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Discovery of the nitric oxide signaling pathway and targets for drug development

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1. ABSTRACT

Nitric oxide is a multifunctional signaling molecule, intricately involved with maintaining a host of physiological processes including but not limited to host defense, neuronal communication and the regulation of vascular tone. Many of the physiological functions first ascribed to NO are mediated through its primary receptor, soluble guanylyl cyclase. Endogenous production of NO is a highly complex and regulated process involving the 5-electron oxidation of L-arginine requiring numerous substrates and cofactors. The production of a highly reactive and diffusible free radical gas further complicates our established concept and model of specific and targeted receptor-ligand interaction to elicit cell signaling events. Hence there are many steps in the endogenous pathway for altered production of NO and subsequent activation of sGC that may be targets for drug development as well as other molecular targets for NO. The following review will highlight the current state of the art of NO-sGC research and illustrate disease processes which may benefit from novel drug development exploiting the NO-sGC pathway as well as NOS & cGMP-independent pathways.

2. INTRODUCTION

The free radical NO is a common air pollutant, a constituent of cigarette smoke, and a toxic gas, which appears in the exhaust of motor cars, causes acid rain, and destroys the ozone layer. The realization of such a molecule acting as an endogenous signaling molecule in biology and normal physiology has revolutionized conceptual reasoning in science and medicine over the past 30 years. Since the identification of endothelial derived relaxing factor as nitric oxide (1, 2), an enormous amount of research has been devoted to unraveling the complex chemistry and biochemistry of this simple molecule. At normal atmospheric pressure and temperature, NO is a gas that is moderately stable in aqueous media and functions as a biological messenger in physiological solutions. Several research fields converged to show that NO functions as a signaling molecule in endothelial and nerve cells and as a killer molecule, released from activated immune cells. Furthermore, NO is also implicated in the pathophysiology of many diseases, whereby either there is decreased bioavailability or production of NO, or there is an enormous prolonged over-production that exposes its toxic,

noxious properties (3). NO is one of the most important signaling molecules in mammalian physiology. To date there are over 86,000 published papers on NO with greater than 99% coming after the seminal discovery by Ferid Murad in 1977 (4). There are over 3000 publications on the NO and sGC, the primary molecular target or receptor of NO. A substantial knowledge of the NO signaling pathway has been gained during the past three decades. It is now appreciated that there are many physiological effects of NO that occur independent of activation of sGC or even independent of nitric oxide synthase, including post-translational modification of proteins, lipids and other biomolecules. We will discuss the current state of the art of NO based research and some of the potential molecular targets for drug development.

3. DISCOVERY OF NO

3.1. Nitric oxide and the nobel prize

On October 12, 1998, the Nobel Assembly awarded the Nobel Prize in Medicine or Physiology to scientists Robert Furchgott, Louis Ignarro, and Ferid Murad for their discoveries concerning nitric oxide as a signaling molecule in the cardiovascular system. In the 1970s, Ferid Murad and his colleagues demonstrated that soluble guanylate cyclase was stimulated by nitrogen-containing compounds, causing an increase in cGMP, which in turn brought about vascular relaxation (4, 5). Murad first showed that the activation of soluble guanylate cyclase by nitrovasodilators could occur via the formation of NO. He was fascinated by the idea that a gas and free radical could regulate smooth muscle function and proposed that hormones and other endogenous factors may also act through NO. Nitric oxide function as a messenger was proposed for first the time (6, 7).

In 1980, Furchgott and his colleagues published an article (8) underlining the obligatory role of endothelial cells in the acetylcholine-induced relaxation of arterial smooth muscle and recognized that vasodilation by bradykinin, histamine and ATP was due to the same relaxing substance, which they named endothelial derived relaxing factor (EDRF). It was Ignarro (1) who went on to conclude that EDRF from the artery and vein is either NO or a chemically related radical species in 1987 after Murad suggested (9) that EDRF was an "endogenous nitrovasodilator". Subsequently, Salvador Moncada's group revealed that NO release accounts for the biological activity of EDRF (2). In 1982, prior to the identification of EDRF, the endogenous activator of sGC in neuroblastoma cells was identified as L-arginine (10) and later it was recognized that NO is formed from L-arginine in the central nervous system (11). Even earlier however, nitrogen oxides were emerging as a central participant in the immune response. The investigations of NO as an immunoreactive compound began with the observation that high concentrations of urinary nitrates were excreted from a patient with infectious diarrhea (12). The source of these nitrogen compounds remained unclear until Stuehr and Marletta (13) demonstrated that serum and urinary nitrates were increased in normal mice after immunostimulation, but not in C3H/HeJ mice, which have a genetic alteration rendering their macrophages resistant to endotoxin. It was through this model that NO was identified as the intermediate compound in the L-arginine to nitrite/nitrate pathway. These observations, together with the discovery of the L-arginine:NO pathway in the vasculature, led to the investigations and subsequent discovery of the existence of this ubiquitous pathway in mammalian physiology. It was really the collective efforts of many research groups working in completely different fields that converged on a single pathway in multiple systems.

3.2. NO generation and Nitric Oxide Synthase

The demonstration of NO formation by an enzyme in vascular endothelial cells in 1987 has since had profound implications in research and medicine. NO was shown to be a potent vasodilator, inhibitor of platelet aggregation, and active species of nitroglycerin (4, 5) before the discovery of EDRF in 1980. Nitric oxide synthase (NOS) enzymes produce ·NO by catalyzing a five electron oxidation of a guanidino nitrogen of L-arginine (L-Arg). Oxidation of L-Arg to L-citrulline occurs via two successive mono-oxygenation reactions producing N^Ghydroxy L-arginine as an intermediate. Two moles of O_2 and 1.5. moles of NADPH are consumed per mole of ·NO formed (14) (Figure 1). NOS enzymes are the only enzymes known to simultaneously require five bound cofactors/prosthetic groups: FAD, FMN, heme, tetrahydrobiopterin and Ca^{2+}-calmodulin. There are three isoforms of NOS, the genetic sequence of each residing on three distinct chromosomes. One type is constitutive, cytosolic, Ca^{2+}/calmodulin dependent and releases NO for short time periods in response to receptor or physical stimulation. The NO released by this enzyme acts as a transduction mechanism underlying several physiological responses. The other enzyme type is induced after activation of macrophages, endothelial cells and a number of other cells by cytokines and once expressed, synthesizes NO for long periods of time. Furthermore, this enzyme is cytosolic, Ca^{2+} independent since calmodulin is already bound to the enzyme, and its induction is inhibited by glucocorticoids (15). Endothelial NOS/eNOS, neuronal NOS/nNOS which are both constitutively expressed in mammalian cells have now been well characterized in the cardiovascular system and nervous system respectively, and an inducible NOS/iNOS which was first believed to be expressed only when activated by an immune response. Now it is appreciated that eNOS is found in other cells and tissues besides the endothelium, iNOS is found constitutively in some tissues, and there are inducible forms of both eNOS and nNOS, adding confusion to the nomenclature as it was first described. In an attempt to clarify the nomenclature, the three different isoforms are now commonly referred to as NOSI, NOSII, and NOSIII for neuronal, inducible and endothelial isoforms, respectively, based on the order in which they were first purified and cloned.

