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Application of nitric oxide in drug discovery and development

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Introduction: It is becoming increasingly clear that many diseases are characterized or associated with perturbations in nitric oxide (NO) production/signaling. Therapeutics or strategies designed to restore normal NO homeostasis will likely have broad application and utility in human health. This highly complex and multi-step pathway for NO production and subsequent target activation provides many steps in the endogenous pathway that may be useful targets for drug development. Important therapeutic areas for NO-based therapies are inflammatory disorders, cardiovascular diseases, erectile dysfunction and metabolic disorders.

Areas covered: The following review will discuss the endogenous NO pathway, highlight the current market and indications for NO-based therapeutics, as well as identify pathway targets currently under drug development. Each step along the NO pathway will be discussed including exogenous sources of NO, use of precursors to promote NO production and downstream pathways affected by NO production with advantages and disadvantages highlighted for each.

Expert opinion: Development of NO-based therapeutics is and will continue to be a major focus of biotech and pharmaceutical companies. Understanding and utilizing dietary and nutritional strategies to restore NO homeostasis could allow for safer, quicker marketing of products that may be just as efficacious as drugs designed against specific targets.

1. Introduction

The discovery in the 1980s of the mammalian biosynthesis of nitric oxide (NO) and its roles in the immune [1,2], cardiovascular [3-5] and nervous [6] systems established a startling new paradigm in the history of cellular signaling mechanisms. Prior to this discovery, it was essentially inconceivable that cells would intentionally produce a toxic molecule as a messenger; NO was previously known as a common air pollutant, a constituent of cigarette smoke and a toxic gas, which appears in the exhaust of motor cars and jet airplanes, causes acid rain and destroys the ozone layer. Amazingly, despite this nasty reputation, it is now known that NO is one of a family of reactive signaling molecules, which includes both reactive nitrogen and reactive oxygen species that performs essential cellular functions in the body. This is, in fact, a hallmark example of the propensity of nature to seek out and exquisitely utilize the unique properties of unusual molecules. NO is one of the most important signaling molecules in the body, and is involved in virtually every organ system where it is responsible for modulating an astonishing variety of effects. NO has been shown to be involved in and affect (just to list a few major examples) neurotransmission, memory, stroke, glaucoma and neural degeneration such as in Alzheimer's disease, pulmonary hypertension, penile erection, angiogenesis, wound

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Article highlights.

- Nitric oxide (NO) is one of the most important molecules produced in the human body.
- Many human diseases are associated with insufficient NO production.
- Strategies or therapeutics designed to restore or enhance NO production will have a profound impact on human disease and public health.
- There are a number of technologies being developed as NO-based therapies.
- Nutritional strategies aimed at preventing the decline in NO production are likely to become first line of defense for chronic disease.

This box summarizes key points contained in the article.

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healing, atherogenesis, inflammation such as arthritis, nephritis, colitis, autoimmune diseases (diabetes, inflammatory bowel disease), invading pathogens, tumors, asthma, tissue transplantation, septic shock, platelet aggregation and blood coagulation, sickle cell disease, gastrointestinal motility, hormone secretion, gene regulation, hemoglobin delivery of oxygen, stem cell proliferation and differentiation and bronchodilation. One can then imagine the host of diseases or conditions that may be caused or affected by the body's dysregulation of NO production/signaling. Maintaining NO homeostasis is critical for optimal health and disease prevention and developing drugs or therapeutics to accomplish this will have a profound effect on public health.

It is appreciated that too little NO is associated with a number of human conditions but also too much NO produced under chronic inflammatory conditions is toxic and harmful. Therefore, maintaining physiological levels of NO is key to restoring NO homeostasis. Although there are several conditions where inhibition of NO production may have therapeutic utility such as sepsis, chronic inflammation, that is, inflammatory bowel disease, etc. and even some neurological disorders, the focus of this review is directed exclusively on problems associated with diminished NO production and the therapeutic effects of NO enhancement through a number of different strategies and technologies.

The discovery of the NO pathway represented a critical advance in the understanding of cell signaling and subsequently into major new advancements in many clinical areas including, but not limited to cardiovascular medicine. This seminal finding was viewed as so fundamentally important that the Nobel Prize in Physiology or Medicine was awarded to its discoverers, Drs. Louis J. Ignarro, Robert F. Furchgott and Ferid Murad in 1998, 11 years after NO was identified. The Swedish Nobel Assembly sagely noted, 'The signal transmission by a gas that is produced by one cell, penetrates through membranes and regulates the function of another cell, represents an entirely new principle for signaling in biological systems'. It was shocking to realize that NO, a colorless, odorless gas, was able to perform such important biochemical functions in selective and specific cell signaling events. Dr. Valentin Fuster, then president of the American Heart Association, noted in a 1998 interview that 'the discovery of NO and its function is one of the most important in the history of cardiovascular medicine'. More than a decade after the Nobel Prize was awarded for the discovery of NO and after more than 120,000 scientific papers have been published on it, we still don't have a firm grasp on its production and regulation or understand all of its biological functions.

What we do know is that continuous generation of NO is essential for the integrity of the cardiovascular system, and decreased production and/or bioavailability of NO is central to the development of many disorders [7]. The production of NO from L-arginine (L-Arg) is a complex and complicated biochemical process involving a 5-electron oxidation with many co-factors and prosthetic groups carried out by a group of enzymes called nitric oxide synthase (NOS). There are three isoforms of NOS: neuronal NOS (nNOS or NOS 1), inducible NOS (iNOS or NOS 2) and endothelial NOS (eNOS or NOS 3). There are many steps and/or factors that may be altered and affect ultimate NO production. Once produced, NO can be quickly scavenged before it has a chance to perform its actions. It is, therefore, a war of attrition when it comes to producing bioactive NO, and is a remarkable feat that this short-lived gas is responsible for so many essential cellular activities.

Despite NO being recognized by the scientific and medical community as one of the most important molecules produced within the body and being named 'Molecule of the Year' by *Science* in 1992 [8] and a Nobel Prize in Physiology or Medicine awarded for its discovery, there are currently only three Food and Drug Administration (FDA)-approved products in the market directly related to NO: i) organic nitrates, such as nitroglycerin for the treatment of acute angina (these have been used for centuries long before the discovery of NO); ii) inhaled NO therapy for neonates for treatment of pulmonary hypertension due to underdeveloped lungs and iii) phosphodiesterase (PDE) inhibitors, such as sildenafil, which do not directly affect NO production but act through affecting the downstream second messenger of NO, cyclic guanosine monophosphate (cGMP). With the knowledge gained in the physiology and pharmacology of NO, better and new drugs are being designed not only for cardiovascular diseases but for neurological and several other disorders as well. NO-based therapies include many conventional as well as new therapies where NO plays a role in mechanism of action.

