# Formation of the NO donors glyceryl mononitrate and glyceryl mononitrite from the reaction of peroxynitrite with glycerol

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Peroxynitrite ($ONOO^-$), formed from the rapid reaction of superoxide ($O_2^{-\bullet}$) with NO, is known to generate stable compounds capable of donating NO on reaction with thiols and molecules containing hydroxy groups. Using glycerol as a model compound for the reactions of $ONOO^-$ with biomolecules containing hydroxy groups, we separated the products and identified them by HPLC/MS. It was shown that both glyceryl mononitrate and glyceryl mononitrite were formed and released NO on incubation with copper and L-cysteine. The compounds were stable over a period of 4 h when shielded from light and kept on ice. Slow spontaneous decomposition occurred in the buffer used for the bioassay, but this was not sufficient to explain the vasorelaxing properties of these NO donors. It is concluded that the stable organic nitrate and nitrite have the capacity to be metabolized by vascular tissues, resulting in vasorelaxation.

## INTRODUCTION

Peroxynitrite ($ONOO^-$), an oxidant derived from the reaction of NO with superoxide ($O_2^{-\bullet}$), is formed in the vasculature in response to hypercholesterolaemia [1–5]. The resulting vascular dysfunction is progressive and partially reversible early in the atherosclerotic process by pharmacological interventions that decrease $O_2^{-\bullet}$ formation, such as treatment with the enzyme superoxide dismutase or allopurinol [2,6–9]. Apart from xanthine oxidase, other sources of $O_2^{-\bullet}$ may include NAD(P)H oxidases and endothelial cell NO synthase under conditions of hypercholesterolaemia and arginine depletion [10–12]. The induction of NO synthase is the probable source of NO in the developing atherosclerotic lesion and a likely locus for the formation of $ONOO^-$ [3,5,13]. As the disease progresses, loss of NO-dependent vasorelaxation becomes irreversible, consistent with the hypothesis that sustained formation of $ONOO^-$ results in permanent damage to the artery wall. The precise biochemical pathways that lead to such damage remain uncertain, but may include nitration of tyrosine residues and possibly other amino acids in the atherosclerotic lesion [3].

Peroxynitrite has a broad spectrum of properties, including the capacity to nitrate and nitrosate proteins, carbohydrates and thiols, resulting in compounds that may act as NO donors [14–18]. Such reactions are a probable explanation for the reported 'NO-like' properties of $ONOO^-$, including relaxation of vascular smooth muscle and inhibition of platelet aggregation [16,17]. These responses are typically mediated by the activation of vascular smooth-muscle-cell guanylate cyclase and the formation of the second messenger cGMP [19,20]. Indeed, it has also recently been shown that $ONOO^-$ treatment of endothelial cells leads to increased levels of cGMP through the intermediate formation of an *S*-nitrosothiol [21,22].

It has been argued that the existence of these pathways will decrease the potential cytotoxicity of $ONOO^-$ [23]. However, exposure of perfused rat hearts to successive doses of $ONOO^-$ resulted in progressive diminution of the vasorelaxant response characteristic of the development of tolerance [17]. The phenomenon of nitrate tolerance is well known and is characterized by a diminished response to nitrovasodilators as the result of chronic usage, which may be attributed to the depletion of cellular thiols [24,25]. The products of the reaction of hydroxylated compounds with $ONOO^-$ are moderately stable species capable of releasing NO on contact with tissues, resulting in NO-dependent vasodilatation and inhibition of platelet aggregation [18]. These compounds are formed on reaction with glucose, mannitol or glycerol and release NO, when incubated with a thiol and transition metal [18]. These characteristics are consistent with the formation of an organic nitrate or nitrite, well-known vasodilators, which are used extensively in the treatment of angina [26–31]. They have been shown to be activated by a diverse range of metabolic pathways in tissues [32–37]. The product(s) of the reaction of $ONOO^-$ with compounds containing hydroxylated groups has not been defined. The goal of the current studies was to characterize the structure and function of a putative NO donor derived from the reaction of glycerol with the oxidant $ONOO^-$.

## MATERIALS AND METHODS

### $ONOO^-$ preparation

$ONOO^-$ was synthesized by the reaction of acidified $NaNO_2$ (1.8 M) with $H_2O_2$ (2.1 M) and quenched with NaOH (4.2 M) [1,18]. Decomposed $ONOO^-$ was prepared by delaying quenching with NaOH for 10 min. Excess $H_2O_2$ was removed from the $ONOO^-$ preparations by passage down an $MnO_2$ column [18]. This preparative reaction typically yields $ONOO^-$ at concentrations of 200–350 mM.

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### Preparation of the products of $ONOO^-$ reaction with glycerol or glucose

$ONOO^-$ (20 mM) or decomposed $ONOO^-$ was mixed with 0–1210 mM glycerol or glucose, in water, and allowed to react for 5–30 min at room temperature. To avoid pH artifacts and the liberation of NO from contaminating nitrite, the $ONOO^-$/glycerol reaction mixture was neutralized to pH 7.4 by the addition of HCl (1 mM) before biological assay. A lack of residual $ONOO^-$ activity was confirmed by the loss of $A_{302}$. A series of stock solutions were prepared resulting in a minimum dilution of the mixture in an isolated tissue bath of 430-fold. These solutions were then used for vessel-relaxation studies. Undiluted samples were used for analysis by HPLC/MS.

