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# NITRIC OXIDE–RELEASING DRUGS

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■ **Abstract** Pharmacological compounds that release nitric oxide (NO) have been useful tools for evaluating the broad role of NO in physiology and therapeutics. NO deficiency has been implicated in the genesis and evolution of several disease states. Both medical needs and commercial opportunities have fostered attempts to modulate NO in the human body for therapeutic gain. Strategies for NO modulation encompass antiinflammatory, sexual dysfunction, and cardiovascular indications. Apart from newly developed drugs, several commonly used cardiovascular drugs exert their beneficial action, at least in part, by modulating the NO pathway. This review discusses the fundamental pharmacological properties and mechanisms of action of NO-releasing drugs. Some of these compounds may enter in the clinical arena providing important therapeutic benefits in human diseases.

## INTRODUCTION

A gaseous nitrogen monoxide radical, nitric oxide (NO), is a ubiquitous signaling molecule able to diffuse readily across cell membranes, modulating a plethora of physiological responses including gene regulation, cytostasis, apoptosis, platelet function, vascular smooth muscle cell (VSMC) relaxation and proliferation, neurotransmission, memory, and immune stimulation [reviewed in (1–3)]. Since the discovery of NO, an increasing number of studies have attempted to address its chemistry and its connection with the biology of this unique mediator. Unlike other biological molecules, the chemistry of NO determines its biological properties. NO is produced by the basic semiessential amino acid L-arginine (2-amino-5-guanidinovaleric acid) in a reaction catalyzed by a family of nitric oxide synthases (NOSs) (1–3). The classical physiological role of NO generally reflects direct activation of guanylate cyclase to generate cGMP, followed by kinase-mediated signal transduction (1–3). However, several other NO-related activities are cGMP-independent. NO-derived reactive nitrogen oxide species (RNOS) can

modify bioactivity of certain molecules such as proteins, lipids carbohydrates, and nucleic acids (4). Indeed, oxygen radicals can enhance the bioreactivity of NO through RNOS formation (1–4). However, reaction of NO with superoxide radical, although antioxidant in the sense that a superoxide radical is removed from the system, can damage cell membranes and cell function by producing peroxynitrite, a highly oxidizing agent capable of perturbing bioactivity, or by consuming regulatory NO (1–4). Detailed consideration of general NO biology and chemistry is obviated by recent presentations elsewhere (1–3, 5). Therefore, appreciation of this "double-edged sword" aspect of NO under conditions supporting mammalian life plays a pivotal role to any attempts at therapeutic NO modulation. Distinction between a beneficial and an adverse body response to NO at the physiological level could be related to tissue redox status, tissue NO concentration, tissue NOS activity, presence of oxygen radicals, formation of RNOS, site of exogenous NO production or scavenging, autooxidation of NO, and interaction of NO with cellular constituents. Moreover, the nature of a given biological response to NO need not influence its ultimate therapeutic gain. Indeed, NO can limit cell proliferation by acting as reversible cytostatic agent (pro-apoptotic effect) or as a nonspecific rank cytotoxin (6).

NO-releasing drugs are pharmacologically active compounds that, in vivo or in vitro, release NO. For a detailed overview of the general role of NO-donors (e.g., direct NO-donors, NO-donors requiring metabolism, and bifunctional NO-donors), the reader is referred elsewhere (7). There are some diseases that result from quantitative or functional NO deficiency. A NO insufficiency may be characterized by a net tissue NO deficit, enhanced NO inactivation, impaired NO availability, or altered NOS catalysis. In all these states, a NO deficiency would limit NO-dependent signal transduction pathways to the detriment of normal cellular function. For example, dysfunction of the normally protective endothelium is found in several cardiovascular diseases, including atherosclerosis, hypertension, heart failure (HF), coronary heart disease (CHD), arterial thrombotic disorders, and stroke (1, 2, 8). Endothelial dysfunction leads to NO deficiency (1, 2, 8), which has been implicated in the underying pathobiology of many of these disorders. In the case of the gastrointestinal tract, NO is a critical mediator of mucosal defense and repair (9). Finally, NO is an essential mediator of penile erection, which is fundamentally a hemodynamic process (10).

Pro-inflammatory effects of NO include vasodilation, edema, cytotoxicity, and the mediation of cytokine-dependent processes that can lead to tissue destruction [reviewed in (11)]. Conversely, the production of NO by endothelial cells may serve a protective, or antiinflammatory, function by preventing the adhesion and release of oxidants by activated neutrophils in the microvasculature (11). In light of this multifaceted pathophysiological scenario, replacement or augmentation of endogenous NO by exogenously administered NO-releasing drugs has provided the foundation for a broad field of pharmacotherapeutics.

Here, we review our current understanding of NO-releasing drugs and of commonly used cardiovascular drugs that modulate the bioactivity of NO.

## SODIUM NITROPRUSSIDE

In sodium nitroprusside (SNP), NO is coordinated as a nitrosyl group liganded to iron in a square bipyramidal complex (12, 13) and is released spontaneously at physiological pH from the parent compound. SNP remains an effective, reliable, and commonly used drug for the rapid reduction of significant arterial hypertension regardless of the etiology, for afterload reduction when blood volume is normal or increased, and for intraoperative-induced alterations of blood pressure (12, 13). SNP has been used effectively for decades for the treatment of hypertension and HF (12, 13). The use of SNP is limited by the need to administer it parenterally, tolerance, and the potential for the development of thiocyanate toxicity with prolonged administration (in rhodanase-deficient individuals). Careful attention to infusion rates, particularly in patients at risk for depleted thiosulfate stores, is mandatory, and the use of other drugs in conjunction with or instead of SNP should always be considered. Despite its toxicity, SNP is popular because it is often the most effective drug in some difficult clinical circumstances.

Mutagenic effects of some NO donors have also been demonstrated. Birnboim & Privora, using a very sensitive detection system, showed that glyceryl trinitrate and SNP appear to promote mutagenesis in a glutathione-dependent manner; in this study, N-acetylcysteine and oxothiazolidine-4-carboxylate paradoxically reduced mutagenicity of the NO donors (14).

