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Nitrogen oxide-induced autoprotection in isolated rat hepatocytes

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Abstract Pretreatment of rat hepatocytes with low-dose nitrogen oxide (addition of SNAP in vitro or induction of nitric oxide synthase in vitro or in vivo) imparts resistance to killing and decrease in aconitase and mitochondrial electron transfer from a second exposure to a higher dose of SNAP. Induction of this resistance is prevented by cycloheximide, indicating upregulation of protective protein(s). Ferritin levels are increased as are non-heme iron-NO EPR signals. Tin-protoporphyrin (SnPP) prevents protection, suggesting involvement of hsp32 (heme oxygenase) and/or guanylyl cyclase (GC). Cross-resistance to H_2O_2 killing is also observed, which is also prevented by cycloheximide and SnPP. Thus, hepatocytes possess inducible protective mechanisms against nitrogen oxide and reactive oxygen toxicity.

Key words: Nitric oxide; Nitrogen oxide; Rat hepatocyte; Reactive oxygen species

1. Introduction

Nitric oxide is a small, widely diffusible dissolved non-electrolyte in aqueous solution for which cellular membranes present no appreciable barrier to diffusion [1,2]. In addition to its functions as a messenger molecule (acting through the heme-mediated stimulation of soluble guanylyl cyclase [3]), •NO is paramagnetic and reacts with metals and with oxygen species [4]. There is much data to suggest that the interaction of •NO with oxygen species can affect oxidative injury, although the actions of •NO can be to either attenuate or potentiate injury. The factors which determine this 'Janus-faced' action of •NO [5] are largely unknown. Much of the contradictory data on the damaging vs. protective actions of NO synthesis may be due to the multiple nitrogen oxide species which are produced in the aerobic, chemically complex biological milieu [4] and it is difficult to ascribe a particular biological effect to a specific derivative of NO.

We report here that either exogenous (addition of the nitrosothiol *S*-nitroso-*N*-acetyl-L-penicillamine; SNAP) or endogenous (in vivo or in vitro enzymatic induction) nitrogen oxide(s) induces a protective response against an otherwise lethal dose of nitrogen oxide(s) in isolated rat hepatocytes. Importantly, this response involves upregulation of protective protein(s). We also present data suggesting possible mechanisms for this protective response. Thus, in addition to radical reactions which occur during the process of oxidative injury by nitrogen and oxygen intermediates, another factor which can dictate the protective vs. damaging actions of nitric oxide production on oxidative injury is the existence of defensive mechanisms which are upregulated by exposure to these agents.

2. Experimental

2.1. Materials

Williams media E, Trypan blue, insulin, penicillin, streptomycin, L-glutamine and HEPES were purchased from Gibco Laboratories (Grand Island, NY). Calf serum was obtained from Hyclone Laboratories (Logan, UT). Sn-protoporphyrin was purchased from Porphyrin Products (Logan). SNAP was synthesized every 2 months as described previously [6], stored frozen as a solid aliquot in the dark and checked for stoichiometric *S*-nitrosothiol content by the method of Saville [7]. Other chemicals were obtained from Sigma unless otherwise stated.

2.2. Isolation and treatment of hepatocytes

Purified hepatocytes were isolated from Sprague–Dawley rats (200 g) and pretreated with either SNAP (as described in the figure legends) or with cytokine mix ('CM') (TNF-α, interferon-γ, interleukin-1β and lipopolysaccharide) ± 0.75 mM N^G-monomethyl-L-arginine (NMMA) [8]. The cells (2.5×10^5 cells/well in 12-well plates) were washed twice with media and then treated with media containing 2 mM SNAP for 12 h. Cell viability was determined after this treatment by Cresyl violet staining [9]. In vivo activated hepatocytes were isolated 5 days after intraportal injection of rats with killed *C. parvum* [10].

2.3. EPR spectroscopy

Hepatocytes (1×10^7 cells) harvested from culture plates [8] were suspended in PBS, transferred to quartz EPR sample tubes and frozen. EPR spectra were obtained at 77 K using a Bruker EP-300 spectrophotometer. All spectra were time-averaged over 10 scans. Instrumental parameters were 3.2×10^5 gain, 1 mW power, 9.44×10^9 Hz microwave frequency, 6.3 G modulation amplitude, 80 ms time constant and 20.97 s (× 10 scans) scan time. Signal intensity was calculated from height from the baseline of the $g = 2.04$ excursion.

