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# CURRENT TRENDS IN NITRIC OXIDE RESEARCH

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**Abstract –** Nitric oxide (NO), a molecule with multidimensional effects has generated exponential amount of research since its identification as a biological messenger almost two decades back. The recent trend in NO research is to explore newer dimensions in the cellular and molecular mechanisms of actions and interactions of NO with various biomolecules and their implications in various pathophysiological states. Advances in our knowledge of the mechanisms by which this pleiotropic molecule regulates the expression of eukaryotic genes has generated considerable excitement and is paving the way for development of novel NO based therapeutic strategies. However, it is still a challenge to understand fully the paradox of beneficial and damaging effects of this exciting molecule. This review will discuss the current trends of research in this area especially highlighting the new insights gained from recent experimental and clinical studies. New approaches to reduce or augment the availability of NO to benefit a wide range of clinical conditions and avenues for future research are also briefly discussed.

**Key words**: Nitric oxide, Cellular and Molecular mechanisms, Stress, Seizures, Therapeutic prospects

## INTRODUCTION

Nitric oxide (NO) is a simple, unique, yet multifunctional molecule involved in the regulation of a wide variety of physiological functions and pathophysiological processes. In 1980, Furchgott and Zadawski first showed that vasorelaxation evoked by acetylcholine was endothelium dependent and the term *endothelium-derived relaxing factor* (EDRF) was coined (36). The discovery of EDRF caused an explosion in biomedical research but it was not until six years later that it was shown that EDRF was actually NO. Later in 1987, Furchgott, Ignarro *et al* and Palmer *et al*. all independently showed that EDRF was actually NO. This started a wave of experimental and clinical research in different areas in medicine and NO was recognized as a startling advance (34, 36, 38). The biological activities of NO are numerous and complex, and it is now known that NO plays a fundamental role at the very beginning of life when NO synthase (NOS) activity in male gametocytes is required for the activation of eggs immediately after insemination (25). The significance of this ubiquitous molecule in biology has been highlighted in 1992 by the prestigious Science magazine, which assigned NO as the 'molecule of the year' and phrases like "NO news is good news" were coined. The importance of NO research became universally known when Robert Furchgott, Louis Ignarro and Ferid Murad were jointly awarded with the Nobel Prize in 'Physiology or Medicine' in 1998 for their pioneering work in this field. The role of NO as a neurotransmitter was also established in the non-adrenergic non-cholinergic (NANC) neurons. The therapeutic significance of NO lies in the fact that organic nitrates exert their beneficial effects in angina and heart failure through NO (30) and the use of sildenafil in male erectile dysfunction stems from its NO modulatory effect. It is now clear that NO is a fundamental component of basal metabolism and cellular function and is implicated in numerous pathophysiological fields including aging, apoptosis, diabetes, inflammation, ischemic preconditioning, neurodegenerative pathology etc.(6). Recent cellular and molecular studies suggest that NO may play a more diverse role in a variety of biological systems than it was initially assigned (38). This paper will focus on some of the current areas of nitric oxide research, recent understanding on the novel actions of this molecule, state of the art in developing drugs that works through NO and speculate on future research directions.

### *Classical and novel actions of nitric oxide*

Classically NO acts as a physiological messenger activating soluble guanylate cyclase(sGC) by binding to the haem group/moiety of the enzyme (36) . The secondary increase in cyclic GMP (cGMP) further activates cyclic GMP dependent protein kinase and causes phosphorylation of respective protein kinases (myosin). This phosphorylated myosin kinase has

reduced afiinity for $Ca^{2+}$-Calmodulin complex and there is less efiicient phosphorylation of proteins. This in turn results in reduced smooth muscle contraction and causes reduction in muscle tone (36). Such effects explain NO effects on vascular, bronchial and gastrointestinal smooth muscles. In some cases NO also causes vasorelaxation by $K^+$ channel activation and hyperpolarisation. The effects of sGC are prevented by inhibitors of guanylate cyclase (ODQ), which are useful investigational tools in this regard. Effects of cGMP are terminated by phosphodiesterase enzymes, and zaprinast and sildenafil are inhibitors of phosphodiesterase type V enzyme. NO mediates other effects apart from those via sGC activation (9), for example : (a) many proteins containing haem or metal centers can constitute the target of NO that thus modulate their functions (eg.cytochrome oxidase, aconitase, ribonucleotide reductase etc.); (b) NO may act as a messenger by reacting with cysteine groups of different proteins and modifying their effects through S-nitrosylation; (c) NO can react with reactive oxygen species (ROS), regulating their intracellular concentrations and/or producing peroxynitrite (ONOO-), a highly reactive moiety that is capable of actively interacting with a variety of biomolecules ( proteins, lipids and DNA). Since NO is labile , direct measurements are difficult and studies on NOS provide information about NO activity. NADPH diaphorase is a widely used marker for NOS and this has shown that NOS is distributed widely in different tissues. The constitutive variety (cNOS) is mainly present in the endothelium, platelets, renal mesangial cells and osteoblasts/osteoclasts. In the CNS , NOS (nNOS) is present in cerebellum, hippocampus, olfactory lobes and in the PNS in GIT, respiratory tract, adrenal glands etc. NO is considered as a neurotransmitter in the noradrenergic noncholinergic (NANC) neurons. Inducible NOS (iNOS) is generated by macrophages, lymphocytes and neutrophils during inflammatory and immunological reactions.