All three isoforms of the enzyme function as a homodimer consisting of two identical monomers, which can be functionally and structurally divided into two major domains: a C-terminal reductase-carboxy domain, and an N-terminal oxygenase-amino domain (14, 16). The

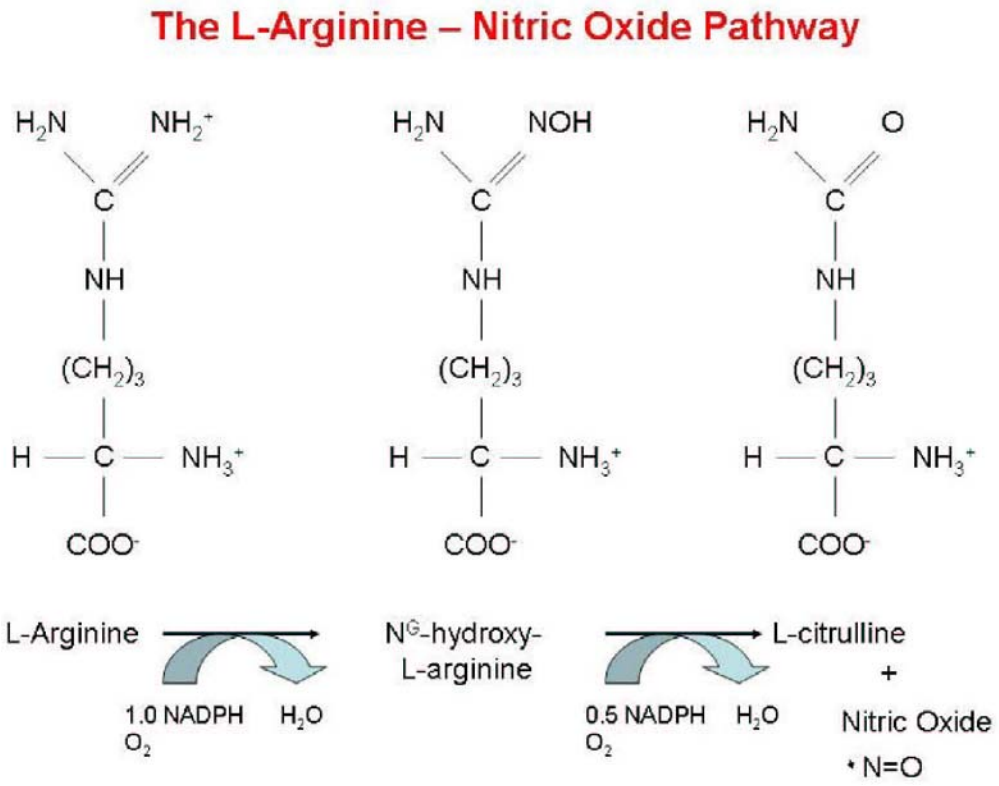

Figure 1. The L-arginine-nitric oxide pathway.

catalytically active isoforms exist as homodimers with tetrahydrobiopterin and heme serving to facilitate dimer formation (17, 18). The carboxy terminal domain has considerable homology between the isoforms, and is homologous to cytochrome P450 (19). However, the amino terminal domain has less homology. The homology of the three isoforms is about 50 to 60% while the homology of a given isoform between species can be as great as 85 to 92%. All isoforms are catalytically self-sufficient provided all required substrates and co-factors are available. Enzyme-bound calmodulin facilitates the transfer of electrons from NADPH to the flavoprotein domain of NOS and also from the flavins to the heme domain of NOS (20). These electrons are used to reduce the iron to the ferrous state so that it can bind oxygen, which is incorporated into the substrate, arginine, to generate NO plus citrulline. CaM also facilitates NADPH dependent reduction of cytochrome *c* and ferricyanide in BH_4 and heme depleted nNOS (20, 21). If any of the co-factors become limiting, then NO production from NOS shuts down, and in many cases NOS then produces superoxide instead. This is indeed a very complex and coordinated effort to enzymatically produce NO which normally proceeds very efficiently. However, in disease characterized by oxidative stress where cofactors become oxidized, NOS uncoupling, or conditions of hypoxia where oxygen is limiting, or increased formation of asymmetric dimethyl arginine occurs this process can no longer maintain NO production. One consequence is endothelial dysfunction where blood vessels make too little NO.

3.2.1.eNOS

NO is perhaps best characterized for its actions in the vasculature. In the vascular endothelium, agonists such as acetylcholine and bradykinin stimulate inositol 1,4,5-triphosphate or IP_3 production by activating the phosphoinositide second messenger system. IP_3 binds to receptors on the endoplasmic reticulum and causes Ca^{2+} release from intracellular stores (22). This transient elevation of intracellular Ca^{2+} promotes calcium binding to calmodulin, forming a complex that is a crucial cofactor for constitutive NOS activity (23). eNOS produces modest amounts of NO until the calcium concentration decreases. This rapid and transient production of NO by eNOS allows NO to function in maintaining basal vascular tone (22). NO, once produced, then diffuses into nearby target cells to interact with specific molecular targets. NO regulates protein activity by reversibly binding to available acceptor functionalities, including heme iron and thiols (24). The interaction between NO and the enzyme guanylyl cyclase, which mediates target cell responses such as vascular smooth muscle relaxation and platelet inhibition, has been well characterized (25, 26). After entering the target cell, NO binds to the heme moiety of guanylyl cyclase and activates the enzyme by inducing a conformational change that displaces iron out of the plane of the porphoryrin ring (27). Guanylyl cyclase then catalyzes the production of cyclic GMP from GTP to elevate cyclic GMP. Cyclic

GMP then triggers a cascade of intracellular events that culminate in a reduction in calcium-dependent vascular smooth muscle tone by inactivating myosin light chain kinase or MLCK (28, 29). MLCK normally phosphorylates the regulatory set of myosin light chain. This phosphorylation event activates cross-bridge cycling and initiates contraction (30). cGMP modulates MLCK activity by activating a cGMP-dependent protein kinase that phosphorylates MLCK (31). Phosphorylation of MLCK diminishes its affinity for calmodulin and, as a consequence, decreases the phosphorylation of myosin light chain, which in turn stabilizes the inactive form of myosin. In this manner, cGMP may induce vasorelaxation by indirectly decreasing myosin light chain-dependent myosin activation. Infusion of a NOS inhibitor causes a sustained hypertension that is reversible by administration of excess L-arginine (32, 33). NO is now considered the endogenous nitrovasodilator. However, the physiological effects of NO extend well beyond the vascular endothelium. Radomski *et al* (34) has shown that human platelets contain a NOS that is activated when platelets are stimulated to aggregate. Thus, platelets themselves also have the enzymatic capacity to synthesize NO with both a constitutive and inducible form of NOS identified in human megakaryoblasts (35). NOS activity increases with platelet activation, and this response appears to modulate platelet aggregation, thereby potentially limiting the self-amplification of platelet thrombus formation *in vivo*. It was also reported early on that human neutrophils inhibit platelet aggregation by releasing an NO-like factor (36). These anti-thrombotic properties of the endothelium may be a consequence of the synergistic action of NO and prostacyclin. Radomski *et al* (37) has shown the synergistic antiaggregatory effects of NO and prostacyclin on platelets. NO and prostacyclin may act in concert to oppose local vasospasm or thrombus formation at sites where platelets aggregate and the coagulation cascade is activated. It has also been proposed that the antiplatelet effects of endothelial-derived NO may prevent thromboembolic events during administration of potent prostacyclin inhibitors such as aspirin (38). In this regard NO acts as an anti-inflammatory molecule.