### Preparation of a saturated solution of NO

A gas-sampling tube was filled with double-distilled water and bubbled with argon gas for 30 min to purge $O_2$ from the solution and the gaseous phase. Gas was allowed to vent through a stopcock at one end of the tube. A gas line from an NO cylinder was next attached to the sampling tube, and the solution was gassed for approx. 5 min. The emission of a brown vapour from the sampling tube was taken to indicate that the solution was saturated with dissolved NO gas. We have found that the concentration of NO in this solution is approximately 1.8–2.0 mM after analysis by a chemiluminescence analyser (Antek Instruments) and a standard curve generated from known concentrations of $NaNO_2$. NO was sampled by piercing a self-sealing cap on the side arm of the tube with a gas-tight Hamilton syringe.

### Measurement of NO release

NO was measured electrochemically using a Clark-type electrode (Iso NO; World Precision Instruments) which was calibrated using anaerobic solutions of pure NO gas in water. Mixtures of the $ONOO^-$ and glycerol or products were diluted into the electrode, and changes in NO concentration measured. Data were calculated relative to the authentic solution of NO and related to maximal release of NO measured. All experiments were performed at 37 °C, and care was taken to exclude light from the chamber.

### Vessel reactivity studies

Isometric tension was measured in isolated rabbit aortic ring segments as described previously [2,6]. The aorta was excised immediately on the death of the rabbit, cleansed of fat and adherent tissue, cut into individual ring segments (3–4 mm in width) and suspended from a force-displacement transducer in a tissue bath. Ring segments were bathed in a bicarbonate-buffered Krebs–Henseleit solution of the following composition (mM): NaCl (118); KCl (4.6); $NaHCO_3$ (27.2); $KH_2PO_4$ (1.2); $MgSO_4$ (1.2); $CaCl_2$ (1.75); disodium EDTA (0.03); glucose (11.1). A passive load of 3 g was applied to all ring segments and maintained at this level throughout the experiments. At the beginning of each experiment, indomethacin-treated (5 $\mu$M) ring segments were depolarized with KCl (70 mM) to determine the maximal contractile capacity of the vessel before pre-contraction to 40 % of the maximal capacity (40 % of KCl response) with phenylephrine (approx. $3 \times 10^{-8}$–$10^{-7}$ M). When tension development reached a plateau, the reaction products from mixing glycerol with $ONOO^-$ were added to achieve a cumulative dose–response curve expressed as the nominal concentration of glycerol to which the tissue was exposed.

Glycerol solutions of various concentrations (0.077–1.21 M) were exposed to a fixed concentration of $ONOO^-$ (20 mM). In independent experiments, ring segments were exposed to cumulative dilutions of each $ONOO^-$/glycerol stock solution to determine relative yields of the NO donor with increasing concentration of glycerol. These responses were compared with dose–response profiles for ring segments exposed to a saturated solution of NO gas. NO was added to the tissue bath using a gas-tight Hamilton syringe to minimize decomposition and exposure to air. Control experiments were performed in which glycerol was exposed to decomposed $ONOO^-$ before bioassay.

Oxyhaemoglobin was prepared by the reduction of human haemoglobin (Sigma) with sodium dithionite and was then oxygenated by separation of the excess reductant on a Sephadex G-25 column. Oxyhaemoglobin concentration (greater than 95 %) was then estimated from the $A_{576}$ using an absorption coefficient of 14.6 $mM^{-1} \cdot cm^{-1}$. The specificity of the relaxation response to the release of NO was then confirmed by inhibition with oxyhaemoglobin (5 $\mu$M). The stability of the putative NO donor was tested by assaying its vasorelaxant effects at time points up to 2 h after reaction of glycerol with $ONOO^-$. The effective concentration of the putative NO donor resulting in a 50 % decrease in developed tension ($ED_{50}$) was used as an index of vessel-relaxation capacity. Real-time data were collected for all experiments and downloaded to an IBM personal computer for later analysis using commercially available software (Experimenter's Workbench).

### Analysis of the reaction products of glycerol and $ONOO^-$

Samples of the reaction mixture (10 $\mu$l) of glycerol and $ONOO^-$ were injected on to a C-8 reversed-phase column (Applied Biosystems RP300; 2.1 mm internal diameter × 100 mm). The mobile phase (10 % methanol/water, 10 mM sodium acetate; flow rate of 0.2 ml/min) was split 1:1, with one-half passed into the electrospray interface of a PE Sciex (Concorde, Ontario, Canada) API III triple-quadrupole mass spectrometer operating in the negative-ion mode. To release NO from organic nitrates or nitrites formed on reaction of $ONOO^-$ with glycerol, samples were incubated with 1 mM cysteine and 10 $\mu$M $CuSO_4$ for 30 min at room temperature and again subjected to analysis.

### Statistical analysis

All results are expressed as means ± S.E.M. Dose–response profiles for the different experimental treatments were analysed and tested to determine differences in relaxation responses using the Sigma Stat statistical analysis program. Unpaired observations were assessed by one-way analysis of variance and multiple range tests.

