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Serial Review: Mechanisms and Novel Directions in the Biological Applications of Nitric Oxide Donors
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NITRATES AND NO RELEASE: CONTEMPORARY ASPECTS IN BIOLOGICAL AND MEDICINAL CHEMISTRY

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Abstract—Nitroglycerine has been used clinically in the treatment of angina for 130 years, yet important details on the mechanism of action, biotransformation, and the associated phenomenon of nitrate tolerance remain unanswered. The biological activity of organic nitrates can be said to be nitric oxide mimetic, leading to recent, exciting progress in realizing the therapeutic potential of nitrates. Unequivocally, nitroglycerine and most other organic nitrates, including NO-NSAIDs, do not behave as NO donors in the most fundamental action: in vitro activation of sGC to produce cGMP. The question as to whether the biological activity of nitrates results primarily or exclusively from NO donation will not be satisfactorily answered until the location, the apparatus, and the mechanism of reduction of nitrates to NO are defined. Similarly, the therapeutic potential of nitrates will not be unlocked until this knowledge is attained. Aspects of the therapeutic and biological activity of nitrates are reviewed in the context of the chemistry of nitrates and the elusive efficient $3e^-$ reduction required to generate NO.

Keywords—Nitrate, Nitric oxide, Biotransformation, cGMP, Signaling, Nitration, Nitrosation, Free radicals

Contents

This article is part of a series of reviews on "Mechanisms and Novel Directions in the Biological Applications of Nitric Oxide Donors." The full list of papers may be found on the home page of the journal.

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INTRODUCTION

The organic nitrate nitroglycerine is well into its second century of use both as a medicinal agent and as an agent of destruction and suffering. In popular culture nitroglycerine is used as a simile for anything that is volatile and explosive under the merest provocation. A search of the Web will generate equal mentions of the use of nitroglycerine in terrorist and sniper attacks and in new therapeutic applications from spider bites, to anal fissures, to restless leg syndrome. Despite its deep history, knowledge of the biological chemistry of nitroglycerine and of nitrates in general is wholly incomplete.

The synthesis of nitroglycerine was first reported in 1846, and 20 years later, the taming of nitroglycerine in the form of dynamite and gun glycerine was the basis of Alfred Nobel's fortune. Nobel's contemporaries, Thomas Brunton, who was studying the effects of organic nitrites in angina pectoris, and William Murrell, who demonstrated that small doses of nitroglycerine taken sublingually provided rapid and remarkable relief from the intense pain of angina, are generally credited with the development of nitroglycerine as a therapeutic agent [1,2]. Nitroglycerine was renamed glyceryl trinitrate (GTN), to avoid the anxiety associated with ingesting a high explosive, and has been used continuously in treatment of angina since 1878. GTN has also been applied in controlled hypotension during cardiac surgery and in congestive heart failure. Decreased cardiac preload due to the selective venodilator response to organic nitrate vasodilators is the basis for their clinical use in treatment of angina; these, and other simple nitrates that have been used only as biological probes, we may class as "classical nitrates" (Fig. 1).

Sinitrodil, FR 46171, and nicorandil represent nitrates containing functionalities, other than simple hydrocarbon or sugar skeletons, which influence biological activity (Fig. 1). Sinitrodil is proposed as an agent with enhanced antianginal and attenuated hypotensive activity relative to classical nitrates due to reduced dilation of the smaller coronary and resistance vessels and the resultant effects on mean arterial blood pressure and heart rate [3,4]. Nicorandil is a nitrovasodilator that is also a K_{ATP} channel opener, which is in preclinical and clinical studies for vascular diseases, including myocardial infarction [5,6]. Nitrate esters have a significant clinical attribute in that a nitrate is, inherently, considerably more lipophilic than its parent alcohol, allowing delivery by routes including sublingual or transdermal, as used in slow-release GTN patches. Oral administration of GTN is ineffective because of rapid first-pass metabolism, but the lone criticism of GTN as a therapeutic agent is the onset of clinical nitrate tolerance during chronic administration. The physiological perturbation that causes tolerance is not known, but most theories have a central role for attenuated nitrate bioactivation (also termed *mechanism-based biotransformation*). The efficient biotransformation of nitrates is also central to the therapeutic activity of nitrate drugs, because it is almost universally assumed that nitrates are in fact NO prodrugs, although the details of this mechanism-based biotransformation of the nitrate moiety ($RONO_2$) to NO remain uncertain.

The pharmaceutical development of nitrates containing adjunct pharmacophores is not a new pursuit. For example, steroid nitrates had been reported over 40 years ago and observed to manifest biological properties beyond those of the parent steroid [7,8]. However, there has been an explosion of activity in the area of hybrid nitrates over the past decade stimulated by the actions of Del Soldato and NicOx SA (France) in developing NO-NSAIDs (NO-donating nonsteroidal anti-inflammatory drugs), sometimes known as CINODs (cyclooxygenase inhibitory NO donors), and in promoting the scientific study of these compounds (Fig. 2) [9–12]. Interest in the further use of nitrates in new cardiovascular applications is increasing, but there is a growing realization that nitrates may represent new therapeutic agents in non-traditional areas, for example, in neuroprotection and neuropathic pain [13–17]. This represents a paradigm shift that has been inspired by discoveries in NO biology and pharmacology, examples being the application of nitrates in dementia [13], the association of nitrates and

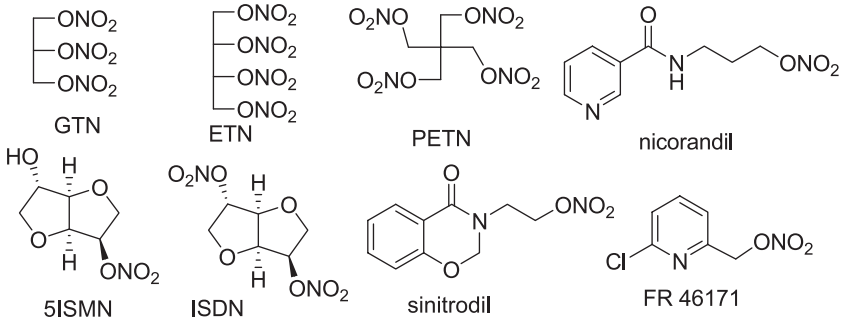


Fig. 1. Nitrate vasodilators, including classical nitrates: erithrityl tetranitrate (ETN), glyceryl trinitrate (GTN), pentaerithrityl tetranitrate (PETN), isosorbide dinitrate (ISDN), isosorbide-5-mononitrate (5ISMN).

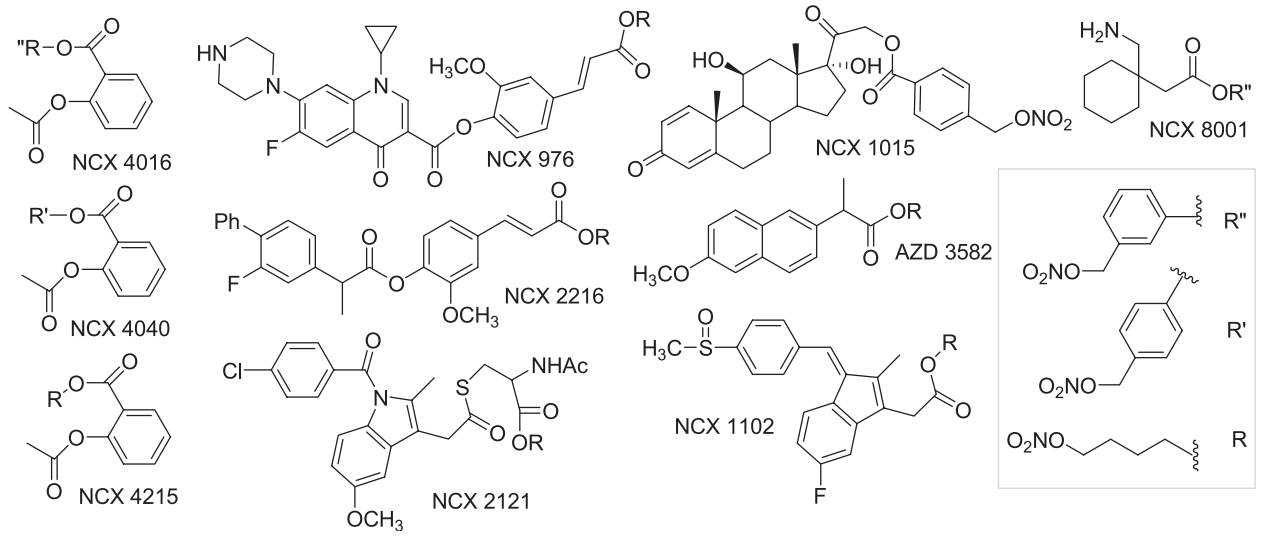


Fig. 2. Selected NO-NSAIDs and hybrid nitrates. These nitrates are all described as NO-donor drugs and are in preclinical or clinical trials for a range of indications; NO-naproxen, AZD 3582, has completed Phase 2 clinical trials.

NO with regulation of glutamate receptors [18–20], and the proposal of hybrid nitrate drugs containing the noncompetitive, open-channel NMDA receptor antagonist memantine [21]. There are two observations that are common to many of the published studies on NO-NSAIDs and hybrid nitrates: (i) the biological activity of the nitrate is greater than that of the parent and (ii) the biological activity observed is not seen for the parent pharmacophore [22]. A further common observation is that neither the chemical mechanism nor the biological apparatus for release of NO is understood.

Nitrate esters or organic nitrates contain the nitrooxy functional group (—ONO_2), almost all examples being aliphatic nitrates, owing to the presumed instability of the aromatic nitrate to rearrangement [23]. Contrary to some descriptions, nitrates are not nitroso compounds. This is not a point of semantics, because nitroso compounds, including nitrosothiols (RS−N═O), nitrosamines (RR′N−N═O), and nitrosoalkanes (RR′R′C−N═O) require only one electron reduction to yield NO. Conversion of the nitrooxy group of nitrates to NO is a three-electron reduction that must involve oxygen atom transfer. To date, no purified protein system has been demonstrated to mediate the direct reduction of nitrates to yield relevant quantities of NO, although it is widely held that the biologically active product of mechanism-based biotransformation of nitrates is NO [24,25]. The present article reviews the potential chemical mechanisms and the biochemical pathways associated with nitrate biotransformation. There is a vast literature on the biological activity of various nitrates, in contrast to a relative paucity of studies on the biological chemistry. Endogenous nitrates may be formed in vivo, for example in lipid peroxidation radical chain termination by nitric oxide [26–28], but nitrates are first and foremost exogenous therapeutic agents and it is the biological chemistry of these compounds that is of primary interest.

