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INVITED REVIEW

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The use of nitric oxide donors in pharmacological studies

Abstract A growing appreciation of the involvement of nitric oxide (NO) in numerous bioregulatory pathways has not only opened up new therapeutic avenues for organic nitrates and other NO donors but also led to an increased use of such compounds in pharmacological studies. By definition, all NO donors produce NO-related activity when applied to biological systems and are thus principally suited to either mimic an endogenous NO-related response or substitute for an endogenous NO deficiency. However, the pathways leading to enzymatic and/or non-enzymatic formation of NO differ greatly among individual compound classes, as do their chemical reactivities and kinetics of NO release. Moreover, since the reaction of NO with oxygen is a function of its concentration, the same absolute amounts of NO generated over different periods of time may lead to substantially different rates of NO_x formation and, consequently, to varying extents of side reactions, such as nitration and/or nitrosation of biomolecules. Matters are further complicated by compound-specific formation of by-products, which may arise during decomposition or metabolism, sometimes in amounts far exceeding those of NO. The term "NO donor" implies that the compound releases the active mediator, NO. Ultimately, this may be true for many different chemical classes of compound, since the principal NO-related species generated may be converted to NO, if not directly released as such. However, in a biological system, the redox form of nitrogen monoxide (NO^+, NO• or NO^-) that is actually released makes a substantial difference to the NO donor´s reactivity towards other biomolecules, the profile of by-products, and the bioresponse. Such considerations are likely to account for much of the discrepancy in experimental results obtained using the same cell or tissue preparation but different NO mimetics. Thus, compound selection is not a trivial issue and the investigator should be aware of the key properties and differences between various NO donor classes in order to avoid misinterpretation of experimental results.

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Introduction and scope

Amyl nitrite and glyceryl trinitrate, two representatives of a pharmacological class of compounds that used to be known as "nitrovasodilators", were introduced as therapeutic agents more than a century ago for relief from acute attacks of angina pectoris. Their vasodilator action is now known to be mediated by the release of nitric oxide (NO) following biotransformation in the target tissue. Thus, from the step of NO formation onward, the mode of action of these xenobiotics is identical to that of the endogenous, endothelium-dependent vasodilators. Besides the regulation of blood vessel diameter NO plays a key role in a variety of other cellular functions. In order to study the involvement of NO in a particular biological mechanism, one can either apply inhibitors of NO synthase to prevent endogenous formation of NO or administer exogenous NO. Because of the somewhat cumbersome nature of delivery of NO gas in solution or by inhalation there is increasing interest in compounds which generate NO or related N-oxide species in a controlled manner (the so-called "NO donors"). This paper provides a brief overview of the most widely used (and often misused) classes of NO donors. For a detailed treatise on this subject, including practical tips for compound handling and preparation of stock solutions, the reader is referred to a recent work published elsewhere (Feelisch and Stamler 1996).

General considerations

The different pathways of NO formation from NO donors

Although all NO donors somehow produce NO-related activity when applied in biological systems (and thus are prin-

cipally suited to mimic an endogenous NO response), the pathways leading to NO formation differ significantly among individual compound classes, as do their chemical reactivities. Some compounds require enzymatic catalysis while others produce NO non-enzymatically. Some NO donors require interaction with thiols in order to release NO, some have to undergo reduction, others oxidation. Matters are further complicated by compound-specific susceptibility to changes in pH, oxygen, light and temperature (which is important not only for the conditions of the actual experiment but also for the preparation of stock solutions), and by the formation of different by-products which arise during decomposition or metabolism. In some cases, a number of metabolites (of which the biological actions may be less well characterized or even unknown) are produced in amounts far exceeding that of NO.

NO flux versus total amounts of NO generated

The product of time of exposure to and concentration of NO largely determines the quality and magnitude of the biological response to exogenously applied NO. It follows that the kinetics of NO release from a given compound is in most cases more important than the absolute amounts of NO released. Thus, short-lived NO donors (like authentic NO) may have to be administered as continuous infusions rather than in bolus form in order to avoid the delivery of only a short burst of NO. In most cases, NO will have to be delivered continuously over the entire period of incubation in order to best mimic the biological mode of release. In aerobic solutions, the resulting NO concentration depends on the rate of NO formation during decomposition/metabolism of the NO donor and the autoxidation rate of NO. As the latter is a function of its concentration (i.e. the rate of reaction of NO with oxygen increases with increasing concentrations of NO; Kharitonov et al. 1994), the same absolute amounts of NO generated from different NO donors over different periods of time may lead to substantially different rates of dinitrogen trioxide (N_2O_3) formation and, as a consequence, to different extents of side reactions, such as nitrosation. When NO release closely follows compound decomposition (as holds true for most NONOates), it is possible to calculate the resulting NO concentration (Ramamurthi and Lewis 1997; Schmidt et al. 1997), provided no other NO-scavenging mechanisms are present. Estimated NO concentrations may, however, greatly deviate from actual concentrations in the presence of cells. For most other NO donors it is, however, difficult, if not impossible, to predict reliably the actual rate of NO formation, owing to the complex nature of decomposition and secondary reactions with, e.g., thiols. Thus, wherever possible, investigators should measure NO release under the prevailing experimental conditions to ensure that NO concentrations are well within the desired range. The mere determination of degradation products or measurement of the formation of nitrite/nitrate (NO_2^-/NO_3^-) as an index for NO formation can be misleading as both may occur without concurrent release of NO.

