NO Generation From Nitrite and Its Role in Vascular Control

Jon O. Lundberg, Eddie Weitzberg

Abstract—NO generated from L-arginine by NO synthases (NOSs) in the endothelium and in other cells plays a central role in several aspects of vascular biology. The biological activity of NO is acutely terminated by oxidation to nitrite and nitrate, and these compounds have long been considered only as inert end-products of NO. However, this dogma is now being challenged because recent research convincingly has shown that the nitrite ion can be recycled back to bioactive NO again in blood and tissues. Nitrite reduction to NO can occur via several routes involving enzymes, proteins, vitamins, or even simple protons. This pathway may serve as a backup system for NO generation in conditions such as hypoxia, in which the NOS/L-arginine system is compromised, but detrimental effects can also be foreseen. With this new knowledge, nitrate and nitrite should probably be viewed as storage pools for NO rather than inert waste products. Here we discuss novel aspects of nitrite-dependent NO generation in vivo and its role in vascular control. (Arterioscler Thromb Vasc Biol. 2005;25:915-922.)

Key Words: nitric oxide ■ nitrite ■ nitrate ■ S-nitrosothiol ■ superoxide ■ xanthine oxidase ■ hemoglobin

NO is transiently released from endothelial cells whereby it serves to regulate important functions in the vessel itself and in circulating cells. When generated (eg, in response to shear stress), NO diffuses radially from the production site near the vessel lumen. Some of this NO will survive unaffected all the way to the underlying smooth muscle cells to promote vasorelaxation, and some will affect cells passing in the blood stream, for example platelets and leukocytes. However, the major part of NO will be destroyed before it ever reaches a target cell because of rapid oxidation either by hemoglobin (Hb) in blood or in tissues. This ensures that the effects of NO are restricted to the close vicinity of its production site, which helps to control and precisely target the effects of this potent biological messenger. However, from an energetic point of view, such “waste” of NO is far from optimal because its generation is performed by complex enzymes in an energy-consuming reaction that requires numerous substrates and cofactors. So, is then the rapid oxidation and inactivation of NO really an irreversible process as long believed? Pathways designed for the reuse of important biological messengers are ubiquitous in biological systems. Here we discuss recent advances in NO biology pointing to the fact that also, NO metabolites can be recycled back to bioactive NO again. The physiological and pathophysiological aspects of this newly recognized salvage pathway are covered with focus on regulation of blood flow.

Conservation of NO Bioactivity in Blood

As stated above, the general view of NO has been that it can only act in a paracrine manner on neighboring cells because of its very short half life. However, more recent studies have suggested that the bioactivity of NO in blood in fact can be conserved, thereby allowing for more distal and sustained effects. As an example, inhaled NO can have vasodilatory effects not only locally in the pulmonary circulation but also in peripheral tissues. In addition, intravascular delivery of authentic NO results in vasodilation distal to the site of injection. How then is this NO transported in blood? Over the last years, several alternative explanations have emerged.

First, it is possible that NO itself can remain intact in the blood vessel for a longer period than initially believed. During normal laminar flow, the red blood cells travel mainly near the center of a vessel, thereby leaving a cell-free zone near the endothelium. Similarly, a second effective diffusion barrier is created by the unstirred plasma layer surrounding the erythrocyte and possibly also by the plasma membrane. It has been suggested that NO can survive unchanged in these zones away from the scavenging Hb inside the red blood cells, thereby allowing for transportation to more distal sites of action.

A second theory that has received much attention is the notion that NO is transported in blood and tissues in the form of S-nitrosothiols (SNOs), which would then act as stable carriers of NO. SNOs are formed when thiol groups react...
with NO or chemically related species, and they can act as donors of NO. One example is S-nitrosylation of albumin, but most recent focus has been on a thiol group in the Hb molecule. According to this theory, NO or a closely related species binds to a cysteine residue in the Hb (cys-β93), thereby forming S-nitroso Hb (SNO-Hb). The binding and subsequent release of free NO is allosterically regulated by the oxygenation of Hb and the redox state so that SNO-Hb formation is favored in well-oxygenated conditions (in the lungs when Hb is in the R-form), and release of NO occurs mainly when oxygen leaves Hb (T-form). In this way, NO is delivered by the red blood cells preferably to less oxygenated tissues where it is most needed. This very attractive theory has gained great interest over the past decade, but more recently, its role in human biology has also been questioned. The main criticism has been the extremely low levels of SNO in human blood reported recently by some groups and the absence of an arteriovenous gradient for SNOs.