NITRATES AS THERAPEUTIC AGENTS

Physiologically, NO is produced enzymatically from the terminal guanidino nitrogen of L-arginine, by nitric oxide synthase (NOS). Endothelial NOS (eNOS) releases NO, which causes inhibition of platelet aggregation and vasodilation via activation of soluble guanylyl cyclase (sGC) in underlying vascular smooth muscle cells. Inducible NOS is found in many tissues and, in macrophages, when induced, produces NO as part of the body's immune response. NO from neuronal NOS is involved in neurotransmission in the central nervous system. These are only some of the multitude of biological roles for NO, which have been amply reviewed elsewhere [29–36]. Because nitrates seem in many respects to act as exogenous NO sources, this would suggest a large number of potential therapeutic applications. Circumventing nitrate tolerance in current cardiovascular therapies would be beneficial, but the newer applications of nitrates outside of angina and cardiovascular indications hold the most exciting promise. A review of the novel nitrate therapies under development is beyond the scope of this article, but it is worthwhile examining the biotransformation of nitrate therapeutics, because if nitrates are indeed NO-donor drugs, these metabolic pathways must involve the chemical generation of the NO free radical.

In addition to GTN, several classical nitrates, and nicorandil, the most extensive published data on nitrate metabolism is for the NO-NSAIDs and related hybrid

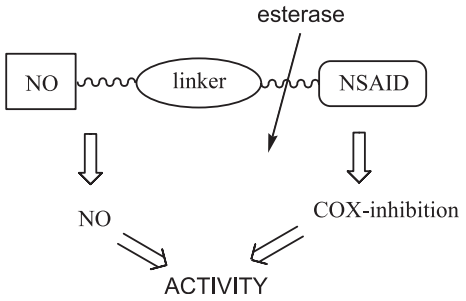


Fig. 3. General depiction of the mode of NO release from NO-NSAIDs and other hybrid nitrates.

nitrates (Fig. 2). The latter two classes of agents are explicitly described in the literature as NO-donor drugs, but it must be emphasized that these compounds do not merely "resemble organic nitrates," they *are* nitrates and will manifest nitrate chemistry. NO-NSAIDs and related hybrid nitrates are proprodrugs, containing an ester moiety that is cleaved by nonspecific esterase action: the general depiction of such NO-donor drugs (using an NO-NSAID as an example in Fig. 3) is of an NO moiety (more accurately a nitrate moiety) connected via a linker to the parent pharmacophore. It is reported that the activity of NO-NSAIDs and hybrid nitrates in vitro is influenced by addition of esterases, for example, NCX 1015 at high micromolar concentrations (100–500 μM) was reported to release increased amounts of NO_2^- on addition of esterase [38]. Unfortunately, the frequent reporting of the Griess test for NO_2^- as a measure of NO release obfuscates the analysis and understanding of the mechanisms of action and of biotransformation, and this, in part, has led to some confusion in the literature as to the metabolic pathway that leads to NO release from these nitrates, with several claims that NO release simply requires the action of an esterase (e.g. [37]).

The action of an esterase or nonenzymatic hydrolysis in the gut does not release NO, but generates a simple aliphatic nitrate, in the case of NCX 4016, hydroxybenzyl nitrate (HBN), or, in the cases of NCX 4215 and AZD 3582, hydroxybutyl nitrate (Fig. 4). Therefore, focusing on the NO-ASA NCX 4016, one must query why a combination therapeutic of ASA and a benzyl nitrate does not provide similar benefits and even a clinical advantage in the ability to titrate the NO and NSAID effects independently (Fig. 4). In reviewing NO-NSAIDs in colon cancer chemoprevention, Rigas poses the same question of the effectiveness of classical nitrates in combination with NSAIDs in such indications [39,40].

Ester hydrolysis of the NO-NSAID yields a primary aliphatic nitrate which may undergo subsequent reductive denitration to inorganic nitrite (NO_2^-) or nucleophilic substitution to generate inorganic nitrate (NO_3^-) (Fig. 4). There is ample evidence that a number of

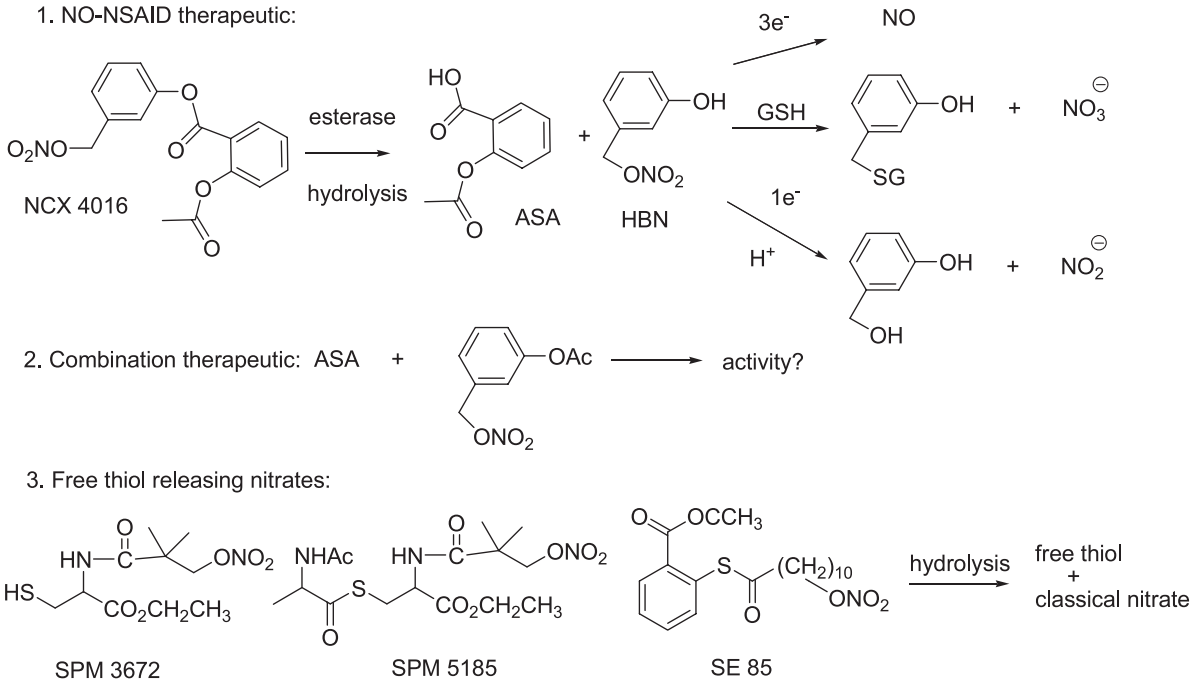


Fig. 4. (1) Biotransformation of an NO-NSAID therapeutic leading to eventual formation of inorganic nitrate, inorganic nitrite, and NO. A large number of enzymes, including cytochrome P450 oxidase, cytochrome P450 reductase, glutathione *S*-transferase, and aldehyde dehydrogenase, have been demonstrated to mediate the reductive denitration of nitrates to NO_2^-. Nucleophiles such as glutathione (GSH) may displace inorganic nitrate via S_N2 substitution. This analysis suggests that (2) a combination therapy might have similar effectiveness. (3) Hybrid nitrates, similarly, are designed to hydrolytically yield free thiol and simple aliphatic nitrate.

diverse enzymes may catalyze the $2e^-$ reduction of nitrates to NO_2^- (vide infra), whereas NO_3^- is a good leaving group for nucleophilic substitution. In contrast, there is no unambiguous evidence for any enzyme catalyzing the direct $3e^-$ reduction of nitrates to NO. There is little evidence for significant biological activity associated with NO_3^-, the levels of which in human plasma and rat aorta are relatively high (~25 and ~50 μM) and fluctuate substantially with diet [41–43]. However, there is renewed interest in the biological activity of NO_2^-, which has been shown to be oxidized by peroxidase to NO_2 [44,45] and to be reduced by ferrous hemoglobin (Hb) to yield NO-Hb or NO [43]. Physiological concentrations of NO_2^- have been reported to range from 0.5 to 25 μM in plasma and tissue and may be higher under pathophysiological conditions [46,47]. The direct addition of NO_2^- has failed to elicit a biological response in several systems [41,48,49]; however, compartmentalization and active transport, combined with the demonstrated reactivity of NO_2^- with transition metal centers, leaves open a biological role for NO_2^-, via its oxidation or its reduction products [43,50–52]. Thus, it is possible that nitrates act, not as NO donors, but as NO_2^--donor drugs, providing intracellular delivery of NO_2^- to specific locations.

The metabolism of the NO-NSAID NCX 4016, has been studied in detail [53–57]. In rats, the intact nitrate, NCX 4016, was not detected in plasma after oral administration, and in rat liver microsomal S9 fractions, metabolism was complete within 5 min. The sole observable nitrate in rat liver fractions was HBN and the glutathione adduct shown in Fig. 4. Compatible with these observations, after administration in rats, plasma NO_2^- levels were unchanged (~1 μM), whereas plasma NO_3^- was elevated from ~20 to ~130 μM over 6 h. Reports of detection of NO itself as a product of in vivo administration of NO-NSAIDs are limited: NO-Hb was observed in rat blood and NO-Mb (myoglobin) in myocardial tissue in vivo and in vitro after administration of NCX 4016, using the sensitive ESR spectroscopic detection technique. Using this method, ISMN and NCX 4016 (1 mM) generated similar levels of NO-Hb (~30 μM at 1 h) in rat blood. Because ferrous-Hb is known to react directly with nitrates, nitrites, and NO_2^- to generate NO-Hb, it is difficult conclude from these experiments that the NO-NSAID nitrates are directly reductively biotransformed to NO in vivo. Thus, the best studied NO-NSAID, NCX 4016, is claimed to be largely absorbed in the small intestine, subject to substantial first-pass metabolism, and to possess little or no systemic bioavailability. Exciting data on the CNS activity of the NO-flurbiprofen NO-NSAIDs have been assembled [15,58], and like NCX 4016, the nitrooxybutyloxy ester of flurbiprofen undergoes rapid, extensive first-pass metabolism in rats, such that: (i) only flurbiprofen could be detected in brain and plasma in significant amounts and (ii) levels of (NO_2^- + NO_3^-) increased in plasma over 8 h, but were unchanged in brain [59]. The hydroxybutyl nitrate metabolite was not quantified in this study.

