Nitric Oxide

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Nitric Oxide (NO) is used by the body as a signaling molecule. It was previously known as EDRF (endothelial derived relaxing factor) and was named the “Molecule of the Year” by Science magazine in 1992 as the importance of the role of NO in the mammalian body became apparent. In 1998 Furchgott, Ignore and Murad won the Nobel Prize for elucidating the role of NO in the cardiovascular system.

Outside of the human body it is produced as the byproduct of industrial processes, especially by internal combustion engines, and by cigarettes. It is a greenhouse gas and is classified by many countries as an extremely hazardous gas.

Chemistry

Nitric oxide is a colorless gas at standard temperature and pressure. It has an *unpaired* electron on the nitrogen atom which makes it highly reactive. Common reactions include:

$2NO + O_2 = 2NO_2$ which is nitrogen dioxide and causes nausea, headaches and impaired immune and respiratory function.

$4NO + O_2 + H_2O = 4HNO_2$ (nitrous acid)

NO is naturally produced by lightning strikes. Since the heat generated is over 2000 degrees Celsius this reaction does not need a catalyst, ie $N_2 + O_2 = 2NO$

Commercially NO is prepared by the oxidation of ammonia at 850 degrees Celsius with platinum as a catalyst. The reaction is $4NH_3 + 5O_2 = 4NO + 6H_2O$

In the laboratory NO is produced by the reduction of dilute nitric acid with copper. $8NO_3 + 3CU = 3Cu(NO_3)_2 + 4H_2O + 2NO$

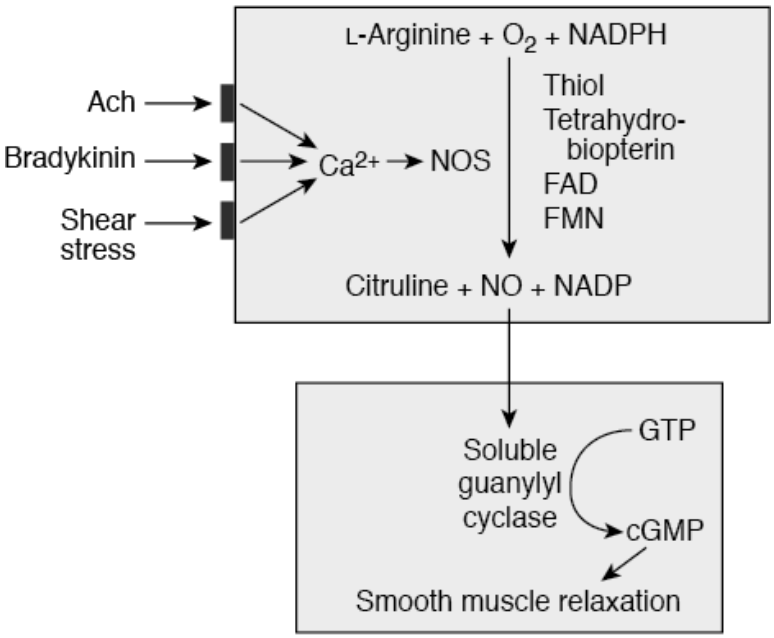
Medical grade NO is produced under carefully controlled conditions, diluted with nitrogen and stored in the absence of oxygen.

Levels are measured by chemoluminescent and electrochemical methods. Chemoluminescence is regarded as the gold standard but electrochemical is also accurate.

Physiology

NO is generated the reaction of L-arginine with oxygen and NADPH. Citrulline, NO and NADP are generated as end products. The reaction is catalyzed by NOS (nitric oxide synthase) which uses Thiol, tetrahydrobiopterin (TH4), FAD (flavin adenine dinucleotide), FMN (flavin mononucleotide) as requisite cofactors.

Nitric Oxide is a gaseous molecule and does not require channels or receptors. It diffuses across cell membranes to activate soluble guanylyl cyclase (sGC) which in turn will catalyze the formation of cGMP (cyclic guanosine monophosphate) from GTP (guanosine 5' triphosphate). cGMP can now activate smooth muscle relaxation.