There are a number of NO-based therapies in development, including technologies designed to activate and promote NO synthesis from NOS, NO donating compounds, therapies designed to modulate post-translational protein modifications through *S*-nitrosation and therapies designed to affect or prolong downstream signaling pathways from NO. The method of delivery of NO and cellular and molecular specificity is of utmost importance. Delivery of NO through

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controlled and enzymatic metabolism of organic nitrates is an effective acute treatment for angina [9], but still not without some adverse effects when used chronically [10,11]. The safe delivery of NO gas through inhalation therapy is also now in practice [12].

A substantial knowledge of the NO signaling pathway has been gained during the past three decades. The current state of the art of NO-based research will be discussed and some of the potential molecular targets for drug development. It is prudent at this juncture to discuss the market for NO-based therapies which is an important consideration given the enormous time and capital investment in developing drugs.

2. Impact of NO-based therapies on international markets

Most of the commercial developments of NO-based products/technologies have taken place in North America with Europe in the second place. Conventional products, primarily dietary supplements or traditional medicines with NO activity are marketed all over the world. As NO-based therapies and products develop and get approved in the USA and Europe, they are expected to be prominent in the international markets in a very short time period. This is due to the international appreciation of NO in health and disease by researchers and physicians. Some of these technologies may not be classified as NO therapies but merged with categories of traditional pharmaceuticals and biotechnology products. For example, NOS gene delivered by gene therapy might be classified under gene therapy. NO-donating non-steroidal anti-inflammatory drugs (NSAIDs) might remain under the categories of traditional NSAIDs with innovative action. In the worldwide pharmaceutical market, share of drugs where NO is involved in the mechanism of action was US$58 billion in 2009 and is projected to rise to US$102 billion in 2014 and US$147 billion in 2019 as new drugs with NO-based mechanisms are introduced into the market [13]. In fact, most large pharmaceutical companies have a division committed to NO-based therapies and many new start-ups are founded on intellectual property around NO.

3. Education of the public

Education of the public about NO not only assumes importance for promotion of health products based on NO, especially non-pharmaceutical products such as dietary supplements, but also assumes importance for pharmaceutical products as many patients want to understand what their physicians are prescribing them. Most studies on NO are published in the scientific literature but not many lay people or consumers are familiar with it. In a survey conducted for Herbalife Inc. by NFO Plog Research Inc. in 2003, 67% of respondents indicated they had heard of NO yet less than 1% of the respondents could correctly identify that NO was a blood circulation aid. Only 5.9% understood that increasing

their NO levels could improve their cardiovascular health and 24% of the respondents confused NO with laughing gas (nitrous oxide) [13]. No recent survey has been reported but the public knowledge of NO is still relatively poor. Public knowledge is likely to increase as new NO-based therapies hit the market.

4. NO generation and nitric oxide synthase

In order to get a sense of the drug targets for the NO pathway, it is important to first describe the pathway for endogenous production. 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. Specifically, NO produced by the endothelial cells is responsible for smooth muscle relaxation. Gaseous NO diffuses across the endothelial cell and into the underlying smooth muscle cell, where it stimulates the pathway of soluble guanylyl cyclase (sGC) to produce vasodilatation [14]. (The demonstration of NO-activating sGC was a seminal discovery that led to the Nobel Prize for Ferid Murad.) 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 [15]. 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 cell biology will be imperative as researchers develop newer compounds that may enhance NO bioavailability within the vasculature.

NO was shown to be a potent vasodilator, inhibitor of platelet aggregation and active species of nitroglycerin [3] before the discovery of endothelium-derived relaxing factor (EDRF) in 1980 [5]. (The discovery of EDRF by Furchgott marked the beginning of the NO field.) The first endogenous pathway to be discovered is through oxidation of L-Arg to nitrite [2] (first demonstration that cells utilized L-Arg for cytotoxicity in the immune system). It was only later realized that NO is an intermediate in that cycle [16] (seminal paper showing production of NO by immune cells). NOS enzymes produce NO by catalyzing a five-electron oxidation of a guanidino nitrogen of L-Arg. Oxidation of L-Arg to L-citrulline occurs via two successive mono-oxygenation reactions producing N^{ω}-hydroxy-L-arginine as an intermediate. Two moles of O_2 and 1.5 moles of NADPH are consumed per mole of NO formed [17]. NOS enzymes are the only enzymes known to simultaneously require six bound co-factors/prosthetic groups: flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), nicotinamide adenine dinucleotide phosphate (NADPH), heme, tetrahydrobiopterin (BH_4) and

Ca^{2+}-calmodulin (CaM). All three NOS isozymes 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 [18]. These electrons are used to reduce the iron to the ferrous state so that it can bind oxygen, which is incorporated into the substrate, L-Arg, to generate NO plus L-citrulline. CaM also facilitates NADPH-dependent reduction of cytochrome c and ferricyanide in BH_4- and heme-depleted nNOS [19].

A simplified illustration of the L-Arg-NO pathway is shown in **Figure 1**. Under healthy normal conditions, NO production typically proceeds quite efficiently. This pathway is illustrated in the top half of **Figure 1**. However, if there is a problem with L-Arg uptake or L-Arg production from L-citrulline, or if any of the co-factors become limiting due to oxidative stress, NOS uncoupling or conditions of hypoxia where oxygen is limited, then NO production from NOS shuts down, and in many cases NOS then produces superoxide instead [20]. Once NO is produced this is only half the battle. NO then has to diffuse to its specific cellular target either acting in an autocrine or paracrine fashion, activating downstream pathway such as sGC or modifying thiols through nitrosation reactions. Regulation of protein nitrosothiols is mediated through *S*-nitrosoglutatione reductase (GSNOR) which was recently identified as a key mechanism for NO-based signaling [21]. When the cell can no longer maintain NO production or regulate NO signaling pathway, the consequences are profound. Strategies to restore NO production and signaling will now be discussed.

5. Delivery of NO

Targeted delivery of NO at precise cellular locations poses an extreme challenge with respect to recapitulating physiological production of NO. There are several methods of delivery of NO which will only be mentioned briefly. The most common and effective for targeted delivery to the pulmonary circulation is inhaled NO [12]. There are also biomaterials being developed for sustained release of NO for topical applications for wound healing, infections, etc [22]. Nanoparticle delivery of NO is an emerging field, particularly in cancer biology [23]. NO-eluting stents [24] or NO-coating of orthopedic implants for preventing biofilm growth and infection [25] is an area of active development. This review will focus primarily on cardiovascular targets for drug development on restoring NO homeostasis rather than the different methods of delivery but should not trivialize the importance of targeted delivery of NO.

