Review

Nitric oxide signaling in health and disease

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SUMMARY

The surprising discovery that the diatomic gas nitric oxide (NO) is generated by mammalian cells and serves to regulate a multitude of physiological processes has continued to fascinate biologists for almost four decades. The biochemistry of NO is complex, and novel insights into the control of NO biosynthesis and mechanisms of signal transduction are continuously emerging. NO is a key regulator of cardiovascular function, metabolism, neurotransmission, immunity, and more, and aberrant NO signaling is a central feature of many major disorders including cardiovascular disease, diabetes, and cancer. Here, we discuss the basics of NO biology emphasizing recent advances in the field including novel means of increasing NO bioactivity with therapeutic and nutritional implications.

INTRODUCTION AND HISTORICAL CONTEXT

Nitric oxide (NO) is an ancient messenger. In fact, NO generated by lightning when earth was anoxic has been suggested as a crucial factor to the origin of life itself (Santana et al., 2017; Wong et al., 2017). Nitrogen is essential for life, but the abundant atmospheric N2 cannot be utilized by organisms unless it is first reduced or oxidized, and NO has been proposed as one of the major sources of utilisable nitrogen in the primitive earth. Later, bacteria and plants started to synthesize NO via nitrate (NO3−) and nitrite (NO2−) reductases; NO is an obligate intermediate in the denitrification part of the nitrogen cycle and can be utilized as an electron acceptor for respiration in the absence of oxygen (Lundberg et al., 2004). The discovery of NO as a secretory product of mammalian cells took much longer. Nevertheless, in the mid-80s, the intense search for the elusive nature of an endogenous vasodilator termed endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980) came to an end when this labile mediator turned out to be NO (Palmer et al., 1987; Ignarro et al., 1987). The identification of EDRF as NO was facilitated by the earlier finding that nitroglycerine, an old vasoactive drug used to treat angina pectoris, acts through the release of NO and activation of soluble guanylyl cyclase (sGC) (Arnold et al., 1987; Katsuki et al., 1977). In sharp contrast to bacteria, NO and activation of O2 enabling reaction with arginine and the biosynthesis of NO. Tetrahydrobiopterine (BH4) is the second cofactor in the oxygenase domain (Benjamin et al., 1994; Lundberg et al., 1994b). Through this cycle, NO and chemically related bioactive nitrogen oxides are formed in a process dependent on commensal bacteria (Lundberg et al., 2008). The biochemistry of NO is intriguingly complex, and this free radical gas mediates its effects either through direct interaction with its cellular targets or through the formation of other reactive nitrogen oxide species, some of which have signaling properties in their own right. Even to this day, many questions remain regarding control of NO biosynthesis and transduction of NO bioactivity. In this review, we discuss the basics of NO biology and highlight recent advances and controversies in our understanding of the molecular mechanisms that govern NO signaling in health and disease, expanding the scope to include novel therapeutic opportunities.

NO BIOSYNTHESIS AND CELLULAR MECHANISMS

NO synthase

In mammals, nitric oxide synthase (NOS) exists in three isoforms, all of which generate NO and L-citrulline from L-arginine, molecular oxygen, and NADPH (Figure 1). NOS is a homodimeric heme-containing enzyme with an N-terminal oxygenase domain linked via a calmodulin (CaM)-binding sequence to a C-terminal reductase domain (Stuehr, 1997). Electrons from NADPH flow through the reductase domain aided by two co-factors FAD and FMN and then pass over to the oxygenase domain in a process that requires the binding of CaM to the mid-section of the enzyme (Stuehr, 1997). The final destination of these electrons is heme iron in the oxygenase domain that is reduced, thereby enabling binding of O2 to start the biosynthesis of NO. Tetrahydrobiopterine (BH4) is the second cofactor in the oxygenase domain. Its role seems to be to act as both a one-electron reductant and later in the catalytic cycle as an oxidant during the two-step oxidation of L-arginine (Stuehr and Haque, 2019). Donation of an electron from BH4 to the heme-bound oxygen results in activation of O2 enabling reaction with arginase and the
intermediate N(G)-Hydroxy-L-arginine (NOHA). Ultimately, when NOHA is oxidized, a ferric heme-NO species is formed that can release NO. How free NO is released from the ferric heme is still not entirely clear although direct measurements of NOS-derived NO gas in the airways or intestines clearly shows that it can occur (Lundberg et al., 1997b). An interesting recent hypothesis suggests that the entire heme-NOS complex is released from NOS rather than free NO and then functions as a signaling entity in its own right through insertion at the heme-binding site of sGC (Kleschyov, 2017) (Box 1). Although there is still an insufficient amount of data available, partial support for this theory was presented recently with the demonstration that NOS-derived NO finds heme in the cytosol, binds to it and then the entire heme-NOS complex is carried to sGC to trigger its assembly and activation (Dai et al., 2022). The main on-off switch for eNOS is binding of CaM to the enzyme which is regulated by changes in intracellular calcium fluxes and any agent capable of mobilizing intracellular calcium, including acetylcholine as classically used by Furchgott (Furchgott and Zawadzki, 1980), would in principle be able to activate eNOS provided that CaM is activated. The sensitivity of CaM toward eNOS can be additionally regulated by heat shock protein 90 (Brouet et al., 2001). Direct in vivo evidence for agonist-induced activation of eNOS comes from measurements of authentic NO gas in exhaled breath of pigs and humans after i.v. administration of various vasoactive drugs (Malmström et al., 2003). A dose-dependent and L-NAME-inhibitable release of exhaled NO, originating from the pulmonary endothelium, was observed for acetylcholine (ACh), bradykinin, and other eNOS activating compounds. Although overwhelming evidence exists for eNOS activation by the above vasoactive compounds, their true role in physiological regulation of NO signaling in the vasculature is less clear. Phosphorylation represents an important means of eNOS regulation and occurs at least at 7 different sites, 4 of which are stimulatory and 3 inhibitory. Protein kinase B (Akt) was described early to phosphorylate a serine (S1177 in humans and S1176 in rodents) on eNOS to activate it (Dimmeler et al., 1999; Fulton et al., 1999). Later studies have identified a number of other kinases including AMPK and protein kinase A, G, and C that can phosphorylate this and other sites (Garcia and Sessa, 2019). The role of Akt-stimulated eNOS regulation in vivo has been confirmed using a variety of mutant mice. As an example, mice in which serine 1176 on eNOS is replaced with an alanine, making it nonresponsive, exhibit reduced endothelial-dependent vasodilatation, increased blood pressure, insulin resistance and increased weight, i.e., a similar phenotype as the global eNOS KO (Atokin et al., 2007; Kashwagi et al., 2013). For the other phosphorylation sites, there is still no clear evidence of a physiologically relevant role in vivo. Importantly, the mere presence of eNOS phosphorylation is not necessarily coupled to a change in eNOS activity as assessed by modulation of sGC activity or vasoactivity. Examples are insulin and vascular endothelial growth factor (VEGF), which induce robust Akt-dependent phosphorylation of S1177 in endothelial cells but no appreciable cGMP formation or vasodilatation (Eroglu et al., 2019; Fleming et al., 2003). A major physiological trigger of vascular eNOS activation is when hemodynamic forces put pressure on the vessel wall (Rubanyi et al., 1986) (Figure 2). This rapid and dynamic shear stress-induced activation of eNOS is mediated by PIEZO1 in endothelial cells (Wang et al., 2015). Activation of this mechano-sensitive anion channel triggers ATP release and activation of the Gq/11/P2Y2 receptor complex (Wang et al., 2015) to initiate an intracellular cascade of events involving activation of Akt and another kinase PKN2 to phosphorylate eNOS at two sites (Jin et al., 2021). A recent report suggests that endothelial PIEZO1 is important also for normal expression of eNOS and in sustaining muscle capillary density (Bartoli et al., 2022). An illustrative example of rapid shear stress-induced NO signaling in vivo is flow-mediated dilatation, a method used in clinical research to evaluate endothelial function where the diameter of an artery is measured with ultrasound before and after a brief period of occlusion. The normal increase in vessel diameter in response to release of the occlusion is attenuated after pretreating with a NOS inhibitor in humans (Joannides et al., 1995) and in global as well as in endothelium-specific eNOS KO mice (Leo et al., 2021). The term constitutive is used frequently for eNOS and nNOS as transcriptional regulation was initially thought to be of minor significance. Gene expression of eNOS can however be induced in various ways.
Box 1. The heme-NO signaling hypothesis

A central dogma in NO signaling is that NO, being a tiny uncharged lipophilic gas, readily diffuses over cell membranes from one cell to another to reach its main target, soluble guanylyl cyclase (sGC). NO then binds to the heme iron of sGC resulting in activation of the enzyme and generation of cGMP (A). Although widely accepted, there are still some outstanding questions surrounding this paradigm. How is the NO released from the heme iron of NOS where it is generated? How is specificity of NO signaling obtained given that NO diffuses randomly in all directions and should have many potential targets? How does the reactive NO radical survive scavenging by other reactive species on its journey to sGC in another cell?

A new paradigm for NOS signaling has been suggested recently. According to this hypothesis, free NO is not even needed because signaling is mediated by release of the entire mobile/exchangeable heme-NO complex from NOS rather than NO itself (B). While this concept still remains to be proven, there are indications both from early studies and from very recent work to support it. Studies from 1978 show that heme-NO activates sGC while heme inhibits the Apo (heme-free) enzyme. Also heme-NO binding to sGC is much stronger than for heme implying that this species can easily replace heme at sGC. Moreover, a recent study shows that NO binds to cytosolic heme attached to GAPDH and then with the help of Hsp90, this heme-NO complex is incorporated into heme-free sGC to activate it (Dai, 2022). In aggregate, heme-NO, a powerful activator of sGC, is formed already at NOS. Why would nature then choose to first decompose it into reactive NO and then reform it again at sGC if instead the entire complex could travel and bind to sGC? In contrast to free NO, the mobile NO-heme complex may be delivered more safely to its target and in a coordinated manner. Such NO-heme could contribute also to additional NOS/NO-related signaling including S-nitrosation.

