Evidence for peroxynitrite formation during *S*-nitrosoglutathione photolysis in air saturated solutions

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Abstract Flash photolysis of *S*-nitrosoglutathione (GSNO) in aerated solutions at pH 10 gave rise to an absorption with a maximum around 310–320 nm. This peak is spectrally similar to that displayed by $ONOO^-$. The decay kinetics of this absorption was compared to that of authentic $ONOO^-$, generated independently. An excellent correlation was obtained. Further proof of $ONOO^-$ generation was provided by HPLC studies showing the production of 3-nitrotyrosine on irradiation of GSNO in the presence of tyrosine at pH 7.4. In addition, the nitration yield was increased ~5-fold in the presence of bicarbonate and totally eliminated with DMPO, indicating the requirement of a radical intermediate for peroxynitrite production during *S*-nitrosothiol photolysis.

Key words: *S*-Nitrosoglutathione; Peroxynitrite; Photolysis; Nitric oxide

1. Introduction

Peroxynitrite ($ONOO^-$) is a product of the reaction of NO with superoxide ($O_2^{\bullet-}$). At physiological pH, the protonated form ONOOH ($pK_a = 6.8$) is significant and rapidly breaks down to ultimately form nitrate (NO_3^-) [1]. The decay process is complex involving the formation of reactive intermediates with nitrating, nitrosating and hydroxylating properties [2,3]. Breakdown of ONOOH in the cellular milieu can therefore result in reactions with proteins, lipids and nucleic acids, leading to irreversible modifications. Biological damage produced in this manner by $ONOO^-$ is employed in mammalian defence mechanisms where macrophages can kill foreign organisms by simultaneous production of large amounts of both NO and $O_2^{\bullet-}$ and resultant peroxynitrite.

In previous studies we showed that GSNO and related compounds release NO in high quantum yield via homolytic bond cleavage on photoexcitation, although the photochemistry of these compounds is complicated by subsequent reactions of the initial radicals produced [4,5]. In this communication, we present evidence for the production of $ONOO^-$ upon photolysis of *S*-nitrosoglutathione (GSNO) in aerated solutions.

2. Materials and methods

2.1. Chemicals

L-Tyrosine, 3-nitro-L-tyrosine, glycine, trifluoroacetic acid and sodium phosphate were purchased from Sigma (St. Louis, MO). GSNO was synthesized according to a procedure by Hart [6], stored at −80°C and protected from light. Peroxynitrite was prepared in a continuous-flow system according to previously described methods [7]. HPLC grade acetonitrile was purchased from Aldrich.

2.2. Experimental procedures

2.2.1. Flash photolysis. A frequency-tripled Quantel YG660 Nd/YAG laser (Continuum, Santa Clara, CA) was used to provide 355 nm excitation (8 ns pulse duration, < 20 mJ/pulse) for flash photolysis experiments, using an apparatus described in detail elsewhere [8,9]. Solutions of GSNO in aqueous buffers of varying pH (pH 6-−8.5 phosphate, 0.1 mol/l; pH 9–11 glycine, 0.1 mol/l) were prepared with an absorption of 0.1–1.0, depending on the experiment. Samples were irradiated in 1×1 cm path length quartz cuvettes and were subjected to no more than 5 laser pulses per sample. Transient absorption spectra were constructed from kinetic traces for individual wavelengths at various time delays with respect to the laser pulse. Samples were sealed with a rubber septum and purged with nitrogen or oxygen, as required.

In another series of experiments, GSNO was irradiated in aerated solution at pH 10 with 50 pulses from a Nd/YAG laser at 355 nm (5 mJ pulse, 5 Hz repetition rate) and the cuvette immediately transferred to a Hewlett Packard HP8451 UV/visible spectrophotometer. The time dependent change in absorbance at 302 nm was then followed over a period of 30 min.