3.2.2. iNOS

It is the inducible isoform of NOS that is responsible for macrophage NO production. Inducible NOS has been found in many cell types including macrophages (39), and neutrophils (40) and is immunologically activated by exposure to bacterial endotoxin or pro-inflammatory cytokines such as interleukin-1, or interferon-gamma (41, 42) and tumor necrosis factor. Inducible NOS activity is regulated at the transcriptional level (43) and is not affected by changes in intracellular calcium concentrations nor is dependent on the cofactors NADPH and tetrahydrobiopterin. iNOS induction after cytokine exposure requires several hours and once induced, can generate far greater amounts of NO per mole of enzyme than the constitutive NOS (44). Macrophages stimulated with interferon gamma, and lipopolysaccharide have NOS messenger ribonucleic acid present by 2 hours, and NOS protein by 4 hours. The presence of NOS-2 message or protein can serve as biomarker for inflammation in tissues. The NOS protein may remain present for several days (45). The expression of the inducible form of NOS, but not nNOS or eNOS is specifically inhibited by glucocorticoids (46). Although cultured macrophages can produce large amounts of NO, multiple cytokines are required to achieve maximal induction. Interferon gamma or LPS alone can induce noncytotoxic levels of NO. However, when cytokines work synergistically, they can induce cytotoxic levels of NO. Priming cell cultures with IFN-gamm prior to exposure to other agents result in enhanced NO production. The synergistic activity between IFN-gamma and microbial products is mediated by tumor necrosis factor-alpha. At these high concentrations and flux rates, NO is cytotoxic and plays a key role in the immune response of macrophages to bacteria and other pathogens. Tumor necrosis factor acts in an autocrine fashion to amplify the synthesis and release of NO by primed macrophages; however, it cannot stimulate NO synthesis by itself. Antimicrobial activity and NO production parallel tumor necrosis factor activity (47). A strong correlation exists between antimicrobial activity and production of L-Arg-derived NO by cytokine-activated cells observed during *in vitro* studies (47, 48). The precise mechanism of NO-mediated bactericidal and tumoricidal activity is unknown, but these observations suggest that macrophage NO production contributes to nonspecific immunity (49). NO from activated macrophages may be responsible for the profound loss of vascular tone seen in septic patients. It is this relative overproduction of NO and the subsequent vasodilation that are thought to mediate NO's pathophysiologic role during sepsis and multiorgan failure during hypovolemia and hypoxia. Because NO is known to directly or indirectly modulate the inflammatory response as well as to play an important role in pain perception, there is also an increasing interest in defining the role that NO may play in the pathogenesis of chronic inflammation and the associated chronic pain (50). Despite the rapid progress in our understanding of the complex physiological and pathophysiological processes involving NO, uncertainties remain with regard to the critical cellular targets of NO cytotoxicity, the relative importance of different NO redox states and carrier molecules, and the importance of the NO antimicrobial system in human phagocytes. Ultimately, the immunoregulatory and vasoregulatory activities of NO may prove to be just as important as its antimicrobial properties during infection.

3.2.3. nNOS

In the central nervous system, NO is a neurotransmitter that underpins several functions, including the formation of memory. Recurrent as in other organ systems, this NO pathway may also play a role in the pathology of the central nervous system. The NOS isoform in the nervous system is activated by glutamate acting on N-methyl-D-aspartate receptors. In a matter of seconds the glutamate-induced increase in intracellular calcium concentration activates NOS via the calcium/calmodulin interaction as previously described. Under most circumstances, eNOS and nNOS are constitutive in the sense that their activation does not require new enzyme synthesis. However, both forms of NOS are inducible in

that new enzyme synthesis occurs primarily under conditions of traumatic or pathological insult. The calcium influx that accompanies prolonged NMDA receptor activation is associated with degeneration of the neurons (51). It is likely that excessive NMDA receptor activation, with the consequent increase in calcium, contributes to glutamate neurotoxicity by enhancing NO which reacts with superoxide and then performs its nitrosative chemistry (52). The dichotomy of both the protective and deleterious actions of NO is again revealed in the nervous system.

In the periphery, there is a widespread network of nerves, previously recognized as nonadrenergic and non cholinergic, that operate through a NO-dependent mechanism to mediate some forms of neurogenic vasodilation and regulate various gastrointestinal, respiratory, and genitourinary tract functions as well as autonomic innervation of smooth muscle in the gastrointestinal tract, the pelvic viscera, the airways, and other systems (53). In the stomach, a decrease in gastric mucosal blood flow has been identified as a prerequisite to the development of acute erosions and stress ulcers (54, 55). NOS has been detected in the gastric mucosa, and NO appears to play a role in protecting the gastric mucosa during physiologic stress by acting as an endogenous vasodilator and thus supporting mucosal blood flow (56). The exact mechanism of NO's protective effect is unclear, but may relate to vasodilation, inhibition of platelet aggregation in the gastric microvasculature, or a protective effect on the epithelial cells themselves (57).

3.3. Nitrovasodilators

In contrast with the short research history of the enzymatic synthesis of NO, the introduction of nitrate-containing compounds for medicinal purposes marked its 150th anniversary in 1997. Organic nitrates are simple nitric and nitrous acid esters of alcohols. Clinically used $RONO_2$ compounds include nitroglycerin or GTN, pentaerythrityl tetranitrate, isosorbide dinitrate, and triethanolamine trinitrate biphosphate. GTN is the most common organic nitrate used clinically and was synthesized by Sombrero in 1847 (58). After his discovery of dynamite, Alfred Nobel routinely suffered from angina pectoris and was prescribed nitroglycerin for his chest pain in 1895 (58). Almost a century later, organic nitrates and their gaseous metabolic end product, NO, were implicated in a vast array of biologically diverse activities (6, 7). GTN has been used for well over a century to treat angina pectoris, myocardial infarction, and heart failure, and continues to remain a mainstay of therapy in the management of these conditions. In 2001, GTN was prescribed for the treatment of angina more than 2 million times in the United States alone (59). In addition to its anti-anginal benefits, GTN has been found to induce ischemic preconditioning (60) a physiologic phenomenon that can protect the heart from lethal ischemia. Recently, GTN has also been demonstrated to be beneficial in noncardiovascular contexts, including pain management (61, 62), treatment of chronic anal fissure (63), preservation of organs for transplantation (64, 65), and overall response and time to progression in patients with inoperable small cell cancer (66). Collectively these reports highlight the multitude of therapeutic applications of NO and the subsequent cell signaling pathways.