Adapted from Kleschyov (2017).
explore a pathogenic role of ADMA in blocking NO formation, thereby contributing the progression of cardiovascular disease. However, although numerous studies show positive correlations between plasma levels of ADMA and cardiovascular and renal disorders (Schwedhelm and Böger, 2011), its role in physiological regulation of NO signaling remains unclear.

The nitrate-nitrite-nitric oxide pathway
Oxidation of NO into inorganic nitrate (NO₃⁻) and nitrite (NO₂⁻) represents a major route for rapid inactivation of this potent biological messenger. For long, these anions were of biological interest mainly as measurable end oxidation products of NO, but research in the mid-90s demonstrated the existence of a reverse pathway where nitrate and nitrite are reduced back to NO again (Benjamin et al., 1994; Lundberg et al., 1994b; Zweier et al., 1995) (Figure 3). Intriguingly, the first step in bioactivation of nitrate is bacteria dependent. Circulating nitrate is actively taken up by the salivary glands and concentrated in saliva (Qin et al., 2012). In the mouth, oral commensal bacteria reduce nitrate to form the more reactive intermediate nitrite (Duncan et al., 1995). Nitrite in turn is swallowed and generates NO and other reactive nitrogen oxides through nonenzymatic disproportionation in the acidic stomach (Lundberg and Weitzberg, 2013). In addition to this, there are a multitude of enzymatic and nonenzymatic mechanisms systemically by which nitrite can be reduced to NO and other bioactive nitrogen oxides in blood and tissues (Lundberg et al., 2008) (Figure 3). Interestingly, these are all greatly accelerated under hypoxic and acidic conditions, i.e., when the oxygen-dependent NOSs may be dysfunctional. Therefore, the nitrate-nitrite-NOS pathway has been viewed as a backup system ensuring NO bioactivity also when NO output from NOS is low. Because nitrate and nitrite are abundant constituents of our everyday diet, much interest has been on the dietary aspects of this pathway, which are discussed in more detail below (Weitzberg and Lundberg, 2013). There is some evidence that not only exogenous dietary nitrate but also endogenous NOS-derived nitrate and nitrite plays a physiologically relevant role in NO signaling. Kapil and colleagues treated healthy subjects with an antiseptic mouthwash twice daily for a week to eliminate bioactivation of nitrate. This procedure caused a stop in oral nitrate conversion to nitrite, a drop in circulating nitrite and intriguingly an increase in blood pressure, which correlated with the decrease in plasma nitrite (Kapil et al., 2013). These findings are partly supported also by epidemiological data indicating that chronic mouthwash use is associated with negative health effects (Glot; 2021; Joshi-pura et al., 2020).

CELLULAR NO SIGNALING AND INACTIVATION
NO signaling is transduced through diffusion from its production site to the target, which may be in the same cell or in an adjacent cell. Paracrine signaling is possible because NO is tiny, uncharged and partly lipophilic, allowing it to freely pass biological membranes. In the target cell, NO canonically activates sGC by nitrosylation of the iron in its heme moiety, which in turn increases the synthesis of cGMP from cellular GTP. Nitrosylation is the direct binding of the NO radical to a transition metal, in this case Fe²⁺ (Heinrich et al., 2013). This activates downstream kinases including cGMP-regulated protein kinases and ion channels, to regulate a variety of physiological processes (Figures 1 and 4). Mechanistically, NO binds to a noncatalytic heme in the N-terminal part of the sGC, with a resulting conformational change in the enzyme (Kang et al., 2019) causing a several 100-fold increase in activity in the C-terminal cyclase domain (Arnold et al., 1977; Katsuki et al., 1977). The enzyme is a heterodimer composed of one α subunit and one β subunit. Although the α₁ and β₁ subunits are widely expressed in many tissues, expression of α₂ is tissue-specific and β2 is possibly not even translated (Koesling et al., 2016). Thus, the physiologically relevant isoforms of sGC are NO-GC1 (α₁/β₁) and NO-GC2 (α₂/β₁), whose functions have been characterized in isofrom-specific knockouts (Koesling et al., 2016). Overall, these enzymes display similar functions
in the cardiovascular system and most other tissues with the exception of the central nervous system where NO-GC1 is expressed presynaptically and NO-GC2 is found postsynaptically. A major overall control mechanism for this pathway is breakdown of the cGMP formed, a reaction catalyzed by various phosphodiesterases (PDE) of which PDE 5, 6, and 9 show the greatest specificity toward cGMP (Bischoff, 2004). Additional control of sGC activity is mediated by the matricellular protein trombospondin-1 (Isenberg et al., 2005). Binding of trombospondin-1 to the cell-surface receptor CD47 causes alterations in intracellular calcium fluxes that lead to inhibition of sGC activity and also negatively affects several other components of the NO signaling cascade, including eNOS and PKG (Rogers et al., 2014). Conversely, very low levels of NO have been shown to down regulate trombospondin-1 expression (Ridnour et al., 2005).

NO signaling is also effectively terminated by its direct oxidation into higher oxides of nitrogen. Nitrate is by far the dominating end oxidation product with levels in human plasma in the 10–50 μM range, whereas nitrite is generally below 1 μM (Lundberg and Govoni, 2004). Two quantitatively important oxidation pathways include reaction of NO with oxy-Hb and reaction with another radical, ultimately forming nitrate. The effectiveness of Hb in scavenging NO in vivo is illustrated by the predominant dilatation of pulmonary vessels in response to inhaled NO, with minimal acute effects on systemic hemodynamics.
2012)( Figure 2). Most of the NO diffusing in the other direction, forms peroxynitrite (Beckman et al., 1990), which can act as an oxidant and nitrating agent after protonation as discussed (Frostell et al., 1991). The reaction between NO and superoxide is a potent NO scavenger, is strategically expressed in the myoendothelial junction of small arteries and arteriole and acts as a “gate keeper,” controlling the diffusion of NO between these cells. The oxidation state of the heme iron in Hb-α determines its NO scavenging ability, and this is regulated by a cytochrome b₅ reductase 3 localized to the same area (Straub et al., 2012) (Figure 2). Most of the NO diffusing in the other direction, i.e., toward the luminal side is rapidly scavenged by the blood.

Some cross talk exists in the NO signaling between the three NOS isoforms and between NOS-dependent and NOS-independent pathways. Mice lacking eNOS have lifelong hypertension and develop metabolic disturbances but other than that the phenotype is not very severe and life span is near normal (Duplicaï et al., 2001; Huang et al., 1995). For life span, the same is partly true also for single gene knockdown of nNOS or iNOS (Morishita et al., 2005). In contrast, if all three NOSs are deleted, a severe cardiovascular and metabolic phenotype develops rapidly with mice dying predominantly from myocardial infarction before 1 year of age (Nakata et al., 2008). Such mice also display exceptionally low circulating levels of nitrate and nitrite. In aged eNOS deficient mice, the metabolic phenotype could be partly reversed by dietary inorganic nitrate (Carlström et al., 2010) and conversely, long-term dietary nitrate deficiency causes metabolic syndrome, endothelial dysfunction, and increased cardiovascular death in normal mice (Kina-Tanada et al., 2017). Lastly, dietary supplementation with nitrate at higher doses in normal rats causes down regulation of eNOS activity and signaling (Carlström et al., 2015). Altogether, this suggests that NOS-dependent and NOS-indepen- dent NO-signaling pathways overlap and can partly substitute for each other.

S-nitrosation and nitration
S-nitrosation refers to the covalent addition of a nitrogen monoxide group (formally NO⁺) to a thiol group (-SH) (Heinrich et al., 2013). Low molecular weight S-nitrosothiols such as S-nitroso cysteine (Cys-NO) or S-nitroso glutathione (GS-NO) are highly bioactive and are as potent vasodilators as NO itself, acting via the sGC-cGMP pathway (Ignarro et al., 1980). Besides being used as NO donor drugs in research, endogenously formed small S-nitrosothiols are also thought to be involved in cellular and inter-cellular physiological signaling e.g., in vascular regulation (Lima et al., 2010) and in transnitrosation reactions, i.e., when a S-nitrosothiol is transferred between two entities. S-nitrosation also occurs at cysteine residues in proteins can alter their function, whereas nitration of unsaturated fatty acids or nucleotides may give rise to novel species with signaling properties.

Questions remain as to how specificity is obtained by a signaling molecule similar to NO that easily passes biological membranes and diffuses randomly in all directions. Specific subcellular localization of NOS to caveolae (Garcia-Cardenas et al., 1996) or in the direct vicinity of a target (Iwakiri et al., 2006) represent two possible mechanisms to achieve this. For NO signaling between the endothelium and vascular smooth muscle cells another mechanism has been proposed. Hemoglobin-alpha (Hb-α), a potent NO scavenger, is strategically expressed in the myoendothelial junction of small arteries and arteriole and acts as a “gate keeper,” controlling the diffusion of NO between these cells. The oxidation state of the heme iron in Hb-α determines its NO scavenging ability, and this is regulated by a cytochrome b₅ reductase 3 localized to the same area (Straub et al., 2012) (Figure 2). Most of the NO diffusing in the other direction, i.e., toward the luminal side is rapidly scavenged by the blood.