2.3. Evidence for 3-nitro-L-tyrosine formation during GSNO photolysis in the presence of L-tyrosine

2 mmol/l GSNO plus 50 μmol/l L-Tyr (in phosphate buffer, pH 7.4, 1 ml total volume) was irradiated for 1 h with 355 nm light (0.085 W/cm^2) in a Shimadzu RF-551 fluorimeter and 200 μl of the irradiated mixture was then chromatographed on a BioLogic HPLC System equipped with a C_{18} column (Microsorb-MV; 50×240 mm). Where indicated, the irradiation mixtures contained either 100 μM $NaHCO_3$ or DMPO. Separation of the components was achieved by a linear gradient that reached 60% CH_3CN, 0.1% trifluoroacetic acid in 30 min (at a flow rate of 0.35 ml/min). The amount of 3-nitro-L-tyrosine was quantified by comparing peak area (retention time 23.11 min) to a standard curve generated using authentic samples of 3-nitro-L-tyrosine subjected to HPLC under identical conditions using a Microsorb-MV (C18; 50×240 mm) HPLC column (Rainin Instrument Co., Woburn, MA).

3. Results

3.1. Evidence for peroxynitrite formation during photolysis

Fig. 1A shows the transient absorption spectra obtained on 355 nm excitation of GSNO in air saturated aqueous solution at pH 10. The initial spectrum is dominated by loss of GSNO absorption as described previously [5]. However, the absorption increases with time at wavelengths below 375 nm. Subtraction of the initial spectrum at 3.5 μs from the spectrum at 34 μs, when the growth is complete reveals the spectrum of the species responsible, as shown in Fig. 1B. This species does not subsequently decay on the time scale of the laser flash photolysis experiments (< 10 ms). The differential spectrum in Fig. 1B shows a maximum around 320 nm which is the difference spectrum between this species and the GSNO precursor. The similarity in spectral profile and kinetic behavior

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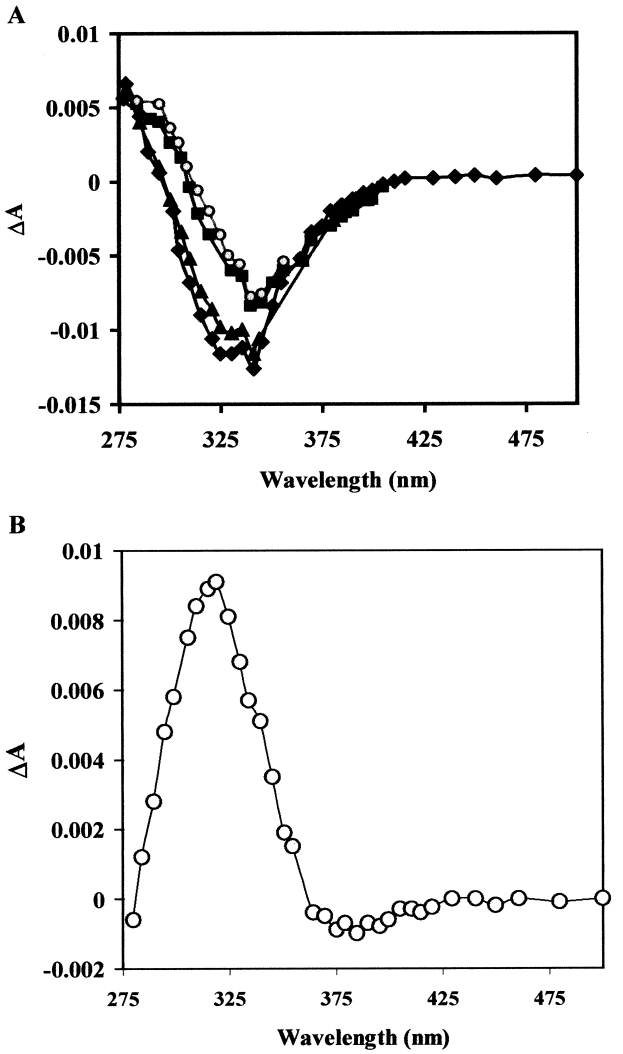

Fig. 1. A: Time dependent absorption spectrum measured for GSNO in air saturated solution at pH 10 at delays of (○) 34 μs, (□) 18.5 μs, (△) 6.0 μs and (◇) 3.5 μs after the laser pulse. B: Differential spectrum obtained by subtraction of initial spectrum in A (◇) from last spectrum (○).

of this species to peroxynitrite prompted us to investigate whether this was actually the case. If peroxynitrite is formed during photolysis of GSNO a species absorbing at 302 nm with the decay characteristics of authentic peroxynitrite should be observed. To test this, the rate constant for decay of authentic $ONOO^-$ was determined under conditions identical to those of the photolysis experiments (i.e. in the presence of GSNO, 0.1 M glycine buffer, pH 10, 25°C). The decay of authentic $ONOO^-$ was characterized by a first order process with a rate constant of $0.013 \pm 2\%\ s^{-1}$ (Fig. 2). A sample of GSNO (0.1 M glycine buffer, pH 10, 25°C) was then exposed to 50 pulses from a Nd/YAG laser at 355 nm (5 mJ pulse, 5 Hz repetition rate), rapidly transferred to a spectrometer and the subsequent absorbance at 302 nm was monitored as a function of time. The resultant decay was also a first order process with a nearly identical decay rate constant ($0.013 \pm 10\%\ s^{-1}$) to that of authentic peroxynitrite. The same procedure was carried out at lower pHs (9.5 and 9) where the lifetime of $ONOO^-$ is shorter and again good agreement was seen in the decay of the absorbance of authentic $ONOO^-$ and that resulting from irradiation of GSNO. Thus, these spectral and kinetic studies support the formation of $ONOO^-$ from irradiation of GSNO.

3.2. *Evidence that GSNO photolysis forms significant amounts of $ONOO^-$ at physiological pH*

In recent work, we have demonstrated that cancer cells in culture can be killed when irradiated in the presence of NO-releasing agents such as GSNO [4] and thionein-NO [11] at physiological pH. The data presented in this communication indicates that peroxynitrite may well be the cytotoxic molecule produced during GSNO photolysis. However, it is not clear whether sufficient amounts would be produced at physiological pH to induce cell killing. In order to test this a chemical trap was required to demonstrate the existence and reactivity of peroxynitrite under these conditions. Nitration is a well-known reaction of the protonated form of peroxynitrite, peroxynitrous acid (ONOOH) which is unstable and fragments to give nitrating intermediates. L-Tyr was employed as a suitable trap for this species, yielding 3-nitrotyrosine as product. The detection of 3-nitrotyrosine has been used previously in biochemical studies as evidence of the involvement of peroxynitrite [12–14]. Thus, a solution containing 2 mM GSNO and 50 μM L-Tyr (in PBS, pH 7.4) was irradiated for 1 h at 355 nm ($0.085\ W/cm^2$). The mixture was then analyzed by reverse phase HPLC. As can be seen from Fig. 3A, the irradiated mixture exhibits a peak corresponding to the retention time of authentic 3-nitrotyrosine. The peak area translated to a 3-nitrotyrosine concentration in the mixture of ~1 μmol/l. These results indicate under the conditions employed there was enough $ONOO^-$ produced at physiological pH to nitrate ~2% of tyrosine that was present. It is well established that $ONOO^-$ rapidly reacts with carbon dioxide to form an intermediate ($ONOOCO_2^-$) which is more reactive in tyrosine activation than $ONOO^-$ itself. Lymar et al. [14] have shown that nitration yields nearly doubled when performed in the presence of $ONOO^-$ plus CO_2. Therefore, if peroxynitrite is produced during GSNO photolysis, the nitration yields should increase in the presence of CO_2. To test this, the irradiations

