Formation of Nitroxy1 and Hydroxy1 Radical in Solutions of Sodium Trioxodinitrate

EFFECTS OF pH AND CYTOOTOXICITY

Juliana Ivanova§, Guy Salama§, Robert M. Clancy¶, Nina F. Scho1, Karen D. Nylander, and Detcho A. Stoyanovský‡

From the §Department of Surgery, University of Pittsburgh, Pittsburgh, Pennsylvania 15213, the ¶Department of Cell Biology and Physiology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, the ¶Department of Rheumatology, New York University School of Medicine, New York, New York 10016, and the §§Departments of Pediatrics, Neurology, and Pharmacology, University of Pittsburgh, and Pediatric Center for Neuroscience, Children’s Hospital of Pittsburgh, Pittsburgh, Pennsylvania 15213

Despite its negative redox potential, nitroxy1 (HNO) can trigger reactions of oxidation. Mechanistically, these reactions were suggested to occur with the intermediate formation of either hydroxy1 radical (‘OH) or peroxynitrite (ONOO‘). In this work, we present further experimental evidence that HNO can generate ‘OH. Sodium trioxodinitrate (Na2N2O3), a commonly used donor of HNO, oxidized phenol and MeSO to benzene diols and ‘CH3, respectively. The oxidation of MeSO was O2 independent, suggesting that this process reflected neither the intermediate formation of ONOO‘ nor a redox cycling of transition metal ions that could initiate Fenton-like reactions. In solutions of phenol, Na2N2O3 yielded benzene-1,2-diol and benzene-1,4-diol at a ratio of 2:1, which is consistent with the generation of free ‘OH. Ethanol and MeSO, which are efficient scavengers of ‘OH, impeded the hydroxylation of phenol. A mechanism for the hydrolysis of Na2N2O3 is proposed that includes dimerization of HNO to cis-hyponitrous acid (HON-N=O=OH) with a concomitant azo-type homolytic fission of the latter to N2 and ‘OH. The HNO-dependent production of ‘OH was with 1 order of magnitude higher at pH 6.0 than at pH 7.4. Hence, we hypothesized that HNO can exert selective toxicity to cells subjected to acidosis. In support of this thesis, Na2N2O3 was markedly more toxic to human fibroblasts and SK-N-SH neuroblastoma cells at pH 6.2 than at pH 7.4. Scavengers of ‘OH impeded the cytotoxicity of Na2N2O3. These results suggest that the formation of HNO may be viewed as a toxicological event in tissues subjected to acidosis.

The biochemistry of nitroxy1 (HNO) has attracted considerable interest in recent years. In cells, the biosynthesis of HNO is believed to proceed via reduction of NO by superoxide dismutase (1) and cytochrome c (2), and reduction of S-nitrosoglutathione by low molecular weight and protein thiols (3–5). It has been suggested that HNO can affect the etiology of various pathophysiological conditions such as inflammation and neurodegenerative diseases, especially when H2O2 and transition metal ions are present (6, 7). Similar to NO and NO+, HNO is a potent inducer of the antioxidant protein heme oxygenase 1 (8), exhibits vasorelaxant properties (9), and modulates the activity of thiol-containing proteins, such as aldehyde dehydrogenase (10, 11) and the N-methyl-b-aspartate receptor (12, 13). In vivo experiments, Paolocci et al. (14) observed that HNO exerts positive inotropic and lusitropic action, which unlike NO and nitrates is independent and additive to β-adrenergic stimulation and increases the release of plasma calcitonin gene-related peptide; these results suggest that donors of HNO are potential prodrugs for the treatment of heart failure (14). At high doses, HNO has been shown to induce DNA single-strand breakage (15, 16) and a concentration-dependent cytotoxicity in murine thymocytes (16). This cytotoxicity was associated with activation of the nuclear nick sensor enzyme poly(ADP-ribose)polymerase, perturbation of the mitochondrial membrane potential, and an increased production of superoxide (16).