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Figure 4. The chemical biology of NO signaling
NO generated by NOS can signal via different mechanisms. Nitrosylation refers to the direct binding of the NO radical to a transition metal, e.g., heme-iron (Fe). This reaction underlies the canonical activation of sGC by NO and the inhibition of cytochrome c oxidase (CCO) in mitochondria. There are a number of additional targets for nitrosylation including iron-sulfur clusters in proteins and non-heme iron. Nitrosation is the covalent binding of an NO⁺ moiety to a sulfhydryl group (-SH) to form an S-nitrosothiol (S-NO). Such post-translational modification is suggested to regulate the activity of a multitude of proteins. Low molecular weight thiols such as cysteine and glutathione may also be nitroso- tated and these compounds may serve as transducers of NO bioactivity or act to nitrosate other targets. Nitration refers to the introduction of a nitro group (-NO₂) into an organic compound and requires intermediate formation of the nitrogen dio- xide radical. Nitration of tyrosine residues in pro- teins can alter their function, whereas nitration of unsaturated fatty acids or nucleotides may give rise to novel species with signaling properties.
major pathway for physiological NO signaling in addition to the sGC/cGMP-pathway (Figure 4). A multitude of proteins are suggested to be regulated by S-nitrosation (Stamler, 1994) both under physiological conditions as well as in pathologies (Liu et al., 2004), e.g., inflammation or cancer when nitrosative stress can be considerable. All rapid physiological signaling pathways require tightly controlled dynamic switches in activity at multiple levels. So, how then is S-nitrosation regulated in vivo? Several enzymes capable of removing the NO moiety from the cysteine have been identified. These are often referred to as denitrosylases and include S-nitrosoglutathione reductase (formally glutathione-dependent formaldehyde dehydrogenase) (Liu et al., 2004), thioredoxin (Benhar et al., 2008), thioredoxin-related protein of 14 kDa (Pader et al., 2014), glutaredoxin (Ren et al., 2019), and more. However, the mechanism by which S-nitrosothiols are formed in vivo is much less clear (Keszler et al., 2010). The sources of nitrosating nitrogen oxides are the three NOSs but also circulating nitrite, derived from oxidation of NO or from dietary nitrate. NO itself is incapable of S-nitrosation (besides a possible reaction with a thylid radical); so, it must first be converted to a nitrosating agent. There may be multiple mechanisms to achieve this including reaction with oxygen, which is second order in NO and, therefore, slow, or intermediate formation of nitrosyl groups in heme- or non-heme iron (Vanin, 2019). In contrast to phosphorylation, which is exclusively enzymatic, most S-nitrosation observed in vivo in mammals is thought to be of a nonenzymatic origin although enzymatic pathways were recently reported in bacteria (Seth et al., 2018). The true physiological regulatory role of S-nitrosation is still a matter of debate. A simple observation of the gross phenotypes associated with deletion of different components of the NO signaling pathways can be quite illustrative. If cGMP/sGC-independent signaling via NOS-dependent S-nitrosation regulates as many vital processes as claimed one would expect vastly different phenotypes in NOS-deficient mice versus mice lacking sGC activity. Thoonen and colleagues developed heme-deficient soluble guanylyl cyclase knock-in mice, which rendered them NO-unresponsive (Thoonen et al., 2015). It seems that the classical physiological functions known to be regulated by NO, including blood pressure, platelet function, gastrointestinal motility, and penile erection (Decaluwe et al., 2017) are affected similarly in these mice compared with the various models of NOS-deficient mice. Life span is shorter (Thoonen et al., 2015) and correlates well to what is seen in mice lacking all three NOSs (Nakata et al., 2008). Overall, this may suggest that cGMP-signaling is in fact the major pathway for physiological transduction of NO bioactivity in mammals. Moreover, for S-nitrosation, questions still remain as to how stable the SNO bond is or whether rapid further oxidation to a disulfide instead is the final product of this reaction (Wolhuter et al., 2018). In fact, any study where a critical cysteine has been replaced with a non-nitrosable amino acid is inherently non-conclusive because the procedure also disables any other oxidative modification of the cysteine. Also, there are issues about specificity and the predicted slow predominantly non-enzymatic in vivo kinetics of S-nitrosation. In contrast to phosphorylation, S-nitrosation is quite promiscuous, in particular at high NO fluxes such as after induction of iNOS or following treatment with in vivo nitrosating agents such as nitrite (Chouchani et al., 2017; Quesnelle et al., 2020). Such massive S-nitrosation may be involved in pathophysiological events but also possibly in therapeutic effects of drugs with NO-like bioactivity.

Nitration is a reaction where a nitro group (-NO2) is introduced into an organic compound (Heinrich et al., 2013). It is utilized in industry, e.g., in the production of explosives; however, interestingly, it also seems to be a relevant reaction in NO biology (Figure 4). In biological systems, nitration is typically mediated by the nitrogen dioxide radical (NO2•), which in turn is formed together with the hydroxyl radical by the spontaneous decomposition of protonated peroxynitrite, by reaction of peroxynitrite with carbon dioxide, oxidation of nitrite, e.g., by a peroxidase or from disproportionation of nitrite in the highly acidic gastric environment (Radi, 2018; Rocha et al., 2011; Tsikas, 2011). Thus, in tissues, nitration likely is most abundant where iNOS is upregulated and a source of superoxide is present to form peroxynitrite. Nitration was initially believed to be an overall harmful reaction causing alterations in protein function. Tyrosine residues in proteins can be nitrated, and detection of this species has been used extensively as a marker of peroxynitrite formation and nitrosative stress in general (Kooy et al., 1997). Interestingly, however, nitration reactions can give rise to other compounds, altering their bioactivity and possibly even forming novel signaling species. Unsaturated fatty acids (FAs) including oleic and linoleic acid can be nitrated to form nitro-lipids (NO2-FAs), which render them electrophilic, allowing reaction mainly with cysteines through Michael addition reactions (Coles et al., 2002). Through this mechanism, NO2-FAs potently antagonize NF-xB and activate NrF2 signaling, two major effects explaining their broad anti-inflammatory and antioxidant activity (Cui et al., 2006). The role of endogenously formed NO2-FAs in regulation of physiological signaling is still unknown, and the site and extent of their formation under normal conditions is unclear. Nevertheless, when given exogenously, protective anti-inflammatory effects have been observed in numerous preclinical animal models of disease, including cardiovascular, pulmonary, and renal fibrosis (Kho and Schopfer, 2019). Nucleotides, such as GTP, can also be nitrated by NO reaction products. When such GTP is further metabolized by sGC, the result is formation of 8-nitro-cGMP (Sawa et al., 2007). Studies in Akaike lab have shown that in addition to classical activation of cGMP-dependent protein kinases, 8-nitro-cGMP also reacts with cysteine residues in various proteins to form S-guanylated proteins, a kind of post-translational modification that induces modification of protein functions (Akaike et al., 2013). Nitro-cGMP also differs from cGMP in that its degradation by PDEs is delayed due to intermediate reactions with persulfide species. Similarly to NO2-FAs, questions still remain regarding control of formation and activity of nitro-cGMP and its role in physiological signaling. The gastric environment offers favorable conditions for nitration in humans as nitrogen dioxide (NO2) is readily formed from the reaction of salivary nitrite and gastric acid (Benjamin et al., 1994; Lundberg et al., 1994b). Studies have shown that pepsin is nitrated and inactivated in the rat stomach, preventing the progression of gastric ulcers. Moreover,
gastric nitration is likely a major route for generation of NO\textsubscript{2}\textsuperscript{-} FAs found systemically (Buchan et al., 2018).

**NO IN PHYSIOLOGY AND DISEASE**

**Cardiovascular function**

The cardiovascular system may be viewed as “the canonical NO system” because the discovery of NO was enabled by experiments using the vascular endothelium, and the first therapeutic NO donor ever used was nitroglycerine to treat angina pectoris. In the vasculature, it is evident that eNOS-derived NO plays an important homeostatic role by regulating tissue blood flow, controlling vascular remodeling, and protecting the endothelium against platelet aggregation and leukocyte adhesion (Daiber et al., 2019). The clearest illustration of NO’s physiological role in vascular control is the vasoconstriction and increase in blood pressure seen after acute administration of a NOS inhibitor (Rees et al., 1989). The essential role of endothelially expressed eNOS in vascular control was finally confirmed recently in endothelial cell-specific eNOS deficient mice whose vascular phenotype is similar to what is seen in the global eNOS knockout (Leo et al., 2021). In most cardiovascular diseases, an impairment of endothelial function is observed, mainly driven by lower bioavailability of NO (Lundberg et al., 2015); however, we are yet to explore whether a correction of this abnormality would delay or prevent disease. There has been a wide interest in investigating if genetic predisposition related to NO signaling is associated with various common diseases. Several studies have found associations between genetic variants related to NOS-sGC signaling and increased risk of cardiovascular disease (Erdmann et al., 2013; Malik et al., 2018). Conversely, genetic predisposition to enhance NO signaling has been associated with reduced risk of coronary heart disease and stroke (Emdin et al., 2018). Future studies will determine the benefit and clinical utility of these findings.

Although the role of NO in vascular control is now well established, its function in the heart is less clear. The finding of specific subcellular localization of eNOS and NOS isoforms in cardiomyocytes indicate different roles of these enzymes in cardiac function (Barouch et al., 2002). These spatial constraints suggest effects coupled to nearby specific effector proteins because diffusion of NO is likely limited by the high amount of myoglobin in cardiac myocytes with NO scavenging properties. However, cardiac myoglobin may have an opposite role in regulating cardiac NO bioavailability, by its nitrite-reducing capacity (Hendgen-Cotta et al., 2008). Activation of eNOS in vitro and in vivo leads to increased cardiomyocyte and ventricular relaxation, which has been attributed to reduced intracellular Ca\textsuperscript{2+} via inhibition of L-type calcium channels and enhanced Ca\textsuperscript{2+} reuptake into the SR as well as by PKG-mediated phosphorylation of troponin I and subsequent reduction in myofilament Ca\textsuperscript{2+} sensitivity (Farah et al., 2018; Shah et al., 1994). The role of NO on contractility and response to β-adrenergic stimulation is more complex with contradicting results. In vivo studies mainly suggest that eNOS localized to caveolae, in vicinity to L-type Ca\textsuperscript{2+} channels and β-adrenergic receptors (Bailjepalli et al., 2006), has been shown to have an inhibitory role on β-adrenergic stimulation (Gödecke et al., 2001; Massion et al., 2004), whereas in vitro studies using cardiomyocytes or muscle preparations give opposite results. This inconsistency may be explained by in vivo paracrine NO signaling from vascular eNOS in regulation of cardiac contractility. Moreover, the vasodilator action of eNOS-derived NO in the coronary vasculature may promote contractility in the in vivo situation (Rassaf et al., 2006). nNOS located at the sarcoplasmic reticulum stimulates Ca\textsuperscript{2+} release via the ryanodine receptor (RyR) and enhances contractility, possibly by S-nitrosation of thiol groups on the RyR (Barouch et al., 2002; Wang et al., 2010; Xu et al., 1998). Other important roles of NO in cardiac function include regulation of O\textsubscript{2} consumption by affecting mitochondrial respiration (Trochu et al., 2000), anti-arrhythmic properties (Reilly et al., 2016), and modulation of autonomic nerve signaling (Schwarz et al., 1995).

