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REVIEW ARTICLE

Biochemistry of Nitric Oxide

Safia Habib · Asif Ali

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Abstract Nitric oxide (NO) a free radical having both cytoprotective as well as tumor promoting agent is formed from L-arginine by converting it to L-citrulline via nitric oxide synthase enzymes. The reaction product of nitric oxide with superoxide generates potent oxidizing agent, peroxynitrite which is the main mediator of tissue and cellular injury. Peroxynitrite is reactive towards many biomolecules which includes amino acids, nucleic acid bases; metal containing compounds, etc. NO metabolites may play a key role in mediating many of the genotoxic/carcinogenic effects as DNA damage, protein or lipid modification, etc. The basic reactions of nitric oxide can be divided as direct effect of the radical where it alone plays a role in either damaging or protecting the cell milieu and an indirect effect in which the byproducts of nitric oxide formed by convergence of two independent radical generating pathways play the role in biological reactions which mainly involve oxidative and nitrosative stress. Nitric oxide is also capable of directly interacting with mitochondria through inhibition of respiration or by permeability transition. Reaction of nitric oxide with metal ions include its direct interaction with the metals or with oxo complexes thereby reducing them to lower valent state. Excessive production of nitric oxide can be studied by inhibiting the synthetic pathway of nitric oxide using both selective or specific nitric oxide synthase inhibitor or non-selective nitric oxide synthase inhibitor with respect to isoforms of nitric oxide.

S. Habib · A. Ali (✉)
Department of Biochemistry, Faculty of Medicine,
Aligarh Muslim University, Aligarh 202002, India
e-mail: asifali_amu@rediffmail.com

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Introduction

The initial studies of the molecule (nitric oxide, NO) dates back to 1772, when Joseph Priestly called it "nitrous air," nitric oxide was first discovered as a colorless, toxic gas. Unfortunately, the tag of toxic gas and air pollutant continued until 1987, when it was shown to actually be produced naturally in the body. By 1987, nitric oxide's role in regulating blood pressure and relieving various heart ailments became well-established. Two years later, research revealed that nitric oxide is used by macrophages to kill tumor cells and bacteria. In 1992, nitric oxide was voted "Molecule of the Year". The importance of the molecule became front page news in 1998 when Louis J. Ignerro, Robert F. Furchgott and Ferid Murad were awarded the Nobel Prize for Medicine and Physiology for identifying nitric oxide as a signaling molecule. The discovery has opened up newer ways of treatment for millions of patients.

Nitric oxide (NO) plays an important role in the protection against the onset and progression of cardiovascular diseases. The cardioprotective roles of NO include regulation of blood pressure and vascular tone, inhibition of platelet aggregation and leukocyte adhesion, and prevention of smooth muscle cell proliferation. Reduced bioavailability of NO is thought to be one of the central factors common to cardiovascular disease, although it is unclear whether this is a cause of, or result of, endothelial dysfunction. Any disturbance in the bioavailability of NO

leads to a loss of cardio protective actions and in some cases may even increase disease progression [1].

NO is composed of an atom each of nitrogen and oxygen such that seven electrons from nitrogen and eight electrons from oxygen are involved to form an uncharged molecule (N≡O). The high reactivity of NO is not due to the fact that it contains an unpaired electron having a half life of 2–30 s. If this were the case, how would tissues survive in presence of molecular oxygen with two unpaired electrons at a concentration of 20–200 μM [2]. Nitric oxide only reacts with those biological molecules that have unpaired orbital electrons e.g., other free radicals or transition metal ions. Since most of the biological molecules have completely filled orbitals, it renders nitric oxide unreactive towards them [3]. The reactivity of NO depends upon its physical properties, such as its small size, high diffusion rate, and lipophilicity. Moreover, the reaction products of nitric oxide, i.e. the related species, also react with biological molecules and may have toxic effect as well [4]. At low levels, NO can protect cells; however, at higher levels, it is a known cytotoxin, having been implicated in tumor angiogenesis and progression [5].

The biological reactions of nitric oxide can be divided into three main pathways [6].

(a) Diffusion

Nitric oxide trespasses the cell membrane by simple diffusion and reacts with cellular components. Once inside the cell, it may react with non-heme iron or quench tyrosyl radical of ribonucleotide reductase which may lead to inhibition of DNA synthesis [7, 8].

(b) Auto-oxidation to form N_2O_3 (nitrous anhydride)

Nitric oxide combines with nitrogen dioxide to form nitrous anhydride as given under:

$$NO + NO_2 \longleftrightarrow N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+ \quad (1)$$

(c) Reaction with superoxide to form peroxynitrite

Reaction of nitric oxide with superoxide in biological media yields peroxynitrite which is not a free radical as the new bond formed involves the free electrons on NO and $O_2^{\bullet -}$. The peroxynitrite formed is a potent oxidant thus reacting with almost all biological molecules [9].

The peroxynitrite anion combine with carbon dioxide to form nitroso peroxycarbonate adduct, a known fastest reaction [10]. On decomposition it forms NO_3^- and CO_2.

$$ONOO^- + CO_2 \rightarrow \{ONO_2 \cdot CO_2^-\} \rightarrow NO_3^- + CO_2 \quad (2)$$

Nitric oxide also reacts with molecular oxygen. The reaction may take place in aqueous or in gas phase. Although the product of both phases is same but its stability differs. The rate of the reaction is second order with respect to NO and first order with respect to O_2 (Eq. 3) [11]. In gaseous phase, NO_2 is a stable product of NO oxidation. But in aqueous solutions, NO_2 give rise to NO, NO_3^- [4]. NO reacts with molecular oxygen, which is present in much higher concentration than nitric oxide, to form peroxynitrite [12].

$$2NO + O_2 \xrightarrow{\text{gas}} 2NO_2 \xrightarrow{\text{aqueous}} NO^- + NO_3^- \quad (3)$$

$NO^- + NO_3^-$ → (O_2) → $ONOO^-$

The nitroxyl anion (NO^-) is also said to be endothelium derived relaxing factor, which is reactive but a short lived species [13]. It reacts with two nitric oxides to form nitrite and nitrous acid [14, 15]. The reaction intermediate ($ONNO^-$) is quite similar to peroxynitrite. The only difference being that $ONNO^-$ will be one electron oxidant whereas peroxynitrite can be both one and two electron oxidant [2].