5.1 NO donating drugs
NO donors were really the first class of NO-based therapies. NO donors comprise a heterogeneous group of different chemical classes of compounds that either decompose spontaneously or are metabolized in cells and tissues to generate NO. As diverse as the chemistries of the individual agents and the pathways that lead to NO formation from them are, so are the differences in their pharmacodynamic, pharmacokinetic and toxicological properties. A common feature of all of these compounds is that they can relax isolated blood vessels *in vitro* (hence, the older designation, 'nitrovasodilators') and, depending on their mode and rate of biotransformation, are principally capable of enhancing blood flow and lowering blood pressure *in vivo*. Nitrovasodilators are used in the management of various acute and chronic cardiovascular pathologies. Their pharmacological effects are mediated biochemically via the release of NO in the vasculature independent of endogenous eNOS [14]. Once liberated, NO activates sGC in the smooth muscle, increases the concentrations of the secondary messenger cGMP, alters calcium flux and ultimately causes relaxation [3]. Only a select few compounds are in clinical use today, however, all these drugs had been introduced into medical practice long before the discovery of NO as a biological signaling molecule. Clinically available NO donors approved for use in the USA in patients with cardiovascular disease include nitroglycerin (GTN), isosorbide dinitrate (ISDN), isosorbide mononitrate (IS-5N), amyl nitrite and sodium nitroprusside (SNP). Pentaerythrityl-tetranitrate (PETN) has been approved for use in the USA for many years, but has been largely replaced by ISDN and IS-5N. Nicorandil and molsidomine (which is converted to the active moiety, 3-morpholinosydnonimine (linsidomine, SIN-1), *in vivo*) are not approved for use in the USA, but, like PETN, are available abroad.

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 USA alone [26]. In addition to its anti-anginal benefits, GTN has been found to induce ischemic preconditioning [27], a physiologic phenomenon that can protect the heart from lethal ischemia. Recently, GTN has also been demonstrated to be beneficial in non-cardiovascular contexts, including pain management [28], treatment of chronic anal fissure [29], preservation of organs for transplantation [30] and overall response and time to progression in patients with inoperable small cell cancer [31]. Collectively these reports highlight the multitude of therapeutic applications of NO and the subsequent cell signaling pathways. The chemical structure of GTN is shown in **Figure 2**.

The beneficial pharmacological effects of nitrovasodilators are severely limited by the rapid development of tolerance to their vasodilatory effects [32]. In order for organic nitrates to maintain their vasodilatory effects when used clinically, an 8 – 12 h nitrate-free period needs to be incorporated. This interruption complicates organic nitrate therapy in patients who may require around-the-clock angina protection. Interestingly, in contrast to the vasodilatory effects, tolerance
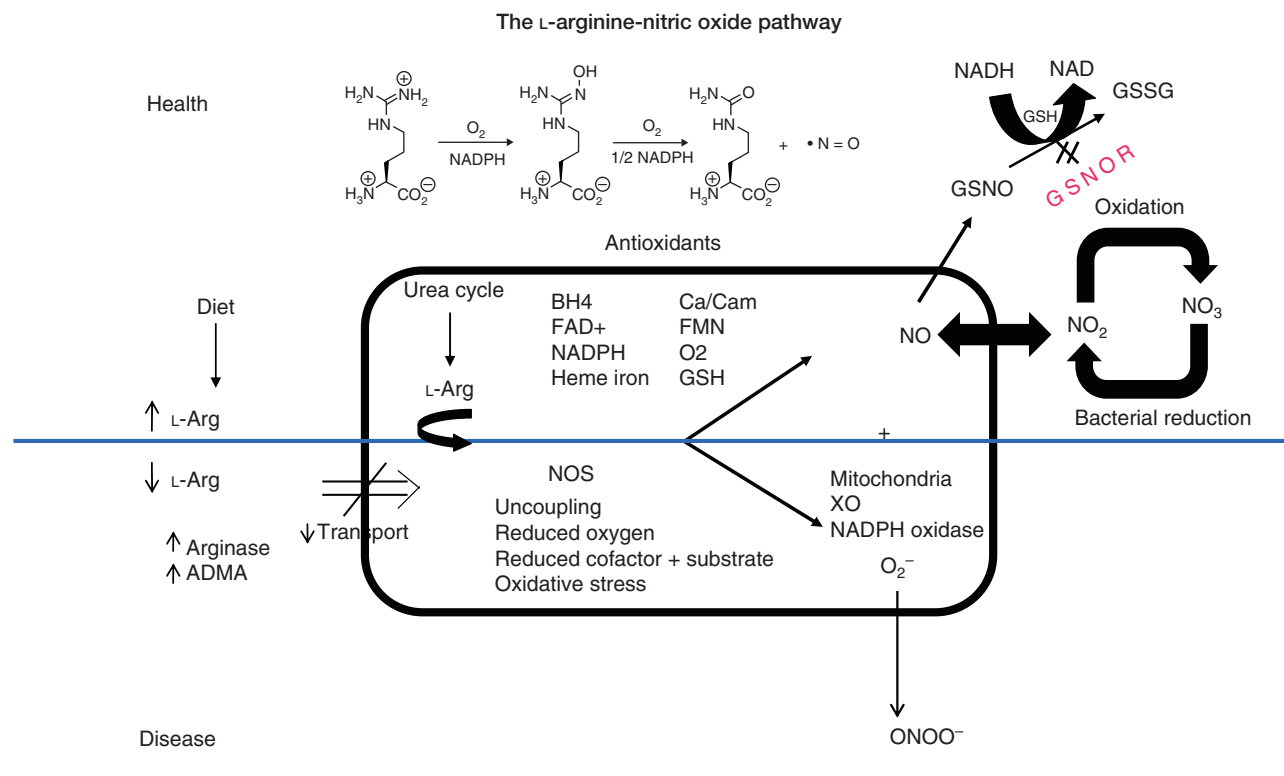
The L-arginine-nitric oxide pathway

Figure 1. The NO pathway.

