S-Nitroglutathione, a Product of the Reaction between Peroxynitrite and Glutathione That Generates Nitric Oxide*

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Peroxynitrite (ONOO−) has been shown in studies on vascular relaxation and guanylate cyclase activation to react with glutathione (GSH), generating an intermediate product that promotes a time-dependent production of nitric oxide (NO). In this study, reactions of ONOO− with GSH produced a new substance, which was characterized by liquid chromatography, ultraviolet spectrosopy, and electrospray tandem mass spectrometry. The mass spectrometric data provided evidence that the product of this reaction was S-nitroglutathione (GSNO2) and that S-nitrosoglutathione (GSNO) was not a detectable product of this reaction. Further evidence was obtained by comparison of the spectral and chromatographic properties with synthetic standards prepared by reaction of GSH with nitrosium or nitronium borofluorates. Both the synthetic and ONOO−/GSH-derived GSNO2 generated a protonated ion, GSNO2H+, at m/z 353, which was unusually resistant to decomposition under collision activation, and no fragmentation was observed at collision energy of 25 eV. In contrast, an ion at m/z 327 (GSNOH+), generated from the synthetic GSNO2, readily fragmented with the abundant loss of NO at 9 eV. Reactions of ONOO− with GSH resulted in the generation of NO, which was detected by the head space/NO-chemiluminescence analyzer method. The generation of NO was inhibited by the presence of glucose and/or CO2 in the buffers employed. Synthetic GSNO2 spontaneously generated NO in a manner that was not significantly altered by glucose or CO2. Thus, ONOO− reacts with GSH to form GSNO2, and GSNO2 decomposes in a manner that generates NO.

Exposure of vascular tissue to peroxynitrite (ONOO−) results in a prolonged relaxation (1) that appears to be mediated through a glutathione (GSH)-dependent regeneration of NO (2). Peroxynitrite has also been observed to stimulate guanylate cyclase activity in a thiol-dependent manner in vascular endothelial and smooth muscle preparations (3, 4). Whereas the reaction of ONOO− with GSH has been reported to form small amounts of S-nitroso-GSH (GSNO) (3, 5), our previous studies detected a different product of this reaction, which was isolated and demonstrated to possess potent vascular relaxant activity (2). Examination of the reaction of nitrogen dioxide (NO2) with GSH detected the formation of what appears to be the same product as that observed in the reaction with ONOO− (6). Because the biologically active metabolite of these reactions co-migrated on HPLC with a product of a reaction between nitrosomonium borofluoride (NOBF3) and GSH, the vascular relaxant detected was suggested to be a nitrated product of GSH (GSNO2) (6). Thus, additional studies are needed to identify the biologically active substances derived from the reaction of GSH with ONOO−.

Peroxynitrite is also known to undergo additional reactions in the presence of physiological buffered systems and GSH. One of the first observed actions of ONOO− on thiols was that it caused oxidation reactions, and an analysis of products of these reactions resulted in a hypothesis that nitrated thiols were a key unstable intermediate formed during these reactions (7, 8). Recent studies have also provided evidence that thiol radicals seem to be one of the major initial products of the reaction of ONOO− with thiols (9, 10). Peroxynitrite appears to react with glucose and other hydroxylated compounds to produce relatively stable products that cause tissue- and thiol-dependent generation of NO and a prolonged relaxation of vascular tissue (11, 12). However, the vasoactive products formed from reactions with glucose seem to be significant only at very elevated levels of ONOO− (13). Peroxynitrite also reacts with CO2/bicarbonate to generate an intermediate that is a potent nitrating agent (14–17). Thus, ONOO− may interact with additional components of tissues and buffers to form biologically active metabolites. The purpose of this study was to determine whether the reaction of ONOO− with GSH generates GSNO2 and to examine aspects of how the formation of GSNO2 potentially participates in the generation of NO from ONOO−.