Dysregulated NOS activity has been implicated in the pathophysiology of heart failure (HF), but the role of different NOS isoforms is complex. Both increased and/or uncoupled NOS activity has been observed in heart failure and has been associated with dysfunctional Ca\textsuperscript{2+} handling and cardiac remodeling (Damy et al., 2004; Haywood et al., 1996; Takimoto et al., 2005). Experimental disruption of the nNOS gene aggravates the development of heart failure (Dawson et al., 2005), and conversely cardiomyocyte-specific overexpression of nNOS leads to some protection in animal models (Loyer et al., 2008). Translocation of nNOS away from the sarcoplasmic reticulum to the sarcolemma has been described, leading to altered nNOS-mediated regulation on Ca\textsuperscript{2+} handling, which by some investigators has been interpreted as an adaptive mechanism to limit effects of chronic β-adrenergic stimulation seen in HF (Damy et al., 2004). Reduced expression of caveolin 3 and eNOS uncoupling have been observed in HF and has been suggested to contribute to hypertrophy and remodeling (Takimoto et al., 2005) (Feiner et al., 2011), whereas overexpression of eNOS has been protective (Jones et al., 2003). In addition, the increased oxidative stress seen in heart failure can negatively affect several steps of the NOS-NO-cGMP-sGC pathway, including oxidation of BH4 and NOS uncoupling, scavenging of NO, and oxidation of sGC (Daiber et al., 2019). In addition, nitrosative stress generated by iNOS induction has also been implicated in HF (Haywood et al., 1996; Schiattarella et al., 2019). This knowledge has stimulated numerous trials targeting the NOS-NO-cGMP-sGC pathway, including oxidation of BH4 and NOS uncoupling, scavenging of NO, and oxidation of sGC (Daiber et al., 2019). In addition, nitrosative stress generated by iNOS induction has also been implicated in HF (Haywood et al., 1996; Schiattarella et al., 2019). This knowledge has stimulated numerous trials targeting the NOS-NO-cGMP-sGC pathway in patients with HF, but, as of today, only the sCG stimulator vericiguat has shown enough efficacy to be approved for clinical use (Armstrong et al., 2020; Borlaug et al., 2018) (Figure 5). In the future, we will most likely see other sGC stimulators and activators emerging in the clinic for a variety of indications.

Numerous studies in models of myocardial ischemia-reperfusion (IR) injury strongly suggest NO-mediated protection. Transgenic overexpression of eNOS (Erod et al., 2006; Janssens et al., 2004) and NO donors (Bice et al., 2016) show protection, whereas NO inhibition aggravates injury (Schulz et al., 2004). However, results from various strains of eNOS KO mice show different effects on myocardial injury (Sharp et al., 2002). Mitochondria have been suggested to be central targets by which NO can ameliorate cardiac IR injury. Via opening of K\textsubscript{ATP} channels, NO may reduce Ca\textsuperscript{2+} overload (Sasaki et al., 2000), by inhibiting opening of mitochondrial permeability transition pores.
it can prevent cytochrome c release and apoptosis (Shiva et al., 2007), and NO may also limit ROS generation through S-nitrosation of complex I (Burwell et al., 2006; Chouchani et al., 2013). In addition, the inhibitory effect of NO on cytochrome c oxidase has been suggested to positively regulate myocardial O2 consumption (Trochu et al., 2000). Other well-described effects of NO may also contribute to protection including dilation of coronary blood vessels, scavenging of superoxide, inhibition of platelet, and leukocyte aggregation and arginase inhibition (Omar et al., 2016). The impressive preclinical data highlighting the beneficial role of NO in IR injury have unfortunately not been followed by positive outcomes in human studies so far. Randomized clinical trials in human cardiac IR injury with organic nitrates (Morris et al., 1995), inhaled NO (Janssens et al., 2018), inorganic nitrite (Jones et al., 2015; Kim et al., 2021; Siddiqi et al., 2014), or nitrate (Eriksson et al., 2021) do not show major positive effects.

The red blood cell and platelets
The red blood cell (RBC) has a unique role in NO biology, both as a major inactivation pathway and, at the same time, a transducer of NO bioactivity (Cosby et al., 2003; Pawloski and Stamler, 2002; Yang et al., 2013). RBCs contain hemoglobin at 5–10 mM, and, when oxygenated, this protein functions as an extremely effective dioxygenase, rapidly metabolizing NO to form nitrate and Met-Hb (Fe3+)(Doyle and Hoekstra, 1981). By this mechanism, RBCs inactivate NO diffusing from the endothelium into the vessel lumen so that free NO is practically nonexistent in the blood. Stamler and colleagues originally proposed that RBCs are in fact also capable of generating and exporting NO bioactivity to elicit vasodilation (Jia et al., 1996). Intriguingly, such release is said to be dynamically controlled by the degree of blood oxygenation, with more NO bioactivity exported under hypoxic conditions, thereby enabling exact matching of blood flow and tissue oxygen demand. Mechanistically, in the arterial oxygenated blood, an NO+ moiety is transferred from nitrosyl-Hb to a highly conserved cysteine residue at position 93 of the b chain of Hb (b93C) to form S-nitroso (SNO)-Hb. In turn, on subsequent deoxygenation, SNO-Hb exports NO bioactivity from the RBC to cause vasorelaxation (Premont et al., 2022). An alternative means of RBC generation and export of NO bioactivity was suggested by Gladwin, Rifkind, and their coworkers, who described enzymatic reduction of nitrite to NO by deoxy-Hb, a reaction also enabling maximal NO export when vasodilatation is most needed (Cosby et al., 2003; Nagababu et al., 2003). Nitrite is an oxidation product of NO; so, this would represent an

| Symptom/condition | Treatment | Drug (generic) |
|-------------------|-----------|----------------|
| Angina pectoris   | Organic nitrates | Glyceryl trinitrate (nitroglycerine) Isosorbid mono nitrate |
| Pulmonary hypertension of the newborn | Inhaled NO gas | |
| Glaucoma          | Hybrid NO donor | Latanoprostene bunod |
| Asthma            | Exhaled NO | |
| Primary ciliary dyskinesia | sGC stimulator | Vericiguat |
| Heart failure     | sGC stimulators PDE inhibitors | Riociguat Sildenafil |
| Pulmonary artery hypertension | sGC stimulators PDE inhibitors | Riociguat Sildenafil |
| Erectile dysfunction Prostate hyperplasia | PDE inhibitors | Sildenafil |

Figure 5. Approved NO-related therapies and diagnostic procedures
Treatment with organic nitrates including nitroglycerine has been used clinically for over 150 years, i.e., long before we knew that NO was a signaling molecule in mammals. These drugs act through the release of NO bioactivity and formation of cGMP to reduce cardiac pre- and afterload and dilate coronary vessels, thereby relieving angina pectoris. Inhalation of authentic NO gas is approved for clinical use in newborn babies with respiratory distress syndrome and pulmonary hypertension. The beauty of inhaled NO is its “double selectivity.” First, because it is administered via inhalation it dilates only vessels supplying well-ventilated parts of the lungs, which increases ventilation-perfusion matching. Secondly, NO is more or less selective to the lung and systemic vasodilatation is minimized due to the rapid scavenging of NO in blood. A hybrid compound linking the glaucoma drug latanoprost with an organic nitrate was recently approved and is used topically. The NO moiety is said to increase the overall efficiency of this drug via improved drainage of aqueous humor. Airway formation of NO is altered in inflammatory airway disease and measurements of exhaled NO in a single breath is an approved method used to diagnose and monitor treatment of allergic asthma. In primary ciliary dyskinesia (PCD), airway levels of NO are markedly reduced, and this test is used in the diagnostic workup for this genetic airway disease. Drugs that stimulate sGC have been approved for use in patients with heart failure as well as in pulmonary artery hypertension. Finally, PDE5 inhibitors inhibit the breakdown of cGMP and are used clinically for erectile dysfunction and benign prostate hyperplasia and have also been approved for pulmonary artery hypertension. The generic names of example drugs are given in the right column.
intriguing mechanism for the recycling or reactivation of this biological messenger and is discussed in further detail below (Gladwin et al., 2000; Modin et al., 2001). The general ability of RBCs to generate and export NO bioactivity has been heavily debated over the years, mainly because NO is not believed to be able to escape rapid destruction by the Hb reaction. However, more recent studies do in fact confirm that export of NO bioactivity can occur albeit not in the form of free NO. Building on the surprising demonstration of a catalytically active eNOS in RBCs (Kleinbongard et al., 2006), Yang and colleagues went on to show that this eNOS controls the release of cardioprotective NO bioactivity in a process regulated by arginase 1 (Yang et al., 2013). Isolated mouse hearts exposed to IR injury were protected if the coronary circulation was perfused with isolated RBCs prior to the onset of ischemia, and the protection was lost if RBCs from eNOS deficient mice were used. Interestingly, the cardioprotective effect is entirely independent on the presence of eNOS in the heart itself because hearts from global eNOS knockouts were protected if perfused with WT RBCs. In a recent publication, Cortese-Krott and coworkers describe the generation of RBC-specific eNOS knockout mice and show increased infarct size and aggravated left ventricular dysfunction after in vivo myocardial infarction, compared to wild-type controls (Cortese-Krott et al., 2022). Moreover, in vitro studies where RBCs and platelets are co-incubated suggest that NO bioactivity can be released upon deoxygenation to affect platelet function (Srihirun et al., 2012). To further dissect the mechanisms behind RBC export of NO bioactivity, mice in which the β93 cysteine of Hb has been replaced with alanine (β93A) were developed. Multiple different experiments both in vivo and in vitro now show that NO bioactivity export from RBCs does not require the presence of a β93 cysteine in Hb, leaving the exact mechanism of the RBC-derived vasodilatation and tissue protection unresolved (Isbell et al., 2008; Sun et al., 2019). Although these mice clearly have a phenotype with pulmonary hypertension and premature death when kept in a low oxygen environment (Zhang et al., 2022), there is no evidence that this is selectively caused by abrogated SNO-Hb signaling. Notably, the Hb β93 cysteine might in fact have an alternative role for example in protecting the heme iron against oxidation as suggested in early studies (Winterbourn and Carrell, 1977). Cortese-Krott and colleagues recently developed tissue-specific eNOS knockout mice lacking this enzyme either in endothelial cells or in RBCs (Leo et al., 2021). Their study nicely confirms the central role for eNOS in the endothelium in control of vascular function and blood pressure and additionally suggest a surprising role also for RBC eNOS in blood pressure control. Indeed, blood pressure was increased in both mutant strains and could be normalized by reintroducing the eNOS gene in the endothelium or in RBCs, respectively. RBCs are currently being established as exporters of NO bioactivity to elicit vasodilatation and tissue protection. Interestingly, the opposite also seems to be true. Thus, under certain circumstances RBCs can mediate oxidative stress, endothelial dysfunction and increased susceptibility toward IR injury (Pernow et al., 2019). RBCs collected from type-2 diabetics induce endothelial dysfunction if co-incubated with healthy vessels (Zhou et al., 2016) and aggravate myocardial injury when perfused in an ex vivo rodent heart model before exposed to global hypoxia (Yang et al., 2018). These effects seem ROS dependent and mediated by increased arginase-1 activity coupled to reduced NOS activity and signaling in the RBC.