## NITROVASODILATORS: ORGANIC NITRATE AND NITRITE ESTERS

The classic nitrovasodilators, organic nitrate and nitrite esters, including nitroglycerin, amyl nitrite, isosorbide dinitrate, isosorbide 5-mononitrate, and nicorandil, have been used for many years in the treatment of cardiovascular diseases (15–17). Indeed, the organic nitrates are a safe and effective choice for the management of ischemic syndromes related to coronary heart disease (CHD). Their principal action is vasorelaxation, mediated by guanylyl cyclase activation and by direct inhibition of nonspecific cation channels in VSMCs. As such, these agents represent the prototypical form of NO-replacement therapy. In addition to their well-established venodilative activity, nitrates are now known to cause vasorelaxation of coronary arteries, coronary stenoses, and coronary collateral vessels and to prevent episodic coronary constriction. A direct antiplatelet effect has also been investigated. Notwithstanding these shortcomings, a careful and controlled use of these agents represents the mainstay of therapy for patients with CHD. The first reports of the clinical use of organic nitrates and nitrite esters was derived from the seminal work of Brunton in 1857 showing the clear benefits of these compounds in the treatment of angina pectoris (18). The rapid- but short-acting nitrate preparations are useful in arresting and preventing acute attacks of angina pectoris, whereas longer-acting oral and transdermal formulations are indicated for the

relief of chronic symptomatic and asymptomatic ischemia (15). The intermittent nitrate dosing regimens introduced in recent years have reduced the likelihood of tolerance, which greatly limited the usefulness of long-acting nitrates in the past. Intravenous infusion of nitroglycerin is particularly appropriate for the management of unstable angina and the early complications of myocardial infarction (MI) (15). In patients with acute MI, intravenous nitroglycerin lowers left ventricular (LV) filling pressure and systemic vascular resistance. At lower infusion rates (less than 50 $\mu$g/min) nitroglycerin is principally a venodilator, whereas at higher infusion rates more balanced venous and arterial dilating effects are seen. Patients with HF demonstrate increased or maintained stroke volumes, whereas patients without HF show a decrease in stroke volume (15). Longer-term infusions (24–48 hours) have resulted in myocardial preservation and positive effects on remodeling, as assessed by global and regional LV function and laboratory indices of infarct size. Comparison of intravenous nitroglycerin and SNP reveals increased intercoronary collateral flow with nitroglycerin, in contrast to a decrease with SNP, compatible with a "coronary steal" (15). Short-term administration of intravenous nitroglycerin with or without chronic administration of long-acting nitrates has been found both to reduce short-term mortality and to have long-term beneficial effects on LV remodeling in patients with anterior transmural MI (15). Current clinical practice would utilize intravenous nitroglycerin as initial therapy for patients receiving intravenous thrombolytic therapy and/or acute percutaneous transluminal coronary angioplasty within four to six hours of the onset of symptoms of acute MI, in order to optimize intercoronary collateral flow until reperfusion can be accomplished. Patients reaching the hospital more than 6 hours but less than 14 hours after symptom onset can still benefit from intravenous nitroglycerin administered for 24–48 hours.

The limitations of this class of agents are well known and include potentially adverse hemodynamic effects, drug tolerance, lack of selectivity, and limited bioavailability. All of the organic nitrate esters are prodrugs requiring enzymatic metabolism to generate bioactive NO. The major enzyme system involved is located within microsomal membranes, has an estimated apparent molecular mass of 160 kDa, and manifests enhanced activity in the presence of reducing equivalents (19). Although the enzyme has not been more specifically characterized, growing evidence suggests that the cytochrome P450 system is required for the linked metabolic processes of denitration and reduction of organic nitrate esters to authentic NO (16, 20–23). Importantly, thiols potentiate the action of organic nitrate esters (16, 20–24).

Tolerance limits the clinical use of organic nitrite and nitrate esters; it is associated with increased angiotensin II (ANGII)-dependent vascular production of superoxide radical from NAD(P)H oxidase and endothelial NOS (eNOS) (25, 26). The superoxide radical generated by these enzymes reacts with NO derived from the NO-donor to form peroxynitrite, as indicated by the finding of increased urinary 3-nitrotyrosine in nitrate-tolerant patients (27). Importantly, nitrate tolerance is also associated with cross-tolerance to endothelium-derived NO (28), both by

the oxidative inactivation of this endogenous NO to peroxynitrite and by "uncoupling" of eNOS activity (29). Low-molecular-weight thiols, ascorbate, L-arginine, tetrahydrobiopterin, hydralazine, angiotensin converting enzyme (ACE) inhibitors, and folate have been successfully used to reverse or prevent nitrate tolerance (30).

## S-NITROSOTHIOLS

Early studies have demonstrated that S-nitrosothiols represent a source of circulating endogenous NO, and may have potential as donors of NO, distinct from currently used agents (16, 21–23). Their stability is influenced by the properties of the R group, heat, light, the presence of transition metal ions (in particular copper), and the presence of other thiols. S-nitrosothiols participate in transnitrosation reactions in which the NO group is transferred to another thiol to form a more stable compound (16, 21–23). Thus, the stability of these compounds in vivo is difficult to predict. Potential interactions of S-nitrosothiols include that with ascorbic acid (vitamin C), which enhances the ability of copper to catalyze the degradation of S-nitrosothiols. S-nitrosothiols, offer advantages over the existing drugs because they do not share the drawbacks of organic nitrates and SNP, including a limited capacity for inducing oxidant stress and tolerance in vascular cells (31). Initial small clinical studies suggest that they may be of benefit in a variety of cardiovascular disorders (32). S-nitrosothiols are a class of naturally occurring NO-donating compounds that spontaneously release NO and nitrosonium ($NO^+$); they may also gain access to the intracellular compartment by the catalytic action of plasma membrane-bound protein disulfide isomerase and associated transnitrosation reactions (33). However, the action of these compounds is linked to NO because $NO^+$ does not activate guanylate cyclase (21–23). Members of this class of agents include S-nitroso-glutathione, S-nitroso-N-acetylpenicillamine, and S-nitroso-albumin (34) (Figure 1). Investigators have chosen to modify existing pharmacological agents with these functional groups in an effort to exploit some