2.4. Measurement of ferritin

For determining the effects of SNAP and SnPP on ferritin levels, hepatocytes (5×10^6 cells/100 mm plate) pretreated as described in the legend to Fig. 4 were washed with ice-cold phosphate buffered saline containing 0.1 mM phenylmethylsulfonyl fluoride and lysed by three cycles of freezing and thawing. Cytosolic fraction was obtained from the supernatant of a 12000 rpm 15 min centrifuge (microcentrifuge) at 4°C. Quantitative ferritin determination was performed by ELISA using polyclonal antibody (Sigma A5762) of horse spleen ferritin and anti-rabbit IgG (Sigma A3687) conjugated with alkaline phosphatase with the standard curve of rat liver ferritin (Sigma P7005) following previously published procedures [11]. Protein concentration was measured by protein assay kit (p5656, Sigma).

2.5. Aconitase assays and respiration measurements

Samples for mitochondrial aconitase assay were prepared and assayed as described by Drapier and Hibbs [12]. Aconitase activity was measured spectrophotometrically at 240 nm by disappearance of *cis*-aconitate. The reaction was started with the addition of 0.2 mM *cis*-aconitate and enzyme activity was determined from the initial reaction rate using an extinction coefficient of 3.4 cm^{-1} mM^{-1} at 240 nm [13] for *cis*-aconitate. Oxygen consumption was measured using a Clark type electrode (YSI Instruments). Hepatocytes were permeabilized with 0.0075% digitonin in respiration medium (250 mM sucrose, 2 mM HEPES, 2.5 mM KH_2PO_4, 210 mM mannitol and 0.5 mM EDTA).

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Effective permeabilization was determined by Trypan blue staining. Measurement of mitochondrial respiration was initiated by adding permeabilized hepatocytes into respiration medium supplemented with 1 mM ADP and 10 mM β-hydroxybutyrate, 2.5 mM $MgCl_2$ and 0.7% BSA and appropriate substrate/inhibitor as described [14].

3. Results

For the data in Fig. 1, hepatocytes with or without various pretreatments were subjected to a 12-h incubation in medium with NMMA alone (solid bars) or 0.75 mM NMMA + 2 mM SNAP (open bars) and viability determined as described in section 2. As shown in 1A, compared with previously untreated hepatocytes ('CTRL'), cells which had previously produced •NO by induction with cytokine mix ('CM') or pretreated with low (100 μM) SNAP are virtually completely resistant to killing. The effect of CM pretreatment is due to •NO production, since it is prevented by the presence of NMMA during CM pretreatment ('CM + NMMA'). SNAP effects (both protection and killing) are due to SNAP-induced nitrogen oxide formation, since the same concentration of the parent compound not containing the thionitrite moiety (NAP) has no effect (see below). In addition, pretreatment with media which had SNAP added 12 h prior does not induce protection (data not shown).

In contrast to hepatocytes isolated from normal animals, preinjection with *C. parvum* 5 days prior to isolation and 16 h preculture are resistant to the toxic effect of SNAP treatment (1B). *C. parvum* treatment has been shown previously to induce a major hepatic inflammatory response, including prodigious •NO synthesis both in vivo and by hepatocytes isolated from the animals [15]. Interestingly, prevention of •NO synthesis by the hepatocytes during the 16-h culture prior to SNAP addition ('NMMA' pretreatment) does not render the cells sensitive, indicating that a protective response induced in vivo by *C. parvum* injection carries over during subsequent culture. Alternatively, the small amount of •NO synthesis in the presence of NMMA may be enough to induce the resistance.

For Fig. 2, SNAP killing was measured for hepatocytes pretreated with the indicated SNAP concentrations for 14 h (2A) or pretreatment with 100 μM SNAP for the indicated times (2B). Maximal protection is induced with 50–100 μM SNAP concentration (2A) and requires 8–12 h to develop (2B, squares)). Pretreatment alone, with no subsequent SNAP exposure (2B, circles) results in no toxicity.