development to the platelet anti-aggregatory effects of organic nitrates does not appear to be significant [33]. The mechanisms of nitrate tolerance have been described as a 130-year-old mystery [34]. Nitrate tolerance exhibits several major features, namely: i) *sulfhydryl (-SH) involvement*: nitrate-induced vasodilation is facilitated by the presence of free -SH groups [35]. Modifications or depletion of free -SH have been proposed as a mechanism of vascular tolerance, [36] widely known classically as the Needleman sulfhydryl depletion hypothesis. However, conflicting findings have been observed based on lack of demonstrated sulfhydryl depletion, as well as incomplete prevention or reversal by exogenous sulfhydryl repletion [37]; ii) *multiple consequences of chronic nitrate exposure*: nitrate tolerance is accompanied by a myriad of events, including reduced organic nitrate metabolism to NO and inactivation of aldehyde dehydrogenase-2 (ALDH2) [38], neurohormonal counter-regulation, presence of withdrawal rebound, presence of vascular oxidative stress, including increased $O_2^{\bullet -}$ accumulation [39], and extensive alteration of gene expression in the rat aorta [40]. Partial avoidance/reversal of nitrate tolerance can be accomplished by a variety of agents such as *N*-acetylcysteine [41], vitamin C [42], vitamin E [43], folic acid [44], L-Arg [45], carvedilol [46], hydralazine [47], etc. Although eNOS uncoupling has been proposed as a mechanism of nitrate tolerance [48], eNOS knockout mice also exhibited this phenomenon [49].

Chronic and long-term organic nitrate therapy has been associated with reduced survival when used in patients with coronary artery disease [50] but the underlying mechanisms are not known. It is clear, however, that use of organic nitrate therapy leads to increased vascular oxidative stress which in turn can produces endothelial dysfunction [51]. Organic nitrate therapy causes an increased production of reactive oxygen species and reactive nitrogen species via several mechanisms including increased activity of NADPH oxidase and uncoupling of both NOS and mitochondrial respiration [51]. As a result, use of these compounds is limited to acute treatments, such as acute angina or for emergency hypertensive crises.

5.2 Diazeniumdiolates

Diazeniumdiolates, compounds of structure R_1R_2NN (O) = NOR_3, which have also been called NONOates, have proven useful for treating an increasing diversity of medical disorders in relevant animal models [52]. NONOates are generated by exposing various nucleophile compounds to NO gas at a few atmospheres of pressure. The resulting compounds are stable as a solid and highly soluble in aqueous solution, releasing 2 moles of NO per mole of donor compound. The simple reaction scheme for diethyl NONOate is shown in **Figure 3**. Decomposition rates are dependent on pH, temperature and the chemical characteristics of the donor compound, generating compounds whose NO generation rate can be predicted and adjusted. NO release from the NONOate complex is not dependent on exogenous thiols. A distinct advantage of this class of NO donors is that they generate NO spontaneously, without any need for electron transfer, co-factors, metabolic
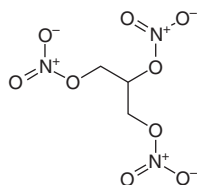
Figure 2. Structure of nitroglycerin.

activation or oxidation–reduction activation. A disadvantage is the spontaneous NO release which presents a challenge for targeted delivery. To provide a means of targeting NO release, photosensitive precursors to diazeniumdiolates have been developed. There are three different classes of photo-triggered diazeniumdiolates: 2-nitrobenzyl derivatives, meta-substituted benzyl derivatives and naphthylmethyl and naphthylallyl derivatives [53]. This photo-triggered diazeniumdiolate derivative may prove useful in future pharmacological investigations. Examples of potential applications include inhibition of restenosis after angioplasty, preparation of thromboresistant medical devices, reversal of vasospasm and relief of pulmonary hypertension. Although these compounds show promise in preclinical studies, they have yet to be tested in human clinical trials.

5.3 NO-hydrid drugs

In the last few years, a revision of the 'one-compound-one-target' paradigm has led pharmacologists and pharmaceutical chemists to develop new classes of molecules which combine different pharmacodynamic properties. This innovative strategy has produced hybrid drugs, with a dual mechanism of action: i) the slow release of NO and ii) another fundamental pharmacodynamic profile. These drugs have been obtained by inserting appropriate NO-donor chemical groups (i.e., nitrate esters, nitrosothiols, etc.), linked to a known drug, by means of a variable spacer moiety. This theory has opened up the possibility of designing new drugs that are capable of delivering NO into tissues and the bloodstream in a sustained and controlled manner. This objective has been achieved by grafting an organic nitrate structure onto existing drugs through chemical spacers, such as aliphatic, aromatic or a heterocyclic chain. The approach has led to the synthesis of several new chemical entities whose pharmacological profile challenges the parent drug, not only on the basis of new properties, but also with respect to a better safety profile. These new pharmacodynamic hybrids present the advantage of combining a basic mechanism of action (e.g., cyclooxygenase (COX) inhibition, beta-antagonism or angiotensin-converting enzyme (ACE) inhibition) with a slow release of NO, which may be useful either to reduce adverse side effects (e.g., the gastrotoxicity of NSAIDs), or to improve the effectiveness of the drug (e.g., conferring direct vasorelaxing and anti-platelet effects on an ACE inhibitor). Leading this charge are the NO-NSAIDs. NSAIDs, including those that are selective for COX-2, are

among the most widely used drugs. However, these drugs produce significant side effects in the gastrointestinal and cardiorenal systems, which greatly limit their utility. In recent years, the new NO-NSAIDs have been developed that appear to offer significant advantages over conventional and Cox-2-selective NSAIDs [54]. NO-NSAIDs are derivatives of conventional NSAIDs, which are able to release NO over prolonged periods of time. The combination of balanced inhibition of the two main isoforms of COX with controlled release of NO yields a series of drugs that exert anti-inflammatory and analgesic activities in a wide range of settings, and have markedly reduced gastrointestinal and cardiorenal toxicity [55]. Recent clinical trials of NO-NSAIDs have provided a 'proof of concept' that is completely consistent with preclinical characterization of these compounds [56]. Many pharmaceutical companies have invested millions of dollars in this strategy and although the preclinical and clinical data looked very promising, in 2010 the US FDA outright rejected the first of its kind naproxcinod Sophia Antopolis, France, a NO-naproxen drug developed by NicOx. The FDA review recommended further trials in humans to assess the safety on a cardiovascular and gastrointestinal level. NicOx spent about 10 years and 100 million euros (US$127.6 million) to fund the US launch of its lead anti-inflammatory drug. This ruling was a major blow to the strategies and pipeline products of big pharma and small biotech companies. It is currently unclear how industry will respond to further development of this technology.