On a molecular level, there are several differences between the reactivity of NO and HNO that may account for the distinct biological effects of the latter species: in contrast to NO, HNO directly interacts with thiols (4, 17), it preferentially binds to Fe(III) complexes (18, 19), and acts as a hydroxylating agent (15, 20, 21). Recently, we have reported that HNO can generate ‘OH in a pH-dependent manner (20). Because of its high reactivity, ‘OH is one of the most toxic species that can be formed in biological systems. This free radical reacts with most cellular molecules at diffusion-controlled rates; thus it cannot diffuse from its site of generation further than the nearest molecules (22). Hence, we hypothesized that the pH-dependent generation of ‘OH from HNO can have toxicological significance, particularly because tissue acidification occurs under various pathological conditions, such as hypoxia, inflammation, and cancer (23–25). In the present work, we provide further experimental evidence that HNO generates ‘OH in an oxygen-independent manner. We also report that HNO exhibits a pH-dependent toxicity to normal human fibroblasts and SK-N-SH neuroblastoma cells that could be impeded by scavengers of radical species.

EXPERIMENTAL PROCEDURES

Reagents—All reagents used were purchased from Sigma. The solutions used in the experiments were prepared in deionized and Chelex 100-treated water or potassium phosphate buffer. Sodium trioxodinitrate was synthesized from Na2N2O3, following a procedure reported by Haddad et al. (26). The solution of sodium trioxodinitrate was prepared by the addition of Na2N2O3 (4.0 M) to deionized and Chelex 100-treated water (2:1), in a ratio of 2:1 to yield a solution with a pH of about 7.4. The solution was then adjusted to the desired pH with pH 1.0 and pH 10.0 to yield the final solution.

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The abbreviations used are: HPLC, high performance liquid chromatography; PBN, N-tert-butyl-α-phenylnitrone; MGD, N-methyl-D-glucamine dithiocarbamate; DMPO, 5,5′-dimethyl-1-pyrolline N-oxide.

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Fig. 1. EPR spectra of ON-FeIII-MGD and DMPO/OH formed in solutions of Na2N2O3, FeIII-MGD, and DMPO. All reactions were carried out at 20°C in either 0.1 M Tris (panel A) or 0.1 M phosphate buffer (panel B). The reactions were initiated by the addition of Na2N2O3 and EPR spectra were recorded 4 min thereafter. A, FeCl3, 0.3 mM; MGD, 1 mM; Na2N2O3, 0.1 mM. B, DMPO, 100 mM; Na2N2O3, 0.3 mM. For each experiment, a fresh stock solution of Na2N2O3 was prepared in 10 mM NaOH.

RESULTS

EPR Analysis of the Hydrolysis of Na2N2O3—In model studies aimed at mimicking the biochemistry of HNO, sodium trioxodinitrato(Na2N2O3; Angeli’s salt) is often used as a donor of this species. Depending on the degree of protonation, the stability of this compound in aqueous solutions follows the sequence N2O3− > HNO2− > H2N2O3−. (1e) (28) and pHK = 3.0 and pHK = 9.35; Scheme 1) (28). 1c is relatively stable in alkaline solutions (pH > 10). However, the rate of decomposition of 1b within the pH interval 4–8 is [H+]−independent and leads to the formation of HNO (Kb = 5.1 × 10−4 M−1 s−1 (29)). The latter species can dimerize to cis-hyponitrous acid (3a), which is unstable and decomposes to H2O and H2O2. The decomposition of 3a is especially fast in aqueous solutions with pH 6–13 (30, 31), which most likely reflects shifts in the equilibrium between 3a and 3b in favor of the latter (Scheme 1).

Bonner and Ravid (29) reported that the hydrolysis of Na2O(NO2)2 at either pH 3.0 or 8.5 yields exclusively 15N2O and NO2, implying that this process follows the reaction sequence presented in Scheme 1 (29). However, these authors noted that the proportions of 15N in the reaction products were different at pH 5.0, suggesting that some modification of pathway cannot be ruled out. At pH < 4, the decomposition rate of 1a increases with increasing acidity with production of NO−.

We recently reported that 1b can convert primary alcohols to aldehydes via the intermediate formation of OH (20). In these experiments, the formation of OH was characterized by EPR spin trapping analysis. However, we could not evaluate the absolute amounts of spin-trapped OH as the resulting nitroxides are readily converted to EPR silent hydroxylamines under reductive conditions (32). Quantitative evaluation of the latter reaction is important because the production of OH from HNO may have toxicological implications. Hence, we have carried out EPR/HPLC-UV/EC spin trapping experiments optimized for the quantitation of OH under reductive conditions.