It has been suggested that multiple pathways contribute to NO formation from organic nitrates *in vivo*; however, the metabolic mechanism is still poorly understood. Many different enzyme systems have been proposed including cytochrome P450, glutathione S-transferase and other glutathione and NADH/NADPH-dependent activities (67-69). It is not until recently, that researchers at Duke University Medical Center have been able to make significant progress in the identification of the enzymatic mechanism of GTN bioactivation, even though the compound's discovery occurred over 150 years ago. Chen *et al* (70) purified a nitrate reductase known as mitochodrial aldehyde dehydrogenase that specifically catalyzes GTN to form 1,2-glyceryl dinitrate and nitrite, which are eventually converted to NO. This finding demonstrates that the biotransformation of GTN occurs predominantly in mitochondria and indicates that attenuated biotransformation of GTN by mtALDH underlies the induction of nitrate tolerance. Although the study has not clarified every aspect of the pathway, patients taking organic nitrates esters for the treatment of acute ischemic syndromes and congestive heart failure will benefit from awareness of the contraindicated effect of certain classes of drugs that inhibit mtALDH activity, such as sulfonylurea antidiabetics, chloral hydrate, and acetaminophen. In addition to the many enzymatic pathways, organic nitrates may also undergo non-enzymatic metabolism reacting with thiol-containing molecules, including cysteine, glutathione, and sulfhydryl proteins (71, 72).

4. NO BASED CELL SIGNALING

4.1. cGMP dependent signaling

Many of the physiological functions of NO in the cardiovascular, neuronal, gastrointestinal and other systems are mediated through its primary receptor, soluble guanylyl cyclase. sGC is a heme-containing, heterodimeric NO receptor. Soluble GC consists of two subunits, α and β, which make up the active enzyme. Four sGC isoforms, products of four genes, have been identified so far: α_1, α_2, β_1 and β_2. Only α_1/β_1 and α_2/β_1 heterodimers are activated by NO (73). The α_1/β_1 sGC is the most abundant isoform and is distributed ubiquitously in mammalian tissues with the highest levels of mRNA in brain, lung, heart, kidney, spleen and muscle (74). Vascular smooth muscle and endothelial cells express predominantly α_1 and β_1 subunits (75). The functional importance of α_1/β_1 sGC was demonstrated by the significantly decreased relaxing effects of major vasodilators such as acetylcholine, NO, YC-1 and BAY 41-2272 in the α_1 sGC knockout mice of both genders. (76). The heme-containing heterodimer sGC converts guanosine triphosphate into the secondary messenger guanosine 3':5'-cyclic monophosphate. Through the production of cGMP, sGC can exert many physiological effects such as mediating vascular smooth muscle tone and motility, phototransduction, and maintaining fluid and electrolyte homeostasis. To do this, cGMP acts directly with downstream effectors such as the family of cGMP-dependent protein kinases, cyclic nucleotide-gated channels, and cGMP-regulated phosphodiesterases (77-79). The sGC activity increases

more than 200 fold in response to NO (80, 81). This signal is quickly removed by the action of phosphodiesterase 5A enzyme.

4.2. PDE inhibitors

Phosphodiesterases (PDEs) are intracellular enzymes that specifically catalyze the hydrolysis of the second messengers cAMP and cGMP to the inactive metabolites AMP and GMP. Among the 11 families of PDEs a number are able to hydrolyze cGMP, but only PDE5 exclusively catalyses the breakdown of cGMP (82). By counterbalancing cGMP production by guanylate cyclases, PDE5 is able to decrease cGMP levels very effectively. Thus PDE5 inhibition increases intracellular cGMP levels and initiates a cGMP-driven cascade of reactions. Ultimately, these pathways decrease intracellular calcium levels, thereby promoting relaxation of smooth muscle cells and a variety of other calcium-dependent processes (77). Since inhibitors of PDE5 raise intracellular cGMP levels, the effects will be much more pronounced under conditions when cGMP formation is already increased. Strong evidence to support this concept is the highly efficacious treatment of erectile dysfunction with PDE5 inhibitors (83). Phosphodiesterase type 5A selectively hydrolyzes cyclic GMP. Inhibitors of PDE5A such as sildenafil are widely used to treat erectile dysfunction, but growing evidence supports important roles for the enzyme in both the vasculature and heart. These agents may also be beneficial in other disorders such as pulmonary hypertension, Raynaud's syndrome, etc.

4.3. Splice forms as novel genetic regulators of sGC

Recently, the vital importance of sGC for mammalian physiology was directly confirmed by generation of sGC knockout mice (76, 84, 85). The absence of sGC protein resulted in a significant increase in blood pressure, complete loss of NO-dependant aortic relaxation and platelet aggregation in knockout animals, which died prematurely at the age of 4 weeks due to severe gastrointestinal disorders (84). sGC function is affected not only by NO, but also by regulation of the expression of sGC subunits at transcriptional and post-transcriptional levels. The steady state mRNA levels of α_1 and β_1 subunits decrease with hypertension, ageing and vary during embryonic development (86). The expression of sGC subunits is regulated by estrogen (87), cAMP-elevating compounds (88, 89), cytokines (90) and NO donors (91). Subcellular localization of sGC and its activity can also be affected in proliferating tissue (92) by protein interactions and phosphorylation (86). In mammals, the alternative splicing for α_2 subunit generates a dominant negative variant (93). Existence of splice forms for β_1 and β_2 subunits has been also demonstrated (95-97). Recently, a shortened α_1 sGC transcript, which lacks the predicted translation site in exon 4, has been found and its expression was correlated with lower sGC activity in several cell lines (97). Gene therapy with α_1 β_1 subunits may provide future therapeutic utility.

4.4. Allosteric effectors of sGC

There are also many allosteric regulators of sGC which provide NO independent activation. Impaired bioavailability and/or responsiveness to endogenous NO has been implicated in the pathogenesis of cardiovascular and other diseases. Current therapies that involve the use of organic nitrates and other NO donors have limitations, including non-specific interactions of NO with various biomolecules, lack of response and the development of tolerance following prolonged administration. Compounds that activate sGC in an NO-independent manner might therefore provide considerable therapeutic advantages (98). The recent discoveries of compounds that stimulate or activate sGC independently of NO release allow this venerable pharmacological target to be approached from a completely different perspective. NO-independent but heme-dependent stimulators of sGC, as well as NO- and heme-independent sGC activators, are emerging as valuable tools that could help to elucidate the physiology and pathophysiology of the NO–sGC–cGMP pathway in more detail. The first group of these compounds comprises the heme-dependent sGC stimulators including YC-1, BAY 41-2272, BAY 41-8543, A-350619 and CFM-1571. These compounds show a strong synergy with NO and a loss of activation after oxidation or removal of the prosthetic heme moiety of sGC. The mechanism of YC-1-dependent activation of sGC is not completely understood because some aspects of it are unsettled. YC-1 alone activates the enzyme only 10-fold, but it potentiates the CO- and NO-dependent activation of sGC, resulting in stimulation of the highly purified enzyme that may be several hundred- to several thousandfold. More recent studies reveal that YC-1 can activate sGC with both heme-dependent and heme-independent mechanisms (99). The second group comprises the sGC activators, including BAY 58-2667 and HMR-1766, which have been found to require neither NO nor heme, and demonstrate even more pronounced action on the oxidized form of sGC (98).