### *Nitric oxide research: recent trends*

In recent years research in nitric oxide field has been enormously expanded, and several newer aspects of the role of NO in bioregulatory and pathophysiological processes have emerged.

### *Nitric oxide and gene expression*

Currently available data illustrates that NO has multiple molecular targets. It can directly influence the activity of transcription factors, and modulate upstream cascades, mRNA stability and translation, as well as processing of the primary gene products (4). However, to date, there is no report for the existence of DNA elements within the promoter region of eukaryotic genes that respond directly to NO. A growing body of evidence suggests that post translational modification of transcription factors serve a regulatory role on gene transcription particularly after changes of redox state of the cell. Zinc fingers are most prevalent transcription – DNA binding motif. As NO is able to S-nitrosate thiols of zinc finger clusters leading to their reversible disruption, this provides a molecular mechanism for the regulation of transcription of genes (24). NO has been found to have both positive and negative influences on gene regulation by hypoxia (1). Experiments performed on rat PC-12 cells using spermine NO (a NO donor) suggest that hypoxia induced cfos mRNA expression and promoter activity were significantly potentiated in presence of sNO whereas this NO donor significantly inhibited tryptophan hydroxylase mRNA and promoter expression during hypoxia.

### *Nitric oxide and mitochondrial function*

Research reports from different laboratories suggest that nitric oxide might play an important role in regulation of mitochondrial function particularly under low oxygen concentrations. There is now growing evidence that NO can be produced within the mitochondria themselves. NO can influence respiratory activity both through direct effects on respiratory chain or indirectly via modulation of mitochondrial calcium accumulation (43). At pathological concentrations, NO can cause irreversible alterations in respiratory function and also interact with reactive oxygen species to form reactive nitrogen species, which can further impair mitochondrial respiration and even lead to opening of mitochondrial permeability transition pores and cell death (43). NO has also been shown to protect mitochondria of anterior pituitary cells and prevent cadmium induced cell death by reactive oxygen species (50). Rintoul *et a*l (55) recently studied the effect of NO on mitochondrial movement and morphology in primary cultures of forebrain neurons using mitochondria targeted enhanced yellow fluorescent protein and concluded that NO is a

novel modulator of mitochondrial trafficking which may act through the inhibition of mitochondrial function. Moncada and coworkers showed that at physiological concentrations NO inhibits mitochondrial enzyme cytochrome-c-oxidase (complex IV) in competition with oxygen. The findings suugested that endogenous NO modulates oxygen consumption under basal and stimulated conditions. Exposure to exogenous NO also suppressed mitochondrial respiration which results in a decrease in intracellular glutathione (GSH) and oxidative stress by mitochondrial free radical generation. This effect was shown to be reversed by GSH and cold light - suggesting that S-nitrosylation of thiols may be involved. Inhibition of respiratory chain can also result in increased superoxide and subsequently peroxynitrite generation (46).

### *Nitric oxide and heat shock proteins*

Heat shock proteins (hsp) are highly conserved intracellular proteins that are upregulated following a wide range of noxious stimuli and their overexpression is associated with cellular resistance to a variety of insults. In brain glial cells, expression of calcium independent NO synthase (NOS-2) is induced following stimulation with bacterial endotoxin (lipopolysaccharide (LPS)) and/or pro-inflammatory cytokines. Studies suggest that hsp70 suppresses astroglial-inducible nitric-oxide synthase expression by decreasing NF kappa beta activation (12). In a similar study it was found that hsp inducer, sodium arsenate, inhibited iNOS expression and attenuated vasoplegia and hypotension in endotoxin challenged rats. In the rostral ventrolateral medulla, the 70-kDa heat shock protein (hsp70), but not hsp90, confers neuroprotection against fatal endotoxemia via augmentation of NO synthase I (NOS I)/protein kinase G signaling pathway and inhibition of NOS II/peroxynitrite cascade. Song *et al* (59) has shown that hsp90 augments neuronal nitric acid synthase activity by enhancing $Ca^{2+}$ /calmodulin binding.