EXPERIMENTAL PROCEDURES

Nitric Oxide Measurements—Head space NO measurements were determined employing a NO chemiluminescence analyzer (model 20B, Sievers Instruments, Boulder, CO), using a slightly modified version of the method of Brien et al. (18) that was adapted for our previous studies on ONOO− and NO2 (2, 6). To quantify the amount of NO produced in the head space gas derived from 0.4 mM ONOO− or 0.1 mM GSNO2, the following protocols were applied: Fernbach flasks (6 ml) containing a 2-ml final volume of 25 mM potassium phosphate or 25 mM bicarbonate buffer (pH 7.4) in the presence or absence of 1 mM GSH and 5.6 mM glucose were incubated at 37 °C equilibrated under an atmosphere of argon or 95% N2/5% CO2, respectively. To the sealed Fernbach flasks, aliquots of deoxygenated ONOO− or GSNO2 were injected, and the flasks containing the various experimental conditions were allowed to accumulate NO in the head space for 30 min. After 30 min, a single 0.5-ml aliquot was taken of the head space gas from each sealed Fernbach flask to quantitate the amount of NO produced from each experimental condition employing the Sievers NO analyzer. Under these severely hypoxic conditions, NO is stable over the 30-min accumulation period. The amount of NO formed was quantitated, after subtraction of an injection artifact blank, based on NO standards and recovery of authentic NO from the Fernbach flask containing 2 ml of buffer.

Chemistry—Peroxynitrite was prepared from sodium nitrite and...
H$_2$O$_2$ in a quenched-flow reactor with minor modifications (19). H$_2$O$_2$ and oxygen were removed from the peroxynitrite solution by adding catalytic amounts of MnO$_2$, followed by bubbling with a gentle stream of dry nitrogen for 1 h. ONOO$^-$ was concentrated by freezing the ONOO$^-$ solution for overnight at $-20^\circ$C, and the concentrated top yellow layer was carefully thawed and transferred to a clean glass tube. The concentration of peroxynitrite was determined spectrophotometrically by measurement of absorbance at 302 nm in 0.1 N NaOH ($A_{302} = 1670$ m$^{-1}$cm$^{-1}$). The stock solution contained 170–190 mM ONOO$^-$, and dilutions were made in 0.1 N NaOH. The reaction of GSH (1 mM) with ONOO$^-$ (1 mM) was carried in 0.5 ml of phosphate buffer (pH 7.4) in a stopper-capped glass tube with constant stirring at room temperature. The aliquots (20 µl) of the reaction mixture were taken at various time points and analyzed by injection into a reverse-phase HPLC column (RP-HPLC). In control experiments, the GSH was reacted with decomposed ONOO$^-$ (30 min at 37°C). The products of the reaction were analyzed by RP-HPLC and electrospray ionization tandem mass spectrometry (ESI/MS/MS).

**Preparation of Standards**—GSNO and GSNO$_2$ were prepared as described previously (6). Briefly, GSNO$_2$ was prepared by addition of thiolic nitrating reagent, nitronium tetrafluoroborate (NO$_2$BF$_4$, 0.1 M) to 25 mM GSH dissolved in 0.5 N phosphate-buffered saline (pH 7.4), and the mixture was the subjected to purification by HPLC. The NO$_2$BF$_4$ was prepared immediately before use as a 1 M solution in 0.1 N HCl. GSNO was obtained via analogous reaction with nitrosonium tetrafluoroborate (NOBF$_4$). Overnight storage of GSNO and GSNO$_2$ at $-20^\circ$C allowed us to obtain enriched preparations of these compounds as concentrated upper pink-colored layers, which were collected, purified by RP-HPLC, and analyzed by ESI/MS/MS.

**ESI/MS/MS**—Mass spectrometry was performed on a triple quadrupole tandem mass spectrometer (TSQ 700, Finnigan-MAT, San Jose, CA) equipped with an ESI interface. Both the electrospray needle and the skimmer were operated at ground potential, whereas the electrospray chamber and metalized entrance of the glass capillary were operated at 2.5 kV in the positive ion mode. The mass spectrometer was operated at unit resolution across its entire mass range. Nitrogen was used for all inlet gases other than the collision gas, which was argon. The collision gas thickness for tandem mass spectrometry experiments was 250–300 × 10$^{-12}$ molecules/cm$^2$. All samples (0.5–1 µmol/µl) were dissolved in deionized water containing 5% acetonitrile and 0.01% acetic acid and infused into the electrospray ion source by using a syringe pump (model 22, Harvard Apparatus, South Natick, MA) at a flow of 1 µl/min for 5 min through a 100 µm-inner diameter fused silica capillary. The capillary transfer tube temperature was kept at 150°C. The curtain gas flow was 1.2 liters/min, and the nebulizer gas flow was held at 0.9 liter/min. Collision-induced dissociations were induced with 1.4 torr, resulting in 50–60% decrease in ion transmission. Collision energy ranged from 8 to 25 eV.