Redox state of the NO-related species released

The term “NO donor” has been used to imply that the compounds release the active mediator, NO. Ultimately, this may be true for many different pharmacological classes of compounds categorized as such, since the principal NO-related species that is generated may be converted to NO, if not directly released as such. However, in a biological system, it makes a substantial difference as to which NO-related species (i.e. NO^+, NO• or NO^-) is actually released. This will impact on the reactivity towards other biomolecules, the profile of by-products formed and the bioresponse. It should be kept in mind that NO donors are not just “donors of NO”, but may be endowed with distinct compound-specific activities. Even when applied at concentrations which will result in a comparable rate of NO release, compounds capable of transferring NO^+ equivalents (such as S-nitrosothiols) may have an action profile that differs substantially from that of, e.g., nitroxyl (HNO/NO^-)-generating agents such as Angeli´s salt. Moreover, the extent of conversion of NO^+ and NO^- generating agents to NO may differ largely among compounds, depending on ambient conditions and enzymatic profile of the tissue or organ preparation used.

“In vitro” versus “in vivo”

When working with isolated cell systems it is important to know whether the cells under investigation are capable of activating the chosen class of NO donor if the latter requires enzymatic biotransformation. For example, the absence of appropriate metabolizing enzymes provides an explanation for the poor anti-aggregatory activity of organic nitrates in washed platelets (Aissa and Feelisch 1992). It should also be kept in mind that the profile of metabolites generated in aqueous buffer systems may differ from that produced by the same compound in the tissue (see SIN-1 as peroxynitrite donor).

In an in vivo setting the situation tends to be even more complex. Most NO donors have limited tissue specificity, leading to NO formation at sites other than the intended one. Note, however, that the vasodilator effect of NO donors is mainly a consequence of the relative richness in tissue content of cytosolic guanylyl cyclase and thus of the high sensitivity of smooth muscle for NO and not the result of a selective biotransformation of the compound in the vascular wall. The vasodilator properties of classical NO donors limit their potential usefulness in non-cardiovascular applications, where lowering of systemic blood pressure often represents an unwanted side effect. In general, NO donors have disparate effects on systemic hemodynamics and local blood flow; these effects are dependent on their relative ability to relax resistance and conductance vessels. At low doses, organic nitrates, for example, predominantly reduce cardiac preload with little or no effect on cardiac output. This so-called venoselective effect of the nitrates clearly distinguishes them from the action of other NO donors. The additional preference of nitrates to dilate coronary conductance vessels is probably due to a lack of bioa-

ctivation in the resistance arteries (Kurz et al. 1991) and may be the reason why nitrates, unlike many other NO donors, do not cause a "coronary steal" (unfavourable redistribution of blood away from ischemic regions). The long-term application of NO donors in experimental animals may result in physiological counterregulations and produce rebound hemodynamic changes upon withdrawal. For example, some NO donors have been shown to decrease the hematocrit, indicative of significant plasma volume expansion. Moreover, a number of compounds (in particular the less lipophilic ones) are likely to penetrate barely the blood-brain barrier, making them less well suited for systemic application in studies aimed at modulating NO-dependent processes in the CNS. The opposite holds true for lipophilic compounds, where effects in the periphery may be confounded by unwanted (counter-regulatory) central actions following leakage of the NO donor from the local site of application into the systemic circulation. In many cases, effects obtained with comparatively high doses of NO donors are opposite to those observed at lower doses, stressing the importance of establishing always a proper dose-response relationship. This, of course, also holds true for studies in isolated cells or organs.