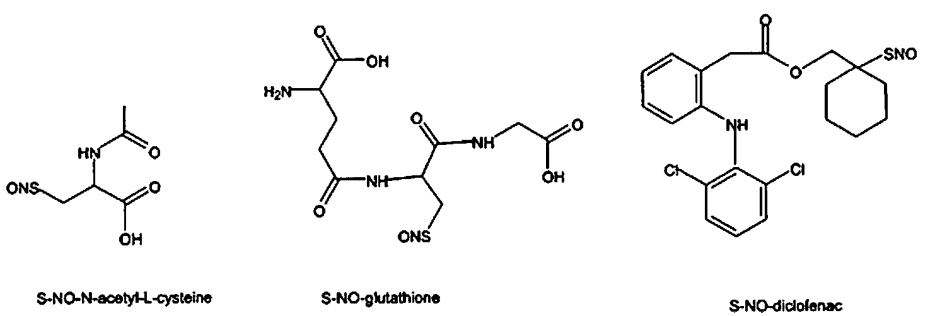


**Figure 1** Structures of selected S-nitrosothiols.

of the beneficial effects of NO without limiting the pharmacological effect of the parent compound. Early work with S-nitroso-captopril represents one such effort (35, 36). A theoretical role of S-nitrosothiols has been suggested in the treatment of asthma and in their potential to be used as agents to treat infectious diseases ranging from the common cold to immunodeficiency diseases.

## NONSTEROIDAL ANTIINFLAMMATORY DRUGS (NSAIDS) AND THE DEVELOPMENT OF NO-NSAIDS

Nonsteroidal antiinflammatory drugs (NSAIDs) are among the most commonly used medications. They are prescribed largely for their antiinflammatory, antipyretic, and analgesic properties. Moreover, low-dose aspirin is increasingly used on a long-term basis for its well-documented antithrombotic effects (37, 38). The major limitation for the use of NSAIDs is their ability to cause gastrointestinal toxicity. Indeed, even low-dose aspirin maintains its ability to damage the gastric mucosa by inducing bleeding ulcers and/or erosions (38, 39). The efficacy and toxicity of NSAIDs appears to be closely related to cyclooxygenase (COX) activity inhibition. The discovery of a second inducible isoform of COX (COX-2) has given a new boost to NSAID research in the attempts to develop COX-2 selective inhibitors. These drugs have been developed in an effort to improve gastrointestinal tolerability and efficacy. However, questions remain about the effectiveness and safety of this new class of drugs (40). Because some biological effects are mainly driven by COX-1 products, as in thrombosis (41), COX-2 inhibitors cannot replace aspirin in antithrombotic therapy because by definition they do not inhibit COX-1, the constitutive form of the enzyme, present in platelets.

Prostaglandins and NO are thought to play a major role in maintaining mucosal integrity. Indeed, NO has cytoprotective properties that derive from its ability to increase local blood flow and to scavenge highly reactive free radicals in the stomach and other organs (42–45). In particular, NO exhibits many of the same actions in the stomach as prostaglandins, such as stimulation of mucus secretion (46) and maintenance of mucosal blood flow (47). These overlapping physiological properties at the gastric level have prompted efforts to design an aspirin-like compound that can release NO, counterbalancing the negative effect due to prostaglandin inhibition at this level. Indeed, local delivery of NO could be a surrogate for prostaglandin effect, restoring the imbalance between destructive and protective factors caused by COX-1 inhibition in the gastrointestinal tract. In addition, because NO plays an important role in pathophysiological conditions where platelets, endothelial, and other blood cells are actively involved and in the inflammatory process, these compounds also have an intense antiinflammatory effect.

New classes of NSAIDs that release NO, called NO-NSAIDs, have been discovered and early clinical evidences suggests that these compounds could be safe and effective alternatives to conventional NSAIDs. These agents comprise two classes of compounds, one that contains a nitrate ester functionality (48) and one that contains an S-nitrosothiol functionality (49).

## NO-RELEASING ASPIRINS

NO-releasing aspirins are nitrate-ester compounds and include 2-acetoxybenzoate 2-(2-nitroxy-methyl)-phenyl ester (NCX-4016) and 2-acetoxybenzoate 2-(2-nitroxy)-butyl ester (NCX-4215) (Figure 2). NCX-4016 is a stable compound that requires enzymatic hydrolysis to liberate NO, and the kinetics of this metabolic processing leads to durable production of NO released at a constant rate (48). Following intragastric administration of NCX-4016, levels of NO are elevated both in gastric content and plasma (48). NO generation by NCX-4215 has also been evaluated by using human platelets and measuring NO generation by chemiluminescence (48). To establish whether NCX-4215 could spontaneously liberate NO, the compound was incubated in the absence or presence of platelets. A significant release of NO was observed only in presence of platelets. The biological activity of NCX-4016 has been evaluated in a variety of experimental models to characterize its antiinflammatory and antithrombotic effects (48).

The antiplatelet activity of nitroaspirins NCX-4215 and NCX-4016 was compared in vitro to aspirin with comparable results for maximal inhibition of arachidonic acid-stimulated platelet aggregation (50). Furthermore, NCX-4016 was more efficient in inhibiting platelet activation induced by thrombin (48, 50). This was reversed by oxyhemoglobin and methylene blue acid, further supporting the NO-related inhibitory effect. NCX-4016 was shown to possess greater antiinflammatory and analgesic activity (51, 52). The antithrombotic activity of NCX-4016 was also present in vivo (53) and reduced pulmonary thromboembolism

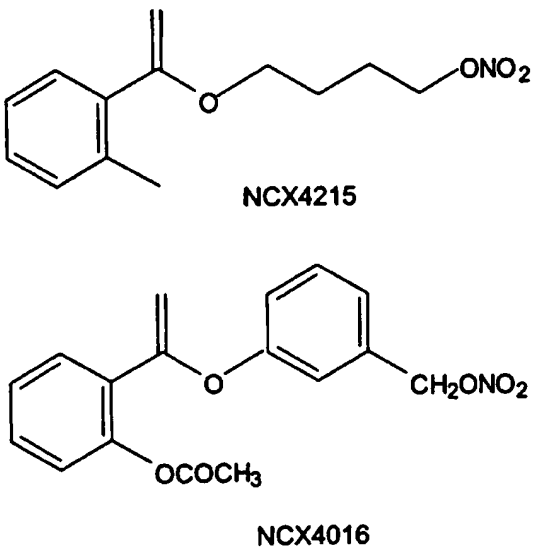


**Figure 2** Structures of nitro-aspirin derivatives.

in several platelet dependent and -independent animal models (54). The compound also has greater protective activity than aspirin in focal cerebral ischemia in the rat (55). NCX-4016 and diethylenetriamine/NO, a NO-donor, raised myocardial production of prostacyclin and thromboxane during MI (56). Interestingly, NCX-4016 also reduces infarct size after myocardial reperfusion injury (57, 58).