**HPLC Analyses**—Samples were injected into a 1050 HPLC system (Hewlett-Packard, Palo Alto CA) equipped with a quaternary pump and a variable wavelength UV detector and operated by a ChemStation data system. The compounds were analyzed on a C18 Hypersil column (250 × 4.6 mm, Beckman Instruments) that was eluted isocratically with acetonitrile (5%, v/v) in water (containing acetic acid, 0.01% v/v),
pH 4) at a flow of 1 ml/min. The effluent from the column was collected in 1-ml fractions using a Gilson FC203B fraction collector. The chromatograms were obtained by monitoring UV absorbance at 215 and 344 nm. Fractions containing UV light-absorbing substances were subjected to further analyses.

**UV Spectroscopy**—Samples (1 ml) containing either GSNO₂ or GSNO, freshly purified by HPLC, were placed in a semimicro-UV 1-ml quartz cuvette (QS 1.000, Fisher Scientific), and the spectra were recorded using a diode array spectrophotometer (Hewlett-Packard HP 8452A). The spectra were taken from 200 to 450 nm at a scan rate of 250 nm/s. Specific absorbance at 334 nm was 980 M⁻¹cm⁻¹ for GSNO₂ and 800 M⁻¹cm⁻¹ for GSNO.

**Statistical Analysis**—The data were analyzed by one- and two-way analysis of variance for multiple comparisons employing a Bonferroni's modified t test to determine statistical significance for all studies. Results are reported as means ± S.E., with n equal to the number of separate determinations in chemical reactions. p < 0.05 was used to determine statistical significance.

**Materials**—Reduced glutathione was from Calbiochem. NO₂BF₄ and NOBF₄ were purchased from Aldrich. All solvents were of highest chromatographic grade.

**RESULTS**

**Properties of NO Generation from Reactions of ONOO⁻ with GSH and from GSNO₂**—Our previous work demonstrated that the release of NO from the reaction of ONOO⁻ with GSH was approximately linear for the first 30 min, and it decreased

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**FIG. 4.** Electrospray tandem mass spectrometry of products a and b obtained by reaction of glutathione with peroxynitrite and purified by RP-HPLC (as shown in Fig. 3). **Top:** mass spectrum of product b, identified as GSNO₂ (collision energy, 25 eV); **Bottom:** mass spectrum of product a, which corresponds with GSH (collision energy, 10 eV).
substantially after this time point (2). This observation was used to design the experiments shown in Figs. 1 and 2. The data in Fig. 1 show that GSH stimulates the release of NO from ONOO\(^-\) as previously reported (2). Generation of NO was significantly lower in CO\(_2\)/bicarbonate buffer than in phosphate buffer. This is consistent with a recent observation that CO\(_2\) catalyzes ONOO\(^-\) decomposition via formation of an unstable intermediate nitrosoperoxycarbonate (ONOOCO\(_2\)\(^-\)), thereby accelerating formation of nitrate (14). Addition of glucose alone had no effect on NO release from ONOO\(^-\). However, as compared with the reaction with GSH alone (in phosphate buffer), about 50% less NO was detected when ONOO\(^-\) was reacted with GSH in the presence of glucose.