The different compound classes

In the following section, the main NO donor classes are briefly discussed with regard to their key chemical features and reactivity towards biologically relevant molecules. Unfortunately, for most compounds, our current knowledge of their reactivity in solution is limited and little or no information about the nature and extent of enzymatic biotransformation is available. The potential user should, however, be aware of the basic physico-chemical properties and health hazards of the compound planned to be used as a source of NO, particularly when it is known to be mutagenic or carcinogenic. Considerable caution should be taken not to inhale fumes of NO donors or to expose the skin (e.g. during weighing) to dust from solid compounds. Not less important is a proper information about the susceptibility of a given compound to, e.g., oxygen, light and changes in pH in order to prevent decomposition occurring in the stock solution. Sydnonimines, for example, are prone to alkaline hydrolysis and can be stabilized by shifting the pH to slightly acidic values (pH 5), whereas stock solutions of NONOates should be kept under strongly alkaline conditions; they will decompose rapidly as one approaches the physiological pH range. In general, it is recommended that stock solutions are made up freshly before use, kept on ice and protected from light (e.g. by use of darkened glass vials wrapped in aluminium foil). Final dilutions should preferably be prepared in assay buffer immediately before application and checked for pH. In some cases the buffer capacity may require adjustment, in particular when strongly acidic (hydrochloride salts) or alkaline (NONOates) stock solutions are used.

Table 1 provides a general overview of the key features and reactivities of different NO donor classes. Individual representatives of a certain compound class may, however, differ greatly in reactivity and basic physiochemical properties (the reader is referred to Feelisch and Stamler 1996 for details). A considerable number of compounds have recently become commercially available. Although this is good news for those researchers lacking proper in-house medicinal chemistry support, one should keep in mind that commercial availability is not necessarily an indication that the NO donor properties have been well characterized.

Organic nitrates

Organic nitrates ($RONO_2$s) are nitric acid esters of mono- and polyhydric alcohols and most representatives are only sparingly soluble in water. Stable stock solutions can be prepared in ethanol or dimethyl sulfoxide and stored properly sealed in brown glass containers for months to years. Organic nitrates are susceptible to alkaline hydrolysis, but are generally stable in neutral or weakly acidic aqueous solution. Clinically used representatives include glyceryl trinitrate (GTN), pentaerythrityl tetranitrate (PETN), isosorbide dinitrate (ISDN), and isosorbide 5-mononitrate (IS-5-N). Even after more than a century of therapeutic use the exact mode of action of organic nitrates remains a mystery. It is now clear, however, that the majority of their biological effects is attributable to the formation of NO and that thiols play a major role in their bioactivation. Whether they serve as cofactors or donors of reducing equivalents is not entirely clear. The partially denitrated metabolites (e.g., the glyceryl dinitrates and mononitrates of GTN) are still pharmacologically active, although considerably less potent than the parent nitrates. For NO release to occur, organic nitrates require either enzymatic or non-enzymatic bioactivation. It is likely that multiple intracellular and extracellular pathways contribute to NO formation from these compounds in vivo, but the relative importance of individual metabolic systems is poorly understood (Ahlner et al. 1991; Taylor et al. 1987; Harrison and Bates 1993; Bennett et al. 1994; Feelisch 1993; Torfgard and Ahlner 1994).

Nonenzymatic formation of NO from nitrate esters requires interaction with sulfhydryl (SH) groups. In the course of this reaction, thiols (RSHs) are oxidized to their respective disulfides (RSSRs) and nitrite (NO_2^-) is released as the major nitrogenous metabolite. Although virtually all thiol compounds decompose organic nitrates to inorganic nitrite, only a select few (i.e. cysteine, N-acetyl-cysteine and thiosalicylic acid, but not glutathione) promote concomitant NO generation as well (Feelisch et al. 1988; Chong and Fung 1991). Most likely, a thionitrate ($RSNO_2$) is formed as an intermediate via a transesterification reaction between the nitrate ester and the thiol (Yeates et al. 1985), giving rise to possible protein nitration reactions (via transfer of a NO_2^+ moiety). The finding that specific thiols are required for the vasorelaxant effect of nitrates and enhance their biological activity contributed to the concept that the impairment of bioactivity after long-term adminis-

Table 1 Key characteristics of different NO donor classes. The N-oxide species generated will vary with reaction conditions, may require oxidative or reducing conditions, and may often not be released in free form, but transferred to an acceptor instead. Sensitivity and/or reactivity to light, thiols etc. is not an indication that bioactivity is enhanced by these interventions as such pathways may also lead to compound inactivation (*GST* glutathione-S-transferase, *Cyt P450* cytochrome P450 enzyme system, *RT* room temperature, *ox* oxidative conditions, *red* reducing conditions)