More recently, a third theory has emerged, which is the main topic of this review. In this pathway, NO is generated from the nitrite ion by simple reduction, and this can occur in blood and in tissues. There are several different pathways for NO formation from nitrite, and each is discussed in detail below.

Nitrite as a Vasodilator

The knowledge that inorganic nitrite can dilate vessels is not new. Furchgott used acidified sodium nitrite to relax preconstricted rabbit aortic strips in 1953. However, in these early experiments, the nitrite concentrations and the pH used were far outside physiological levels, and the mechanism of dilatation (NO generation) was unknown. Other studies have shown that ingestion of fairly large amounts of inorganic nitrite can reduce blood pressure in spontaneously hypertensive rats, which is suggestive of systemic NO generation.

Very recently, Tsuichiya et al showed that ingestion of nitrite resulted in rapid appearance of nitrosylhemoglobin in blood, which is highly indicative of systemic NO formation from the nitrite. They also found that coadministration of nitrite with Nω-nitro-L-arginine methyl ester for 3 weeks attenuated the hypotension induced by this NO synthase (NOS) inhibitor. Thus, nitrite could partly compensate for the loss of NOS-derived NO, thereby counteracting a rise in blood pressure.

Nitrite Sources In Vivo

There are at least 3 sources of nitrite in mammals. First, nitrite is an oxidation product of NOS-derived NO. Second, nitrite is present in some food stuffs, for example in processed meat, in which this anion is used to prevent botulism. Third, nitrite is generated from commensal bacteria in the digestive system as a result of nitrate reduction. The relative contribution from these different sources of nitrite during normal conditions is variable and therefore difficult to judge. During fasting conditions with a low previous intake of nitrate/nitrite, the NOS/L-arginine pathway probably dominates and even more so during systemic inflammation when much additional NO is generated from inducible NOS. However, with a high intake of nitrate, the plasma levels of nitrite increase greatly as discussed below.

<table>
<thead>
<tr>
<th>Basal Blood/Plasma Levels and Half Lives of Some NO-Related Compounds</th>
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<tbody>
<tr>
<td><strong>Blood/Plasma Levels, nmol/L</strong></td>
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<tr>
<td>Nitrate</td>
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<td>Nitrite</td>
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<tr>
<td>NO</td>
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<tr>
<td>Hb-NO*</td>
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<tr>
<td>S-nitroso-Hb*</td>
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<td>S-nitroso-albumin*</td>
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Values are approximated from recent studies in humans.

A common and simple way of estimating body formation of NO is to measure its more stable oxidation products nitrate and nitrite in plasma or other biological fluids. This is convenient because direct in vivo measurements of NO can be very difficult because of the extremely low levels and its short half life. When combined measurements of nitrate/nitrite are made, this is usually denoted by the term NOx. In simple aqueous solution, NO is oxidized almost exclusively to nitrite, whereas in whole blood in the presence of oxygenated Hb, nitrite is rapidly further oxidized to nitrate. Therefore, plasma nitrate is normally much higher than nitrite, and as a consequence, NOx is almost identical to nitrate. Plasma nitrite levels are usually in the range 0.1 to 0.5 μmol/L (Table), whereas tissue concentrations are typically 1 to 2 orders of magnitude higher.

The nitrate content in food can be very high, for example, in green leafy vegetables and sometimes in drinking water. In addition, the plasma half life of nitrate is fairly long (~6 hours). Because of this, one cannot rely on nitrate measurement as an adequate index of systemic NO generation. On the other hand, nitrite measurements are thought to much better reflect ongoing endothelial NOS (eNOS) activity. In a study by Kleinbongard et al, it was concluded that ~70% of plasma nitrite is derived from eNOS in the endothelium. However, recent studies show that ingestion of nitrate can influence not only plasma nitrate but also nitrite. This is very surprising because it requires reduction of nitrate to nitrite, a reaction that cannot be catalyzed by mammalian enzymes. The answer lies in the enterosalivary circulation of nitrate. When nitrate is ingested, as much as 25% is activatedly taken up by the salivary glands and secreted into saliva. In the oral cavity, much of this nitrate is reduced to nitrite by commensal bacteria, and this nitrite enters the circulation when saliva is swallowed.

Nitrite Reduction to NO In Vivo

In 1994, 2 independent groups reported that nitrite can be a substrate for NOS-independent generation of NO in vivo. This was first demonstrated in the stomach, where nitrite-derived NO seems to play an important role in host defense and in...
regulation of gastric mucosal integrity. It is now clear that reduction of nitrite can occur not only locally on epithelial surfaces such as in the stomach, in the oral cavity, on the skin, or in urine, but also systemically in blood and tissues (Figure 1). Several alternative pathways for NO generation from nitrite have been described, which are discussed below.

