The Nitrovasodilators
New Ideas About Old Drugs

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The nitrovasodilators are a diverse group of pharmacological agents that produce vascular relaxation by releasing nitric oxide. The mechanisms by which these compounds release nitric oxide vary, depending on their chemical structure. Compounds with lower oxidation states of nitrogen such as nitroprusside, nitroamines, and nitrosothiols release nitric oxide nonenzymatically. In the case of nitroprusside, this involves a one-electron reduction that may occur upon exposure to a variety of reducing agents and tissues such as vascular smooth muscle membranes. In the case of the organic nitrates, which have higher oxidation states of nitrogen, the release of nitric oxide in vascular tissue occurs predominantly by a poorly understood enzymatic process. This interesting property of nitroglycerin is important because it “targets” its effect to vascular tissues that are capable of this enzymatic process. In the case of the coronary circulation, nitroglycerin predominantly dilates the larger coronary arteries while having a minimal effect on coronary resistance vessels <100 μm in diameter. This prevents the development of coronary steal, which is often encountered with agents that produce intense vasodilation of the coronary resistance vessels. In this review, the mechanisms by which the nitrovasodilators (particularly nitroglycerin) release nitric oxide will be considered, and recent studies of nitroglycerin bioconversion in various-sized coronary vessels will be discussed in detail. (Circulation 1993;87:1461–1467)

KEY WORDS • coronary arteries • microcirculation • cysteine • N-acetylcysteine • methionine • glutathione

During the past decade, a great deal has been learned about the mechanisms of action of the pharmacological class of agents known as the nitrovasodilators. One of the fascinating aspects of this research is that these drugs for the most part produce their biological effects by releasing nitric oxide1-4 and thus in many respects mimic the endothelium-derived nitric oxide. The most commonly used of these include nitroglycerin, sodium nitroprusside, and the long-acting nitrate isosorbide dinitrate. Other pharmacological donors of nitric oxide are isosorbide mononitrate (recently introduced in the United States), SIN-1 (used principally in Europe), amyl nitrite, and some of the newer compounds (nicoradil and others) that have additional properties such as the capacity to open vasodilating K+ channels in vascular smooth muscle. It has become rather fashionable to state that these pharmacological agents “replace” the endogenous arginine/nitric oxide pathway, which may be defective in a variety of diseases. Although this statement is true in its broadest interpretation, there are important differences between the endogenous nitric oxide and exogenous nitrovasodilators that should be familiar not only to investigators interested in vascular biology but also to the clinician who uses these drugs. These involve variations in how these compounds are metabolized to free nitric oxide and differences in how they behave in various tissues. This review will summarize some of these newer, important concepts.

Chemistry of the Nitrovasodilators

Nitrogen has five electrons in its eight-electron valence shell, which means that in compounds it can have oxidation states of $-3$ to $+5$.6 Although oxidation state is a purely theoretical concept and not a direct measure of any physical property of an atom, it is a useful concept in the discussion of redox reactions. Unlike many other elements, nitrogen readily forms compounds in each of its possible oxidation states. Examples of each of these states are presented in Table 1.

As apparent from Table 1, most biological nitrogen-containing compounds have nitrogen in its most reduced form. This includes all amino acids, peptides, nucleic acids, organic bases, amines, and more than 99% of all the nitrogen present in all organisms. Aside from the ecological nitrogen cycle carried out mostly by bacteria, biochemical reactions generally do not change the oxidation state of nitrogen from $-3$.

One of the most exciting physiological discoveries of the past decade, however, is the only established exception to this rule in mammalian biochemistry. It is now well known that many cells, especially endothelial cells, neurons, and hematopoietic cells, can oxidize a guanidino nitrogen in arginine (oxidation state, $-3$) to nitric oxide (oxidation state, $+2$) (for reviews, see References 7 and 8). A related discovery first made a decade earlier
TABLE 1. Compounds With Various Oxidation States of Nitrogen