Direct Effect of NO

The kinetically fast reactions occurring physiologically are considered relevant [16]. NO does not react rapidly with amines or thiols but its reaction with metal complexes can be considered as relevant [16]. It reacts with metal complexes to form metal nitrosyls, e.g. Fe–NO complex which is quite stable. The radical is also capable of reacting with metallooxo as well as metal oxo complexes [16]. NO can also directly interact with hypervalent complex formed by agents such as H_2O_2 [17] and can reduce it to lower valency state.

$$Fe^{+(2,3)} + H_2O_2 \rightarrow Fe^{+(4,5)} = O + H_2O \quad (4)$$

$$Fe^{4+} = O + NO \rightarrow Fe^{3+} + NO_2^- \quad (5)$$

The presence of NO results in scavenging superoxide which besides preventing enzyme inactivation also converts any ferrous oxyadducts to active ferric state. It is also reported that at low concentration of NO, direct effects will predominate, while at higher concentration indirect effects mediated by $NO/O_2^{\bullet -}$ [16].

NO protects tissue from peroxide mediated damage by scavenging metal oxo species [18]. It has been shown to inhibit lipid oxygenase activity by reacting with non-heme iron at the active site [19]. A heme protein,

cyclooxygenase, involved in the conversion of arachidonic acid to prostaglandin, and other related enzymes is also influenced by NO radical reactions and metal –NO interaction [19]. A possible mechanism accounted for cyclooxygenase inhibition by superoxide involves the reduction of ferric form to the inactive ferrous state. It has also been reported that NO generation results in nitrosative reactions at nucleophilic centers resulting in the formation of *S*-nitrosothiols [20]. Excess production of NO has been shown to mediate glutamate induced neuronal toxicity in cortical and striatal neurons culture [21]. NO mediated apoptosis has also been reported in murine peritoneal macrophages [22]. NO also react with oxyhemoglobin and results in the formation of met-Hb and NO_3^-.

$$\begin{aligned} &\mathrm{Hb{-}Fe^{2+}(O_2) + NO \rightarrow Hb{-}Fe^{3+} + NO_3^-} \\ &\quad \text{Rate constant } \mathrm{H} = 3 \times 10.7\ \mathrm{M^{-1}\ S^{-1}} \end{aligned} \tag{6}$$

NO also interacts with deoxy-Hb and met-Hb by binding to heme ion center. The binding is hindered by a water molecule coordinated to heme Fe^{3+} atom is case of met-Hb and it has been reported that the association rate constant is 100 fold less for ferrous deoxy-Hb [23]. Besides these reactions, NO plays important roles in tackling diseases of cardiovascular system by improving blood supply to heart muscle [24]. The anti-impotence drug Viagra exploites the signalling role of NO and increases blood flow into the *corpus cavernosum* of penis. NO acts as a signaling molecule in smooth muscle cell and neurons. The effect is due to activation of soluble guanyl cyclase (SGC). The formation of guanosine 3′-5′ monophosphate (cGMP) from guanosine 5′-triphosphate is catalyzed by SGC which act as an intracellular messenger that connects the NO signal to the cellular response by activating specific protein kinases, phosphodiesterases and ion channels [9]. It has also been reported that binding of NO to heme moiety of soluble guanyl cyclase results in a pentacoordinate complex and the bond to the proximal histidine is lost [23] (Fig. 1).

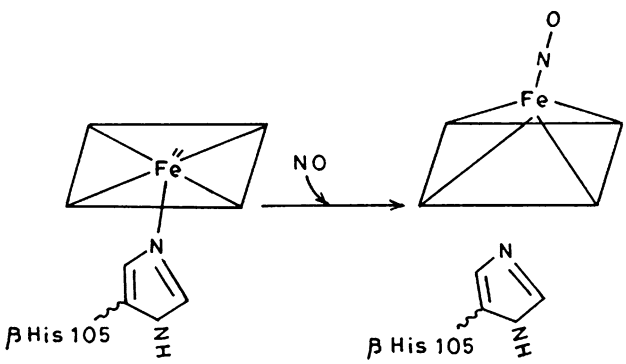

Fig. 1 Formation of pentacoordinate complex in heme moiety of guanyl cyclase

The production of nitric oxide in brain is very well established and it is quite different from other neurotransmitters like acetylcholine. The later, after the release from synapses, lasts for few milliseconds whereas NO persist for seconds, coupled with its rapid diffusion, enables it to encompass several million synapses [24].

The toxic effects of NO generally involve its oxidation products whereas NO alone is not capable of damaging DNA or ribosylate glyceraldehyde-3-phosphate dehydrogenase [25]. The radical can reversibly inhibit enzymes containing transition metal ions or free radical intermediate in their catalytic state [2]. NO in the micromolecular range can also reversibly inhibit cytochrome c oxidase [26, 27] which may result is leakage of superoxide from electron transport chain.

P53 is a protein involved in maintaining genome stability. Following exposure to DNA damaging agents rapid increase in p53 level occurs. Normally p53 has a short half life but DNA damage results in its accumulation in cells [28]. Following exposure to NO generating agents it has been shown that p53 is induced in both RAW 264.7 macrophages and RINm5F cells [29]. P53 accumulation proceed DNA fragmentation and hence apoptosis. NO inhibitors such as NMMA prevent both p53 accumulation and inducible NO generation thus leading to apoptosis [28]. NO mediates DNA damage by three mechanisms.

(a) Formation of nitrosoamines.
(b) Inhibition of DNA lesions repair systems which is also mediated by other genotoxic systems.
(c) Modification of DNA not directly by NO but by its oxidation products [30].

It has been reported that NO production is increased in patients with Systemic lupus erythematosus (SLE) [31]. The disorder is characterized by immune activated state where Inducible nitric oxide synthase (iNOS) level is increased in tissues like macrophages, splenic and renal tissues and consequently NO production [32]. Induction of iNOS occurs is response to cytokine production which is a non-specific event occurring in a wide variety of conditions like ulcerative colitis [33], psoriasis [34], arthritis [35], etc. Increased production of NO is not specific for SLE rather it represent an activated state of immune system.

To relate the role of NO in SLE, its site of production and quantum is of relevance. Patients with SLE showed upregulation of iNOS in normal appearing vascular endothelium. These endothelium also over express the soluble vascular adhesion molecules Intracellular cell adhesion molecule (ICAM-1), E-Selectin and Vascular cell adhesion molecule (VCAM) [36] as have been reported in active SLE [37]. Therefore, it appears that endothelium plays an active role in the localization and propagation of leukocyte and antibody-mediated inflammation [38]. Histological examination of ICAM-1 deficient mice revealed a

significant reduction in glomerulonephritis and vasculitis of the kidney, lung and skin [39].