Platelets were shown early to be affected by NO (Radomski et al., 1987). These cells harbor their own eNOS (Sase and Michel, 1993) and may also import NO bioactivity, with the main effect being to inhibit platelet activation in a cGMP-dependent fashion (Freedman et al., 1999). This is illustrated by the shortened bleeding time observed after inhibition of NOS (Remuzzi et al., 1990) and in eNOS deficient mice (Freedman et al., 1999), and enhanced experimental thrombus formation also seen in these animals (Shultz and Raji, 1992). Later studies revealed a more complex regulatory role of platelet NO/cGMP-signaling involving its downstream target PKG. The events consist of an early stimulatory response that promotes platelet activation followed by a delayed platelet inhibition that serves to limit the size of platelet aggregates (Li et al., 2003).

Metabolic regulation

NO is a physiological regulator of mitochondrial respiration by inhibition of cytochrome c oxidase (CCO) at its binuclear heme/copper site (Brown and Cooper, 1994; Carr and Ferguson, 1990; Cleeter et al., 1994). The reaction with reduced heme is competitive with oxygen and is predominantly occurring at high electron fluxes. At lower electron fluxes and oxidized copper, the reaction with NO generates a cupric-nitrite complex, which cannot react with oxygen and is, therefore, not truly competitive. The inhibitory effect of NO is higher at low PO2, meaning that during hypoxic conditions lower levels of NO are needed, but at the same time under such conditions NO activity would be reduced due to less available oxygen (Erusalimsky and Moncada, 2007). Experimental data show that low nanomolar concentrations of NO, resembling physiological concentrations, raise Km for oxygen substantially, thereby affecting respiration (Brown and Cooper, 1994). Components of the electron transport chain (complex III, cytochrome c, and CCO) can generate NO by reduction of inorganic nitrite that may contribute to regulation of mitochondrial respiration (Basu et al., 2008; Castello et al., 2006; Kozlov et al., 1999). However, the regulatory role of NO in vivo has been more difficult to establish. Animal experiments show that systemic administration of NOS inhibitors increases oxygen consumption in various tissues as well as whole-body oxygen consumption (Shen et al., 1994). In humans, however, the effects of systemic NOS inhibition on oxygen consumption show conflicting results (Heinonen et al., 2011; Schrage et al., 2010). Interestingly, human studies show that inorganic nitrate reduces oxygen cost during rest (Larsen et al., 2014) and exercise (Larsen et al., 2007) and has been shown to increase skeletal muscle mitochondrial efficiency (P/O ratio) due to down regulation of uncoupling protein 3 and ATP/ADP translocase, leading to less uncoupling (Larsen et al., 2011).

There are several theories aiming to explain the physiological rationale for NO-mediated CCO inhibition. One suggestion is that inhibition by NO leads to redistribution of intracellular oxygen to other enzymes with higher Km for oxygen, exemplified by NO-dependent increase in prolyl hydroxylase-degradation of HIF-1α (Hagen et al., 2003; Trimmer et al., 2001). Conversely, the incomplete reduction of O2 at the electron transport chain
promotes generation of superoxide and hydrogen peroxide that can participate in physiological signaling to reduce prolyl hydroxylase-degradation and activate HIF-1α-dependent downstream signaling (Sarkela et al., 2001). The same mechanism has been proposed to affect redox-sensitive transcription factors, such as NF-κB and Nrf2. Other interpretations suggest that NO-mediated CCO inhibition extends the O2 gradient from capillary to more distant cells (Thomas et al., 2001) and regulates cardiac O2 consumption (Trochu et al., 2000). However, higher levels of NO generation, which is seen after iNOS induction may lead to irreversible CCO inhibition, increased ROS and reactive nitrogen species (RNS) formation and mitochondrial damage, which has been a proposed mechanism partly explaining mitochondrial dysfunction in sepsis (Brealey et al., 2002).

Mitochondrial biogenesis is an adaptive response to several stimuli, most importantly exercise (Egan and Zierath, 2013) and caloric restriction (Civitarese et al., 2007). Early in vitro studies by Nisoli and coworkers could show eNOS-cGMP-dependent mitochondrial biogenesis mediated by PGC1-α in mammalian cells (Nisoli et al., 2003) and later during caloric restriction in mice (Nisoli et al., 2005). However, subsequent studies in transgenic mice or with NOS inhibition show varying results related to tissue and type of NOS isoform to the importance of NO for mitochondrial biogenesis (Schild et al., 2006; Wadley and McConell, 2007). Moreover, the importance of NO in this context seems different between rest and after exercise (Wadley et al., 2007).

The described effects of NO on mitochondrial function and redox reactions are of importance for metabolic homeostasis. This is exemplified by the development of metabolic dysfunction with dyslipidemia, impaired glucose tolerance and deficient mitochondrial β-oxidation in mice lacking eNOS (Carlström et al., 2010; Le Gouill et al., 2007) and the association between eNOS polymorphisms and diabetes (Monti et al., 2003). Physiological NO signaling is involved in several pathways related to carbohydrate and lipid metabolism and has been shown to modulate insulin secretion and glucose uptake (Bahadoran et al., 2020). The role of NO in insulin secretion upon glucose stimulation is complex with both stimulatory and inhibitory effects, the latter suggested by some investigators to reduce excessive insulin secretion to protect the β cells (Henningsson et al., 2002; Laffranchi et al., 1995; Lajoix et al., 2001). The different roles of eNOS and nNOS in enhancing glucose uptake in skeletal muscle, liver, and adipose tissue are not fully elucidated, but a common mechanism is by increasing glucose transporter 4 (GLUT 4) expression and its translocation to the membrane, which partly involves AMPK phosphorylation (Li et al., 2004; Lira et al., 2007). Glucose uptake is also facilitated by the vasodilatory action of eNOS-derived NO. In addition to the peripheral effects of NO described above, data suggest involvement also in central regulation and sensing of glucose homeostasis (Fioramonti et al., 2010). Although these mechanisms are still unclear, NO-dependent modulation of release of hypothalamic-pituitary hormones as well as vagal nitrergic signaling have been suggested (Murphy et al., 2009).

Reduced NO bioavailability due to a combination of diminished synthesis, eNOS uncoupling, and increased oxidative stress has been described in obesity and diabetes and has been implicated in metabolic and cardiovascular dysfunction (Bahadoran et al., 2020). However, iNOS induction has been observed in diabetes and obesity and even if the role of iNOS is unclear, high NO levels seem to negatively affect β cell function (Corbett et al., 1993) and induce insulin resistance in peripheral tissues (Perreau and Marette, 2001). This raises questions regarding dosing, timing, and other aspects when exploring beneficial effects of NO donating substances to improve metabolic function.

**Neurotransmission**

Nitric oxide is profoundly involved in central and peripheral neuronal signaling, regulating a multitude of modalities including control of movement, energy homeostasis, learning, memory, sleep, feeding, anxiety, reproduction, and regulation of cerebral blood flow (Garthwaite, 2019). nNOS is the most abundant isoform in the central nervous system (CNS), distributed in most regions of the brain but with some histological dominance in cerebellar cortex, the olfactory bulb, the striatum, the hypothalamus, and the hippocampus and found mostly in GABAergic and glutaminergic neurons (Chachlaki and Prevot, 2020). The expression in both excitatory and inhibitory neurons can explain the multiple roles of NO in neuronal communication, supported by data from several studies using nNOS gene disrupted mice (Tanda et al., 2009; Wetzdorfer et al., 2004). In addition, the three-dimensional release of NO and the ability to diffuse through cell membranes allows multimodal signaling and synaptic crosstalk via retrograde transsynaptic activity, as well as neurone-astrocyte communication (Figure 1). Complexity is further compounded by the fact that vascular eNOS in proximity to neurons can modulate nerve transmission and that NO also signals to heterogeneous non-neuronal NO targets such as blood vessels (Garthwaite, 2019). The most common nNOS splice variant in the CNS is nNOSz, which contains a PDZ domain that allows for physical interaction with N-methyl-D-aspartate receptors (NMDAR) in complex with postsynaptic density protein 95 (PSD95) (Brenman et al., 1996). Glutamate activation of the NMDAR leads to influx of intracellular Ca2+ and subsequent nNOS activation, which can be further enhanced by phosphorylation of serine 1412 by AKT (Adak et al., 2001). The main signaling mode in the CNS is via the NO-eGC–cGMP axis with subsequent phosphorylation of several possible molecular targets. However, excessive glutaminergic NMDAR activation and increased NO generation, contributes to several pathological processes such as stroke, epilepsy, and neurodegenerative disorders (Zhou and Sheng, 2013). It is obvious that regulation of NMDAR activity is critical for normal brain function and avoidance of brain damage and an inhibitory feedback role of NO has been suggested by S-nitrosation of NMDAR-associated ion channels (Choi et al., 2000; Raju et al., 2015).