## RESULTS

### Vascular responses to the reaction products of glycerol and $ONOO^-$

The biological detection of compounds with NO-donor properties can be achieved with great sensitivity. The minimum detection limits depend on the specific NO donor, but can be as low as 10 nM when an isolated blood-vessel bioassay system is used. Chemical detection and characterization of these products is more difficult, requiring much higher amounts of compound. To determine whether the conditions selected for the reaction of glycerol with $ONOO^-$ resulted in compounds capable of donating NO, the reaction products were added to an organ bath containing precontracted rabbit aortic ring segments. As depicted in Figure 1, the $ONOO^-$/glycerol reaction products produced a

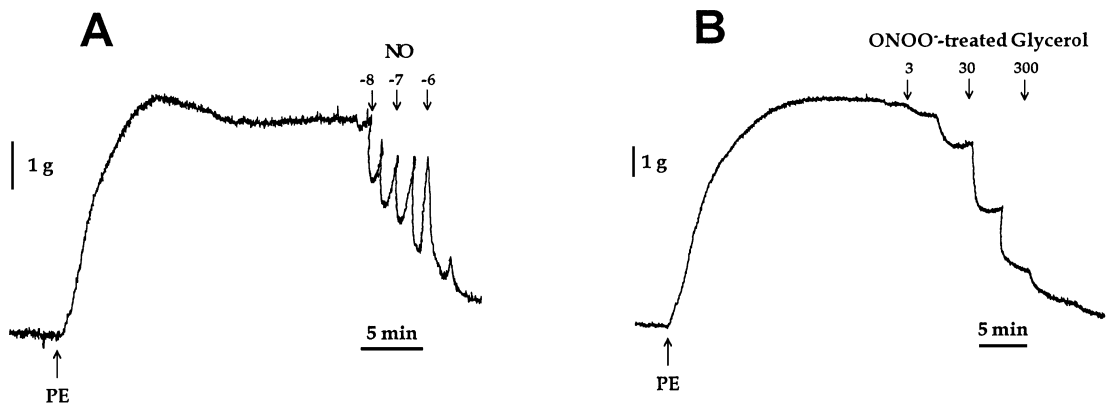


**Figure 1 Sample traces depicting the relaxation of rabbit aortic ring segments in response to the $ONOO^-$/glycerol reaction products and authentic NO**

Rabbit aortic rings were precontracted with phenylephrine (PE) and exposed to NO gas in solution (**A**) or the products of the $ONOO^-$ reaction with glycerol (**B**). The NO was added to give the final nominal molar concentration indicated. Since the concentrations of materials with NO-donating properties in the glycerol/$ONOO^-$ mixture are not known, concentrations are expressed in terms of $\mu$l of the reaction product per l, taking into account the final dilution in the organ bath. Differences in the kinetics of relaxation are evident from the rapid relaxation and recovery found on addition of NO and the delayed and sustained response elicited by the $ONOO^-$/glycerol reaction products.

slowly developing and sustained decrease in tension, which is in contrast with the rapid relaxation stimulated by authentic NO. The rapid recovery observed with vessels exposed to authentic NO reflects, in part, its loss due to equilibration with the air/buffer surface in the organ chamber. This differential response of NO and the $ONOO^-$/glycerol mixture is consistent with the time required for the metabolic conversion of the $ONOO^-$/glycerol product into a physiologically active species, presumably NO.

Relaxation in response to the NO donor was concentration-dependent (Figure 2A). Since the concentration of the putative NO donor is unknown, the data are represented as the dilution of the glycerol/$ONOO^-$ reaction mixture. Incubation of more concentrated solutions of glycerol with a fixed amount of $ONOO^-$ (20 mM) produced greater yields of the NO donor, as reflected by the leftward shift in dose–response profiles (Figure 2). The maximum relaxation response occurred with exposure of ring segments to dilutions of an $ONOO^-$/glycerol solution (20 mM $ONOO^-$, 605 mM glycerol) equivalent to an $EC_{50}$ of $17 \pm 1$ $\mu$l/l (Figure 3). This compares with an $EC_{50}$ for authentic NO of $121 \pm 38$ nM. Under these conditions, exposure of ring segments to oxyhaemoglobin (5 $\mu$M) prevented relaxation, providing further evidence that NO was released from the $ONOO^-$/glycerol product (results not shown). Incubation of glycerol with decomposed $ONOO^-$ did not result in vasorelaxation over the concentration range tested (Figure 3A), nor did exposure of ring segments to serial dilutions of glycerol alone (results not shown). As a measure of the stability of the NO-donating compounds, vasorelaxation mediated by the glycerol/$ONOO^-$ mixtures at ratios of 3.8, 15.1 and 60.5 were re-assessed at a period 2 h after the reaction had occurred. The potency of the compounds showed a small decrease when compared with values obtained immediately after reaction of $ONOO^-$ with glycerol, suggesting that slow decomposition was occurring (Table 1).

### Comparison of the stability of NO-donating compounds from the glycerol/or glucose/$ONOO^-$ reaction

The vasorelaxation studies indicated that products of the reaction of glycerol and $ONOO^-$ were capable of releasing NO and were much more stable than either NO or $ONOO^-$ itself. Similar results have been reported for glucose [18]. To compare the stability of the NO-donating compounds with those formed with glycerol, $ONOO^-$ was mixed with 300 mM glycerol or glucose, followed by the addition of 70 mM potassium phosphate buffer, pH 7.4. To elicit NO release from the compounds, aliquots were added to the sample chamber of an NO electrode in the presence of 100 $\mu$M $CuSO_4$ and 200 $\mu$M L-cysteine [18]. A 50-fold dilution of the glycerol/$ONOO^-$ reaction product into 0.2 M potassium phosphate buffer resulted in a maximal steady-state concentration of $0.37 \pm 0.09$ $\mu$M NO (Figure 4A). After preparation, the samples were kept on ice and protected from light. Samples were then taken and assayed for NO release in the presence of copper and cysteine. The results are in good agreement with our vessel-relaxation studies an showed a 10–15 % decrease in activity over the first 2 h, after which little change in NO release occurred (Figure 4B).