Indirect Effect of NO

The indirect effect of NO involves the reactions between superoxide and NO which leads to the production of peroxynitrite, a powerful oxidant (rate constant—7×10^9 M^{-1} S^{-1}). Formation of $ONOO^-$ is governed by the relative amount of NO and superoxide produced and also on reaction of these radicals with other biological components. In presence of excess NO or superoxide, peroxynitrite gets converted to nitrogen dioxide, an inactive entity. An intracellular source of $ONOO^-$ is mitochondria where aerobic respiration results is production of superoxide and as NO concentration is higher in lipid layers than in cytosol [16], most $ONOO^-$ formed is in hydrophobic region. Its production is controlled by manganese, SOD as well as other antioxidants. It has also been reported that catechol-estrogens adducts or complexes are oxidized to quinones which can reduce oxygen to generate $O_2^{\bullet -}$ ion or in presence of NO releasing compound leads to the production of $ONOO^-$ [40]. Similarly, polyhydroxy aromatic compounds such as pyrogallol, and 1,4-hydroquinone autooxidize easily to form semiquinone radicals that react with dioxygen to generate $O_2^{\bullet -}$ which in combination with the NO releasing compound like SNP, DEA-NO, SPER-NO results in production of $ONOO^-$ [41] which is responsible for DNA damage (Fig. 2). The damaged DNA has been reported to be highly immunogenic, implicating its role in the production of antibodies in diseases like SLE and Cancer [42–44].

It has also been reported that quinone derivative of catechol-estrogen, which is produced by NO-mediated oxidation may form covalent adducts with nucleophilic groups of DNA [45]. Since human uterus and breast are site for hydroxylation of estrogens to form catechol-estrogens [46], therefore a possible mechanism of hormonal carcinogenesis associated with these organs can be related with increased production of 4-hydroxyestradiol [47–49].

The peroxynitrite can also influence protein and enzyme function, this occurs by nitration of tyrosine residues in tissues thus contributing to pathological dysfunction [9]. The formation of 3-nitrotyrosine is contributed by many nitrogen oxide species such as peroxynitrite, nitrogen dioxide, nitrous acid, nitronium ion, etc. NO alone is not capable of nitrating tyrosine [50]. In addition to this, another way to tyrosine nitration involve myeloperoxidase which is secreted by monocytes and polymorphonuclear neutrophils under inflammatory conditions. Myeloperoxidase catalyzes

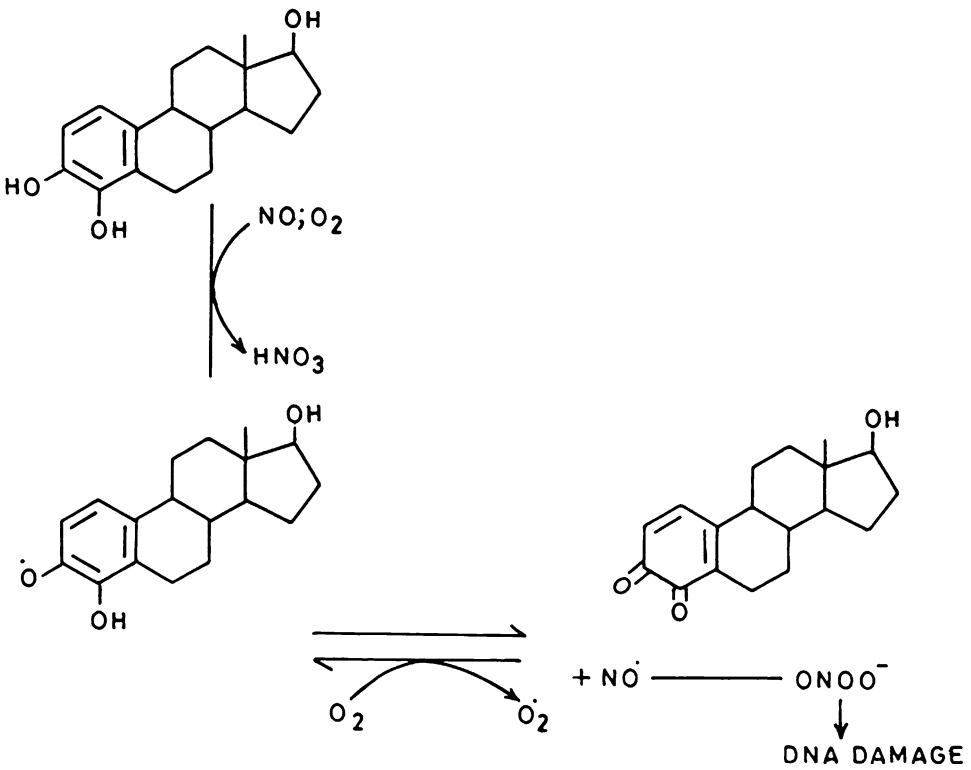

Fig. 2 Synergistic action of nitric oxide and catechol estrogen on DNA molecule

the formation of hypochlorous acid which is capable of reacting with NO_2^- to form NO_2Cl (nitryl chloride) which is a potent nitrating agent [51].

The nitrated product 3-nitrotyrosine is found in many disease states such as chronic inflammation, atherosclerosis, acute lung injury, etc. [52, 53]. Nitration of cardiac actin impairs contractile function of heart in myocarditis. Whereas, if human neurofilament gets nitrated it interferes in the filament polymerization in amylotrophic lateral sclerosis [2]. The signal transduction cascade that depends on reversible phosphorylation of tyrosine is also disrupted by tyrosine nitration. Thus the occurrence of nitrotyrosine-containing proteins in vivo should be regarded as a general indication of tissue damage induced by reactive nitrogen species such as peroxynitrite [54].

The indirect effect of NO can be further divided as oxidation and nitrosation. Those reactions in which reactive nitrogen oxide species (RNOS) donate NO to nucleophilic groups e.g. thiols and amines, lead to formation of nitrosonium adducts known as nitrosation reactions and condition termed as nitrosative stress. Whereas, when removal of electrons or hydroxylation reactions occurs, similar to those for reactive oxygen species (ROS), leading to oxidative stress, they are termed as oxidation reactions [55]. Both these reactions have different effects on biological systems (Fig. 3).