In the presence of FeIII and N-methyl-D-glucamine dithiocarbamate (MGD), the hydrolysis of 1b was paralleled by the appearance of the characteristic EPR spectrum of ON-FeIII-MGD formed via the interaction of HNO and FeIII-MGD (Fig. 1A; Scheme 2) (18). The formation of ON-FeIII-MGD was H−-independent within the pH interval of 4.5 to 7.4 (Fig. 1A), which is in agreement with previous findings (28) that the rate of 1b hydrolysis at these proton concentrations is constant. The substitution of FeIII-MGD with 5,5′-dimethyl-1-pyrolline N-oxide (DMPO) resulted in the appearance of the typical EPR spectrum of DMPO/OH (6, Fig. 1B) (33), suggesting that the hydrolysis of 1b resulted in the formation of OH. In contrast to the formation of HNO, the generation of OH from 1b was strongly affected by the acidity of the reaction solutions. The latter implies that OH was not directly derived from 1b but rather followed the release of HNO (Fig. 1, panel A compared with panel B; Fig. 4D).
Nitroxyl-dependent Generation of Hydroxyl Radical

The low stability of OH-derived nitroxides (t_{1/2} \sim 30 \text{ s} to 15 \text{ min} (34, 35)) is a limiting factor for quantification of OH. To solve this experimental difficulty, we used an HPLC protocol for quantification of OH that is based on the oxidation of Me$_2$SO (32). The latter is oxidized by OH to CH$_3$, which forms relatively stable nitroxides (t_{1/2} > 48 \text{ h}) with PBN. The hydrolysis of 1b in the presence of Me$_2$SO and PBN produced the typical EPR spectrum of PBN/CH$_3$ (Fig. 2 trace 2) (33). However, both 1b and HNO could act as reductants (36, 37), suggesting that the EPR spectrum of 4 may not reflect the real amount of OH and CH$_3$ formed in this reaction system. In the presence of reductants, nitroxides can be readily reduced to the corresponding EPR silent hydroxylamines (32). In support of the latter assumption, the addition of K$_3$[Fe(CN)$_6$] to an extract resulted in a pronounced increase of the EPR signal of 4. To solve this experimental difficulty, we used an HPLC protocol of a reaction solution consisting of Na$_2$N$_2$O$_3$, PBN, and Me$_2$SO. The latter is oxidized by hydroxylamine effect most likely reflected the oxidation of the EPR silent nitroxides (36). The efficiency of this reaction system, however, cannot be estimated as the concentration of 1b was analyzed by HPLC with electrochemical detection, the latter assumption, the addition of K$_3$[Fe(CN)$_6$] to an extract was confirmed by coinjections of authentic HPLC standards as described previously (32, 38).

The formation of 4 and 5 in solutions of 1b, PBN, and Me$_2$SO was well controlled and with an yield of 7.5% of the initial concentration of 1b (Fig. 4). The actual production of OH in this reaction system, however, cannot be estimated as the efficiency of the OH-dependent oxidation of Me$_2$SO and the subsequent spin trapping of CH$_3$ are undefined. Under anaerobic conditions, the reaction profile remained unchanged (Fig. 4A, open circles), which attests that the generation of OH from HNO was O$_2$-independent and reflected neither the intermediate formation of ONOO$^-$ (Scheme 4) (21, 39) nor the occurrence of Fenton-like reactions. Maximal production of 4 and 5 was observed within the pH interval of 4 to 6. At pH 6, the production of 4 and 5 was 1 order of magnitude higher than that at pH 7.4.

Hydroxylation of Phenol in Aqueous Solutions of Na$_2$N$_2$O$_3$—Phenol is often used as a molecular probe to discriminate free OH from other oxidizing species. For example, radiolytically generated OH reacts with phenol via either abstraction of the hydrogen atom from its -OH function ($k_H = 2.1 \times 10^9 \text{ m}^{-1} \text{ s}^{-1}$) or arylation addition ($k_{\text{aryl}} = 6.6 \times 10^9 \text{ m}^{-1} \text{ s}^{-1}$) to give a phenol phenoxyl (10) or dihydroxycyclohexadienyl (8) radical, respectively (40, 41). Under aerobic conditions, 8 interacts with O$_2$ to form benzene diols (9) and the superoxide anion radical; benzene-1,2-diol and benzene-1,4-diol, forming at a ratio of 2:1, are the main isomers generated in this reaction (41). In contrast, oxidation of phenol by Fenton-type reagents such as L$_3$Fe$^{II}$-O$_2$H leads to the formation of benzene-1,2-diol and benzene-1,4-diol at a ratio that depends on the ligand of the corresponding iron complex and ranges from 11 to $\sim$ 41 (41). It should be noted that 8 and 10 can follow several reaction pathways, implying that the formation of 9 can only be a qualitative marker for free OH. For example, the hydroxylation of phenol by OH in the absence of oxygen (or other electron acceptors, such as K$_3$[Fe(CN)$_6$] and quinones (42)) predominantly leads to the formation of biphenyl diols (11 (41, 43)) (Scheme 3).