6. cGMP-dependent signaling

Many of the physiological functions of NO in the cardiovascular, neuronal, gastrointestinal and other systems are mediated through its primary receptor, sGC. sGC is a heme-containing, heterodimeric NO receptor. Soluble GC consists of two subunits, α and β, which make up the active enzyme. The heme-containing heterodimer sGC converts guanosine triphosphate (GTP) into the secondary messenger cGMP. 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 [57]. To do this, cGMP acts directly with downstream effectors such as the family of cGMP-dependent protein kinases (PKGs), cyclic nucleotide-gated channels (CNGs) and cGMP-regulated PDEs [58-60]. The sGC activity increases more than 200-fold in response to NO [61]. This signal is quickly removed by the action of PDE 5A enzyme. However, in order for sGC activation and downstream signaling to occur, there must be at least some level of bioactive NO produced to bind to target. In conditions of NO insufficiency, this pathway is not activated at all or is severely compromised.

To circumvent this problem, many allosteric regulators of sGC which provide NO-independent activation have been developed. Impaired bioavailability and/or responsiveness to endogenous NO has been implicated in the pathogenesis of cardiovascular and other diseases [7]. Current therapies that
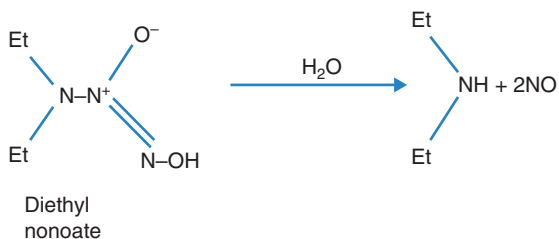
Figure 3. Structure of diethyl NONOate, a simple diaze-niumdiolate.

involve the use of organic nitrates and other NO donors have limitations, as discussed previously 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 [62]. 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 [63]. 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 [64]. While these compounds are gaining popularity in drug development, these targets will obviously only affect the NO–cGMP-dependent pathways. As more cGMP-independent pathways of NO are uncovered, this approach will have its limitations.

Along the same line of reasoning, PDE inhibitors were developed first as treatments for hypertension and angina, conditions of NO insufficiency. PDEs are intracellular enzymes that specifically catalyze the hydrolysis of the second messengers cyclic adenosine monophosphate (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 [65]. By counterbalancing cGMP production by guanylyl 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 [58]. Since inhibitors of PDE5 raise intracellular cGMP levels, the effects will be much more pronounced under conditions when cGMP formation is already increased. When it was realized these drugs could induce penile erections, the indication changed from hypertension and angina to erectile dysfunction. In 1998, the FDA approved the use of sildenafil for erectile dysfunction. There are now other PDE5 inhibitors, tadalafil and vardenafil that compete with sildenafil. Collectively, these drugs have annual sales of several billion dollars in the USA alone. These agents may also be beneficial in other disorders such as pulmonary hypertension, Raynaud's syndrome, etc. However, due to the non-selective inhibition of all PDE, there are a number of adverse side effects. The most common adverse effects of PDE inhibitor use include headache, flushing, dyspepsia, nasal congestion and impaired vision, including photophobia and blurred vision. Some report cases of cyanopsia or seeing everything tinted blue. Rare but serious adverse effects found include priapism, severe hypotension especially when taken with other anti-hypertensives, myocardial infarction, arrhythmias, stroke, increased intraocular pressure and sudden hearing loss. These side effects demonstrate the ubiquitous and multi-system involvement of the NO–cGMP pathway. Targeted delivery of PDE inhibitors may provide a more safe and effective drug without the unwanted systemic side effects.

7. *S*-nitrosothiols (RSNO)

S-nitrosothiols are thio-esters of nitrite with the general structure R-S-N = O (RSNO); naturally occurring examples include *S*-nitrosocysteine, *S*-nitrosoglutathione (GSNO) 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 NO or nitrous acid in extremely acidic condition (pH < 3) [66]. *S*-nitrosation is a ubiquitous redox-related modification of cysteine thiol which transduces NO bioactivity [67] (Dr. Stamler's group was the first to elucidate and describe post-translational protein modification by NO as a cGMP-independent signaling pathway for NO). As early as 1981, Ignarro's group demonstrated that the bioactivities of certain pharmacological nitrogen oxide (NOx) donors were attributed to reactions with cellular thiols [68], 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 RSNO as an intermediate in NO-dependent but sGC-independent signaling processes. *S*-nitrosothiols may be

NO carriers that mediate vasodilation [69] and may also be integral to the regulation of platelet aggregation [70]. Interest in *S*-nitrosothiols was heightened by the discovery that NO can react with thiol groups *in vivo* to form *S*-nitrosothiols such as *S*-nitrosocysteine and GSNO [66]. In addition, it can exist in the plasma as an *S*-nitroso adduct of circulating albumin [70]. Consequently, it has been generally assumed that naturally occurring *S*-nitrosothiols act as *in vivo* storage sites for NO, which can be released on demand. *S*-nitrosothiols are susceptible to decomposition by numerous mechanisms, giving rise to NO and the corresponding disulfide. The rate of decomposition is influenced by a number of factors, including metal ions (particularly Cu$^+$), transnitrosation, that is, the transfer of NO from *S*-nitrosothiols to other thiols, enzymatic decomposition, photochemical decomposition, thermal decomposition and reaction with ascorbate. *S*-nitrosothiols are actively metabolized by cells [71]. Some *S*-nitrosothiols (*S*-nitrosocysteine, *S*-nitrosohomocysteine) can be taken up into cells via amino acid transport system L, whereas others (GSNO, *S*-nitroso-*N*-acetylpenicillamine) are not directly transported, but require the presence of cysteine and/or cystine before the nitroso functional group is transported [72]. *S*-nitrosothiols are biologically active as vasodilators and inhibitors of platelet aggregation [73]. Due to the fact that these low molecular weight nitrosothiols are similar to EDRF with a longer circulating half-life than NO, make them an ideal candidate for drug development. However, the therapeutic use of *S*-nitrosothiols such as SNAP (*S*-nitroso-*N*-acetyl-D,L-penicillamine) has been limited by their potent vasodilatory effects leading to hypotension.

7.1 GSNO reductase inhibitors

Reactive protein thiols are regarded as major intracellular target of NO [74]. Dysregulation of protein *S*-nitrosation is associated with a growing list of pathophysiological conditions [74] and altered blood levels of RSNO have been associated with endothelial dysfunction and impaired NO production [75]. Although RSNOs such as SNAP and GSNO are commercially available, none of them has been used therapeutically due to the unpredictable rate of decomposition in the body. By contrast, an increasing number of proteins have been found to undergo *S*-nitrosation (or nitrosylation as sometimes referred to) *in vivo*. These *S*-nitrosothiol proteins have demonstrated an important role in many physiological as well as pathological processes [76].