Activated macrophages derived NO and its oxidative metabolite, peroxynitrite were reported to play key notes is hepatocyte injury during inflammation and cause subsequent DNA damage is surviving hepatocytes. Stimulated macrophages in vivo express iNOS which produce high amount of NO and in turn RNOS, which is capable of mediating nitrosation of amines [56, 57], a condition encountered during chronic inflammation. Thus nitrosative stress under certain in vivo conditions can result in the formation of carcinogenic nitrosoamines [58, 59].

Both mammalian and bacterial cells when exposed to NO lead to deamination of guanine, cytosine and adenine

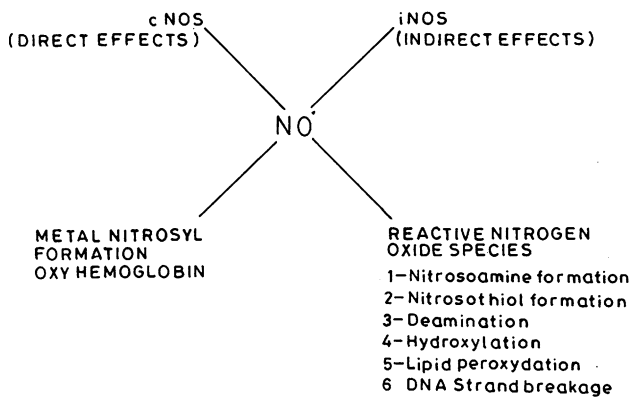

Fig. 3 Nitrosative and oxidative reactions of nitric oxide

via nitrosative chemistry. This would lead to the conversion of cytosine to uracil, guanine to xanthine, adenine to hypoxanthine, methyl cytosine to thymine [60].

DNA damage also occurs by RNOS formed under oxidative stress. This damage is primarily caused due to formation of $ONOO^-$ [61]. It has been reported that SIN-1, a donor which is capable of generating both NO and superoxide, oxidizes guanine to OH-dG [62]. Thus, there occurs a balance between oxidative and nitrosative stress [63]. It has also been reported that NO enhances the oxidative stress caused by H_2O_2 [64] and also reacts with other radicals such as lipid peroxides [2]. The endogenous production of NO leading to DNA damage generates both oxidative and nitrosative stress [65] (Fig. 4).

Nitric oxide can also play a protective role against oxidative damage by converting reactive species such as hydroxyl radical to less damaging and easily detoxified products [65]. Action of NO as pro- or antioxidant depends on relative production of NO and $O_2^{\bullet -}$ [66].

Anti-tumor Effect of NO

Though the direct or indirect reactions mediated by NO leading to modulation/damage to biological molecules have been summarised, the better side of NO should not be neglected. It acts as an immune effector generated by iNOS in macrophages, neutrophils, etc. in large quantities which kills or inhibits the growth of many pathogens including bacteria, fungi and parasites. NO is also capable of eliminating intracellular pathogens and blocking viral replication. It has also been reported that NO derived from leukocytes showed anti-tumor effect, it also upregulates tumor suppressor p53 gene [67]. Cells exposed to NO did not show appreciable mutation in p53 gene [68]. NO donors are capable of inhibiting angiogenesis, metastasis and tumor growth. Nitric oxide also inhibits DNA damage mediated by ROS and also inhibits hydroxylation reactions. Since high risk carcinogenic sites are those exhibiting prolonged expression of iNOS during chronic inflammation, NO generated from microphages, Kupffer cells, and NK cells is capable of inhibiting replication along with anti-tumor effect in the target cell [69, 70]. Murine embryonic liver cells, BNL CL.2, are capable of expressing iNOS in response to IFN-γ thus accumulating NO.

In humans few tumors are caused by viruses e.g. liver cancer is caused by hepatitis B virus, cervical cancer by human papilloma virus, Adult T cell leukemia by human T cell leukemia virus. Interferon γ is particularly important in limiting the spread of certain viral infections and capable of expressing iNOS. Increased concentration of NO in or near the target cell may act as tumoricidal since NO is capable of eliminating intracellular pathogens and blocking