**Acidic Reduction**

When nitrite (NO2⁻) is acidified, it yields nitrous acid (HNO₂, reaction 1), which spontaneously decomposes to NO and other nitrogen oxides (reactions 2 and 3):

\[
\begin{align*}
1 & \quad \text{NO}_2^- + H^+ \rightarrow \text{HNO}_2 (\text{pKa} 3.2 \text{ to } 3.4) \\
2 & \quad 2\text{HNO}_2 \rightarrow \text{N}_2\text{O}_4 + \text{H}_2\text{O} \\
3 & \quad \text{N}_2\text{O}_4 \rightarrow \text{NO} + \text{NO}_2
\end{align*}
\]

The chemistry of acidified nitrite is very complex, and the amounts of NO generated from nitrite is dependent not only on pH and nitrite concentrations but also on the presence of other reducing agents (e.g., vitamin C and thiocyanate), proximity to heme groups, proteins, thiols, and the oxygen tension. Reduction of nitrite is quantitatively most significant in the stomach, where the extremely low pH in combination with a very high nitrite content (0.1 to 1 mmol/L from saliva) results in NO levels sometimes exceeding 100 parts per million (∼4 μmol/L; i.e., >4 orders of magnitude higher than the levels required for vasodilation). These extremely high NO levels (and possibly other nitrogen oxides generated from acidified nitrite) help to kill luminal bacteria and even diffuse through the entire mucus layer to enhance mucus generation and blood flow in the gastric mucus. Interestingly, studies have indicated that acid-catalyzed nitrite reduction to NO can also take place in blood vessels and tissues already at a moderately low pH and within nitrite concentrations normally present in vivo. In 1995, Zweier et al demonstrated systemic generation of NO from nitrite. They found that N¹⁵-labeled nitrite was reduced to NO in ischemic rat hearts, and this production was not blocked by NO synthase inhibitors. During global ischemia in the heart, pH fell to ∼5.5, and under these conditions, reduction of nitrite to NO was greatly accelerated. Modin et al showed that physiological levels of nitrite relaxed rat aortic rings when the acidity of the buffer solution was adjusted to pH just <7, which is commonly seen in tissues during ischemia (Figure 2). This relaxation was blocked by an inhibitor of soluble guanylyl cyclase (ODQ) and paralleled by NO generation in head space gas, supporting that NO was mediating the effects. Interestingly, NO generation from nitrite and relaxation was further increased by vitamin C. This antioxidant is known to enhance NO-mediated vasorelaxation, and several alternative explanations for this effect have been put forward. Scavenging of superoxide (an NO destroyer) has been suggested, as well as direct stimulation of eNOS activity. However, Jackson et al showed that effective scavenging of superoxide occurred only at very high concentrations of vitamin C, so the mechanism of action still remains incompletely understood. It is possible that some of the effect is related to enhancement of NO generation from nitrite.

**Reduction by Xanthine Oxidase**

Xanthine oxidase (XO) is a ubiquitous enzyme involved in a variety of physiological and pathophysiological processes. It has a critical role in purine and pyrimidine catabolism, but it also reduces oxygen to superoxide and hydrogen peroxide, thereby contributing to oxidative cellular injury. The ability of XO to generate reactive oxygen species has led to widespread interest in the pathogenic role of this enzyme in...
ischemia-reperfusion injury. The expression and activity of XO is induced by hypoxia and proinflammatory cytokines, whereas hyperoxia has the opposite effect. Interestingly, XO is structurally related to bacterial nitrate and nitrite reductases, and early studies have indeed shown that this enzyme can reduce nitrate and nitrite. More recently, it has been shown that NO is formed by XO-catalyzed reduction of nitrite or by a 2-step reduction of nitrate to NO. Nitrite reduction by XO is greatly enhanced at low oxygen tensions and acidic conditions such as those seen during ischemia. It has been shown that under conditions of severe ischemia, myocardial XO and nitrite levels are sufficient to generate NO at levels exceeding those from maximally activated NOS. Webb et al recently studied XO-dependent NO generation from nitrite in perfused rat hearts subjected to ischemia. They found that large amounts of NO were generated from nitrite by XO during ischemia, and this NO was strongly cardioprotective, with a marked reduction in infarct size. In the presence of an NO scavenger, the protective effect was completely blocked. A likely location of the XO is the endothelium of blood vessels in the heart, as demonstrated by functional and histochemical studies. These new findings challenge the notion that XO is only damaging in ischemia-reperfusion. The factors that determine whether the net effect of a certain enzymatic pathway is beneficial or harmful may in part be related to the substrate supply. If XO is presented with nitrite as an alternative substrate, the protective effects of the NO generated seems to dominate, whereas in other situations, more superoxide is generated with possible harmful effects. Also, when NO and superoxide combine another potentially harmful product, peroxynitrite is formed. Interestingly, it is now clear that XO and NOS can generate either NO or superoxide, depending on the substrate supply and the oxygen status (Figure 3). Although NOS requires oxygen to generate NO from L-arginine, XO-catalyzed nitrite reduction to NO is greatly enhanced by hypoxia. Superoxide generation from NOS is enhanced when the supply of arginine or cofactors is scarce, whereas XO generates superoxide, for example, during reperfusion after ischemia. The balance between substrate supply and tissue oxygenation may determine whether the net effects of these combined enzyme systems are beneficial or harmful in a particular situation. Thus, the dominating species generated could be NO, oxygen radicals, or their reaction products.