5. cGMP INDEPENDENT SIGNALING

5.1. Nitrite and nitrate

Inorganic nitrite (NO_2^-) and nitrate (NO_3^-) are known predominantly as undesired residues in the food chain or as inert oxidative end products of endogenous NO metabolism. However, from research performed over the past decade, it is now apparent that nitrate and nitrite are physiologically recycled in blood and tissues to form NO and other bioactive nitrogen oxides (100-104). As a result, they should now be viewed as storage pools for NO-like bioactivity to be acted upon when enzymatic NO production from NOS is insufficient. The recognition of this mammalian nitrogen cycle has led researchers to explore the role of nitrate and nitrite in physiological processes that are known to be regulated by NO (105). Inorganic nitrite and nitrate should not be confused with organic nitrites and nitrates described above. Inorganic nitrite and nitrate are naturally occurring salts, whereas organic nitrites and nitrates are synthetic compounds with an ONO and ONO_2 functional group respectively to a parent molecule. Inorganic nitrite and nitrate are simply referred to as nitrite and nitrate. Nitrite is an oxidative breakdown product of NO that has been shown to serve as an acute marker of NO flux/formation (106). Nitrite has recently moved to the forefront of NO biology (107), as it

represents a major storage form of NO in blood and tissues (108). In addition to the oxidation of NO, nitrite is also derived from reduction of salivary nitrate by commensal bacteria in the mouth and gastrointestinal tract (109, 110) as well as from dietary sources such as meat, vegetables and drinking water. Much of the recent focus on nitrite physiology is due to its ability to be reduced to NO during ischemic or hypoxic events (108, 111-113). Nitrite reductase activity in mammalian tissues has been linked to the mitochondrial electron transport system (114-116), protonation (112), deoxyhemoglobin (117), deoxymyoglobin (118) and xanthine oxidase (119-121). Nitrite can also transiently form nitrosothiols (RSNOs) under both normoxic and hypoxic conditions (111) and a recent study by Bryan *et al* demonstrates that steady state concentrations of tissue nitrite and nitroso are affected by changes in dietary nitrite and nitrate intake (101). Furthermore enriching dietary intake of nitrite and nitrate translates into significantly less injury from heart attack (100). Previous studies demonstrated that nitrite therapy given intravenously prior to reperfusion protects against hepatic and myocardial I/R injury (121, 122). Additionally, experiments in primates revealed a beneficial effect of long-term application of nitrite on cerebral vasospasm (123). Moreover, inhalation of nitrite selectively dilates the pulmonary circulation under hypoxic conditions *in vivo* in sheep (124). Topical application of nitrite improves skin infections and ulcerations (125). Furthermore, in the stomach, nitrite-derived NO seems to play an important role in host defense (126, 127) and in regulation of gastric mucosal integrity (128). All of these studies together along with the observation that nitrite can act as a marker of NOS activity (106) opened a new avenue for the diagnostic and therapeutic application of nitrite, especially in cardiovascular diseases, using nitrite as marker as well as an active agent. Oral nitrite has also been shown to reverse L-NAME induced hypertension and serve as an alternate source of NO *in vivo* (129). In fact a recent report by Kleinbongard *et al.* (130) demonstrates that plasma nitrite levels progressively decrease with increasing cardiovascular risk. Since a substantial portion of steady state nitrite concentrations in blood and tissue are derived from dietary sources (101), modulation of nitrite and/or nitrate intake may provide a first line of defense for conditions associated with NO insufficiency (108).

The bioactivation of nitrate from dietary or endogenous sources requires its initial reduction to nitrite, and because mammals lack specific and effective nitrate reductase enzymes, this conversion is mainly carried out by commensal bacteria in the gastrointestinal tract and on body surfaces (131, 132). Dietary nitrate is rapidly absorbed in the upper gastrointestinal tract. In the blood, it mixes with the nitrate formed from the oxidation of endogenous NO produced from the NOS enzymes. After a meal rich in nitrate, the levels in plasma increase greatly and remain high for a prolonged period of time (plasma half-life of nitrate is 5–6 hours). The nitrite levels in plasma also increase after nitrate ingestion (133). Although much of the nitrate is eventually excreted in the urine, up to 25% is actively taken up by the salivary glands and is concentrated up to 20-fold in saliva (131, 133). One in the mouth, commensal facultative anaerobic bacteria reduce nitrate to nitrite during respiration by the action of nitrate reductases (132, 134). Human nitrate reduction requires the presence of these bacteria — suggesting a functional symbiosis relationship — as mammalian cells cannot effectively metabolize this anion (Figure 2). The salivary nitrate levels can approach 10 mM and nitrite levels 1–2 mM after a dietary nitrate load (131). When saliva enters the acidic stomach (1–1.5 liter per day) much of the nitrite is rapidly protonated to form nitrous acid, HNO_2; pKa ~ 3.3., which decomposes further to form NO and other nitrogen oxides (103, 104). Most recently it has been reported that dietary nitrate reduces blood pressure in healthy volunteers (135, 136). For a comprehensive review on this pathway, please see Lundberg et al (105). Nitrite and nitrate therapy or supplementation may restore NO homeostasis from endothelial dysfunction and provide benefit in a number of diseases characterized by NO insufficiency. If so, this will provide the basis for new preventive or therapeutic strategies and new dietary guidelines for optimal health. From a public health perspective, we may be able to make better recommendations on diet and dramatically affect the incidence and severity of cardiovascular disease and the subsequent clinical events.

The juxtaposition of the physiologic requirement for NO or nitrite as a pleiotropic signaling molecule and the toxic effects of either excessive local NO overproduction or formation of carcinogenic nitrosamines necessitate defining the contexts of these actions. The ascription of risk to excessive nitrate and nitrite consumption, as observed with nitrate/nitrite contaminated water from agricultural sources or from nitrate-preserved meats, presumes enhanced risk at normal dietary consumption levels. However, consideration of dietary sources of nitrates and nitrites indicates that humans may consume amounts in greater concentrations than those considered to increase risk of carcinogenic nitrosamine formation, >500 ppm. The primary dietary sources of nitrates and nitrites include plants, vegetables and a few fruits, processed and cured meat, fish and poultry to which nitrites have been added. Plant foods are the primary sources of nitrate, while processed and cured meats are the primary sources of nitrites (137). While estimates of daily nitrate and nitrite intake are reported to vary between 53 and 350 mg/day and between 0 and 20 mg/day, respectively, unpublished analyses (Bryan, NS, Hord, N) indicate these may dramatically underestimate intakes of nitrates from specific fruit and vegetable juices and food supplements. As such, reconsideration of normal or "usual" nitrate and nitrite consumption levels is warranted. In light of these findings, the risks associated with these intake levels should be balanced against the potential preventive and therapeutic benefits of nitrates and nitrites from plant and human food sources. The potential benefits and risks of these dietary components should be understood within specific healthy and at-risk populations rather than relegating these essential substrates for physiologic signaling to categorically toxic compounds. Careful consideration of the physiologic contexts in which these certain foodstuff produce NOx metabolites will yield a more enlightened view of their essentiality for cardiovascular function and potential risks of carcinogenicity due to nitrosamine formation. Although