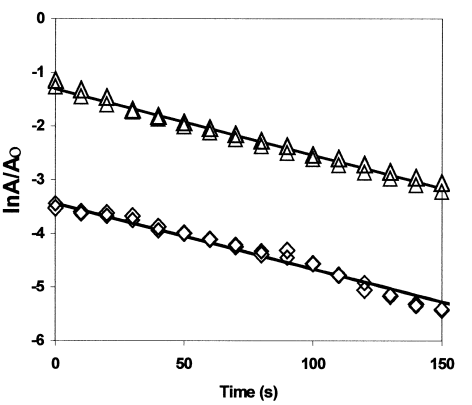

Fig. 2. Decay rates for authentic and photogenerated $ONOO^-$. Decay at 302 nm of authentic $ONOO^-$ (0.18 mM, in 0.1 M glycine buffer, pH 10, 25°C) (▲); and GSNO (0.1 mM, in 0.1 M glycine buffer, pH 10, 25°C) which was exposed to 50 pulses from a Nd/YAG laser at 355 nm (5 mJ pulse, 5 Hz repition rate) then rapidly transferred to the spectrometer (◇). The solid lines represent the best fit of the data to the first order decay equation. Data from both experimental trials are included. The reported errors are standard deviations ($n = 2$).

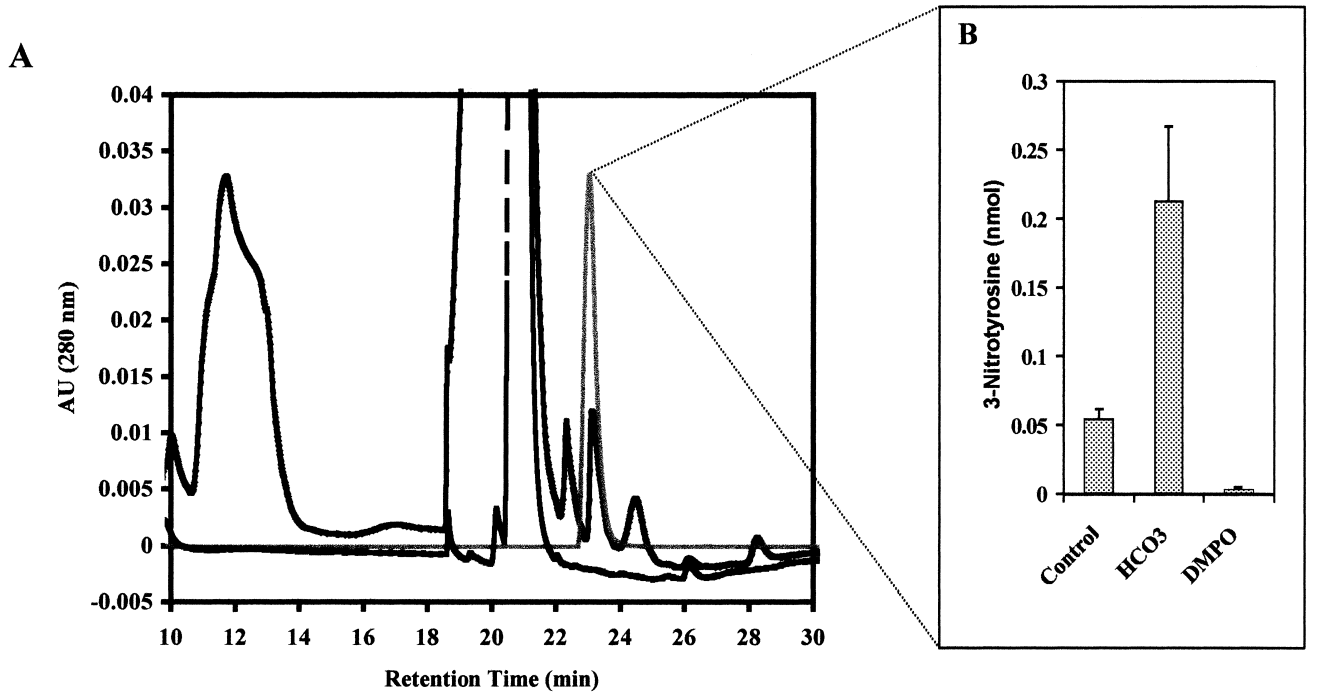

Fig. 3. A: Reverse phase (C_{18}) HPLC elution profiles of authentic 3-nitro-L-Tyr (5 μM) (dotted line), L-Tyr (50 μM) (dashed line) and 2 mM GSNO and 50 μM L-Tyr which was irradiated for 1 h at 355 nm (0.085 W/cm^2) (solid line) in PBS, pH 7.4. B: 3-Nitrotyrosine peak area expressed as nmol (as determined from a standard curve with authentic 3-nitrotyrosine) for irradiations performed as in Fig. 2A. Control or in the presence of 100 μM $NaHCO_3$ or DMPO.

were performed in the presence of phosphate buffer containing 100 μM $NaHCO_3$. As shown in Fig. 3B, nitration yield increased ~3.9-fold when the irradiation was performed in the presence of a CO_2 source. This is further evidence that peroxynitrite is formed during GSNO photolysis.

In order to test the involvement of GS$^\bullet$ or GSOO$^\bullet$ in peroxynitrite formation the GSNO was photolyzed in the presence of L-Tyr plus the radical quencher 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). As shown in Fig. 3B nitration was drastically quenched in the presence of DMPO indicating the possible requirement of GS$^\bullet$ or GSOO$^\bullet$ for $ONOO^-$ production during GSNO photolysis.

4. Discussion

This study was prompted by the observation of the appearance of a new absorption band around 310 nm (Fig. 1), upon photolysis of aerated GSNO solutions at high pH (vide infra). Our interest was heightened as a similar band at 302 nm corresponds to the λ_{max} of authentic peroxynitrite [10]. The several lines of