The slow onset of relaxation could be elicited by a slow spontaneous decay of the compound. To determine whether this was occurring, 40 $\mu$l aliquots of the reaction mixture (20 mM $ONOO^-$, 300 mM glycerol) were added to the NO electrode chamber containing the Krebs–Henseleit buffer used in the vessel-relaxation experiments. Under these conditions, the maximal amount of NO released was $0.23 \pm 0.03$ $\mu$M, which was 23 % ($0.99 \pm 0.12$ $\mu$M) of the amount released in the presence of cysteine and $CuSO_4$.

Nitration of aromatic amino acids by $ONOO^-$ is enhanced by $CO_2$ [38]. $CO_2$ is present in biological samples in equilibrium with bicarbonate. The reaction of glycerol (300 mM) with $ONOO^-$ (20 mM) was performed in the absence and presence of 30 mM bicarbonate, which is in the upper physiological range. The amount of NO released from the NO donors formed in the reaction was determined as described above in the product-stability experiments. The maximum concentration of NO formed in the presence of bicarbonate was $0.303 \pm 0.11$ $\mu$M, compared with $0.37 \pm 0.16$ mM (mean $\pm$ S.E.M., $n = 3$). This result indicates that $CO_2$ does not have a significant impact on the reactions leading to the formation of NO donors from glycerol and $ONOO^-$.

### Identification of the reaction products of glycerol and $ONOO^-$

To identify the $ONOO^-$/glycerol reaction products, the mixtures, without dilution, were separated by HPLC and the eluate was

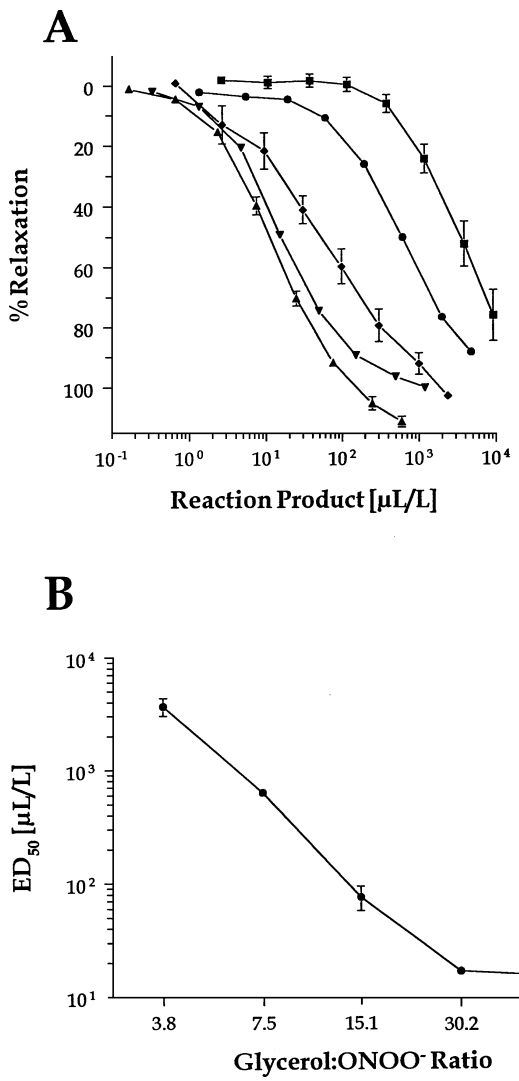


**Figure 2 Concentration-dependence of $ONOO^-$/glycerol reaction products on the relaxation of rabbit aortic ring segments**

(**A**) Increasing concentrations of glycerol stock solution (0.077–1.210 M) were treated with $ONOO^-$ (20 mM) for 30 min to give the following molar ratios of glycerol/$ONOO^-$: 3.8 (■), 7.5 (●), 15.1 (◆), 30.2 (▼), 60.5 (▲). Rabbit aortic rings were precontracted with phenylephrine, and the products of the $ONOO^-$/glycerol reaction were added cumulatively to the tissue bath. Dose–response curves were shifted leftward with increasing concentration of glycerol in the original reaction with $ONOO^-$. The more potent the product, the greater the relaxation at a given dilution. Data are presented as the percentage of maximal relaxation. (**B**) Relationship between the $EC_{50}$ for relaxation and ratio of glycerol/$ONOO^-$ in the reaction mixture. Reaction of 20 mM $ONOO^-$ with increasing concentrations of glycerol resulted in an enhanced production of vasorelaxant metabolites, as revealed by a significant decrease in the $EC_{50}$ for relaxation. The maximum yield of NO donor was achieved under conditions where glycerol was allowed to react with $ONOO^-$ at a molar ratio of 15.1. The data are expressed as $\mu$l of reaction product per l and represent the mean ± S.E.M.