The wealth of exciting biological data being generated on NO-NSAIDs and on hybrid nitrates demonstrates enhanced and additional biological activity over the parent pharmacophores, which would be very difficult to explain simply by enhanced bioavailability deriving from either the increased lipophilicity of the nitrate derivative or the increased local blood flow due to nitrate vasodilation. The published data show that NO-NSAIDs are rapidly metabolized to simple aliphatic nitrates, and in the case of NCX 4016, the major NO_x metabolite is NO_3^-. Classical nitrates are themselves simple aliphatic nitrates: GTN is rapidly metabolized to yield further simple aliphatic nitrate metabolites and NO_2^-. Therefore, metabolism studies confirm that nitrates, whether classical or modernist, are efficiently metabolized to NO_2^- and NO_3^-.

In contemporary nitrate therapeutics, hybrid nitrates in which the nitrooxy group is ancillary to a primary pharmacophore have generated the majority of reports; however, Schwarz Pharma scientists were the first to describe hybrid nitrates that contain a cysteine moiety, designed to be metabolized to free cysteine and a free, simple aliphatic nitrate (Fig. 4) [60,61]. This design is based upon the postulates either that free thiol is needed for biotransformation of nitrates or that thiol overcomes nitrate tolerance. A natural extension of this design is a hybrid nitrate which will release a free thiol other than cysteine, for example thiosalicylic acid (TSA), which like cysteine is an “active thiol” toward nitrates (vide infra) (Fig. 4) [62,63].

Hybrid nitrates are uniformly designed to metabolically degrade to yield a simple aliphatic nitrate (and thence NO), and observations show that much of the therapeutically beneficial biological activity derives from the aliphatic nitrate moiety of such drugs. It is therefore reasonable to explore the structure–activity relationships of aliphatic nitrates themselves toward NO mimetic biological activity and toward metabolism or degradation to NO, by design of novel nitrates through incorporation of other functional groups into the organic nitrate [26,64,65]. These nitrates are predicted to show a different spectrum of NO mimetic activity to simple or classical nitrates, which has been borne out in literature reports. The disulfanyl *S*-nitrate GT 715 has been reported: (a) to reduce the cerebral infarct in the rat middle cerebral artery occlusion model of ischemic stroke by 60–70% when delivered 4 h after the onset of ischemia [66] and (b) to reverse the scopolamine-induced cognitive deficit in the Morris water task rat

behavioral model of dementia [13]. Interestingly, these studies showed that the *S*-nitrate was more effective and relatively more potent than GTN in activating hippocampal sGC and demonstrated greatly attenuated peripheral vasodilatory responses compared to GTN. A member of the *S*-nitrate family is currently in clinical trials for Alzheimer's disease.

NITRATE BIOTRANSFORMATION AND TOLERANCE

Mechanism-based biotransformation of nitrates has been defined as the pathway generating a proximal activator of sGC in vascular smooth muscle cells, which is presumed to be NO, whereas the term clearance-based biotransformation has been coined to describe pathways that lead to nitrate metabolism *without* activation of sGC, often presumed to be via NO_2^- [67,68]. The biotransformation of GTN yields the dinitrate metabolites glyceryl-1,2-dinitrate (1,2-GDN) and glyceryl-1,3-dinitrate (1,3-GDN) as products. The vascular biotransformation of GTN is regioselective for formation of 1,2-GDN [69–74], and it is this isomer that is formed exclusively during the initial exposure of tissues to GTN [75]. In tolerant cultured cells and tissues, the regioselectivity is lost and GTN biotransformation is attenuated [76–79]. Several proteins have been identified that are capable of mediating the denitration of GTN, yielding GDN and NO_2^- as products, including Hb, Mb, xanthine oxidoreductase (XOR), old yellow enzyme, glutathione *S*-transferase (GST), cytochrome P450 oxidase (CYP), and cytochrome P450 reductase [73,74,78,80–87]. More recently, it was confirmed that ALDH2 was capable of mediating denitration of classical nitrates [88–90].

Long-term administration of nitrates is characterized by a decrease in their vasodilator and antianginal effectiveness, which is termed nitrate tolerance. Clinical tolerance can be simulated in animal models by continuous delivery of GTN, but not by repetitive bolus administration [91]. There have been proposed two major hypotheses for nitrate tolerance: blood vessel-dependent and vessel-independent mechanisms. The blood vessel-dependent hypothesis accounts for the inability of vascular smooth muscle cells to adequately respond to nitrates, whereas the vessel-independent hypothesis accounts for the activation of neurohormonal counter-regulatory mechanisms (e.g., intravascular volume expansion, increase in catecholamine levels, and activation of the renin–angiotensin system) [92]. A major criticism of the involvement of neurohormonal mechanisms is that tolerance can be induced in isolated vessels in which the presence of circulating vasoconstrictor hormones is excluded, although the possibility exists that in vitro tolerance models do not reflect clinical nitrate tolerance [93].

Several blood vessel-dependent tolerance mechanisms have been suggested: (i) attenuation of nitrate bioactivation [67,68,94,95], (ii) generation of free radicals during nitrate biotransformation [69,92,95–97], (iii) upregulation of phosphodiesterase activity with the subsequent reduction in intracellular levels of cGMP [98], (iv) loss of sGC responsiveness to NO [99–101], and (v) intracellular depletion of thiol equivalents [102,103]. The observation that spontaneous NO donors (e.g., nitroprusside, nitrosothiols, and diazeniumdiolates) are not subject to tolerance or to cross-tolerance toward nitrates has been argued to support the attenuated mechanism-based biotransformation of nitrates in tolerant vessels as the mechanism underlying tolerance. The thiol depletion hypothesis of Needleman and Johnson proposed that tolerance might result from depletion of critical thiols which are involved in the metabolism of nitrates [102]. However, a causal role in clinical nitrate tolerance due to reduced bioavailability of sulfhydryl groups was not confirmed by more recent investigations [104,105].

A proposed free radical hypothesis of nitrate tolerance is based on the observation that administration of GTN increases the endothelial production of $O_2^{\bullet-}$, although this observation does not seem to hold for nitrates in general [72,106]. The elevation of $O_2^{\bullet-}$ can limit the bioavailability of both endogenous and exogenous NO through the near diffusion-controlled reaction of $O_2^{\bullet-}$ with NO yielding peroxynitrite ($ONOO^-$), which has been reported to be formed during the uninterrupted administration of nitrates in both animals and humans [95,106,107]. Peroxynitrite is an oxidizing and nitrating cytotoxin that has been assigned roles in nitrate tolerance through interference with the normal function of NOS and cGMP-dependent protein kinase I [94,95].

The loss of regioselectivity for GTN denitration in tolerant tissue is dramatic and might be taken to suggest that the mechanism-based biotransformation pathway is regioselective. However, it is quite reasonable to expect clearance-based metabolism also to show regioselectivity and for both pathways to be attenuated in tolerant tissue. For example, the selective flavoprotein inhibitor diphenyleneiodonium sulfate (DPI) was shown to attenuate the regioselectivity for formation of 1,2-GDN, to inhibit cGMP accumulation in blood vessels, and not to inhibit tissue relaxation induced by spontaneous NO donors, but DPI inhibited GTN-induced relaxation to the same extent in aortae from naïve and from GTN-tolerant animals [71,78]. These observations force the conclusion that flavoproteins are important for nitrate metabolism and may participate in biotransformation leading to vasodilation, but their alteration cannot be the basis for nitrate tolerance. Regioselectivity is not therefore, by itself, a

sufficient criterion for identification of mechanism-based biotransformation. ALDH2 activity in hepatic mitochondria also shows significant regioselectivity toward 1,2-GDN formation from GTN, which is lost in mitochondria from tolerant tissue [88,108]. Owing to the perceived importance of ALDH2 in GTN metabolism, the data on ALDH2 need further discussion [109].

In rabbit aorta in which in vitro tolerance was induced by soaking of tissues in high GTN concentration, ALDH activity was reduced, and in addition, compounds that inhibit ALDH activity (cyanamide, aldehydes and their hydrates, at 1 mM) inhibited GTN biotransformation, relaxation, and GTN-induced cGMP accumulation in isolated blood vessels [88]. One might reasonably take these data to suggest a role for ALDH2 in nitrate bioactivation. In vivo studies are confounded because the inhibitors currently used are metabolically and redox labile (including aldehydes/hydrates; isoflavones, e.g., daidzein; and thiocarbamate inhibitors, e.g., disulfiram, benomyl): many require biotransformation themselves and are not specific for ALDH2, having been shown to inhibit other sulfhydryl-dependent enzymes and metalloenzymes including CYPs [110–115]. The lack of specificity of enzyme inhibitors has been commented on as a general problem, not limited to studies on ALDH2, in the identification of the enzyme responsible for nitrate biotransformation and tolerance [68].

Continuous delivery of GTN via a transdermal patch in rats provides an in vivo model of clinical nitrate tolerance, validated by the markedly reduced blood pressure response to GTN observed in the intact animal [116]. Both propionaldehyde and cyanamide attenuated the regioselectivity of GTN biotransformation in hepatic mitochondria, and both compounds inhibited tissue relaxation induced by GTN in naïve aortae; importantly, however, both compounds also inhibited relaxation in tolerant aortae to the same extent as in naïve aortae (Fig. 5) [108]. These and other data are compatible with a role for ALDH2 in mechanism- and clearance-based nitrate biotransformation, but are incompatible with a dominant role for ALDH2 in nitrate tolerance [108]. The actions of cyanamide and aldehydes on blood pressure and tissue relaxation were taken to reflect a nonspecific inhibitory action on GTN-induced relaxation. Furthermore, the numerous biochemical pathways that potentially contribute to nitrate tolerance would suggest that this is likely a multifactorial phenomenon, which includes attenuation of one or more mechanism-based biotransformation pathways.