Two critical intracellular targets for the toxic effects of nitric oxide formation are the iron-containing enzymatic activities aconitase and mitochondrial electron transfer (most especially complexes I and II) [16], including the isolated rat hepatocyte [17]. Fig. 3 shows the effects of SNAP pre- and posttreatment on these activities. Hepatocytes were pre- and posttreated as described in Fig. 1, except that the duration of the second SNAP treatment was 4 h instead of 12 h. During this abbreviated time there was insignificant cell lysis (not shown), but significant decrease in activities of aconitase and upstream segments of mitochondrial electron transfer. As shown in Fig. 3A, pretreatment with low-level SNAP results in substantial protection against subsequent high-level SNAP-induced decrease in aconitase activity. Interestingly, SNAP pretreatment, alone, appears to induce an approximately 30% increase in aconitase activity, perhaps via upregulation of internal iron homeostatic

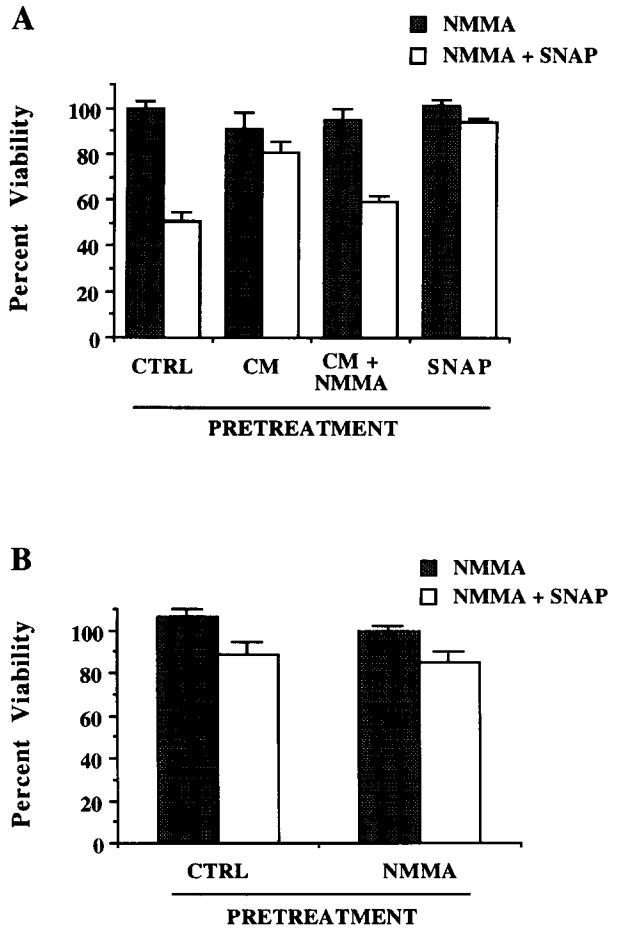

Fig. 1. High concentration SNAP-mediated cytotoxicity toward (A) hepatocytes pretreated in vitro with either CM ± NMMA or with low concentration SNAP and (B) hepatocytes from *C. parvum*-treated rats. (A) Normal hepatocytes were precultured for 20 h with either no addition ('CTRL'), CM, CM + 0.75 mM NMMA or with 100 μM SNAP for 14 h. These cultures were then exposed to NMMA alone (solid bars) or NMMA + 2 mM SNAP (open bars) for 12 h and cell viability was determined as described in section 2. (B) Hepatocytes isolated from *C. parvum*-treated rats (5 days) were precultured in the absence ('CTRL') or presence ('NMMA') of 0.75 mM NMMA for 16 h, then exposed for 12 h to NMMA alone (solid bars) or NMMA+2 mM SNAP (open bars) and viability was determined as described above.

regulatory mechanisms [18,19]. A similar protective effect is observed on inhibition of mitochondrial electron transfer through complexes I+III+IV and II+III+IV (3B). There is no effect of SNAP on Complex IV activity. As is also true for cytotoxicity (Fig. 2B), pretreatment alone ('SNAP/None') results in no appreciable decrease in activity.