The observation of a prolonged release of NO from the interaction of ONOO\(^-\) with GSH suggests that ONOO\(^-\) reacted with GSH producing an intermediate compound, possibly GSNO\(_2\) (2), which released NO. This hypothesis is supported by experiments shown in Fig. 2, which demonstrate that synthetic GSNO\(_2\) spontaneously releases NO in the buffer systems employed. In preliminary experiments, it was confirmed that the amount of NO formed from GSNO\(_2\) at 15 min was approximately half that detected at 30 min. Data in Fig. 2 indicate that the presence of glucose and CO\(_2\)/bicarbonate had minimal inhibitory effects of NO release from GSNO\(_2\), whereas the data in Fig. 1 demonstrate that the presence of glucose or CO\(_2\)/bicarbonate suppresses the generation of NO from ONOO\(^-\). Thus, the actions of glucose and CO\(_2\)/bicarbonate on NO release seem to originate primarily from an action of these buffers on the ONOO\(^-\) and not on the stability of GSNO\(_2\).

The maximal yield of NO from GSNO\(_2\) in the absence of added GSH at the 5 min time point was approximately 30–40%, and this was observed to decrease on addition of increasing concentrations of GSH. In separate experiments, which determined the amount of NO released from GSNO\(_2\) in the absence and presence of 1 mM GSH, it was confirmed that NO generation at 30 min was reduced by 43% in the presence of GSH relative to the control. A reaction of GSH with GSNO\(_2\) resulted most likely in the formation of nitrite and disulfide (GSSG). This explanation is based on a previous observation that was suggested as an explanation for the observed decreased formation of NO from tert-butyl thionitrate via a competitive reaction with cysteine (20). Whereas CO\(_2\)/bicarbonate buffer did not affect the production of NO from GSNO\(_2\) in the presence of GSH and glucose, NO formation in the absence of glucose was further decreased by an additional 46% in CO\(_2\)/bicarbonate buffer (Fig. 2). A strong additional inhibitory effect of GSH on NO release from GSNO\(_2\) in the presence of CO\(_2\)/bicarbonate and in the absence of glucose (Fig. 2, p < 0.05) may explain the minimal release of NO from ONOO\(^-\) under the same conditions (Fig. 1).

**Characterization of GSNO\(_2\)**—Treatment of GSH with peroxynitrite at pH 7.4 followed by RP-HPLC analysis using a mobile phase containing water and 0.1% acetonitrile revealed the presence of several substances (Fig. 3). Compound \(a\) was confirmed to be unreacted glutathione because it coeluted with GSH, and its ESI/mass spectrum (Fig. 4) was identical with that of intact GSH. A novel compound \(b\), eluting at 7.1 min., was detected as a product of ONOO\(^-\) reaction with GSH, and its chromatographic mobility was similar to that previously observed (6). The relative concentration and extent of this product formation was dependent upon the initial concentration of ONOO\(^-\) and GSH. This product also showed a strong chromatographic peak when the HPLC analysis was performed with monitoring of UV absorbance at 334 nm, typical for nitro- and nitroso-thiols (2). Product \(b\) was not formed when GSH was treated with ONOO\(^-\) at pH greater than 8 or with decomposed ONOO\(^-\), indicating that peroxynitrous acid was essential for formation of this product. A peak at 2.5 min and a broad peak at 5 min in the chromatogram on Fig. 3 (top) originated from the decomposition of ONOO\(^-\). Freezing of the ONOO\(^-\)/GSH reaction mixture at \(-20^\circ\text{C}\) for 2–3 h markedly increased the abundance of product \(b\) near the upper frozen layer, via a freeze distillation, which allowed us to collect sufficient amounts of this material for mass spectrometric analyses. Product \(b\), as well as synthetic GSNO and GSNO\(_2\), displayed sharp and reproducible chromatographic peaks on an RP-HPLC column; however, these three compounds had almost the same retention time and could not be separated on several HPLC systems. Thus, the identification of \(b\) based on retention time comparison with synthetic standards was not conclusive.

Ultraviolet spectra of GSNO\(_2\) and GSNO showed minimal differences (Fig. 5). Both compounds displayed a relatively strong UV absorbance at 334 nm. The spectrum of GSNO\(_2\) showed a stronger deflection than the spectrum of GSNO at 218 nm. Because it was difficult to establish the structure of ONOO\(^-\)/GSH product based on comparison with synthetic standards using UV and HPLC, we employed electrospray tandem mass spectrometry to elucidate the structure of this product.