One central role of NO in the brain is its role in control of presynaptic function, which has bearing on synaptic plasticity and long-term potentiation related to memory function (Hardingham et al., 2013). From work mainly in glutaminergic and GABAergic neurons, it is evident that NO generated postsynaptically signals retrogradely by diffusion across the synaptic cleft, resulting in increased presynaptic function. This may be achieved by several concerting mechanisms including direct stimulation of neurotransmitter release, vesicle recycling, effects on the releasable
pool of vesicles and presynaptic growth with both short- and long-term effects on neuronal signaling (Arancio et al., 1996). An important role for NO in cerebral function involves regulation of cerebral blood flow. The vasodilatory response of cerebral vessels to hypercarbia, hypoxia, and alterations in systemic blood pressure (autoregulation) involves NO generated from vascular eNOS (Carter et al., 2021). However, NO is not the sole mediator in these responses, and there is still some controversy on the relative importance of NO in these processes. The physiological mechanism that couples nutritional blood flow to brain neuronal activity and metabolism is termed neurovascular coupling (NVC). Full mechanistic insight into how NVC works is still lacking, but experimental data strongly indicate that NO is a key intermediate mediator (Lourenço and Laranjinha, 2021). Although other substances are involved such as prostanoids, purines, and K^+, experiments using NO inhibitor reduce the NVC response by approximately 60% (Hosford and Gourine, 2019). Recent publications using systemic administration of the NOS inhibitor L-NMMA confirm the role of NO in human NVC (Holland et al., 2020). Both glutamatergic and GABAergic neurons have been implicated in NVC, but recent findings also show the ability of endothelial cells to directly sense neuronal activity via NMDAR expressed on the basolateral endothelial membranes, thereby regulating functional hyperemia via eNOS (Hogan-Cann et al., 2019). Abnormalities in NVC have been suggested as a preclinical manifestation of dementia, which is reasonable because functional hyperemia is not only providing nutrients but also enhance clearance of neuronal waste products (Ruitenberg et al., 2005).

As mentioned above, altered NO homeostasis has been coupled to several cerebral pathologies ranging from acute traumatic brain injury and stroke to psychiatric disorders and progression of dementia. Both excessive NO generation due to iNOS induction as well as reduced NO bioavailability due to oxidative stress have been implicated. However, the initially high expectations that modulation of NO signaling would be of therapeutic value has unfortunately waned, partly due to the lack of clinically useful specific NOS inhibitors, especially for nNOS and iNOS, or failure to show effect of NO donors (Bath et al., 2017; ENOS Trial Investigators, 2015; RIGHT-2 Investigators, 2019; Krishnan et al., 2016).

In the peripheral nervous system (PNS), non-adrenergic, non-cholinergic (NANC) inhibitory and excitatory nerves participate in regulation of smooth muscle tone in the gastrointestinal, respiratory, and urinary tracts and in the corpus cavernosum (Sanders and Ward, 1992). Together with nNOS-derived NO, various candidates have been implicated in NANC signaling including vasoactive intestinal polypeptide (VIP), substance P, calcitonin gene-related polypeptide (CGRP), ATP, and carbon monoxide (Xue et al., 2000). In the gastrointestinal tract, the so called nitricergic signaling is involved in gastric adaptive relaxation in response to food intake and gastric emptying (Desai et al., 2003), and targeted disruption of nNOS leads to stomach enlargement, hyper trophy of the pyloric sphincter, and dysfunctional gastric motility (Huang et al., 1993) with similar findings in sGC-1 KO mice (Vanneste et al., 2007). Moreover, in the small and large intestine, NANC signaling modulates motility and peristalsis (Sanders and Ward, 2019). The post junctional cells in the gut responding to nitricergic signals are smooth muscle cells, interstitial cells of Cajal (ICC), and PDGFRα+ cells, together often referred to as the SIP synectium (Gronenberg et al., 2013).

Early findings by Ignarro and coworkers suggested NO-sGC-cGMP-dependent relaxation of rabbit corpus cavernosum and hypothesized that NO was involved in penile erection evoked by NANC inhibitory nerves (Ignarro et al., 1990). Later, it was shown that nNOS-derived NO is the main nitricergic transmitter behind this effect, although some evidence suggests co-participation of eNOS located the sinusoidal endothelium (Trigo-Rocha et al., 1993). Both are activated by calcium influx leading to subsequent activation of the sGC-cGMP axis and relaxation. Another mode of action has been suggested, involving an inhibitory role of NO on the vasoconstrictive effects of the RhoA/Rho-kinase pathway (Mills et al., 2002). Erectile dysfunction is caused by a variety of pathogenic factors, involving impaired formation and action of NO and animal experiments suggest androgen dependency of nNOS mRNA levels and activity (Penson et al., 1996). The use of PDE 5 inhibitors to stabilize cGMP levels to treat erectile dysfunction is since long an established therapy and one of few directly related to NO signaling (Figure 5).

**NO in host defense and inflammation**

The discovery of iNOS occurred independently and in parallel with the identification of EDRF as NO. Building on studies showing endogenous generation of nitrate in germ free mice (Green et al., 1981), Stuehr and Marletta described the formation of nitrate and nitrile by LPS-activated murine macrophages (Stuehr and Marletta, 1985). Hibbs and colleagues went on to show that the cytotoxicity of these cells required L-arginine (Hibbs et al., 1987), and, soon after, NO was identified as an intermediate in the process of L-arginine oxidation to nitrate and nitrile (Marletta et al., 1988). The subsequent purification, characterization (Stuehr et al., 1991), and cloning (Xie et al., 1992) of iNOS revealed that this enzyme differs considerably from the constitutive NOSs, in particular in terms of NO output and regulation of activity and expression. Because iNOS binds calmodulin (CaM) more tightly than eNOS and nNOS, less calcium is needed for its activation, making it practically calcium-independent. Instead, a major regulation of iNOS occurs at the expression level. This means that once iNOS is expressed and sufficient amounts of co-factors and substrates are present, it continues to generate large NO fluxes over prolonged periods of times (Nathan and Xie, 1994).

Expression of iNOS can occur in any nucleated cell, but much focus has been placed on the immune cells where it was first discovered. Macrophages, T cells, and other immune competent cells express iNOS and generate NO in response to bacterial products, such as LPS or proinflammatory cytokines, including interleukin-1 (IL-1), tumor necrosis factor α (TNF-α), and IFN-γ (Nathan and Xie, 1994). These bind to cell-surface receptors and activate kinases, leading to the activation of transcription factors, including nuclear factor κB (NF-κB) and nuclear factor 1 transducer and activator of transcription 1α (STAT-1α), which translocate into the nucleus, where they bind to the promoter region of the iNOS gene and induce iNOS expression (Kleinert et al., 2003). A general problem in research on iNOS is translating findings from rodents to humans. Rodents readily express a
highly active iNOS in macrophages and other immune cells after activation, but it has been notoriously difficult to detect appreciable iNOS activity in these cells in humans (Schneemann et al., 1993). Epithelial cells, on the other hand, are readily capable of iNOS expression and NO formation also in humans, as shown, for example, in the airways and the large intestine during inflammation or infection (Alving et al., 1993; Lundberg et al., 1994a). In the healthy human paranasal epithelium, iNOS is constitutively expressed and generates high fluxes of NO (Lundberg et al., 1995), a surprising observation given that the general belief was that iNOS would only be induced after proinflammatory stimuli (Nathan and Xie, 1994).

There is evidence to suggest that iNOS in the upper airways is a part of innate immunity and defense against pathogens. Patient groups prone to chronic sinusitis (Lundberg, 2005) and instillation of an NOS inhibitor in the nose can trigger acute sinusitis (Lundberg, 2005). A puzzling observation is that nasal airway iNOS in humans seems resistant to corticosteroids (Lundberg et al., 1996c), a class of drugs known to down regulate iNOS in various models.

Inhibition of bacterial growth by NO and its reaction products in vitro was known before the discovery of mammalian NO synthesis and iNOS. NO gas inhibits the growth of aerobic bacteria (Mancinelli and McKay, 1983), and nitrite added to meat prevents the growth of Clostridia (Reddy et al., 1983). In vivo support for the role of NO in mammalian host defense came from early studies in iNOS-deficient mice, which failed to restrain the replication of the intracellular pathogen Listeria monocytogenes (MacMicking et al., 1995). Antiviral effects of NO have also been reported (Croen, 1993). In general, when discussing NO effects on pathogens, it is more relevant to use the term RNS, as the majority of effects observed are likely not caused by NO alone but one or several reaction products. If iNOS is co-localized with a source of reactive oxygen species, e.g., NADPH oxidase, a concerted burst of antibacterial compounds may result. RNSs directly interfere with redox-sensitive cysteine residues or metal centers of key enzymes involved in DNA replication and repair, metabolism, and more (Fang, 1997) (Figure 1).