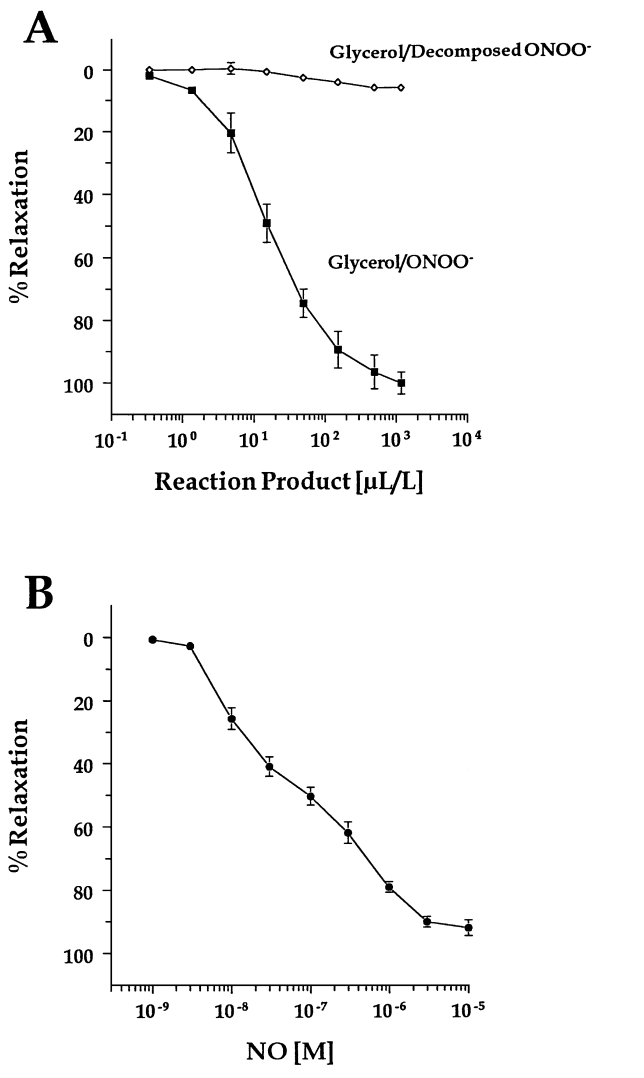


**Figure 3 Cumulative dose–response profiles to the $ONOO^-$/glycerol reaction products and authentic NO**

Rabbit aortic rings were precontracted with phenylephrine, followed by cumulative addition of the products of the $ONOO^-$ (20 mM)/glycerol (605 mM) reaction (**A**). Addition of glycerol that had previously been incubated with decomposed $ONOO^-$ did not induce relaxation of rabbit aortic ring segments. As a control, ring segments were exposed to cumulative concentrations of authentic NO (**B**). As indicated, the relaxation of ring segments induced by NO occurred at much lower concentrations. Data are means ± S.E.M.

analysed by electrospray ionization MS, scanning a $m/z$ range between 60 and 300. Creation of the selected ion chromatogram for the $(M-H)^-$ ion of glycerol ($m/z$ 91) when glycerol was mixed with decomposed $ONOO^-$ at a concentration equivalent to 20 mM of the active species resulted in the elution profile shown in Figure 5(A). Under these conditions glycerol was eluted as a trailing peak (due to sample overload) at between 6.5 and 11 min. Other ions in the control samples were adducts of glycerol with methanol ($m/z$ 124), chloride ($m/z$ 127), formate

**Table 1 Stability of the glycerol/$ONOO^-$-derived NO donors measured by relaxation of aortic rings**

Reaction conditions for the preparation of the glycerol/$ONOO^-$ mixtures and addition to the vessels in the organ bath were identical with those described in Figure 2. The data are represented as the $EC_{50}$ for the relaxation of the vessels from the computed fit of the sigmoidal cumulative dose–response curve. The values represent mean ± S.E.M. for the fit that was defined by a minimum of 11 determinations.

| Glycerol/$ONOO^-$ (molar ratio) | $EC_{50}$ ($\mu$l/l) | |
|---|---|---|
| | Relaxation at time zero | Relaxation at time 2 h |
| 3.85 | 3667 ± 666 | 3355 ± 274 |
| 15.1 | 75 ± 18 | 584 ± 2 |
| 60.5 | 15 ± 1 | 19 ± 1 |

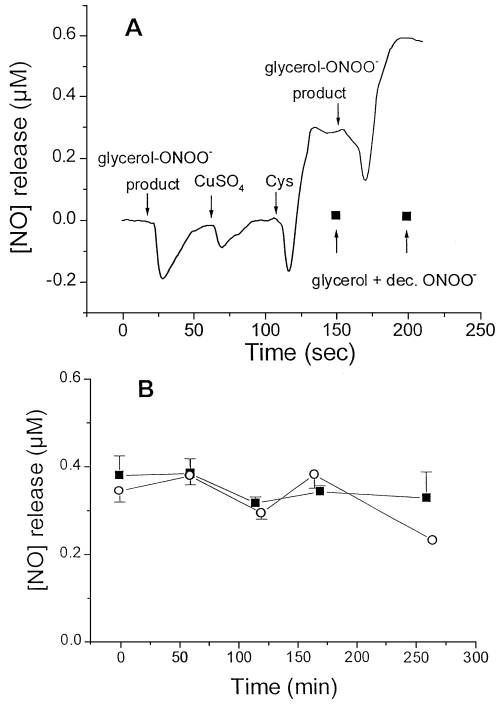


**Figure 4 Stability of the NO donors formed on reaction of glycerol or glucose with $ONOO^-$**

The reaction products for 300 mM glycerol or glucose and $ONOO^-$ (20 mM) were prepared as described in the Materials and methods section and kept under the same conditions as those used for the vessel-relaxation studies (i.e. on ice and shielded from light). Aliquots (40 $\mu$l) were then taken at the time intervals shown and added to the chamber of an NO electrode containing 2 ml of 0.2 M sodium phosphate buffer, pH 7.4, 200 $\mu$M L-cysteine and 100 $\mu$m $CuSO_4$ at 37 °C. (**A**) shows first the addition of the glycerol/$ONOO^-$ mixture followed by $CuSO_4$ and cysteine as indicated by the arrows. The decrease in the signal on addition of compounds is due to temperature fluctuation. NO released from the glycerol/$ONOO^-$ mixture after two successive additions to the chamber of the NO electrode is shown. The mixture of decomposed (dec.) $ONOO^-$ and glycerol did not result in NO release (equivalent concentrations shown as ■). Measurement of the maximal production of NO was then determined as the stable plateau of NO release and converted into the concentration of NO using an authentic NO standard. (**B**) shows the maximal NO release from the glycerol/ (■) or glucose/ (○) $ONOO^-$ mixtures measured for the times shown after the reaction. The data represent the means ± S.E.M. ($n$ = 3–4 determinations).