ESI mass spectrum of the ONOO\(^-\)/GSH-derived product \(b\) contained a prominent ion at \(m/z\) 353 (Fig. 4), which presumably corresponds to a protonated form of the molecule. Thus, the molecular mass of this product was 352 atomic mass units, suggesting that product \(b\) had a structure consistent with \(S\)-nitroglutathione. This product displayed unusual stability un-
under collision-induced activation tandem mass spectrometry, where ions were dissociated as a result of interaction with a neutral gas, argon. Our attempts to decompose ion $m/z$ 353 at an energy of 25 eV caused a decrease in the detection of this ion by more than 90%, without producing any detectable ion fragments. In particular, ions that might be expected to originate from the loss of NO, NO from the GSNO$_2$, or decomposition of a GSH backbone could not be detected. Treatment of GSH with NO$_2$BF$_4$ yielded a compound that also produced a protonated GSNO$_2$ ion at $m/z$ 353, and it was also resistant to collisional activation (Fig. 6). Although the spectrum of ONOO$^-$/GSH product was identical to that of synthetic GSNO$_2$, the spectrum of synthetic GSNO was very different (Fig. 6). Collision-induced dissociation of the protonated GSNO ion ($m/z$ 337) resulted in a loss of nitric oxide, producing an abundant fragment ion at $m/z$ 307 (GSH$^+$). The GSNOH$^+ \rightarrow$ GSH$^+$ fragmentation occurred readily, and therefore, the cleavage of the S-NO bond required relatively mild conditions. In the absence of additional ion activation, the mass spectrum of GSNO showed an ~8% relative abundance of the $m/z$ 307 ion, suggesting that GSNOH$^+$ ion was releasing NO (not shown). Additional loss of NO radical was observed under condition of a collision-induced dissociation of GSNOH$^+$ ion at 9 eV, where approximately 80% of the S-NO bonds appear to

FIG. 6. Electrospray tandem mass spectra of synthetic standards. S-nitroglutathione (GSNO$_2$) (collision energy, 25 eV); S-nitrosoglutathione (GSNO) (collision energy, 9 eV).
have been broken (Fig. 6). Several minor ions were also observed in the mass spectrum of GSNO that originated from dissociation of the GSH backbone. Our mass spectrometric data provided evidence that the reaction of GSH with ONOO⁻ produces a S-nitro derivative of GSH, and the protonated GSNO₂⁻ ion is considerably more stable to collision-activated dissociation than the protonated GSNO ion.

The abundance of the HPLC peak corresponding to GSNO₂⁻ relative to GSH was 6.3% following a mixing of 1 mM GSH with 400 μM ONOO⁻ for 5 min, suggesting that about 16% of the ONOO⁻ appears to have been converted to GSNO₂⁻. Thus, our studies identified the biologically active substance derived from the reaction of GSH with ONOO⁻ as GSNO₂⁻ and GSNO was not a detectable product of ONOO⁻/GSH reaction.

**DISCUSSION**

The results of the present study provide evidence for our recent suggestion (6) that GSNO₂⁻ is a somewhat stable product of the reaction of ONOO⁻ with GSH. We have previously observed that a product of the reaction between ONOO⁻ and GSH has the properties of a substance that slowly releases NO, and a product of this reaction was isolated and shown to cause what appeared to be a NO-mediated relaxation (2). In the present study, GSNO₂ was synthesized, and its ability to spontaneously release NO was demonstrated. The data obtained also suggest that nitrated derivatives of glucose or carbon dioxide do not seem to be primary species contributing to the generation of NO from ONOO⁻ under the conditions examined. Thus, GSNO₂⁻ may be a key participant in the generation of NO from ONOO⁻ in the presence of GSH (Fig. 7).