Aerobic bacteria seem more sensitive to cytochromes of the electron transport chain are particularly vulnerable to nitrosylation by RNSs, thereby blocking respiration. Anaerobes, on the other hand, frequently respire on nitrate and nitrite and are, therefore, heavily exposed to RNSs through their own metabolism. In fact, the enzymes involved in such denitrification, including nitrite- and NO-reductases, represent important parts of defense against exogenous RNIs (Lundberg et al., 2004). This is illustrated during anaerobic growth of the denitrifier Pseudomonas strutzeri, which thrive in nitrite-rich medium, whereas a mutant lacking the NO reductase gene is killed due to the buildup of NO (Braun and Zumft, 1991). A broad antibacterial activity is created when nitrate is acidified due to the spontaneous formation HNO2 and other RNIs under these conditions (Lundberg et al., 2004). Intriguingly, this chemistry is happening in vivo when nitrite-rich saliva meets the acidic gastric milieu (Benjamin et al., 1994; Lundberg et al., 1994b) and also in infected nitrite-containing urine if acidified (Lundberg et al., 1997a). Studies suggest that this gastric system forms a part of our first line defense against swallowed pathogens (Björne et al., 2006; Dykhuizen et al., 1996).

NO participates in host defense also by modulating immunity. iNOS is protective for the host during experimental infection with Tuberculosis, but the primary role of NO was found to be anti-inflammatory and not related to any direct antimicrobial activity (Mishra et al., 2017). Lack of iNOS increased susceptibility through increased neutrophil recruitment to provide a growth-permissive milieu for the pathogen. The authors suggested that the mycobacterium exploits the increased accessibility of nutrients in granulocyte-rich lesions. In contrast, the opposite is true for infection with S. Aureus, where iNOS promotes accumulation of granulocytes in the lungs, in this case to enhance host defense (Urbano et al., 2018). Not surprisingly, bacteria have developed means of protection against nitrosative stress. This occurs via upregulation of various enzymes that scavenge or metabolize RNSs and includes nitrite- and NO-reductases described above, the hmp-encoded flavohemoglobin as well as cytochrome bd (Bang et al., 2006; Davis et al., 2015; Jones-Carson et al., 2016). Some pathogens including salmonellae, mycobacteria, and fransicella can downregulate host iNOS expression via various mechanisms including RNA interference or suppression of IFNγ-induced STAT-1 signaling (Fang and Vázquez-Torres, 2019; Parsa et al., 2008). In still other situations, the potential gut pathogen E. coli may utilize the nitrate generated by iNOS in the host mucosa for their own respiration to outcompete other gut commensals (Winter et al., 2013). Besides iNOS, a role for eNOS in regulation of immunity has also been suggested. In T cells, eNOS is rapidly activated upon antigen presentation to modulate the synthesis of cytokines including IFN-γ and IL-2 (Ibiza et al., 2006).

The overall role of iNOS in inflammation is still very unclear, and its impact on pathophysiological processes likely depend on the timing and duration of iNOS induction, where it is expressed and other factors including the local redox environment. A role for iNOS in wound healing was suggested early, and iNOS-deficient mice display a delayed would closure (Yamasaki et al., 1998). The same authors managed to reverse this by application of an adenoviral vector containing human iNOS cDNA. In septic shock, which is characterized by systemic inflammation and severe hypotension, a pathogenic role of iNOS (MacMicking et al., 1995) was suggested. The belief was that massive amounts of NO are generated by iNOS in the vascular wall during systemic inflammation with an overwhelming sGC activation and resulting vascular collapse. However, already at this time, other studies indicated no impact of iNOS on survival in a septic shock model (Laubach et al., 1995) and potential deleterious effects of systemic iNOS inhibition related to cardiac failure and disturbances in the microcirculation (Weitzberg et al., 1995). Nevertheless, a non-specific NOS inhibitor was eventually tested in a clinical phase III trial. Disappointingly, this trial had to be terminated prematurely due to excess mortality in the treatment arm (Lopez et al., 2004). A multitude of more specific iNOS inhibitors have been developed and used successfully in preclinical models of chronic inflammation where NO plays a proposed deleterious role. Some of these inhibitors have been tested in clinical trials (Cinelli et al., 2020) with focus on inflammatory pain and different forms of arthritis, but, overall, the results have been
disappointing and to date no iNOS-inhibitor has been approved for clinical use.

Although therapeutic attempts to dampen the increased iNOS activity in inflammatory diseases have failed, the diagnostic use of measuring such activity has been successful. It started with the discovery of NO gas in exhaled breath (Gustafsson et al., 1991) of humans. Soon after, it was shown that the levels of exhaled NO are increased in asthmatics (Alving et al., 1993) and reduced following treatment with anti-inflammatory corticosteroids (Lundberg et al., 1996b). Studies in allergic asthma show that iNOS is upregulated in bronchial epithelial cells following challenges with allergens with a concomitant increase in exhaled NO (Roos et al., 2014). Today, measurements of exhaled NO have become clinical routine in the management of allergic asthma (Dweik et al., 2011)(Figure 5). Moreover, it was found that the levels of NO in the nasal airways were near absent in patients with primary ciliary dyskinesia (Lundberg et al., 1994c), and this test is now used at specialized centers in the diagnostic workup for this rare disease (Manna et al., 2015). The principle of measuring mucosal release of NO gas to detect inflammation in a hollow organ (Lundberg et al., 1997b) has been applied also in the gastrointestinal tract in inflammatory bowel disease (Lundberg et al., 1994a) as well as in the urogenital tract in cystitis (Lundberg et al., 1996a) and vaginitis (Sioutas et al., 2008). These methods, however, are yet to reach the clinic.

Cancer

It is not surprising that a molecule that dilates vessels and stimulates angiogenesis, while at the same time having cytotoxic and immune-modulatory properties, will have a complex role in cancer. Most researchers would agree that the role NO is playing in cancer will depend on where and when it is generated, by which cells, its cellular concentration, the redox milieu, the abundance of NO target elements, as well as the tumor microenvironment. With a small reactive molecule, such as NO, an additional level of complexity is added. Not only do effects differ with varying concentrations as classically seen with signaling molecules, but NO can also react to form entirely new bioactive species. Wink and colleagues created a simplified model to explain the diverse effects of NO throughout its physiological concentration ranges (Miranda et al., 2021; Wink et al., 1998). Low fluxes of NO, similar to those generated by the constitutive NOSs, mainly signal via nitrosylation of sGC to increase cGMP. Besides vaso-dilation, cGMP also mediates angiogenesis and neovascularization mainly through activation of HIF1α and VEGF signaling (Papapetroupolous et al., 1997), both of which may promote tumor growth and spread. In support of this, Schenk and colleagues recently demonstrated upregulation of sGC during progression of small cell lung cancer that correlated with acquired chemotherapy resistance in vivo (Schenk et al., 2021). At higher concentrations, for example, when generated by iNOS, NO or its reaction products can interact with nonheme-iron or critical thiols in proteins to modulate their activity and also directly interfere with cellular respiration through inhibition of cytochrome c oxidase (Sen et al., 2013) and nitrosation of complex 1 (Moncada, 2015), which in turn affects HIF1α-dependent pathways. Overall, this can lead to activation of major oncogenic pathways that enhance survival, proliferation, and metastasis in aggressive tumors. Eyler and colleagues found that iNOS expression is increased in glioma stem cells (GSCs), which correlated with decreased survival in glioma patients. Inhibition of iNOS slowed glioma growth in mice and growth and tumorigenicity of GSCs in vitro were decreased with pharmacological iNOS inhibition, iNOS knockdown, or with scavenging of NO. The authors suggested suppression of the cell cycle inhibitor CDK1 along with pro-angiogenic effects of NO as the mechanisms underlying these effects (Eyler et al., 2011). In some cancers, the growth accelerating effects of iNOS are further enhanced by the prostaglandin-generating enzyme COX2 (Basudhar et al., 2017), suggesting that combined use of an iNOS-inhibitor and an NSAID might be of therapeutic value. Another strategy that is gaining interest is to attempt to normalize tumor vasculature to improve tumor oxygenation and enhance the delivery and efficacy of anti-tumor drugs (Jain, 2005). Studies suggest that this can be achieved by modulating endogenous and exogenous NO signaling (Kashiwagi et al., 2008). The cellular source of NO synthesis seems to be of importance in relation to cancer. Studies suggest that when tumor infiltrating immune cells express iNOS and generate NO, they contribute to tumor killing (Klug et al., 2013; Marigo et al., 2016), whereas iNOS expression within the tumor cells is coupled to enhanced tumor growth, metastasis, and poor survival in a variety of cancer forms (Basudhar et al., 2017; Glynn et al., 2010; Heinecke et al., 2014). In a small clinical phase1/2 trial, Chung and colleagues recently examined effects of L-NMMA, a non-specific NOS inhibitor, in combination with docetaxel in women with chemoresistant triple-negative breast cancer. They found that the drug combination was well tolerated and noted indications of an enhanced chemotherapy response as assessed by reduction in tumor size and a decrease in phenotypically tumor-promoting neutrophils (N2) (Chung et al., 2021). When NO fluxes are very high, a variety of reactive nitrogen oxides with promiscuous nitrosating and nitrating capacity are generated. This can occur, e.g., during chronic inflammation and promote mutagenesis through deamination (Wink et al., 1991) or oxidation of DNA bases, strand breaks, or cross-linking (Khan et al., 2020). A classic example of a nitrosation product is N-nitrosoamines, which are formed after prolonged iNOS upregulation or with chronic exposure to exogenous nitrogen oxides from cigarette smoke, pollution, or consumption of processed meat (Lundberg and Weitzberg, 2013). These can be metabolized to strong alkylating electrophiles with mutagenic effects. Moreover, nitration of guanine bases by NO-derived NO2 can lead to depurination and cause transversion mutations (Ramezanian et al., 1996). If RNIs are instead generated by activated immune cells invading an already established tumor, the very same species may instead be directed toward the cancer cells to cause apoptosis and cell death. Drugs that release NO or NO-like bioactivity have been tested experimentally with the purpose of directly affecting tumor growth or by enhancing delivery and anti-tumor activity of other therapies (Huang et al., 2017; Mintz et al., 2021). Barsoum and colleagues showed that hypoxia-induced expression of the immune inhibitory molecule programmed cell death ligand-1 (PD-L1) in cancer cells was inhibited by glyceryl trinitrate, and, when administered transdermally to mice, this drug also attenuated tumor growth (Barsoum et al., 2021).
et al., 2014). Numerous challenges remain when attempting to modulate NO signaling events in the treatment of cancer. Given the central role of NO in almost all parts of human physiology, any drug interfering with this system should be precisely targeted both spatially, temporally, and concentration wise. To this end, Sung and colleagues recently created a nanodelivery system enabling steady state-targeted release of NO in mice with experimental hepatocellular carcinoma (Sung et al., 2019). Treatment with this system normalized tumor vessels, which improved the delivery and efficacy of various treatment modalities while also reprogramming the immunosuppressive tumor microenvironment toward an immunostimulatory phenotype. It is important to appreciate that compounds typically classified as “NO donors” differ vastly in their pharmacokinetics and signaling properties. In fact, for some compounds, including the di-nitrosyl iron complexes (DNICs) used in the study cited above, it is not clear if their main bioactivity is transduced through release of free NO.