Inhibitory effects on caspases 1 and 3 (59, 60), T lymphocyte activation (60), cytokine release (60), stimulation of apoptosis (60), and IL-1B$\beta$ converting enzyme (61) have been described for NCX-4016. In very recent studies, NCX-4016 reduces the degree of restenosis after arterial injury in low-density lipoprotein-receptor-deficient mice (62) and in aging rats (63). This protective effect was associated with reduced VSMC proliferation and macrophage deposition at the site of injury. This response appears to agree very well with the potent inhibitory properties on VSMC proliferation possessed by NCX-4016 (64). Thus, a NO-releasing–aspirin derivative could be an effective drug in reducing restenosis following PTCA, especially in the presence of hypercholesterolemia or advancing age.

Another therapeutic goal of NO-aspirin was to achieve lower gastrointestinal toxicity in comparison to aspirin (48). NCX-4215 does not produce macroscopically visible or histological damages in the rat stomach when administered up to 300 mg/kg, whereas 100 mg/kg aspirin produces widespread hemorrhagic damage (65, 66). These protective effects were also seen in the stomach of aged rats treated with NCX-4016 (63). NCX-4016 produced an equipotent inhibition of mucosal $PGE_2$ generation in the stomach as compared with aspirin (67), suggesting that gastrointestinal damage is not linked to a lack of effect on COX enzymes.

Leukocyte adherence is not affected by pretreament with NCX-4016, whereas aspirin causes a fivefold increase in neutrophil adherence (68). Furthermore, whereas aspirin has no effect on human umbilical vascular endothelial cell (HUVEC) apoptosis induced by TNF$\alpha$, NCX-4016 causes a concentration-dependent inhibition of TNF$\alpha$-induced apoptosis (59). The hypothesis that nitroaspirin is also able to positively modulate change in gastrointestinal damage was challenged by testing the ability of NCX-4016 to prevent gastric damage in a rat model of shock (68). Oral administration of NCX-4016 or glyceryl trinitrate or depletion of circulating neutrophils with antineutrophil serum significantly reduced the extent of gastric damage induced by hemorrhagic shock, whereas aspirin had no effect. Recent data also showed the lack of gastric toxicity of NCX-4016, but not aspirin, in the stomach of diabetic rats (69).

The effects of NCX-4016 and aspirin on the release of thromboxane; TNF-$\alpha$; interleukin-6; and expression and activity of tissue factor (TF) in stimulated, adherent human monocytes were recently investigated (70). These data showed that NCX-4016 inhibits thromboxane generation, cytokine release, and TF activity in human monocytes via NO-dependent mechanisms. Moreover, NCX-4016 also reduces blood pressure in hypertensive rats, not simply through the direct vasodilatory actions of the NO released by this compound, but also through possible interference with endogenous pressor compounds (71). These properties, added to its antithrombotic effects, suggest that NCX-4016 may be a safer alternative to aspirin for use by hypertensive patients.

## S-NITROSO-NSAIDS

S-nitroso-diclofenac (Figure 1), as a prototype of the S-nitrosoester class of NSAIDs that release NO, possesses peculiar properties (49). This agent is orally bioavailable as a prodrug, producing significant levels of diclofenac in plasma within 15 min after oral administration to mice. In addition, S-nitroso-diclofenac has equipotent antiinflammatory and analgesic properties as diclofenac, but is gastric-sparing compared with the parent NSAID. Thus, S-nitrosothiol esters of diclofenac and other NSAIDs [reviewed in (72)] comprise a novel class of NO-donating compounds with antiinflammatory and analgesic properties but a markedly enhanced gastric safety profile. The use of NO-NSAIDs for general analgesic and antiinflammatory purposes or for primary or secondary cardiovascular prevention awaits the results of ongoing long-term clinical trials.

## NO-DELIVERY SYSTEMS

There has been considerable long-term interest in developing NO delivery systems that can be used to target drug action and modulate the kinetics of drug release. Drug-eluting vascular stents with a variety of coatings, including fibrin, heparin, and multiple polymers that contain NO-donors, have been tested with variable effects (73, 74). NO-containing crosslinked polyetheleneimine microspheres that release NO (half-life of 51 hours) have been applied to vascular grafts (75) in order to prevent thrombosis and restenosis. Similarly, the [N(O)NO] group has been incorporated into polymeric matrices synthesized to modulate the time-course of NO release (76). This approach shows potent antiplatelet activity in baboons (76).

Bovine serum albumin (BSA) can be modified covalently to bear multiple S-NO groups, which possess vasodilatory and antiplatelet properties (77). Poly-S-nitrosated BSA applied locally to a site of vascular injury reduced restenosis in a rabbit model (78). Local delivery of poly-S-nitrosated BSA induced a 50%–70% reduction in platelet attachment and surface activation, together with a 40% reduction in neointimal area when compared to BSA (79). The advantages to the use of this agent include the avidity of the subendothelial matrix for albumin; its long half-life in vivo; and its ability to serve as a local depot of NO, requiring trans-S-nitrosation reactions (thiol-S-nitrosothiol exchange) to deliver NO via low-molecular-weight S-nitrosothiol intermediates (80).

## CARDIOVASCULAR AGENTS MODULATING THE NO-PATHWAY

### Calcium Channel Blockers

1,4-Dihydropyridine calcium channel blockers (CCBs) have been used for many years in the treatment of angina pectoris and hypertension (81). Their mechanism of action is based on inhibition of the smooth muscle L-type calcium current, thereby