### *Nitric oxide and cell adhesion molecules*

Cell adhesion molecules are proteins located on cell surface involved with binding with other cells or with extracellular matrix in the process called cell adhesion. Leucocyte adhesion to mesothelium is an important step during peritonitis, which is mediated by adhesion molecules including vascular cell adhesion molecule-1 (VCAM-1). Lee *et al* (27) investigated the effect of exogenous NO on VCAM–1 expression in cultured peritoneal mesothelial cells and suggested that NO inhibits VCAM-1 expression via suppression of NF-κβ through a cAMP independent pathway. NMDA receptor and NO synthase activation has been shown to regulate polysialated neural cell adhesion molecule (PSA-NCAM ) expression in adult brain stem synapses (5) . Addition of nitric oxide donors, 3-morpholinosydnonimine (SIN-1) or sodium nitroprusside (SNP), significantly suppressed IL-1β induced ICAM-1 expression in a dose dependent manner in rat glomerular mesengial cells. Waldow *et al* (68) has recently shown that NO applied by inhalation or released from NO donors reduce the expression of cell adhesion molecules and ameliorate other consequences of ischemia/reperfusion injury.

### *Nitric oxide and synaptic plasticity*

Learning, memory, and restoration of lost sensorimotor functions, all involve synaptic reorganization that requires diffusible messenger-mediated communication between pre-synaptic and post-synaptic structures and NO has been identified as a candidate molecule to accomplish this function. During synaptic remodelling in the adult CNS, NO acts as a signal for synaptic detachment and inhibits synapse formation by cGMP-dependent and probably, S-nitrosylation-mediated mechanisms, respectively (62). Calcium dependent activation of nNOS and NO generation via stimulation of the NMDA receptor in cultured neurons activates the Ras/ERK (extracellular signal receptor kinase) pathway (39). Since this calcium dependent activation of Ras-MAPK pathway is considered to be a major pathway of neural activity dependent long-term changes in the nervous system, NO may be a key mediator linking activity to long-term plasticity. NO contributes to late phase long-term potentiation in the hippocampus (21) by stimulating guanyl cyclase and cGMP dependent protein kinase which acts in parallel with PKA to increase phosphorylation of CREB.

### *Nitric oxide and neuro-immunomodulation*

### *NO and Natriuretic peptides*

Different studies now suggest that natriuretic peptides may play a regulatory role in vascular remodeling via the production of large amounts of NO. Iimura *et al* ( 20 ) found that atrial natriuretic peptide enhances IL-1β stimulated NO production in cultured rat vascular smooth muscle cells and suggested that cAMP-

dependent kinase (PKA) activity may be partially involved in this enhancement whereas, in another study, ANP and BNP were found to up-regulate IL-1β-induced iNOS expression in cardiac myocytes. Induction of NO synthase regulates atrial natriuretic peptide receptors in vascular smooth muscle cells. Montoliu *et al* (37) has recently suggested correlation of NO and atrial natriuretic peptide changes with altered cGMP homeostasis in patients of liver cirrhosis.

### *NO and neuropeptide Y (NPY)*

Neuropeptide Y (NPY) is one of the most abundant and widely distributed neuropeptides in the mammalian central nervous system (CNS). NPY and its receptor analogs differentially modulate the immunoreactivity for neuronal or endothelial NO synthase in rat brain following focal ischemia with reperfusion (8). The possible involvement of NO in the regulation of intestinal ion transport induced by NPY was investigated by evaluating the effects of NG-methyl-L-arginine (L-NMA), L-arginine and S-nitroso-N-acetylpenicillamine (SNAP) on NPY activity in mouse ileum mounted in Ussing chambers in vitro. The results showed that L – Arginine and SNAP blocked NPY mediated changes in ion transport, suggesting that NO may play a role in the regulation of NPY-mediated ion transport in the mouse ileum. Studies by Garcia-Villar et al (14) has provided functional evidence for NO-synthase activation by substance P through a mechanism not involving classical tachykinin receptors in guinea-pig ileum *in vitro.*