The concentration–response curves shown in Fig. 5 represent typical data on the effect of in vivo GTN tolerance on GTN-induced tissue relaxation. In GTN-tolerant tissue, potency is reduced by 5- to 10-fold, but the potency of GTN still exceeds that of other nitrate

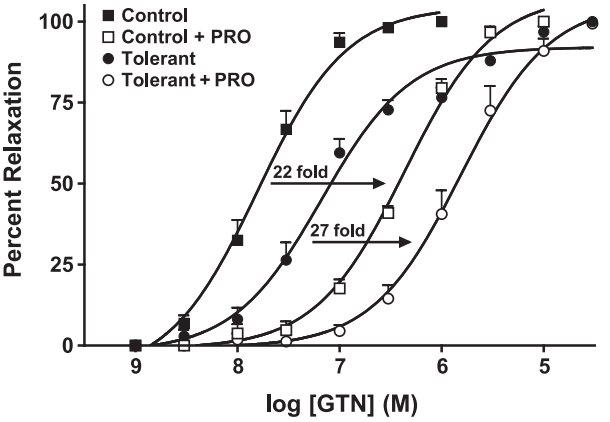


Fig. 5. Effect of in vivo GTN tolerance, 1.0 mM propionaldehyde (PRO), or both on GTN-induced relaxation of isolated rat aorta. The EC_{50} values for GTN-induced relaxation were Control, 19.1 ± 8.3 nM; Tolerant, 81.4 ± 57 nM; Control + PRO, 0.42 ± 0.06 μM; Tolerant + PRO, 2.2 ± 1.5 μM. Each value represents the mean ± SEM (n = 3 or 4). All EC_{50} values were significantly different from each other ($p < .001$, one-way ANOVA). Arrows represent fold-shifts on treatment with propionaldehyde in naive and tolerant tissues.

vasodilators, such as ISDN, in nontolerant tissue. It is therefore tempting to envision multiple biotransformation pathways, with GTN utilizing a high-potency or high-affinity pathway, sensitive to tolerance, in addition to the low-affinity pathway open to all other nitrates. Biphasic concentration–response curves have been reported for GTN-induced relaxation and these have supported a dual-mechanism hypothesis for GTN, with high-affinity and low-affinity components [117–119]. It is perplexing that erithrityl tetranitrate, which incorporates the GTN moiety in its structure, is reported to be 10-fold less potent, to manifest no biphasic activity; and to abolish the putative high-affinity pathway for GTN [120].

If (a) attenuated mechanism-based biotransformation is responsible for nitrate tolerance and (b) nitrate activity is mediated through NO release, the enzyme or agent responsible for this biotransformation must be able to mediate chemical reduction of nitrates to NO.

NITRATES AS NITRIC OXIDE DONORS

Detection and quantification of NO in complex biological systems and under physiological conditions remain challenging. A variety of methods are used for detecting NO, for example: quantification of cGMP; chemiluminescence detection; fluorescence detection using 4,5-diaminofluorescein; conversion of Fe(II)-oxyHb to metHb; spin trap/ESR detection (using, e.g., Fe-diethylthiocarbamate ($Fe(DETC)_2$), Fe(II)Hb, Fe(II)Mb, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide); electrochemical detection (amperometric NO-specific and porphyrinic electrodes); Griess assay of

NO_2^-, NO_3^-, or (NO_2^- + NO_3^-); and immunoassays using anti-nitrotyrosine antibodies. Many of these methods are indirect and have been queried, for example, because of lack of specificity for NO and interference from other components of biological systems [121–125].

The release of NO from nitrates in complex biological systems can often be observed using a variety of different methods, for example, exhaled NO levels were observed to be increased after high-dose i.v. bolus administration of GTN to coronary artery bypass patients [126]. In human plasma, NO release was observed from solutions of nitrates and TSA (0.5–1.0 mM) using electrochemical detection [122]. In plasma, the series of 1,2-dinitrooxy propane derivatives ($O_2NOCHCH_2ONO_2CH_2X$; X = F, OCF_2CF_2H, Cl) including GTN (X = ONO_2) was observed to release NO in the presence of TSA with an initial rate of ~4–80 nM s^{-1} at 0.25–0.5 mM nitrate concentration. Above detection limits, NO was not observed for any reaction in phosphate buffer (pH 7.4) nor in plasma from reactions of GTN with either DTT or glutathione (GSH) nor in the absence of thiol [127]. These data are in general accord with a detailed study using chemiluminescence detection, in which no measurable quantity of NO could be detected from GTN + thiol in phosphate buffer, except under anaerobic conditions with the addition of superoxide dismutase [127]. Even under these conditions, NO generation from GTN + cysteine in buffer was 5% of that observed from GTN in plasma.

Incubation of GTN (~1 mM) with a subcellular fraction obtained from bovine artery smooth muscle cells resulted in the production of NO as detected by chemiluminescence headspace detection [128]. Further studies performed with a microsomal fraction obtained from bovine artery and vena cava smooth muscle cells treated with GTN (150–900 μM) showed that NO production is higher in venous smooth muscle cells [129]. In another study worthy of mention, again using headspace chemiluminescence detection, bovine pulmonary artery rings were pretreated with the thiol-alkylating agent *N*-ethylmaleimide (NEM) before addition of GTN [130]. GTN (30 μM) was observed to generate NO at the rate of ~4 pmol min^{-1} g^{-1} tissue, but this was completely abolished by pretreatment with NEM, although tissue relaxation in response to GTN (10 μM) was diminished by only 20%. These authors queried whether nitrates were NO prodrugs or, alternatively, whether NO was released subsequent to sGC activation [131].

The high potency of GTN, its rapid onset of action, and the short half-life of NO have traditionally created obstacles in studying NO release from GTN under therapeutically relevant conditions, and these have often simply been circumvented by use of suprapharmacological concentrations of GTN. If the vasodilator response to GTN is mediated via NO, the efficiency of conversion of nitrates to NO must be high, because EC_{50} values measured for tissue relaxation range between 2 and 20 nM in different preparations (e.g., see Fig. 5) [67,78], and the biologically active concentrations of NO are thought to range between 10 and 100 nM [132]. A recent paper has addressed this issue by study of NO release in blood vessels from pharmacologically relevant concentrations of GTN and ISDN (0.1–1.0 μM), wherein NO was *not* detected [133]. In this work, NO was detected only at much higher concentrations of nitrates (>10 μM) and, impressively, from eNOS in tissue stimulated with acetylcholine. A skeptic's response to this questioning of the dogma of GTN as an NO donor and NO prodrug is to quote studies in which oxyHb, an NO trap, is shown to quench the biological activity of GTN. However, the published data are not so clear-cut; for example, in measurement of coronary vascular resistance in the isolated Langendorf heart, oxyHb did not inhibit the relaxation induced by GTN, but abolished the relaxation due to SIN-1 (a compound that alternately acts as an NO donor or a peroxynitrite generator in various biological environments) [134,135]. The question of the sensitivity and tissue permeability of the spin trap, colloidal $Fe(DETC)_2$, have been addressed [135]. Interestingly, $Fe(DETC)_2$ also traps NO from nitrosothiols, seemingly ruling out biological activity mediated by nitrosothiol formation from nitrates at therapeutically relevant concentrations [133,136].

NITRATES, NO, AND sGC ACTIVATION

The vascular effects of nitrates are believed to be mediated primarily via activation of sGC, which converts GTP to the secondary messenger, cGMP. Levels of cGMP are regulated by sGC activation and by phosphodiesterase-mediated hydrolysis. Elevation of cGMP within vascular smooth muscle results in vasodilation mediated by cGMP-dependent protein kinases. NO/cGMP signaling is important in other tissues, for example, in the hippocampus, mediating learning and memory [137,138]. The mechanism of sGC activation by NO involves binding of NO to a ferrous-heme center, which is bound reversibly to the protein, inducing activation via conformational change, but detailed understanding of the enzyme mechanism and topology is hindered by the lack of a crystal structure for sGC [139,140].

The most important observation in discussing sGC in the context of nitrates is the disparity between sGC activity in broken cell preparations or purified protein and activity in intact tissue. Sodium nitroprusside, nitrosothiols (e.g., GSNO, SNAP), and other nitrovasodilators all activate sGC above basal levels; however, GTN and simple organic nitrates, in contrast, are incapable of activating sGC above basal levels, in vitro,

in the absence of thiol adjuvants. A large number of papers have appeared on in vitro activation of crude preparations of sGC and purified or recombinant proteins by nitrates, in particular GTN, in the presence of thiols [63,65,76,140–151]. We have recently compared activation of sGC by six nitrosothiol and NONOate (diazeniumdiolate) NO donors with that by GTN in the presence of cysteine, using chemiluminescence to measure NO release [67]. An excellent correlation was observed between the rate constant of NO release (k_{NO}) and potency for sGC activation (EC_{50}) for the NO donors. In contrast, although GTN does react with cysteine in simple aqueous buffer to release NO, the low rate constant of NO release did not fit the correlation of k_{NO} with EC_{50}. Furthermore, nitrates behave as partial agonists: maximal enzyme activity for nitrates is 4- to 5-fold lower than for NO donors. *Unequivocally, GTN and most other organic nitrates, including NO-NSAIDs, do not behave as NO donors in the most fundamental action: activation of sGC to produce cGMP.*

The Fe^{II}-heme site of sGC is a high-affinity NO receptor, and formation of sGC–NO by binding of one or two molecules of NO to this site induces conformational changes that result in an increase in V_{max}/K_m and a several hundred-fold increase in enzyme activity [140,152]. Although carbon monoxide also activates sGC, the different structure at the heme of sGC–CO results in a much smaller increase in V_{max}/K_m. The NO-independent sGC activator 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1) is thought to bind to an allosteric site on sGC, possibly in the region of Cys238 and Cys243 on the α_1 subunit [153]. The combination of YC-1 allosteric binding and CO binding to the heme site conformationally triggers an increase in V_{max}/K_m approaching that for sGC–NO, although the potency of CO remains orders of magnitude below that of NO. The actions of YC-1 on sGC raise the possibility of endogenous sGC allosteric modulation, although the activity of YC-1 itself does not seem to be specific to sGC activation [139,154].