**DIETARY ASPECTS**

There are several dietary aspects related to the generation of NO and other nitrogen oxides in the body. Before the discovery of endogenous NO signaling, the focus was mainly on the potentially harmful role of dietary nitrate and nitrite as precursors of carcinogenic N-nitrosamines (Tannenbaum et al., 1974). In the acidic stomach, salivary nitrite is protonated and generates nitrogen oxides that can nitrosate dietary secondary amines. Mainly from early animal research, these N-nitrosamines were associated with various types of cancer and this led to recommended restrictions in human exposure to nitrate and nitrite that are still present. However, recent statements from international agencies do not find an association between dietary nitrate in vegetables and cancer and that high intake of nitrate with cancer is equivocal (EFSA, 2008; Speijers and van den Barandt, 2003). Other constituents in vegetables such as vitamin C and E and polyphenols may counteract gastric N-nitrosamine formation from salivary nitrite, which could explain the lack of association between dietary nitrate and human cancer.

The discovery of L-arginine as a substrate for NO generation (Palmer et al., 1988) naturally triggered the idea of using this amino acid to enhance NOS signaling in various CVDs. Theoretically, L-arginine substitution would not work because plasma levels are 20- to 30-fold higher than its Km for eNOS, but several studies show enhanced NO generation (Lundberg et al., 1996c) and improved endothelial function after L-arginine substitution (Gambardella et al., 2020; Imaizumi et al., 1992). The mechanism(s) underlying this “L-arginine paradox” has not been unraveled but cellular compartmentalization of L-arginine, substitution overriding increased arginase activity, or counteracting increased asymmetrical dimethylarginine (ADMA) have been proposed. The latter explanation has promoted evaluation of plasma L-arginine/ADMA ratio to identify subjects that would benefit from L-arginine substitution (Bode-Böger et al., 2007). It has been suggested that administration of L-citrulline instead of L-arginine to increase systemic L-arginine levels might be advantageous because it escapes metabolism by gut bacteria and liver arginase (Solomonson et al., 2003). Clinical trials in CVD have shown diverging results, and there is currently not enough evidence to support L-arginine substitution (Bednarz et al., 2005; Hadi et al., 2019). Other dietary means to support NOS output in CVD have been proposed, where administration of the BH4 precursors folate or sepiapterin has been tested in small clinical trials, but data are inconclusive and larger trials are needed to clarify any beneficial role of BH4 analogs (De Maria et al., 2014; Gori et al., 2001).

The discovery of the nitrate-nitrite-NO pathway in mammals ignited an interest in using dietary nitrate to increase systemic NO bioactivity (Figure 3). The dietary implications were obvious because green leafy vegetables and beetroot contain high amounts of this anion. Beetroot juice has been used in numerous studies because it is a convenient natural source to provide nitrate and because a nitrate-depleted beetroot juice has been available as placebo (Gilchrist et al., 2014). Early human studies in this field could show vasodilation and blood pressure reducing properties of nitrate in healthy subjects (Larsen et al., 2006; Webb et al., 2008) and later in hypertensives (Kapil et al., 2015). In addition to vasodilation, other mechanisms may contribute to the vasorelaxant effects, such as central and peripheral sympatholysis (Guimarães et al., 2019; Notay et al., 2017), reduction of NADPH oxidase activity (Gao et al., 2015; Montenegro et al., 2011), and modulation of angiotensin II receptor signaling (Hezel et al., 2016). Other effects of dietary nitrate in human studies with relevance to CVD include improved endothelial function (Kapil et al., 2013; Rodriguez-Mateos et al., 2015), reduced arterial stiffness (Rammos et al., 2014), inhibition of platelet aggregation (Webb et al., 2008), and mobilization of circulating angiogenic cells (Heiss et al., 2012). Within these entities, there are varying results that may depend on several factors, such as study populations, dosing, and temporal aspects of administration. Because oral bacteria are central in reducing nitrate to nitrite, variations in the human oral microbiome could also influence the outcome (Kapil et al., 2018). Moreover, stomach acidity has been shown to be of importance in the blood pressure-lowering effects (Montenegro et al., 2017; Pinheiro et al., 2015). In animal models of myocardial infarction, inorganic nitrate and nitrite have been generally beneficial, which has prompted a few human phase II trials with dietary nitrate or nitrite (Eriksson et al., 2021; Jones et al., 2015; Siddiqui et al., 2014). The results from these have shown neutral or limited beneficial effects but newer trials are ongoing (clinicaltrials.gov), which will shed more light on the role of these anions in ameliorating IR injury.

Another field of major interest related to dietary nitrate is the ergogenic effects of inorganic nitrate, where both acute and chronic intake in humans has been shown to reduce oxygen cost (Larsen et al., 2007) and enhance performance (Jones et al., 2018; Maughan et al., 2018). There are most likely parallel mechanisms contributing to the oxygen sparing and performance enhancing effects, including improved mitochondrial efficiency (Larsen et al., 2011), enhanced blood flow to the working muscle (Lee et al., 2015), and increased muscle contractile efficiency (Bailey et al., 2010). Interestingly, fast twitch type II muscle type seems to be especially responsive to the blood flow and contractile effects of dietary nitrate (Ferguson et al., 2013; Hernández et al., 2012). Translation of these effects into diseases
with reduced exercise capacity, such as heart failure (Egebeeen et al., 2016), peripheral artery disease (Kenjale et al., 2011), and COPD (Pavitt et al., 2020) has shown varying results and larger clinical trials are clearly needed to confirm a relevance for nitrategeneration in these conditions.

Promising preclinical data show benefit of dietary nitrate in genetic or diet-induced animal models of the metabolic syndrome or type 2 diabetes. In these models, several features of metabolic disturbance have been positively affected by dietary nitrate, including glucose tolerance, insulin sensitivity, dyslipidemia, hepatic lipid accumulation, and inflammation (Lundberg et al., 2018). There are several proposed molecular targets where the nitrate-nitrite-NO pathway may signal, converging at the central metabolic regulator AMPK (Cordero-Herrera et al., 2019; Lai et al., 2016). Inhibition of mitochondrial respiration and reduced generation of reactive oxygen species via inhibition of mitochondrial complex I as well as NADPH oxidases have been proposed to activate/phosphorylate AMPK. This will lead to reduced fatty acid synthesis, increased fatty acid oxidation, and GLUT4 translocation with increased glucose uptake. Interestingly, some of these molecular events are also suggested to underlie the effect of biguanides, such as metformin (Cordero-Herrera et al., 2020).

In addition to this signaling pathway, nitrate and nitrite have been suggested to increase insulin secretion (Nyström et al., 2012) and induce browning of fat (Roberts et al., 2015). Human studies have, to date, not been able to show convincing salutary effects of dietary nitrate in patients with type 2 diabetes, and the reasons are still unclear (Bahadoran et al., 2021; Gilchrist et al., 2013).

These above-mentioned features of dietary nitrate have raised the question of whether inorganic nitrate is responsible, at least in part, for the described health effects of certain diets, such as the DASH, Mediterranean, and traditional Japanese diets (Appel et al., 1997; de Lorgeril et al., 1999). Large epidemiological investigations specifically associate green leafy vegetables with a reduced risk of CVD (Hung et al., 2004; Joshipura et al., 1999) and type 2 diabetes (Carter et al., 2010). However, studies more specifically designed to pinpoint nitrate as a beneficial constituent in a green diet have not been conclusive and the question is still out for debate (Siervo et al., 2020; Sundqvist et al., 2020).

PERPECTIVES

After the discovery of EDRF by Robert Furchgott in 1980, it took 6 more years until this mysterious mediator was finally identified as NO. A major reason for this delay was that it just seemed too unlikely that a gas merely known as an environmental pollutant could be generated in our bodies to control vital cellular functions. Nevertheless, after this highly unexpected discovery, the field virtually exploded, much aided by the immediate availability of pharmacological NO synthase inhibitors. NO also opened the door to research on endogenous carbon monoxide (CO) and hydrogen sulfide (H2S) creating a family of novel signaling molecules commonly referred to as gasotransmitters. How these systems interact is currently an area of active research. In many ways, the early discoveries in the NO field still hold true today; eNOS in the endothelium is a major regulator of cardiovascular function and many of NO’s effects are mediated through the sGC/cGMP pathway. Other NO-derived products generated by nitrosation and nitration reactions are also involved in physiological and pathophysiological signaling, but, even today, questions remain about how these species are formed and how signaling is conveyed. The output in terms of novel therapeutic modalities has been somewhat disappointing given the enormous efforts put down. Early trials with NOS inhibitors in sepsis failed, as did trials using selective iNOS inhibitors for various inflammatory conditions. Nevertheless, iNOS is now instead emerging as a promising target in cancer and larger trials are in the pipeline. The development of sGC-stimulating drugs has been a success, and new indications can be foreseen besides pulmonary hypertension and heart failure, for which they are already approved. Finally, boosting of NO-like signaling by dietary means is emerging as a viable strategy to improve cardiovascular and metabolic function in health and disease.

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DECLARATION OF INTERESTS

Lundberg and Weitzberg are named inventors on patents and patent applications relating to the medical uses of inorganic nitrate and nitrite and co-directors of HeartBeet.

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