The formation of 4 in solutions of 1b and phenol in 0.1 M phosphate buffer (pH 4.0), 1b caused a time- and dose-dependent hydroxylation of phenol to 9 with an overall yield of 80% (Fig. 6, A and B). Within the pH interval of 4 to 7 (pH 6, 0.5), the ratio between benzene-1,2-diol and benzene-1,4-diol was 2.2 + 0.24 ($n = 5$;
Nitroxyl-dependent Generation of Hydroxyl Radical

Effects of pH on the Cytotoxicity of Na₃N₂O₃—The pH-dependent production of ·OH from Na₃N₂O₃ suggests that this compound may exert a H⁺-amplified cytotoxicity. This possibility is interesting because acidification of tissues occurs under various pathological conditions, such as ischemia, inflammation, and cancer (24, 47, 48). For example, the intense metabolism of glucose to lactic acid leads to acidification in the microenvironment of tumor tissues. In actively glycolyzing tumors, the extracellular pH is in the range of 6.0 to 7.0 (reviewed in Ref. 24), whereas the extra- and intracellular milieu of most tissues has a pH of 7.4.

Fig. 9 depicts the cytotoxic effect of Na₃N₂O₃ on normal human fibroblasts and SK-N-SH neuroblastoma cells. A marked cytotoxicity was observed at pH 6.2, as compared with pH 7.4, respectively. The toxic effect of Na₃N₂O₃ was pronounced several hours after the treatment of the cells. Delayed cell death induced by exposure to oxidative stress has been described in a number of other cell systems and has usually been attributed to apoptosis (49, 50). The cytotoxicity of Na₃N₂O₃ was pH-dependent as suggested by the protective effect of ascorbic acid, α-(4-pyridyl-1-oxide)-N-tert-butylisotriazole, and Me₃SO, which are efficient scavengers of ·OH (Fig. 9, B and C) (32, 34). No toxicity was observed when cells were treated with acidic buffers that did not contain Na₃N₂O₃ (data not shown).

DISCUSSION

The distinct cellular responses that HNO can trigger have made this species the subject of intense research. However, in contrast to NO⁺, for which there is a relatively well-established mechanistic and derivative chemistry, our knowledge of HNO remains sparse. HNO is a weak acid whose pK value and redox potential have recently been corrected from 4.5 and −0.3 V to 11.4 and −0.8 V, respectively (37, 51, 52). Although HNO is a strong reductant, it has been established that this species triggers reactions of oxidation. Mechanistically, these reactions were suggested to occur with the intermediate formation of either ·OH (15, 20, 21) or (an isomer of) (39) ONOO⁻ (Scheme 4) (21, 53). However, there are conflicting reports regarding the formation of ONOO⁻ in acidic-to-neutral solutions of HNO. The potential of Na₃N₂O₃, a commonly used donor of HNO, to generate ONOO⁻ via the intermediate formation of singlet NO⁻ ("NO⁺") was the focus of a series of studies. Upon relaxation of "NO⁺", a triplet state of NO⁻ ("NO⁺") is formed that reacts with O₂ to form ONOO⁻ (51, 54). Various substrates of ONOO⁻ were reported to undergo oxidation in neutral aqueous solutions of Na₃N₂O₃ with yields of the corresponding reaction products ranging from 1.9 to 65% (21, 53).

On the other hand, Donald et al. (55) reported that ONOO⁻ and its product of decomposition, NO₃⁻, are not formed in neutral solutions of Na₃N₂O₃. In experiments aimed at assessing the Na₃N₂O₃-dependent hydroxylation of phenol (pH 5–7.4), we could not observe the formation of 4-nitrophenol, which is the expected product of the interaction of phenol with ONOO⁻ (56, 57). Shafirovich and Lymar (51) have recently pointed out that nitrogen (+1), if formed in biological systems, must be present in the form of HNO (pKₐ = 11.4); direct addition of O₂ to HNO that could yield ONOO⁻ is spin forbidden and cannot be rapid, if it occurs at all (51). Measurable amounts of ONOO⁻, however, could be observed in alkaline solutions of Na₃N₂O₃, which led to the cautionary notes that the use of aged stocks of this compound may result in contaminations with ONOO⁻ (51, 55).

The formation of ·OH in solutions of Na₃N₂O₃ was first suggested by Hughes and Wimbledon (28), who observed that at pH < 3.0 H₂N₂O₃ (1a) decomposes via a free radical chain reaction that could be inhibited by ethanol (28). Because at pH < 3 H₂N₂O₃ generates HNO₃ and nitric oxide, the genera-
tion of OH was proposed to occur via the intermediate forma-
tion of 1d (Scheme 4) (28). Buchholz and Powell (58) suggested
a similar mechanism for the formation of /H18528 OH in acidic solu-
tions of 3a (3a - 3c - 'OH). However, direct detection of OH
in these reaction systems has not been presented.

Recently, Wink et al. (59) reported that 1b is toxic to Chinese
hamster V79 lung fibroblast cells. At a molecular level, 1b
exposure resulted in DNA double strand breaks in whole cells
(59). This observation is in agreement with the studies of Ohs-
hima et al. (15, 60) who reported that HNO caused DNA strand
breakage and base oxidation via an HNO-dependent genera-
tion of OH. Scavengers of OH, metal chelators, superoxide
dismutase, and catalase impeded the 1b-dependent oxidation
of DNA, indicating that superoxide anion radical, H2O2, and
free OH were involved in this process. These results led to the
thesis that OH could be generated via an HNO-dependent
reduction of O2 to superoxide anion radical (HNO + O2 → NO−+
H+ + O2) (15). However, the latter mechanism was not
supported by the studies of Nelli et al. (61), who reported that
in the presence of chelators of metal ions HNO does not un-
dergo O2-dependent oxidation to NO− (Scheme 4). It should be
pointed out that in solutions of NaN2O3 the occurrence of
Fenton-like reactions via redox cycling of transition metal ions
cannot be ruled out; recently, Al-Ajlouni and Gould (36) re-
ported that HN2O3 (1b) can directly reduce iron complexes
(Scheme 4).

In this work, we present further experimental evidence that
1b can generate 'OH. In aqueous solutions of 1b, phenol and
Me2SO were oxidized to benzene diols and 'CH3, respectively.
Because the oxidation of Me2SO was O2-independent (Fig. 4A),
it could be concluded that this process reflected neither the
intermediate formation of ONOO− nor a redox cycling of trans-
sition metal ions that could initiate Fenton-like reactions. The
treatment of aqueous solutions of phenol with 1b yielded ben-
zene-1,2-diol and benzene-1,4-diol at a ratio of 2:1, which is

FIG. 4. Kinetics of formation of the nitroxide and hydroxylamine forms of PBN/CH3 in solutions of NaN2O3, PBN, and Me2SO. All reactions were carried out for 30 min in 0.1 M phosphate buffer (pH 5; 40 °C) containing PBN (50 mM), Me2SO (0.5 M), and NaN2O3 (panels A, C, and D, 10 mM). In selected experiments, the acidity of the reaction solutions was adjusted with either NaOH or HCl (panel D). The total production of PBN/CH3 (Scheme 2, 4 plus 5) in the presence (closed circles) or absence (open circles) of oxygen is presented in panel A. Anaerobic conditions were achieved as described under *Experimental Procedures.* Each experimental point represents the mean of three experiments ± S.E.
consistent with the generation of free 'OH (Fig. 6C). At pH 4, the overall production of benzene diols was 14% of the initial concentration of 1b (Fig. 7). However, the production of 'OH could be higher, as it is unlikely that the hydroxylation of phenol proceeded quantitatively. The oxidation of phenol and Me₂SO was optimal at pH 4–5, implying that 'OH was not directly released from 1b; the formation of HNO within the

Fig. 6. Kinetics of formation of benzene diols in solutions of Na₂N₂O₃ and phenol. Reactions were carried out for 50 min in 0.1 M phosphate buffer (pH 4; 35 °C) containing phenol (10 mM) and Na₂N₂O₃ (10 mM). The overall production of benzene diols was assessed by HPLC-EC. A, phenol in the presence (closed circles) or absence (open circles) of Na₂N₂O₃. B, consecutive additions (time interval, 50 min) of equimolar amounts of Na₂N₂O₃ in phosphate buffer containing phenol. Throughout the incubation period, the reaction solution was purged with a stream of air. C, benzene-1,2-diol, benzene-1,3-diol, and benzene-1,4-diol formed in 0.1 M phosphate buffers containing Na₂N₂O₃ and phenol (incubation time, 30 min; n = 3; mean ± S.E.).