NO-donor nitrosothiols and NONOates activate partially purified sGC, via release of NO, with varying potency but with the same maximal enzyme activation of up to 100-fold above basal levels, which is of similar magnitude compared to values observed for purified enzyme [67,140]. YC-1 is an NO-independent activator of sGC, with modest potency and efficacy in vitro, that exerts a small effect on NO-dependent activation of sGC, as exemplified by the small increase in potency of NONOate in the presence of YC-1 shown in Fig. 6 [67,151,155–157]. Classical nitrates, in the presence of cysteine (1–10 mM), activate sGC with low potency, but more remarkably, maximal enzyme activation is 4- to 5-fold less with nitrates than with NO. EC_{50} values for the NO donor DEA/NO and for GTN + Cys are the same (100–200 μM). The

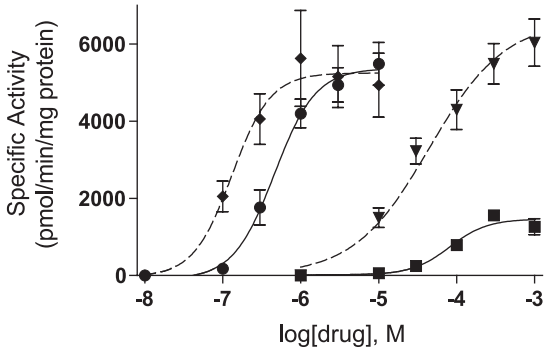


Fig. 6. Activation of sGC as a function of concentration of (1) NO donor (DEA/NO) and (2) GTN (+ cysteine), showing potentiation by YC-1: solid lines, without YC-1; dashed lines, with added YC-1. (1) DEA/NO (●, without, and ◆, with YC-1 (100 μM). (2) GTN + Cys (2 mM) (■, without, and ▼, with YC-1 (100 μM)). Values for enzyme activity in the presence of YC-1 alone have been subtracted in order to show only the potentiation by YC-1 of NO-dependent and GTN-dependent activation of sGC. Data points shown are means ± SEM.

effects of YC-1 are also much more profound on nitrate activation of sGC than on NO activation. YC-1 potentiates activation of sGC by GTN + Cys such that activity is observed at the maximal levels seen for NO and NO donors, although this potentiation is achieved with little or no increase in the potency of GTN (Fig. 6). YC-1 potentiates the activation of sGC by GTN + Cys by a factor comparable to that reported for potentiation of the activity of sGC-CO (3- to 5-fold), and greater than that for potentiation of the activity of sGC-NO (1.0- to 1.2-fold, see Fig. 6) [148,155,157]. In accord with these data, YC-1 has been shown to potentiate GTN-induced tissue relaxation in both naïve and tolerant aortic tissue [17]. Again in accord with these results, data on cGMP accumulation in PC12 cells in response to NCX 8001, a hybrid nitrate drug, show that activity is profoundly amplified by YC-1 [158].

The partial agonist activity of GTN + Cys toward sGC might indicate that GTN contributes to both an activating and an inhibitory pathway. The potential inhibitory properties of GTN toward sGC–NO were studied by incubation of sGC with an NO-donor NONOate after preincubation with GTN alone, which demonstrated a rightward shift in the concentration response curve for sGC activation by the NO donor [17]. Furthermore, at a high concentration of GTN (1 mM), inhibition of the maximal activity of sGC–NO was also observed. The rightward shift in the concentration–activity curve for NO donor in the presence of GTN suggests a reversible antagonist action for GTN, but is also compatible with either nitrate binding to and deactivation of sGC–NO or inhibition of NO binding to sGC.

Inhibition of sGC via heme or thiol oxidation is a viable inhibitory mechanism for GTN and for nitrates in general. Oxidative inhibition of sGC by 1*H*-(1,2,4)oxadiazole(4,3-

a)quinoxaline-1-one (ODQ), the best studied inhibitor of sGC, results from binding of ODQ at the ferrous-heme and oxidation of the Fe^{II} center [159,160]. GTN reacts very rapidly with deoxyHb, leading to oxidized metHb, GDN, and NO_2^- [81], and reactions of N,O-compounds with reactive Fe^{II}/Fe^{III}-heme and cysteine sites on hemoglobin are well documented [161,162]. Protein thiol oxidation by GTN has been reported [163]. Oxidation of one or more cysteine residues of sGC by GTN can be proposed as an inhibitory mechanism, because modification of cysteine residues of the β_1 subunit has been shown to inhibit NO-dependent activation of sGC [164]. In accord with the ferrous-heme oxidation mechanism for sGC inhibition, study by ESR and UV–Vis spectroscopy of the interactions of recombinant sGC with GTN, and with ferricyanide, concluded that there was no evidence for a Fe^{II}–sGC–NO complex in incubations of GTN or GTN + Cys with sGC, but that there was substantial evidence for oxidation of Fe^{II}–sGC by both GTN and GTN + Cys to ferric–sGC [165]. Importantly, (a) ferric–sGC was not activated by cysteine and (b) no evidence for a nitrate radical anion ($GTN^{\bullet -}$) could be obtained, leaving the mechanism of activation of sGC by GTN + Cys, in vitro, unknown. The in vitro observations of an NO-independent mechanism for sGC activation, amplified by an allosteric potentiator, require that such a mechanism for mediation of nitrate biological activity be considered.

NO RELEASE FROM NITRATES: MODELS AND MECHANISMS

Unfortunately, a substantial number of publications purport to quantify NO release from nitrates, by using assays that do not measure NO, such as the Griess test for NO_2^- (for example, [166]). It has been elegantly shown that assay of NO_2^- (but not NO_3^-) provides a reliable biomarker for endogenous NO production [167], but measurement of NO_2^- as a marker for NO is not suitable for nitrates, which are rapidly metabolized directly to NO_2^- by clearance-based biotransformation. The chemical conversion of nitrate to NO is a $3e^-$ reduction that can be achieved by a number of possible $2e^-$ plus $1e^-$ pathways, some of which can be drawn to involve initial liberation of NO_2^-:

$$RONO_2 + 2e^- + H^+ \rightarrow ROH + NO_2^-,$$

$$NO_2^- + e^- + H^+ \rightarrow HO^- + NO, \text{ or}$$

$$NO_2^- + M^{n+} \rightarrow M^{(n-1)} + {}^{\bullet}O + NO.$$

The seminal Ignarro hypothesis posits entry of GTN into the smooth muscle cell, where it is then converted to NO_2^- by reaction with cysteine; nitrite then liberates NO via nitrous acid; NO combines with thiol to generate a nitrosothiol, which activates sGC [145]. Recent work on ALDH2-mediated biotransformation of GTN to NO_2^- has resuscitated this pathway [145]. Although NO_2^- is the predominant N,O-containing species formed during the vascular biotransformation of GTN, it has low vasodilator potency and high endogenous levels, arguing against involvement of NO_2^- in nitrate activation of sGC [24,48,49,108]. The NO_2^- concentration in rat aorta has recently been reported to be 25 μM [42], and pharmacokinetic studies have demonstrated that NO_2^- is distributed evenly between plasma and erythrocytes and between the intravascular and the extravascular compartments [168]. The pharmacologically effective concentration of GTN for vascular smooth muscle relaxation is in the nanomolar range, thus the NO_2^- derived from GTN would only incrementally increase the intracellular concentration of NO_2^- and thus would not be expected to exert pharmacological actions.

Compartmentalized conversion of NO_2^- to NO in mitochondria subsequent to nitrate denitration, followed by facile translocation of NO to its site of action, has been proposed [88]. Disproportionation of nitrous acid (HONO), akin to the Ignarro hypothesis, even in the proton-rich environment of the mitochondrial intermembrane space, is still problematic because of the high potency of GTN and incremental increase in NO_2^- concentration. At pH 4, the rate of NO formation from NO_2^- is estimated at 0.01% per second [169]; therefore nanomolar concentrations of NO_2^- derived from GTN could generate only localized, subpicomolar concentrations of NO in a relevant time frame for GTN-induced vasodilation (seconds). Mitochondrial $1e^-$ reduction of NO_2^- to NO-utilizing components of the mitochondrial electron transport chain including the bc_1 complex of the respiratory chain and cytochrome *c* oxidase have been proposed [170,171]. However, NO release from NO_2^- by the mitochondrial electron transport chain occurs under anaerobic conditions and not under conditions of physiological oxygen tensions, under which conditions reduction of oxygen yields the efficient NO scavenger $O_2^{\bullet -}$. Cytochrome oxidase reduction of NO_2^- to NO has recently been questioned [172], and indeed, under aerobic conditions NO is expected to be oxidized to NO_2^- with cytochrome oxidase serving to remove NO from the mitochondria [173]. Under low electron flux, cytochrome oxidase uses the ferric–heme–Cu^I complex to catalyze oxidation of NO:

$$NADH + 3H^+ + 1/2O_2 + NO \rightarrow NAD^+ + H_2O + 2H^+ + NO_2^-.$$

Nitrate biotransformation pathways via NO_2^- cannot be dismissed, because reduction of NO_2^- to NO has been

observed in biological systems, but given the very low pharmacological concentrations of GTN, a pathway via direct reduction to NO is attractive. Hints on the mechanism of such a pathway must be gleaned from the chemistry of nitrates.

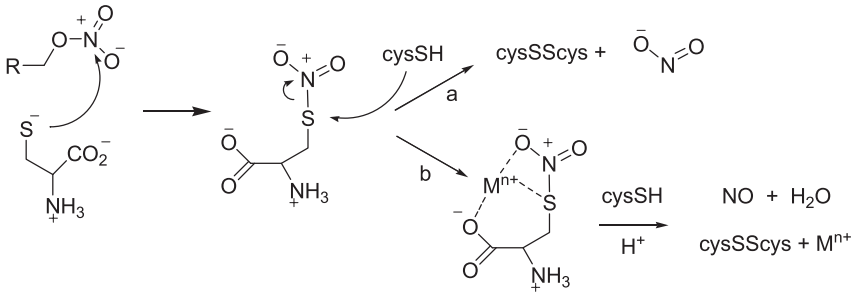
Nitrate esters are subject to ionization in concentrated sulfuric acid, giving rise to the production of nitronium ions [174]. Organic nitrates in the presence of both Bronsted and Lewis acids may act as nitrating agents toward nucleophiles, such as arenes, with the implication that biological transition metal catalysts may be able to mediate nitration using nitrates. However, nitrate esters are stable in dilute acid [163]. In strong alkaline solution, nitrate esters are known to undergo solvolytic decomposition via S_N2 nucleophilic substitution at carbon, β-hydrogen elimination, and α-hydrogen elimination [175,176]. In the case of *vic*-dinitrates or β-hydroxy nitrates, oxirane formation is also possible under strongly alkaline conditions. NO_3^- as the conjugate base of a strong acid is a good leaving group, leading to the use of organic nitrates in solvolytic studies on the S_N2/S_N1 continuum [177–179]. But, whereas many nitrate solvolytic reactions involve C–ONO_2 fission, evidence for O–N fission exists in the presence of a number of nucleophiles, including the N-nucleophiles hydrazine and hydroxylamine, as demonstrated by isotope labeling and stereochemical studies [180–184].