<table>
<thead>
<tr>
<th>Oxidation State</th>
<th>Compound Description</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>-3</td>
<td>Ammonia (NH₃), amines (RNH₂), amides (RCONH₂), most biological nitrogen compounds</td>
<td></td>
</tr>
<tr>
<td>-2</td>
<td>Hydrazine (N₂H₄)</td>
<td></td>
</tr>
<tr>
<td>-1</td>
<td>Hydroxylamine (NH₂OH)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Nitrogen (N₂)</td>
<td></td>
</tr>
<tr>
<td>+1</td>
<td>Nitrous oxide (N₂O)</td>
<td></td>
</tr>
<tr>
<td>+2</td>
<td>Nitric oxide (NO)</td>
<td></td>
</tr>
<tr>
<td>+3</td>
<td>Organic and inorganic nitrates (RONO and NO₂⁻)</td>
<td></td>
</tr>
<tr>
<td>+4</td>
<td>Nitrogen dioxide (NO₂)</td>
<td></td>
</tr>
<tr>
<td>+5</td>
<td>Organic and inorganic nitrates (RONO₂ and NO³⁻)</td>
<td></td>
</tr>
</tbody>
</table>

is that nitric oxide is the major modulator of the enzyme-soluble guanylate cyclase, the source of much and in some cases all of the cGMP inside many cells. Since cGMP is involved in many important regulatory pathways, these discoveries have focused much attention on the study of biological nitric oxide.

One result of these studies is a new understanding of the mechanism of action of some old drugs, the nitrovasodilators. A common feature of these drugs is that they contain nitrogen in an oxidation state higher than -3, and the action of these drugs is dependent on the release of this nitrogen as nitric oxide. The mechanisms by which nitric oxide release occurs is by no means similar between the various compounds, and at least in some instances, may occur via several mechanisms in the same compound. For some nitroso compounds with an oxidation state of +3, release of the nitric oxide occurs via a simple one-electron reduction. In contrast, compounds with higher oxidation states almost certainly require enzymatic reduction to release nitric oxide. This property of nitroglycerin and related organic nitrates almost certainly is one of the major reasons for some of the unique properties discussed in this review.

Figure 1 illustrates the structure of a variety of nitrogen-containing compounds that are either nitrovasodilators or closely related compounds. Also shown are their oxidation states. Figure 2 illustrates the chemical structure of nitroglycerin for comparison. Some of the nitrovasodilators are readily recognized by the clinician, including amyl nitrite, sodium nitroprusside, the organic nitrates, and nitric oxide. Others may be less familiar. The thionitrites have been proposed as an intermediate of nitroglycerin bioconversion, as have the nitrosodiethionates. Some of these compounds such as the nitrosodiethionates and nitrosamines are potent vasodilators but are not used clinically because of chemical instability or potentials for toxicity that have not been resolved.

**Biotransformation of Nitroprusside**

An example of a nitroso compound with an oxidation state of +3 that is a potent vasodilator is sodium nitroprusside. Despite the fact that this drug has been widely used clinically for several years, the precise mechanism by which it released nitric oxide has only recently been elucidated. It had been reported previously that sodium nitroprusside "spontaneously" released nitric oxide, but the studies demonstrating this point were performed in the presence of light. This concept seemed unlikely, particularly in view of the fact that solutions of sodium nitroprusside remain relatively stable when protected from light for prolonged periods. To address this issue, we examined the release of nitric oxide from solutions of sodium nitroprusside using chemiluminescence. In contrast to prior reports, when protected from light, sodium nitroprusside did not release detectable levels of nitric oxide. Exposure to even low levels of light readily elicited the release of nitric oxide from sodium nitroprusside, and this effect of light was dependent on intensity. Because of the oxidation state of sodium nitroprusside, we reasoned that nonenzymatic one-electron reduction would probably release nitric oxide. Indeed, when solutions of sodium nitroprusside were exposed to a variety of reducing agents including dithiothreitol, glutathione, or preparations of hepatic microsomes (which generate reducing

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![Figure 1](image1.jpg)

**FIGURE 1.** Chart shows comparison of various nitroso compounds including common nitrovasodilators. Oxidation states of the nitrogen eventually released as nitric oxide are shown.

![Figure 2](image2.jpg)

**FIGURE 2.** Chemical structure of nitroglycerin (glycerol trinitrate).
equivalents), large quantities of nitric oxide were released. Even red cell membranes and smooth muscle cell membranes could serve this reducing function. Another interesting aspect of this process was that cyanide loss from the sodium nitroprusside molecule appeared to precede nitric oxide loss, suggesting that even after reduction, several chemical transformations of the nitroprusside anion were required before nitric oxide could be released. The concept that cyanide loss accompanies nitric oxide release from nitroprusside is not new.\textsuperscript{10,12,13} The nitric oxide released is such a powerful vasodilator, however, that nitroprusside is effective at doses that do not produce toxic amounts of cyanide. However, very high doses used to induce intraoperative hypotension have caused death by cyanide intoxication.\textsuperscript{14} Similarly, the use of nitroprusside at high concentrations in experimental situations could lead to levels of cyanide that may impact on the experimental outcome.