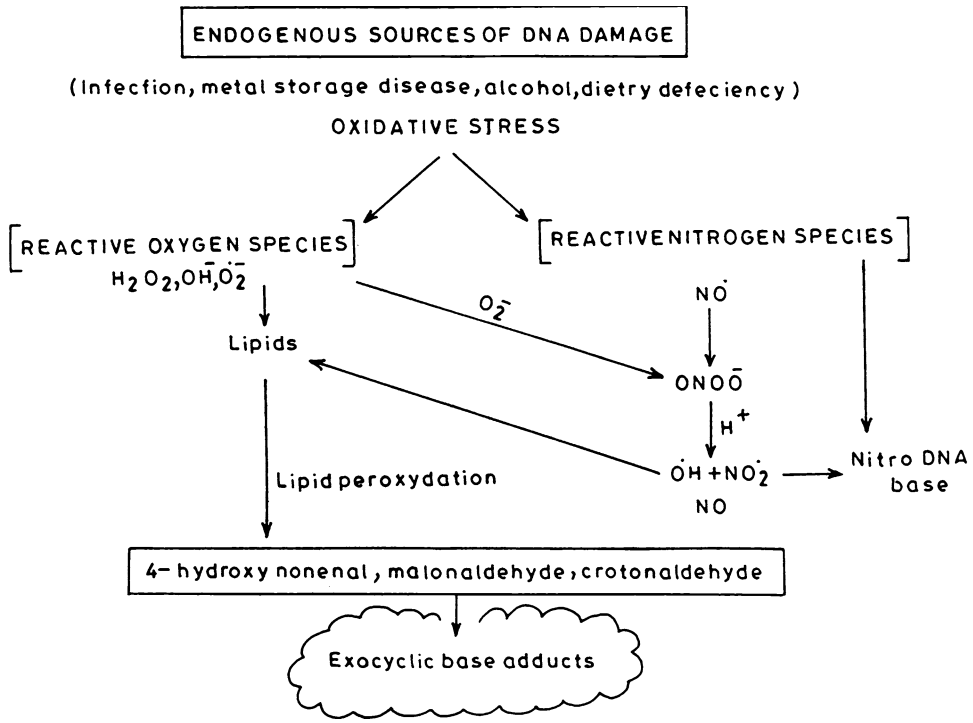

Fig. 4 Endogenous sources of DNA damage leading to oxidative stress

viral replication [71]. Nitric oxide is capable of protecting cell from apoptosis or mediating apoptosis depending upon the cell type e.g. NO protects rat ovarian follicles from atretic generation on one hand while on the other it induces apoptosis in tumor cells like in mastocytoma, sarcoma, melanoma [16]. NO is also reported to protect tissue form peroxide mediated damage by scavenging metallooxo species which are formed by oxidation of metal species or metal–oxygen species by H_2O_2 [72]. It is also been reported that animal subjects having tumorous growth acquire the ability through which their tumour tissues suppress the expression of iNOS and thus reduce the concentration of NO [73, 74]. Tumor growth is enhanced by accelerated angiogenesis by down regulating the production of vascular endothelial growth factor which is the mediator of angiogenesis [75]. Nitric oxide is also capable of suppressing metastasis by reducing intracellular stores of GSH [76, 77] or by blocking the adhesion of tumor cells to venular side of microcirculation [78]. It has also been reported that NO produced in vasculature of brain limits the spread of colon cancer to the brain [79]. Liver endothelial cells produce NO which curbs the metastases of melanoma cells to the lungs [80]. Though excessive production of NO is associated with tissue injury, it has been reported that endothelial NO production plays protective role in microvasculature [81]. NO is also reported to inhibit platelet aggregation and it reduces platelet adhesion to endothelial monolayers [82]. The defensive properties accounting for beneficial effect of NO in IL-2 induced injury include protection against tissue injury in myocardial ischemia reperfusion and adult respiratory distress syndrome [83, 84].

It is also reported that when cells were exposed to NO it resulted in DNA single strand breaks [85]. However, when purified DNA was exposed to NO at concentrations as high as 1.0 M, single stand breaks were not observed [86]. Nitric oxide is also reported to protect DNA against oxidative stress by inhibiting Fenton reaction of hydrogen peroxide which leads to single strand generation [87]. It has also been reported that NO in presence of a flavonoid having orthotrihydroxy group namely epigallocatechingallate or quercetogelin inhibits DNA damage by preventing formation of 8-nitroguanine in calf thymus DNA.

Pro-tumor Effect of NO

Carcinogenesis can be defined as a malignant transformation of a cell or group of cells. This process can be divided broadly into two stages, initiation and promotion. The initiation phase involves an irreversible modification of the genetic material of the cell caused by single exposure to any carcinogenic agent whereas promotion requires multiple exposures to the promoters to alter gene expression and produce a tumor. The promotion stage of carcinogenesis is irreversible. Cancer has multifactorial aetiology

which includes genetic and environmental factors. The common environmental factors are tobacco chewing, smoking, high fat diet, contaminants, also the occupational exposures to agents like, arsenic, cadmium, chromium, etc. Other environmental factors include sunlight, radiation, pesticides, steroidal medications, etc. It is well known that most of the exogenous carcinogens act via production of reactive oxygen or reactive nitrogen species. The reactive oxygen species are generated both physiologically and pathologically in mammals and induce many kind of cellular damage [88] including DNA damage [89]. Since DNA plays a central role in information transfer, attention has focussed on oxidative damage as significant source of mutations that lead to cancer and other human pathologies [90]. The possible role of ROS modified human DNA in cancer has been indicated from our studies and it has been found that binding of circulating antibodies in cancer sera was much stronger with ROS modified DNA than native DNA. ROS modified DNA has been shown to be a better inhibitor of naturally occurring antibodies in majority of cancer patients than native human DNA [91], reiterating the enhanced recognition of ROS-DNA.

Excess production of NO has been linked to endogenous human carcinogenesis. Induction of apoptosis by NO has also been observed in culture of macrophages and pancreatic β cells. Macrophages exposed to nitric oxide exhibited typical morphology and showed DNA fragmentation indicating apoptotic cell death [28]. NO also induced cell death and showed toxic effects in two different cell lines, viz., CHO-AA8 (Chinese hamster ovary cells) and TK6 (human lymphoblastoid cells) highlighting the role of NO in the onset of mutagenesis and cell death and the involvement of these processes in cancer and inflammatory diseases [92]. Nitric oxide also showed genotoxic effects by abolishing cell growth which is a valuable parameter sensitive to different kinds of damage, e.g. membrane damage, energy depletion, organelle damage and enzyme release, etc. [93]. Peroxynitrite is short lived with $t_{1/2} < 1$ s. and is capable of oxidative damage of wide range of biological molecules e.g. nucleic acids, lipids, thiols, etc. [94]. At pH 6.8 its conjugate acid ONOOH can diffuse through membranes and cause damage at a distance from its site of synthesis [95].

It is well known that solid tumors require tumor angiogenesis for their growth i.e. the tumorous cells should be well supplied with oxygen, nutrients and growth factors. Another important feature of malignancy is enhanced vascular permeability which is regulated by endothelial cell production of vasoactive substances. The endothelial cells synthesize NO by Endothelial nitric oxide synthase (eNOS) which helps in vascular permeability and relaxation [96]. Various tumours over express NOS [97, 98]. A study between the relationship of malignancy and eNOS expression in endothelial cells of tumor vessels showed that astrocytic tumor vessels possess higher level of nitric oxide than do normal vessels and found that there was significant correlation with the proliferative potential and eNOS expression in tumor vessels [99]. Several studies have suggested that NO and its reactive derivatives e.g. $ONOO^-$ were found to be elevated in infection and inflammation and plays important role in carcinogenesis [100, 101]. The carcinogenic effect of NO may be due to its cytotoxic potential which lead to reduced cell viability [93]. As stated earlier, NO per se is not capable of reacting with biomolecules; only its reaction products lead to the production of RNOS e.g. N_2O_3 or $ONOO^-$ which can result in DNA lesions.

The type of mutation under oxidative and nitrosative stresses differ [102]. Oxidative stress mediate DNA damage by the formation of peroxynitrile. It has been shown that DNA strand breaks were generated when plasmid DNA was incubated with NO-donor compound and polyhydroxy aromatic compounds. On autooxidation of polyhydroxy aromatic component produce semiquinones which react with dioxygen to generate $O_2^{\bullet -}$

Nitrosative stress leads to the formation of nitrosoamines e.g. RNOS generated from acidic nitrite is potentially carcinogenic in stomach [103, 104]. Nitrosamines are formed under conditions of inflammation which can lead to cancer [100]. NO also enhances tumor production by increasing the production of prostaglandin, PGE2 which increases the permeability of tumor vasculature and thus facilitate angiogenesis. Further tumor growth is supported by increased uptake of nutrients [105, 106]. Cells lacking Cu, Zn–SOD are reported to be more susceptible to NO and $ONOO^-$ [107, 108]. It has been reported that large amount of $ONOO^-$ causes necrosis where as small amounts produce apoptosis [109]. Nitric oxide also causes cellular energy deficit through DNA damage [110]. NO inhibits ribonucleotide reductase which results in impaired DNA synthesis as deoxyribonucleotides are no more available [111]. Since $ONOO^-$ causes DNA breaks this leads to activation of poly (ADP-ribose) polymerase to repair the damaged DNA [112, 113]. The activated PARP transfers about 100 ADP ribose moieties from NAD^+ to nuclear proteins e.g. histones and PARP itself [112, 114]. ADP ribose polymers thus formed are degraded by glycohydrolases followed by NAD^+ resynthesis which is a futile cycle and depletes ATP [114].

Reactive nitrogen oxide species have high affinity towards amino acid with thiol groups and therefore are capable of inhibiting enzymes having thiol residues required for their function [115] e.g. DNA alkyl transferases, required for repair of DNA lesions induced by alkylation, are inhibited by nitrosating its thiol residue in the active site [116, 117]. Other DNA repair enzyme inhibited by RNOS is DNA ligase. T4 DNA ligase contains

lysine in partially deprotonated state which is nitrosated by NO [30]. Other DNA repair enzyme inhibited by nitrosating thiols in formamidopyrimidine glycosylase which has zinc finger motif required for its activity. Upon nitrosation, Zn is removed resulting in enzyme inactivation [118].

Effect of NO on Mitochondria

Interaction of NO with mitochondria occurs is many ways. NO synthesis may occur in organelle itself or NO produced outside may diffuse inside. NO may affect mitochondria by three main pathways.

(a) Inactivation of mitochondrial enzymes which is irreversible
(b) Induction of mitochondrial permeability transition
(c) Inhibition of respiration which is reversible [110].

Therefore, effect on mitochondria mediated by NO occurs both by irreversible and reversible means. It has been shown that NO is capable of nitrosating critical thiol residues on creatinine phosphokinase which is irreversible and de-energizes mitochondria thus disrupting its ATP supply [119–121]. Nanomolar concentrations of NO are capable of reversible binding to heme as of cytochrome c oxidase [122]. This direct interaction of NO with cytochrome c is due to cNOS inhibition of mitochondrial respiration [26, 123]. Under aerobic conditions it has been shown that electron transport chain participate in forming superoxide which can then react with NO to form $ONOO^-$ [123]. In addition, $ONOO^-$ formed outside mitochondria diffuses into the matrix. The increased concentration of $ONOO^-$ inactivates Mn–SOD resulting in increase of $O_2^{\bullet -}$ level and hence activating a destructive cascade of NO [124] which includes:

(1) Irreversible damage of enzymes of citric acid cycle e.g. aconitase, iron-sulphur centers, etc. [125, 126].
(2) Inhibition of glycolysis by inactivating glyceraldehyde-3-phosphate dehydrogenase thus impairing ATP synthesis.
(2) Under inflammatory conditions, NADH:ubiquinone oxidoreductase (complex I) and succinate:ubiquinone oxidoreductace (complex II) are irreversibly inhibited by NO [127].

It has been found that NOS is present in mitochondria and that under normal conditions, production of NO is well regulated [2]. Induction of mitochondrial permeability transition is mainly caused by $ONOO^-$ which oxidizes thiols and NADPH of mitochondria and induces Ca^{2+} efflux [128, 129] along with oxidative efflux. As a consequence, Ca^{2+} homeostasis is disrupted [130, 131]. Mitochondrial decrease in membrane potential in induction of permeability transition leads to increase in cytoplasmic Ca^{2+} [131, 132]. This permeability transition is accompanied by the formation of a protein pore in mitochondrial inner membrane resulting is the leakage of its contents [131, 133].

Current evidence indicates that irreversible modification of iron binding cysteine residues releases metal and disrupts FeS structure. This is caused by $ONOO^-$ rather than by NO [126, 134]. Peroxynitrite damages membranes by hydrogen abstraction from polyunsaturated fatty acids which results is lipid peroxidation [135]. $ONOO^-$ also consumes biological antioxidants [136] enhancing the chances of mitochondrial damage.

The role of NO in membrane damage and necrosis was also demonstrated and the leakage of lactate dehydrogenase in TKG and CHO-AA8 cell lines both treated with NO has been observed. However, no LDH leakage was observed before 24 h in TK6 cell and 48 h in CHO-AA8 cells [137]. It has been reported that inactivation of mitochondria by $ONOO^-$ in vitro is remarkably similar to the respiratory inhibition observed in cultured tumor cells caused by activated macrophages [138]. If the internal nitric oxide concentration approaches that of mitochondrial superoxide dismutase the superoxide is reported to largely react with the nitric oxide and form peroxynitrite that would injure mitochondria irreversibly [139, 140].

Interaction with Metal Ions

Nitric oxide is capable of directly reacting with metal complexes or oxo complexes. The product of reaction is metal nitrosyls. Reaction of NO with metals may be categorized as:

(A) Direct reaction of NO with metals
(B) Reaction of NO with dioxygen metal complexes
(C) Reaction of NO with oxo-complexes.

The reaction of nitric oxide to form metal nitrosyls is involved is both regulatory and cytotoxic actions [141]. Nitric oxide reacts into oxyhemoglobin. (oxy-Hb) to form met-Hb and NO_3^- (Eq. 7). The reaction is second order having a rate constant of $3 \times 10^{-7}\ M^{-1}\ S^{-1}$.

$$Hb{-}FeII\ (O_2) + NO \rightarrow Hb{-}FeIII + NO_3^- \quad (7)$$

A variety of cellular components present inside the cell e.g. cytochrome c oxidase and cytochrome P450 is able to bind with NO which may result in inhibition of mitochondrial respiration. Since nitric oxide synthase is also present in mitochondria, it may increase the production of NO under inflammatory stressed state. NO has great affinity for iron and heme proteins due to the presence of porphyrin ligands [141]. Reactions of NO with zinc and

copper containing proteins have also been reported, an important class of DNA binding protein induce zinc finger motifs [142]. Since zinc finger motif contain two to four cysteine residues and up to two histidine residues e.g. formamidopyrimidine-DNA glycosylase (Fpg protein). The protein repairs oxidative damage to guanine. NO under aerobic conditions inhibits Fpg [30]. Another example of reaction of NO with metal centers is reaction of soluble guanyl cyclase which is activated by binding of NO to ferrous heme iron resulting in generation of cGMP and transduction of NO signal [141].

Nitric oxide forms iron nitrosyl complexes by binding to iron-sulfur clusters e.g. NO is capable of inactivating aconitase due to its direct interaction with the enzymes heme iron nitrosyl complex [9]. These reactions of NO with metals generally involve covalent interactions. In addition, various metal oxygen complexes e.g. reaction of NO and oxyhemoglobin to form met-hemoglobin and nitrate (Eq. 7) also occur. This is one of the primary detoxification mechanisms of NO.

Nitric oxide also rapidly reacts with hypervalent metal complexes i.e. metallooxo species which are formed by the oxidation of metals or metal oxygen complexes by e.g. H_2O_2 [30]. Nitric oxide reacts with these hypervalent species and results in conversion of hypervalent complex to lower valent state thereby scavenging the metallooxo species and protecting cells from peroxide mediated damage [18]. Formation and scavenging the metallooxo species can be presented as under:

$$Fe^{(2,3)} + H_2O_2 \rightarrow Fe^{(4,5)+} = O + H_2O_2 \quad (8)$$

$$Fe^{4+} = O + NO \rightarrow Fe^{3+} + NO_2{}^- \quad (9)$$

The high reactivity of NO with diverse chemical groups and with transition metal ions is used as a guide line for designing drugs used as NO scavengers [143]. Many coordination complexes that are Ruthenium complexes are proposed as efficient NO scavengers e.g. JM-1226 a Ruthenium coordination complex has been shown in the recovery of rat from sepsis, another NO scavenger used is 2 Phenyl-4,4,5,5 tetramethylimidazolin-1-oxyl-3 oxide (PTIO).

NOS Enzymes

Biosynthesis of nitric oxide takes place by the conversion of L-arginine to L-citrulline catalyzed by nitric oxide synthases. The enzyme has three isoforms. NOS1 or neuronal nitric oxide synthase (nNOS). NOS2 or inducible nitric oxide synthase (iNOS) and NOS3 endothelial nitric oxide synthase (eNOS). Among these, the first isoform has been purified and cloned [30]. Each isoform is a product of distinct gene [144]. Broadly speaking isoforms of nitric oxide synthase may be categorised as constitutive (cNOS) and inducible nitric oxide synthase (iNOS). Constitutive NOS is calcium dependent and continuously present whereas iNOS is Ca^{2+} independent and is expressed only after cytokine exposure [145]. Based on this category nNOS and eNOS are constitutively expressed and require elevated levels of Ca^{2+} along with activation of calmodulin to produce NO for brief period of time [146]. iNOS is induced by cytokines in almost every cell and generates locally high level of NO for prolonged periods of time [30]. It is mainly expressed in macrophages, neutrophils and epithelial cells. It has also been reported that in mice, estrogen and progesterone-induced iNOS expression is different in different cell population i.e. estrogen induces it in myometrial mast cell whereas progesterone induces it in epithelial cells.

It is also reported that cNOS are employed in physiological regulation within cardiovascular and nervous system where they produce low concentration of NO—1.0 μM in cardiovascular and 5 μM in neurological systems whereas iNOS produce NO achieving concentrations equal to 10 μM [141].

Inducible NOS

This is type 2 NOS and is induced by inflammatory stimuli e.g. cytokines or lipopolysaccharides. It is mainly expressed is macrophages and possess tightly bound calmodulin [141]. It is also reported that iNOS is not only present is activated macrophages but its synthesis can be induced in glial cells, liver and cardiac muscle.

Endothelial NOS

It is constitutively expressed in endothelial lining of blood vessels and depends on Ca^{2+}. The NO produced by eNOS diffuses into smooth muscle cells of blood vessel and elicits cGMP dependent smooth muscle relaxation and thus increasing blood flow.

Neuronal NOS

Neuronal nitric oxide synthase is constitutively expressed in post synaptic terminals of neurons and is Ca^{2+} dependent. It is activated by Ca^{2+} influxes caused by binding of neurotransmitter glutamate, to receptor in cell membrane. nNOS is also activated by membrane depolarization through opening of voltage gated Ca^{2+} channels [9].

The synthesis of NO by nitric oxide synthases takes place by conversion of L-arginine to L-citrulline via the formation of N^G-hydroxy-L-arginine (Fig. 5). Therefore, NOS utilize L-arginine, NADPH and oxygen to produce nitric oxide. Nitric oxide synthase enzyme is an heme iron

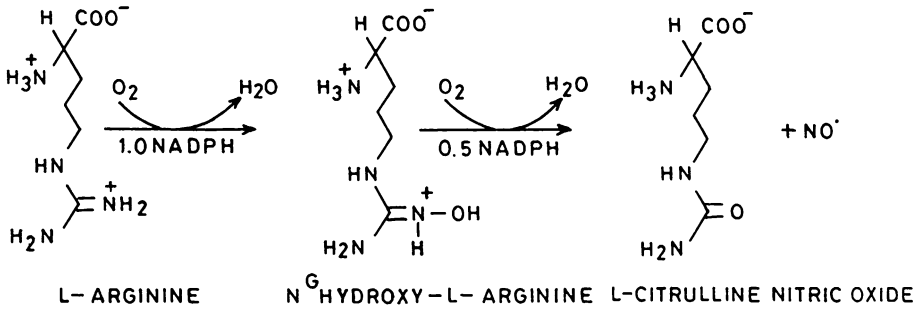

Fig. 5 Synthesis of nitric oxide from L-arginine

dependent tetrahydrobiopterin enzyme where tetrahydrobiopterin binds far away and on the wrong side of porphyrin to act as hydroxylating cofactor at distal side of heme–iron center [9]. Other enzymes that use tetrahydrobiopterin are e.g. phenyl monooxygenase, tyrosine-3-monooxygenase, tryptophan-5-monooxygenase, etc. In these cases the coenzyme is directly involved in hydroxylation of substrate and which then gets oxidized to dihydrobiopterin. The dihydrobiopterin is recycled to tetrahydrobiopterin by dihydropteridine reductase.

Nitric oxide synthase enzyme in its active form is a homodimer where each subunit is composed of C-terminal reductase and N-terminal oxygenase domain. It is also reported that the isolated oxygenase domain remain homodimeric whereas isolated reductase domain is monomeric indicating that the two subunits are joined by their oxygenase domains. The oxygenase domain contains a heme group and one binding site for tetrahydrobiopterin and L-arginine. Whereas, the reductase domain of nitric oxide synthase has binding sites for either of the one molecule of Flavin mononucleotide (FMN), Flavin adenine dinucleotide (FAD) and NADPH [144]. Between the reductase and oxygenase domain there is a binding site for calmodulin. In case of nNOS and eNOS only Ca^{2+}-calmodulin complex can activate the enzyme whereas is case of iNOS, it is already bound to calmodulin and is fully active. Calmodulin is reported to improve electron flow from NADPH to flavins and also facilitates electron transfer from FMN to heme [8]. The flow of electrons in nitric oxide synthase domains are represented in Fig. 6.

Inhibitors of NO

In order to study the over production of NO, inhibitors of NOS have been synthesized and investigated for their probable role in controlling the over production of NO under disease condition. Since L-arginine is a naturally occurring substrate for NO, analogs of L-arginine have been used as competitive NOS inhibitors. These inhibitors were non-selective with reference to isoforms of nitric oxide synthase e.g. *N*-monomethyl-L-arginine and nitro-L-arginine methyl ester [143]. Among the synthetic analogs of L-arginine only homo L-arginine and agmatine are active (Fig. 7).

It is reported that removal of α-aminopentanoic acid part of N^G methyl-L-arginine (L-NMA), N^G amino-L-arginine

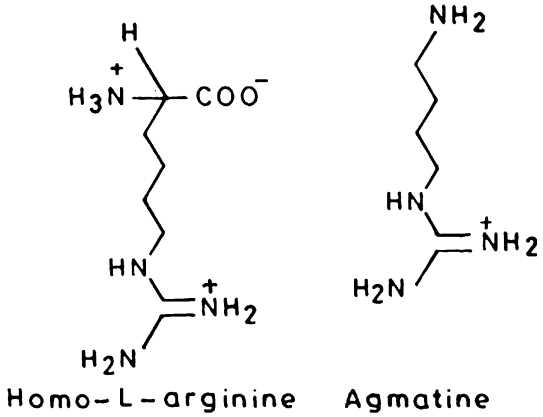

Fig. 7 Competitive inhibitors of nitric oxide synthase enzyme

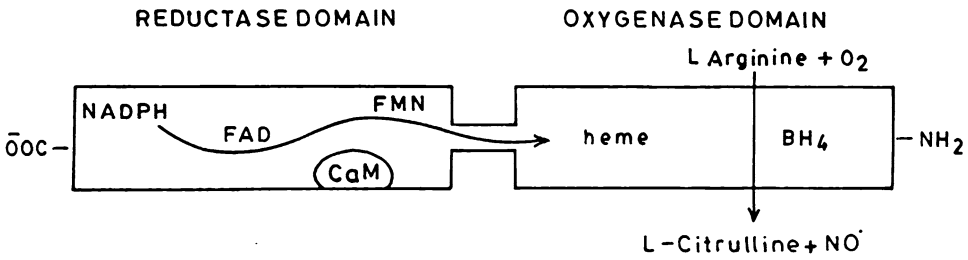

Fig. 6 Flow of electrons in nitric oxide synthase domain from FMN to heme

Table 1 Characteristics of NOS inhibitors

Inhibitor	Specificity	Clinical importance
L-NIL	iNOS	Effective in arthritis and leishmaniasis
S-methylisothiourea, 4-amino-tetrahydrobiopterin	iNOS	NOS is most sensitive towards inhibition during de novo protein synthesis
Galaxo-Wellcome compound 1400 W	iNOS	Rapid reversibility and low potency Ki $\approx$ 2–50 μM
1-Amino-*S*-methylisothiourea	iNOS	–
7-Nitroindazole	nNOS	Experimental stroke
Astra Arcus Compound ARL 17477		Infracts caused by temporary occlusion of middle cerebral artery in rats

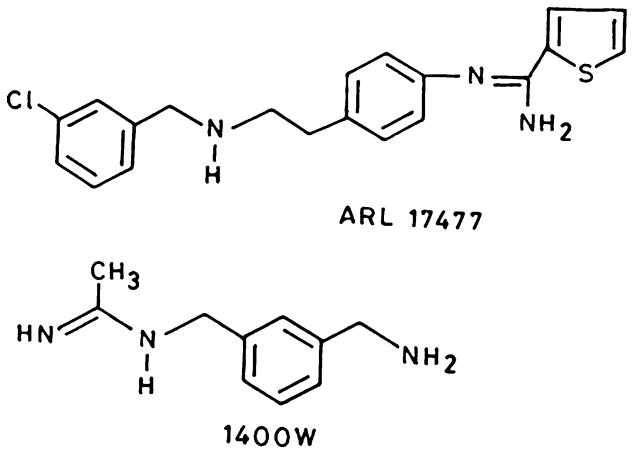

Fig. 8 Specific inhibitors of neuronal nitric oxide synthase

(L-NAA) and N^G nitro-L-arginine (L-NNA), also the analogs of L-arginine, results in the formation of guanidine which looses binding affinity [9]. Some of the analogs of L-arginine also exhibit irreversible inactivation of NOS in presence of oxygen and NADPH e.g. L-NMA, N^{ε} iminoethyl-L-Lysine (L-NIL), N^8 iminoethyl-L-ornithine (L-NIO) i.e. inhibition is mechanism based.

Along with the non-specific inhibitors of NOS, many isoform specific inhibitors are also available. e.g. 7-nitroindazole and aminoguanidine are inhibitors of nNOS and iNOS, respectively [143]. The importance of these isoenzyme specific inhibitors is that they inhibit selective NO biosynthesis rather than suppressing whole NO biosynthesis (Table 1). L-NIL is an exception with 30 folds more specificity towards iNOS [147]. Among these inhibitors Galaxo Wellcome compound 1400 w (*N*-(3-aminomethyl) benzyl) acetamide) is most selective iNOS inhibitor and Astra Arcus compound ARL 17477 is most selective for nNOS (Fig. 8).

In higher vertebrates nitric oxide has key roles in maintaining homeostasis and in vascular smooth muscle, neurons and the GI tract. It has a definite role in regulating all aspects of our lives from walking, digestion, sexual function, pain perception and pleasure, memory recall and sleeping. Finally, the way it continues to function in our bodies will influence how we degenerate with age. It has a likely role in our deaths through cardiovascular disease, stroke, diabetes and cancer. Our ability to control NO signaling and to use NO effectively in therapy must therefore have a major bearing on the future quality and duration of human life [148].

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