evidence, obtained here, support the hypothesis that the photolysis of GSNO leads to the formation of peroxynitrite. The strongest of these is the fact that, the decay kinetics of this absorption band obtained upon GSNO photolysis was nearly identical to that obtained with of authentic $ONOO^-$, generated independently. Further proof of $ONOO^-$ generation was provided by HPLC studies showing that the irradiation of GSNO in the presence of tyrosine at pH 7.4 yielded 3-nitrotyrosine. It is well established that CO_2 enhances $ONOO^-$ induced nitration yields [14]. Thus, when the irradiation of GSNO was performed in the presence of a CO_2 source, ~4-fold increase in the nitration yield was observed. On the other hand the tyrosine nitration was nearly eliminated in the presence of the radical trap DMPO, indicating the requirement of a radical intermediate for peroxynitrite production during *S*-nitrosothiol photolysis.

The above results provide spectroscopic and chemical evidence for the formation of peroxynitrite on irradiation of GSNO in aerated buffer solution. By what mechanism then does peroxynitrite formation occur? In a previous study on GSNO [5] we demonstrated a complex photochemistry with the possible involvement of many different transient species, depending on irradiation conditions. Following bond homolysis NO and GS$^\bullet$ are formed. GS$^\bullet$ in aerated solutions reacts with oxygen giving GSOO$^\bullet$ (λ_{max} 550 nm). GS$^\bullet$ may also react with $GSNO+H_2O$ to yield $GSSG^{\bullet-}+NO_2^-$, $GSSG^{\bullet-}$ can reduce O_2 to superoxide ($O_2^{\bullet}-$) [15,16]. $NO^\bullet+O_2^{\bullet-}$ would then react to form $ONOO^-$.

This work shows through spectroscopy, kinetics and product studies that the highly reactive species, $ONOO^-$, can be a product of irradiation of an innocuous NO precursor such as GSNO. This is very significant as it may provide an explanation for the previously demonstrated light-dependent cytotoxicity of GSNO and other photolabile *S*-nitrosylated proteins towards cancer cells in culture [4,11].

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