### Biotransformation of Nitroglycerin

When nitroglycerin is exposed to many mammalian tissues, (most relevantly vascular smooth muscle), nitric oxide is released, generally from the C-3 carbon.\textsuperscript{15} The precise biochemical process responsible for this has not been defined. Because nitroglycerin requires a three-electron reduction to release nitric oxide, it has been repeatedly suggested that cellular thiols are involved in this process.\textsuperscript{1,16-18} Needelman et al\textsuperscript{17,18} and subsequently Ignarro et al\textsuperscript{1} first proposed that thiols were involved in the biotransformation of nitroglycerin and that the formation of a nitrinosothiol intermediate was likely. Indeed, most\textsuperscript{19-21} but not all\textsuperscript{22} studies have shown that manipulation of thiols either in vivo or in vitro influence responses to nitroglycerin. Whether or not a nitrinosothiol is an intermediate in this process is not shown conclusively by these observations. Sulphydryl groups may have other important roles such as serving as cofactors or donating reducing equivalents that lead to the formation of nitric oxide directly from nitroglycerin.

One consideration is whether the release of nitric oxide from nitroglycerin is enzymatic or nonenzymatic. Exposure of nitroglycerin to large concentrations of various thiol-containing compounds will lead to the spontaneous hydrolysis of nitroglycerin to glycerol dinitrate and nitric oxide.\textsuperscript{1,23,24} The most active thiols in this reaction included cysteine, dithiothreitol, N-acetylcysteine, mercaptosuccinic acid, thiosalicylic acid, and methylthiosalicylic acid.\textsuperscript{23} Glutathione, the major intracellular source of thiols, is relatively ineffective in releasing nitric oxide from nitroglycerin in this nonenzymatic manner.\textsuperscript{1,23,25} The intracellular concentrations of the thiols identified to catalyze this reaction are very low or in most instances nonexistent. In preliminary studies, we have found that homogenates of vascular smooth muscle, heated to inactivate all enzyme systems, lose their capacity to release nitric oxide from nitroglycerin. Finally, Chung and Fung\textsuperscript{23} have provided strong evidence that nitroglycerin releases nitric oxide via an enzyme system attached to the cellular surface membrane. Thus, it would appear that the release of nitric oxide from reasonable concentrations of nitroglycerin and thiols almost certainly involves an enzymatic process. The need for sulphydryl-donating compounds is not to serve as a substrate for nonenzymatic interac-

tions but more likely as a cofactor in this enzymatic process.

Related to this issue is whether or not nitroglycerin tolerance is caused by depletion of intracellular thiols. Commonly, the efficiency of nitroglycerin markedly decreases after 18–24 hours. This is in part due to a decrease in formation of nitric oxide from nitroglycerin, and several have attributed this to depletion of intracellular sulphydryl groups.\textsuperscript{20,26,27} This concept has recently been challenged, because contrary to what was initially expected, vascular sulphydryl groups are not decreased during the development of nitroglycerin tolerance.\textsuperscript{28,29} The administration of thiols does enhance vasodilation produced by nitroglycerin both in vitro and in vivo, but this is not specific for tolerance. Thiol-donating compounds enhance the effect of nitroglycerin even in the nontolerant state.\textsuperscript{30,31}

Thus, the role of thiols in the metabolism of nitroglycerin remains quite controversial. Most studies have suggested that they play an important role in the cellular biotransformation, but the precise mechanism by which they work remains unclear. It is unlikely that nitroglycerin tolerance is mediated by thiol depletion.

