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Selective modifiers of glutathione biosynthesis and 'repriming' of vascular smooth muscle photorelaxation

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1 Photorelaxation of vascular smooth muscle (VSM) is caused by the release of nitric oxide (NO) from a finite molecular store that can be depleted by irradiating pre-contracted arteries with visible light. The ability of an 'exhausted' vessel to respond to a further period of illumination is lost temporarily but then recovers slowly as the photosensitive store is reconstituted in the dark. The recovery process, termed repriming, displays an absolute requirement for endothelium-derived NO and is inhibited by pre-treating arteries with ethacrynic acid, a thiol-alkylating agent. Here we demonstrate that agents that up- or down-regulate glutathione (GSH) biosynthesis influence the extent to which the store is regenerated in the dark.

2 Isolated rat tail arteries (RTAs) were perfused internally with Krebs solution containing phenylephrine (PE; mean [PE] ± s.e.mean: 5.78 ± 0.46 μM) and periodically exposed to laser light ($\lambda = 514.5$ nm, 6.3 mW cm^{-2} for 6 min). Photorelaxations of control RTAs were compared with those from either (a) vessels taken from animals previously injected i.p. with buthionine sulphoximine (BSO), an inhibitor of γ-glutamylcysteine synthetase (three injections, 100 mg kg^{-1} at 8 h intervals); or (b) isolated RTAs that were perfused *ex vivo* with oxothiazolidine (OXO), a precursor of cysteine (10^{-4} M OXO for 60 min). RTAs from BSO-treated animals exhibited attenuated photorelaxations: the mean (± s.e.mean) amplitude of the response recorded after 72 min recovery in the dark was 12.4 ± 1.6% versus 21.4 ± 2.9% for control arteries ($n = 5$; $P < 0.01$). Conversely RTAs treated with OXO and allowed to recover for a similar period showed enhanced photorelaxations, 32.6 ± 6.3% as compared to 21.4 ± 2.9% for control arteries ($n = 5$; $P < 0.01$). A hyperbolic curve fit to repriming curves for BSO-treated and control arteries returned asymptote values (maximum photorelaxations) of (mean ± s.e.mean) 24.2 ± 3.2% and 55.2 ± 8.5%, respectively.

3 The level of GSH in RTA extracts was measured by high-pressure liquid chromatography (HPLC). Injecting animals with BSO decreased GSH to 85% of control levels ($P < 0.05$) while treatment of isolated vessels with OXO resulted in a 31% increase above control levels ($P < 0.05$). Thus, drug-induced changes in RTA GSH levels were positively correlated with altered photorelaxations.

4 The results lead us to postulate that the photosensitive store in VSM is generated, at least in part, from intracellular GSH which becomes converted to *S*-nitrosoglutathione (GSNO) by nitrosating species that are formed ultimately from endothelium-derived NO. The possible physiological significance of a photolabile store of NO in VSM is discussed briefly.
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Abbreviations: BSO, buthionine sulphoximine; eNOS, endothelial nitric oxide synthase; GSH, glutathione; GSNO, *S*-nitrosoglutathione; GSSG, glutathione disulphide; HPLC, high pressure liquid chromatography; NO, nitric oxide; PE, phenylephrine hydrochloride; RTA, rat tail artery; SNAP, *S*-nitroso-*N*-acetylpenicillamine

Introduction

Furchgott *et al.* (1961) showed that pre-contracted vascular smooth muscle (VSM) relaxes when exposed to light. The discovery that endothelial cells synthesize and release a potent relaxing factor (Furchgott & Zawadzki, 1980), subsequently identified as nitric oxide (NO; Palmer *et al.*, 1987), led to the suggestion that photorelaxation might also be due to endogenous NO, liberated from a photodegradable molecular 'store' of NO contained within the vessel wall (Matsunaga & Furchgott, 1989).