S-nitrosation has since been implicated in the control of a wide array of protein functions and cell activities [74,77]. 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 [74]. Through *S*-nitrosation of these proteins, NO has been shown to regulate apoptosis, G-protein-coupled receptor-based signaling, vascular tone and inflammatory responses [77-80]. 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 NO inside the body, denitrosation pathways inside the cells terminate the cellular effects of NO. Denitrosation of SNO-proteins in cells can be accomplished by simple chemistry, wherein intracellular glutathione (GSH) or other intracellular thiols, including other protein Cys residues, act as acceptors and effectively remove nitrosyl groups via transnitrosation reactions. Decomposition can also be enhanced through heat, UV light and metal ion (Cu^{2+}), which results in the formation of NO and the corresponding disulfide (RSSR). The reaction can be enhanced by the presence of ascorbate, thiols, high oxygen tension and pH >3. Additionally, ascorbate and metal ions can promote SNO-protein decomposition [81,82]. In this system, the rate of SNO-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 [76].

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 (GSNOR) [21] (first demonstration of the enzymatic denitrosation and regulation of nitrosothiols as a transient post-translational modification). GSNOR, a member of alcohol dehydrogenase family, has been shown to be the primary pathway through which cells denitrosate intracellular proteins [21]. 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 (NADH), 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 SNO proteins [21] (this finding identifies the first biologically relevant mammalian denitrosase and confirms that levels of GSNO determine intracellular levels of SNO-proteins). GSNOR brings about denitrosation of intracellular proteins by the reduction of GSNO, a NO metabolite arising from the reaction of glutathione with *S*-nitrosated proteins or NO [83-85]. Owing to its ability to regulate the *S*-nitros(yl)ation of intracellular proteins, GSNOR has become an important target for developing agents that modulate NO 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 hyper-responsivity owing to higher GSNO concentrations in bronchial fluid and diminished tachyphylaxis to β-agonists owing to *S*-nitrosation of G-protein coupled receptor kinase [79,86]. Development of specific drugs which modulate the steady-state levels of RSNO will likely have therapeutic
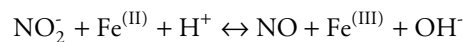
benefit. Several compounds have now been identified that act as inhibitors of GSNOR [87].

8. 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 [88-91]. As a result, they should now be viewed as storage pools for NO-like bioactivity to be acted on when enzymatic NO production from NOS is insufficient. Nitrite is an oxidative breakdown product of NO that has been shown to serve as an acute marker of NO flux/formation [92]. Nitrite is in steady-state equilibrium with S-nitrosothiols [88,93] and has been shown to activate sGC and increase cGMP levels in tissues [88]. Therefore, nitrite is an ideal candidate for restoring both cGMP-dependent and -independent NO signaling.

In addition to the endogenous oxidation of NO, nitrite is also derived from reduction of salivary nitrate by commensal bacteria in the mouth and gastrointestinal tract [94,95] as well as from dietary sources such as meat, vegetables and drinking water. 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 mouth and gastrointestinal tract and on body surfaces [96] (first demonstration of NOS-independent NO production). Nitrate from the diet 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 h). The nitrite levels in plasma also increase after nitrate ingestion [97]. 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 [97,98]. Once in the mouth, commensal facultative anaerobic bacteria reduce nitrate to nitrite during respiration by the action of nitrate reductases [96,99]. Human nitrate reduction requires the presence of these bacteria – suggesting a functional symbiosis relationship – as mammalian cells cannot effectively metabolize this anion. The salivary nitrate levels can approach 10 mM and nitrite levels 1 – 2 mM after a dietary nitrate load [97]. When saliva enters the acidic stomach (1 – 1.5 l/day), much of the nitrite is rapidly protonated to form nitrous acid (HNO_2; pK_a ~3.3), which decomposes further to form NO and other nitrogen oxides [90,91]. This human nitrogen cycle is illustrated in **Figure 4**. Once nitrite is absorbed and circulated, it is taken up by peripheral tissues and can be stored in cells. The one-electron nitrite reduction to NO can occur in a much simpler mechanism than the two-electron reduction of nitrate by bacteria. The one-electron reduction of nitrite can occur by ferrous heme proteins (or any redox active metal) through the following reaction:

$$NO_2^- + Fe^{(II)} + H^+ \leftrightarrow NO + Fe^{(III)} + OH^-$$

This is the same biologically active NO as that produced by NOS, with nitrite rather than L-Arg as the precursor and is a relatively inefficient process [100]. Much of the recent focus on nitrite physiology is due to its ability to be reduced to NO during ischemic or hypoxic events [101-103]. Nitrite reductase activity in mammalian tissues has been linked to the mitochondrial electron transport system [104,105], protonation [102], deoxyhemoglobin [106] and xanthine oxidase [107,108]. Nitrite can also transiently form nitrosothiols (RSNOs) under both normoxic and hypoxic conditions [101] and a recent study by Bryan *et al.* demonstrates that steady-state concentrations of tissue nitrite and nitroso are affected by changes in dietary NOx (nitrite and nitrate) intake [88] (this study demonstrates that nitrite had unique signaling properties independent of NO). Furthermore, enriching dietary intake of nitrite and nitrate translates into significantly less injury from heart attack [109] (first study to show that dietary nitrite and nitrate could reduce injury from heart attack). Previous studies also demonstrated that nitrite therapy given intravenously prior to reperfusion protects against hepatic and myocardial ischemia–reperfusion (I/R) injury [110]. Additionally, experiments in primates revealed a beneficial effect of long-term application of nitrite on cerebral vasospasm [111]. Moreover, inhalation of nitrite selectively dilates the pulmonary circulation under hypoxic conditions *in vivo* in sheep [112]. Topical application of nitrite improves skin infections and ulcerations [113]. Furthermore, in the stomach, nitrite-derived NO seems to play an important role in host defense [96] and in regulation of gastric mucosal integrity [114]. All of these studies together along with the observation that nitrite can act as a marker of NOS activity [92] 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 (NG-nitro-L-arginine methyl ester)-induced hypertension and serve as an alternate source of NO *in vivo* [115]. In fact, a report by Kleinbongard *et al.* [116] 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 [88], modulation of nitrite and/or nitrate intake may provide a first line of defense for conditions associated with NO insufficiency [103]. In fact, it has been reported that dietary nitrate reduces blood pressure in healthy volunteers [117,118] (both of these publication demonstrate the effects of inorganic nitrate on NO-dependent regulation of blood pressure).