Compound class	N-oxide species generated/donated				N-oxide generation at physiological pH		Sensitivity/reactivity to				Water solubility
	NO	NO^-	NO^+	NO_2^+	Non-enzymatic (conditions/cofactors)	Enzymatic (enzymes)	Thiols	Light	Oxygen	Hydrolysis (pH)	
Organic nitrates	+	–	–	+	+ (Red, specific thiols)	+ (GST, Cyt P_{450}, others)	+	–	–	+ (>7)	+/–
S-Nitrosothiols	+	+	+	–	+ (Enhanced by thiols, light and transition metals)	+ (Unknown)	+	+	+	+ (>2)	+/–
Sydnonimines (SIN-1)	+	?	–	–	+ (Enhanced by light, ox and pH >5)	+ (Molsidomine requires enz. hydrolysis)	–	+	+	+ (>5)	++
SIN-1A	+	–	(+)	–	+ (Ox, light, metals)	?	–	+	+	–	+
NONOates	+	(+)	(+)	–	+ (Acid-catalyzed)	? (Prodrugs require enz. hydrolysis)	+/–	(+)	+	+ (<10)	++
Sodium nitroprusside	+	(+)	+	–	+ (Light, red, thiols, nucleophils)	+ (Unknown)	+	+	+	–	++

tration of organic nitrates in vivo (nitrate tolerance) may be caused by intracellular thiol depletion. Measurement of plasma and intravascular thiol levels, however, have failed to confirm this hypothesis (Boesgard et al. 1994; Gruetter and Lemke 1985). The mechanisms involved in the phenomenon of nitrate tolerance are complex and will not be discussed here. It should be noted, however, that a dissociation between systemic hemodynamic and coronary arterial as well as anti-platelet effects has been reported (Kaltenbach and Schneider 1986; Hebert et al. 1997; Miyauchi et al. 1993), suggesting that tolerance may not necessarily occur to the same extent in all tissues.

Despite increasing efforts in recent years, the site of enzymatic biotransformation of organic nitrates has not yet been identified. Moreover, results from studies using enzyme inhibitors have been contradictory due, in part, to the lack of specificity of those compounds. The biphasic profile of NO formation from GTN and the differential susceptibility of tissues that support bioactivation of GTN to heat inactivation (Feelisch and Kelm 1991) suggest involvement of at least two distinct sites of biotransformation, one of which may be a membrane-bound enzyme (Seth and Fung 1993), although cytosolic enzymes are likely to be involved as well. Two enzyme systems have been proposed to account for the bioactivation of organic nitrates – an NADPH-dependent cytochrome P450 pathway (Schröder 1992; McDonald and Bennett 1993; McGuire et al. 1994) and certain isoenzymes of the glutathione S-transferase (GST) family (Kenkare et al. 1994). However, the contribution of these pathways to nitrate ester bioactivation has been questioned repeatedly (Liu et al. 1993; Haefeli et al. 1993; Kurz et al. 1993; Chung et al. 1992). In small coronary microvessels it has been demonstrated that administration of L-cysteine markedly enhances GTN-induced vasodilation. This effect is the result of L-cysteine serving as a precursor for intracellular glutathione synthesis. Thus, glutathione is likely to participate in the intracellular enzymatic bioconversion of GTN to NO (Wheatley et al. 1994). Alternatively, GST activity may represent an inactivation pathway for organic nitrates in the vasculature.

The requirement for specific thiols and/or enzymatic bioactivation steps for NO release to occur renders organic nitrates less than ideal compounds for the generation of predictable rates of NO in vitro; indeed NONOates appear to be far better suited for this purpose. However, organic nitrates possess clear advantages over other NO donor classes for use in animal studies since, with most representatives, their in vivo effects are reasonably well documented, the compounds are generally fairly stable and can be administered i.v., i.p. or i.m., transdermally or, in the case of PETN, ISDN and IS-5-N, also via the oral route. The latter two are long-lasting NO donors which can be administered in drinking water over periods of weeks to months.

S-Nitrosothiols

S-Nitrosothiols (RSNOs) are colored solids or liquids which are obtained by S-nitrosation of primary and secondary (pink or red) or tertiary (green) thiols. The cysteine residue of a number of proteins can also be nitrosated (Stamler et al. 1992a). Protein nitrosothiols are usually more stable than the S-nitroso derivatives of low-molecular-weight thiols and small peptides. Both S-nitrosylated low-molecular-weight thiols (such as S-nitrosoglutathione) and protein thiols (in particular S-nitrosoalbumin) have been detected in biological fluids as a consequence of endogenous N-oxide metabolism and exogenous administration of NO (Gaston et al. 1993; Stamler et al. 1992b). It has been proposed that S-nitrosothiol groups in proteins serve in the metabolism of NO and in regulation of cellular functions such as the transport and targeting of the NO group to specific, thiolregulatory effector sites, including enzymes and signaling proteins (Stamler 1995).