decreasing intracellular calcium concentration and inducing smooth muscular relaxation. 1,4-Dihydropyridine CCBs (nifedipine, nitrendipine, and lacidipine) can also induce the release of NO from the vascular endothelium (81). CCBs counteract the effects of ANG II and endothelin-1 at the level of vascular smooth muscle by reducing $Ca^{2+}$ inflow and facilitating the vasodilator effects of NO. Indeed, these compounds can reverse impaired endothelium-dependent vasodilation in different vascular beds, including the subcutaneous, epicardial, and peripheral arteries of the forearm circulation. In the forearm circulation, nifedipine and lacidipine can improve endothelial dysfunction by restoring NO availability (81). Furthermore, in several experimental preparations, including micro- and macrovascular studies, the sensitivity of the vasorelaxing effect of the 1,4-dihydropyridines to inhibitors of NOS, such as L-$N^G$-nitroarginine or L-N-nitro-argininemethylester, has been clearly demonstrated. These studies show that the NO-releasing effect is not unique to nitrendipine but is a class phenomenon shared by 1,4-dihydropyridine CCBs and several nondihydropyridine CCBs (81). More importantly, 1,4-dihydropyridine CCBs also have a potent antioxidant activity in vivo (82). Thus, the above effects on NO could also be due to this property. Indeed, the underlying mechanism of NO release evoked by these drugs is not entirely clear but may also involve modulating endothelial membrane potential via a myo-endothelial interaction (83), upregulating eNOS expression (84), increasing activity of endothelial superoxide dismutase(s) (85), and enhancing flow-mediated release of endothelial NO via VSMC relaxation and vasodilation. These dual modes of action, i.e., the direct relaxing effect of inhibiting smooth muscle L-type calcium current and the indirect relaxing effect of releasing NO from vascular endothelium, may help explain the beneficial antihypertensive effect of the 1,4-dihydropyridine CCB class.

## Ace-Inhibitors and ANGII Type 1 Receptor Antagonists

ANGII and bradykinin levels within the vascular wall are controlled by ACE (86). ACE degrades bradykinin (87) and generates ANGII; in turn, bradykinin stimulates the endothelium to release vasodilating substances, in particular, NO. Thus, by potentiating bradykinin, ACE-inhibitors may promote the release of endothelial NO (88). Indeed, ACE-inhibitors exert some of their beneficial pharmacological effects by increasing vascular NO activity (86, 89, 90). Moreover, due to the significant constitutive expression of NOS in the juxtaglomerular apparatus, NO appears to act as a tonic enhancer of renin secretion via cGMP-dependent inhibition of cAMP degradation [reviewed in (91)]. This effect may also revert to an inhibitory effect compatible with the inhibition of renin secretion by cGMP-dependent kinase(s). Moreover, ANGII can stimulate superoxide production, which reduces the bioavailability of NO (92), an event that can be blocked by ACE inhibitors.

In patients with high cardiovascular risk, chronic ACE inhibition improves endothelial function (86, 88). This may explain why patients treated with ACE inhibitors experience a greater cardiovascular benefit than is attributable to the decrease in blood pressure. Indeed, ACE inhibitors improve endothelial function

in the subcutaneous, epicardial, and renal circulation, but are ineffective at potentiating the blunted response to acetylcholine in the forearm of patients with essential hypertension [reviewed in (86)]. In addition, ANGII type 1 (AT-1) receptor antagonists can restore endothelium-dependent vasodilation to acetylcholine in the subcutaneous tissue, but not in the forearm microcirculation (90). Treatment with an AT-1 antagonist can improve basal NO release and decrease the vasoconstrictor effect of endogenous endothelin-1 (90). Thus, drugs interfering with the renin-angiotensin-aldosterone pathway may affect NO signaling by several mechanisms; however, the molecular mechanisms involved in the relationship between ACE inhibitors and the NO-pathway via bradykinin are still unclear (93).

There is mounting evidence that (*a*) ACE efficiently catabolizes kinins; (*b*) ANG-derivatives such as ANG-(1-7) exert kinin-like effects; (*c*) kallikrein probably serves as a prorenin-activating enzyme; (*d*) the protective effects of ACE inhibitors are at least partly mediated by a direct potentiation of kinin receptor response on bradykinin stimulation; and (*e*) studies on AT-1 antagonists, which do not directly influence kinin degradation, and studies on ANG-receptor transgenic mice have revealed additional autocrine interactions among the NO, kinins, prostaglandins, cyclic GMP, and ANGII receptor effects. The beneficial effects of ACE inhibitors or AT-1 antagonists are reportedly mediated by NO in HF. It was recently hypothesized that in the absence of eNOS, both LV dysfunction and myocardial remodeling would be more severe after MI, and the cardioprotective effect of ACE inhibitors or AT-1 antagonists would be diminished or absent in mice with HF after MI (94). eNOS knockout mice and wild-type C57BL/6J mice were subjected to MI by ligating the left coronary artery. One month after MI, each strain was treated with vehicle, enalapril or valsartan, for five months (94). ACE inhibitors improved cardiac function and remodeling in wild-type mice, as evidenced by increased LV ejection fraction and LV shortening fraction, and decreased diastolic LV dimension, mass, myocyte cross-sectional area, and interstitial collagen fraction, but these benefits were absent or diminished in eNOS knockout mice. Interestingly, AT-1 antagonists had benefits similar to those of ACE-inhibitors. These interesting data suggest that the absence of NO does not alter the development of HF after MI; however, it significantly decreases the cardioprotective effects of these drugs.

## $\beta$-Blockers

Some $\beta$-blockers may also interfere with the NO-pathway. For example, nebivolol, a $\beta$1-blocker and a racemic mixture of (S,R,R,R) and (R,S,S,S) enantiomers (88, 95), was found to induce endothelium-dependent arterial relaxation in dogs in a dose-dependent fashion (96). However, its hemodynamic effects differ from those of classical $\beta$-adrenoceptor antagonists as a result of a vasodilating action. Indeed, the endothelium-dependent relaxation induced by nebivolol is abolished by L-NAME, an inhibitor of NOS.

Nebivolol and atenolol have been compared in phenylephrine preconstricted dorsal hand veins of healthy men (97, 98). Nebivolol caused venodilation, which

was antagonized by N(G)-monomethyl-L-arginine (LNMMA), whereas atenolol did not, suggesting that such a mechanism could also operate in human veins. Venodilation could be functionally important in reducing cardiac pre-load. $\beta$2-adrenoceptor stimulation increases forearm blood flow (FBF) by activating the L-arginine/NO pathway, but nebivolol lacks direct $\beta$2-adrenoceptor agonist activity. Resistance vessel function has been studied by measuring FBF by venous occlusion plethysmography in healthy men during brachial artery infusions of racemic nebivolol and its enantiomers, atenolol, carbachol (a stable analogue of acetylcholine that vasodilates this vascular bed, in part, by activating the L-arginine/NO pathway), SNP, and LNMMA (97, 98). Nebivolol increased FBF by 91 $\pm$ 18% ($p < 0.01$), whereas an equimolar dose of atenolol had no significant effect. LNMMA inhibited responses to nebivolol and carbachol to a significantly greater extent than it reduced responses to SNP. Antagonism of nebivolol by LNMMA was abolished by L-arginine. The (S,R,R,R) and (R,S,S,S) enantiomers caused similar increases of FBF.