Nitric oxide signaling pathway and its targets

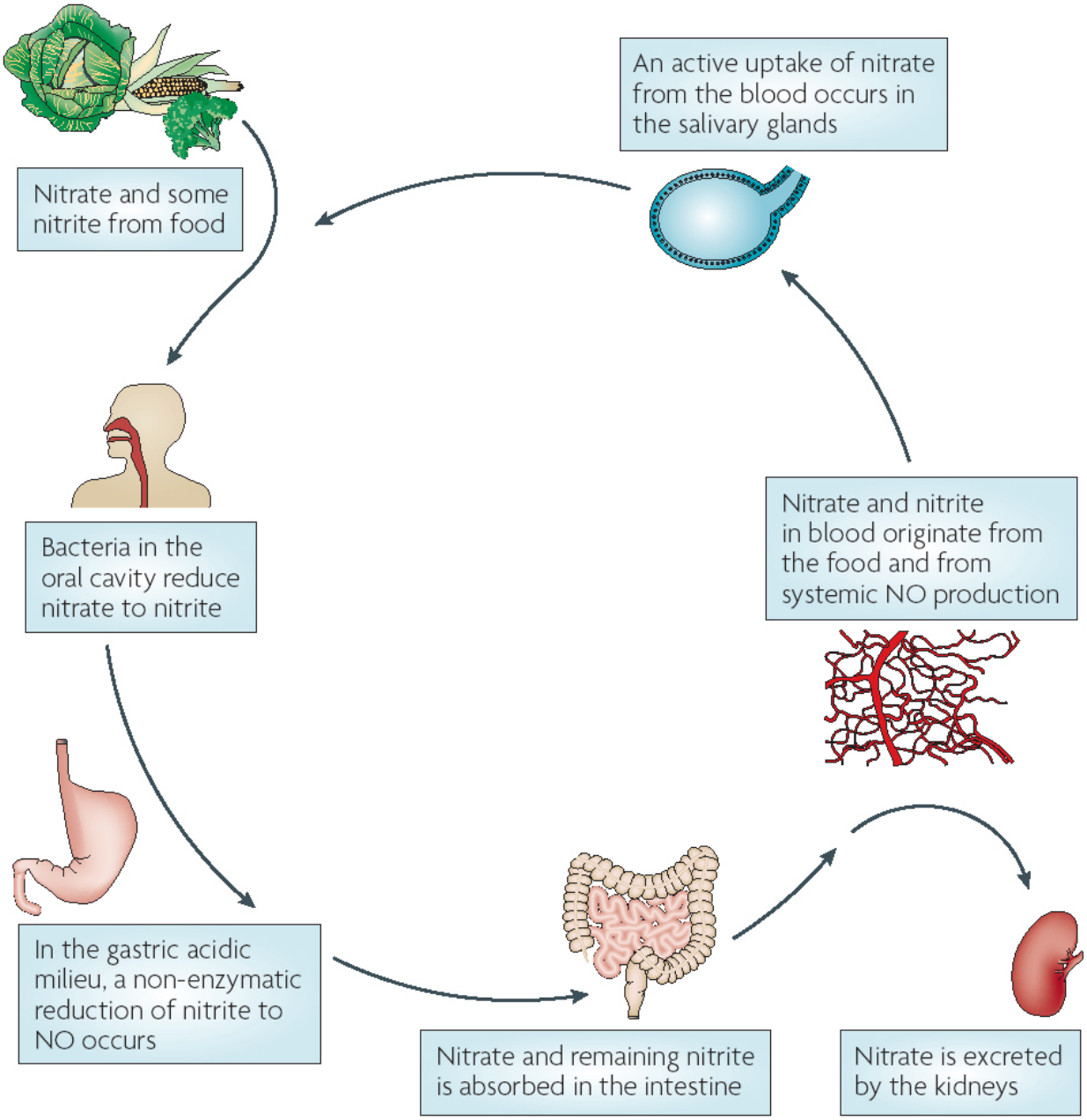

Figure 2. Ingested inorganic nitrate from dietary sources is rapidly absorbed in the small intestine. Although much of the circulating nitrate is eventually excreted in the urine, up to 25% is actively extracted by the salivary glands and concentrated in saliva. In the mouth, commensal facultative anaerobic bacteria effectively reduce nitrate to nitrite by the action of nitrate reductase enzymes. Nitrate reduction to nitrite requires the presence of these bacteria, as mammalian cells cannot effectively metabolize this anion. In the acidic stomach, nitrite is spontaneously decomposed to form nitric oxide and other bioactive nitrogen oxides, which regulate important physiological functions. Nitrate and remaining nitrite is absorbed from the intestine into the circulation and can convert to bioactive NO in blood and tissues under physiological hypoxia. Reproduced with permission from 105.

there have been numerous reports on the association of N-nitrosamines and human cancers (138, 139) a causative link between nitrite exposure and cancer is still missing (140). Furthermore, a two years study on the carcinogenicity of nitrite by NIH has conclusively found that there was no evidence of carcinogenic activity by sodium nitrite in male or female rats or mice (141). In fact increasing steady state nitrite and nitrate

concentrations in blood is a natural, adaptive, physiological response in humans (142).

5.2. S-nitrosothiols

S-nitrosothiols are thio-esters of nitrite with the general structure R-S-N=O; naturally occurring examples include S-nitrosocysteine, S-nitrosoglutathione and S-nitrosoalbumin, in which R is an amino acid, polypeptide and protein respectively. S-nitrosothiols can be synthesized from the reaction between thiols and nitrous acid in extremely acidic condition. S-nitrosation is a ubiquitous redox-related modification of cysteine thiol which transduces NO bioactivity (143). As early as 1981, Ignarro's group demonstrated that the bioactivities of certain pharmacological nitrogen oxide donors were attributed to reactions with cellular thiols (144), which is several years before making the observation that NO is actually synthesized endogenously in mammalian cells. There is now a large body of literature that implicates S-nitrosothiol as an intermediate in nitric oxide–dependent and guanylyl cyclase–independent signaling processes. Reactive protein thiols are becoming regarded as major intracellular target of nitric oxide (145). Nitrite is in steady state equilibrium with S-nitrosothiols (101, 146). Dysregulation of protein S-nitrosation is associated with a growing list of pathophysiological conditions (145) and altered blood levels of RSNO have been associated with impaired clinical outcome in patients with CVD (147, 148). Although RSNOs such as S-nitroso-N-acetyl-penicillamine and S-nitrosoglutathione are commercially available but none of them has been used therapeutically due to the unpredictable rate of decomposition in the body. In contrast, an increasing number of proteins have been found to undergo S-nitrosylation *in vivo*. These S-nitrosothiol proteins have demonstrated an important role in many physiological as well as pathological processes (149).

Reaction of NO, or more correctly, any of several NO-derived species with Cys residues in target proteins is termed s-nitrosation. This post-translational protein modification was already recognized 10 years ago for albumin, by authors who portended its potential generality and importance in cell-signaling (150). *S*-nitrosation has since been implicated in the control of a wide array of protein functions and cell activities (145, 151), Among the growing list of proteins whose activities are regulated by s-nitrosation are included, ion channel proteins, kinases, proteolytic enzymes, transcription factors and proteins involved in energy transduction (145). Through s-nitrosation of these proteins, nitric oxide has been shown to regulate apoptosis, G-protein-coupled receptor based signaling, vascular tone and inflammatory responses (151-154). However cellular signaling events are dictated by specificity and a transient modification that can quickly and specifically be inactivated to turn off the signal. Whereas s-nitrosation produces the effects of nitric oxide inside the body, denitrosation pathways inside the cells terminate the cellular effects of nitric oxide. Denitrosation of *S*-NO-proteins in cells can be accomplished by simple chemistry, wherein intracellular glutathione or other intracellular thiols, including other protein Cys residues, act as acceptors and effectively remove nitrosyl or nitroso groups via transnitrosation reactions. Decomposition can also be enhanced through heat, UV light and metal ion, particularly copper, dependent, which results in the formation of NO or NO^+ and the corresponding disulphide. The reaction can be enhanced by the presence of ascorbate, thiols, high oxygen tension and pH > 3. Additionally, ascorbate, and metal ions (155) can promote *S*-NO-protein decomposition (156, 157). In this system, the rate of *S*-NO-protein decomposition would be modulated by changing levels of intracellular thiols; in other words, conditions that promote glutathione oxidation in cells would enhance steady-state levels of protein *S*-nitrosation. This mechanism would put protein *S*-nitrosation under the control of environmental changes that affect the intracellular redox milieu (149).

New knowledge and recent discoveries in the NO field provide insights as to how specificity for *S*-nitrosation of mammalian cell proteins is achieved through formation to degradation by S-nitrosoglutathione reductase (158). S-nitrosoglutathione reductase or GSNOR, a member of alcohol dehydrogenase family, has been shown to be the primary pathway through which cells denitrosate intracellular proteins (158). This enzyme that is evolutionarily conserved in bacteria and humans and has been shown to catalyze the selective reduction of GSNO at the expense of reduced nicotinamide adenine dinucleotide, forming glutathione disulfide and ammonia. Deletion of the gene encoding GSNOR in both mice and yeast resulted in increased levels of both intracellular GSNO and *S*-NO-proteins. This finding identifies the first biologically relevant mammalian denitrosase and confirms that levels of GSNO determine intracellular levels of *S*-NO-proteins. GSNOR brings about denitrosation of intracellular proteins by the reduction of s-nitrosoglutathione, a nitric oxide metabolite arising from the reaction of glutathione with s-nitrosated proteins or nitric oxide (159-161). Owing to its ability to regulate the s-nitrosation of intracellular proteins, GSNOR has become an important target for developing agents that modulate nitric oxide bioactivity inside the cells. The therapeutic potential of preventing the breakdown of s-nitrosothiols via inhibition of GSNOR has been demonstrated in the mice model of asthma. Mice lacking GSNOR were found to resist airway hyperresponsivity owing to higher GSNO concentrations in bronchial fluid and diminished tachyphylaxis to β-agonists owing to s-nitrosation of G-protein coupled receptor kinase (153, 162). Development of specific drugs which modulate the steady state levels of RSNO will likely have therapeutic benefit.

5.3. Nitrotyrosine

Vertebrates constantly generate reactive oxygen species which include superoxide anion, hydroxyl radicals and hydrogen peroxide as a consequence of aerobic metabolism. The overproduced ROS have detrimental effects on cellular function in large part through the oxidation of proteins. The discovery of nitric oxide (163) focused attention on reactive nitrogen species, which includes NO that can under go interconversion to form NO^+ or nitrosonium and NO^- or nitroxyl anion. NO reacts with $O_2^{\bullet-}$ to form peroxynitrite that can further form peroxynitrous acid, a very unstable and reactive oxidizing

species. Involvement of ONOO is the most widely studied mechanism of protein nitration (164), and the formation of NO_2-Tyr has been detected in various pathological conditions including atherosclerosis, myocardial infarction, myocarditis, heart failure, shock, diabetic complication and neurodegenerative and inflammatory disorders (165). Approximately 80 key proteins have been reported to be modified by peroxynitrite with potential relevance to various human diseases. Peroxynitrite may also be an important trigger of cell death, both apoptotic and necrotic (164). Substantial evidence has emerged which revealed a very close association between the formation of NO_2-Tyr and the presence of activated granulocytes containing peroxidases, such as MPO (166-169). MPO-generated oxidants enhance selective cleavage of plasmalogens, a predominant membrane constituent in many cells of the cardiovascular system, and result in the release of both α-chloro fatty aldehyde species, such as 2-chlorohexadecanal that may act as a phagocyte chemoattractant, and unsaturated lysophosphatidylcholine, an as-yet unrecognized proatherogenic molecule because it elicits increased surface expression of P-selectin on human coronary arteries (170). MPO-generated reactive species participate in the induction of foam cell formation, endothelial dysfunction, and development of vulnerable plaque.

Involvement of $ONOO^-$ in inflammatory diseases has been determined by detection of nitrotyrosine formation in various inflamed tissues (171, 172). Nitrite is another major oxidation product derived from NO, and can be oxidized by peroxidase to form a reactive nitrogen intermediates such as nitrogen dioxide that is capable of nitrating tyrosine (173). MPO and MPO derived hypochlorous acid have been implicated in the pathogenesis of atherosclerosis (174). Carr and Frei have revealed that physiological concentrations of nitrite inhibit MPO mediated modification of LDL (175) providing a means to interrupt the process. These data also demonstrate the first line of evidence of nitrite acting in an "antioxidant" capacity in atherosclerosis. Nitrite dependent decrease in oxidative modification of LDL may translate into decreased LDL uptake by macrophages, thus attenuating the formation of lipid-laden foam cells, the hallmark of atherosclerotic lesions. This may represent a novel mechanism by which metabolites of NO may exert an antiatherogenic effect (175). MPO and other peroxidase are also able to use halides and pseudohalides as co-substrates to generate the reactive intermediate hypochlorous acid, which further forms nitryl chloride that results in formation of NO_2-Tyr (169). Thus, it is likely that multiple pathways participate in tyrosine nitration.

The average adult human body contains ~3-4 g of iron, and 65% of it is bound to hemoglobin. Ten percent is a constituent of myoglobin, cytochromes, and iron-containing enzymes, and the rest is bound to the iron storage proteins (176). The higher contents of heme iron in certain tissues such as heart and vascular smooth muscle cells could serve as a biological base for iron toxicity on free radical-mediated tissue damage, including formation of nitrotyrosine. The first evidence (177) that muscle contraction can be altered by nitration of key proteins is suggested by the study of skeletal muscle sarcoplasmic-reticulum Ca^{2+}-ATPase isoforms 2, which suggests that tyrosine nitration may affect Ca^{2+}-ATPase activity. We have tested the hypothesis that both free heme and iron play a key role in NO_2-Tyr formation and demonstrated that isolated heme and free metals are capable of tyrosine nitration in the presence of hydrogen peroxide and nitrite (178). Summarizing current information briefly, hemin or chelated iron reacts with H_2O_2 to form the ferryl π-cation radical complex, which then oxidizes both nitrite and tyrosine to form nitric dioxide radical and tyrosine radical, respectively. These nitrating species nitrate tyrosyl residues in proteins to form NO_2-Tyr. In the presence of heme, H_2O_2 and NO_2^-, considerable protein nitration was observed in homogenates of heart and skeletal muscle but not in the brain, liver, and kidney. Increasing the exogenous heme concentration, however, promoted nitration in the brain, liver, and kidney. To further elucidate the role of endogenous heme in NO_2-Tyr formation, we compared protein nitration in homogenates of heart under conditions of with or without exogenous heme. A significant protein nitration could be induced by application of NO_2^- and H_2O_2 alone into the heart homogenate, and the addition of exogenous heme did not further increase the level of NO_2-Tyr formation. Thus, hemoprotein rich tissues such as cardiac muscle are vulnerable to protein nitration in pathological conditions characterized by the overproduction of H_2O_2 and NO_2^- or nitric oxide. Our understanding of this molecular process is allowing us to adopt principles of anti-oxidation therapy that may be more beneficial for patients with cardiovascular disorders. Recognizing and isolating specific molecular targets for drug development against protein nitration will require a better understanding of more specific mechanisms in individual disease processes.

6. RATIONALE DESIGN AND DEVELOPMENT OF THERAPEUTICS

6.1. Endothelium Centered healthy environment for vasculature

Our understanding of the process leading to cardiovascular diseases is allowing us to adopt principles of therapy that may be more beneficial for patients. For instance, hypertension, particularly in high-risk patients, is a result of loss of balance and the absence of the ability to vasodilate normally. The interaction between the endothelial cell and the smooth muscle cell is very important in this process. The endothelium is a group of cells that produce compounds that are important in regulating vascular homeostasis by elaborating factors such as angiotensin II, NO, endothelin, and prostaglandins. Specifically, NO is found in endothelial cells responsible for smooth muscle relaxation. Normal endothelium maintains vascular tone and blood viscosity, prevents abnormal blood clotting and bleeding, limits inflammation of the vasculature, and suppresses smooth muscle cell proliferation. Abnormal endothelium causes increased inflammation and hypertrophy of the smooth muscle cells, promotes thrombosis and vasoconstriction, leading to the rapid growth of atherosclerotic plaques. Therefore,

understanding endothelial function will be imperative as researchers develop newer compounds that may enhance NO bioavailability within the vasculature. These may include compounds that promote the production of or extend the biological half life of NO or enhancing the cellular targets of NO.

6.2. NO-cGMP dependent vasodilator extracted from plants

There are hundreds of chemical substances that have been derived from plants for use as drugs and traditional medicines. During the past several years, the Murad Research Center in Shanghai has focused its efforts on identification of some plant extracts with antihypertensive and vasodilator effects. The strategy adopted by the Murad Research Center is to review the literature and use of plant extracts that were commonly used in Traditional Chinese Medicine for improving circulation. After the effective ingredients are extracted, the cell signaling pathways are analyzed with cultured cells and platelets and isolated tissues as well as whole animals (179, 180). The Center has been functioning for three years and to date more than 20 ethanol or water extracts from various plants demonstrate endothelium dependent or independent vasodilatory effects, and some exhibit inhibition of platelet aggregation or nitrite/nitrate production via conditioned cell culture media. More interestingly, the extracts exert different inhibitory effects on vasoconstriction by phenylephrine, serotonin, dopamine and prostaglandin F2α, which further suggest that multiple components are present in the extracts (181). These preliminary studies are encouraging and additional studies are necessary to fully characterize the active materials and their mechanisms of action to develop novel cardiovascular agents acting via the NO/cGMP signaling pathway and illustrate the importance of natural substances that may be found in the diet.

6.3. Inflammatory diseases & NOS-2 selective inhibition

The activation of NOS-2 and the subsequent production of large amounts of free radical gas NO is an important anti-infectious and anti-tumor mechanism of innate immunity. However, overproduction of NO has been implicated in several inflammation centered pathological conditions that include but are not limited to: 1) Tissue injury and various inflammatory disorders (182-184); 2) Neuronal disease (185, 186); 3) Auto-immune diseases (187-189); 4) Cancer or tumor cell proliferation (190-192); 5) Angiogenesis and related pathological changes (193, 194); and 6) Diabetes mellitus (195, 196). Thus, selective inhibition of NOS-2 may have therapeutic potential for treatment of diseases mediated by the overproduction of NO. It is not surprising that non-selective inhibition of NOS, which blocks constitutive isoforms of NOS as well as NOS-2, has deleterious effects. It follows then that selective inhibitors of NOS-2 will have considerable therapeutic potential. So far, two major categories of NOS-2 inhibitors have been developed: 1) L-arginine analogues, which show limited isoform selectivity and affect the substrate for all NOS enzyme and 2) guanidine inhibitor which has some selectivity towards NOS-2 inhibition, but has low efficacy and causes severe side effects. The recent discovery made by us indicates that an alternative approach is possible (197, 198). We have found NOS-2 expression can be selectively down regulated by Trichinella spiralis infection and the features of this inhibition include: a) Systemic: local jejunal infection by *T. spiralis* induces systemic inhibition of NOS-2 expression in the ileum, colon, kidney, lung and uterus. b) mRNA level inhibition: inhibition of NOS-2 expression appears to be regulated at gene transcriptional level. This serves as a very attractive mechanism by which we can develop a novel and selective NOS-2 inhibitor since expression of NOS-2 requires *de novo* synthesis in most cells and is primarily regulated at the transcriptional level. c) Potent: the effect of inhibition can override endotoxin-stimulated NOS-2 expression that is the major cause of septic shock and multiple organ failure. d) Selective: the inhibition does not extend to the expression of other isoforms of NOS; to paxillin, a housekeeper protein; or to cyclo-oxygenase-2, another inducible protein by proinflammatory cytokines. Our work with a variety of genetically modified mice has demonstrated that inhibition of NOS-2 expression by *T. sprialis* infection is dependent on the signaling pathway that includes the IL-4 receptor alpha subunit, receptor-associated kinases, Janus tyrosine kinase, and Stat6 in the suppression of NOS-2. Furthermore, the serum levels of IL-13 during infection are not consistent with the change of NOS-2. Thus, we propose that a yet undefined signal or alternative IL4Rα ligand could be involved in the IL-4Rα/Stat6 stimulating pathway which plays an important role in helminth provoked host immunoresponses. Further elucidation of this pathway could lead to the development of new therapies for inflammatory conditions characterized by overproduction of nitric oxide, but could also offer more information to the hygiene hypothesis that has been very influential in directing strategies to prevent allergic diseases.

7. SUMMARY AND PERSPECTIVE

Nitric oxide research has expanded rapidly in the past 30 years and the roles of NO in physiology and pathology have been extensively studied. The pathways of NO synthesis, signaling and metabolism in vascular biological systems have been a major area of research resulting in the 1998 Nobel Prize in Medicine or Physiology. As a gas and free radical with an unshared electron, NO participates in various biological processes. The rapid growth of NO research has generated more than 80,000 publications in the field of NO signaling. The interaction between NO and proteins may be roughly divided into two categories. In many instances, NO mediates its biological effects by activating guanylyl cyclase and increases intracellular cyclic GMP synthesis from GTP. However, the list of cGMP-independent effects of NO is also growing at a rapid rate. In this review, a brief history of the medical usage of nitric oxide is introduced. The importance and relevance of overproduction of NO in cardiovascular pathology has been stressed. The utilization of intact cell cultures, tissues and cell-free preparations with the use of pharmacological, biochemical and molecular biological approaches to characterize, purify and reconstitute these NO regulatory pathways should lead to the development of new therapies for various pathological conditions characterized by an un-balanced production of NO.

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Abbreviations: NO: nitric oxide, ROS: reactive oxygen species, RNO: reactive nitrogen species, IL: interleukin, MPO: myeloperoxidase, EDRF: endothelium dependent relaxing factor, BH4: tetrahydrobiopterin, mtALDH: mitochodrial aldehyde dehydrogenase, GSNO: S-nitrosoglutathione, cGMP: guanosine 3':5'-cyclic monophosphate

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