Only a few RSNOs have been isolated in solid form, including S-nitroso-N-acetyl-DL-penicillamine (SNAP; Field et al. 1978), S-nitrosoglutathione (GSNO; Hart 1985), and S-nitrosoalbumin (J.S. Stamler, unpublished). As a solid, the first can be stored for years at room temperature, provided it is kept dry and protected from light. GSNO can be stored desiccated in the dark at –20°C for at least 6 months. The more unstable nitrosothiols must be prepared in situ shortly before use (for preparation see Stamler and Feelisch 1996). In physiological buffers, many RSNOs undergo relatively rapid decomposition to yield the corresponding disulfide and NO by homolytic fission of the S-N bond. Thiyl radical intermediates (RS•) are believed to be responsible for the mutagenicity of S-nitrosothiols in the Ames test (Carter and Josephy 1986). Stability in solution varies as a function of temperature, pH, oxygen pressure, nucleophiles, redox-active species and trace metal content of the incubation solution. The half-life of CysNO, for example, can vary from seconds to hours at pH 7.4, depending on the nature of the buffer. It has recently been shown that homolytic decomposition of S-nitrosothiols, yielding thiyl radicals (RS•) and NO, is catalyzed by trace amounts of cuprous (Cu^+) and ferrous (Fe^{2+}) ions (McAninly et al. 1993), and that the biological activity of some RSNOs can be significantly reduced by chelation (e.g. with EDTA or DTPA) of copper ions (Gordge et al. 1995). Further studies have revealed that the effects of thiols and other reductants on the effect of RSNOs can be explained by reduction of Cu^{2+} (present in the water used to prepare the buffer solution) to Cu^+ (Dicks et al. 1996; Gorren et al., 1996). Thus, careful control of the metal content of buffers is required to achieve reproducible results with S-nitrosothiols. In addition to homolytic cleavage, nitrosothiols can also decompose heterolytically with formation of NO^+ or NO^- (Arnelle and Stamler 1995), a pathway of decomposition that appears to predominate in biological systems. Thus depending on the reaction conditions, nitrosothiols can act as donors of NO, NO^- or NO^+. Indeed, pharmacologcial activities of S-nitrosothiols do not correlate with lipophilicity, charge, stability in solution or with the rate of "spontaneous" NO release from individual compounds (Gaston et al. 1994; Kowaluk and Fung 1990; Mathews and Kerr 1993; Salas et al. 1994). Other important processes to consider with S-nitrosothiols are transnitrosation reactions, i.e. the transfer

of bound NO from one thiol group to another (including the reversible blockade of critical thiol groups in enzymes). This feature clearly distinguishes S-nitrosothiols from other NO donor classes. In fact, there is growing appreciation that some of the pharmacological actions of RSNOs are not mediated by the release of NO but are a consequence of nitrosation reactions at cellular proteins. Although individual compounds may have very different stabilities in vitro, their pharmacodynamic behaviour is often remarkably similar. The in vivo application of a given RSNO is thus likely to lead to rapid transnitrosation reactions with tissue and plasma thiols. The contribution of metal-catalyzed NO formation from S-nitrosothiols in biological systems is unclear at present as the effects of chelating agents have been shown to vary with the type of the bioassay, the chelator and nature of the thiol (Gordge et al. 1995).

RSNOs are photosensitive, but stock solutions can usually be kept on ice in the dark for several hours. If compatible with the assay, metal chelating agents such as EDTA or DTPA may be added to increase stability. Highly unstable compounds such as S-nitrosocysteine should be prepared freshly and diluted in buffer immediately before use.

Sydnonimines

The most studied representative of this class of heterocyclic NO donor is molsidomine (N-ethoxycarbonyl-3-morpholino-sydnonimine), which is in clinical use as an anti-anginal drug in several countries. Molsidomine is a prodrug which is converted by liver esterases to the active metabolite, SIN-1 (3-morpholino-sydnonimine; linsidomine). Whereas molsidomine itself is only poorly vasoactive in vitro, SIN-1 is a potent vasorelaxant and anti-platelet agent. These activities are thought to be mediated largely by the release of NO. Soluble guanylyl cyclase activation by sydnonimines is generally independent of the presence of thiols and can differ by a factor of 1000 among different compounds of this class (Feelisch et al. 1989; Noack and Feelisch 1989). However, mechanisms of NO release from sydnonimines cannot be generalized as other molsidomine-related compounds, e.g. metabolites of CAS 936 (pirsidomine) appear to be subject to enzymatic degradation (Mülsch et al. 1993). Furthermore, not all the biological effects of sydnonimines can be attributed to NO. CAS 936, for example, was shown to relax potassium-depolarized vascular tissue in a hemoglobin-insensitive manner (Bohn et al. 1991) and SIN-1-induced suppression of neutrophil degranulation was shown not to be mediated by cGMP, NO or $ONOO^-$, but by the NO-lacking metabolite, SIN-1C (Kankaanranta et al. 1997).