To determine whether brachial artery infusion of nebivolol causes vasodilation in the forearm resistance vasculature of patients with essential hypertension and to investigate the possible involvement of the L-arginine/NO pathway, healthy volunteers with uncomplicated essential hypertension were also studied (99, 100). Nebivolol caused similar vasodilatation as in normotensive subjects, and these responses were sensitive to inhibition by LNMMA. If acute effects of nebivolol on the L-arginine/NO pathway persist during chronic treatment of patients with hypertension or HF, this could reduce cardiac after-load as well as pre-load, improve organ perfusion, and reduce atherogenesis and thrombosis.

Finally, experimental studies have established that nipradilol, another NO-releasing beta-adrenergic blocker, enhances postischemic recovery and limits infarct size (100a). Moreover, there is a clear involvement of NO in the ocular hypotensive action of nipradilol (100b). These studies should be carried out in humans before a conclusion is made.

## Hydroxymethylglutaryl-CoA Reductase Inhibitors

The efficacy of the widely prescribed hydroxymethylglutaryl (HMG)-CoA reductase inhibitors (statins) in decreasing the incidence of cardiac events and mortality is likely enhanced by their possible antioxidant and other unknown properties (101).

In 1997, it was demonstrated that statins prevent hypoxia-induced down-regulation of eNOS in normocholesterolemic cells by stabilizing eNOS mRNA, leading to an increase in NO production by endothelial cells (102). Subsequently, it was shown that statins exert their salutary effects on eNOS expression predominantly by posttranscriptional mechanisms that are mediated by blocking geranylgeranylation of the small GTP-binding Ras-like protein Rho due to inhibition of the biosynthesis of geranylgeranylpyrophosphate (GGPP) (103). By inhibiting L-mevalonate synthesis, statins also reduce the synthesis of farnesylpyrophosphate (FPP) and

GGPP inducing important posttranslational modification of a variety of proteins, including eNOS and Rho. Inhibition of Rho results in a threefold increase in eNOS expression and nitrite generation, and the effects of statins on eNOS expression are reversed by GGPP, but not by FPP or LDL cholesterol (103). Thus, an important non-cholesterol-lowering effect of statins is the upregulation of eNOS expression via inhibition of Rho. A recent study sheds light on additional mechanisms involved in the statin-induced upregulation of eNOS activity by demonstrating that simvastatin rapidly activates the serine/threonine kinase AKT in endothelial cells, which in turn leads to phosphorylation of eNOS, resulting in an increase in its activity and enhanced NO production (104). This may serve as an additional beneficial mechanism in individuals with atherothrombotic disease. The potential clinical importance of these observations was underscored by the finding that prophylactic treatment of normocholesterolemic mice with statins increased cerebral blood flow, reduced cerebral infarct size, and improved neurological function via a NO-mediated mechanism (105). Other experimental studies in animal models (106, 107) and human cells (108) have confirmed the statins' lipid-independent ability to upregulate eNOS expression. Notably, the increase of eNOS protein in endothelial cells in response to statin therapy was established to be associated with enhanced release of NO (109). Moreover, statins prevent the downregulation of eNOS induced by TNF-$\alpha$ (110). A very recent study also demonstrated that statins preserve the structure of coronary adventitial vasa vasorum in experimental hypercholesterolemia independent of lipid lowering (111). These effects may play an important role in the setting of chronic statin therapy for the primary and secondary prevention of CHD. Furthermore, simvastatin preserves endothelial function in experimental porcine hypercholesterolemia in the absence of any lipid lowering effect (101). In accordance with these results, statins improved coronary endothelial function in cynomolgus monkeys, which were pretreated with an atherogenic diet for two years, independent of serum lipoprotein concentrations (112). These experimental data are also associated with beneficial effects of statins on endothelial function in patients with CHD (8, 113). Thus, the statins can now be considered as agents that both enhance the bioactivity of NO and improve endothelial function in patients with coronary plaques.

## Antioxidants and L-Arginine

Atherogenic lipids, particularly oxidized low-density lipoprotein (oxLDL), are responsible for a wide range of cellular dysfunctions within the arterial wall (114, 115). Oxidative modification of LDL plays a pivotal role in human early atherogenesis (116, 117). Concerning the regulation of vascular tone, oxLDL may disturb relaxation or act directly against vasodilating substances [reviewed in (115)]. Native and oxLDL can uncouple eNOS (118, 119). OxLDL may also induce a decreased uptake of L-arginine (119). Interestingly, physiological differences can affect arterial segments from different regions. For example, oxLDL impairs contraction and endothelium-dependent relaxation in carotid but not in basilar

arteries (120), suggesting that intracranial arteries may be relatively protected from atherosclerosis via endothelial resistance to oxidative injury. NO produced by inducible NOS in VSMCs inhibits oxidation of LDL (121). Thus, NO release via inducible NOS action induced by cytokines in VSMC may play a protective role during LDL oxidation.

Although antioxidants and L-arginine are not considered classical drugs but perhaps as dietary supplements, the L-arginine hypothesis and increased oxidative stress may actually fit together and are not mutually exclusive. L-arginine administration partially restores endothelium-dependent vasodilation in hypercholesterolemia (122, 123) and dilates coronary stenoses in patients with CHD (124). L-arginine supplementation for six months also improves coronary small-vessel function in association with a significant improvement in symptoms (125). Therefore, L-arginine administration could be a therapeutic option for patients with endothelial dysfunction and nonobstructive CHD (125, 126). The effects of intracoronary administration of L-NMMA and L-arginine were studied in patients with normal angiograms and in patients with CHD (127). L-arginine reversed the effect of L-NMMA and caused greater dilation of the diseased arteries, indicating that there is a deficiency of L-arginine. Moreover, there was a significant clinical improvement in more than 70% of patients, which was associated with a significant decrease in proinflammatory cytokines (128). Thus, L-arginine may have clinical benefits in patients with intractable angina. Furthermore, L-arginine improved myocardial perfusion during exercise in patients with angina and normal coronary arteries (129). The value of L-arginine as adjunctive therapy to improve endothelial function in patients with advanced CHD maintained on medical therapy has also been investigated (130). Oral L-arginine therapy did not improve NO bioavailability in patients with CHD and thus may not benefit this group of patients. However, it is possible that a more prolonged period of L-arginine treatment and fewer coronary lesions are necessary in order to see clinical improvement in CHD patients. The effect of exogenous L- and D-arginine on coronary stenosis vasomotion in relation to stenosis morphology in patients with CHD and stable angina was also examined (131). During intracoronary infusion of L-arginine, but not D-arginine, a larger proportion of complex stenoses than smooth stenoses dilated by 10% ($p < 0.01$). Irrespective of the type of morphology, there was a positive correlation ($p < 0.01$) between the severity of stenoses and the magnitude of vasodilatation to L-arginine. This finding is consistent with a deficiency of L-arginine at the site of coronary stenoses (131).