Reduction by Deoxyhemoglobin
Hb can interact with NO and related compounds in many ways (Figure 4). The traditional view is that Hb in the red blood cells is an extremely effective NO scavenger, and in many ways, this notion still holds true. Oxygenated Hb reacts rapidly with NO to form nitrate and methemoglobin (met-Hb), and this reaction is near–diffusion limited. However, other interactions between NO and Hb have been characterized, including formation of nitrosyl-Hb and SNO-Hb, as discussed above. In 1981, Doyle et al described a specific reaction between nitrite and deoxyhemoglobin (deoxy-Hb) by which met-Hb and NO is formed (Figure 4). These observations were made before the biological significance of NO was revealed. More recently, several authors have expanded on the role of this reaction in NO physiology. It is suggested that nitrite is recycled back into bioactive NO by this mechanism and that this ensures an autoregulated NO generation in regions of poor oxygenation where deoxy-Hb predominates. Cosby et al showed that...
amounts released earlier in the vascular tree may be concentrations of NO are needed for vasorelaxation, so the control of blood flow. On the other hand, only minute maximal, but these vessels are not actively involved in to be greatest in the veins where deoxy-Hb levels are low. A compartmentalized NO production red blood cell and how NO is exported without being trapped key question is by which mechanism nitrite is taken up by the enzyme most likely responsible for carrying out this reaction. Using inhibitors of the respiratory chain for chemical sequestration of respiratory segments, Kozlov et al identified the ubiquinone/cyt bc1 couple as another reductant site where nitrite is recycled. Nitrite requires respiring mitochondria under conditions established during ischemia, when electron carriers are highly reduced.

The magnitude of NO generated from nitrite by mitochondrial enzymes in vivo and its physiological or pathophysiological role needs to be clarified. The high affinity of NO to the heme-iron of cytochrome oxidase may support a detrimental role because NO has been shown to impede the energy-linked respiration and to trigger mitochondrial generation of superoxide radicals. On the other hand, beneficial effects of NO on mitochondrial function have also been described. For example, NO stimulates mitochondrial biogenesis in vitro and in vivo, which results in increased mitochondrial function and enhanced ATP formation.

### Nitrate and Nitrite Reduction by Commensal Bacteria

Some bacteria are highly effective in reducing nitrate and nitrite. They use these substrates as alternative electron donors during oxygen starvation or in protein synthesis for incorporation in biomass. Nitrite reduction to NO is a part of the nitrogen cycle in nature and is performed by denitrifying anaerobic bacteria in soil and sediments. Nitrogen generation also involving commensal bacteria was reported recently by Sobko et al. Luminal levels of NO were measured along the gastrointestinal tract of rats and were found to be high in conventional rats but almost absent in germ-free animals. NO generation by gut commensals is likely a combination of nitrite reduction by bacterial nitrite reductases and acidification (eg, by lactic acid–producing bacteria resulting in nonenzymatic reduction of nitrite; J.O. Lundberg, unpublished observation, 2005). Interestingly, bacteria may participate not only in local generation of NO in the gastrointestinal tract but also systemically. In fact, the oral microbiota is required to obtain an increase in plasma nitrite after ingestion of nitrate. This is because ingested nitrate has to pass the salivary glands into saliva before it is reduced to nitrite by oral bacterial enzymes. Indeed, if a test person avoids swallowing after ingestion of nitrate, the increase in plasma nitrite is abolished. With the recently described identification of systemic nitrite generation from inorganic nitrate, a reverse pathway for regeneration of NO from nitrate is completed. Thus, the nitrate ion could be considered a major vascular storage pool for NO rather than an inert waste product. In an extension, this implies that generation and activity of NO in the cardiovascular system can be influenced by dietary intake of nitrate.