Molsidomine and related sydnonimine compounds are stable solids which can be stored, protected from light, at room temperature. Stock solutions can be prepared in DMSO or DMF, respectively, and may be applied i.v. or orally. The hydrochloride salts of SIN-1 and related sydnonimines are stable in solid form when stored dry and protected from light at 4°C. SIN-1 is highly water-soluble and stable in acidic solution. Aqueous stock solutions of SIN-1 made up in deoxygenated distilled water adjusted to pH 5 can be used throughout the entire day of an experiment. At physiological pH, and more so at alkaline pH, SIN-1 undergoes rapid non-enzymatic hydrolysis to the ring-open form SIN-1A, which chemically is a nitrosamine. Under strictly anaerobic conditions SIN-1A is stable at pH 7.4, provided that the solution is protected from light. Traces of oxygen promote oxidative conversion to a cation radical intermediate which, upon NO release and deprotonation, undergoes cleavage to the corresponding N-morpholino-aminoacetonitrile, SIN-1C. In the course of this reaction stoichiometric amounts of superoxide anions (O_2^-) are formed as a result of oxygen reduction (Feelisch et al. 1989). Oxygen consumption may become important when these compounds are applied to biological systems. In particular, when high sydnonimine concentrations are used under oxygen-limiting conditions, this can quickly render such incubates hypoxic or even anoxic. Besides O_2^-, protons (H^+) are generated during sydnonimine decomposition, which may lead to a considerable decreases in pH if unbuffered aqueous solutions of SIN-1 or SIN-1A are allowed access to room air. As NO is known to react with O_2^- at almost diffusion-controlled rate (Huie and Padmaja 1993), peroxynitrite ($ONOO^-$) production is inevitable. Formation of $ONOO^-$ from SIN-1 has been shown to promote lipid peroxidation (Hogg et al. 1992; Darley-Usmar et al. 1992). This and other observations have led to the general misconception that SIN-1 is in fact an $ONOO^-$ rather than an NO donor. Whereas $ONOO^-$ formation may occur on decomposition of SIN-1 in aqueous buffer solution, NO release from this NO donor is not restricted to reaction with molecular oxygen (Bohn and Schönafinger 1989). Oxidants other than O_2 and certain redox-active enzymes can promote oxidation of and NO release from SIN-1A. For example, oxidation of SIN-1 is supported by cytochrome c in a superoxide-independent manner under anaerobic conditions (Werringloer et al. 1990). Under such conditions an enzyme, rather than molecular oxygen, promotes oxidation and NO release, and no $ONOO^-$ is formed. Such reactions are likely to be involved in the vascular bioactivation of these compounds. Although some “free” NO from SIN-1 may escape the reaction with O_2^- due to scavenging of the latter by tissue SOD, it would otherwise be difficult to understand why SIN-1 is approximately three log-orders more potent a vasodilator than peroxynitrite (M Feelisch, unpublished).

Solid SIN-1A, which can be prepared from SIN-1 with aqueous bicarbonate under argon, is best stored at –80°C under an inert and dry atmosphere. However, even under these conditions stability is limited to a few months. This drawback can be overcome by complexation of SIN-1A with cyclodextrins (CDs; Vikmon et al. 1995). SIN-1A/CD inclusion complexes were found to be exceptionally stable, allowing storage at room temperature for several years without significant decomposition. In aqueous solution, the complex instantaneously generates NO in predictable amounts at constant rates and for prolonged periods of time. NO release is increased in the presence of oxygen and transition metals, but is hardly influenced by the presence of thiols (M. Feelisch, unpublished observations).

NONOates

NONOates are adducts of NO with nucleophiles (X^-; usually an amine, but other reaction partners, such as sulfite, are known as well) in which an NO dimer is formally bound to the nucleophilic residue via a nitrogen atom, thus forming a functional group of the structure X-[N(O)NO]$^-$. The in vitro vasorelaxant activities of a series of different NONOates was found to correlate well with the measured rates of NO release and the same may apply under in vivo conditions. It is thought that NONOates generate NO by acid-catalyzed dissociation with regeneration of the free nucleophile and NO, although enzymatic metabolism in vivo cannot be excluded. The rate of release appears to be largely unaffected by biological reactants. This important property, in addition to their predictable rates of NO release, make NONOates a valuable tool in NO research. NO liberation properties of individual compounds are determined by the structure of the nucleophile, the pH and the temperature of the incubation solution (Morley and Keefer 1993). The decomposition of NONOates is pH-dependent, proceeding at an extremely slow rate at values >9, a moderate rate at physiological pH and almost instantaneously at acidic pH. The reaction follows first-order kinetics, yielding up to 2 moles of NO per mole NONOate. At higher concentrations of NONOates and at physiological pH, the yield is usually less than this. However, batch to batch variations in the stoichiometry of NO release may occur, again stressing the importance of *measuring* rather than *estimating* NO concentrations using mathematical models (Schmidt et al. 1997). Reported half-lives at pH 7.4 and 37°C range from 2 min for diethylamine NONOate (DEA/NO) to 20 h for diethylene triamine NONOate (DETA/NO; Mooradian et al. 1995; Maragos et al. 1991). This difference in stability is mirrored by the potency difference in biological test systems. By variation of the nucleophile structure, NONOates with custom-tailored NO time-release profiles have been synthesized and a large number of different compounds are now commercially available. The most important criteria for selection of the most appropriate NONOate for a certain pharmacological investigation is the rate of dissociation of NO and the properties of the products generated during decomposition. Potential effects of the nucleophile should always be controlled for, especially as they may be present as impurities in commercially available batches. Indeed, polyamines have well-known biological activities, and some receptor systems are regulated by polyamine binding.