**Table 2 Predicted masses of the negative ions of mononitrated or nitrosated adducts of glycerol**

| | Glycerol | Glyceryl mononitrate | Glyceryl mononitrite |
|---|---|---|---|
| $(M-H)^-$ | 91 | 136 | 120 |
| Glyceryl–formate adduct | 137 | 182 | 166 |
| Glyceryl–acetate adduct | 151 | 196 | 180 |

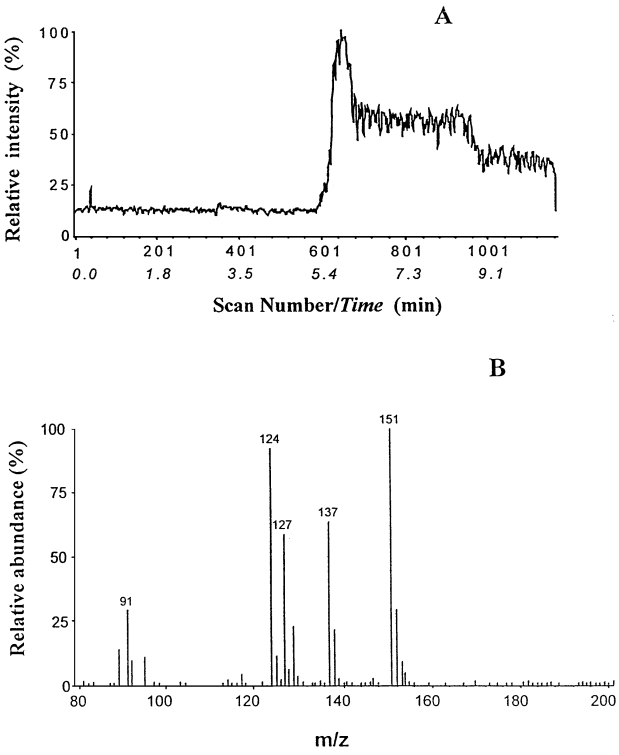


**Figure 5 Glycerol is detected as the formate and acetate adducts by HPLC/electrospray MS**

$ONOO^-$ (20 mM) was mixed with HCl (72 mM, pH 7.4) for 30 min at room temperature, protected from light, before the addition of glycerol (600 mM). The mixture of products (10 $\mu$l) was then injected on to the HPLC column and the eluate subjected to MS analysis. (**A**) Selected ion chromatogram for the negative ion of glycerol ($m/z$ 91); (**B**) mass spectra for 5.55 min with background due to the mobile phase subtracted.

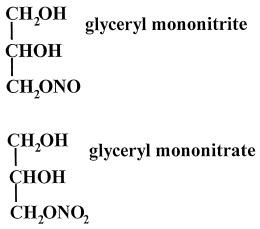


**Figure 6 Structures of glyceryl mononitrate and glyceryl mononitrite**

($m/z$ 137) and acetate ($m/z$ 151) (Figure 5B). Essentially identical results were obtained if unmodified glycerol was injected without separation by HPLC analysis and after incubation with decomposed $ONOO^-$ (result not shown). Chloride is introduced into the sample as a consequence of neutralization of the alkaline solutions that arise on mixing glycerol with $ONOO^-$; methanol and acetate are major components, and formate is a contaminant, of the mobile phase used for HPLC analysis. The assignment of the negative ions with $m/z$ 137 and 151 was confirmed by MS/MS analysis of these species, which yielded the formate and acetate daughter ions respectively (results not shown).

The molecular masses of the negative ions of the predicted compounds arising from the formation of the formate and acetate adducts of glyceryl mononitrate and glyceryl mononitrite are shown in Table 2 and the structures in Figure 6. Figure 7 shows the mass chromatogram of the reaction products of $ONOO^-$ and decomposed $ONOO^-$ with glycerol, monitored at an $m/z$ of 180, the molecular mass of the acetate adduct of glyceryl mononitrite (Table 2). A trailing peak was detected with a maximum intensity at a retention time of 6.9 min, whereas no significant ions of this mass were detected above background

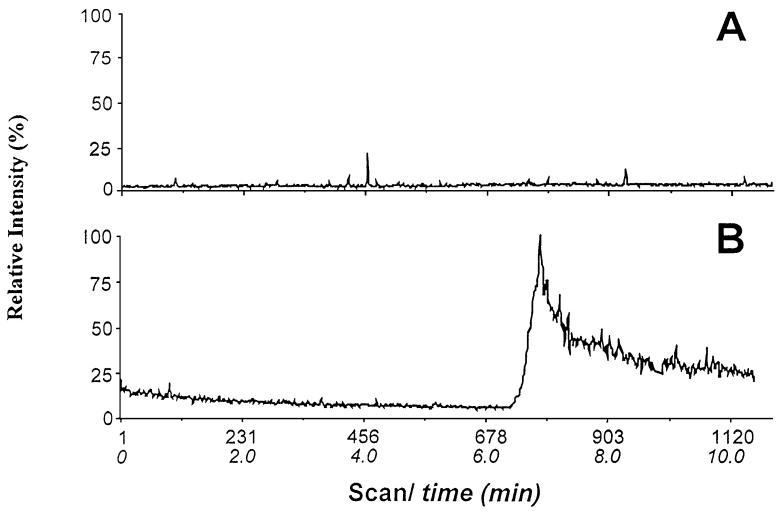


**Figure 7 Formation of new compounds from the reaction of $ONOO^-$ with glycerol**

Selected ion chromatograms (*m/z* 180) are shown for the mixing of glycerol (130 mM) with 20 mM decomposed $ONOO^-$ (**A**) or $ONOO^-$ (**B**) followed by neutralization.

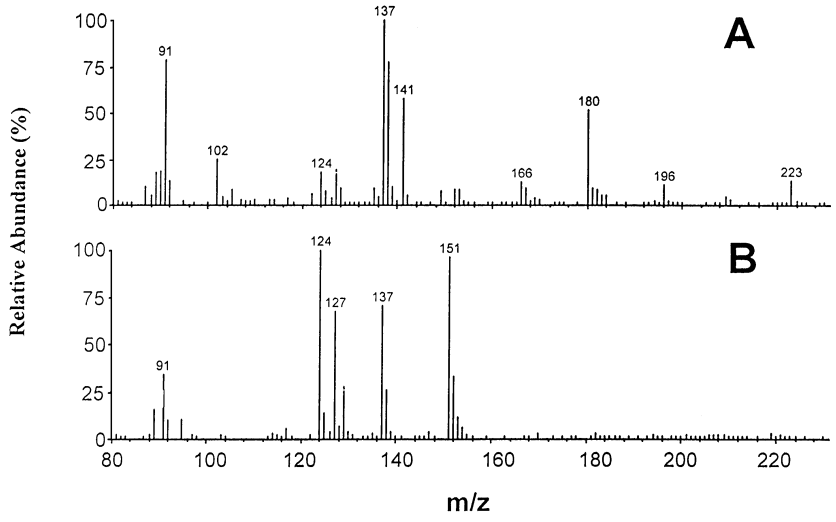


**Figure 8 Mass spectra of the products of reaction of glycerol with $ONOO^-$**

The mass spectrum for the mixing of glycerol (130 mM) with $ONOO^-$ (20 mM) at a retention time of 6.93 min (**A**) and decomposed $ONOO^-$ (**B**) is shown. The masses of the negative ions of the novel products and unchanged glycerol are marked.

with decomposed $ONOO^-$ and glycerol (Figure 7). The mass spectra at 6.9–7.2 min of the $ONOO^-$ and decomposed $ONOO^-$/glycerol mixtures from the chromatogram shown in Figure 7 are shown in Figure 8. They revealed a range of new compounds of *m/z* 166–223 only in the samples containing authentic $ONOO^-$. The peaks at *m/z* 166, 180, 182 and 196 correspond to the *m/z* of the negative ions of the acetate and formate adducts of both glyceryl mononitrate and glyceryl mononitrite (Table 2). To confirm the assignment of these species, MS/MS was performed on the parent *m/z* 196 and 180 ions and resulted in the formation of the acetate ion (*m/z* 59) and nitrate (*m/z* 62) daughter ions in the case of the 190 ion, and acetate ion only for the 180 ion (Figure 9). Since NO is not a negative ion, it would not be detected in the negative-ion mode of the spectrometer. It is likely that similar compounds are formed from the reaction of glucose with $ONOO^-$. However, the putative organic nitrates or nitrites formed on reaction of glucose with $ONOO^-$ could not be detected by MS (result not shown). The reasons for this are not clear, but it is probable that the compounds do not readily form molecular ions under these conditions.

## DISCUSSION

Previous studies have shown that both glucose and glycerol react with $ONOO^-$ to form compounds that cause relaxation of isolated vascular preparations [18]. This result was confirmed in the present study by the addition of increasing concentrations of the mixture of glycerol and $ONOO^-$ to rabbit aortic rings *in vitro*

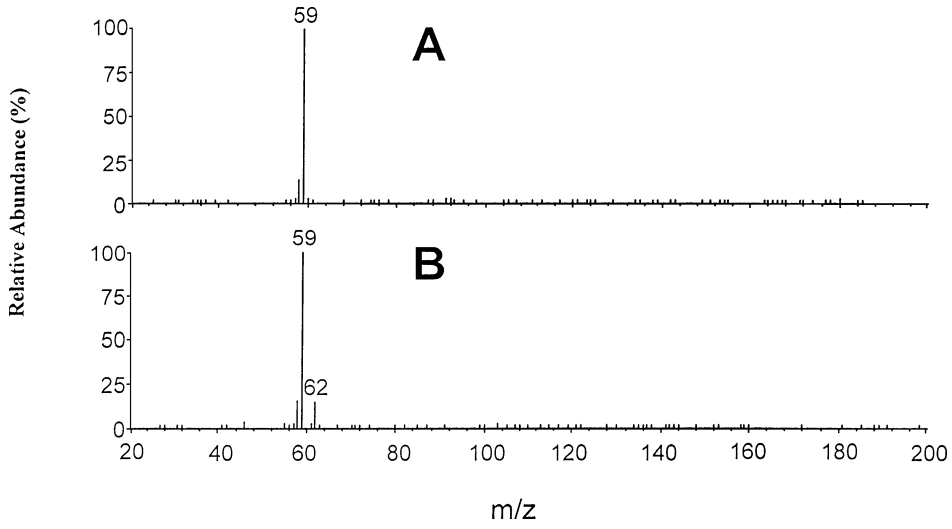


**Figure 9 MS/MS of the acetate adducts of the $ONOO^-$/glycerol products**

The peaks corresponding to the ions of *m/z* 180 (**A**) and *m/z* 196 (**B**) were subjected to fragmentation. The resulting acetate ion and nitrate anions are shown.