A corollary of the oxidation hypothesis of atherogenesis is that antioxidants may reduce the progression of the disease (114). Antioxidants present in LDL, including alpha-tocopherol, and antioxidants present in the extracellular fluid of the arterial wall, including ascorbic acid (vitamin C), inhibit LDL oxidation (132), and this action is extended to multiple oxLDL-mediated signaling pathways (133). Vitamin C may potentiate NO activity and normalize vascular function in patients with CHD and classical risk factors (132). Thus, NO may restore endothelial dysfunction and ameliorate vascular remodeling in several clinical correlates to experimental

models (1–5). The hypothesis that the plasma concentration of alpha-tocopherol is associated with the preservation of NO-mediated endothelium-dependent vasomotion was tested in humans (134). Patients who were not taking vitamin supplements were studied using coronary angiography. Coronary endothelium-dependent and -independent vasomotion was assessed by intracoronary infusions of acetylcholine and nitroglycerin. Plasma alpha-tocopherol was significantly correlated with the acetylcholine response but not the nitroglycerin response. Thus, alpha-tocopherol may preserve endothelial vasomotor function in patients with coronary atherosclerosis. However, the results of clinical intervention trials using antioxidants have been contradictory. The CHAOS trial carried on patients with CHD (135) and the SPACE trial designed for patients with severe end-stage renal diseases (136) showed clinical beneficial effects of antioxidants. However, two clinical trials (GISSI-Prevenzione and HOPE trials) (137, 138), using adequate doses of vitamin E, demonstrated no effect on a composite endpoint of nonfatal infarction, stroke, or death from cardiovascular causes. The experimental data on which these trials were based deal primarily with the evaluation of early atherosclerotic lesions (fatty streaks). This pathophysiological scenario does not necessarily provide a rational basis for predicting what antioxidant intervention will do in patients with advanced atherosclerotic lesions, particularly when the end-points used relate to unstable atherosclerotic plaques and fatal thrombosis. Moreover, the same antioxidants (and doses) used successfully in animals may not be effective in humans. Negative clinical trials with antioxidants, in patients with advanced CHD and lasting only a few years, should not be taken as refutation of the oxidation hypothesis of atherogenesis. Conversely, results from several observational and experimental studies consistently support an effect of vitamin E supplementation on reducing risk of cardiovascular events [reviewed in (139, 140)]. The evidence suggests that the major effect, if any, is found at supplemental intake levels at or greater than 100 IU/day. Moreover, oxLDL is already present in early atherosclerotic lesions in human fetuses and children (116, 117). Perhaps different types of human trials are needed, trials in which the development of newly formed lesions is measured, in order to test whether antioxidants can decrease the rate of initiation and progression of atherosclerosis as they do in experimental models (140). In this regard, the Physicians' Health Study-II (PHS II) is a randomized, double-blind, placebo-controlled trial (141), testing alternate day beta-carotene, alternate day vitamin E, daily vitamin C, and a daily multivitamin in the prevention of total and prostate cancer, cardiovascular diseases, and the age-related eye diseases. The PHS-II trial is the only primary prevention trial testing the balance of benefits and risks of vitamin E on cancer and cardiovascular diseases in apparently healthy men. However, by age 55 there are already advanced atherosclerotic lesions in apparently healthy men. Thus, in this study the impact of antioxidants on early lesion formation and major cardiovascular events could be missed and the benefits of the approach underestimated.

Whereas low concentrations of vitamin E may improve endothelial function, high concentrations may worsen endothelial function (140). If confirmed in

further long-term trials done in young adults [or at earlier age in order to adverse in utero programming events, reviewed in (142)], the net benefit of antioxidant supplementation among populations with existing CHD may be substantial, although the current clinical results are insufficient to warrant a change in broad policy recommendations.

## Phosphodiesterase Inhibitors

Sildenafil is a selective inhibitor of phosphodiesterase type-5 that is orally effective in the treatment of erectile dysfunction. Its pharmacological actions are a consequence of prolonging the signaling actions of NO because this drug prevents cGMP hydrolysis by inhibition of a cGMP phosphodiesterase V subtype enriched in penile smooth muscle (1, 10, 143).

Diabetic men have a more than threefold increased prevalence of erectile dysfunction compared with nondiabetic men (1). Erectile function is primarily a vascular phenomenon, triggered by neurologic controls and facilitated by appropriate hormonal and psychological components (1). Recent advances in the understanding of the physiology of penile vasculature and its role in male sexual performance have influenced the clinical approach to erectile dysfunction (1). The pathophysiological alterations leading to impotence in diabetic men include vasculogenic, neurogenic, and hormonal etiologies. A clinical work-up, including a thorough history and physical examination, is an important aspect of erectile dysfunction management. Oral medications acting through phosphodiesterase type-5 inhibition in penile vasculature have revolutionized the treatment of impotence. The long-term safety and efficacy of vacuum-constriction devices, intraurethral suppositories, intracavernosal injections, and other therapies are still under investigation. However, in patients with stable angina coadministration of sildenafil with isosorbide mononitrate or nitroglycerin produces significantly greater reductions in blood pressure than nitrates alone (144). This issue was further investigated in a randomized, double-blind, placebo-controlled crossover trial conducted at a ambulatory-care referral center among 105 men with a mean age of 66 years who had erectile dysfunction and known or highly suspected CHD (145). All patients underwent two symptom-limited supine bicycle echocardiograms separated by an interval of one to three days after receiving a single dose of sildenafil (50 or 100 mg) or placebo one hour before each exercise test. In men with stable CHD, sildenafil had no effect on symptoms, exercise duration, or presence or extent of exercise-induced ischemia, as assessed by exercise echocardiography (145). However, in men with chronic HF, sildenafil citrate reduces vagal modulation and increases sympathetic modulation, probably through its reflex vasodilatory action (146). The autonomic system changes induced with sildenafil citrate could alter QT dynamics. Both changes could favor the onset of lethal ventricular arrhythmias (146). Therefore, before prescribing sildenafil for erectile dysfunction in patients with known cardiac disease or multiple cardiovascular risk factors, physicians should discuss the potential cardiac risk of sexual activity and perform a complete medical assessment.