There is emerging evidence that many of the physiological effects and perhaps part of the mechanism of action for nitrite involves sub-cellular interaction within the mitochondria [119]
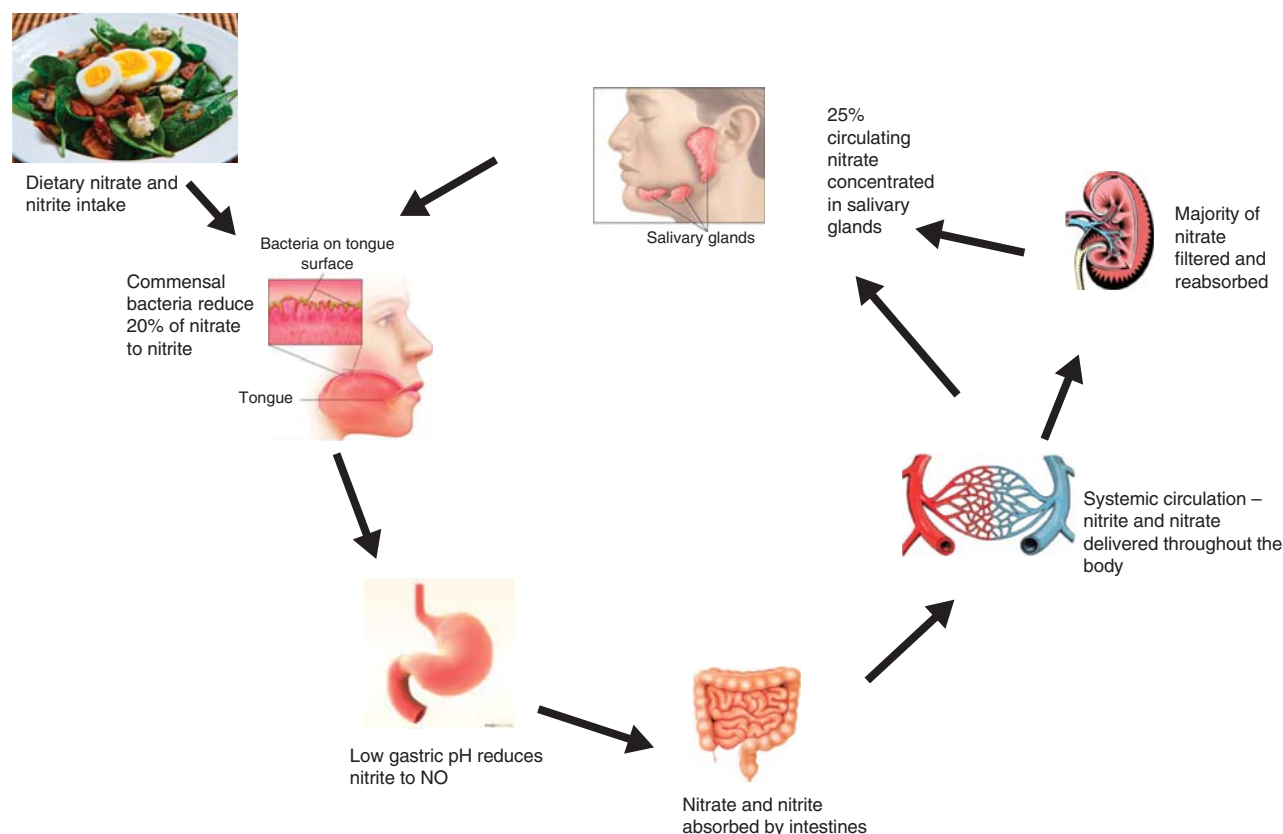
Figure 4. Human nitrogen cycle.

(this study demonstrates a unique mechanism of action for nitrite). We have known for over a decade that NO affects mitochondria energetics and biogenesis [120,121] (these studies by Moncada's group were the seminal papers on effects of NO on mitochondrial function). NO competes with oxygen for binding to cytochrome *c* oxidase in the mitochondrial electron transport chain, leading to inhibition of mitochondrial respiration [122]. This reversible inhibition is thought to extend to oxygen gradient in tissue and protect from ischemic damage [123]. These NO-mediated events also act as triggers by which mitochondria modulate signal transduction cascades involved in the induction of cellular defense mechanisms and adaptive responses, particularly in response to hypoxia and other environmental stressors [124]. Since nitrite can be reduced to NO by components of the mitochondrial electron transport chain [105,119,125], nitrite may exert NO-like effects on mitochondria. This suggests that nitrite could play a role in regulating cellular energetic and oxygen utilization as well as mitochondrial biogenesis, especially in conditions of physiologic hypoxia [126]. Nitrite may be an important therapeutic consideration in mitochondrial disorders such as mitochondrial myopathy, encephalopathy, lactic acidosis with stroke-like episodes (MELAS) syndrome.

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 [127,128] (edited textbooks exclusively devoted to nitrate–nitrite–NO biochemistry and physiology). 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.

9. Summary

In 2006, total healthcare expenditures in the USA exceeded US$2 trillion, or US$6700 per person [129]. This trend is expected to increase over the coming years, reaching US$4 trillion in 2015. Currently, costs associated with chronic diseases such as obesity, diabetes, hypertension, coronary artery disease account for 75% of the nation's annual healthcare costs. According to the American Heart Association, an estimated 81 million people had one or more forms of cardiovascular disease in the USA in 2006, including hypertension, coronary artery disease, myocardial infarction, angina pectoris, stroke and heart failure. Most, if not all, of the chronic conditions mentioned above are the result of a dysfunctional endothelium and inability to produce NO and/or maintain NO homeostasis

and signaling. Understanding and developing new strategies to restore NO homeostasis will have a profound impact on public health and on the healthcare system.

