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Nitric oxide in health and disease – its role in the practice of medicine

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Summary Nitric oxide (NO), originally discovered as an endothelium-derived relaxing factor, is now known to participate in the physiologic processes of the nervous, renal, gastrointestinal, and cardiovascular systems. NO is an important mediator of vasodilation and is a potent inhibitor of platelet aggregation and endothelial adhesion. Endothelial dysfunction, which is characterized by a deficit in endothelial NO, is associated with cardiovascular risk factors and is a harbinger of impending cardiovascular disease. The association of reduced NO and cardiovascular disease has led to research into drugs that might enhance the activity of endogenous NO or that can donate exogenous NO to vulnerable tissues. Drugs that donate NO include nitroglycerin, which has been used for more than 100 years in the management of angina pectoris. Drugs used to treat erectile dysfunction act primarily by inhibiting the degradation of the second messenger of NO, thus enhancing and prolonging its action. A novel approach to delivering exogenous NO is to link a NO-donating moiety to an existing drug to improve its safety profile. An example of this strategy is naproxcinod, which in clinical trials for osteoarthritis has demonstrated analgesic equivalence with the parent drug, naproxen, while attenuating some of the gastrointestinal and cardiovascular adverse effects. It is anticipated that the practice of medicine will continue to be affected as new drugs are developed that exploit the important pathways modulated by NO.

In the 1970s, nitric oxide (NO) was known only as a noxious gas and was considered an environmental pollutant that was found in cigarette smoke, automobile exhaust, and smog.[1] The negative reputation of this molecule began to change in 1977, when Ferid Murad showed that NO increased tissue cyclic guanosine monophosphate (cGMP) levels, suggesting that NO might regulate enzyme activity.[2] However, NO had never been identified in mammalian cells, and the physiological importance of this observation was not apparent. The initial observation of a physiologic role for NO is readily traced to 1980, when Robert Furchgott and John Zawadzki demonstrated that a substance released by vascular endothelial cells was responsible for the relaxation of vascular smooth muscle cells in response to acetylcholine.[3] This unknown substance, called *endothelium-derived relaxing factor* (EDRF), was further characterized as being diffusible[3] and having a short half-life in solution, on the order of seconds.[4] This substance was finally identified as NO by Louis et al in 1987.[5] Furchgott, Murad, and Ignarro were honored for their discoveries and were awarded the Nobel Prize in Physiology or Medicine in 1988.

The discovery of EDRF and its identification as NO stimulated interest and research into this molecule (Fig. 1). In addition to being a potent vasodilator, NO participates in many and varied physiologic and pathologic processes. For example, NO is a potent inhibitor of platelet aggregation[6] and adherence to vascular endothelium.[7] NO acts as a neu-

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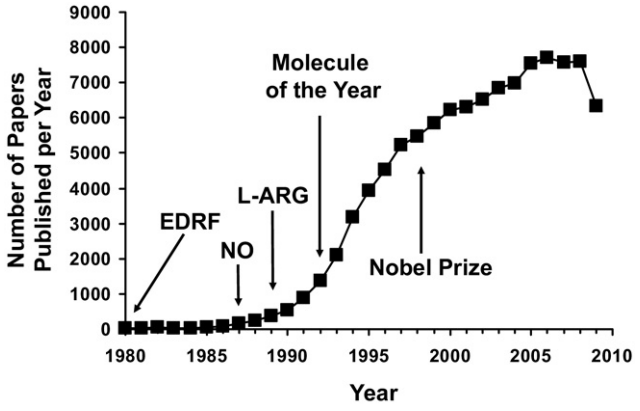


Figure 1 The number of NO-related articles published between 1980 and 2010. PubMed was searched for all papers that were indexed with the term "nitric oxide."

rotransmitter and neuromodulator in the central and peripheral nervous system.[8] In the kidney, NO has important roles in the regulation of renal function, with the net effect of promoting natriuresis and diuresis.[9] NO helps maintain the integrity of the gastric epithelium by stimulating gastric mucus production.[10] NO has also been shown to contribute to several pathological processes, including inflammatory joint disease[11] and asthma.[12]

In the remainder of this review, we will present information about the endogenous production of NO, with emphasis on the relationship between NO and cardiovascular risk. Current therapeutic agents that either provide exogenous NO or influence the actions of endogenous NO will be explored. Finally, the development of novel NO-donating drugs that have the potential to enhance the safety profile of existing agents will be presented.

Endogenous NO

NO is produced from L-arginine by the enzyme nitric oxide synthase (NOS).[13] Three major isoforms of NOS have been identified: neuronal NOS (nNOS or type 1 NOS), inducible NOS (iNOS or type 2 NOS), and endothelial NOS (eNOS or type 3 NOS).[14] Neuronal NOS is present in neurons where NO functions as a neurotransmitter and may be involved in learning and memory.[15] Inducible NOS is present in macrophages and produces NO in high concentration (μM).[14] Inflammatory joint disease is mediated in part by NO produced by iNOS.[11] As is denoted by its name, eNOS is present in vascular endothelium and produces NO in pM to nM concentrations. Endothelial NO functions as a vasodilator and inhibits thrombosis by inhibiting platelet aggregations and adhesion.[6,7] Endothelial NO activates soluble guanylate cyclase, which then converts guanosine triphosphate to cGMP.

NO and cardiovascular disease

The seminal research into the physiology and pharmacology of NO focused on its effect on the cardiovascular system and served to emphasize the importance of endothelial function in the maintenance of cardiovascular health. In addition to NO, the endothelium produces other vasoactive substances, including the arachidonic acid metabolite, prostacyclin, and endothelin, which are all involved in the regulation of vascular tone. The endothelium influences thrombosis, primarily through cell surface adhesion molecules that control interactions with circulating leukocytes, monocytes, and platelets. The endothelium is also involved in the response to vascular injury and modulates smooth muscle cell migration and proliferation seen during the formation of atherosclerotic plaques. Endothelial-produced NO influences each of these functions: NO is antithrombotic,[6,7] antiproliferative,[16] and antiatherogenic.[17,18]

Endothelial dysfunction

Perturbation of any of the principal functions of the endothelium results in a state of endothelial dysfunction and is associated with an increased risk of atherosclerosis and incidence of major cardiovascular events. The primary mechanism of endothelial dysfunction is a reduction in the bioavailability of endothelial NO. Reduced bioavailability of NO may be caused by decreased expression of eNOS,[19] a deficit in substrate or cofactors of eNOS, or an increased rate of degradation of NO by reactive oxygen species.[20] Endothelial dysfunction is now thought to be the first sign of impending cardiovascular disease and occurs in advance of clinically detectable atherosclerosis.

Endothelial function is assessed by monitoring the vascular responses to stimuli known to act via endothelial NO. For example, monitoring the changes in forearm blood flow during intra-arterial infusions of acetylcholine is one way to assess vascular endothelial function.[21] However, this test is invasive, requiring arterial catheterization. A method more commonly used is termed *flow-mediated dilation* (FMD), in which brachial artery diameter is measured by ultrasound during a period of reflow after a period of arterial occlusion with a blood pressure cuff.[22] Anderson and colleagues have shown that the noninvasive FMD method of detecting endothelial dysfunction correlates well with coronary artery endothelial dysfunction detected with the intra-arterial acetylcholine.[23]

Endothelial dysfunction and cardiovascular risk factors

Endothelial dysfunction has been shown to be associated with many of the classic cardiovascular risk factors, for example, hypertension, serum cholesterol level, and advancing age. Linder and colleagues compared the blood flow response to intra-arterial infusions of acetylcholine in 2 groups of subjects: one group with uncomplicated hyperten-

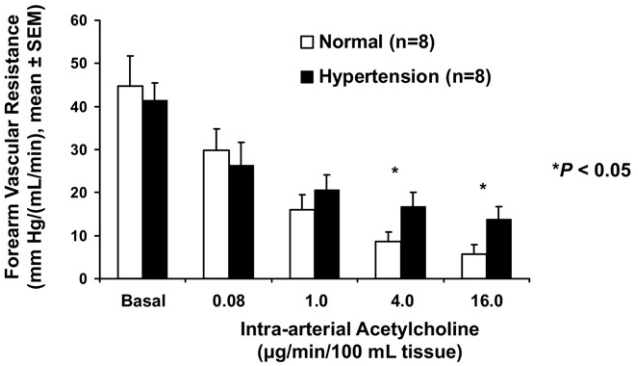


Figure 2 Changes in forearm vascular resistance during intra-arterial infusion of acetylcholine in subjects with and without hypertension. Reprinted from Linder L, Kiowski W, Buhler FR, Luscher TF: Indirect evidence for release of endothelium-derived relaxing factor in human forearm circulation in vivo. Blunted response in essential hypertension. Circulation 81:1762-1767, 1990, with permission from The American Heart Association, Inc.[21]

sion (defined as casual diastolic blood pressure >95 mm Hg) and the other group without hypertension (defined as casual diastolic blood pressure <90 mm Hg).[21] As shown in Figure 2, the hypertensive group demonstrated a blunted blood flow response (presented as change in forearm vascular resistance to account for the difference in mean arterial blood pressure in the two groups). Importantly, the vascular response to the endothelial-independent vasodilator, sodium nitroprusside, was not statistically different between the two groups, demonstrating that the blood vessels in both groups had a similar capacity to vasodilate. The relationship between hypertension and endothelial function has also been demonstrated in coronary arteries. Treasure et al used quantitative angiography to assess the epicardial coronary artery responses to intra-arterial acetylcholine in patients with and without hypertension who were undergoing diagnostic catheterization for chest pain.[24] In normal appearing coronary arteries (e.g., having a smooth appearance with no obvious atherosclerosis), vasoconstriction was observed in response to acetylcholine in patients with hypertension ($p = .0001$), whereas no significant change in mean luminal diameter was seen in normotensive patients ($p = .18$). The vasodilation response to nitroglycerin was similar in both groups. In a similar study, Zeiher and coworkers reported a highly, statistically significant, negative correlation between advancing age or total serum cholesterol and the vascular responses to intracoronary infusions of acetylcholine.[25] Endothelial dysfunction has also been shown to be associated with metabolic syndrome,[14] diabetes,[26] and obesity.[27]

Endothelial dysfunction and cardiovascular events

The presence of endothelial dysfunction is a predictor of future major cardiovascular events. Schächinger et al studied 147 consecutive patients who had undergone diagnostic coronary angiography for chest pain or for single-vessel percutaneous transluminal coronary angioplasty (PTCA).[28] The coronary vascular responses of normal appearing coronary arteries to intra-arterial acetylcholine, papaverine, adenosine, or nitroglycerin, or to sympathetic activation by cold pressor testing was assessed by quantitative coronary angiography. Over a median 7.7-year follow-up period, the patients or their families were queried annually about the incidence of major cardiovascular events, including cardiovascular death, unstable angina pectoris, myocardial infarction, PTCA, coronary artery bypass grafting, ischemic stroke, or revascularization of peripheral arteries. During the follow-up period, 16 patients had at least one major cardiovascular event. As shown in Figure 3, patients who had an event had abnormal vascular responses consistent with endothelial dysfunction during the baseline evaluation. For example, patients who had an event had significantly greater vasoconstrictor response to intra-arterial acetylcholine and a decreased flow-mediated vasodilator response at baseline. Kaplan-Meier analysis according to the occurrence of vasodilation (34% of patients) or vasoconstriction (66% of patients) during acetylcholine testing clearly showed that the incidence of cardiovascular events was higher in patients who demonstrated vasoconstriction compared with patients who demonstrated vasodilation ($p = .022$). An individual example is shown in Figure 4. The left anterior descending artery (LAD) appeared normal during the baseline evaluation (Fig. 4A) but demonstrated vasoconstriction in response to acetylcholine infusion (Fig. 4B). Nitroglycerin elicited minimal vasodilation in the segment of interest, thereby revealing a developing atherosclerotic lesion (Fig. 4C). After 3.7 years of follow-up, the patient was admitted to the hospital with acute coronary syndrome, and an angiogram revealed the presence of a mature atherosclerotic lesion in the same segment of the LAD that had demonstrated endothelial dysfunction at the time of the initial study (Fig. 4D).

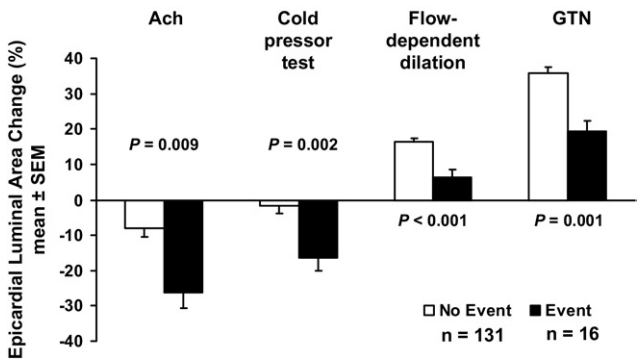


Figure 3 Baseline coronary vascular responses to vasoreactivity tests in patients with and without major cardiovascular events during long-term follow-up.[a] *Ach*, intra-arterial acetylcholine; *GTN*, glyceroltrinitrate or nitroglycerin. [a]Flow-dependent dilations were assessed during intra-arterial infusion of papaverine or adenosine downstream from the artery segment of interest. Reprinted from Schächinger V, Britten MB, Zeiher AM: Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. Circulation 101:1899-1906, 2000, with permission from The American Heart Association, Inc.[28]

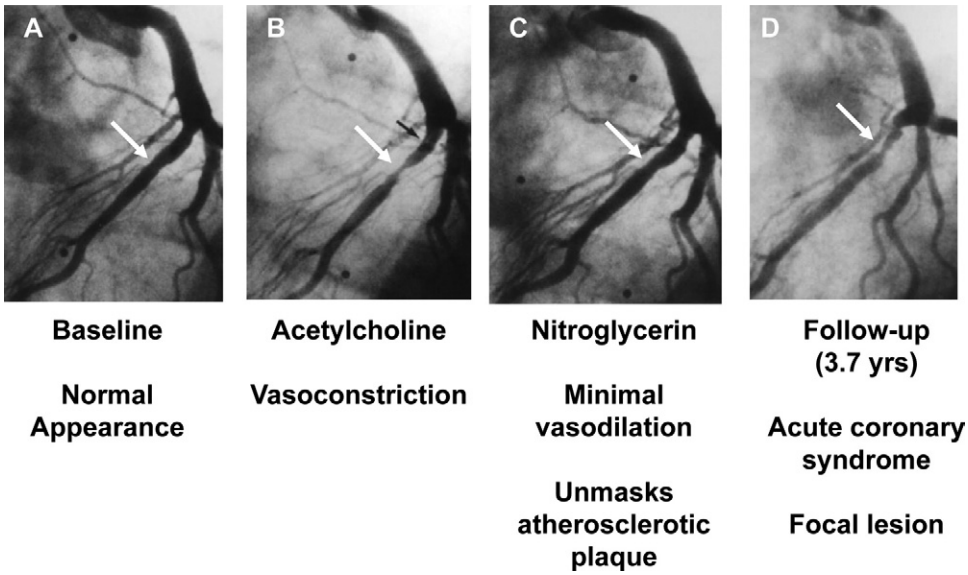


Figure 4 Coronary artery vasoreactivity and the progression of atherosclerosis in a single patient. Panels A, B, and C are angiograms of the LAD taken at baseline before vasoreactivity testing (**A**), during acetylcholine infusion (**B**), and during nitroglycerin infusion (**C**). Panel D is an angiogram performed after 3.7 years of follow-up. The white arrows indicate the same segment of the LAD in each angiogram. The black arrow in Panel B shows the location of the tip of the acetylcholine infusion catheter. Reprinted from Schächinger V, Britten MB, Zeiher AM: Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. Circulation 101:1899-1906, 2000, with permission from The American Heart Association, Inc.[28]

Systemic endothelial dysfunction is also predictive of future cardiovascular events. Brachial artery FMD was determined in a prospective study of 435 healthy subjects (65% men, mean age = 54 years).[29] During the mean 32-month follow-up period, major cardiovascular events occurred more frequently in the group with FMD below the median values of 10.7% than in the group with FMD above the median (11.8% vs 4.7%, respectively, $p = .007$). Importantly, after multivariate analysis that included conventional coronary artery disease risk factors, median FMD was the best independent predictor of long-term risk of major cardiovascular events (odds ratio = 2.70, 95% confidence interval = 1.16 to 6.32, $p = .011$).

NO—therapeutic interventions

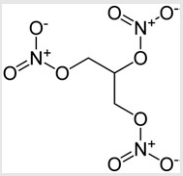
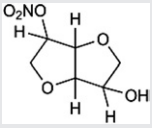
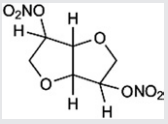
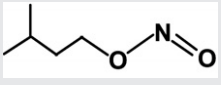
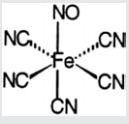
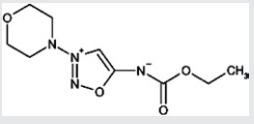
The fact that a relative deficit of endothelial NO resulting in endothelial dysfunction is associated with many of the classic cardiovascular risk factors would suggest that therapy directed at enhancing the availability of NO might be beneficial in treating or preventing cardiovascular diseases.[30,31] Indeed, drugs that provide or "donate" NO have been used for many years to treat or prevent angina pectoris pain or to manage hypertensive crises.

NO-donating drugs

NO-donating drugs that are currently marketed in the United States are presented in Table 1. The primary uses of these drugs are to prevent or treat angina pectoris and to manage hypertensive crises. Two of these agents, nitroglycerin and amyl nitrite, represent some of the oldest drugs currently in use. Both amyl nitrite and nitroglycerin were introduced into clinical practice for the treatment or prevention of angina pectoris in the 19th century. Amyl nitrite was initially put forward as a treatment for angina in 1867 by the English physician T. Lauder Brunton after self-experimentation and clinical observations.[32] He reported a rapid relief of pain in patients upon inhalation of amyl nitrite and postulated that this effect was caused by a reduction in blood pressure. Similarly, William Murrell studied the effects of nitroglycerin on himself and volunteers and reported on what may be the first placebo-controlled clinical study.[33-36] He treated four patients with angina pectoris for one week with a substance that had no effect (the placebo) followed by oral doses of a 1% solution of nitroglycerin in ethanol three or four times per day starting with a dose of a single drop. All patients reported a reduction in severity or elimination of anginal pain, and two patients reported that taking their dose during an attack would cut it short. Tolerance to nitroglycerin, as evidenced by the requirement for an increased dose, was also reported in this study. Despite its clinical utility, it would take another 100 years to elucidate the biochemical mechanism of the effect of nitroglycerin.

Nitroglycerin exerts its anti-anginal action via its vasodilator properties, which are a result of the enzymatic release of NO.[37] In vascular smooth muscle, mitochondrial aldehyde dehydrogenase converts nitroglycerin to 1,2-glyceryl dinitrate and a thionitrite intermediate that releases NO_2, which is quickly reduced to NO. Tolerance to the

Table 1 NO-donating drugs marketed in the United States

Drug	Structure	Indication	Formulation	Adverse Events
Nitroglycerine		Acute angina Prevention of angina	Buccal spray Buccal tablets Transdermal patch Topical ointment Intravenous	Hypotension Headache Tolerance
Isosorbide mononitrate		Acute angina Prevention of angina	Buccal tablets Oral tablets	Headache Dizziness Palpitation Weakness Tolerance
Isosorbide dinitrate		Acute angina Prevention of angina	Buccal tablets Oral capsule	Headache Dizziness Palpitation Weakness Tolerance
Amyl nitrite		Acute angina	Inhalation	Headache Dizziness Palpitation Weakness
Sodium nitroprusside		Treatment of hypertensive crises	Intravenous	Excessive hypotension Cyanide toxicity Methemoglobinemia
Molsidomine		Prevention and treatment of angina	Oral tablets	Headache Dizziness Palpitation Weakness

effects of nitroglycerin and the other organic nitrates develops quickly with continuous use. Although thiol depletion is thought to be involved in the development of tolerance, a full understanding of the biochemical mechanisms responsible for tolerance has yet to be elucidated. Intermittent use of the organic nitrates is indicated to minimize the development of tolerance.

Drugs that enhance the actions of NO

NO is an important player in the normal physiology of penile erection. Neuronally-derived NO activates soluble guanylate cyclase, resulting in a local increase in cGMP, which in turn relaxes smooth muscle necessary for blood flow and engorgement of the penis.[38] The activity of cGMP is normally terminated by its hydrolysis to 5′-GMP via the enzyme phosphodiesterase type 5 (PDE5). The erectile dysfunction drugs sildenafil, tadalafil, and vardenafil are inhibitors of PDE5 and act to prolong the effect of cGMP.[39] Thus, the effectiveness of this class of drugs is not based on a direct effect on NO or its synthesis, but rather on the ability to inhibit the breakdown of the second messenger responsible for the smooth muscle relaxation. Because of the potential to also inhibit the metabolism of systemic endothelial cGMP, patients are cautioned to not use PDE5 inhibitors if they are also being treated with organic nitrate vasodilators.

Drugs that stimulate the production of endogenous NO

Nebivolol is a third-generation beta-blocker that is as effective as carvedilol in the treatment of heart failure[40] and is the only beta-blocker that has demonstrated efficacy in elderly patients with heart failure.[41,42] Nebivolol has vasodilator properties that are the result of its ability to stimulate the production of vascular NO.[43] This activity appears to be mediated through the beta-3 receptor.[44] In a crossover study of 12 hypertensive patients, nebivolol reversed endothelial

dysfunction compared with both placebo or atenolol.[45] This action may provide additional cardiovascular protection in addition to that expected by lowering blood pressure.

Novel approaches to providing exogenous NO

A new class of NO-donating drugs is being developed to address safety and tolerability aspects of traditional nonsteroidal antiinflammatory drugs (NSAIDs).[46] These NSAIDs (e.g., naproxen, ibuprofen, indomethacin) are widely used in the treatment of pain of osteoarthritis, but gastrointestinal side effects limit their tolerability, and increases in blood pressure raise safety concerns.[47] These issues have led to the development of a novel class of agents that are designed to combine the efficacy the antiinflammatory and analgesic efficacy of traditional NSAIDs with the potential to improve the safety and tolerability by adding an NO-donating moiety.[48] These agents are called *cyclooxygenase inhibiting nitric oxide donators* (CINODs).

Naproxcinod (24-[nitrooxy]butyl-[2*S*]-2-[6-methoxy-2-naphtyl]propanote) is the first example of the CINOD class and is in the late stages of Phase III clinical testing. Naproxcinod combines the traditional NSAID naproxen with an NO-donating moiety in a single molecule. After oral dosing, the molecule is hydrolyzed in the liver, releasing naproxen and the NO-donating moiety.[49] NO is released enzymatically after tissue absorption of the NO-donating moiety.

Naproxcinod was originally developed to address gastrointestinal safety, with the hypothesis that the exogenous NO would be gastroprotective. In a six-week, double-blind clinical trial comparing placebo, naproxen (500 mg twice daily), and naproxcinod (750 mg twice daily, a dose that provides equimolar dose of naproxen) in patients with osteoarthritis of the knee or hip, naproxcinod was similar in efficacy with naproxen for reducing osteoarthritis pain and tended to reduce the incidence of endoscopically-detected gastroduodenal ulcers (the primary endpoint) compared with naproxen (9.7% vs 13.7%, $p = .07$).[50] Naproxcinod was also associated with significantly reduced mucosal injury as judged by most of the prespecified secondary endpoints, including the percent incidence of significant gastroduodenal damage as assessed by Lanza scores 3 and 4[51] (naproxcinod, 32.2%; naproxen, 43.7%; $p < .05$).[50]

The NO-donating moiety of naproxcinod appears to reduce the hypertensive liability of the parent compound, naproxen. In a 13-week, double-blind, placebo-controlled clinical trial of patients with osteoarthritis of the knee, naproxcinod (375 or 750 mg twice daily) was compared with naproxen (500 mg twice daily) or placebo.[52] Both doses of naproxcinod were statistically superior to placebo in their effect on pain ($p < .0001$), and both doses of naproxcinod were found to be noninferior to naproxen on the reduction in pain.[52] However, analyses of blood pressure during the study revealed important differences among the treatment groups. All groups demonstrated a mean decrease in systolic blood pressure during the study, with the smallest decrease at week 13 seen in the naproxen 500-mg group (–1.0 mm Hg) and the largest seen in the naproxcinod 750-mg group (–3.9 mm Hg, $P = .015$ compared with naproxen 500 mg [Table 2]). The difference in the blood pressure responses between these two groups was even larger in the subgroup of patients who were treated with renin-angiotensin blocker drugs during the study, a finding that is consistent with the notion that these drugs depend on vascular NO and/or prostacyclin for some of their efficacy.[53]

Table 2 Effect of naproxen, naproxcinod, and placebo on systolic blood pressure in patients with osteoarthritis of the knee after 13 weeks of treatment

	All patients		Patients taking renin-angiotensin blockers	
	n	Change in SBP	n	Change in SBP
Placebo	222	−3.1	54	−2.7
Naproxen 500 mg	225	−1.0	44	+1.6*
Naproxcinod 750 mg	229	−3.9†	53	−4.9‡
Naproxcinod 375 mg	240	−2.9	56	−4.6

Based on White et al.[59]
*$p = .24$ compared with placebo.
†$p = .015$ compared with naproxen 500 mg.
‡$p = .011$ compared with naproxen 500 mg.

Meta-analyses from the 1990s showed traditional NSAIDs increased blood pressure, especially in patients with hypertension.[54,55] Although this effect appears modest, it has been well established that small increases in blood pressure are associated with an increased risk of major cardiovascular events.[56,57] For example, one estimate of the effect of a 5-mm Hg difference in systolic blood pressure suggests that risk of stroke or coronary heart disease would be 7% higher in the group with higher systolic blood pressure.[58] Thus the difference in blood pressure response between naproxen and naproxcinod would be expected to reduce the risk of cardiovascular events in patients with osteoarthritis who were treated with naproxcinod rather than naproxen or other traditional NSAIDs.[47]

Conclusions

Our understanding of the roles of endogenous NO has greatly expanded since its discovery and identification. Because a deficit in the activity of NO is associated with pathologic effects in the cardiovascular, renal, and gastrointestinal systems, methods of enhancing the activity of endogenous NO or of providing exogenous NO to vulnerable tissues have been proposed. Indeed some of the earliest useful therapeutic agents, the organic nitrates, exert their benefit by delivery of NO. A new approach in which an NO-donating moiety is linked to an existing drug may

provide a mechanism for using exogenous NO to modify the safety profile of existing pharmaceutical products.

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