To date, the actions of sildenafil in vascular disorders distinct from that of erectile dysfunction have yet to be studied adequately. For example, oral sildenafil is an effective and specific pulmonary vasodilator in patients with pulmonary arterial hypertension (146a).

On the other hand, an alternative, NO-based approach for erectile dysfunction therapy has recently been suggested by evidence that pathways inhibiting erection and favoring smooth muscle contraction are mediated by adrenergic nerves (147). S-nitrosated-$\alpha$-adrenergic receptor antagonists have been developed that contain an S-nitrosothiol functionality linked to an $\alpha$-adrenergic receptor antagonist (yohimbine and moxisylyte) by an inert, organic-ester tether. The rationale behind the development of this agent is that it prompts early (immediate) vasodilation, whereas the $\alpha$-adrenergic blocker maintains the vasodilator effect. Pharmacological demonstration of these NO-donor properties in relaxing human penile smooth muscle, their $\alpha$-adrenergic antagonism, and their ability to induce erection in laboratory animals (148) suggest that NO-releasing adrenergic receptor antagonists may be useful as bifunctional agents for local treatment of erectile dysfunction. However, experimental models to study the effect of agents on penile erection usually include in vitro models (poor models when transferred to in vivo conditions) and electrical stimulation of peripheral nerves in anesthetized animals combined with systemic or intracavernous injection of drugs. In contrast, conscious rabbits can be used as a simple and quantitative model for the assessment of compounds that show potential for the treatment of erectile dysfunction (149). Erection was assessed by measuring the length of uncovered penile mucosa before and after the intravenous administration of agents. Animals did not require anesthesia during the course of the study. The phosphodiesterase type-5 inhibitors vardenafil and sildenafil were given intravenously, and measurements were taken for 0–5 hours (149). The effects of phentolamine and milrinone were also evaluated. Vardenafil induced dose-dependent penile erections in conscious rabbits. The efficacy of vardenafil was potentiated, and the minimal effective dose was reduced significantly to 0.01 mg/kg by simultaneous administration of SNP. Administration of the NO-synthase inhibitor L-NAME abolished the effect. Sildenafil was also effective in this model. Phentolamine induced erections with a slower tmax compared with vardenafil and sildenafil. Intravenous administration of milrinone was less effective than vardenafil (149). Further studies are warranted in this model.

## CONCLUSIONS

NO-releasing drugs can elicit beneficial actions relevant to cardiovascular disorders. Figure 3 shows pathways by which these drugs can interfere with NO release. Although more than 20 years have passed since the identification of NO as an endogenous substance produced by the cardiovascular system, attempts toward developing accepted therapeutic approaches for modulating endogenous NO activity have progressed slowly until recently. For example, recent data have contradicted the notion that NO acts solely as a negative inotrope and have shown positive inotropic activity in both isolated rodent and human ventricular myocytes

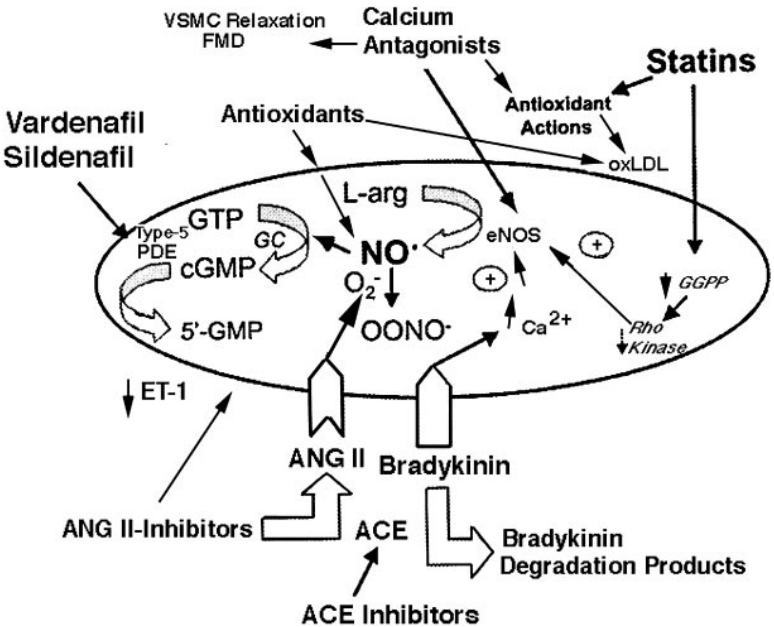


**Figure 3** Mechanisms of cardiovascular agents that indirectly modulate endogenous NO activity.

in response to a range of NO donors [reviewed in (150)]. Recent advances in gene transfer technology and the cloning of the inducible NOS gene have led to the development of strategies for gene therapy to increase NO production for the treatment of disorders ranging from vascular restenosis to impaired wound healing. Different NO donors or NO-releasing drugs have different NO release kinetics and may generate a range of nitrogen monoxide species that may interact at a number of subcellular targets. In the case of cardiac in vitro preparation, the observed response to a NO donor represents the net effect of activation of different effector targets and may explain the contradictory reported effects of NO (150). The use of inhaled nitric oxide (INO) allows selective pulmonary vasodilatation and it is effective in the acute management of reversible pulmonary hypertension and is also useful in assessing the pulmonary vasodilator capacity in patients with chronic pulmonary hypertension [reviewed in (151)]. The clinical use of INO in cardiac failure, postoperative cardiac patients, patients with congestive cardiac failure, or congenital heart disease can be also hypothesized (151). However, to realize the complete therapeutic potential of NO requires specific targeting at the subcellular level. Understanding the complex pathophysiological role of NO and its transduction responses, developing new targeted therapies for delivery of NO, and choosing the optimal adjunctive therapies that potentiate the benefits of NO-releasing drugs are issues that require additional preclinical and clinical controlled studies.

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