### *NO and Oxytocin*

The effects of neuronal, endothelial, or inducible NO synthase gene disruption on the expression of oxytocin gene were examined in the hypothalamus (paraventricular, supraoptic, suprachiasmatic, and anterior commissural nuclei) and extrahypothalamus (bed nucleus of the stria terminalis). The oxytocin mRNA levels in the anterior commissural nucleus of neuronal NO synthase knockout mice were significantly higher than in control mice, but not in endothelial or inducible NO synthase knockout mice. In contrast, no significant effects of neuronal, endothelial, or inducible NO synthase gene disruption on oxytocin messenger RNA levels in the other hypothalamic and extrahypothalamic nuclei were observed. These results suggest that neuronal NO-synthase-derived NO may be involved in the regulation of oxytocin gene expression in the anterior commissural nucleus (42). It has been reported that upregulation of NO synthase mRNA in an integrated forebrain circuit involved in oxytocin secretion. Interaction between NO and oxytocin has been reported to influence luteinizing hormone-releasing hormone (LHRH) release from the hypothalamus (7). Results indicate that oxytocin releases LHRH by stimulating NOS via NE, resulting in an increased release of NO, which increases prostaglandin E2 (PGE2) release that in turn induces LHRH release. Furthermore, the released NO can act back on oxytocinergic terminals to suppress the release of oxytocin in an ultrashort-loop negative feedback.

### *NO and Opioids*

Endogenous opioid peptides and their receptors are widely expressed throughout brain and play important modulatory role in neural, behavioral, immune and gastrointestinal function. NO derived from the increased expression of iNOS is implicated in the enhanced effects of morphine and in the upregulation of morphine gene transcription observed during intestinal inflammation (49). NO is a key mediator of morphine antinociceptive tolerance. It has been reported that neuronal nitric oxide modulates morphine antinociceptive tolerance by enhancing constitutive activity of the μ-opioid receptor. Studies suggest that biochemical mechanisms related to NO release are involved, at least in part, in morphine effects on the eye. Since the μ3 opioid receptor subtype is able to release NO and is sensitive to inactivation by GSH, it may be possible that μ3 opioid receptors are involved in morphine-induced miosis and reduction in intraocular pressure. Evidence from pharmacologic inhibition and gene knockout mice studies (22) suggest that inducible NO synthase mediates delayed cardioprotection induced by morphine in vivo.

### *NO and Neurosteroids*

NO-dependent mechanism may be involved in the beneficial and antiamnesic effects of the neurosteroids, pregnenolone sulfate (PS) and dehydroepiandrosterone sulfate (DHEAS) on the aging- and dizocilpine-induced impairment of learning and memory processes (54). PS and DHEAS at 1-20 mg/kg, s.c., significantly improved the passive-avoidance and plus-maze performances and significantly attenuated dizocilpine (0.1 mg/kg, i.p.)-induced amnesia in mice, whereas, preadministration of L-NAME

(10 and 20 mg/kg, i.p.), at doses that did not disrupt cognition alone in either young or aged mice, significantly blocked the beneficial and antiamnesic effects of neurosteroids. Chronic administration of N-omega-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase, diminished the ability of 5alpha-pregnan-3alpha-ol-20-one, a neurosteroid, to potentiate the [3H]muscimol (5 nM) binding in the rat hippocampus but not in the cerebellum or cerebral cortex. suggesting that NO may be involved in some of the effects of neurosteroids in hippocampus. Simoncini *et al* (58) has suggested that DHEA administration to human endothelial cells triggers NO synthesis, due to enhanced expression and stabilization of endothelial NO synthase (eNOS). Additionally, DHEA rapidly activates eNOS, through a nontranscriptional mechanism that depends on ERK1/2 MAPK but not on phosphatidylinositol 3-kinase/Akt.

### *NO and Estrogen*

Estrogen has been found to modulate endothelial and neuronal NO synthase expression via an estrogen receptor beta dependent mechanism in hypothalamic slice cultures (15). On the other hand Wycoff et al (71) has suggested that eNOS stimulation by estrogen requires plasma membrane estrogen receptor alpha coupling to G alpha 1 and that G alpha1 mediates requisite downstream signaling events. The fact that treatment with 17-beta estradiol increases NO synthase expression and number of NO producing neurons in several regions of the brain suggest that NO may paly an important role in mediation of estrogen's effects in neuronal tissues. A positive correlation between circulating estrogen levels and levels of plasma NO has been described in humans. A recent study has shown that estrogen treatment of mice markedly upregulates the levels of iNOS mRNA, iNOS protein, and NO in activated splenocytes (23) This upregulation of NO is in part mediated through interferon-gamma (IFN-γ), a pro-inflammatory cytokine that is enhanced by estrogen. These findings are important considering that estrogens are not only involved in regulation of normal immune responses, but also are implicated in many autoimmune and inflammatory diseases.

### *NO and Melatonin*

Melatonin is a pineal hormone involved in regulation of diurnal rythmicity. Recent studies have shown that in addition to its neuromodulatory and antioxidant effect, melatonin may act as modulator of NO. This is achieved by scavenging of peroxynitrite which is produced due to interactions between excessively produced NO and reactive oxygen species (2). However melatonin may reduce function of NO both in vitro and in vivo by inhibition of n NOS and iNOS and also mitochondrial NOS (10,74). The effects of melatonin on the generation of nitric oxide in stimulated murine macrophages were studied by electron paramagnetic resonance techniques and it was found that melatonin inhibits the expression of inducible nitric oxide synthase by modulating the nuclear factor kappaB pathway (10). Our studies have shown that melatonin may interact with NO during iatrogenic seizures induced by aminophylline and isoniazid in experimental animals. Melatonin alone and in combination with NO synthase inhibitor L-NAME attenuated aminophylline induced a) seizure susceptibility b) elevated brain nitric oxide levels. Interestingly these melatonin induced changes was also associated with reduction of lipid peroxidation in the brain (16, 52).

### *NO and cytokines*

NO plays a key role in inducing the changes in the release of hypothalamic peptides induced in infection by cytokines. Cytokines, such as IL-1 beta, also act in the anterior pituitary gland, at least in part, via induction of inducible NOS. The NO produced alters the release of anterior pituitary hormones (33). Gadek-Michalska (13) has reported that nitric oxide mediates the interleukin-1beta- and nicotine-induced hypothalamic-pituitary-adrenocortical response during social stress and NO generated by eNOS, but not nNOS, is involved in the stress-induced alterations of HPA axis activity by nicotine. In a recent study it has been shown that stress-induced elevations of TNF-α and lowered IL-4 levels, and these were reversed by L-arginine pretreatment. L-NAME, on the other hand, aggravated these effects of stress on the cytokine profiles (unpublished data).

### *Nitric oxide and stress*

Stress is defined as any external/internal stimulus capable of disrupting the physiological homeostasis and the ability or lack of it to cope with such aversive inputs are crucial for health and disease. Complex neurochemical pathways in the CNS have been implicated in stress

mechanisms and it has recently been shown that NO may exert a significant regulatory influence during stress related biological responses (11). Experimental data showed that stress-induced neurobehavioral, endocrinal and immunological changes were pharmacologically modulated by NO ergic agents. Restraint stress (RS) induced suppressions in behavioral activity in both elevated plus maze (EPM) and open-field (OF) tests, which were attenuated by the NO precursor, L-arginine and NO releaser, isosorbide dinitrate. On the other hand, these responses were further aggravated by NO synthase inhibitors, L-NAME and 7-nitroindazole. Stress-induced elevations of plasma corticosterone were also influenced by the NO modulators in a similar manner. Further, in rats immunized with SRBC, RS suppressed markers of both humoral and cell-mediated immunity and these were reversed by the NO mimetics and aggravated by NO depletors (30,32). RS also induced elevations in plasma TNF-α levels and lowered IL-4 levels, which were pharmacologically modulated in a manner which was suggestive of an NO ergic involvement in these effects (unpublished data). Repeated exposure to RS, however, tended to induce adaptive effects in that the behavioral effects of acute RS were returned to near baseline levels. Brain biochemistry revealed that the behavioral effects of acute and repeated RS were closely paralleled by suppressions and elevations in brain NOx levels respectively (18). These studies indicate that NO may act as an endogenous anti-stress agent or adaptogen in CNS and that NO ergic mechanisms could play crucial modulatory role in stress induced neurobehavioral and other effects (52). Stress-induced gastric ulcer formation has also been shown to be under the regulatory influence of NO. Cold restraint stress induced gastric ulcerogenesis was attenuated by L-arginine whereas, L-NAME aggeravated this phenomenon. Further, “high emotional” rats were more susceptible to such gastric ulcers and NO-ergic modulation as compared to their “low emotional” counterparts (18). It was concluded that the NO was an important regulator of the brain-gut-axis for such stress ulcerogenesis. Thus, the general consensus appears to support a protective role for NO during stress.

### *Nitric oxide and seizures*

Recent studies indicate that free radicals and nitric oxide may be involved in excitotoxicity. Reactive nitrogen species (RNS) like NO has been shown to be an important neuromodulator in the CNS and studies have suggested both neuroprotective and neuroexcitatory roles for NO. Nitric oxide has been implicated as an endogenous anticonvulsant on one hand, and is shown to sensitize / promote the convulsive effects on the other. For example, L-arginine increased seizure severity in response to subconvulsive dose of NMDA, suggesting NO to be a proconvulsant, whereas, NOS inhibitor doubled the duration of seizures in bicaculline-induced model. Similarly, NOS inhibition has been found to increase the severity of seizures and mortality in response to kainic acid (47), whereas, attenuation of convulsion in response to tacrine by L-NAME has also been reported (3). Our recent studies have shown that NO synthase inhibitors, L-NAME and 7-nitroindazole, significantly blocked the incidence of seizures and post-ictal mortality in response to aminophylline administration, suggesting that NO plays a role in drug induced seizures. Interestingly, pretreatment with the NO mimetics, L-arginine and glyceryltrinitrate potentiated the proconvulsant effects of subthreshold doses of aminophylline and precipitated the seizures in most animals (16,17). Nevertheless, other studies have also reported protective effects for NO synthase inhibitors i.e. attenuation of seizures induced by a combination of subthreshold electroshock and subconvulsive dose aminophylline, and these were closely paralleled by changes in brain NOx activity (17). In support of the procolvulsant role for NO, another recent study has shown that potentiation of subthreshold PTZ seizures were also blocked by NO synthase inhibitors. Further, in the PTZ-kindling model, the onset of kindling was delayed by L-NAME co-administration (unpublished data). In another study, seizures induced by the anti-tubercular agent, INH, and INH+elctroshok, were also antagonized by pretreatment with L-NAME or 7-nitroindazole (69). It thus appears that the NO synthase inhibitors have a clearcut profile in relation to various seizure models, with NO lowering agents being protective in most situations. Interactions between ROS and RNS are reported in different situations and a balance between the two is a critical determinant of several disease processes. In our study, combined treatment of sub-effective doses of melatonin and NOS-inhibitors showed a synergistic effect against aminophylline seizures and mortality, which is suggestive of involvement of both ROS-RNS interactions in

this phenomenon (53) Assay of biochemical markers showed that MDA and NOx levels were elevated in the brain homogenates during convulsiogenesis, and pretreatment with melatonin (an antioxidant/peroxynitrite scavenger) and L-NAME, reverted these changes. The role of endothelial nitric oxide (NO) in partial seizures was investigated in transgenic mice (deleted for the endothelial NO synthase), and in their paired wild-type (WT) congeners. The results suggested that endothelial NO could mediate the cerebrovascular response within the epileptic focus and participate in the maintenance of local cerebral blood flow in distant areas (48). Luszczki et al (29) showed that 7-nitroindazole potentiates the anticonvulsant action of some second-generation antiepileptic drugs in the mouse maximal electroshock-induced seizure model, whereas, long-term L-NAME treatment potentiated the blood-brain barrier disruption during PTZ-induced seizures in rats.

### *Nitric oxide and Cancer*

Several reports have suggested the involvement of NO in tumor initiation and progression but the mechanisms are not yet clearly understood. For example, while on one hand, NO has been implicated in the mechanisms involved in promoting tumor growth (61) , on the other hand, NO derived from leukocytes has been shown to play a crucial role in tumoricidal activity. In the therapy of cancer, NO also has diverse effects, in that, it can enhance the cytotoxic efficacy of some chemotherapeutic agents as well as radiation (in vitro) and also can provide whole body protection against the same agents. NO has also been shown to participate in the complicated process of carcinogenesis by mediating DNA damage in early phases of tumorigenesis, as well as support tumor progression through the induction of angiogenesis and suppression of the immune response. A very recent study (44) has suggested that NO-donating aspirin prevents pancreatic cancer in a hamster tumor model. Further, the activation of hypoxia-inducible factor (HIF-1α), a transcription factor whose widespread presence in cancer is a result of tumour hypoxia and its relationship to NO has also been reported. Studies on human oral squamous carcinoma and cell lines has suggested that NO-induced activation of HIF-1α is a free radical-dependent action, thus linking three mechanisms considered to be significant in the progression and resistance to cancer treatment, namely free radicals, NO and HIF-1α.

### *Nitric oxide and Infection*

The role of NO in infection and immunity has been widely speculated and its involvement in bacterial, partasitic and viral infections have been reported. For example, some earlier studies implicated NO in malaria, leishmaniasis and viral infections and its modulation of specific immune responses have been documented(28,63,75). Recently, the role of NO and cytokines like TNF-α have been suggested in M.Tuberculosis infections, and studies have indicated that this free radical may act a useful marker for disease in both in vitro and in vivo experiments, in both drug sensitive and MDR-TB (56, 57). In another clinical study the association between smoking, NO and pulmonary tuberculosis has been suggested. It was shown that smokers and non-smokers with pulmonary TB have differential plasma NOx concentrations, and NO metabolites were significantly altered after anti-tubercular drug therapy and clinical improvement (Ray et al., unpublished). The overall impression from these studies is that NO may act as a useful molecule in relation to pathophysiology, diagnosis and treatment of M Tuberculosis infections. In several studies, both in vitro and in vivo, overproduction of NO has been observed in the presence of HIV-1 infection (26, 65). Furthermore, increased NO production has also been reported to contribute to the pathogenesis of HIV-1-associated dementia. The mechanisms of virus infection mediated by NO may be related to: (a) direct antiviral effects of NO; (b) impairment of antiviral defence mediated by Th-1 immune response by suppressing Th-1 functions; (c) NO-induced cytotoxic effects by oxidative injury with cellular and organ dysfunctions; and (d) NO-induced oxidative stress leading to rapid viral evolution with productions of drug-resistant and immunologically tolerant mutants. By contrast, there is some evidence of NO activity (directly, indirectly, or both) in the decreasing or blocking HIV-1 replication, through inhibition of viral enzymes, such as reverse transcriptase, protease, or cellular nuclear transcription factor (NF-k B) and long-terminal repeat-driven transcription(65). Therefore, although NO surely plays an important part in HIV-1 infection, that role is sometimes helpful and at other times damaging to the host.

*Nitric oxide – cytotoxicity versus cytoprotection: a question of balance*

NO can function as a beneficial physiological agent utilized for essential functions or can mediate several cytotoxic and pathological effects. Whether NO is harmful or helpful depends on a variety of factors (70) such as (a) the cellular environment in which NO is released , (b) the rate of NO flux as determined by which NOS enzyme is activated and (c) the array of second messenger cascades available for beneficial / toxic cell signaling. Interactions between RNS and ROS generate potentially cytotoxic agents which mediate some of the pathology associated with Parkinson's diseases, chronic inflammation and atherosclerosis. Thus, NO can serve as a chain terminating antioxidant by reacting with chain carrying peroxyl radicals. The ratio of NO to ROS may be all important and it has been shown that a 1:1 ratio of superoxide to NO generates peroxynitrite and induces lipid peroxidation whereas an excess of NO can inhibit lipid peroxidation by scavenging peroxyl radicals (45). The major differences between cNOS and iNOS activities do not reside in the concentration of NO generated per enzyme but rather in the duration of NO produced. Cytotoxicity usually correlates with the product of iNOs and not with the product of two cNOSs (possible exception of brain injury). Thus, regulated versus constant unregulated NO synthesis differentiates between the messenger and killer properties of NO (45).

*Advances in nitric oxide based drug therapy*

The therapeutic modulation of NO system has generated considerable interest as a new strategy for the treatment of disease processes. Pharmacologically this can be achieved by providing NO precursors, NO donors/releasers or NO synthase inhibitors.

*NO donors*

These can be divided into different groups that include organic nitrates (eg. glyceryl trinitrate or GTN, isosorbidedinitrate or ISDN), inorganic nitroso compounds (eg sodium nitroprusside or SNP ) and, sydnonimines (eg molsidomine, SIN-1). All these compounds exert their pharmacological actions after their metabolism into NO and therefore the name NO donors has been adopted for this class of drugs (35). Recently, a wide range of novel NO donor classes have emerged, viz. the diazeniumdiolates (NONOates like DETA NONOate, SPER NONOate etc.) , S-nitrosothiols ( eg S-nitroso-N-acetylpenicillamine [SNAP]) and mesoionic oxatriazoles.(40) Another development has been the hybridization of NO donor moieties with currently available cardiovascular drugs. Examples of such compound are: nitrosocaptopril (SNOCap), NCX-4016 etc. SNOCap combines NO donor properties with an inhibitory effect on angiotensin converting enzyme.(ACE) whereas, NCX-4016 is an aspirin/nitrate hybrid (35, 40). Dermal application of GTN-releasing patches has been shown to alleviate the inflammatory symptoms of thrombophlebitis.

*NO mimetics*

These are small molecules that mimic the biological activity of NO. Such NO mimetics display cGMP dependent and cGMP independent activity and may operate via multiple biochemical signaling pathways both to ensure survival of neurons subjected to stress and also to provide cognition enabling pathways to circumvent dementia. GT 1061 is one such NO mimetic currently in clinical trials for Alzheimer's disease (64).

*Inhalation of NO*

Nitric oxide gas, when inhaled at 5-80 ppm, has been shown to reverse persistent pulmonary hypertension of the newborn, pulmonary hypertension induced by hypoxia or after surgery and chronic pulmonary hypertension(61). The beneficial effects of NO lasts throughout the inhalation period and in some cases persist even after termination of the treatment.

*Enhanced generation or efficacy of endogenous NO*

The physiological generation of NO by eNOS is activated by a variety of chemical and physical stimuli. It may be possible to develop agonists that cause long term activation of the L-arginine :NO pathway. Indeed angiotensin converting enzyme (ACE) inhibitors, besides preventing the generation of angiotensin II, act indirectly in this way, by preventing the breakdown of bradykinin, which in turn, stimulates the synthesis of NO(32). Another way in which the physiological activity of endogenous NO may be increased is through preservation of cyclic GMP and type V phosphodiesterase inhibitors like zaprinast or

sildenafil that are especially helpful in this regard(35).

### *NOS inhibitors*

A number of analogs of L-arginine, including $N^G$-monomethyl-L-arginine (L-NMMA), $N^G$nitro-L-arginine (L-NA) and its methyl ester (L-NAME), N-iminoethyl-L-ornithine (L-NIO) and $N^G$amino-L-arginine (L-NAA) act as competitive and in some cases irreversible inhibitors of both constitutive and inducible NO synthase (66). Glucocorticoids have also been shown to inhibit directly the induction of iNOS. Although selective inhibitors of the inducible NOS are currently being developed , so far all experimental and clinical studies in which NO synthase is inhibited has been carried out using drugs that affect both the inducible and constitutive enzyme. Such inhibitors can reverse or prevent the hypotension induced in animals by LPS or in those with haemorhagic or anaphylactic shock. Some compounds such as S-methyl-L-thiocitrulline and S-ethyl-L-thiocitrulline, exhibit a degree of selectivity in vitro towards nNOS when compared with eNOS. If such compounds display the same selectivity in vivo, they may prove to prevent the neurotoxic actions of NO without interfering with the protective actions of eNOS. This may be achieved with compounds such as 7-NI which inhibits cerebellar NOS (66) *in vitro* and exhibits antinociceptive activity in vivo in mice without affecting blood pressure.

### *NOS gene therapy*

Studies are in progress to manipulate NOS gene(s) using DNA expression vectors or antisense oligonucleotides designed to enhance or modify NOS activity for clinical /therapeutic benefit (67). In vivo gene transfer of either eNOS or iNOS can consistently inhibit the development of a wide range of experimental vascular diseases, including restenosis, atherosclerosis, vein graft diseases and transplant vasculopathy. Aerosolised eNOS gene transfer can act as a selective pulmonary vasodilator and represents an attractive therapeutic approach to treat pulmonary hypertension. Targetting NOS by a 'loss of function' approach is aimed at inhibiting the expression of specific NOS isoform gene(s) using antisense technology and antisense oligonucleotides to iNOS - a strategy that may be used to treat sepsis. The main benefit of NOS gene transfer is that it may enable the achievement of therapeutic concentration of NO locally in target tissue, without the potential adverse effects of excessively high blood levels that occur when using systemic therapy (67).

### *NO releasing coating to prevent infection of implanted devices*

Medical implants such as catheters, artificial organs and sensors placed under the skin are critical to curing illness but they also raise the risk of serious infections. Researchers have found that they can store NO in sol-gel based materials that could be used to coat implants. NO, a natural antibacterial agent, is slowly released by coating when placed in blood or tissue and reduces the tendency of the bacteria to stick to implants and form living films that lead to infection (73).

## CONCLUSIONS

Nitric oxide has an important role in health and disease and while many of its wide ranging effects in physiology and medicine are well known, several newer areas are fast emerging. The discovery of NO has helped to explain the mechanism of action of currently used nitrovasodilators (NO donors) and phosphodiesterase inhibitors eg. sildenafil. Further, novel pharmacodynamic and pharmacokinetic aspects in relation to NO effects are being forwarded and concepts like endogenous NO synthase inhibitors, polymorphisms in NO synthase isoforms, NO in stem cell research, target specific NO delivery, etc. Have surfaced newer approaches to reduce or augment the availability of NO are being evaluated as potential new therapeutic approaches for a wide range of pathophysiological states. Some important areas in physiology and medicine in which NO could have a considerable impact are: cellular respiration, apoptosis, pharmacokinetics, stress and anxiety, cardiovascular diseases and infectious diseases.

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