Reactions of nitrates with thiols

The observation that tolerance associated with prolonged exposure to GTN was accompanied by a decrease in the levels of tissue thiols led to the "thiol receptor model" for GTN biotransformation that posited a sulfhydryl species as essential for biotransformation, the oxidation of which to a disulfide was the cause of tolerance [185]. There is evidence supporting and contradictory to the tolerance hypothesis [127,186,187], but regardless, this model was seminal for later hypotheses of nitrates acting as NO donors through the chemical reaction of a thiol with the nitrate group. Variants of these hypotheses use a free thiol, a protein thiol, or the glutathione cofactor of GST [68,88,188–190]. Of relevance to the free thiol hypothesis are observations that in vitro activation of sGC by GTN requires addition of "active" thiols (cysteine, *N*-acetylcysteine, TSA), whereas other thiols not containing a β-carboxylate group (e.g., DTT) do not elicit activation of sGC by GTN [127,149].

Early studies on the chemical reaction of S-nucleophiles with the nitrate group reported that alkaline polysulfides and hydrogen sulfide reacted with alkyl nitrates to yield NO_2^- or, with a greater excess of sulfide, to give ammonia, via initial reaction at the N or O of the nitrate group, and an intermediate of the form $S_x(NO_2)^-$ [182,191]. In contrast, benzyl nitrate was shown to undergo substitution at C with thiophenolate as nucleophile [179,192]. Nitrates are relatively stable toward reaction with nucleophiles in neutral, aqueous solution.

At physiological pH, the reactivity of sulfur nucleophiles toward nitrates is dependent on thiol pK_a and for biologically relevant thiols is slow, for example, only 10% reaction of GTN (1 mM) with cysteine (2 mM) is observed in aerobic, aqueous solution at pH 7.4, 37°C [67]. This reaction does not produce sufficient NO for detection using earlier electrochemical devices; chemiluminescence is required to detect NO at the very low levels produced in these mixtures. Fung and co-workers analyzed NO release from GTN and thiols in plasma and reported that NO was not detectable from the reaction of GTN with cysteine in phosphate buffer, but that low concentrations of NO were detectable after addition of superoxide dismutase (SOD) [127]. Recently, SOD has been shown to participate in reactions other than removal of superoxide, for example, reacting with nitrosothiols to generate NO [193]. Brien et al. measured NO concentration in headspace gas at 5 min from the anaerobic reaction of GTN (0.2 mM) with cysteine (5 mM) in phosphate buffer and did observe NO [194]. In agreement with this observation, a rate of release of NO of 3 nM/s was measured for an anaerobic solution of GTN (1 mM) with cysteine (2 mM), representing <10% of the entire reaction flux [67]. The rate of NO release was reduced 100-fold in aerobic solution and quenched by metal ion chelator. In addition, NO_2^- was shown not to be a kinetically competent intermediate for NO release from



Scheme 1.

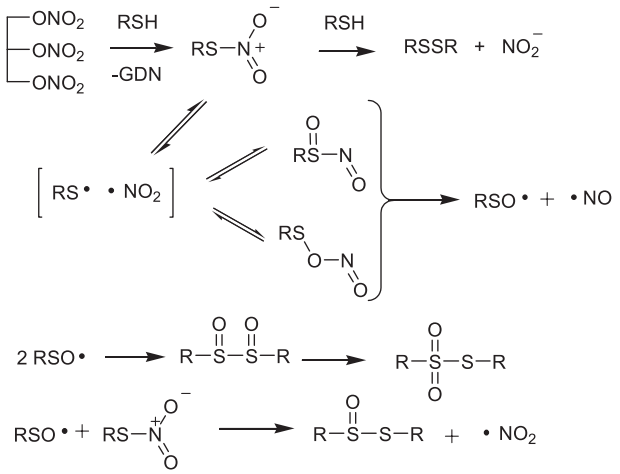


Fig. 7. Thionitrate rearrangement mechanism for sulfhydryl-dependent reduction of nitrates to NO, via either sulfinyl (RS(O)NO) or sulfenyl (RSONO) nitrite intermediates, generating thiol sulfinate and sulfonate products.

GTN, although in the presence of cysteine (2 mM), NO_2^- (0.1 mM) did generate nanomolar concentrations of NO over 10 min. These data are compatible with a minor anaerobic pathway for thiol-mediated release of NO from nitrates that is dependent on reduction by trace metal ions. A possible mechanism illustrating major (a) and minor (b) reaction pathways is illustrated in Scheme 1. The aerobic production of NO from aqueous cysteine/GTN solutions, as assessed by headspace chemiluminescence, is too low to be consistent with activation of sGC via NO in vitro [67].

Thionitrates and sulfenyl nitrites

The GST-mediated reaction of GSH with GTN yields glutathione disulfide as the final product and in the presence of the good thiolate nucleophile mercaptoethylamine ($^-SCH_2CH_2NH_3^+$), a mixed disulfide was detected [195]. These data were interpreted as requiring an organic thionitrate ($GSNO_2$) intermediate. Similarly, observations on thiol loss and NO_2^- formation from reaction of thiols with nitrates in neutral aqueous solution were explained by initial formation of thionitrate intermediates [196]. Yeates was the first to publish a well-described chemical mechanism that identified an organic thionitrate ($RSNO_2$) as the common intermediate in a mechanism leading to either NO_2^- or NO [189]. It was proposed that this thionitrate ester could undergo isomerization to a sulfinyl nitrite, homolytic decomposition of which would lead to the formation of NO. Yeates assigned a pivotal role for GST-mediated biotransformation of GTN in vivo and also proposed an alternative mechanism whereby GST could additionally catalyze the conversion of the sulfinyl nitrite to a nitrosothiol (RSNO), possibly *S*-nitrosoglutathione (GSNO).

Other reports mentioned thionitrates in the context of nitrate biotransformation with some confusion over the structures of the sulfinyl (RS(O)NO) and sulfenyl (RSONO) nitrite isomers. In 1995 and 1996, a computational and experimental approach yielded a cohesive thionitrate rearrangement pathway, shown in Fig. 7 [197]. The generalized conclusions from the theoretical study of the isomers of $RSNO_2$ (R = CH_3, H) were (see Fig. 8): (1) Thionitrate and sulfenyl nitrite isomers are close in energy and of lower energy than the sulfinyl nitrite isomer. (2) Homolysis of the thionitrate and in-cage rearrangement of the geminate radical pair {$RSO^{\bullet}$ $^{\bullet}NO_2$} provides a pathway to the sulfenyl or sulfinyl nitrite. Concerted rearrangement was calculated to be a high-energy pathway. (3) Homolysis of either sulfenyl or sulfinyl nitrites yields NO with an O–S bond dissociation energy of approximately 18 kcal/mol. Gas-phase calculations hold

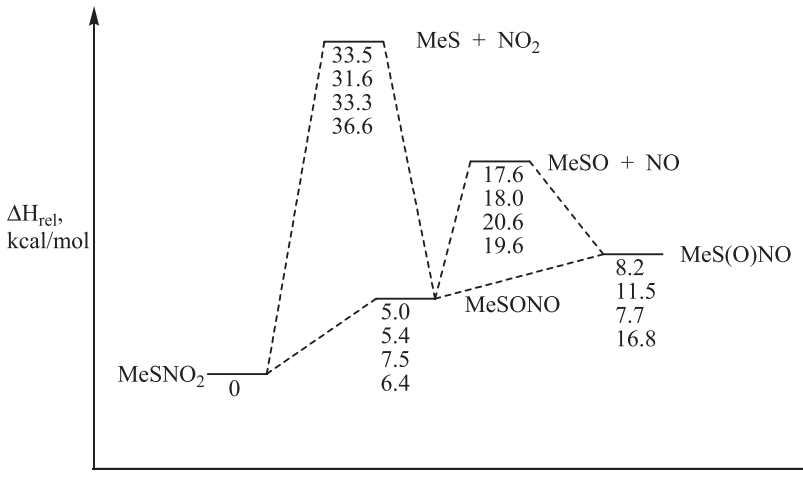


Fig. 8. Relative enthalpies at 298.7K for species involved in the thionitrate rearrangement pathway yielding NO, calculated using (from top to bottom) BLYP, EDF1, MP2, MP2//HF/6-31G* level methodologies and the 6-311 + G** basis set (unless otherwise stated) on Spartan (Wavefunction, Inc.).

the corollary that solvation may alter solution kinetics and thermodynamics: for example, the higher bond dipoles of the sulfinyl isomer will result in stabilization in polar solvents, meaning that this isomer's relevance cannot be ruled out.

In 1932 *tert*-butyl thionitrate was synthesized by oxidation of the corresponding nitrosothiol employing fuming nitric acid as the oxidant: it was reported that the resulting thionitrate was more stable than the initial nitrosothiol [198]. In 1978, an alternative synthesis via reaction with N_2O_4 was published [199], the same group later reporting the synthesis of the unstable aryl thionitrate esters and data on thermolytic decomposition of thionitrates [200]. More recently, an elegant synthesis and crystal structure of a stable aryl thionitrate was reported, exploiting a highly sterically hindered thiol in organic solvent [201]. Thionitrates have characteristic IR spectra (1530 cm^{-1}; NO_2 sa), but are colorless in contrast to the pink or green colors of the corresponding nitrosothiols [202–204]. Neither sulfenyl nor sulfinyl nitrites have been isolated, but these compounds would all be readily distinguishable on account of very different spectroscopic characteristics, including IR [204] and UV–Vis spectra: $MeSNO_2$ (λ_{max} 262 nm), MeSNO (209, 313, 497 nm), MeSONO (227, 316, 426 nm), and MeS(O)NO (216, 287, 418 nm) from DFT calculations at the B3LYP/6-31+G* level.

