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RESEARCH ARTICLE

NITRIC OXIDE (NO) A FREE RADICAL AND A MESSENGER MOLECULE

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ABSTRACT

Nitric Oxide (NO) is a lipophilic free radical diffusible gas that mediates significant and diverse signaling functions in almost every organ system in the body. It is formed from L- arginine a semi- essential amino acid present in the body with the help of three isoform of nitric oxide synthase (e.g. eNOS, nNOS and iNOS), eNOS and nNOS in cardiovascular system and nervous system respectively and inducible form produced during various body stress situations. Pharmacological compounds that release NO have been useful tools for evaluating the broad role of NO in physiology and therapeutics. NO deficiencies have been implicated in the generation and evolution of several disease states. Apart from newly developed drugs e.g. NO- Aspirin, several commonly used cardiovascular drugs exert their useful action, at least in part by modulating the NO pathway. This review discusses the fundamental pharmacological properties and mechanism of action of NO, their physiological effect in various system of the body and pathophysiological role in various disease process e.g. hypertension, coronary artery disease, stroke, shock and neurodegenerative diseases, either because of deficiency or excess of NO.

INTRODUCTION

NO a free radical lipophilic gas and endogenous cell signaling molecule formed in the atmosphere during lightning storms and in mammals in an enzyme – catalyzed reaction between molecular oxygen and L-Arginine (Loscalzo *et al*., 2000, Ignarro *et al*., 1999 and Rang *et al*., 2001). NO acts as a key signaling mechanism (intracellular and cell to cell messenger) e.g.-Cardiovascular, Nervous system and Host defense. It Modulates various physiological responses e.g. Platelet function, vascular smooth muscle (VSM) relaxation and proliferation, neuromodulation, memory, immune stimulation, cytostasis and apoptosis. (Loscalzo *et al*., 2000, Murad, 1998 and Ignarro *et al*., 1999). First introduced by Furchgott, Ignarro and Murad (for which they have been awarded Nobel prize in 1998). Initial observation of biological role was seen in rodent macrophages and neutrophils, second observation was made by Furchgott and Zawadski in 1980 that on stimulation with acetylcholine the endothelium releases a short lived vasodilator substance –EDRF (Endothelium derived relaxing factor in isolated smooth muscle preparation). Later it was told that EDRF works through release of NO from VSM cells. Other workers told that EDRF and NO are the same entity (Furchgott *et al*., 1980, Ignarro *et al*., 1987 and Palmer *et al*., 1987). NO is the endogenous activator of soluble guanylate cyclase, it leads to the formation of cGMP which acts as a second messenger in various cells e.g.- Nerves, Smooth muscles Monocytes and Platelets (Ignarro *et al*., 1999, Loscalzo *et al*., 2000, Murad, 1998 and Rang *et al*., 2001). NO shows the properties of both nitrogen and oxygen, shares many properties with O2 in particular- e.g. increased affinity for haeme and other iron sulphur groups. This property leads to the activation of guanylate cyclase which contains haeme group and which is also important for inactivation of NO by hemoglobin.

Biosynthesis and Control of NO

Control of NO biosynthesis is regulated by NOS (NO Synthase, an enzyme). Following are three known isoforms of NOS-(Ignarro *et al*., 1999, Loscalzo *et al*, 2000, Murad, 1998 and David *et al* 2006) :

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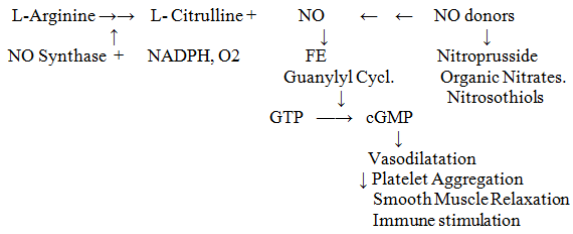
iNOS (mNOS or NOS-II)- expressed in macrophages, neutrophils, fibroblast, vascular smooth muscles and endothelial cells in response to pathological stimuli e.g. microbial infection. It is an Inducible form

eNOS (ecNOS or NOS-III) present in endothelium, cardiac myocytes, renal
mesengial cells, osteoblasts, osteoclasts and in small amounts in platelets.

nNOS (ncNOS or NOS- I) present in neurons.