It has been suggested that the cytosolic glutathione-S-transferases are responsible for the biotransformation of nitroglycerin to nitric oxide. This is based on the observation that the glutathione-S-transferases can degrade nitroglycerin (glycerol trinitrate) to glycerol dinitrate\textsuperscript{32,33} and that certain isomers of glutathione-S-transferase with this activity are present in vascular smooth muscle.\textsuperscript{34} Bromosulfophthalein, an inhibitor of glutathione-S-transferase, impairs vascular relaxations to nitroglycerin in isolated vessels.\textsuperscript{35} This effect is not specific because bromosulfophthalein also inhibits relaxations to a nitrosothiol, which clearly does not require enzymatic bioprocessing. The biochemistry of the reaction between nitroglycerin and the glutathione-S-transferases is of substantial interest. According to well-established biochemical reactions involving glutathione-S-transferases, this enzyme class catalyzes the attachment of glutathione to one of the nitrate groups of nitroglycerin and eventually forms an unstable thionitrate.\textsuperscript{36} This unstable thionitrate is then attacked by a second glutathione molecule to yield glycerol dinitrite, a nitrogen oxide, and oxidized glutathione (GSSG). Whether the nitrogen oxide is released in vascular smooth muscle as nitric oxide or as nitrite (which is not vasoactive except at very high concentrations) has, however, not been established. In recent studies, we have found that purified preparations of the glutathione-S-transferases release only nitrite from nitroglycerin and do not yield nitric oxide. Furthermore, manipulations that increase glutathione-S-transferases activity in vascular homogenates increase the yield of nitrite while decreasing the yield of nitric oxide from nitroglycerin.\textsuperscript{37}

### Effect of Nitroglycerin on the Coronary Microcirculation

Some insight regarding the biotransformation of various nitrovasodilators has been gained from studies of the coronary microcirculation. One of the fascinating aspects of the pharmacology of nitroglycerin is its effect on the coronary circulation. In commonly used concentrations, nitroglycerin potently dilates the larger coro-
Circulatory microvessels while producing only a submaximal and brief effect on coronary flow. Because coronary perfusion is predominantly regulated by the coronary microcirculation, these findings suggest that nitroglycerin has only a minor effect on the smaller coronary vessels; however, the mechanisms underlying this phenomenon remain unclear. Potential explanations include autoregulatory influences from the adjacent myocardium, which counteract the vasodilatation produced by nitroglycerin, an absence of guanylate cyclase in the vascular smooth muscle of coronary microvessels, and finally, an inability of the smaller coronary microvessels to convert nitroglycerin to its active vasodilator intermediate.

In an effort to address these possible explanations for the heterogeneous effect of nitroglycerin on the coronary circulation, we studied several different sizes of coronary microvessels using an in vitro microvascular imaging apparatus. This allowed us to control a number of factors that may influence vascular responsiveness such as distending pressure, degree of preconstricted tone, and presence or absence of other vasoactive agents. In addition, because the vessels were studied in vitro, removed from the myocardium, autoregulatory influences of the myocardium could not have participated in modulation of nitroglycerin's responses.

The results of these initial experiments are depicted in Figure 3. Interestingly, the response to nitroglycerin was markedly dependent on vessel diameter. Vessels >200 μm in diameter were very responsive to nitroglycerin, whereas those <100 μm in diameter dilated only minimally to the highest concentrations of nitroglycerin. Vessels between 100 and 200 μm in diameter demonstrated an intermediate response to nitroglycerin. Thus, these in vitro experiments showed that the lack of effect of nitroglycerin in the coronary microcirculation was not simply due to autoregulatory influences imparted by the myocardium on these smaller vessels but that this phenomenon occurred in vessels dissected free from the myocardium and studied in vitro.

We sought to determine whether this lack of effect of nitroglycerin was related to an inability of smaller coronary microvessels to convert nitroglycerin to its active vasodilator metabolite. We reasoned that if the smaller vessels, which do not respond to nitroglycerin, diluted to either nitric oxide or a nitrosodithiol, two putative metabolites of nitroglycerin, this would provide evidence that these vessels simply could not convert nitroglycerin to these vasodilator substances. The results of these experiments are shown in Figure 4. Unlike nitroglycerin, both nitric oxide and S-nitrosocysteine elicited potent relaxations of small coronary microvessels. An important observation in these experiments was that responses of coronary microvessels <100 μm in diameter to nitric oxide and S-nitrosocysteine were markedly inhibited by LY83583, a compound that blocks elevations of cGMP after activation of guanylate cyclase. Thus, these experiments disproved the hypothesis that diminished responsiveness of coronary microvessels <100 μm diameter to nitroglycerin is due to an absence or decrease responsiveness of guanylate cyclase. Because these vessels could respond to putative metabolites of nitroglycerin, it seemed likely that these vessels were incapable of converting nitroglycerin to these more vasoactive substances.