An experimental study of the hydrolysis of *tert*-butyl thionitrate reported a half-life of approximately 10 min for decomposition at neutral pH in a reaction that showed buffer catalysis, generated NO, and cleanly yielded *tert*-butyl sulfinyl and *tert*-butyl sulfonyl products, as characterized by NMR, GC-MS, and ES-MS (Fig. 8) [204]. Release of NO, measured electrochemically, was quenched by addition of equimolar cysteine, and the thionitrate was observed not to activate partially purified sGC from aortic homogenates containing DTT, probably because of the high reactivity of thionitrates toward thiols. The experimental and computational data on thionitrate reactivity are in accord, suggesting that organic thionitrates are reasonable intermediates in nitrate biotransformation, in particular if the thionitrate is formed in an environment, such as an enzyme active site, which excludes a second thiol. A later study on the reaction of GSH with bolus peroxynitrite reported the production of the thionitrate ester of glutathione ($GSNO_2$) as product, resting on ES-MS for identification [205]. In this study, the compound reported to be a thionitrate displayed the characteristics of the nitrosothiol GSNO (pink coloration, λ_{max} 334 nm, identical RP-HPLC retention time).

A problem with nitrate bioactivation via thionitrate rearrangement is the low reactivity of the nitrate group toward free thiols at physiological pH. This would argue for a requirement for enzyme catalysis or acceleration of the reaction of nitrates with GSH or a protein-thiol, although it is also possible that a cysteine residue outside of an enzyme active site could provide the required rate acceleration. The concept of intramolecular reactions providing models for enzyme-mediated processes is closely associated with Kirby and Jencks [206,207]; thus GT 150 provides a model for the reactivity of GTN bound at a protein binding site adjacent to a cysteine thiol functionality (Fig. 9). In aqueous solution, GT 150 is a spontaneous NO donor, generating fluxes of NO comparable to NONOates and other NO donors and displaying the expected properties of a genuine NO donor in both activating sGC and inhibiting lipid peroxidation [26,64]. GT 150 represents only one model for biotransformation of nitrates to NO, because more than one mechanism can be drawn for NO release from a nitrate (Fig. 9).

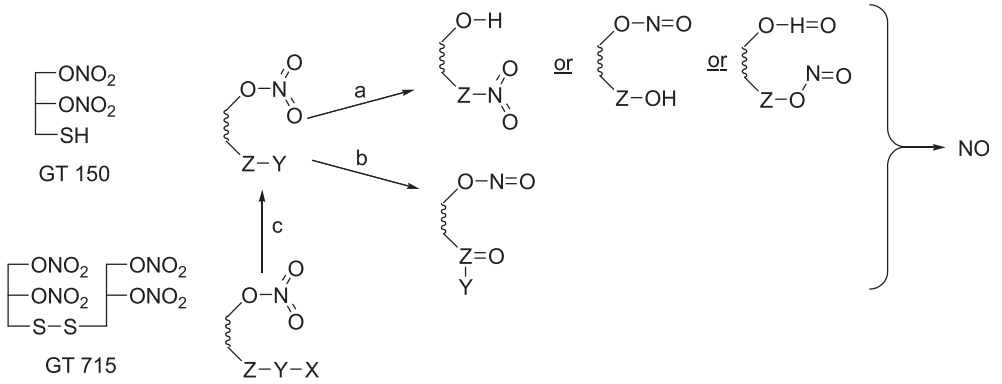
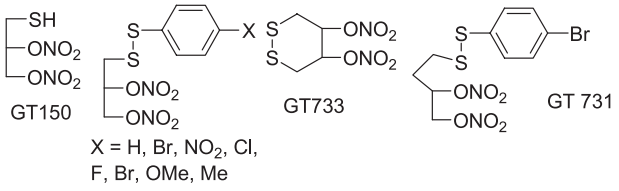
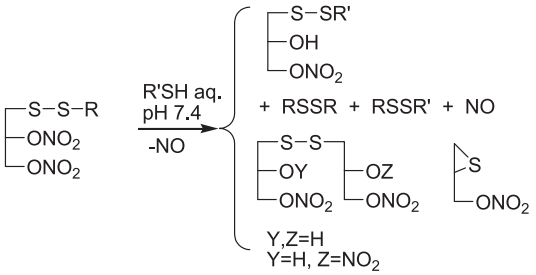


Fig. 9. Compounds that provide intramolecular models of nitrate biotransformation juxtapose a reactive functional group in proximity to the nitrate moiety. (Path a) Pathways for intramolecular nitrate group or oxygen atom transfer (Z–Y = S–H) proceeding via thionitrate, nitrite, and sulfenyl nitrite intermediates, respectively. (Path b) Pathway for intramolecular oxygen atom transfer from nitrate to an oxygen atom acceptor (Z = S, Se, P, N; Y = groups other than H). (Path c) Nitrates may be designed to provide precursors, for example GT 715, that require activation or bioactivation to expose a reactive functional group, for example in GT 150.

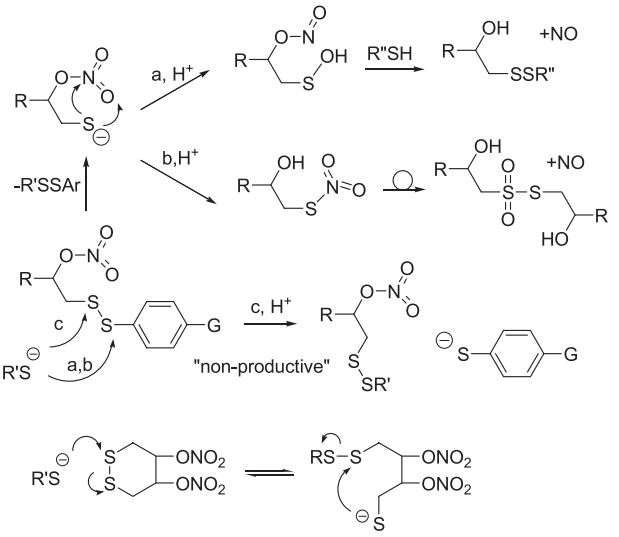
The disulfanyl *bis*-dinitrate, GT 715, and the related aryl disulfanyl dinitrates (Scheme 2) do not spontaneously generate NO in aqueous solution, but liberate NO and a variety of by-products, in the presence of added thiols, such as cysteine and thiophenol [64]. The related dinitrates, GT 733 and GT 731, did not react with thiols to release measurable NO. These observations are compatible with an intramolecular reaction via a 5- or 6-membered ring transition state (Scheme 3). Other reductants studied did not facilitate NO release from the disulfanyl dinitrates, reaction was unaffected by O_2, and no evidence was obtained for sulfonyl or sulfinyl products (S═O containing). These data argue against an outer-sphere electron-transfer mechanism, but leave open the possibility of an inner-sphere electron-transfer process via an intermediate in thiol–disulfide exchange. The simplest mechanism compatible with observations is rapid thiol–disulfide exchange liberating the thiolate of GT 150, which may cyclize by attack of S at C or at the nitrate group (Scheme 3): nitronium is an ambident electrophile; therefore bond forming between S and O to form a sulfenate is a chemically reasonable mechanism. The organic nitrite (RONO) thus generated may liberate NO either via formation of a nitrosothiol or spontaneously.

We have attempted to explore the reactivity of the S-centered radicals, including thiyl and disulfide anion, with nitrates, employing photochemical methods, but such studies are hampered by the photolysis of the nitrates themselves to yield NO_2. Nitrates and thionitrates, unlike nitrites and nitrosothiols, have very weak absorbance above 300 nm, but photochemical reactions of nitrates are known [208–210]. Photolysis of mixtures of thiols and nitrates yields the products of thionitrate decomposition, which would be expected from the reaction of NO_2 with thiol generating thiyl radical, followed by radical combination with NO_2 [211].





Scheme 2.



Scheme 3.

Goto's intriguing stable thionitrates provide support for the alternative pathway (b) in Fig. 9: phosphines and selenides were observed to cleanly reduce a thionitrate to a nitrosothiol by oxygen atom transfer [212]. Biotransformation of nitrates themselves via oxygen atom transfer to methionine or selenomethionine would yield an organic nitrite (RONO), but would likely require catalysis. The role of organic nitrites as intermediates in nitrate biotransformation is attractive, because nitrites are vasodilators, require only a $1e^-$ reduction to yield NO, and react rapidly with thiols to generate nitrosothiols [213–215]. Nitrites spontaneously generate NO in neutral aqueous solution in the presence of metal ion chelators and in the dark [26,216,217]. This observation is perplexing, because homolysis of nitrites is thermodynamically less favorable than that of nitrosothiols in the gas phase and organic solvents [203,216]. The detailed mechanism of NO release requires further study, but may involve either the reaction of the nitrite with a species formed on initial heterolysis or weakening of the O–N bond by stabilization of the strong nitroso dipole in aqueous solution, akin to that observed for the O–H bond [218].

Reactions of nitrates with metal ion assistance

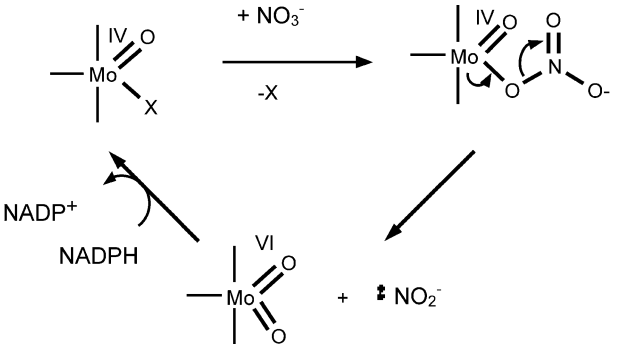
Reductive biotransformation of GTN has been demonstrated for a variety of metalloproteins, including CYPs and XOR [68,80,219]. Metalloproteins represent prime candidates for mediating the $3e^-$ reduction of nitrates, because nitrates have been demonstrated to coordinate with and be reduced by transition metals. GTN reacts with the ferrous-heme moieties of hemoglobin and myoglobin to give GDN regioisomers, and GTN is rapidly consumed by deoxyHb to give NO_2^- [81,220]. A synthetic Fe(II)–tetraphenylporphyrin–*N*-methylimidazole complex was

observed to rapidly reduce GTN to GDN and NO under anaerobic conditions in organic solvents [217].