Recent studies support this hypothesis. Venturini *et al.* (1993) showed that repeated exposure of endothelium-denuded rabbit aortic strips to polychromatic light resulted in a progressive loss of photosensitivity, suggesting that fractionated irradiation caused the 'stepwise' depletion of a finite source of NO. A similar conclusion was reached by Kubaszewski *et al.* (1993) who used endothelium-denuded aortic strips isolated from both normotensive and hypertensive rats. These authors also demonstrated u.v. light-induced release of NO directly using a porphyrinic sensor. Megson *et al.* (1995) studied both intact and endothelium-denuded tail arteries from the rat. Their experiments showed that the store was depleted rapidly by exposure to visible ($\lambda = 514.5$ nm) laser light and that irradiated vessels were refractory to a further period of illumination when this was delivered soon (< 1–2 min) after the first. However, the photosensitivity of functionally intact (but not endothelium-denuded) arteries spontaneously recovered in the dark ($t_{1/2} = ca.$ 100 min). The mechanism responsible for regenerating the store, termed repriming, displayed an absolute requirement for endothelium-derived NO. Thus, it was prevented by (a) treating vessels with N^G-monomethyl-L-

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arginine, an inhibitor of NO synthase (Rees *et al*., 1990); (b) perfusing arteries with oxyhaemoglobin, an NO scavenger (Martin *et al*., 1985); or (c) mechanically removing the endothelium. Conversely, repriming was accelerated following brief treatment (*ca*. 300 ms, followed by a 20 min 'washout' period) with either *S*-nitroso-*N*-acetylpenicillamine (SNAP), a NO donor, or authenic NO, both administered as bolus injections (10 μl) into the vessel lumen. The same procedures restored photorelaxations to arteries in which repriming by endogenous NO was prevented by removing the endothelium. Megson *et al*. (1995) also showed that repriming of the store could be prevented by pre-treating intact arteries with ethacrynic acid, a thiol alkylating agent. Taken together, these observations led them to postulate that the photosensitive store of NO could be an intracellular nitrosothiol (RS-NO). Lovren & Triggle (1998) have since provided further evidence in support of this hypothesis.

The tripeptide glutathione (γ-glut-cysH-gly, or GSH) is the most abundant non-protein thiol in mammalian cells, present at concentrations of $\sim$0.1–10 mM (Meister, 1988), and its *S*-nitrosated adduct, *S*-nitrosoglutathione (GSNO), is known to release NO in a photochemical reaction (Sexton *et al*., 1994; Singh *et al*., 1996). The aim of the present study was to investigate the effects of pharmacological agents that selectively perturb the biosynthesis of GSH (buthionine sulphoximine and oxothiazolidine) on the repriming process. We elected to use functionally intact, perfused rat tail arteries as our experimental model, in preference to either arterial 'rings' or endothelium-denuded preparations, for two reasons. First, because repriming depends upon endothelium-derived NO; and second, because fluid shear stress associated with flow is a potent regulator of endothelial nitric oxide synthase (eNOS) activity (Dimmeler *et al*., 1999). Thus, in a functionally-intact artery, flow is likely to be a critical determinant of both the rate and extent to which the photosensitive store is regenerated in the dark. The results of our study lead us to postulate that photorelaxation is due, at least in part, to the photochemical release of NO from GSNO, formed ultimately from endothelium-derived NO and intracellular GSH.

Methods

Preparation

Experiments were performed on isolated segments of tail arteries (RTAs, 8–12 mm long) taken from adult male Wistar rats (mean ($\pm$s.e.mean) weight 319.7$\pm$3.1 g; n=40). Animals were sacrificed by cervical dislocation. Arteries were dissected free, connected to a plastic cannula and perfused (2 ml min^{-1}) with pre-warmed, oxygenated Krebs' solution (composition (mM): NaCl, 118; KCl, 4.7; $NaHCO_3$, 25; NaH_2PO_4, 1.15; $CaCl_2$, 2.5, $MgCl_2$, 1.1; glucose, 5.6; gassed with 95%O_2/5%CO_2 to maintain pH 7.4; temp. 31–33°C). The outer surface of the vessel was superfused with warm Krebs' solution (8 ml min^{-1}). The perfusion pressure was monitored throughout by a differential pressure transducer (Sensym SCX 150NC; Farnell Electronic Components, Leeds, U.K.) located just upstream of the artery. The output of the transducer was fed to a Macintosh LCIII microcomputer *via* a MacLab type 4e AD converter. RTAs were pre-contracted with phenylephrine hydrochloride (PE). The mean ($\pm$s.e. mean) concentration of PE used was 5.78$\pm$0.46 μM, which generated active (total – passive) pressures of 99.8$\pm$2.3 mmHg (n=40 arteries).

Experimental protocols

An argon ion laser (Spectra Physics type 168–09) was used to irradiate vessels periodically (6.3 mW cm^{-2}; λ=514.5 nm; duration, 6 min). This caused a transient vasodilator response: the perfusion pressure fell initially but then recovered fully (>96%) during the period of illumination. 'Depleted' vessels were again exposed to laser light after spending different time intervals (ΔT=20, 40, 72 and 150 min) in the dark. These data were used to construct repriming curves. The amplitudes of photorelaxant responses for normal (control) vessels were compared with those for vessels taken from animals previously injected with BSO, or with vessels which had been perfused (*ex vivo*) with Krebs' solution containing OXO.

Drug treatment

Seven adult male Wistar rats were each given three injections (i.p.) of BSO (dose: 100 mg kg^{-1}) at 8 h intervals, the last of which was administered 2 h before sacrifice. The tail artery was dissected free and perfused with Krebs' solution containing 10^{-4} M BSO throughout the experiment.

In other experiments, vessels were first isolated from untreated animals post mortem and then perfused with OXO. Control photorelaxant responses (ΔT=72 min) were recorded first. OXO (10^{-4} M) was then added to the Krebs' solution and arteries were perfused for 60 min followed by a 12 min washout period in Krebs solution only (ΔT=72 min) before being irradiated again with laser light. The same procedure was repeated, but on the second occasion, oxyhaemoglobin (5 μM) was added to the internal perfusate throughout the 12 min washout period and also during laser irradiation.

Preparation of RTA extracts

BSO-treated and control rats (n=7 and 11 animals, respectively) were killed by cervical dislocation. Immediately after sacrifice, tail arteries were dissected free, rapidly frozen and kept at −70°C until required. Frozen arteries were later homogenized in ice-cold phosphate buffer (composition (mM): Na_2HPO_4, 60; NaH_2PO_4, pH 7.4; 200 μl), then centrifuged at 10,000$\times g$ for 10 min. The supernatant was used for the determination of tissue protein and GSH (reduced plus oxidized) concentrations. RTAs treated *ex vivo* with OXO were similarly extracted.

GSH measurements by HPLC

The procedure used was a modification of the method of Daskalakis *et al*. (1996). An internal standard of 2-mercaptoethylamine was added to RTA extracts to a final concentration of 100 μM. Disulphides were reduced with tri-n-butylphosphine (5%) in dimethylformamide, after which the samples were deproteinized using trichloroacetic acid (10%) and then reacted with 7-fluorobenzo-2-oxa-1,3-diazole sulphonic acid at 60°C for 1 h. Derivatized thiols were separated by HPLC (C_{18} column, Waters Symmetry; mobile phase: 6.5% acetonitrile in 0.1 M KH_2PO_4 adjusted to pH 2.15 with H_3PO_4, flow rate, 0.8 ml min^{-1}) and detected in a fluorescence detector (Perkin Elmer LS30; excitation wavelength=385 nm; emission wavelength=515 nm). Chromatograms were analysed using computer software (JCL 6000 Chromatography Data System). The ratio of the GSH peak area (retention time: 4.8 min) to the 2-mercapoethylamine (internal standard) peak area (retention time: 2.5 min) varied linearly with exogenous GSH in 'spiked' samples of RTA extracts (range: 5–60 μM;

$r^2 = 0.997$). GSH concentrations are expressed throughout in nmole.mg^{-1} protein.

Protein assay

Protein was measured using the Boehringer Mannheim Protein Assay ESL kit. RTA extracts were diluted 1 : 8 with phosphate buffer (pH 7.4; composition as above). The diluted extracts (25 μl) were incubated with alkaline copper tartrate (50 μl; reagent A) for 5 min at room temperature and then treated with ascorbic acid plus bathocuproine disulphonic acid (500 μl; reagant B). The resulting solutions were left for 30 s at room temperature and their absorbance was measured at 485 nm (Philips 8720 u.v./vis spectrophotometer). Protein concentration was determined by comparison with a standard calibration curve constructed using bovine serum albumin.

Source of drugs and reagants used

2-mercaptoethylamine, tri-n-butylphosphine, trichloroacetic acid, 7-fluorobenzo-2-oxa-1,3-diazole sulphonic acid, bovine serum albumin, bovine haemoglobin, buthionine sulphoximine and phenylephrine hydrochloride were all obtained from Sigma-Aldrich Co Ltd (Poole, Dorset, U.K.). Dimethylformamide and acetonitrile were supplied by Merck Ltd (Poole, Dorset, U.K.). Oxothiazolidine was obtained from Bachem Inc. (Saffron Walden, Essex, U.K.).

Data analysis

Pressure recordings were analysed using CHART software. The amplitude of the photorelaxant response, used here as a measure of the extent to which the store has been reconstituted in the dark, is given as the maximum drop in pressure expressed as a percentage of the perfusion pressure immediately before laser irradiation ($\Delta P/P$). All data points represent mean values $\pm$ s.e.mean (except for Figure 1A, where for clarity standard error bars are shown for every 20th data point).

Repriming curves were fitted to a hyperbolic function of the form $Y = B.X/(C + X)$, where

$Y = \Delta P/P$

$B = \Delta P/P_{max}$, the maximum photorelaxation attainable

$C = T_{1/2}$ for $\Delta P/P$ to reach 50% of its maximum value

and $X = \Delta T$

using an iterative statistical procedure (Graphpad Prism software) that considered each replicate observation individually. The data for both control and BSO-treated vessels showed convergence and the calculation for each curve was halted when consecutive iterations changed the sum of squares by $< 0.01\%$.

Statistical comparisons between groups of vessels treated differently were made using a two-tailed, unpaired Student's *t*-test, 2 factor, repeated dose ANOVAs, or by the *post hoc* Bonferroni test, as appropriate ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). Results were considered significant for $P < 0.05$.

Results

GSH measurements

The HPLC assay measured both reduced and oxidized (disulphide) forms of glutathione (i.e. GSH and GSSG, respectively) and our results therefore reflect the total amount of GSH and GSSG in RTA extracts. The intracellular [GSSG] is extremely small in comparison to the [GSH] ($\sim 0.2\%$; Gilbert, 1990) but in the interest of accuracy and brevity, we will refer to 'total GSH and GSSG' measured by HPLC as the 'GSH pool'.

Table 1 summarises the results obtained. BSO treatment reduced the GSH pool to 85% of control level, while in isolated vessels that were treated with OXO, the GSH pool was increased by 31% above the level of control arteries. These results are statistically significant at the $P < 0.05$ level (BSO vs control and OXO vs control).

Effects of BSO and OXO on repriming

Figure 1A shows computer averaged photorelaxations that were recorded 72 min after depleting the store. The symbols

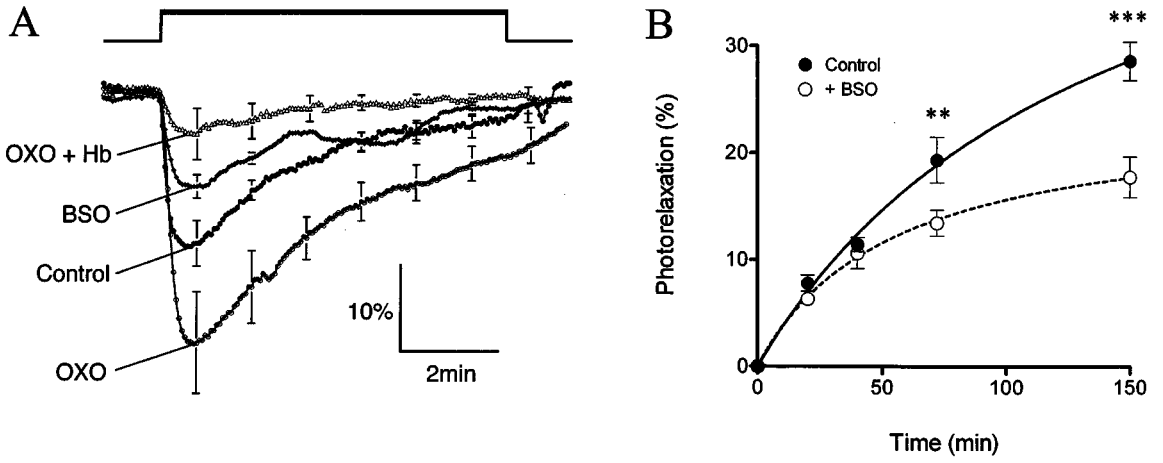

Figure 1 (A) Time-averaged photorelaxations recorded 72 min after depleting the store for control arteries ($n = 5$), arteries taken from BSO-treated rats ($n = 5$), OXO-treated arteries ($n = 5$) and for OXO-treated vessels irradiated in the presence of 5 μM Hb ($n = 4$). Horizontal bar denotes period of laser irradiation (= 6 min). (B) Repriming curves for control (untreated) arteries ($n = 5$) and for arteries taken from animals injected with BSO ($n = 5$). The 72 and 150 min responses for BSO-treated arteries are significantly different from controls ($P < 0.01$ and 0.001, respectively). $\Delta P/P_{max}$ values are estimated to be 55.2% (control) and 24.2% (BSO-treated arteries). The times required to reach 50% maximal responses ($t_{1/2}$ values) are 138 and 54 min, respectively.

Table 1 GSH pool in RTA extracts

	[GSH+GSSG] (nmol.mg^{-1} protein)	*Number of samples*
Control	12.0±0.4	11
BSO treated	10.2±0.6*	6
OXO treated	15.7±1.6*	7

*Two-tailed, unpaired Students' *t*-test. Significance estimated with respect to corresponding control values (*$P<0.05$).

are mean values ($n=4$ or 5) sampled at 3 s intervals. The vertical lines are the standard errors of mean values calculated for every 20th data point.

Photorelaxant responses were attenuated in vessels from BSO-treated animals as compared to control (untreated) vessels: the peak amplitudes were 12.4±1.6% and 21.4±2.9% ($n=5$; $P<0.01$), respectively. In contrast, pretreating isolated arteries with OXO enhanced photorelaxations, from 21.4±2.9% to 32.6±6.3% ($n=5$; $P<0.01$). The potentiating effect of OXO was eliminated by adding Hb (5 μM) to the internal perfusate.

The time course of the repriming process for RTAs taken from BSO-treated animals and from untreated (control) animals is compared in Figure 1B. In each case, the amplitude of the photorelaxant response increases with ΔT, rising asymptotically towards a maximum value. Two-way ANOVA showed that the two sets of data were significantly different ($P<0.0001$). A *post hoc* Bonferroni test revealed that there was no difference in the rate of repriming initially, for $\Delta T<40$ min, whereas the 72 min and 150 min values were significantly greater for control vessels than for BSO-treated vessels ($P<0.01$ and $P<0.001$, respectively). Both curves were well fitted by a hyperbolic function (see Methods) that returned asymptote values ($=\Delta P/P_{max}$; mean±s.e.mean) of 55.2±8.5% and 24.2±3.2% for control and BSO-treated arteries, respectively. The corresponding $t_{1/2}$ values were estimated to be 138±36 min (control) and 54±17 min (BSO-treated).

Discussion

The mechanism of repriming

BSO and OXO are selective modifiers of GSH biosynthesis. Each agent targets a critical step in the γ-glutamyl cycle (Meister, 1988): BSO inhibits γ-glutamylcysteine synthetase, the rate-limiting enzyme in GSH synthesis, and reduces intracellular [GSH], whereas OXO, after conversion to cysteine by 5-oxoprolinase, increases GSH synthesis. The results obtained here establish a positive correlation between altered intracellular GSH pools and modified photorelaxations. Thus, BSO treatment significantly reduced the GSH pool in RTAs and this was associated with impaired photorelaxations, while OXO increased the GSH pool and enhanced photorelaxations.

These results are consistent with the idea that intracellular GSH becomes nitrosated during the repriming process and that endogenous GSNO contributes to the molecular store of NO in VSM cells. GSNO is a relatively stable S-nitrosothiol but it decomposes when exposed to either visible or u.v. light (Sexton *et al*., 1994), releasing NO with formation of the glutathionyl ($GS^{\bullet}$) radical (Singh *et al*., 1996). Reactions of the latter result in the production of glutathione disulphide (GSSG).

The study by Megson *et al*. (1995) demonstrated that the endothelium is the ultimate source of NO required to reprime the store, though it is well-documented that the NO radical *per se* will not react with tissue thiols directly (Kharitonov *et al*., 1995; Wink *et al*., 1994; Butler *et al*., 1995). However, certain other oxides of nitrogen (N_xO_y, e.g. NO_2, N_2O_3, N_2O_4 and $ONOO^-$), produced from NO by reaction with dissolved oxygen or with oxygen-derived radicals, are strong nitrosating agents (More *et al*., 1995; Butler *et al*., 1995) and can generate GSNO from GSH. Thus, Kharitonov *et al*. (1995) showed that N_2O_3 readily nitrosates GSH and they conclude that almost all N_2O_3 formed under physiological conditions is consumed in this manner. An important point to note here is that N_xO_y will not nitrosate GSSG, the end product of the photolysis of GSNO, so disulphide formed during the period of irradiation must first be reduced to GSH if it is to be re-cycled back into the store. This could be achieved by glutathione reductase, the enzyme that catalyses the NADPH-dependent reduction of GSSG to GSH in the γ-glutamyl cycle.

Anti-oxidant properties of GSH and repriming

GSH is a powerful anti-oxidant and consideration must be given to the possible consequences of reducing its levels on both the synthesis and fate of endothelial NO. Ghigo *et al*. (1996) established a correlation between altered intracellular GSH levels and eNOS activity and concluded that GSH has a protective role, preventing inhibition of the enzyme by NO. Thus, lowered GSH could result in impaired NO synthesis. Moreover, oxidative stress, resulting from reduced intracellular [GSH], could serve to reduce the bioavailability of NO *per se, via* its reaction with the O_2^- anion to form peroxynitrite.

We think it unlikely that either of these factors, acting alone or together, can explain our results. A statistical comparison of the kinetics of the repriming process for control and BSO-treated vessels (Figure 1B) shows that there is no significant difference in the rate ($\sim$0.3% min^{-1}) at which the store is resynthesized during the first 40 min or so. This implies that repriming of BSO-treated vessels is not limited by an inadequate supply of endothelium-derived NO, or by reduced availability of NO, since either or both would be expected to slow the rate of recovery. In addition, peroxynitrite is a powerful nitrosating agent (More *et al*., 1995) that would be expected to accelerate the formation of GSNO and result in a faster rate of recovery but this is not what we see. Instead, our results show that the repriming curve for BSO-treated vessels levels off earlier than that for control arteries and approaches a maximum value ($\Delta P/P_{max}$) which is estimated to be $\sim$44% of that for untreated vessels. This is consistent with reduced availability of the target reactant for the endothelium-derived nitrosating species. The overall result is to reduce the capacity of the store.

Can NO be released from the store in a 'dark' reaction?

The maximum amplitude of photorelaxations produced by irradiating an artery with visible light (*ca*. 55%) is at least comparable in size with vasodilator responses obtained by injecting RTAs with maximal doses of either authentic NO ($\sim$70%) or SNAP ($\sim$85%. See Megson *et al*., 1995). However, the functional significance of a molecular store of NO in vascular smooth muscle, evidently capable of releasing comparatively large amounts of NO momentarily, is still not clear. It seems improbable that its photosensitivity can be of any direct physiological relevance, since only the most

superficial vessels are ever exposed to light, yet photorelaxations can be elicited from 'deep' vessels too, which would not be illuminated ordinarily. The photodegradable nature of the store should perhaps be viewed instead as an interesting (but experimentally useful) side issue. A more important question that should be addressed is: can NO be released from the store in the dark, by as yet unidentified biochemical pathway(s), and if so, can the underlying mechanism(s) be manipulated pharmacologically in the search for novel therapeutic strategies? Certain metal ions, especially Cu^+, are effective catalysts for the release of NO from *S*-nitrosothiols *in vitro* (Dicks *et al*., 1996) and recent evidence suggests that this may be important physiologically (Al-Sa'doni *et al*., 1997; Gordge *et al*., 1996; Mayer *et al*., 1998). The study by Mayer *et al*. (1998) showed that GSH can increase basal soluble guanylate cyclase activity and also enhance activation of the enzyme by 'mixed' NO/O_2^- generators (e.g. peroxynitrite and Ca^{2+}-calmodulin-activated NOS). They propose that both actions are indirect, due to: (a) initial oxidation of GSH to the glutathionyl radical ($GS^{\bullet}$), which reacts with NO to form GSNO; followed by (b) Cu^+ -catalyzed decomposition of GSNO to GSH and NO.

Nikitovic & Holmgren (1996) showed that GSNO is an NADPH-oxidizing substrate for the enzyme thioredoxin reductase, which generates stoichiometric amounts of GSH and NO by homolytic cleavage. The breakdown of GSNO by this route is of particular interest in the present context, since light is reported to activate thioredoxin reductase in chloroplasts, leading to the reduction of disulphide groups on several important enzymes (Gilbert, 1990), resulting in their activation (including, *inter alia*, fructose-1,6-bisphosphatase, NADP-malate dehydrogenase, 1,7-bisphophatase and NADP-glyceraldehyde-3-phosphate dehydrogenase). A further pathway for the bioconversion of GSNO to nitrite was reported recently in cultured cells and human platelets by Gordge *et al*. (1998), who suggested the designation 'GSNO lysase' for the enzyme involved.

In summary, agents that selectively up- or down-regulate GSH biosynthesis clearly influence the extent to which the photosensitive store of NO is reconstituted in the dark. The effects on the repriming process correlate with alterations to the GSH pool and therefore support the idea that GSNO is an important component of the photodegradable store.

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