Both eNOS and nNOS are **Constitutive forms**

Constitutive form generate small amounts of NO .whereas activity of inducible form is thousand times greater than constitutive form. NOS are dimeric enzymes having similarity with Cytochrome P-450 enzymes and having binding sites for L- arginine, NADPH and Calcium–calmodulin. In contrast to Constitutive isoform of NOS , activity of inducible isoform is independent of Ca++ .They are induced by bacterial lipopolysaccharide , cytokines and interferon y (Rang HP *et al*., 2001). This induction is inhibited by glucocorticoids and many cytokines e.g. transforming growth factor β (TGF β).



Degradation and Carriage of NO: (Rang HP *et al*., 2001)

$2NO + O2 \rightarrow N2O4$
$N2O4 + H2O \rightarrow NO3^{-} + NO2^{-} + 2H+$
$NO2^{-} + HbO \rightarrow NO3^{-} + Hb$.
NO reacts rapidly with even low concentration of super oxide anion ($O2^{-}$) to form $ONOO^{-}$(Peroxy nitrite anion). $ONOO^{-}$ is responsible

for some of the toxic effects. It has tissue damaging moity and affinity with sulfhydryl groups which inactivates several enzymes. Formation of $ONOO^-$ is regulated by cellular content of glutathione which interacts with NO to generate S- nitrosoglutathione, more stable form of NO (which serves as carrier of NO). Vascular glutathione is decreased in diabetes mellitus and atherosclerosis and may cause increased incidences of cardiovascular complications. Carrier mechanism allow NO to act at a distance from its site of biosynthesis. In the absence of O2, NO bound to hemoglobin (Hb) which is relatively stable. In presence of O2, NO immediately converted to NO3 and haeme iron oxidized to met Hb. NO can also bind reversibly to globin part of Hb through the reactive –SH groups of the cysteine residue nitrosothiols which are stable. It is said that resulting S-nitrosylated Hb may be involved in the transduction related activities e.g. – control of vascular resistance and blood pressure.

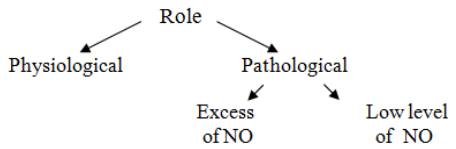
Deactivation

1. by combination of NO with haeme of Hb.
2. by oxidation to nitrite and nitrate which gets excreted in urine.

Effects of NO

Some physiological and pathological roles of NO—

System



Cardiovascular System

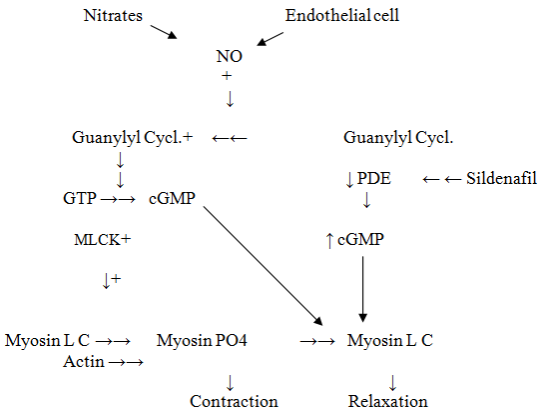
Endothelium. / Vascular smooth-muscle	Control. of B.P.	Hypotension (Shock)	Atherosclerosis Thrombosis Vasospasm (Hypertension, Hypercholeste-rolemia, Diabetes mellitus)
Platelets	Limitation of adhesion. and aggregation.		
Host Defense (Macrophages., Neutrophil. and Leukocytes)	Defense against microbials , Fungi and Protozoa		
Nerves			
Central	Neuromodulation LTP, Plasticity (? Memory, appetite control. and nociception)	Exitotoxicity (Ischemia, Stroke and Neurodegeneration)	
Peripheral	Neuromodulation. e.g.- GIT and Penile erection.		Hypertrophic.-pyloric stenosis, Impotence. in Diabetes mellitus

Physiological effects of low concentration of NO produced by constitutive enzymes are mediated by cGMP. Cytotoxic/ cytoprotective effects of high concentration of NO usually produced by inducible NOS enzymes and is due to its chemical nature of free radical. NO can activate guanylate cyclase in the same cells where it is produced or more commonly diffuses from its site of action and activate guanylate cyclase of neighboring cells which increases cGMP, activate protein kinases, cyclic phosphodiesterases and ion channels which causes inhibition of response to Ca ++ contractile and proaggregatory effects in respective VSMs and platelets. In addition NO causes hyper polarization of VSMs in some circumstances as a consequence of K+ channel activation. Moreover, increase cGMP levels inhibits monocyte adhesion. and migration , as well as inhibition of smooth muscle and fibroblast proliferation which contributes to antiatherogenic role of NO

Vascular effects

Tonically active endothelial-L- arginin / NO pathway exists in resistance vessels leads to-

- Physiological vasodilatory mechanism (Katjung, 2001).
- Decreases peripheral vascular resistance (Husang P L *et al*., 1995) leads to hypotension (by increase in cGMP synthesis).
- (*Mutant mice which are deficient in Gene colony for eNOS become hypertensive*)
- Decreases neutrophil adhesion to vascular endothelium.
- Protects against ischemia- reperfusion mediated endothelial dysfunction.
- Thus it can prevent cardiovascular diseases (Yoram and Timothy, 2001; Evan and Ignarro, 2005)



Mechanism of Vascular Smooth Muscle Relaxation

Platelet

Platelet activation is associated with platelet aggregation and adhesions which leads to increased incidence of thrombotic events. NO causes inhibition of platelets, neutrophil and monocyte adhesion and aggregation (Platelet contains iNOS and eNOS but less than that of endothelium). This protects against atherogenesis and thrombosis and also causes beneficial effects on coagulation (by increase in fibrinolysis).

Atherosclerosis

Plaque formation is due to decrease NO formation and thus inhibition of vasodilatory response (Napoli C *et al*., 2001 and Kelly R A *et al*., 1996). NO carriers, donors and cGMP analogs decreases smooth muscle proliferation. It also acts as antioxidant and therefore blocks oxidation of low density lipoprotein (LDL) which prevents formation of foam cells in the endothelium.

Host Defense

Cytoprotective to host and cytotoxic to microbials. Non specific host defense against numerous pathogens and tumor cells .Mechanism of action is Nitrosylation of nucleic acids and combination with haem containing enzyme involved in microbial cell respiration .

Septic Shock

Lipopolysaccharide (LPS) a component of bacterial cell wall which activate iNOS ,causes excessive production of NO and leads to severe hypotension ,which causes shock and death. It can be reversed by NOS inhibitors and Methylene blue.

Hypertension Associated with Pregnancy

Preeclampsia which leads to inhibition of normal physiological response (may be due to combined deficiency of NO and Prostaglandins) and causes increase in blood pressure and increase capillary permeability, disseminated intravascular coagulopathy (DIC) which ultimately decreases organ perfusion and intra uterine growth

retardation (Katjung, 2001). (*In these cases high doses of L-arginine supplementation may be effective*)

Respiratory Disorders

Given in new born by inhalation in cases of pulmonary hypertension and in acute respiratory distress syndrome (ARDS). NO acts by decreasing pulmonary artery pressure and increases blood oxygenation, also relaxes airway smooth muscle. (Rossaint R *et al*., 1993 and Steudel W *et al*., 1999).

Organ Transplantation

NO prevents rejection by decreasing platelet and neutrophil adhesion to vasculature, decreasing free radical injury, ischemic reperfusion injury and myointimal proliferation at graft area. (Rossaint *et al*., 1993) (*L-arginine inhibits atherosclerosis. and prevents organ graft rejection*).

Neuronal Effect

NO is Non Adrenergic Non Cholinergic (NANC) neurotransmitter / neuromodulator in many tissues e.g. – GIT, upper respiratory airways and Penile muscle. It acts as neuromodulator of ligand gated receptors. Targets are pre and postsynaptic terminals and also modulates release of neurotransmitters at other brain sites. It activates NMDA receptors postsynaptically and further release of NO (increase synthesis by activation of nNOS) and initiates presynatic release of Glutamate (Excitatory neurotransmitter). This can be blocked by NOS inhibitors e.g. L- NMMA and enhanced by L-arginine. Therefore having a role in long and short term potentiating effects on excitatory amino acids which is involved in brain development and learning. Prolonged NMDA receptor activation causes degeneration of neurons (due to increase in Ca++ influx) which causes further activation of nNOS and leads to increased NO formation (sustained) which in turn causes increased free oxygen radical formation or generation of secondary radicals like $ONOO^-$ (peroxide radical) which leads to increase in Ca-ATPase resulting in increase Ca++ accumulation which ultimately causes neurodegeneration e.g. – Amylotropic Lateral Sclerosis, Alzheimer's disease and Huntington's disease. This also causes destruction of Photoreceptor cells in Retina and have the role in epileptic seizures. (Katjung, 2001 and Bredt and Snyder, 1994)

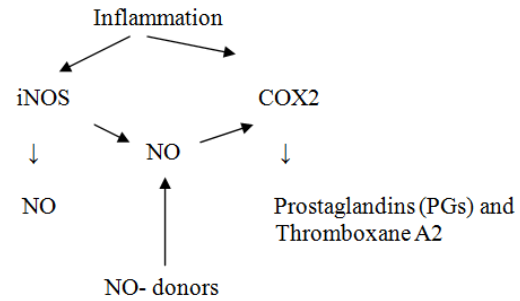
Peripheral Nervous System

Noradrenergic noncholinergic (NANC) nerves are widely distributed in peripheral tissues especially GIT and reproductive tracts. In GIT it causes increased mucosal release and host defense (Katjung, 2001). In Penile regulation – it causes penile erection which is thought to be due to NO release from NANC neurons and promote relaxation of smooth muscle of corpora cavernosa. (NOS inhibitors prevents erection in rats). Two mechanisms for penile erection are proposed –

1) Local PGE1 secretion in corpora cavernosa.
2) Inhibition of PDE-5 which increases cGMP levels and therefore leads to increased NO formation (mechanism of Sildenafil, a drug used in impotency).

Inflammation

NO has a role in acute and chronic inflammation (NOS inhibitors has dose dependent protective effect). NO promotes edema and vascular permeability and also causes increased synthesis of prostaglandins by activating COX2 (Katjung, 2001) (*decrease of NO formation by NOS inhibitors has benefit in inflammatory-joint diseases* (Mackenzie *et al*., 2008).



Therapeutic Uses

Controlled low concentration of NO when inhaled causes vasodilatation and also smooth muscle relaxation, this is limited to pulmonary circulation and acts on ventilated alveoli only. These features can benefit ARDS and pulmonary hypertension (Inhalation of high concentration of NO, when cylinders of N2O for anesthesia get contaminated, causes acute pulmonary edema and met - haemoglobinaemia).

Diagnostic uses

(i) In cardiac catheterization.
(ii) Assessing the severity of asthma and respiratory tract infection (RTI).

As Nitric Oxide Donors

NO releasing drugs (in vivo or vitro) are pharmacologically active compounds and can be direct NO donors or require metabolism (Napoli and Ignarro 2003).

Sodium Nitroprusside (SNP)

Drug for rapid reduction of blood pressure but use is limited by need to administer parentally. Mechanism of action is through release of NO which causes K+ channel activation and leads to hyperpolarization of the cells, which ultimately causes relaxation of VSMs.

Organic Nitrates-(e.g.Nitroglycerine, amyl nitrate, Iso-mono and dinitrate)- Represents the prototypical form of NO replacing therapy. Safe and effective method for the management of coronary heart disease (CHD). All the organic nitrate esters are pro-drugs and requires enzymatic metabolism to generate NO (Loscalzo, 2001). Limitation- drug tolerance which can be decreased by

1. Intermittent dosing.
2. Thioles, ascorbate , l-arginine, ACEIs and folate.

(*They can be used successfully to reverse or prevent nitrate tolerance*)

S- Nitrosothioles: It is a source of circulating endogenous NO. Naturally occurring NO donating compounds spontaneously release NO and nitrosonium (NO*) e.g.- S- nitrosoglutathione, S- nit.-N-acetyl penicillamine and S- nitroso albumin. Role- Treatment of asthma and infective diseases (Theoretically). (Grutter *et al*., 1980 and Ewing *et al*., 1977)

NSAIDS

Prostaglandins and NO maintain mucosal integrity of GIT and other organs. NO has cytoprotective properties (Lowenstein,1994; Moncada, 1991; Gupta *et al*., 1998, Takeuchi *et al*., 1998, Brown *et al*., 1992 and Whittle, 1993) as follows:

i) Ability to increase local blood flow.
ii) Scavenge highly reactive free radicals.
iii) Like PGs it also increases mucus secretion and maintain mucus blood flow.

Aspirin like compounds are so designed that can simultaneously release NO, therefore counterbalance negative effects due to PG inhibition and also act as anti-inflammatory agent. They are called as NONSAIDs (Saldato *et al*., 1999) e.g.- NCX-4016 and 4215 also having anti platelet activity due to inhibition of arachidonic acid stimulated platelet - aggregation which leads to antithrombotic activity. (*Increased production of prostacycline and thromboxane by giving aspirin in MI causes inhibition of infarct size and ischemic reperfusion injury*).

Cardiovascular agents:

Calcium Channel Blocker (CCB)

It counteracts the effect of Angiotensin II and Endothelin.-I at the level of VSM by inhibiting Ca++ inflow and facilitating vasodilatory effects of NO. Act by up- regulation of eNOS, increasing activity of endothelial super oxide dismutase and flow mediated release of NO. (Dhein *et al*., 1999, Mombouli and Vanhoutte, 1999, Vanhoutte 1998 and Schiffrin, 1998)

Angiotensin Converting Enzyme Inhibitor (ACEI) and AT II type -I Receptor blockers:

ACEIs -potentiate bradykinin which stimulate endothelium to release vasodilator substance like NO and increases constitutive expression of NOS in Juxtaglomerular apparatus which causes release of NO which in turn causes increased release of renin. Angiotensin- II stimulate superoxide production which causes decreased bioactivation of NO and this is blocked by ACEIs. Angiotensin-I receptor antagonist e.g. Losartan improves basal release of NO and decreases vasoconstrictive effect of Endothelin –I released from endothelium.

β Blockers: (Gao *et al*., 1991)

Some interfere with NO path way e.g.-Nebivolol- found to induce dose dependent arterial relaxation in Dogs. (*This effect of Nebivolol is blocked by L- NAME an inhibitor of NOS*)

Hydroxy Methyl Glutaryl (HMG) Co A- Reductase Inhibitors (Statins): (Laufs *et al*., 1997)

Prevent hypoxia induced down regulation of eNOS by stabilizing eNOS mRNA , this causes increased NO formation by endothelial cells which is mediated by blocking geranylgeranylation of the small GTP- binding Ras like protein Rho due to decreased biosynthesis of geranylgeranyl pyrophosphate (GGPP) (Laufs *et al*., 1998) Thus important noncholesterol lowering effect of statins is the up - regulation of eNOS expression of Rho. Simvastatin activate serine threonine kinase (AKT) in endothelial. Cells and causes phosphorylation of eNOS which in turn causes increased production. of NO (Kureishi *et al*., 2000). Statins also prevent down regulation of eNOS induced. By tumor necrosis factor (TNF). (*These effects may play significant role in setting of chronic Statin therapy for prevention of CHD.*)

Anti-oxidants and L- arginine -

Atherogenic lipid e.g.- oxidized LDL is responsible for wide range of cell dysfunction with in arterial wall and plays pivotal role in atherogenesis. Oxidized –LDL uncouples eNOS and induce decreased uptake of L-arginine (Witztum, 1994, Jessup, 1996, Napoli *et al*., 1997, Napoli *et al*., 1999, Pritchard *et al*., 1995 and Vergnani *et al*., 2000). (*NO produced by iNOS in VSMCs induced by cytokines decreases oxidation of LDL which may play protective role*) α-Tocopherol and Vitamin- C also causes inhibition of LDL oxidation. Therefore antioxidants and L-arginine are not considered as classical drugs but as a dietary supplement. Vitamin- C may potentiate NO activity and normalize vascular functions in patients of CHD. (Frei, 1999)

Inhibition of Synthesis of NO: (Rang *et al*., 200)

By L-arginine analogues e.g.

i) NG-Monomethyl L-arginine (L- NMMA)
ii) NG- Nitro-L- arginine methyl ester (L- NAME)
iii) PIN- an endogenous Protein Inhibitor of nNOS.

Intravenous L-NMMA increases blood pressure. In several species including humans and 7-nitroindazole inhibit nociception. without altering blood pressure.

Clinical Co-relation with NO

Clinical conditions related to NO

Either increased or decreased production could play part in disease states.

Under production of NO –

- Reported in babies with hypertrophic- pyloric-stenosis.
- Reduced NO production-in patients of hypercholesterolemia. and may contribute to atherogenesis.

Over production– may be important in Neuro- degenerative diseases and septic shock.

Status in Therapeutics

Sepsis- which leads to Multiple Organ Failure (NO is of benefit in host defense by contributing to microbial killing). Subsequent excessive NO production causes hypotension (*Inhibition of NO synthesis by L-NMMA may be of therapeutic value in severe hypotension and multiple organ failure*).

Hypercholesterolemia and other diseases that predispose atherosclerosis e.g- Diabetes Mellitus can be controlled by treatment of hyperlipidemia or by supplementation of L- arginine.

Conclusion

Thus it has been concluded that NO is involved and play important role in various physiological systems and pathological states. It is an important molecule and target for treating various highly prevalent diseases in which it is involved e.g.-Hypertension, severe Hypotension. Atherosclerosis, CHD, Host defense and ARDS. On the other hand it could play important role in pathogenesis of- Shock, Hypertrophic Pyloric Stenosis in child, Neurodegenerative diseases and Organ graft rejection etc (So NO is an double edged sword)

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