The mechanism of decomposition of the sulfite NONOate (SULFI/NO) forms a notable exception as it disproportionates to sulfate and N_2O rather than to sulfite and NO. The formation of N_2O suggests that SULFI/NO is a donor of nitroxyl (HNO/NO^-), which possesses biological activity in its own right. At least one other compound, the propylamino-propylamine/NONOate (PAPA/NO), was shown to form nitrosylmyoglobin from metmyoglobin (Maragos et al. 1993), indicative of NO^- production. Furthermore, Angeli´s salt, formally a NONOate where O^{2-} serves as the nucleophile (OXI/NO), is regarded as a "classical" nitroxyl donor. Thus, the formation of NO^- from certain NONOates is an important consideration.

Almost all NONOates are highly soluble in water. Aqueous stock solutions can be prepared in cold deoxygenated 1–10 mM NaOH (pH 12) just prior to use. Alkaline stock solutions are stable for several hours if kept on ice in the dark. Their characteristic UV absorbance can be used for the quantification of aqueous stock solutions as well as for spectrophotometric monitoring of their decomposition in vitro. To initiate NO release from NONOates, an aliquot of the alkaline stock solution is rapidly mixed with an excess of physiological buffer.

The first-order decomposition of NONOates makes these compounds well suited as general-purpose NO generators for in vitro studies but represents a clear limitation for their application in vivo. Therefore, attempts have been directed at designing prodrugs that cannot release NO unless enzymatically converted to the corresponding NONOate in the target tissue. The same approach has been applied to develop representatives which are subject to liver-specific biotransformation (Saavedra et al. 1997). O-alkylation of NONOates of secondary amines leads to derivatives with considerably enhanced stability (Saavedra et al. 1992). While DEA/NO has a half-life of 2 min at 37°C, pH 7.4, its ethylated derivative shows no decomposition at all. At the same time enzymatic bioactivation occurs in vivo as evidenced by blood pressure-lowering activity. However, the decomposition products of these derivatives are less predictable than those of the non-alkylated species, and formation of N-nitrosamines has to be considered a possibility.

Sodium nitroprusside

The most widely studied of the iron nitrosyls is sodium nitroprusside (SNP; sodium pentacyanonitrosyl ferrate(II)), an inorganic complex where iron is in the ferrous state (Fe^{2+}) and NO is formally bound as NO^+. The compound is used clinically to reduce blood pressure, e.g. in hypertensive emergencies. The nitroprusside anion undergoes addition reactions with a variety of sulfur-, oxygen-, nitrogen- and carbon-centered nucleophiles some of which have served as the basis of analytical tests. Despite intensive studies of its chemical reactivity, in particular with thiols, the mechanism of NO release remains incompletely understood. It is clear, however, that SNP requires either irradiation with light or one-electron reduction to release NO. Earlier reports of "spontaneous" release of NO were probably due to ambient light or to the light source of the spectrophotometer.

In biological systems, both non-enzymatic and enzymatic NO release from SNP may occur. Attack of thiolate anions (RS^-; membrane-bound or free) may lead to decomposition of the complex with formation of disulfide (RSSR), NO and cyanide (CN^-). Upon addition of SNP to tissues, formation of nitrosyl-iron complexes with thiols have been detected (Rochelle et al. 1994; Kleshchev et al. 1985), which may be in dynamic equilibrium with S-nitrosothiols and dinitrosyl-iron complexes. Indeed, nitrosation reactions

are sustained by SNP, although the exact mechanism of RSNO formation is not known. A membrane-bound enzyme may be involved in the generation of NO from SNP in biological tissues, and either NADH or NADPH appears to be required as a cofactor (Bates et al. 1991; Kowaluk et al. 1992; Rao et al. 1991; Mohazzab-H et al. 1992).