(Figure 1). Treatment of glycerol with decomposed $ONOO^-$ failed to produce an NO-donating metabolite. The $EC_{50}$ for vasorelaxation of the $ONOO^-$/glycerol mixture (glycerol/$ONOO^-$ ratio = 15.1) was $75 \pm 18$ $\mu$l/l, which compares with a value of $121 \pm 38$ nM for authentic NO added to the organ bath. The value for NO is probably overestimated, since it is rapidly purged from the tissue bath during the experiment (Figure 1). The $EC_{50}$ for relaxation of rat aortic ring segments to the organic nitrite, amyl nitrite, is 1.1 $\mu$M [32]. Glyceryl trinitrate is reported to have an $EC_{50}$ of approx. 0.07 $\mu$M under these experimental conditions [24]. Assuming an efficiency of NO donation to the vascular tissue similar to that of the organic nitrates, then $75 \pm 18$ $\mu$l of the NO donor formed by the reaction of glycerol and $ONOO^-$ is equivalent to 70 nM glyceryl trinitrate. It is then estimated that the concentration of putative NO donors formed as products of the reaction of glycerol with $ONOO^-$ is approx. 1–1.68 mM. This represents an approx. 5% yield for the NO-donating compound in the reaction mixture with respect to $ONOO^-$. Since $ONOO^-$ is capable of both nitration (the addition of $NO_2$) and nitrosation (the addition of NO) reactions, the products formed on reaction with glycerol are probably either glyceryl mononitrite or glyceryl mononitrate [3,33].

It has previously been shown that the reaction of the products of the mixture of glycerol and $ONOO^-$ with cysteine and $CuSO_4$ results in the formation of NO [18]. This result was interpreted as evidence in favour of the hypothesis that a stable organic nitrate or nitrite had been formed. This experiment was repeated in the current study with two concentrations of $ONOO^-$/glycerol mixtures, and the chromatogram extracted for the negative ions of *m/z* 166, 180 or 196. The current at maximum peak height was determined and decreased 60–40% for the species of *m/z* 166 and 180, with little change for the species of *m/z* 196. This is consistent with the known ability of organic nitrites to be converted into nitrosothiols and their subsequent decomposition to release NO under these conditions [34–36].

The slow prolonged relaxation induced by the $ONOO^-$/glycerol product may be due to the time required for the metabolic activation of the NO donor. This is in contrast with the rapid decrease in tension induced by authentic NO. Alternatively, the slow spontaneous decomposition of an NO donor may account for the observed vasorelaxation. To assess this, NO formation by the glycerol/$ONOO^-$ mixture was measured in the same buffer as used for the organ-bath studies. From this experiment, we can calculate that the dilution of the glycerol/$ONOO^-$ mixture yielding a maximal relaxation (i.e. 8000-fold) would result in the addition of approx. 2 nM NO. This would not be sufficient NO to cause significant relaxation of the vessels (Figure 3). Furthermore the NO-donating compounds formed on reaction with $ONOO^-$ with either glucose or glycerol showed a negligible rate of decomposition, indicating that spontaneous decomposition rates are slow (Figure 4).

The conversion of organic nitrates and other NO-donating compounds into their physiologically active forms has been ascribed to a variety of cellular enzyme systems. The cellular mechanisms by which organic nitrates and nitrites are converted into NO-donating species may be quite different [32]. The metabolism of glyceryl trinitrate to NO appears to be dependent on the activation of a membrane-associated enzyme [31,32]. Evidence suggests that glutathione S-transferase and the NADPH-dependent cytochrome *P*-450 system may play important roles in the conversion of organic nitrates into their physiologically active forms [35,37]. The biotransformation of organic nitrites such as amyl nitrite has recently been ascribed to both a membrane-bound glutathione S-transferase [36] and an uncharacterized cytosolic enzyme [32].

In this study, the concentrations used for both $ONOO^-$ and glycerol far exceed those that can be expected physiologically, but were necessary to achieve sufficient amounts of product to be detected and characterized using the HPLC/MS methodology described here. The biological responses to the organic nitrates and nitrites formed from these reactions do, however, occur at concentrations that are some 10000-fold lower than required for detection by direct chemical methods. For example, significant vasorelaxation occurred after a 10000-fold dilution of the glycerol/$ONOO^-$ mixture (Figure 1). Furthermore, vasorelaxation and inhibition of platelet aggregation occurred after addition of low concentrations of $ONOO^-$ (30 $\mu$M) to physiological concentrations of glucose [18]. If these compounds were formed pathologically, they could contribute to vascular dysfunction by inducing tolerance to the effects of NO on prolonged

exposure. This raises the intriguing possibility that organic nitrates and nitrites, which have been used for many years as therapeutic agents, may be produced endogenously and contribute to the pathophysiology associated with chronic inflammation.

We gratefully acknowledge support from the National Institutes of Health Grants no. HL 48676-04 and HL 54815. The mass spectrometer was purchased by funds from an NIH Instrumentation grant (S10RR06487) and from this institution. Operation of the UAB Comprehensive Cancer Center Mass Spectrometry Shared Facility has been supported in part by an NCI Core Research Support Grant to the UAB Comprehensive Cancer Center (P30 CA13148).

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Received 4 April 1997/4 July 1997; accepted 30 July 1997