### Nitrite and Nitroglycerine

The mechanism behind the vasodilatory action of organic nitrates (eg, nitroglycerine, glyceryl trinitrate [GTN]) remained a mystery for almost 100 years before Murad et al showed in 1978 that they act through release of NO via activation of soluble guanylyl cyclase. However, still today, the exact way by which NO is formed in vivo from...
these agents remains controversial. Interestingly, several observations suggest that the major obligate intermediate in this process is in fact nitrite (Figure 1). Chen et al showed that a mitochondrial aldehyde dehydrogenase catalyzed formation of nitrite from GTN in smooth muscle cells. In this study, the further reduction to NO from nitrite was not characterized. Hepatocytes generate nitrite from GTN by glutathione–S-transferase, and in these cells, nitrite is further reduced to NO by an enzyme of the cytochrome P-450 family. Other candidates for the final step in GTN-derived nitrite reduction to NO are the mitochondrial enzymes and the XO discussed above. Considering the potent vasorelaxatory effects of organic nitrates, this again illustrates that highly efficient intracellular pathways exist to generate NO from nitrite. Because the nitrite ion itself is charged, it traverses biological membranes more slowly and less effectively than organic compounds (eg, nitroglycerine and amyl nitrite), and this probably explain why inorganic nitrite is a much less potent vasodilator than organic nitrates/nitrates.

Summary and Future Directions

Oxidation of NO to nitrite and nitrate acutely terminates its biological activity. However, as discussed here, several distinct pathways exist in vivo whereby nitrite can be reduced back into bioactive NO. Interestingly, these reactions are all greatly enhanced during ischemia with low oxygen tension, a situation in which NO synthesis by NOS is repressed. The different pathways work in concert to ensure sufficient NO synthesis along the entire oxygen gradient. When oxygen supply is sufficient, NO generation from NOS will dominate. However, with falling oxygen tensions and ischemia, the nitrite-dependent pathway comes into play. In blood, deoxy-Hb seems to be the predominant catalyzer of nitrite reduction to NO, whereas in a situation of tissue hypoxia, XO-derived NO also becomes important. Finally, in situations of global ischemia when pH drops dramatically in the tissues, NO generation can occur via simple nonenzymatic acidic reduction of nitrite, a reaction that is accelerated by vitamin C and other reducing agents. One could view this as a salvage pathway for NO, where much of the spill over from NOS-derived NO is being recycled when most needed. A major future challenge for the researchers working in this field will be to finally settle the exact significance of nitrite-dependent NO in normal physiology as well as pathological states for example during tissue hypoxia. Because diet can have a great influence on systemic levels of nitrate and nitrite, it will be very interesting to study the long-term effects of nitrate-rich food (eg, vegetables) on systemic nitrite-derived NO generation. Indeed, it is tempting to speculate that the well-known cardioprotective effects of a vegetarian diet in part could be attributed to a continuous low-grade generation of NO from nitrate in the diet via formation of nitrite. A study by Richardson et al actually supports the notion that inorganic nitrate can have systemic effects in humans. They found that ingestion of sodium nitrate in an amount equivalent to 300 g of spinach inhibited platelet function, an effect that might be attributed to NO.

It is possible that increasing knowledge about nitrite-derived NO can result in development of new drugs to be used in cardiovascular medicine. Nitrite-based pharmaceuticals that selectively deliver NO only to ischemic areas are indeed an interesting alternative approach. Theoretical advantages with such compounds in relation to traditional organic nitrates could be fewer side effects (eg, headache) and possibly less development of “nitrate tolerance.” As discussed here, several recent studies suggest a protective role of nitrite-derived NO, but in certain situations (eg, ischemia-reperfusion), a massive NO generation from NOS-independent sources could instead be harmful, for example, if it coincides with generation of superoxide, thereby promoting formation of oxidizing radicals.

Besides nitrite-derived NO, which is the main focus of this review, other means of preserving NO activity in blood have been suggested, including SNO formation and transport of free NO, and there is an intense ongoing debate as to which pathway is the most significant in vascular biology. These controversies aside, today, there is no longer any doubt that NO activity in blood can be preserved for much longer than originally believed. In addition, nitrite, the oxidation product of NO, can be recycled into bioactive NO again in blood and tissues. These novel aspects of NO biology will provide further important insights into the mechanisms of blood flow regulation, which could lead to novel strategies for treatment and prevention of cardiovascular pathologies.

Acknowledgments

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References

Lundberg and Weitzberg

NOS-Independent NO Generation From Nitrite