Fig. 7. Effects of oxygen and pH on the formation of benzene diols in solutions of Na₂N₂O₃ and phenol. All reactions were carried out for 50 min at 35 °C in 0.1 M phosphate buffers containing Na₂N₂O₃ (10 mM) and phenol (10 mM). The formation of benzene diols was assessed by HPLC-EC. The final reaction solution in selected experiments (closed circles) was purged with a stream of air throughout the incubation period. Each experimental point represents the mean of three experiments ± S.E.

same pH interval was H⁺-independent (Fig. 1, panel A compared with B, and Fig. 4D) (28, 29). Furthermore, GSH and Fe³⁺-MGD, which are efficient scavengers of HNO, markedly inhibited the hydroxylation of phenol (data not shown). These results allowed us to propose a reaction mechanism for the hydrolysis of 1b that includes dimerization of HNO to HO-N=N-OH (3a) with a concomitant azo-type homolytic fission of the latter acid to N₂ and 'OH (Scheme 4). A similar mechanism was established for the decomposition of dialkyl esters of 3a to RO' and N₂ (62). Within this mechanism, the effects of increasing concentrations of H⁺ on the formation of
'OH can be explained with a shift in the equilibrium between 3a and 3b in favor of 3a (Scheme 1). The impeded production of 'OH from 1b at pH < 4 most likely reflected the lack of formation of HNO; at pH < 4, 1b hydrolyzes to NO (28).

With regard to the effects of pH on the 'OH production from HNO, it is interesting to speculate that this phenomenon may have a toxicological significance. The HNO-dependent production of 'OH is 1 order of magnitude higher at pH 6.0 than at pH 7.4. It could be generalized that metabolic hyperactivity or limited oxygen supply can cause a decrease of tissue pH. Acidosis is characteristic for such disease states as sepsis, arthritis, ischemia, and cancer. In these diseases, the intra- and/or extracellular pH of the affected tissues typically decreases from control values of 7.4 to -6.0 (23, 47, 48, 63, 64). Hence, we hypothesized that HNO can exert selective toxicity to cells subjected to acidosis via a mechanism that includes an H+ -amplified generation of 'OH. In support of this thesis, 1b was markedly more toxic to human fibroblasts at pH 6.2 than at pH 7.4. The cytotoxicity of 1b was 'OH-dependent as suggested by the protective effect of scavengers of free radicals. In comparative experiments with human fibroblasts and SK-N-SH neuroblastoma cells, 1b exhibited a similar toxicological pattern, which leads to the conclusion that the extracellular pH was the predominant mediator of the toxic effect. 1b is a highly hydrophilic compound that has a partition coefficient for n-octanol/water of CLogP = -1.332 (estimated with ChemDraw; CambridgeSoft.com, Inc., Cambridge, MA). Hence, in biological systems the 1b-dependent generation of HNO will predominantly occur in the extracellular milieu. Shafirovich and Lymar (51), however, proposed that the diffusibility and membrane permeability of HNO is similar to that of NO (51), which implies that the intracellular interactions of HNO formed from 1b will also contribute to the toxicological effect of this compound. In an in vivo model of myocardial ischemia and reperfusion injury, Ma et al. (65) reported that 1b exhibited a marked necrotic effect, whereas S-nitrosoglutathione minimized muscle injury (65). The mechanisms responsible for these opposite effects are not well understood. It is tempting to speculate that the cytotoxicity of HNO released from 1b could be amplified by its conversion to 'OH in ischemia-acidified tissue (64). However, further studies are needed to understand the effects of HNO in biological systems, as well as to advance the mechanistic algorithms for interpretation and prediction of the cytotoxicity of HNO-releasing prodrugs such as Na2N2O3, derivatives of nitrosobenzene (10), benzisothiazol (66), chloropropamide (67), and N-hydroxybenzene carboximidate (68).

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Nitroxyl-dependent Generation of Hydroxyl Radical

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