SNP, which is a brownish-red crystalline salt of the composition $Na_2[Fe(CN)_5NO] \times 2H_2O$, can be stored for years at room temperature when kept dry and protected from light. Commercially available freeze-dried material for clinical use can be stabilized with sodium citrate. SNP in solution is extremely photosensitive. Slight acidification (pH 3–5) and exclusion of oxygen can enhance stability. Aqueous solutions of SNP should be made freshly before use and kept in the dark. NO release is usually enhanced by thiols, but the kinetics are not straightforward. As SNP reduction and subsequent decomposition of the nitrosyl complex is accompanied by cyanide release (a maximum of five equivalents of CN^- per mole SNP), controls with this anion should be routinely included. SNP has a balanced action on resistance and capacitance vessels. The rapid restoration of blood pressure after cessation of infusion is indicative of rapid compound degradation. The fact that it can only be given intravenously and CN^- may accumulate during long-term application, however, limits its usefulness as NO donor for in vivo studies.

Specificity of NO donor actions

In general, NO donors are applied as pharmacological tools in the understanding that most, if not all, of their biological effects are mediated by NO. This, however, may not always be the case as metabolites or decomposition products of the respective compound may have biological effects in their own right. It is advisable, therefore, always to include proper controls which confirm that the respective action was indeed elicited by NO. This can be achieved in several ways.

The best positive control is a comparison with authentic NO. Often this will not be possible due to technical application problems or simply due to lack of access to aqueous NO solution. A description of the handling and preparation of aqueous solutions of NO is beyond the scope of this chapter (descriptions can be found in Feelisch 1991; Beckman et al. 1996), but readers should be advised that the mere bubbling of NO gas into aerobic buffers does not represent an appropriate alternative. Under those conditions, gaseous NO will react with oxygen dissolved in the buffer solution and lead to the formation of higher N-oxides at the gas/liquid interface promoting nitrosation reactions.

Where a comparison with authentic NO cannot be performed scavengers of NO should be applied. There are numerous compounds available which differ in specificity and also in their ability to gain access to the site of NO action. Oxygenated hemoglobin (HbO_2) is generally a good choice, and concentrations of 5–10 μM are usually sufficient to scavenge NO effectively. The reaction between NO and HbO_2 is rapid and stoichiometric, resulting in the formation of methemoglobin and nitrate (NO_3^-; Doyle and Hoekstra 1984). Because of its size HbO_2 does not cross cell membranes and thus can only scavenge that portion of the NO which is generated in the extracellular/interstitial space. Thus, if the respective NO donor requires intracellular bioactivation to release NO, as do organic nitrates, HbO_2 is likely to have less of an effect than with compounds such as NONOates which generate NO even in the extracellular compartment. HbO_2 does not inhibit transnitrosation reactions. The lack of inhibition by HbO_2 of a biological effect of an NO donor such as a nitrosothiol may indicate the involvement of S-nitrosation rather than the release of "free" NO as the principal mode of action.

Where access to intracellular sites is mandatory, compounds can be used which are able to cross cell membranes and promote the release of O_2^-. Compounds of this type include the dye methylene blue (Wolin et al. 1990) and the Lilly compound LY83583 (Mülsch et al. 1989), both of which are also inhibitors of soluble guanylyl cyclase, and pyrogallol. Note that all these compounds will only work under aerobic conditions as molecular oxygen is used for O_2^- generation. Another class of compounds, the nitronyl nitroxides, which were introduced earlier for the quantification of NO by ESR (Yoshida et al. 1994), can also be used as NO scavengers. These phenyl-tetramethylimidazoline-oxyl-oxides (PTIOs) inactivate NO by oxidative transformation to NO_2. The reaction between NO and PTIOs is believed to be stoichiometric, but proceeds at a considerably slower rate than the reaction between NO and HbO_2. One of the limitations of PTIOs for their use in biological systems is that they are susceptible to non-specific reduction by various reducing agents such as ascorbate, thiols and O_2^-. Moreover, direct cytotoxic effects have been observed in cultured cells. Furthermore, the NO_2 generated as the principal end-product of NO inactivation by PTIOs can support nitrosation reactions.

Conclusions

The general considerations about the application of NO donors and the specific differences between individual compounds discussed above may account for much of the discrepancy in experimental results obtained using the same cell or tissue preparation but different NO mimetics. The complexity of the underlying biochemistry shows that compound selection is not a trivial issue. As crucial as the design of the experiment is the awareness of investigators of the key properties and differences between various NO donor classes so that they are able to select the most appropriate compound for their pharmacological studies and avoid misinterpretation of experimental results.

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