Nitric oxide has a half-life of 2-6 seconds and is rapidly inactivated by blood. This makes its action highly specific to the area within which it is generated. Nitric oxide is mainly inactivated by binding to oxyhaemoglobin, forming methaemoglobin. Haemoglobin in turn will be regenerated forming NO_3^- (nitrate). A lesser proportion of NO will bind to deoxyhaemoglobin forming nitrosohaemoglobin or will dissolve with oxygen (O_2) to form NO_2^- (nitrite).

An alternative pathway to the formation of NO exists in the GIT. Nitrate rich vegetables (leafy greens) are metabolized by the nitrate reductase enzyme present in the GIT (mainly in the mouth) to nitrite (NO_2^-). The low pH in the stomach will then reduce nitrite to NO.

In the presence of hypoxia the nitrate-nitrite-nitric oxide pathway can also be activated intracellularly by deoxyhaemoglobin or deoxymyoglobin.

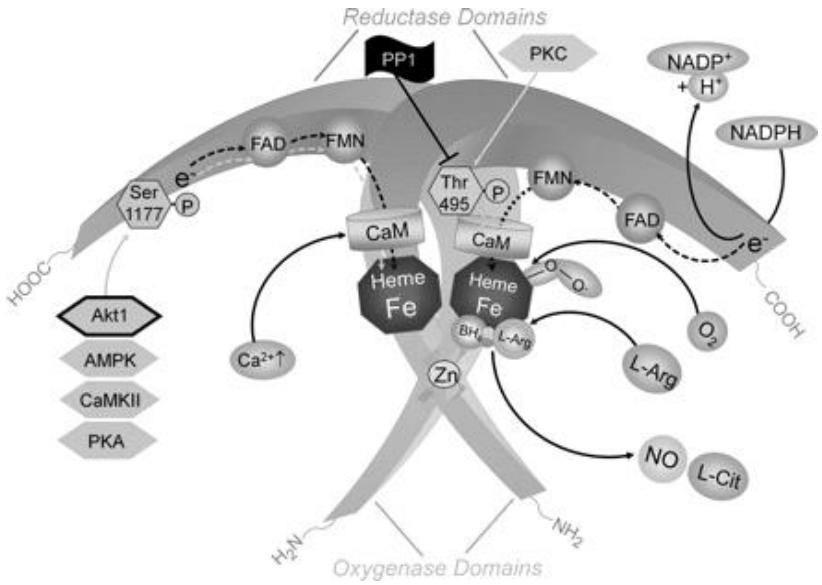
Primarily however the majority of the NO present in the body is catalyzed by NOS and therefore I will focus in the enzyme.

Nitric Oxide Synthase

Recommended reading: Nitric Oxide Synthases: Regulation and function by Forstermann and Sessa in European Heart Journal of April 2012

Three isoforms of the enzyme exist. NOS 1 is constitutive and present in neuronal cells. It is also known as nNOS. The expression of NOS2 is induced by factors such as bacterial endotoxins and cytokines. It is therefore known as iNOS, and when induced can be found in multiple cells, most notably in macrophages and neutrophils. NOS3 (eNOS) is constitutive and is present in multiple cells, but is well known for its function in endothelium and platelets. Constitutive NOS enzymes (1 and 3) are normally present in cells and are responsible for multiple baseline functions. They produce NO under normal physiological conditions in picomols. Induced NOS is produced in times of physiological stress and then larger amounts (nanomols) are produced. All NOS contain haem which is essential to their function. NOS also contains zinc but it is a structural element and does not catalyze reactions.

NOS enzymes are homodimers. NOS transfers electrons from NADPH via the flavins in FAD and FMN in the reductase domain to the haem in the oxygenase domain. The oxygenase domain binds BH4, O_2 and L-arginine. Now NOS will hydroxylate L-arginine. Secondly NOS oxidizes the N-hydroxy-L-arginine formed in the previous step to L-citrulline and NO.



Calmodulin binds to all isoforms of NOS and regulates their function. In nNOS and eNOS calmodulin binding occurs when there is an increase in intracellular calcium. This will then facilitate electron transfer, increasing levels of NO and cause smooth muscle relaxation. Calmodulin can bind to iNOS at low intracellular calcium levels.

NOS1

nNOS can be found in brain tissue, the spinal cord and some peripheral nerves. Nerves containing nNOS are known as nitrergic nerves. NO acts as a neurotransmitter in the CNS and autonomic nervous system. NO is involved in learning and memory by being involved in long term neuronal inhibition and potentiation of signals. In this way NO seems to help neurons “remember” previous signals. NO is not used by the CNS as an “acute” neurotransmitter. NO plays a role in the modulation of the state of arousal, pain perception, neurogenesis and apoptosis. NO is also present the medulla and hypothalamus, thereby playing a role in the central regulation of blood pressure. Furthermore nitrergic nerves innervate smooth muscle in the periphery, such as vascular smooth muscle, leading to vasodilation.

Pathyphysiologically abnormal NO signaling has been implicated in neurodegenerative conditions such as Alzheimers, Parkinsons and multiple sclerosis. In the presence of an influx of intracellular calcium (such as after a cerebrovascular incident) NMDA activation leads to cell death. NO participates by forming peroxynitrite ($OONO^-$) on reaction with the superoxide anion (O_2^-). Peroxynitrite is a potent oxidant and will cause cellular damage.

Nitrergic nerves also assist with the control in regional blood flow in the corpus cavernosum. eNOS is also present in penile endothelium. NO is therefore critical to penile erections.

nNOS can also be found in adrenal glands, kidney macula densa cells, pancreatic islet cells and epithelial cells of various organs.

NOS2

NOS2 is not normally found in cells. NOS2 expression is induced by substances such as cytokines and bacterial endotoxins. Corticosteroids can inhibit the formation of NOS. NOS2 is used by several cell types. In neutrophils and macrophages NO is formed which is toxic to some pathogens and tumour cells by causing direct DNA damage as well as degradation of the iron sulphur centers of enzymes critical for mitochondrial electron transport, cis-aconitase and ribonucleotide reductase. The high levels of NO produced can also damage surrounding healthy cells.

NOS2 expression can also be induced in multiple other cells such as myocytes and endothelial cells. In endothelial cells it has been implicated in the hypotension and capillary leak found in septic shock. In reperfusion injuries NO has been implicated in cellular damage via the formation of peroxynitrite ($OONO^-$) when NO reacts with superoxide. Peroxynitrite will harm the surrounding cells.

NOS3

As mentioned previously calmodulin regulates the activity of this enzyme. In addition heat shock protein 90 (hsp90) can activate eNOS and caveolin-1 is a tonic inhibitor of eNOS activity. Caveolin-1 can be displaced from eNOS by calmodulin and hsp90.

eNOS can also be activated by shear stress. This is achieved by the phosphorylation of the enzyme. This is achieved in several ways. Shear stress itself activates protein kinase A (PKA). Akt and AMPK (AMP activated protein kinase) are activated by insulin. Bradykinin activates Ca++/calmodulin dependent protein kinase II (CaMKII). Oestrogen and vascular endothelial growth factor (VEGF) also activate Akt. Enzyme phosphorylation produces increased electron flux in the reductase domain and increases the calcium sensitivity of eNOS.

Protein kinase c (PKC) can inhibit eNOS function by causing the phosphorylation of a different part of the enzyme.

NOS3 is constitutively present in multiple cell lines, including the following:

1) Endothelial cells

Here NO generated in the endothelium diffuses across to the vascular smooth muscle. By activating sGC, cGMP is formed. cGMP in turn activates protein kinase G which phosphorylates a number of proteins involved in smooth muscle relaxation. This will result in a drop in intracellular calcium and the inactivation of myosin light chain kinase.

In the pulmonary vasculature NO also inhibits hypoxic pulmonary vasoconstriction. NO will enhance flow in well ventilated areas which will improve the ventilation/ perfusion matching. Elevated levels of pulmonary NO play a role in attenuating hypoxia at high altitude.

In the vascular tree NO has anti-Inflammatory and anti-atherosclerotic effects

- Inhibits leucocyte adhesion and vascular inflammation (early stages of atherosclerosis)
- Prevents fibrous plaque formation by controlling smooth muscle proliferation (later stages of atherosclerosis)
- During oxidative stress NOS is “uncoupled” and will produce superoxide instead of NO. This is done by the oxidation of BH4, L-arginine depletion, accumulation of ADMA (asymmetric dimethyl arginine) which competes with L-arginine and S-glutathionylation of eNOS. Thereby patients with cardiovascular risk factors produce inadequate NO and instead produce reactive oxygen species.

NO also stimulates angiogenesis postnatally and after ischaemic events. NO is also thought to active endothelial progenitor cells in the bone marrow.

2) Platelets

NO increases permeability of the K+ pump which will hyperpolarize the cell membrane which inhibits contraction. In this way NO prevents platelet aggregation and adhesion. NO also prevents the release of platelet derived growth factors. These growth factors stimulate smooth muscle proliferation. eNOS can therefore play a role in preventing angiogenesis. NO production by eNOS is essential to adaptive vascular remodeling to chronic flow changes.

3) GIT

NO determines motility and modulates morphine induced constipation.

4) Kidneys

NO plays a role in sodium homeostasis in the kidney. NO also increases renal blood flow and GFR.

PHARMACOLOGY

Inhaled NO (iNO)

The lungs have high levels of NO. They arise from various cell types, especially endothelial cells and nitrergic nerves, but also from neutrophils, macrophages, epithelial cells, fibroblasts and smooth muscle cells. NO plays a critical role in the lungs. It is responsible for maintaining low pulmonary artery pressure via NO production by pulmonary eNOS. NO also opposes the pulmonary response to endogenous and exogenous vasoconstrictors, opposes hypoxic pulmonary vasoconstriction, controls pulmonary blood flow distribution and has an important immune function when expressed in neutrophils. NO may control bronchomotor tone via nitrergic nerves. In pulmonary hypertension decreased expression of eNOS is found.

iNO causes direct relaxation of the pulmonary vasculature therefore decreasing pulmonary vascular resistance, pulmonary artery pressure and RV afterload. As NO is subsequently rapidly deactivated by the circulating blood to form methaemoglobin, it has no effect on the systemic blood pressure. Thus it has no effect on coronary perfusion. By improving RV performance the LV filling pressure may increase. This is only problematic in patients with severe left ventricular dysfunction when pulmonary edema may result.

NO opposes hypoxic pulmonary vasoconstriction and controls pulmonary blood flow distribution, redirecting blood to well ventilated areas, thereby improving ventilation/ perfusion matching and decreasing right to left shunting of deoxygenated blood. iNO can improve oxygenation via these methods.

iNO may cause some bronchodilatory effects, thereby decreasing alveolar dead space. iNO can have anti-inflammatory (decreasing capillary leak) and antiproliferative effects. It will also decrease platelet aggregation and adhesion.

There have been concerns that NO may cause hypotension and bleeding (e.g. intraventricular haemorrhage in neonates), but this has been unfounded. 70% of the iNO dose will be excreted in the urine within 48hours as nitrate.

Side effects of iNO do exist though, namely: methaemoglobinaemia, pulmonary toxicity and rebound hypertension. Oxyhaemoglobin inactivates NO to form methaemoglobin. In adult patients the levels of methaemoglobin formed are not high enough to cause problems if the iNO dose remains less than 40ppm. In patients with a glucose 6 phosphate dehydrogenase deficiency however, methaemoglobinaemia levels may become high enough to impair oxygenation. It should also be noted that fetal Hb is more readily oxidized to methaemoglobin by NO.

In the presence of oxygen, nitrogen dioxide is formed ($2NO + O2 = 2NO2$). Nitrogen dioxide is a respiratory irritant, causing chest pain, bronchospasm and pulmonary edema. This can become problematic if high doses of iNO are administered, or a high FiO2 is used (most institutions will try and keep it under 60%).Neonates, the elderly, patients with respiratory and cardiac failure are more susceptible. Therefore it is imperative that the amount of nitrogen dioxide and NO in the circuit is monitored during iNO administration. NO on its own can also cause DNA damage to the lung when peroxynitrite is formed.

While administering iNO, endothelin 1 is upregulated and NOS is downregulated. If iNO is rapidly discontinued, rebound pulmonary hypertension and/or hypoxia will occur. Therefore iNO will always be weaned off slowly in a stepwise fashion. If the patient needs to be disconnected from the ventilator for transport or for ventilation by hand for incidents such as worsening hypoxia, the iNO must be continued.

iNO has a dosing range of 5-80ppm, but doses over 20ppm have been shown to have little additional benefit.

Other disadvantages of iNO are that is requires specialized expensive equipment, is expensive and cumbersome to administer, makes transporting the patient difficult, delivery system errors may occur, and it can pose an environmental hazard to hospital staff (headaches and respiratory irritant).

South Africa has set the OEL (occupational exposure limit) for NO at 25ppm and for nitrogen dioxide at 3ppm. At levels of 100ppm NO is regarded as immediately dangerous to life and health.

Uses of iNO:

a) Adults

ARDS (Acute Respiratory Distress Syndrome)
In ARDS iNO improves oxygenation and decreases shunting via aforementioned actions. In a small number of patients with a patent foramen ovale their pulmonary artery pressure can be high enough to shunt blood through the PFO. iNO can decrease the pulmonary artery pressure enough to reverse this.

iNO will improve oxygenation more if alveolar recruitment is improved. Therefore iNO is used with maneuvers to improve this, such as optimal PEEP, high frequency oscillatory ventilation or proning.

Initial studies have demonstrated the improvement in hypoxia but could not show an effect on mortality. They also showed an unexpected risk of AKI (acute kidney injury). The risk of AKI was initially put down to poor study design, but a Cochrane Database Review published in Anaesthesia in 2017, which included more recent studies, have confirmed this risk. They have concluded that iNO improves the PaO2 at 24 hours, but not at 48 or 72 hours. It also makes no difference in ventilator free days, duration of the ventilation, resolution of multiorgan failure, length of stay in ICU or hospital or quality of life. iNO also makes no difference in mortality. Furthermore the use of iNO significantly increases the risk of AKI. They found insufficient evidence to recommend the use of iNO in ARDS.

The mechanism of AKI is unclear. Some put it down to the formation of reactive nitrogen species, others to the increase in FiO2 required in ARDS. Both can produce a pro-inflammatory response with renal vasoconstriction.

Munshie and Adhikan point out in Critical Care of 2017 that it is difficult to design a good study in life threatening hypoxaemia in ARDS patients in ICU due to the multiple variables present (FiO2, ventilator strategies, different antibiotics, differing disease aetiology and imaging with contrast among others).

COPD
iNO will not improve gas exchange, and may worsen it.

Pulmonary Hypertension
iNO is used perioperatively for pulmonary hypertension. It decreases the need for postoperative ECMO. Cases in which it has proven useful include mitral valve surgery with significant pulmonary hypertension, heart transplant and LVAD (LV assist device) implantation. It can also be useful in adults with congenital cardiac disease associated with pulmonary hypertension. iNO has been used as salvage therapy in cases such as acute pulmonary embolism with right heart failure. There are varying responses to iNO in pulmonary hypertension due to different degrees of vascular remodeling and smooth muscle hypertrophy.

b) Paediatrics

Persistent Pulmonary Hypertension of the Newborn (moderate or severe)
This can be idiopathic/ primary or secondary due to diseases like meconium aspiration syndrome, pneumonia from group B strep or prematurity with respiratory distress syndrome. iNO reduces the need for ECMO. It is contraindicated in neonates dependent on right to left shunting of blood (certain congenital cardiac lesions).

Prematurity with Respiratory Distress Syndrome
It has been used in the past to improve oxygenation. It was also hoped the anti-inflammatory properties of NO along with its role in stimulating angiogenesis and activating endothelial progenitor cells would help to decrease the development of BPD (bronchopulmonary dysplasia) and neurodevelopmental injury. Studies have therefore looked at the routine and rescue use, both early and late. The Cochrane database review published in January 2017 has concluded that it is not effective as rescue for the critically ill premature infant. Early routine use does not prevent serious

brain injury or improve survival without BPD. Later use of iNO may be effective but the difference is small and requires further study. There were concerns that the effect of NO on platelets may increase intraventricular haemorrhage but this is not the case.

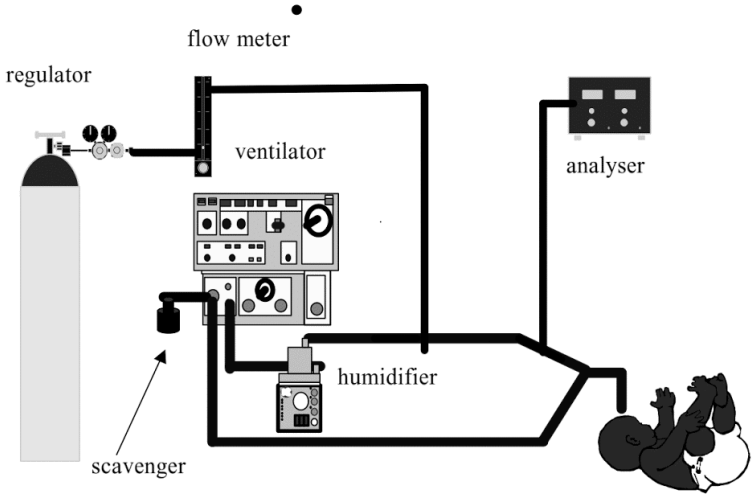
Congenital Diaphragmatic Hernia
NINOS trial demonstrated that although iNO was effective in improving oxygenation initially, it did not reduce the need for ECMO or decrease mortality.

Perioperative
iNO can be useful perioperatively as salvage therapy when there are problems with pulmonary hypertension, for example when coming off cardiac bypass with congenital cardiac lesions associated with pulmonary hypertension, such as AVSD.

c) Diagnostic

iNO has been used to diagnose reversibility with pulmonary hypertension.

Administration of iNO:



- **Cylinder**
  The cylinder contains medical grade NO with 1000ppm in nitrogen. It is under high pressure.
- **Pressure Gauges and Regulator**
  The regulator decreases the pressure from the cylinder. The first gauge demonstrates the pressure in the cylinder while the second one reflects the output pressure.
- **Flowmeters**
  Two flowmeters to determine the flow and therefore dose of iNO. The first one is for flow of 0-600ml and is used for larger adjustments of flow. The second one is for flow of 0-100ml and is used for smaller incremental adjustments of the iNO.
- **T-connector**
  Connects onto the inspiratory limb of the ventilator or oscillator circuit. Connect after the humidifier. It is important to connect it close to the patient in order to minimise formation of NO2. Do not connect closer than 20cm from the patient however, as then there is inadequate time for the NO to mix with the ventilator gases. Flush the delivery system before use to rid it of nitrogen dioxide/ nitric acid/ water or high oxygen levels. The ventilator or oscillator must have a constant flow rate to result in reliable levels of NO. If not the delivered dose of iNO is unpredictable. If the ventilator does not use a constant flow rate specialised injector systems need to be used.
- **Water trap**
  When combined with H2O nitrogen dioxide forms nitric acid which is also toxic.
- **Monitoring**
  Sidestream electrochemical monitoring. Calibrate before use. It is esssential to monitor both NO and NO2 levels as both can be toxic.
- **Scavenging filter**
  Removes NO and NO2 and ensures safe working environment for staff. When in use environmental levels of NO2 are usually less than 1ppm.

DRUGS ACTING INDIRECTLY ON THE NITRIC OXIDE PATHWAY

NO donors

Drugs such as nitroglycerin, isosorbide dinitrate and sodium nitroprusside. Please see your pharmacology textbook.

Phosphodiesterase 5 inhibitors (Sildenafil, Tadalafil, Vardenafil)

Phosphodiesterases are the enzymes responsible for the breakdown of cGMP to GMP. By inhibiting this enzyme these drugs raise the amount of cGMP in the cell and enhance the effect of NO. Phosphodiesterase 5 is the predominant isoform of the phosphodiesterase enzyme in penile tissue. These drugs are well known for their effect in enhancing penile erection (nNOS and eNOS present in the corpus cavernosum).There must be residual nNOS for the drugs to have any effect.

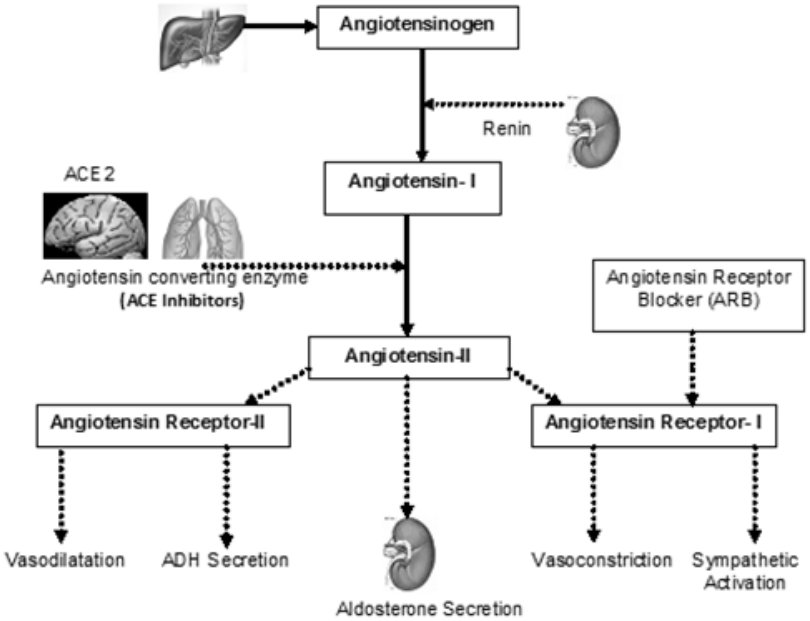
Phosphodiesterase 5 is also well represented in pulmonary arteries, and sildenafil and tadalafil are used in the treatment of pulmonary arterial hypertension. Side effects include headache, flushing, dyspepsia, nasal congestion and visual disturbances. More severe effects include priapism, hypotension (especially when administered with other drugs acting on the NO pathway), myocardial infarctions, arrhythmias, cerebrovascular incidents, increased intraocular pressure and sudden hearing loss.

These drugs can also potentiate and prolong the effect of iNO.

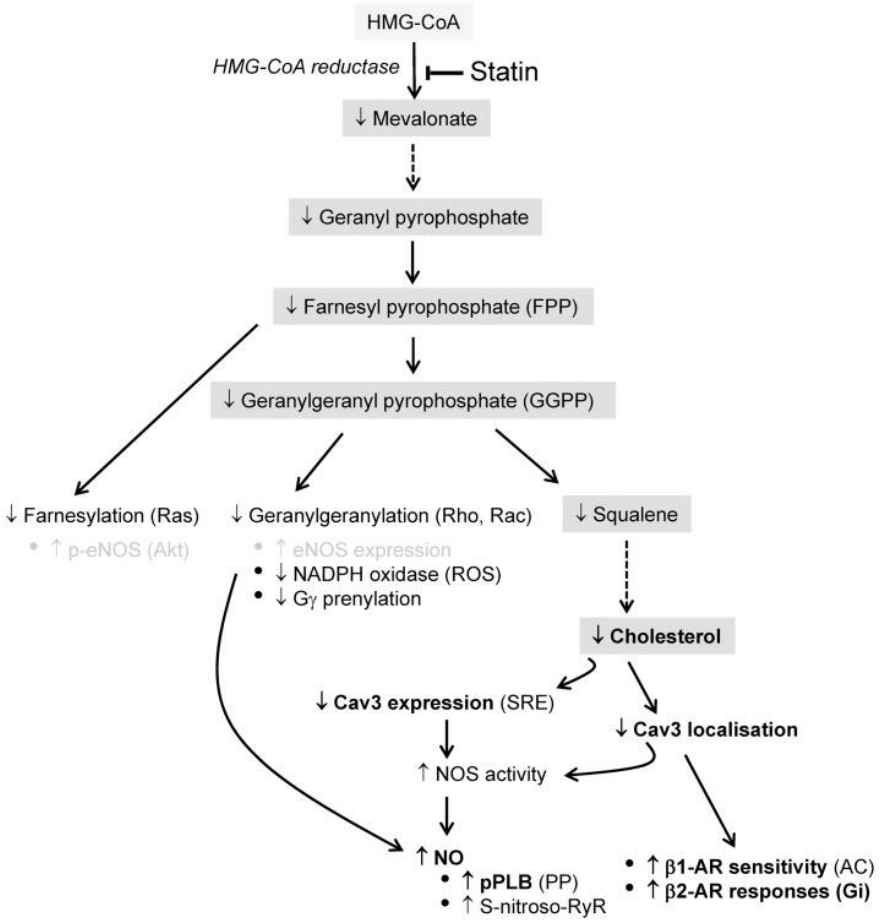
Pleiotropic Drugs

These drugs prevent eNOS uncoupling and in this way prevent endothelial dysfunction. They are regarded as cardioprotective as they prevent endothelial dysfunction. These include drugs that act on the renin- angiotensin- aldosterone pathway and statins.

ACE Inhibitors and Angiotensin Receptor Blockers
They lower blood pressure via their action on the renin/angiotensin/aldosterone pathway. Their pleiotropic effect is not their main mechanism of action but may be very important. These drugs are allosteric enhancer of the bradykinin receptors. This results in phosphorylation of eNOS and will then increase NO levels. AT1 receptors are upregulated by increased levels of LDL. Angiotensin 2 activates NADPH oxidases via AT1 receptors. Therefore ACE inhibitors and ARB's can prevent activation of NADPH oxidase and uncoupling of eNOS. These drugs can also increase the activity of superoxide dismutase (SOD3) which will scavenge superoxide. They may increase expression of GTP cyclohydrolase1 which is the rate limiting enzyme for BH4 synthesis, thereby increasing BH4 levels.



Statins
Their pleiotropic effects include increasing the expression of eNOS, enhancing the activity of eNOS by decreasing caveolin levels and activation of the phosphatidylinositol 3-kinase/ Akt pathway. Statins also decrease expression and activity of NADPH oxidase by preventing isoprenylation of p21Rac. Simvastatin more than doubles superoxide dismutase activity. Statins increases BH4 levels by increasing GTP cyclohydrolase1 mRNA expression in endothelial cells. This means that statins can assist in plaque stabilization, decrease thrombogenic responses, improve endothelial function and inhibit oxidative stress and inflammation.



As demonstrated above statins lower cholesterol levels by reversible inhibition of HMG-CoA reductase. This enzyme is the rate limiting step in cholesterol synthesis by the liver. Statins also inhibit the generation of isoprenoid intermediates such as FFP and GGPP. These isoprenoids can form lipid attachments on various proteins, a process known as isoprenylation. These include G-proteins and GTP binding proteins such as Rac,Ras and Rho. Statins therefore inhibit isoprenylation of these proteins, which results in accumulation of inactive Rac, Ras and Rho. This plays a large part in increasing NO levels.

Corticosteroids

Glucocorticoids will bind to the glucocorticoid receptor (GR) which will stimulate phosphatidylinositol 3-kinase and protein kinase Akt. This will cause activation of eNOS and NO dependent vasorelaxation. This is a non nuclear action and is a cardiovascular protective effect of high dose corticosteroids.
On the other hand, the nuclear actions of corticosteroids inhibit the expression of iNOS which is anti-inflammatory and may assist with the treatment of capillary leak and hypotension in septic shock.

sGC Stimulators

Riociguat is a drug available in the US which stimulates sGC. This will increase cGMP resulting in vasodilation. It is licensed for use for inoperable or persistent recurrent chronic thromboembolic pulmonary hypertension postoperatively, the treatment of pulmonary artery hypertension, and is being

investigated for use in Raynaud's disease and systemic sclerosis. It is contraindicated in pregnancy and pulmonary arterial hypertension from idiopathic interstitial pneumonia. Side effects include hypotension, bleeding, headaches and GI disorders. Drug interactions are with the nitrates and PDE5 inhibitors. Smoking will decrease Riociguat's levels.

Cinaciguat is an experimental drug for the use of acute decompensated heart failure.

OTHER USES

Exhaled NO can be used as an "inflammometer" in asthma. It has also been used to predict kids at risk of perioperative adverse respiratory events. Ramgolam et al in Australia studied this and concluded that an accurate history assessing risk factors remains the most appropriate tool to diagnose children at risk for adverse respiratory events.

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