Mass spectra of a key product that results from the reaction of GSH with ONOO⁻ detected an ion with an m/z of 353 that had a mass spectrum comparable with that of synthetic GSNO₂ produced from the reaction of GSH with NO₂BF₄. The ONOO⁻/GSH-derived product was clearly different from synthetic GSNO, which was not a detectable product of this reaction. Interestingly, collisional activation of GSNO₂ derived from either ONOO⁻ or NO₂BF₄ with 25 eV did not produce fragment ions, whereas the ion with an m/z at 337 of GSNO readily fragmented at 9 eV. Although the actual origin of the gas phase stability of protonated form of GSNO₂ is not yet known, previously reported (21) molecular orbital calculations on model thiol nitrates (RSNO₂⁻) suggest that they have a very low barrier for rearrangement to sulfenyl nitrates (RSONO). Thus, it is possible that rapid equilibration between these species provides a level of stabilization sufficient to prevent the fragmentation of GSNO₂. The relative stability of GSNO₂⁻ in the electrospray conditions could be also influenced by the attachment of proton to GSNO₂⁻, a process that is less likely to occur in solution at neutral pH. In a buffered solution, other factors, such as solvation and ionic strength, may contribute to the release of NO from GSNO₂⁻. Further studies are needed to establish whether the protonation of GSNO₂ enhances its stability in solution.

It has been previously reported that RSNO₂⁻ (R = tert-butyl) species release detectable amounts of NO (20). The rate of this decomposition is relatively low (about 10⁻⁶ s⁻¹) and pH-independent in the neutral pH region of acetate/nitrite/phosphate buffer at room temperature (20). As a result of molecular orbital calculations, it was suggested that the rearrangement of thiol nitrates (RSNO₂⁻) to sulfenyl nitrates (RSONO) followed by the spontaneous decomposition of RSONO was a potential mechanism of NO generation (20, 21). It was also previously observed that tert-butylthionitrat releases NO through a mechanism that was inhibited by thiols, and this inhibitory effect of glutathione was seen in the present study with purified GSNO₂⁻. A reaction of thiols with RSNO₂ results in the formation of nitrite and disulfide was suggested as an explanation for the observed decreased formation of NO (20). Thus, the release of NO from GSNO₂ has similarities with previous observations of NO release from tert-butylthionitrate. Although it has been suggested that nitrated thiols are very unstable species (7, 8), the observed release NO form GSNO₂ over a prolonged period suggests that these substances may be more stable than they were previously considered to be. Because the release of NO₂ via a homolytic cleavage of the S-N bond is not likely to occur in thionitrates (21), a question that remains to be answered is whether NO derives from sulfynyl nitrite (GSi/O/NO) or sulfenyl nitrite (GSNO) after rearrangement of the initially formed thionitrate (GSNO₂⁻) (Fig. 7).

The observation that glucose- and CO₂/bicarbonate-containing buffers reduced the amount of NO generation from ONOO⁻ in the presence of GSH and the absence of an identifiable effect of these buffer components on the release of NO from GSNO₂ are consistent with previous observations on the chemistry of ONOO⁻. Whereas ONOO⁻ reacts with CO₂/bicarbonate to form an intermediate that enhances the efficiency of some of the nitration reactions caused by ONOO⁻ (14–16), data in the present study suggest that this process does not enhance the formation of the key NO-generating species, which appears to be GSNO₂⁻. Although ONOO⁻ reacts with glucose (and other hydroxylated substances) to produce nitrovasodilators (11, 12), it appears that the products that form may have less of a potential to release NO in the presence of excess GSH than the thiol-derived NO-releasing species that form in the absence of glucose. Thus, the NO-releasing species hypothesized in the present study to be GSNO₂ appears to result from a reaction of GSH with ONOO⁻ that is not catalyzed by glucose or CO₂/bicarbonate. Because thiol radicals and nitrogen dioxide seem to be key initial products of the aqueous chemistry of ONOO⁻ and thiols (8–10), perhaps thiol nitration is a result of a radical-radical reaction between these two reactive species.

Observations made in the present study on the reaction of ONOO⁻ with GSH are consistent with GSNO₂ being a key intermediate that participates in the generation of NO. Our recent studies on bovine coronary (22) and pulmonary (23) arteries have already provided evidence that concentrations of NO as low as 50 nM cause a level of intracellular ONOO⁻ formation that produces a prolonged relaxation of these vascular segments as a result of a thiol-dependent process that participates in the regeneration of NO. In addition, GSNO₂ was hypothesized to be a key metabolite of nitroglycerin over 20 years ago (24). Therefore, GSNO₂ may be an important biolog-
ically active metabolite of NO oxidation and nitrovasodilator
drug action.

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