10. Expert opinion

Development of NO-based therapies has been slow and largely unsuccessful given the number of FDA-approved NO therapies, the demand for such technologies and the number of disease states that can be affected. Due to the ubiquitous nature of the NO pathway in virtually all biological systems and the precise spatially and temporally controlled regulation and production of NO, it will be challenging to develop targeted NO therapeutics that can recapitulate endogenous endothelial NO production in select tissues. The fact that NO reacts primarily with transition metals and other free radicals, including thiyl radicals to form nitrosothiols as a means to modulate protein structure and function provides finite candidates for drug targets. Most of the disease conditions associated with NO insufficiency is due to dysfunctional NOS or enhanced scavenging of NO once produced or a combination of both. Strategies designed to restore NO production and prevent NO scavenging that allow NO to reach and activate its cellular targets will likely have the most beneficial effects. As we learn more about the downstream targets of NO, effective and specific therapeutics can be developed to activate those pathways that may be able to overcome insufficient NOS production of NO. Development of NO donors is an unlikely strategy for chronic treatment of conditions due to NO insufficiency, although acutely they may be very effective. The recent FDA ruling of not approving NO hybrid drugs due to long-term safety concerns corroborates my opinion. Activators of sGC or inhibitors of PD5 still are an attractive area of research but until targeted delivery of these agents to the specific tissue bed or organs can be achieved, there will continue to be unwanted systemic side effects.

In my opinion, strategies designed to provide cells or tissue beds with substrate or precursors of NO that can be activated under specific conditions should be the goal of NO-based therapies. This puts the activation and production of NO under local regulation analogous to the endogenous physiological pathway. Most physiological systems are rich in redundancy, allowing backup systems to support the primary system. Until now, scientists have operated under the paradigm of the L-Arg–NO pathway by NOS enzymes as the only pathway to produce NO. The emergence of a redundant pathway for maintenance of NO homeostasis by dietary nitrite and nitrate provides a new mode of intervention and a new paradigm for restoring NO homeostasis. The provision of nitrate and nitrite as sources of NO may then be viewed as a system of redundancy. This paradigm opens up a form of complementary and alternative medicine which aims to prevent and treat disease with substances which are natural to the body. Linus Pauling championed this approach many years ago and advocated 'the right molecules in the right

amounts'. Pauling indicated that the right molecules are 'substances that are normally present in the human body' and that same premise can be applied to the NO biochemistry of patients. Preclinical and clinical studies now reveal that nitrite can replete NO metabolites and correct conditions associated with NO insufficiency, as commonly measured by nitrite. We know nitrite is in steady-state equilibrium with nitrosothiols and nitrite can be reduced back to NO under appropriate conditions which can then activate NO-dependent targets such as sGC. One-electron reduction of nitrite to NO is energetically and kinetically favorable to a five-electron oxidation of L-Arg and the inherent nitrosative chemistry of nitrite serves to regulate nitrosothiols. Therefore, nitrite can theoretically activate the same pathways as NO, but under different regulations based primarily on redox status and oxygen tension. Nitrite then can not only act as a diagnostic or biomarker for NO availability but also act as an agent to restore NO homeostasis. The ultimate question is: Is nitrite 'drugable' and can it be developed and classified as a drug? Although efforts are underway to assess the potential usefulness of inorganic nitrite in a number of clinical research studies at the US National Institutes of Health and other institutions, it is unlikely that nitrite can be developed as a drug. Intellectual property claims related to simple, naturally occurring inorganic compounds are legally difficult to defend and the material itself is cheap and readily available. Furthermore, nitrite and nitrate have been classified by the FDA as 'Generally Recognized as Safe' for use as food additives and they are naturally occurring in many foods [130] and is present in relatively high concentrations in breast milk [131]. Regulation of nitrite and nitrate as a drug would be extremely arduous and complicated since they have been part of our food supply for centuries and may account for the health benefits of the Mediterranean and DASH (dietary approaches to stop hypertension) diets [130]. What is clear though is the undisputed health benefits of inorganic nitrite and nitrate in the proper context which provides justification for their use as active agents [127]. Many over-the-counter dietary supplements, particularly green food complexes are enriched in nitrite and/or nitrate [130]. In fact, use of a rationally designed nitrite- and nitrate-enriched dietary supplement has been shown in a clinical trial to restore NO homeostasis and modify cardiovascular risk factors such as hyperlipidemia [132].

Rather than a drug, nitrite may serve an even more important role. One cannot help but notice the emerging physiological data on nitrite are strikingly analogous to a vitamin. Vitamin or vital amine was the term coined by Casimir Funk (1884 – 1967) for the unidentified substances present in food which could prevent the diseases such as scurvy, beri-beri and pellagra. A vitamin is by definition any of a group of organic substance essential in small quantities to normal metabolism, found in minute amounts in natural foods or sometimes produced synthetically: deficiencies of vitamins produce specific disorders. We may have identified a new

vitamin, perhaps a Vitamin N. We know that nitrite is produced in relatively small quantities in normal metabolism of L-Arg and reduction of nitrate and is found in minute amounts in natural foods. Many animal studies reveal that nitrite insufficiency exacerbates I/R injury and increases mortality from I/R [109]. There are a host of diseases which are associated with decreased NO availability as measured by nitrite. Becoming more evident is the enormous benefit of exogenous dietary nitrite and nitrate in a number of disease models in animals and even in humans.

As we move forward with paradigm shifts and medical discoveries, the scientific community's main objective is to understand mechanisms of disease development to the extent needed to design rational therapies but with the ultimate goal of developing strategies for the *prevention* of human diseases. One could make a strong argument that diet should be a first target for disease prevention. Very little can affect our health more than what we choose to eat and our daily lifestyle habits. The realization of a nitrate–nitrite–NO pathway suggests that NO can be modulated by the diet independent of its enzymatic synthesis from L-Arg, for example, the consumption of nitrite- and nitrate-rich foods, such as leafy vegetables [133]. Antioxidants, such as vitamin C and polyphenols, can positively affect NO production from both pathways. First,

they help protect the essential co-factors for the NOS pathway, such as BH_4, from becoming oxidized and, therefore, promote L-Arg conversion to NO. Second, the presence of antioxidants protects NO from being scavenged once it is produced. Third, vitamin C and polyphenols can facilitate the reduction of nitrite to NO in the presence of an electron acceptor, thereby providing a recycling pathway. Although a very effective strategy at restoring NO homeostasis, the nitrate–nitrite–NO pathway may disrupt commercial development of NO drugs. Diet and nutrition may be the key to NO therapies. After all it was Hippocrates who said 'Let food be thy medicine and medicine be thy food'.

Declaration of interest

Nathan S Bryan has a financial interest in Neogenis, Inc. as a paid consultant and stockowner. Bryan also has stock options in SAJE Pharma and has received honoraria for consulting services to Bristol-Myers Squibb. His financial and research conflicts of interest are managed by UTHSCH Conflicts of Interest Management Plans, developed from and reviewed by the Research Conflicts of Interest Committee and approved by the Executive Vice President for Research at the University of Texas Health Science Center at Houston.

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