In view of the suggested critical role of thiols in the biotransformation of nitroglycerin (discussed above), one reasonable explanation for the inability of coronary microvessels <100 μm in diameter could have been a...
relative deficiency of reduced sulphydryl groups in the vascular smooth muscle of these vessels. To test this hypothesis, we preincubated coronary microvessels <100 μm and >200 μm in diameter with L-cysteine.\(^4\) Surprisingly, L-cysteine markedly increased the response of smaller coronary microvessels to nitroglycerin. This effect seemed somewhat specific because L-cysteine had no effect on the response of larger coronary vessels to nitroglycerin (Figure 5).

In a subsequent study, Kurz et al.\(^1\) examined the response of various-sized coronary microvessels to nitroglycerin using a preparation that allows study of the coronary microcirculation in vivo. As in the in vitro studies, nitroglycerin dilated arterioles with diameters >100 μm while having virtually no effect on smaller coronary microvessels. Furthermore, as in the in vivo experiments, L-cysteine markedly increased responses to nitroglycerin in the smaller vessels while having no effect in larger vessels. Interestingly, unlike L-cysteine, D-cysteine had no effect. We were concerned that nitroglycerin might have nonenzymatically combined with cysteine to form a nitrosothiol extracellularly and that the uptake of the nitrosothiol might be stereospecific. To examine this possibility, we examined responses of coronary microvessels to both S-nitroso-L-cysteine and S-nitroso-D-cysteine. Both isomers produced similar potent relaxations of small coronary microvessels, showing that the effect of L-cysteine was not simply related to the nonenzymatic formation of a nitrosothiol (if it had been, one would have expected that D-cysteine, which also could have nonenzymatically formed S-nitroso-D-cysteine, would have augmented responses to nitroglycerin). In subsequent preliminary experiments, we have found that the effect of L-cysteine and N-acetyl-cysteine can be prevented by inhibiting production of glutathione.\(^3\) These preliminary experiments suggest that the thiol enhanced by the administration of these agents is probably intracellular glutathione and that glutathione is probably critical in the bioconversion of nitroglycerin. In these studies, we also found that the ability of smaller and larger coronary microvessels to convert L-cysteine to glutathione was similar. Thus, there does not appear to be major differences between the formation of glutathione in these different-sized microvessels. One potential explanation relates to accelerated oxidation of thiols in smaller vessels, which, being more intimately associated with the myocardium than larger vessels, are potentially exposed to greater oxidative stress.

These experiments examining the effect of nitroglycerin and related compounds on the coronary microcirculation contribute to our understanding of how nitroglycerin interacts with vascular smooth muscle to produce its vasodilatory effect. By studying a tissue that normally does not dilate to nitroglycerin, we have been able to provide cofactors that are potentially critical for the metabolism of this agent to nitric oxide. These studies strongly support an important role for intracellular thiols (probably glutathione) as a cofactor in an enzymatic process. As discussed above, the precise roles of glutathione and other thiols have not been defined. A summary of some these interactions are presented in Figure 6.

Notwithstanding the numerous unanswered questions left to be addressed about nitroglycerin's biotransformation, it is clear that the unique nature of this compound targets its action in the coronary circulation and probably in other circulations. This property of the drug is almost certainly important for its antianginal effects. Vasodilators that act on the smallest coronary microvessels notoriously induce angina and in fact are often used to elicit myocardial ischemia as an adjunct to cardiac imaging.\(^4\) The mechanism by which these compounds induce ischemia probably relates to redistribution of myocardial perfusion from ischemic regions (the so-called coronary steal phenomenon).\(^4\) Because nitroglycerin is selectively converted to its vasoactive metabolite in larger vessels and to a lesser extent in the smallest microvessels, it does not share this capacity of inducing coronary steal. This property probably has implications regarding the development of newer antianginal nitrovasodilators. It is likely that potent vasodilators of coronary microvessels will be less useful than substances that require an enzymatic biotransformation similar to nitroglycerin. Compounds with higher oxidation states of the nitrogens eventually converted to nitric oxide will be more likely to share this profile of nitroglycerin's lack of effect on the coronary resistance circulation. Finally, these observations may have implications regarding strategies of preventing nitroglycerin tolerance. Several groups have suggested using thiol-donating compounds like N-acetyl-cysteine to prevent or reverse nitroglycerin tolerance. Our data demonstrate that the primary site of interaction of these.
thiol-donating compounds with nitroglycerin is at the level of the smaller coronary microvessels, and use of these converts nitroglycerin from a predominant large vessel dilator to a small vessel dilator. Such an intervention should be viewed with caution, as the resultant small vessel dilatation may contribute to a coronary steal phenomenon similar to that observed with other small vessel dilators.

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