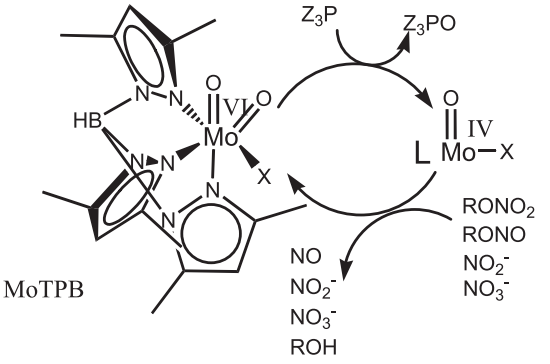
Doyle studied the reaction of organic nitrites with hemoglobin and noted binding at the heme site and reduction to NO and alcohol [162]. Thus, direct reaction of ferrous-heme proteins with organic nitrates to yield NO is not unreasonable. Nitrates have been reported to oxidize oxyHb to metHb in the presence of thiols at a rate that correlates with vasodilation potency, but that does not correlate with NO release [122,149]. Hemoglobin contains reactive Cu-binding, cysteine, and lysine sites, in addition to the Fe-heme site, which may have a role in the direct reactions observed with N,O-containing compounds, including organic nitrites and nitrosothiols and presumably also nitrates. Mixed metalloprotein–sulfhydryl pathways can also be envisioned to provide the three electrons required for nitrate reduction to NO.

Bacterial nitrate reductase, a molybdoenzyme, catalyzes the $2e^-$ reduction of NO_3^- to NO_2^- and might be seen as a lead in identifying proteins responsible for nitrate biotransformation (Scheme 4). Therefore, mammalian molybdoenzymes, including aldehyde, sulfite, and XOR, which catalyze both oxygen atom transfer and e^- transfer, are logical candidates. Molybdoenzymes participate in a wide variety of redox reactions including hydroxylation and oxygen atom transfer, utilizing a variety of redox active centers, including flavins and iron–sulfur clusters. Molybdoenzymes are generally grouped into three families according to the structures of their oxidized Mo(VI) centers: the molybdenum hydroxylases, the eukaryotic oxotransferases, and the prokaryotic transferases. Over 50 molybdoenzymes have been identified to date, but XOR is the most studied, in particular as regards interactions with N,O species [219,221].

The use of XOR as a generator of $O_2^{\bullet -}$ is routine, and xxtherefore NO may be scavenged in solutions of XOR and substrate; however, under anaerobic conditions using NADH or xanthine, XOR has been shown to be capable of reduction of both NO_2^- and NO_3^- to yield NO [51,222]. Reduction occurs at the Mo center and is inhibited by O_2 and high purine substrate



Scheme 4.



X = Cl, SPh, $SC_6H_4O(CH_2)_6CH_3$; Z = Ph, OEt, $C_6H_5SO_3^-$

Scheme 5.

concentrations, but nevertheless provides an interesting alternative biological pathway to NO under hypoxic conditions [219]. Interestingly, the nitrates GTN, ISDN, and ISMN were also observed to be reduced by XOR using NADH or xanthine under anaerobic conditions [80]. Nitrate reduction occurs at the flavin site, and in the presence of NADH, the generation of NO was observed. These studies show low rates of NO production from nitrates, via NO_2^- as intermediate, nevertheless this represents the only purified enzyme system for which significant NO production has been observed. XOR is inactivated in the process of GTN degradation, but it is unlikely that XOR plays a primary role in mechanism-based nitrate biotransformation or nitrate tolerance, because the specific XOR inhibitor, allopurinol, does not influence nitrate-induced tissue relaxation [78]. Nitrites are reduced relatively rapidly by XOR directly to NO at the flavin site of the enzyme

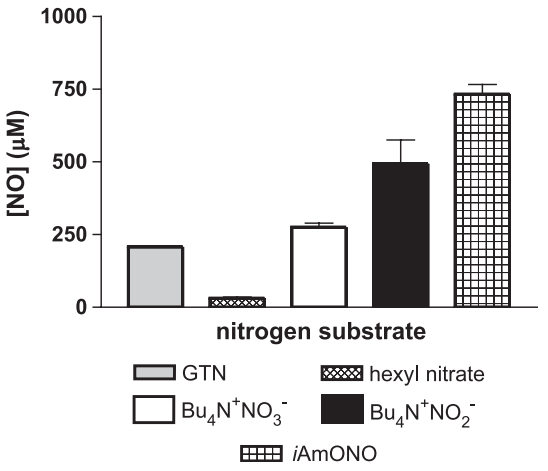


Fig. 10. Cumulative NO production after 30 min as measured by chemiluminescence using repetitive sampling of reaction headspace from a sealed reaction vessel. Injection of various substrates (10 mM) for MoTPB (1 mM) and Ph_3P (20 mM) in stirred, anaerobic CH_2Cl_2 in septa-sealed, water-jacketed reaction vials at 25°C was assessed. Error bars represent standard errors of the mean.

under anaerobic conditions in the presence of xanthine [223]. To summarize, XOR anaerobically catalyzes the NADH or xanthine dependent on: (a) $2e^-$ reduction of nitrates to NO_2^- at the flavin-site, (b) $1e^-$ reduction of NO_2^- to NO at the Mo site, and (c) $1e^-$ reduction of nitrites to NO at the flavin-site.

In order to test the potential for molybdoenzymes, in general, to catalyze the $3e^-$ reduction of nitrates to NO, functional Mo complexes were synthesized, based upon the earlier work of Enemark and others [224–228]. The development of a model system requires a catalytically active molybdenum hydrotris-(3,5-dimethyl-1-pyrazolyl)borate complex, MoTPB, and a reducing cofactor, R_3P (Scheme 5) [227,228]. The substrates employed with this model system included nitrates (GTN, GT 150, hexyl nitrate), nitrites (*i*-amyl nitrite, 2-phenoxyethyl nitrite), and the $Bu^n_4N^+$ salts of NO_2^- and NO_3^-. Reaction products were assayed as a function of time using headspace chemiluminescence for NO, the Griess assay for NO_2^- and NO_3^-, GC-EC for N_2O, HPLC-UV for organic components, and ^{31}P NMR to quantify MoTPB catalysis of oxygen transfer from GTN to phosphine or phosphite. Reactions were observed to be catalytic in MoTPB; reaction rates were dependent on substrate and reducing cofactor concentration, and all N,O substrates studied reacted via reduction and oxygen atom transfer. Only P(III) compounds were able to act as cofactors, with no significant activity seen for potential S, Se, or N cofactors. GTN underwent reductive degradation catalyzed by MoTPB/Z_3P to yield products including GDN and NO. The $1e^-$ reductions of nitrites and NO_2^- to NO were observed to be more facile than the $3e^-$ reductions of GTN and NO_3^- to NO (Fig. 10), with uncatalyzed NO release being detected from both inorganic and organic nitrite. Both organic nitrite and NO_2^- were observed to be kinetically competent intermediates in reduction of nitrates to NO.

It was inferred from the Mo model studies that molybdoenzymes have the catalytic apparatus to mediate reductive denitration and for catalysis of mechanism-based biotransformation of nitrates to generate NO. Furthermore, because MoTPB efficiently catalyzed reduction of NO_2^- to NO in aerobic organic solvents, NO_2^- should not be discounted as an intermediate in mechanism-based nitrate biotransformation [227].

CONCLUSIONS

After 130 years, there is a contemporary renaissance in nitrate medicinal chemistry, which is proceeding almost undetected, because the new nitrate investigational drugs are universally described as NO donors. The irony of course is that we do not know the biological mechanism of NO donation by nitrates; we do not know details of the mechanism of action of nitroglycerine, the grandfather of all nitrates; and questions have been raised as to whether nitroglycerine delivers its therapeutic benefits via NO donation. If the biological activity of nitrates is not mediated via reductive biotransformation to NO, what are the alternative mechanisms? Direct ligand binding to a recognition element is unlikely given the structural diversity of nitrates and their metabolic lability. Nitrate reduction is accompanied by oxidation of metal, S, Se, and other centers and with metal ion catalysis, nitration of biomolecules may even be possible, thus enzyme inhibition and cell signaling via oxidation, nitration, and redox pathways are possibilities [229,230]. We have mentioned the increased interest in the biology of NO_2^- linked with a view of nitrates as NO_2^- donors. The alternative product of $2e^-$ reduction of a nitrate is a nitrite (RONO): nitrites are outstanding nitrosating agents, in particular toward nitrosothiol formation, suggesting intracellular $2e^-$ reduction of nitrates to nitrites as another potential pathway of enzyme modification and of cell signaling. The biology of thionitrates and sulfenyl nitrites remains to be explored. Clearly, there is a rich nitrate biological chemistry yet to be discovered, and given the history of nitrates, the odds are that this will assist in the design of exciting new therapeutic agents.

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ABBREVIATIONS

ALDH — aldehyde dehydrogenase
cGMP — guanosine-3′,5′-cyclic monophosphate
CYP — cyctochrome P450 oxidase
DTT — dithiothreitol
ES-MS — electrospray mass spectroscopy
$Fe(DETC)_2$ — Fe-diethylthiocarbamate
GC-EC — gas chromatography–electron capture detection
GDN — glyceryl dinitrate
GSNO — *S*-nitrosoglutathione
GTN — glyceryl trinitrate
HBN — hydroxybenzyl nitrate
Hb — hemoglobin
ISDN — isosorbide dinitrate
ISMN — isosorbide mononitrate
NEM — *N*-ethylmaleimide
nitrate — organic nitrate, nitrate ester, nitrooxy derivative
nitrite — organic nitrite, nitrite ester, nitrosooxy derivative
nitrosothiol — thionitrite ester, nitrososulfanyl derivative
NO-ASA — NO-donating acetylsalicylic acid
NONOate — diazeniumdiolate salt
NO-NSAID — NO-donating nonsteroidal anti-inflammatory drug
NOS — NO synthase
ODQ — 1*H*-(1,2,4)oxadiazole(4,3-a)quinoxaline-1-one
RP-HPLC — reverse-phase HPLC
sGC — soluble guanylyl cyclase
SNAP — *S*-nitroso-*N*-acetylpenicillamine
thionitrate — nitrothiol, nitrosulfanyl derivative
TPB — hydrotris-(3,5-dimethyl-1-pyrazolyl)borate
TSA — thiosalicyclic acid
YC-1 — 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole