. The compounds produced from NO by the described reactions are believed to play important roles in biological effects of NO and are hence under intensive investigation.

1.3. Molecular Targets of NO

Analysis of molecular targets has shed considerable light on some mechanisms of action of NO in homeostasis and host defense. The known NO targets are diverse and include both low-molecular-weight species and macromolecules. These can be either activated or inhibited as a consequence of reacting with NO.

A cardinal mechanism of action of NO is currently thought to be activation of soluble guanylyl cyclase by nitrosation of its heme[20]. This mechanism is important especially in vascular smooth muscle cells where such stimulated increase in cGMP results in vascular dilatation. ADP-Ribosyltransferase[21] and cyclooxygenase-1 and -2 (ref.[22]) are other heme-containing enzymes activated by NO.

Before macrophages were shown to release NO, their cytotoxic actions on tumor cells were linked to inhibition of tumor cell NADH : ubiquinone oxidoreductase, NADH : succinate oxidoreductase, and *cis*-aconitase, all Fe–S enzymes[23]. Recently, some of these target cell lesions have been recapitulated with NO (ref.[24]) and EPR complexes consistent with nitrosylated Fe–S complexes have been detected in NO-producing macrophages[25] and macrophage-injured target cells[26]. The inhibition of these enzymes by NO is associated with the release of iron from iron–sulfur centers[23].

Macrophage-derived NO was also found to release Fe from ferritin[23,27] leading subsequently to promoted lipid peroxidation.

Nitrogen oxide (NO) has been reported to directly activate some G proteins in association with an increase in GTPase activity[28]. C-Kinase may also be subject to inhibition by NO (ref.[29]). These observations suggest that NO can influence activity of multiple proteins *via* alterations in phosphorylation status.

A prominent action of macrophage-derived NO on tumor cells is, however, inhibition of their DNA synthesis apparently *via* inhibition of mitochondrial respiration and hence ATP synthesis[23,24]. In addition, NO can react with and inactivate ribonucleotide reductase, an enzyme catalyzing deoxyribonucleotide synthesis, thereby inhibiting DNA synthesis and cell proliferation[23,30–34].

A very important target for NO is superoxide anion (see Chapter *4.4.*).

2. NO-SYNTHASES

In 1990, the first NO-synthase (NOS) has been isolated from rat cerebellar homogenates[35]. From that time the name NO-synthase persists in spite of the fact that the enzyme does not belong to synthases but it is an oxidoreductase with E.C. 1.14.13.39. More recently it has been established that mammalian NO synthesis is mediated by at least three NOS isoforms[36]. Sequence data show that distinct, constitutive Ca^{2+}/calmodulin-dependent NOS isoforms are associated with EDRF (NOS-III) and with signal transduction in central and peripheral neurons (NOS-I). The third NOS isoform, originally isolated[37,38] and sequenced[39] from murine macrophages contains calmodulin but is independent on increase of intracellular Ca^{2+} ions[40]. Although isoforms of NOS may differ in their expression and regulation, the overall catalyzed reaction is the same, *i.e.* an electron oxidation of L-arginine, in which molecular oxygen and NADPH (nicotinamide adenine dinucleotide phosphate, reduced form) are required as well as flavine adenine dinucleotide (FAD) and flavine mononucleotide (FMN) for the transport of electrons within the chain[41,42], with L-citrulline and NO as products of this oxidation. Similarly, all isoforms contain one equivalent each of tetrahydrobiopterin (THB) and heme (iron protoporphyrin IX) per monomer when fully active[1].

2.1. Catalytic Mechanism of NOS

In the early nineties it was established: (a) N^G-hydroxy-L-arginine (OH-L-Arg) is a tightly bound intermediate in the NOS reaction, (b) the hydroxylated nitrogen of OH-L-Arg is processed to NO, (c) formation of L-citrulline and NO from L-arginine or OH-L-Arg requires 1.0 or 0.5 equivalents of NADPH, respectively, and (d) the ureido oxygen of citrulline derives from O_2 rather than from H_2O (refs[43,44]). It was subsequently shown that hydroxyl oxygen of OH-L-Arg is retained in NO and the intermediacy of OH-L-Arg was confirmed[45,46].

The reaction sequence shown in Fig. 1 represents two consecutive monooxygenase reactions (not dioxygenase reaction); both steps are mixed function oxidations. Step 1 is the *N*-hydroxylation of one of the two equivalent guanidino nitrogens of L-arginine. This reaction exhibits all characteristics of classical P450-dependent monooxygenations, with the consumption of one mole of NADPH and O_2 and incorporation of one oxygen atom from O_2 into arginine. The second step is a three-electron oxidation of intermediate OH-L-Arg and involves an oxidative cleavage of the C=N–OH bond with formation of citrulline and NO (ref.[1]). Although step 1 is conventional hydroxylation, step 2 has no obvious biological precedent as it consumes one mole of O_2 and only 0.5 mole of NADPH (ref.[42]). This problem is discussed in detail by Masters[47].

The role of THB in the catalysis is not yet clear. Several findings suggest that THB is more likely to have an allosteric than a catalytic role[48–50]. However, the finding reported by Hevel and Marletta[51] that only redox-active THB analogues stimulate the NOS activity indicates that THB is more than a simple allosteric activator and suggests that it contributes in some way to NOS catalysis.

2.2. General Structure and Properties of NOS

As mentioned above, two distinct groups of NOS are now well identified. One is termed constitutive and its activation in response to receptor or physical stimulants at level of neuronal (NOS-I) or endothelial (NOS-III) cells results in the release of small amounts of NO (picomoles) for short periods of time. The other one is termed inducible and its activation in the liver of rats treated by lipopolysaccharide and in macrophages (NOS-II) by LPS or lymphokines results in the release of large amounts of NO (nanomoles) for a long period of time. Thus, even if these two forms of NOS are quite similar in terms of substrate and final reaction products, they are not identical as revealed by purification of several isoforms[52] and as confirmed by their cloning[53–57].

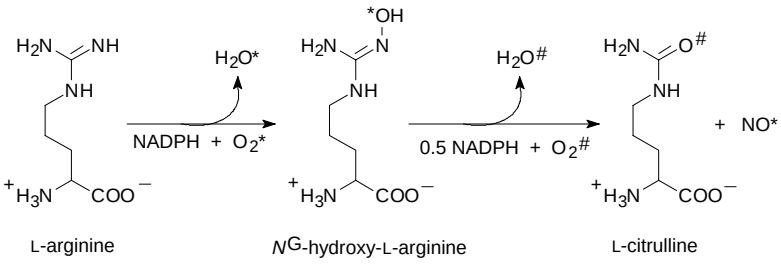

FIG. 1
L-arginine/NO pathway catalyzed by NO-synthase. The symbols (*, #) determine the origin of oxygen

The primary structure of NOS, revealed by molecular cloning, indicates that the enzyme is an α-helical protein with well-defined sites (Fig. 2) for binding of NADPH, FMN and FAD and among the sequenced mammalian proteins NOS only displays a close homology with cytochrome P450 reductase. The two proteins exhibit similarities in the C-terminal half of NOS – 58% homology over 641 amino acids[58]. Recent findings also indicate that NOS is a complex and unique enzyme since, in addition to the presence of FAD and FMN binding sites and striking sequence homology with NADPH-cytochrome P450 reductase, NOS also contains a heme domain on the same polypeptide. Thus, NOS can be considered as the first example of mammalian cytochrome P450 reductase-type hemoprotein where reducing equivalents from NADPH could be shuttled directly through the flavins in the reductase domain to the heme center during hydroxylation of L-arginine to OH-L-Arg (ref.[59]). The complex of the reduced form of NOS with CO exhibits spectral characteristics similar to analogical complex of cytochrome P450. Heme iron is also bound to mercaptide sulfur of cysteine[60].

All isoforms possess a consensus site for phosphorylation by protein kinase and NOS type II contains a calmodulin consensus binding site as does NOS type I, although its activity is Ca^{2+}-independent. The polypeptide chain of NOS type II is shorter than NOS type I (1 144 *vs* 1 429 amino acids), primarily due to the lack of about 200 amino acids at the amino terminus, 15 amino acids at the carboxyl terminus and a 40-amino acid deletion in the interior of the molecule.

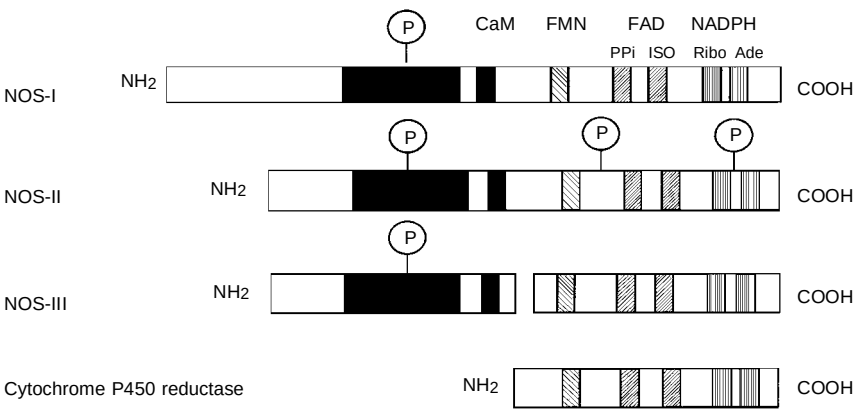

FIG. 2

Relationship among the sequence for NOS isoforms and cytochrome P450 reductase. Consensus sequence for the binding of the cofactors NADPH (adenine and ribose), FAD (isoalloxazine and pyrophosphate), FMN and of calmodulin (CaM) are labelled. The darkened *N*-terminal region shows 65–71% sequence identity between the three types of NOS. This region contains the putative L-arginine binding region and probably the binding site for THB and heme. The gap in the NOS-III sequence represents a 40 amino acid deletion (modified from ref.[65])

A striking homology exists between NOS type I (brain) and III (endothelial cells) and explains why a NOS-like immunoreactivity is detected in endothelial cells by an antiserum raised against brain NOS (ref.[61]). However, NOS type I is soluble whereas NOS type III is partially particulate due to the presence of myristylation site and they are encoded by distinct genes[62].

Recently, it has been demonstrated that fully active NOS is a homodimer[50]. The reconstitution of this homodimer from two monomers requires the presence of a heme, substrate (L-arginine) and a cofactor (THB; Fig. 3).

2.3. NOS Isoforms and Their Tissue Distribution

During the last two years, several isoforms responsible for NO formation have been characterized and purified from different cell types and tissues and recent molecular cloning has confirmed and extended our knowledge about NOS isoforms[52].

Four isoforms of NOS are now individualized. They differ by their regional distribution, mode of regulation, molecular weight and the fact that they are produced by distinct genes (Table II).

First of all, NOS types I and III are constitutive (although their expression can be regulated) whereas type II is inducible. Inducible NOS-II (present for example in LPS or lymphokine-activated macrophages) does not require calmodulin for activity although a calmodulin consensus binding site exists on its primary sequence (Fig. 2). The findings of Iida *et al.*[63] have partially clarified this confounding observation. They demonstrated the induction of a calmodulin-dependent NOS in the liver of rats treated with LPS. The reported efficient conversion of this calmodulin-dependent NOS (termed NOS-IV) to the calmodulin-independent (NOS-II) by incubation with calmodulin, strongly suggests

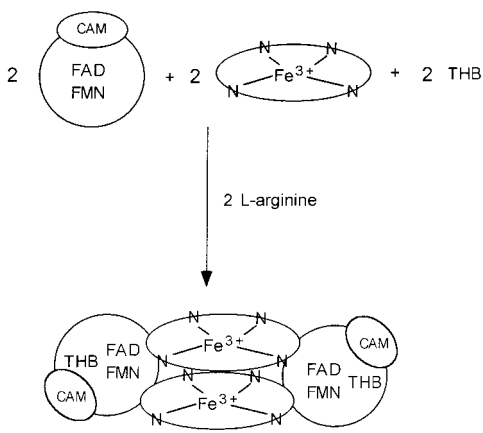

FIG. 3
Proposed model for the reconstitution of the NOS (modified from ref.[50])

that the latter is derived from the former. Further purification and/or molecular cloning of these isoenzymes is necessary to establish the exact relationships between NOS type II and IV.

The tissue distribution of the NOS isoforms under normal and pathophysiological conditions has received considerable attention. Originally identified in a subset of central neurons[61], NOS-I has now been identified by immunological staining in a variety of cells including peripheral nonadrenergic noncholinergic (NANC) neurons, skeletal muscle, pancreatic islet cells, kidney macula densa cells and certain epithelial cells[64–66]. Endothelial NOS expression previously appeared to be restricted almost exclusively to the vascular endothelium[65]. Although NOS-III activity was found in most organs and tissues, such activity was generally attributable to the vascular endothelium contained in those tissues. However, NOS-III was recently identified immunohistochemically in kidney tubular epithelial cells[67,68], CA1 neurons[69] and cardiac myocytes[70]. In CA1 neurons, NOS-III apparently plays a role in long-term potentiation, a memory-related phenomenon; in cardiac myocytes it mediates NO-dependent parasympathetic signaling; its role in kidney epithelium is unknown.

Following exposure to inflammatory cytokines or LPS, NOS-II has been identified in murine macrophages[37,38,71,72], liver[73], vascular endothelial[74] and smooth muscle cells[75–77], chondrocytes[78], myocardium[79] and other tissue and cell types (for reviews, see refs[36,64,66]). It is now assumed that any nucleated cell type is able to express NOS-II if the proper cytokine or LPS stimulus is delivered.

TABLE II
Isoenzymes of NOSs and their tisue distribution

NOS isoforms	Regulated by	Molecular weight of denaturated protein (SDS/PAGE)	Location
NOS-I Cytosolic Constitutive	Ca^{2+}/calmodulin	155 000 native dimer	brain, cerebellum, NANC neurons, skeletal muscle, pancreatic islet cells, epithelial cells
NOS-II Cytosolic Inducible	induced by cytokines and endotoxin (LPS) Ca^{2+} independent	125 000–135 000 native dimer	macrophages, hepatocytes, kupfer cells, smooth muscle cells, endothelial cells
NOS-III Particulate Constitutive	Ca^{2+}/calmodulin	135 000	endothelial cells, kidney epithelial cells, CA1 neurons, cardiac myocytes
NOS-IV Particulate Inducible	induced by cytokines and endotoxin (LPS) Ca^{2+} independent	?	liver

Although it is difficult to reproducibly obtain NOS-II expression in human macrophages or monocytes, human lung macrophages have been shown to express NOS-II *in vivo*[80].

2.4. NOS Activity Regulation

Contrary to the cytochrome P450 reductase, NOS is a highly regulated enzyme. Purified NOS are phosphorylated in a stoichiometric manner by cAMP-dependent protein kinase, protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase II (ref.[53]) and consensus sequences for phosphorylation of the enzyme have been identified on serine sites (Fig. 2). In kidney cells transfected with NOS-cDNA, it has been demonstrated that activation of PKC by phorbol ester leads to a rapid phosphorylation of NOS and a more than 50% reduction in enzyme activity. This finding is consistent with previous physiological data demonstrating that phorbol esters inhibit acetylcholine and calcium ionophore-stimulated EDRF release[81]. Thus, stimulation of inositol 1,4,5-trisphosphate (IP_3) turnover in a NOS-containing cell should have two opposing effects on NOS activity: on the one hand, the increase in intracellular Ca^{2+} due to the IP_3 production activates NOS through calmodulin whereas, on the other hand, the increase in diacylglycerol stimulates PKC and reduces NO synthesis. This down-regulation of NOS activity by PKC is an important site for "cross-talk" between the IP_3 and NO signalling system.

In the case of inducible NOS (iNOS), a number of cytokines including transforming growth factor (TGF-β), interleukin IL4 and IL10 (ref.[82]), interferon-gamma[83,84] and several corticosteroids[85] are known to inhibit the expression of iNOS (for review, see ref.[66]).

Recently, it was demonstrated by several groups that NO can act as a negative feedback signal on NOS activity, indicating that a self-regulatory mechanism is also operative[86–88].

2.5. NOS Inhibitors

The fact that several N^G-methyl- or N^G-nitro-L-arginine derivatives stereoselectively inhibit EDRF formation *in vitro* and *in vivo* through NOS inhibition has been a crucial step in the identification of the L-arginine/NO pathway[89]. N^G-Monosubstituted analogues are a large and useful class of NOS inhibitors. These analogues exhibit variable potencies in their ability to inhibit NOS activity *in vitro* and *in vivo* although recent reports have outlined other important differences in their pharmacological profile. N^G-Nitro-L-arginine derivatives such as N^G-nitro-L-arginine methyl ester (L-NAME) simultaneously inhibit NO and superoxide ($^{\bullet}O_2^-$) formation during conversion of L-arginine to L-citrulline by NOS whereas N^G-methyl-L-arginine derivatives such as N^G-monomethyl-L-arginine (L-NMMA) only inhibit NO synthesis[90]. This finding could explain why superoxide dismutase (SOD) attenuates the vasoconstrictor effect of L-NMMA and

suggests that superoxide may participate in the vascular effects of L-NMMA (ref.[91]). Alternatively, superoxide dismutase may exert its effect by decreased quenching of NO by superoxide[92,93] or by another, so far unknown, mechanism[94]. Moreover, some inhibitors show modest selectivity among NOS isoforms[95,96].

Initial inhibition by all arginine antagonists is competitive with L-arginine, which indicates that they occupy the arginine/citrulline binding site of the enzymes. The extent of inhibition is diminished by L-arginine and, at least initially, inhibition can be reversed by L-arginine. For some of the arginine analogues, NOS inhibition becomes, however, irreversible or poorly reversible with time. Recent studies with L-NMMA show that it is metabolized in an arginine-like fashion by NOS to N^G-hydroxy-N^G-methyl-L-arginine (NOH-NMMA) and eventually to citrulline, NO and other products[97,98]. Although it is clear that L-NMMA is a mechanism-based inactivator of NOS, it is not clear which, if any, of the metabolites of L-NMMA and NOH-NMMA causes irreversible inactivation.

Other irreversible inhibitors of NOS include N^G-amino-L-arginine and L-N5-(1-iminoethyl)ornithine. In contrast, L-NAME is not a mechanism-based inhibitor but is rather a tight-binding, slow-dissociating ligand of the arginine binding site[99,100].

Newer classes of NOS inhibitors include *S*-methyl-L-thiocitrulline[101], L-thiocitrulline[102], both based on arginine/citrulline analogues, and isothioureas[103,104] and guanidine derivatives such as 1-amino-2-hydroxyguanidine[105].

In addition to L-arginine analogues, NOS can be inhibited by flavin antagonists[1,106], by heme binders (*e.g.*, CO; ref.[59]) or by depleters of THB (ref.[107]). Constitutive but not inducible NOS can be inhibited by calmodulin binders[35,38]. On the contrary, inducible NOS can be specifically inhibited by aminoguanidine, a bifunctional molecule containing the guanidine moiety of L-arginine, [H_2N–C(NH)–NH–NH_2], and its derivatives[108,109].

3. N^G-HYDROXY-L-ARGININE

3.1. N^G-Hydroxy-L-arginine and Its Metabolism to NO by NOS

N^G-Hydroxy-L-arginine (OH-L-Arg) was shown to be an intermediate in the biosynthesis of nitric oxide from L-arginine[43]. OH-L-Arg is further converted into NO and L-citrulline by NOS involving heme in catalysis. The apparent *Km* for L-arginine is lower than that for OH-L-Arg (ref.[46]). This can explain the fact that OH-L-Arg is present in the incubation medium of cells in which inducible form is expressed[110]. It is hence speculated that OH-L-Arg can be liberated under some condition from active site of NOS, be released into the extracellular space and circulation and then it can serve as a stable NO donor and exert paracrine effects. This speculation is supported by recent observation that in rats treated with LPS, the OH-L-Arg level in blood is markedly increased[111].

3.2. N^G-Hydroxy-L-arginine and Its Metabolism to NO by other Enzymes than NOS

In 1992, the OH-L-Arg transformation into L-citrulline and nitrogen oxides (NO, NO_2^-, NO_3^-) was found to be catalyzed in the presence of NADPH by rat liver microsomes. This reaction was not inhibited by NOS inhibitors L-NAME and L-NMMA. In contrast, cytochrome P450 inhibitors, CO and miconazole strongly inhibited production of nitrogen oxides[112]. Among different P450 isoforms, P450 3A subfamily with a very close homology with NOS in the heme-binding domain was found to be the most potent[113]. Nevertheless, also other oxidases (horseradish peroxidase, lipoxygenase) were found to catalyze the same reaction, *i.e.*, conversion of OH-L-Arg into citrulline and nitrogen oxides[114,115]. This suggests that oxidation of OH-L-Arg by hemoproteins is a general reaction and OH-L-Arg can be considered an endogenous precursor of NO.

3.3. The Mechanism of NO Formation upon Oxidative Cleavage of C=N(OH) Bond by NOS and Cytochromes P450

Recent findings led to the consideration of superoxide as a molecule which is involved in OH-L-Arg decomposition to nitrogen oxides and citrulline. The transformation of compounds containing C=N(OH) moiety (*e.g.*, 4-chlorobenzamidoxime and 4-chlorophenylguanidoxime) to nitrogen oxides and corresponding urea derivatives by NADPH and O_2, catalyzed by P450 3A was markedly inhibited by SOD (ref.[116]). Inhibitory effects of SOD were also observed during microsomal oxidation of *N*-hydroxydebrisoquine (a guanidine derivative[117]) and *N*-hydroxypentanimidamide[118]. However, it is noteworthy that in this case, a low activity (about 30%) remained even in the presence of large concentrations of SOD.

These results would be in favor of a key role of superoxide in microsomal P450-dependent oxidative cleavage of >C=N(OH) bonds. They are in agreement with another result (related to a study of reactions between superoxide and amidoximes and guanidoximes) published by Sennequier *et al.*[119]. Potassium superoxide dissolved in DMSO rapidly reacted with 4-chlorobenzamidoxime leading to a very selective formation of 4-chlorobenzamide and nitrite.

Concluding, it is now clear that both NOS and P450 3A are able to catalyze the NADPH-O_2 oxidation of OH-L-Arg to citrulline and NO. However, SOD inhibits the P450-dependent oxidation of OH-L-Arg to a great extent whereas it only slightly inhibits the NOS-dependent oxidation of L-arginine to citrulline[94,120]. As suggested by Mansuy *et al.*[116], the very different effects of SOD on P450- and NOS-dependent formation of nitrogen oxides and citrulline could be related to very different coupling mechanisms of the electron transfer from NADPH and the reaction between the Fe(II)–O_2 complex and OH-L-Arg. In NOS, it is likely that the OH-L-Arg substrate is very well positioned in the active site in order to rapidly react with Fe(III)–$OO^{\bullet}$, leading to stoichiometric formation of NO and citrulline for 0.5 mol of NADPH consumed to reduce

NOS–Fe(III) to NOS–Fe(II). In contrast, in the active site of P450 3A, it is likely that OH-L-Arg, which is obviously not a high-affinity substrate for this P450 involved in the oxidative metabolism of a large number of xenobiotics, is not so well positioned to rapidly react with P450–Fe(III)–OO•. This iron–dioxygen complex has another possible fate, the dissociation of its iron–O_2 bond with formation of P450 Fe(III) and O_2^- (ref.[121]). Then, reaction of OH-L-Arg with superoxide itself would lead to citrulline and NO, as similar >C=N(OH) bond cleavages have been observed upon reaction of superoxide with amidoximes and guanidoximes[116].

The possible mechanisms for NO production from OH-L-Arg by cytochrome P450 and NOS involving role of superoxide proposed by Mansuy *et al.*[116] are presented in Fig. 4.

3.4. Other Alternative Pathways for NO Biosynthesis from N^G-Hydroxy-L-arginine

Besides OH-L-Arg transformation into NO and L-citrulline by NOS (ref.[122]), several other pathways for NO production from OH-L-Arg have been proposed.

1) Some years ago, two groups independently reported hydroxylamine as a potent vasorelaxant acting apparently *via* NO or some related compound[123,124]. Both groups

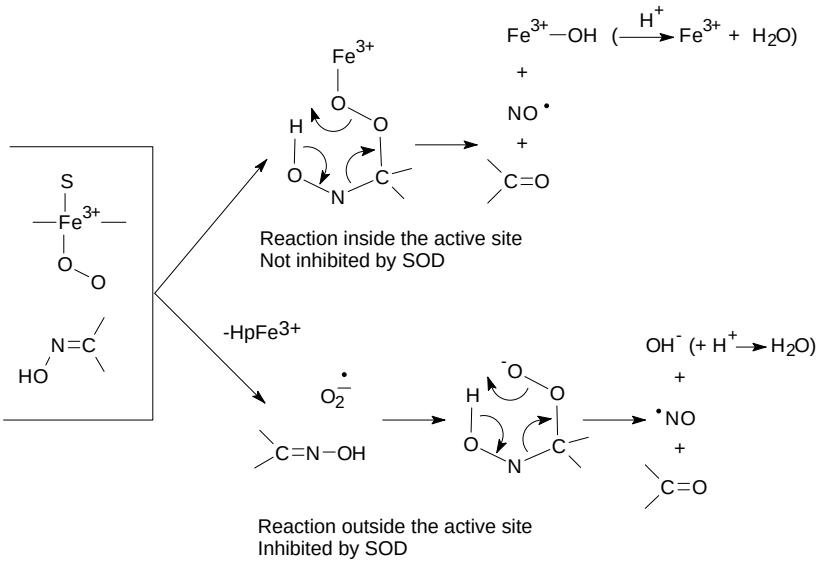

FIG. 4

Possible mechanisms for the oxidative cleavage of C=N(OH) bonds by P450s and NOSs: contribution of $^{\bullet}O_2^-$ in P450-dependent reactions. –HpFe^{3+} means dissociation of the hemoprotein–Fe^{3+}–O–O• complex to hemoprotein–Fe^{3+} and $^{\bullet}O_2^-$; this reaction is especially important in P450-dependent oxidations of C=N(OH) bonds (modified from ref.[116])

found hydroxylamine-induced endothelium-independent vasorelaxation of noradrenaline precontracted aortic strips. However, the conclusions of these two groups differed. Thomas and Ramwell[124] did not find correlation between hydroxylamine-induced relaxation and NO/NO_2^- production from this compound and they, therefore, concluded that EDRF is hydroxylamine and that the hydroxylamine-induced relaxation is not directly related to NO. On the contrary, DeMaster *et al.*[123] considered hydroxylamine as a precursor of NO. Since hydroxylamine conversion to NO by catalase was described[125,126] (see below) and since DeMaster *et al.*[123] found significant levels of catalase activity in homogenates of intact endothelium-denuded aortae, they considered catalase as an enzyme converting hydroxylamine into NO. They proposed a new, less complex, metabolic pathway for NO biosynthesis from L-arginine with hydroxylamine as an intermediate (Fig. 5). The oxidation of L-arginine to nitric oxide involves a loss of five electrons from one of its guanidino nitrogens, *i.e.*, a change in oxidation state from –3 for guanidino nitrogen to an oxidation state +2 for the nitrogen of nitric oxide. *N*-Hydroxylation of one of the guanidino nitrogens yielding OH-L-Arg is a two-electron oxidation[1]. Therefore, they assume that the final three-electron oxidation could occur after OH-L-Arg hydrolysis to hydroxylamine and L-citrulline. Hydroxylamine can be

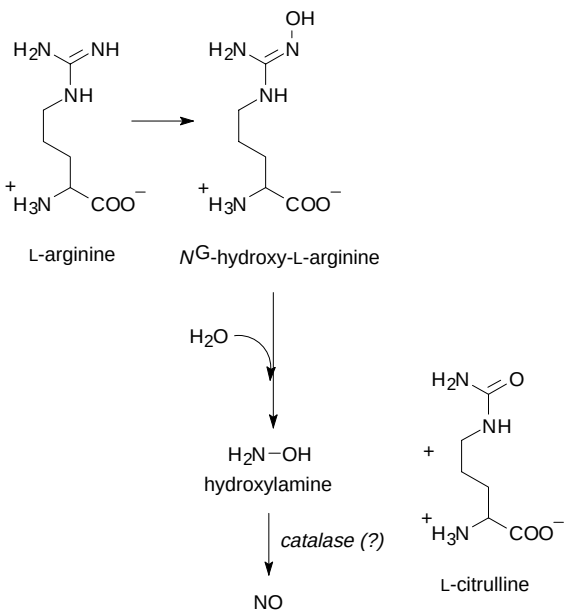

FIG. 5
Proposed pathway for the conversion of L-arginine to nitric oxide through a hydroxylamine intermediate (modified from ref.[123])

then oxidized in a three-electron oxidation to nitric oxide by catalase (Fig. 5) as already described[125,126]. This is achieved through the following series of reactions:

$$E[Fe^{III}] + H_2O_2 \longrightarrow E[Fe^{V}{=}O] + H_2O \qquad (A)$$

$$E[Fe^{V}{=}O] + NH_2OH \longrightarrow H_2O + E[Fe^{III}HNO] \longrightarrow E[Fe^{II}NO] + H^+ \qquad (B)$$

$$E[Fe^{II}NO] \longrightarrow E[Fe^{II}] + NO^{\bullet} \qquad (C)$$

$$E[Fe^{II}] + O_2 \longrightarrow E[Fe^{III}] + {}^{\bullet}O_2^- \qquad (D)$$

In this reaction, $E[Fe^{V}{=}O]$ oxidizes hydroxylamine to nitric oxide through a ferricatalase-nitroxyl intermediate, $E[Fe^{III}HNO]$. Ferrocatalase, $E[Fe^{II}]$, is converted back to ferricatalase ($E[Fe^{III}]$) by molecular oxygen.

Alternatively, the ferricatalase-nitroxyl intermediate could liberate free nitroxyl (and ferricatalase) and free nitroxyl, a known potent vasorelaxant by cGMP-dependent mechanism[19,127], would then be oxidized to nitric oxide by one-electron oxidation as reported[128]. Since cyanamide, a substrate for catalase that is known to yield free nitroxyl, did not elicit a vasodilatary response, the authors[123] concluded that free nitroxyl apparently was not a precursor for nitric oxide. However, direct evidence for hydroxylamine as an intermediate of the proposed pathway is still lacking. Nevertheless, it was recently shown that hydrolysis of OH-L-Arg to L-citrulline and hydroxylamine is possible[129]. It was reported[129] that this reaction is possibly nonenzymic at pH ≥ 9. The nonenzymic attack of hydroxylamine by superoxide ion can obviously also account for the production of NO.

2) Another pathway for NO synthesis from OH-L-Arg was reported by Zembowicz *et al.*[130]. These authors found OH-L-Arg-induced endothelium-dependent relaxation that was not blocked by NOS inhibitors. In contrast to OH-L-Arg, L-arginine did not affect endothelium-dependent relaxation and the authors therefore concluded that the hydroxyguanidino moiety is responsible for relaxation induced by OH-L-Arg (refs[131,132]). However, the precise mechanism of such induced relaxation is not clear so far.

3) As previously demonstrated[133], cultured rat vascular smooth muscle cells converted OH-L-Arg to nitrite in the absence of NOS activity. The nitrite production was augmented with substrate independently of the LPS presence. An inhibitor of NOS, L-NAME, did not affect nitrite accumulation in the incubation medium. On the contrary, miconazole, an inhibitor of cytochrome P450 3A subfamily, suppressed nitrite

production to the control values. These findings suggest the involvement of cytochrome P450 in this transformation instead of NOS. However, direct evidence is lacking and the exact mechanism remains unknown.

4) Recently it was proved[129] that not only hydroxylamine but also OH-L-Arg itself can interact with superoxide ion to produce nitrite and nitrate, which are the stable breakdown products of NO in water. It was demonstrated with superoxide ion produced either chemically or in biological system. L-Citrulline was simultaneously formed from OH-L-Arg. These results partially differ from those of Everett *et al.*[134]. They do not suppose that oxidative denitrification of OH-L-Arg by superoxide ion will prove a major free radical pathway to NO. Furthermore, they suppose production of cyclized carbodiimide instead of citrulline. On the other hand, results of Vetrovsky *et al.*[129] were confirmed by Modolell *et al.*[135]. However, these authors used a different method of superoxide production and different biological material.

3.5. Physiological Properties of N^G-Hydroxy-L-arginine

The physiological properties of OH-L-Arg have not been intensively studied so far. Wallace *et al.*[136] showed that OH-L-Arg induces endothelium-dependent and -independent relaxation of bovine pulmonary artery. Both endothelium-dependent and -independent relaxation were inhibited by NOS inhibitors. This is quite surprising in the case of endothelium-independent relaxation. Since in vascular smooth muscle cells NOS is not expressed under normal physiological conditions[76], the results of Wallace *et al.* suggest that OH-L-Arg was not converted to NO *via* NOS but by another enzyme that is, however, sensitive to L-NAME as well. Unfortunately, the authors did not provide any other information explaining this phenomenon. Nevertheless, the finding that OH-L-Arg is a potent endothelium-dependent vasorelaxant in vessels with intact endothelium[136], whereas L-arginine is not[5,137], is very interesting.

Gibson *et al.*[138], studying mouse anococcygeus, reported that OH-L-Arg but not OH-D-Arg is able to abolish vasoconstrictor effects of N^G-nitro-L-arginine.

As mentioned in Chapter *3.4.*, Zembovicz *et al.*[130] observed that OH-L-Arg produces relaxation by three different mechanisms. First, it is a substrate for the constitutive NOS present in endothelial cells. Second, it reacts with NO generated by this enzyme to form a vasodilator which is more potent and more stable than NO itself, and third, it releases from endothelial cells an NO-like relaxing factor, the formation of which is not blocked by inhibitors of NOS. The latter mechanism suggests that OH-L-Arg is metabolized by an alternative enzymic pathway.

The antagonism of the hemodynamic effects of NO biosynthesis inhibition *in vivo* by OH-L-Arg was demonstrated by Walder *et al.*[139].

More recently, OH-L-Arg has been shown independently by two groups[140,141] to be a potent inhibitor of liver arginase. Since substantial amounts of OH-L-Arg appear to be liberated from the active site of NOS (refs[46,110]), it is conceivable that OH-L-Arg may

act as an endogenous arginase inhibitor in NO-producing cells, such as macrophages. This hypothesis was then studied by Hecker *et al.*[142]. The authors demonstrated that LPS-activated macrophages release substantial amounts of OH-L-Arg which can indeed affect arginase activity. Since exposure of macrophages to LPS may lead to co-induction of NOS and arginase, the increased level of OH-L-Arg could limit arginase activity and direct L-arginine utilization towards an enhanced synthesis of NO. Moreover, as suggested by Hecker *et al.*[142], macrophage-derived OH-L-Arg may exert cytostatic effect, *e.g.*, on tumor cells[110].

4. SUPEROXIDE ION ($^{\bullet}O_2^-$)

4.1. Formation of Superoxide and Hydrogen Peroxide by Cellular Systems

Despite the existence of a variety of mechanisms controlling the cellular formation of superoxide, there are examples in which this free radical is generated during enzymic aerobic metabolism. In 1968, McCord and Fridovich[143] reported that superoxide was produced during the oxidation of purines by xanthine oxidase. In the following years other enzymes, including NOS (ref.[90]), have been found to generate this free radical as the result of oxidative metabolism. In fact, up to 2% of oxygen reduction by normal cells has been shown to occur *via* univalent pathway[144].

At physiological pH, superoxide rapidly dismutates to hydrogen peroxide. Therefore, in the presence of redox-active metal ions, hydroxyl radical can be produced *via* the metal-ion-catalyzed Haber–Weiss reaction:

$$^{\bullet}O_2^- + {}^{\bullet}O_2^- + 2\ H^+ \longrightarrow H_2O_2 + O_2 \qquad (E)$$

$$^{\bullet}O_2^- + Fe^{3+} \longrightarrow Fe^{2+} + O_2 \qquad (F)$$

$$Fe^{2+} + H_2O_2 \longrightarrow HO^{\bullet} + HO^- + Fe^{3+}\ . \qquad (G)$$

Because of the potential of these reduction products of oxygen to cause toxicity, cells have evolved pathways to eliminate superoxide and hydrogen peroxide, thereby limiting formation of hydroxyl radical. These include superoxide dismutase (SOD) for superoxide and peroxidases and catalases for hydrogen peroxide[145].

$$^{\bullet}O_2^- + {}^{\bullet}O_2^- + 2\ H^+ \longrightarrow H_2O_2 + O_2 \quad \text{(superoxide dismutase)} \qquad (H)$$

$$H_2O_2 + H_2O_2 \longrightarrow 2\ H_2O + O_2 \quad \text{(catalase)} \qquad (I)$$

$$H_2O_2 + RH_2 \longrightarrow 2\ H_2O + R \quad \text{(peroxidase)} \qquad (J)$$

Preventing metal ions from redox cycling is an alternative mechanism to inhibit hydroxyl radical formation. This may explain, at least in part, why intracellular and extracellular iron is carefully regulated by binding to amino acids and biological macromolecules including ferritin, transferrin and lactotransferrin[146]. In spite of these antioxidant mechanisms, there are numerous pathological conditions under which, as supposed, endogenous or exogenous sources of these oxygen reduction products contribute[147].

4.2. Leukocyte-Derived Superoxide and Hydrogen Peroxide

It has been shown[148] that binding of a wide variety of compounds to cell-surface receptors of neutrophils and other phagocytes (*e.g.*, macrophages) leads to an assembly of the NADPH-oxidase complex in plasma membrane with resulting extracellular secretion of superoxide utilizing intracellular NADPH. As mentioned, such produced superoxide is a precursor for reactive oxygen intermediates (ROI) including hydrogen peroxide and hydroxyl radical. ROI are believed to be involved in the killing of certain microorganisms during phagocytosis[149,150]. There is also an indirect evidence suggesting that ROI are also important in the killing of tumor cells by mononuclear phagocytes[151,152].

Both NADPH-oxidase and iNOS activity can be induced in macrophages but the secretion of superoxide and NO by macrophages does not occur simultaneously and was shown to be independently regulated[82]. Independent regulation occurs in spite of the fact that γ-interferon primes macrophages for the synthesis of both superoxide and NO (ref.[153]). Independent regulation also avoids the reaction between superoxide and NO, which forms peroxynitrite (see below).

Interestingly, Martin and Edwards[154] reported a change in ROI/NO ratio produced by human blood monocytes with time of their maturation to the macrophages. The changes that monocytes undergo in culture may be similar to those changes that occur after their entry into the tissues after leaving the peripheral blood[155] and therefore cultured human monocytes are thought to resemble human tissue macrophages. Martin and Edwards[154] have shown that previously reported initial decline in cytotoxic properties of monocytes that occurs with increasing age of cells in culture[156] is transient as tumoricidal competence returns after a few days in culture. On the basis of their results, they concluded that ROI are involved in monocyte-mediated cytotoxicity whereas NO is employed by macrophages in tumor cell killing.

4.3. Superoxide Generation by Non-Phagocyte NAD(P)H-Oxidase

Recently it has been shown that some non-phagocytic cells, *e.g.*, fibroblasts[157], endothelial cells[158], vascular smooth muscle[159,160] or neurons[161], can also produce superoxide. Superoxide formation seems to be mediated by another enzyme, NAD(P)H-oxidase, which is different from those found in phagocytic cells. This NAD(P)H-oxidase, found in cells that are not involved in defense, produces significantly lower amounts of superoxide. Activation can be triggered by the binding of a specific peptide to a trimeric G-protein-coupled receptor[162] in the same way as in the case of the superoxide generating NADPH-dependent oxidase of phagocytic cells. The endothelial enzyme is, however, distinguished by its constitutive activity. Thus, in functional terms, the NAD(P)H-oxidase found in non-phagocytic cells may be analogous to the constitutive NOSs (NOS-I and NOS-III), whereas the respiratory burst (NADPH) oxidase is analogous to iNOS.

The toxicity of superoxide produced by non-phagocytic NAD(P)H-oxidase is still debated. On the one hand, small amounts of superoxide produced by the non-phagocytic NAD(P)H-oxidase may act as an intracellular or intercellular signal in controlling cellular functions[163], on the other hand, an activation of membrane-associated neuronal NADPH-oxidase by β-amyloid leads to the injury of neuronal cells[161].

In addition to the NADH-oxidase, which is a major source of superoxide in vascular endothelial and smooth muscle cells[158,160], there are some other minor sources including mitochondria, xanthine oxidase, autoxidation of certain tissue metabolites, cytochrome P450 enzymes such as NOS, cycloxygenase and lipoxygenase[164–170]. Although metabolic processes that use oxygen are common in all cells, a disruption of such processes is characteristic of degenerative diseases. Atherosclerosis, or age-related disease of the vasculature, is associated with an oxidant stress that attenuates endothelium-dependent relaxations (for reviews, see refs[171,172]).

4.4. Reaction Between NO and Superoxide Anion

Experiments have shown that superoxide readily reacts with NO (refs[92,173]) generating peroxynitrite ($ONOO^-$). Beckman *et al.*[93] proposed that peroxynitrite being in the *trans* configuration, decomposes to give hydroxyl radical by the following mechanism:

$$NO^{\bullet} + {}^{\bullet}O_2^- \longrightarrow ONOO^- \qquad (K)$$

$$ONOO^- + H^+ \longrightarrow ONOOH \qquad (L)$$

$$ONOOH \longrightarrow HO^{\bullet} + NO_2^{\bullet} \qquad (M)$$

This represents a novel mechanism of hydroxyl radical generation, which is not dependent on redox-active transition metals. The significance of this finding is still discussed. Firstly, the products of a cleavage of protonated peroxynitrite ($HO^{\bullet}$ and $NO_2^{\bullet}$) were considered as compounds which can produce significant and irreversible damage to both microbes and host cells[174–177]. However, the lack of toxic effects of peroxynitrite reported more recently by Assreuy *et al.*[178] throws a new light on peroxynitrite toxicity. Moreover, it was also reported that nitric oxide protects against cellular damage and cytotoxicity from ROI (refs[179,180]). Thus, NO can possibly serve as a scavenger of toxic superoxide[160]. Whether this is due to the formation of *cis* configuration of peroxynitrite which rearranges to form non-toxic nitrate[93], is not known so far. If so, then it will be important to determine the conditions for the formation of the *cis* and *trans* isomers of peroxynitrite in biological systems. On the other hand, since the formation of *cis*-per-

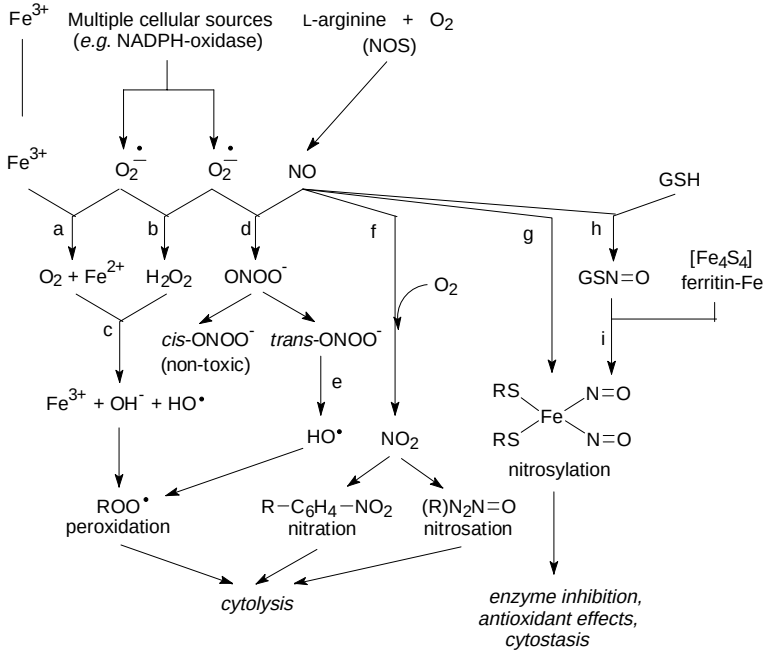

FIG. 6

Schematic representation of the interactions between iron (Fe^{3+}), superoxide ion ($^{\bullet}O_2^-$), nitric oxide (NO) and thiols (GSH). NADPH-oxidase produces $^{\bullet}O_2^-$ (pathways a–c), which is utilized by the neutrophil for defense of the extracellular environment. NO-synthase (NOS) produces NO (pathways g–i), which functions in defense of the intracellular environment. Reactions between O_2, $^{\bullet}O_2^-$ and NO (pathways d–f) produce highly reactive molecules ($ONOO^-$, $HO^{\bullet}$ and NO_2), which indiscriminately react with cellular components and may cause uncontrolled damage to the host cells. GSH may not react directly with NO flowing through pathway h but with NO metabolites such as NO^+, N_2O_3 or N_2O_4 (modified from ref.[218])

oxynitrite neutralizes the cytotoxicity of both superoxide and NO, it can provide an alternative reason for separate regulation of the synthesis of these two molecules.

Possible interactions between superoxide and NO are drawn in Fig. 6.

5. ROLES OF NO IN MAMMALIAN PHYSIOLOGY

5.1. Cardiovascular System

The identification of NO as EDRF (refs[2,3]) initially left impression that NO arises in the vascular wall exclusively from a constitutive NO-synthase (cNOS) activity in endothelial cells. Recently it has become clear that inflammatory stimuli can induce an inducible NO-synthase (iNOS) activity both in endothelium[74,181] and in vascular smooth muscle cells[75–77]. The amounts of NO produced under these conditions is far greater than those produced by agonist-triggered endothelial cells. Thus in addition to regulation of basal blood presure[182,183], NO of vascular origin may play a role in the hypotension associated with the administration of LPS (refs[184,185]) or cytokines[186], and perhaps in septic shock.

The effects of NO of vascular origin were originally described as inhibition of the contraction of smooth muscle, of the adhesion and aggregation of platelets[20,89] and of the smooth muscle cell proliferation[187]. Each of these effects has the potential to play an important physiological and pathophysiological role. Endothelial NO production is enhanced by agonists locally released from platelets (such as ATP/ADP, serotonin, thrombin) or produced by endothelial cells (bradykinin, endothelin); for reviews, see refs[89,188]. It is also enhanced by shear stress, resulting in decreased vascular resistance and enhanced blood flow[189].

Dysregulation of NO production is implicated in the pathogenesis of some cardiovascular diseases. Impaired NO-related, endothelium-dependent vasorelaxation has been observed in, and may contribute to essential hypertension[190], the endothelial dysfunction of hypercholesterolemia[191], diabetes[192] and the pulmonary arterial hypertension of chronic obstructive lung disease[193].

5.2. Immune System

Recent studies have demonstrated a crucial role of NO in the antimicrobial and tumoricidal activity of macrophages[23,194,195]. Large amounts of NO produced by activated cells serve to protect the host from viruses[196,197], bacteria, protozoa, helminths[198] and tumor cells[6]. The biochemical basis for the cytotoxicity induced by nitric oxide is described in Chapter *1.3*. The role of NO in the immune system with respect to the interaction with superoxide ion is discussed in Chapter *4.4*.

5.3. NO in Central Nervous System

One of the physiological roles proposed for NO in the central nervous system is the long-term potentiation or depression (LTP or LTD, respectively) induced in response to stimulation of excitatory amino acid receptors. Both LTP and LTD are considered to be elements of experience-driven synaptic network remodelling that may underlie learning and memory. As an example, LTP induction in the hippocampus requires the activation of *N*-methyl-D-aspartate (NMDA) receptor channels and subsequent NO formation is enhanced as a result of an increase in Ca^{2+} into post-synaptic elements. Thereafter, NO plays the role of a retrograde messenger capable of diffusing from post- to neighbouring presynaptic neurons and then to potentiate their response[199]. NOS inhibitors blunt LTP in a stereospecific and L-arginine-dependent manner[200].

Alternatively, NO production induced by activation of NMDA receptor may result in negative feedback regulation of NMDA receptors which may be considered as a protective mechanism against calcium overload triggered by excessive NMDA channels activation[201]. However, depending on the redox state of neurons, NO production may also cause cytotoxic effects *via* interaction with superoxide radicals resulting in generation of peroxynitrite[202]. This concept is discussed above (Chapter *4.4.*).

5.4. NO in the Peripheral Nervous System

Nitrogen oxide (NO) is now clearly identified as a transmitter or a modulator of the inhibitory autonomic nonadrenergic noncholinergic (NANC) innervation of smooth muscle in the gastrointestinal tract, pelvic viscera, airways, *etc*.[203–207]. Although the exact nature of the relaxing NANC neurotransmitters released by these nerves has not been clearly established (adenosine, ATP, vasoactive intestinal peptide, substance P, *etc*.), experimental arguments are now accumulated indicating that NO is released simultaneously (blockade by NOS inhibitors or hemoglobin) and that part of the final response is attributable to NO-induced stimulation of guanylate cyclase in these smooth muscles. Interestingly, hydroxocobalamine is a scavenger of NO (forming nitrosocobalamine) released from the endothelium but not from NANC nerves[208]. This property may serve as an original tool for investigating the source of NO-mediated pharmacological processes (endothelium *vs* NANC nerves) since, in contrast to hydroxocobalamine, classical NOS inhibitors antagonize the release of NO regardless of its origin. Whether NO acts directly on the target cells as a neurotransmitter released from "nitroxidergic" neurones or indirectly from non-neuronal cells containing NOS and stimulated by NANC nerves remains a matter of debate[209].

Special attention has been paid to the penile corpus cavernosum smooth muscle in rabbits and humans which contains a NANC inhibitory pathway[210]. Electrically evoked relaxation of corpus cavernosum is antagonized by NOS-inhibitors and mimicked by nitric oxide donors[211–213]. Thus, nitric oxide is probably the final common mediator of

penile erection. Some forms of impotence could result from defects in the NO-mediated smooth muscle relaxation in corpus cavernosum[214].

5.5. NO in the Modulation of the Uterine Contractility

In 1993 Yallampalli *et al.*[215] demonstrated the presence of L-arginine/NO pathway in the rat uterus where it inhibited uterine contractility during pregnancy but not during labor. Histochemical and biochemical assays recently revealed the presence of NOS in the pregnant rat uterus and a significant decline in enzyme activity at parturition[216]. The total NOS activity was reduced by nearly 70% in laboring rat uterus.

However, the precise mechanism of the uterine contractility modulation remains unclear. On the one hand, the NOS activity that underwent the greatest decline between the quiescent and laboring state of pregnancy was calcium-independent. This activity corresponds to the isoform that has been found in macrophages, suggesting that macrophages could play a role in NO mediation of uterine quiescence during pregnancy and uterine contraction at parturition. This is supported by the fact that the number of macrophages in the mouse decidua basalis during pregnancy is increased with subsequent regression in the number of these cells at term[217]. On the other hand, the calcium-sensitive activity (corresponding to neuronal and endothelial NOS, see Chapter *2.3.*) significantly decreased when the uterus was actively contracting, too. It may correspond to the NOS demonstrated in nerve fibers and varicosities. These nerve fibers stained avidly for NOS during pregnancy but lack of NOS staining at term may reflect down regulation of NOS production in the nerve or degeneration of the nerve itself[216]. Nevertheless, the presently available findings demonstrate that NO plays a key role in the maintenance of the uterine quiescence during pregnancy and that reduced capacity for its production could trigger the initiation of labor at term.

The authors are deeply indebted to Mrs M. Spundova for an excellent technical assistance in the preparation of the manuscript.

REFERENCES

1. Stuehr D. J., Griffith O. W.: Adv. Enzymol. Relat. Areas Mol. Biol. *65*, 287 (1992).
2. Furchgott R. F. in: *Vasodilatation: Vascular Smooth Muscle, Peptides, Autonomic Nerves, and Endothelium* (P. M. Vanhoute, Ed.), p. 401. Raven, New York 1988.
3. Ignarro L. J., Byrns R. E., Buga G. M., Wood K. S.: Circ. Res. *61*, 866 (1987).
4. Palmer R. M. J., Ferrige A. G., Moncada S.: Nature *327*, 524 (1987).
5. Palmer R. M. J., Ashton D. S., Moncada S.: Nature *333*, 664 (1988).
6. Hibbs J. B., Taintor R. R., Vavrin V.: Science *235*, 473 (1987).
7. Stamler J. S., Singel D. J., Loscalzo J.: Science *258*, 1898 (1992).
8. Furchgott R. F., Khan M. T., Jothianandan K. D. in: *Endothelium-Derived Relaxing Factor* (G. M. Rubanyi and P. M. Vanhoute, Eds), p. 8. Karger, Basel 1990.

9. Kharitonov V. G., Sundquist A. R., Sharma V. S.: J. Biol. Chem. *269*, 5881 (1994).
10. Ignarro L. J., Fukuto J. M., Griscavage J. M., Rogers N. E., Byrns R. E.: Proc. Natl. Acad. Sci. U.S.A. *90*, 8103 (1993).
11. Saran M., Michel C., Bors W.: Free Radical Res. Commun. *10*, 221 (1990).
12. Doyle M. P., Hockstra J. W.: J. Inorg. Biochem. *14*, 351 (1981).
13. Hidalgo E., Demple B.: EMBO J. *13*, 138 (1994).
14. Liu X., Gillespie J. S., Martin W.: Br. J. Pharmacol. *111*, 1287 (1994).
15. Iyengar R., Stuehr D. J., Marletta M. A.: Proc. Natl. Acad. Sci. U.S.A. *84*, 6369 (1987).
16. Bonner F. T., Dzelkalns L. S., Bonnuci J. A.: Inorg. Chem. *17*, 2487 (1978).
17. Hecker M., Boese M., Schini-Kerth V. B., Mulsch A., Busse R.: Proc. Natl. Acad. Sci. U.S.A. *92*, 4671 (1995).
18. Eisenberg R., Meyer C. D.: Acc. Chem. Res. *8*, 26 (1975).
19. Fukuto J. M., Chiang K., Hszieh R., Wong P., Chaudhurs G.: J. Pharmacol. Exp. Ther. *236*, 548 (1992).
20. Ignarro L. J.: Annu. Rev. Pharmacol. Toxicol. *30*, 535 (1990).
21. Brune B., Lapetina E. G.: J. Biol. Chem. *264*, 8455 (1989).
22. Salvemini D., Misko T. P., Masferrer J. L., Seibert K., Currie M. G., Needleman P.: Proc. Natl. Acad. Sci. U.S.A. *90*, 7240 (1993).
23. Hibbs J. B., Taintor R. R., Vavrin V., Granger D. L., Drapier J.-C., Amber I. J., Lancaster J. R. in: *Nitric Oxide from* L*-Arginine: A Bioregulatory System* (S. Moncada and E. A. Higgs, Eds), p. 189. Elsevier, Amsterdam 1990.
24. Stuehr D. J., Nathan C. F.: J. Exp. Med. *169*, 1543 (1989).
25. Lancaster J. R., Hibbs J. B.: Proc. Natl. Acad. Sci. U.S.A. *87*, 1223 (1990).
26. Drapier J.-C., Pellat C., Yann H.: J. Biol. Chem. *266*, 10162 (1991).
27. Reif D. W., Simmons R. D.: Arch. Biochem. Biophys. *283*, 537 (1990).
28. Lander H. M., Sehajpal P. K., Novogrodsky A.: J. Immunol. *151*, 7182 (1993).
29. Gopalakrishna R., Chen Z. H., Gundimeda U.: J. Biol. Chem. *268*, 27180 (1993).
30. Ignarro L. J.: Biochem. Pharmacol. *41*, 485 (1991).
31. Kwon N. S., Stuehr D. J., Nathan C. F.: J. Exp. Med. *174*, 761 (1990).
32. Lepoivre M., Chenais B., Yapo A., Lemaire G., Thelander L., Tenu J.-P.: J. Biol. Chem. *265*, 14143 (1990).
33. Lepoivre M., Fieschi F., Coves J., Thelander L., Fontecave M.: Biochem. Biophys. Res. Commun. *179*, 442 (1991).
34. Lepoivre M., Flaman J.-M., Henry Y.: J. Biol. Chem. *267*, 22994 (1992).
35. Bredt D. S., Snyder S. H.: Proc. Natl. Acad. Sci. U.S.A. *87*, 682 (1990).
36. Sessa W. C.: J. Vasc. Res. *31*, 131 (1994).
37. Hevel J. M., White K. A., Marletta M. A.: J. Biol. Chem. *266*, 22789 (1991).
38. Stuehr D. J., Cho H. J., Kwon N. S., Weise M., Nathan C. F.: Proc. Natl. Acad. Sci. U.S.A. *88*, 7773 (1991).
39. Lyons C. R., Orloff G. J., Cunningham J. M.: J. Biol. Chem. *267*, 6370 (1992).
40. Cho J. H., Xie Q. W., Calaycay J., Mumford R. A., Swiderek K. M. *et al.*: J. Exp. Med. *176*, 599 (1992).
41. Mayer B., John M., Heinzel B., Werner E. R., Wachter H., Schultz G., Bohme E.: Fed. Eur. Biol. Soc. *288*, 187 (1991).
42. Marletta M. A.: J. Biol. Chem. *268*, 12231 (1993).
43. Stuehr D. J., Kwon N. S., Nathan C. F., Griffith O. W., Feldman P. L., Wiseman J.: J. Biol. Chem. *266*, 6259 (1991).

44. Kwon N. S., Nathan C. F., Gilker C., Griffith O. W., Matthews D. E., Stuehr D. J.: J. Biol. Chem. *265*, 13442 (1990).
45. Pufahl R. A., Nanjappan P. G., Woodard R. W., Marletta M. A.: Biochemistry *31*, 6822 (1992).
46. Klatt P., Schmidt K., Uray G., Mayer B.: J. Biol. Chem. *268*, 14781 (1993).
47. Masters B. Sue S.: Ann. Rev. Nutr. *14*, 131 (1994).
48. Giovanelli J., Campos K. L., Kaufman S.: Proc. Natl. Acad. Sci. U.S.A. *88*, 7091 (1991).
49. Klatt P., Schmid M., Leopold E., Schmidt K., Werner E. R.: J. Biol. Chem. *269*, 13861 (1994).
50. Baek K. J., Thiel B. A., Lucas J., Stuehr D. J.: J. Biol. Chem. *268*, 21120 (1993).
51. Hevel J. M., Marletta M. A.: Biochemistry *31*, 7160 (1992).
52. Forstermann U., Schmidt H. H. H. W., Pollock J. S., Sheng H., Mitchell J. A., Warner T. D., Nakane M., Murad F.: Biochem. Pharmacol. *42*, 1849 (1991).
53. Bredt D. S., Ferris C. D., Snyder S. H.: J. Biol. Chem. *267*, 10976 (1992).
54. Lamas S., Marsden P. A., Li G. K., Tempst P., Michel T.: Proc. Natl. Acad. Sci. U.S.A. *89*, 6348 (1992).
55. Lowenstein C. J., Glatt C. S., Bredt D. S., Snyder S. H.: Proc. Natl. Acad. Sci. U.S.A. *89*, 6711 (1992).
56. McMillan K., Bredt D. S., Hirsch D. J., Snyder S. H., Clark J. E., Masters B. Sue S.: Proc. Natl. Acad. Sci. U.S.A. *89*, 1141 (1992).
57. Sessa W. C., Harrison J. K., Barber C. M., Zeng D., Durieux M. E., D'Angello D. D., Lynch K. R., Peach M. J.: J. Biol. Chem. *267*, 15274 (1992).
58. Bredt D. S., Hwang P. M., Glatt C. E., Lowenstein C., Reed R. R., Snyder S. H.: Nature *351*, 714 (1991).
59. White K. A., Marletta M. A.: Biochemistry *31*, 6627 (1992).
60. Sari M.-A., Booker S., Jaouen M., Vadon S., Boucher J.-L., Pompon D., Mansuy D.: Biochemistry *35*, 7204 (1996).
61. Bredt D. S., Hwang P. M., Snyder S. H.: Nature *347*, 768 (1990).
62. Janssens S. P., Shimouchi A., Quertermous T., Bloch D. B., Bloch K. D.: J. Biol. Chem. *267*, 14519 (1992).
63. Iida S., Ohshima H., Oguchi S., Hata T., Suzuki H., Kawasaki H., Esumi H.: J. Biol. Chem. *267*, 25385 (1992).
64. Bredt D. S., Snyder S. H.: Annu. Rev. Biochem. *63*, 175 (1994).
65. Forstermann U., Nakane M., Tracey W. R., Pollock J. S.: Eur. Heart J. *14* (Suppl. 1), 10 (1993).
66. Nathan C.: FASEB J. *6*, 3051 (1992).
67. Tracey W. R., Pollock J. S., Murad F., Nakane M., Forstermann U.: Am. J. Physiol. *266*, C22 (1994).
68. Ujiie K., Yuen J., Hogarth L., Danziger R., Star R. A.: Am. J. Physiol. *267*, F296 (1994).
69. O'Dell T. J., Huang P. L., Dwason D. M., Dinerman J. L., Snyder S. H.: Science *265*, 542 (1994).
70. Balligand J. L., Kobzik L., Han X., Kaye D. M., Belhassen L., O'Hara D. S., Kelly R. A., Smith T. W., Michel T.: J. Biol. Chem. *270*, 27580 (1995).
71. Yui Y., Hattori R., Kosuga K., Eizawa H., Hiki K.: J. Biol. Chem. *266*, 12544 (1991).
72. Noda T., Amano F.: J. Biochem. (Tokyo) *121*, 38 (1997).
73. Geller D. A., Lowenstein C. J., Shapiro R. A., Nussler A. K., Di Silvo M., Snyder S. H.: Proc. Natl. Acad. Sci. U.S.A. *90*, 3491 (1993).
74. Kilbourn R. G., Bellone I.: J. Natl. Cancer Inst. *82*, 772 (1990).
75. Busse R., Mulsch A.: FEBS Lett. *275*, 87 (1990).
76. Fleming I., Gray G. A., Schott C. A., Stoclet J.-C.: Eur. J. Pharmacol. *200*, 375 (1991).

77. Fleming I., Gray G. A., Julou-Schaeffer G., Parrat J. R., Stoclet J.-C.: Biochem. Biophys. Res. Commun. *171*, 562 (1991).
78. Charles I. G., Palmer R. M. J., Hickery M. S., Bayliss M. T., Chubb A. P., Moncada S.: Proc. Natl. Acad. Sci. U.S.A. *90*, 11419 (1993).
79. Schulz R., Nava E., Moncada S.: Br. J. Pharmacol. *105*, 575 (1992).
80. Kobzik L., Bredt D. S., Lowenstein C. J., Drazen J., Gaston B., Snyder S. H.: Am. J. Respir. Cell Mol. Biol. *90*, 371 (1993).
81. Rubanyi G. M., Desiderio D., Luisi A., Johns A., Sybertz E.: J. Pharmacol. Exp. Ther. *249*, 858 (1989).
82. Ding A. H., Nathan C. F., Stuehr D. J.: J. Immunol. *141*, 2407 (1988).
83. Arany I., Brysk M. M., Brysk H., Tyring S. K.: Cancer Lett. *110*, 93 (1996).
84. Salkowski C. A., Detore C., McNally R., Vanrooijen N., Vogel S. N.: J. Immunol. *158*, 905 (1997).
85. Di Rosa M., Radomski M., Carnuccio R., Moncada S.: Biochem. Biophys. Res. Commun. *172*, 1246 (1990)
86. Rogers N. E., Ignarro L. J.: Biochem. Biophys. Res. Commun. *189*, 242 (1992)
87. Assreuy J., Cunha F. Q., Liew F. Y., Moncada S.: Br. J. Pharmacol. *108*, 833 (1993).
88. Rengasamy A., Johns R. A.: Mol. Pharmacol. *44*, 124 (1993).
89. Moncada S., Palmer R. M. J., Higgs E. A.: Pharmacol. Rev. *43*, 109 (1991).
90. Pou S., Pou W. S., Bredt D. S., Snyder S. H., Rosen G. M.: J. Biol. Chem. *267*, 24173 (1992).
91. Thomas G., Ramwell P. W.: J. Pharmacol. Exp. Ther. *260*, 676 (1992).
92. Blough N. V., Zafinou O. C.: Inorg. Chem. *24*, 3502 (1985).
93. Beckman J. S., Beckman T. W., Chen J., Marshall P. A., Freeman B. A.: Proc. Natl. Acad. Sci. U.S.A. *87*, 1620 (1990).
94. Hobbs A. J., Fukuto J. M., Ignarro L. J.: Proc. Natl. Acad. Sci. U.S.A. *91*, 10992 (1994).
95. Gross S. S., Stuehr D. J., Aisaka K., Jaffe E. A., Levi R., Griffith O. W.: Biochem. Biophys. Res. Commun. *170*, 96 (1990).
96. Lambert L. E., Whitten J. P., Baron B. M., Cheng H. C., Doherty D. S.: Life Sci. *48*, 69 (1991).
97. Feldman P. L., Griffith O. W., Stuehr D. S.: J. Med. Chem. *36*, 491 (1993).
98. Olken N. M., Marletta M. A.: Biochemistry *32*, 9677 (1993).
99. Furfine E. S., Harmon M. F., Paith J. E., Garvey E. P.: Biochemistry *32*, 8512 (1993).
100. Klatt P., Schmidt K., Brunner F., Mayer B.: J. Biol. Chem. *269*, 1674 (1994).
101. Narayanan K., Spack L., Hayward M., Griffith O. W.: FASEB J. *8*, A360 (1994).
102. Frey C., Narayanan K., McMillan K., Spack L., Gross S., Griffith O. W.: J. Biol. Chem. *269*, 26083 (1994).
103. Garvey P. E., Oplinger J. A., Tanoury G. J., Sherman P. A., Fowler M., Marshall S., Marmon M. F., Paith J. E., Furfine E. S.: J. Biol. Chem. *269*, 26669 (1994).
104. Szabo C., Southan G. J., Thiemermann C.: Proc. Natl. Acad. Sci. U.S.A. *91*, 12472 (1994).
105. Ruetten H., Southan G. J., Abate A., Thiemermann C.: Br. J. Pharmacol. *118*, 261 (1996).
106. Stuehr D. J., Olufunmilayo A. F., Kwon N. S., Gross S. S., Gonzales J. A., Levi R., Nathan C. F.: FASEB J. *5*, 98 (1991).
107. Werner-Felmayer G., Werner E. R., Fuchs D., Hausen A., Reibnegger G., Wachter H.: J. Exp. Med. *172*, 1599 (1990).
108. Corbett J. A., Tilton R. G., Chang K., Hasan K. S., Ydo Y.: Diabetes *41*, 552 (1992).
109. Misko T. P., Moore W. M., Kasten T. P., Nickols G. A., Corbett J. A.: Eur. J. Pharmacol. *233*, 119 (1993).
110. Chenais B., Yapo A., Lepoivre M., Tenu J.-P.: Biochem. Biophys. Res. Commun. *196*, 1558 (1993).

111. Hecker M., Schott C., Bucher B., Busse R., Stoclet J.-C.: Eur. J. Pharmacol. *275*, R1 (1995).
112. Boucher J.-L., Genet A., Vadon S., Delaforge M., Henry Y., Mansuy D.: Biochem. Biophys. Res. Commun. *187*, 880 (1992).
113. Renaud J.-P., Boucher J.-L., Vadon S., Delaforge M., Mansuy D.: Biochem. Biophys. Res. Commun. *192*, 53 (1993).
114. Boucher J.-L., Genet A., Vadon S., Delaforge M., Mansuy D.: Biochem. Biophys. Res. Commun. *184*, 1158 (1992).
115. Boucher J.-L., Chopard C., Delaforge M., Mansuy D.: *Biology of Nitric Oxide 3rd Int. Meeting, October 3–6, 1993*, Abstr. No. 65. CNRS, Cologne 1993.
116. Mansuy D., Boucher J.-L., Clement B.: Biochimie *77*, 661 (1995).
117. Clement B., Schultze-Mosgau M. H., Wohlers H.: Biochem. Pharmacol. *46*, 2249 (1993).
118. Clement B., Jung F.: Drug Metab. Dispos. *22*, 486 (1994).
119. Sennequier N., Boucher J.-L., Battioni P., Mansuy D.: Tetrahedron Lett. *36*, 6059 (1995).
120. Griscavage J. M., Fukuto J. M., Ignarro L. J.: J. Biol. Chem. *269*, 21644 (1994).
121. Oritz de Montellano P. R.: *Cytochrome P450: Structure, Mechanism and Biochemistry*. Plenum Press, New York 1986.
122. Korth H. G., Sustmann R., Thater C., Butler A. R., Ingold K. U.: J. Biol. Chem. *269*, 17776 (1994).
123. DeMaster E. G., Raij L., Archer S. L., Weir E. K.: Biochem. Biophys. Res. Commun. *163*, 527 (1989).
124. Thomas G., Ramwell P. W.: Biochem. Biophys. Res. Commun. *164*, 889 (1989).
125. Nicholls P.: Biochem. J. *90*, 331 (1964).
126. Craven P. A., DeRubertis F. R., Pratt D. W.: J. Biol. Chem. *251*, 8213 (1979).
127. Fukuto J. M., Hszieh R., Gulati P., Chiang K. T., Nagasawa H. T.: Biochem. Biophys. Res. Commun. *187*, 1367 (1992).
128. Fukuto J. M., Hobbs A. J., Ignarro L. J.: Biochem. Biophys. Res. Commun. *196*, 707 (1993).
129. Vetrovsky P., Stoclet J.-C., Entlicher G.: Int. J. Biochem. Cell Biol. *28*, 1311 (1996).
130. Zembowicz A., Hecker M., MacArthur H., Sessa W. C., Vane J. R.: Proc. Natl. Acad. Sci. U.S.A. *88*, 11172 (1991).
131. Zembowicz A., Swierkosz T. A., Southan G. J., Hecker M., Gryglewski R. J., Vane J. R.: J. Cardiovasc. Pharmacol. *20* (Suppl. 12), S57 (1992).
132. Zembowicz A., Swierkosz T. A., Southan G. J., Hecker M., Vane J. R.: Br. J. Pharmacol. *107*, 1001 (1992).
133. Schott C. A., Bogen C. M., Vetrovsky P., Berton C. C., Stoclet J.-C.: FEBS Lett. *341*, 203 (1994).
134. Everett S. A., Dennis M. F., Patel K. B., Stratford M. R. L., Wardman P.: Biochem. J. *317*, 17 (1996).
135. Modolell M., Eichmann K., Soler G.: FEBS Lett. *401*, 123 (1997).
136. Wallace G. C., Gulati P., Fukuto J. M.: Biochem. Biophys. Res. Commun. *176*, 528 (1991).
137. Mitchel J. A., DeNucci G., Warner T. D., Vane J. R.: Br. J. Pharmacol. *103*, 1295 (1991).
138. Gibson A., Babbedge R., Brave S. R., Hart S. L., Hobbs A. J., Tucker J. F., Wallace P., Moore P. K.: Br. J. Pharmacol. *107*, 715 (1992).
139. Walder C. E., Thiemermann C., Vane J. R.: Br. J. Pharmacol. *107*, 476 (1992).
140. Daghigh F., Fukuto J. M., Ash D. E.: Biochem. Biophys. Res. Commun. *202*, 174 (1994).
141. Boucher J.-L., Custot J., Vadon S., Delaforge M., Lepoivre M., Tenu J.-P., Yapo A., Mansuy D.: Biochem. Biophys. Res. Commun. *203*, 1614 (1994).
142. Hecker M., Nematollahi H., Hey C., Busse R., Racke K.: FEBS Lett. *359*, 251 (1995).
143. McCord J. M., Fridowich I.: J. Biol. Chem. *243*, 5753 (1968).

144. Boveris A. N., Oshino N., Chance B.: Biochem. J. *128*, 617 (1972).
145. Fridovich I.: Science *201*, 875 (1978).
146. Halliwell B., Gutteridge J. M. C.: *Free Radicals in Biology and Medicine*, 2nd ed. Clarendon Press, Oxford 1989.
147. Cross C. E. B., Halliwell B., Borish E. T., Pryor W. A., Saul R. L., McCord J. M., Harman D.: Ann. Int. Med. *107*, 526 (1987).
148. Clark R. A.: J. Infect. Dis. *161*, 1140 (1990).
149. Babior B. M.: Blood *64*, 959 (1984).
150. Babior B. M.: J. Clin. Invest. *73*, 599 (1984).
151. Reed S. G., Nathan C. F., Phil D. L., Rodricks P., Shanebeck P. J., Conlon P. J., Grabstein K. H.: J. Exp. Med. *166*, 1734 (1987).
152. Nathan C. F., Prendergast T. J., Wiebe M. J., Stanley E. R., Platzer E., Remold H. G., Welte K., Rubin B. Y., Murray H.: J. Exp. Med. *160*, 606 (1984).
153. Tennenberg S. D., Fey D. E., Lieser M. J.: J. Leukocyte Biol. *53*, 301 (1993).
154. Martin J. H. J., Edwards S. W.: J. Immunol. *150*, 3478 (1993).
155. Becker S.: Cell Immunol. *84*, 145 (1984).
156. Davies B., Edwards S. W.: Br. J. Cancer *66*, 463 (1992).
157. Meier B., Jesaitis A. J., Emmendorffer A., Roesler J., Quinn M. T.: Biochem. J. *289*, 481 (1993).
158. Mohazzab-H K. M., Kaminski P. L., Wolin M. S.: Am. J. Physiol. *266*, H2568 (1994).
159. Hecker M., Preiss C., Schini-Kerth V., Busse R.: FEBS Lett. *380*, 224 (1996).
160. Stamler J. S.: Nature *380*, 108 (1996).
161. Behl C., Davis J. B., Lesley R., Schubert D.: Cell *77*, 817 (1994).
162. Griendling K. K., Minieri C. A., Ollerenshaw J. O., Alexander R. W.: Circ. Res. *74*, 1141 (1994).
163. Morre D. J., Brightman A. O.: J. Bioenerg. Biomembr. *23*, 469 (1991).
164. Chance B., Sies H., Boveris A.: Physiol. Rev. *59*, 527 (1979).
165. Cross A. R., Jones O. T. G.: Biochim. Biophys. Acta *1057*, 291 (1991).
166. Freeman B. A., Crapo J. D.: Lab. Invest. *47*, 412 (1982).
167. Holland J. A., Pappolla M. A., Wolin M. S., Pritchard K. A., Rogers N. J., Stemerman M. B.: J. Cell. Physiol. *143*, 21 (1990).
168. Kinnula V. L., Mirza Z., Crapo D. J., Whorton A. R.: Am. J. Respir. Cell Mol. Biol. *9*, 603 (1993).
169. Kukreja R. C., Kontos H. A., Hess M. L., Ellis E. F.: Circ. Res. *59*, 612 (1986).
170. Ratych R. E., Chuknyiska R. S., Bulkley G. B.: Surgery *102*, 122 (1987).
171. Munro J. M., Cotran R. S.: Lab. Invest. *58*, 249 (1988).
172. Nilsson J.: Cardiovasc. Res. *27*, 1184 (1993).
173. Huie R. E., Padmaja S.: Free Radical Res. Commun. *18*, 195 (1993).
174. Halliwell B., Gutteridge J. M. C.: Methods Enzymol. *186*, 1 (1990).
175. Prutz W., Monig H., Butler J., Land J.: Arch. Biochem. Biophys. *243*, 125 (1985).
176. Zhu L., Gunn C., Beckman J. S.: Arch. Biochem. Biophys. *298*, 452 (1992).
177. Beckman J. S., Crow J. P.: Biochem. Soc. Trans. *21*, 330 (1993).
178. Assreuy J., Cunha F. Q., Epperlein M., Noronha-Dutra A., O'Donnell C. A., Liew F. Y., Moncada S.: Eur. J. Immunol. *24*, 672 (1994).
179. Wink D. A., Hanbauer I., Krishna M. C., DeGraff W., Gamson J., Mitchell J. B.: Proc. Natl. Acad. Sci. U.S.A. *90*, 9813 (1993).
180. Harbrecht B. G., Billiar T. R., Stadler J., Demetris A. J., Ochoa J., Curran R. D., Simmons R. L.: J. Leukocyte Biol. *52*, 390 (1992).

181. Radomski M. W., Palmer R. M. J., Moncada S.: Proc. Natl. Acad. Sci. U.S.A. *87*, 10043 (1990).
182. Aisaka K., Gross S. S., Griffith O. W., Levi R.: Biochem. Biophys. Res. Commun. *160*, 881 (1989).
183. Rees D. D., Plamer R. M. J., Moncada S.: Proc. Natl. Acad. Sci. U.S.A. *86*, 3375 (1989).
184. Thiemermann C., Vane J.: Eur. J. Pharmacol. *182*, 591 (1990).
185. Kilbourn R. G., Jubran A., Gross S. S., Griffith O. W., Levi R., Adams J., Lodato R. F.: Biochem. Biophys. Res. Commun. *172*, 1132 (1991).
186. Kilbourn R. G., Gros S. S., Jubran A., Adams J., Griffith O. W., Levi R., Lodato R: Proc. Natl. Acad. Sci. U.S.A. *87*, 3629 (1990).
187. Nakaki T., Nakayama M., Kato R.: Eur. J. Pharmacol. *189*, 347 (1990).
188. Furchgott R. F., Vanhoute P.: FASEB J. *3*, 2007 (1989).
189. Lamontagne D., Pohl U., Busse R.: Circ. Res. *70*, 123 (1992).
190. Panza J. A., Quyyumi A. A., Brush J. E., Epstein S. E.: New Eng. J. Med. *323*, 22 (1990).
191. Drexler H., Zeiher A. M., Meinzer K., Just H.: Lancet *338*, 1546 (1991).
192. Calver A., Collier J., Vallace P.: J. Clin. Invest. *90*, 2548 (1992).
193. Dinh-Xuan A. T., Higenbottam T. W., Clelland C. A., Pepke-Zaba J., Cremona G., Butt A. I., Large S. R., Wells F. C., Walwork J.: New Eng. J. Med. *324*, 1539 (1991).
194. Drapier J.-C.: Res. Immunol. *142*, 553 (1991).
195. Green S. J., Nacy C. A., Meltzer M. S.: J. Leukocyte Biol. *50*, 93 (1991).
196. Croen K. D.: Clin. Invest. *91*, 2446 (1993).
197. Karupiah G., Xie Q.-W., Buller M. L., Nathan C., Duarte C., MacMicking J. D.: Science *261*, 1445 (1993).
198. Nathan C. F., Hibbs J. B.: Curr. Opin. Immunol. *3*, 65 (1991).
199. Garthwaite J., Charles S. L., Chess-Williams R.: Nature *336*, 385 (1988).
200. Bon C., Bohme G. A., Doble A., Stutzmann J. M., Blanchard J. C.: Eur. J. Neurol. *4*, 420 (1992).
201. Manzoni O., Prezean L., Marin P., Deshager S., Bockaert J., Fagni L.: Neuron *8*, 653 (1992).
202. Lipton S. A.: Nature *364*, 626 (1993).
203. Gillespie J. S., Liu X., Martin W.: Ref.[23], p. 147.
204. Stark M. E., Szurszewski J. H.: Gastroenterology *103*, 1928 (1992).
205. Jorens P. G., Vermeire P. A., Herman A. G.: Eur. Respir. J. *6*, 258 (1993).
206. Dewinter B. Y., Boeckxstaens G. E., Deman J. G., Moreels T. G., Herman A. G., Pelckmans P. A.: Br. J. Pharmacol. *120*, 464 (1997).
207. Tomita R., Kurosu Y., Munakata K.: J. Gastroenterol. *32*, 1 (1997).
208. Rajanayagam M. A. S., Li C. G., Rand M. J.: Br. J. Pharmacol. *108*, 3 (1993).
209. Rand M. J.: Clin. Exp. Pharmacol. Physiol. *19*, 147 (1992).
210. Burnett A. L., Lowenstein C. J., Bredt D. S., Chang T. S. K., Snyder S. H.: Science *257*, 401 (1992).
211. Ignarro L. J., Bush P. A., Buga G. M., Wood K. S., Fukuto J. M., Rajfer J.: Biochem. Biophys. Res. Commun. *170*, 843 (1990).
212. Holmquist F., Hedlund H., Andersson K. E.: Acta Physiol. Scand. *141*, 441 (1991).
213. Kim N., Azadzoi K. M., Goldstein I., Saenz de Tajada I.: J. Clin. Invest. *88*, 112 (1991).
214. Rajfer R., Aronson W. J., Bush P. A., Dorey F. J., Ignarro L. J.: New Engl. J. Med. *326*, 90 (1992).
215. Yallampalli C., Garfield R. E., Byam-Smith M.: Endocrinology *133*, 1899 (1993).
216. Natuzzi E. S., Ursell P. C., Harrison M., Buscher C., Riemer R. K.: Biochem. Biophys. Res. Commun. *194*, 1 (1993).
217. De M., Wood G.: J. Leukocyte Biol. *50*, 381 (1991).
218. Hibbs J. R. in: *The Biology of Nitric Oxide* (S. Moncada, M. A. Marletta, J. B. Hibbs and E. A. Higgs, Eds), Vol. 2, p. 201. Portland Press, London–Chapel Hill 1992.