We have shown recently that one effect of endogenous •NO synthesis in isolated rat hepatocytes is upregulation of the heat shock protein hsp32, heme oxygenase (HO), as a result of nitrogen oxide-induced liberation of enzyme-bound (primarily cytochrome P450) heme [20,21]. We were thus interested to know whether HO may play a role in SNAP-induced induction of an autoprotective response. As shown in Fig. 3A, tin-protoporphyrin IX ('SnPP'), which is a potent inhibitor of HO [22], indeed prevents the protective effects of low-SNAP pretreatment on subsequent high-SNAP inhibition of aconitase activity ('SNAP+SnPP'). SnPP alone does not alter the sensitivity of

Table 1
Effects of various pretreatments on high concentration SNAP and H_2O_2 cytotoxicity and on intensity of EPR-detectable dithioldinitrosyl iron complex EPR signal (g = 2.04)

Addition during Pretreatment	Addition during Postreatment	Viability (%)	g = 2.04 Signal (arb. U/mg)
None	None	–100–	UD
None	SNAP	51.8 ± 4.9	0.20 ± 0.01
SNAP	None	102.1 ± 6.3	UD
SNAP	SNAP	87.4 ± 5.2	0.32 ± 0.01*
NAP	SNAP	54.2 ± 5.2	0.22 ± 0.01
SNAP + CHX	SNAP	49.0 ± 7.4	0.19 ± 0.02
CHX	SNAP	45.0 ± 6.0	0.18 ± 0.01
SNAP + SnPP	SNAP	51.3 ± 5.8	0.22 ± 0.01
SnPP	SNAP	48.0 ± 3.9	0.19 ± 0.04
None	H_2O_2	44.5 ± 4.7	ND
SNAP	H_2O_2	80.1 ± 6.4	ND
NAP	H_2O_2	46.7 ± 3.2	ND
SNAP + CHX	H_2O_2	39.6 ± 4.4	ND
CHX	H_2O_2	37.9 ± 6.2	ND
SNAP + SnPP	H_2O_2	40.6 ± 4.0	ND
SnPP	H_2O_2	43.6 ± 2.7	ND

Hepatocytes were incubated with the indicated pretreatments for 14 h. For viability measurements, posttreatment with or without SNAP (2 mM) was for 12 h and with or without H_2O_2 (5 mM) was for 3 h. The EPR measurements were performed in cells harvested after 4 h posttreatment. Details are described in section 2. UD, undetectable; ND, not determined. *P 0.002 vs. none/SNAP.

non-pretreated cells to high-SNAP ('SnPP'). Protection against loss of mitochondrial electron transfer activities is also prevented by SnPP, which also has no effect alone (3B). It is important to point out, however, that SnPP also inhibits soluble guanylyl cyclase [23] (which potently responds to •NO in hepatocytes, increasing cGMP [15,24]) and so based on this data alone the protective effect of low-SNAP pretreatment could also be due to cGMP elevation.

We also examined the results of these and other treatments on the appearance of the 'g = 2.04' electron paramagnetic resonance (EPR) signal in hepatocytes (Table 1), indicative of the formation of dithioldinitrosyl iron complexes (DNIC)

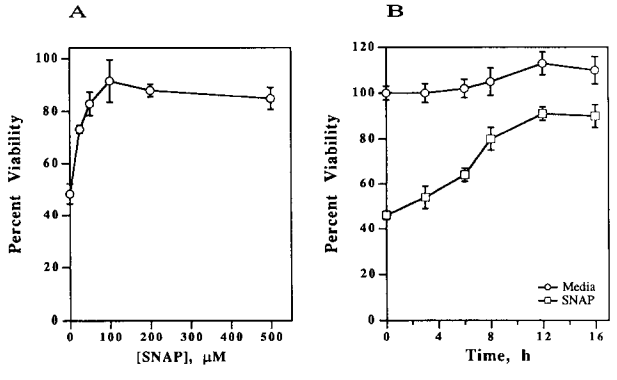

Fig. 2. Dose (A) and time (B) response of low concentrations of SNAP pretreatment on subsequent high concentration SNAP-induced cytotoxicity. (A) Dose response of the indicated increasing SNAP concentrations (14 h preexposure) on subsequent cytotoxicity of 12 h treatment with 2 mM SNAP. Viability was determined as described in section 2. (B) Time course of the development of resistance toward 2 mM SNAP; hepatocytes were pretreated with 100 μM SNAP for the indicated times and then washed and incubated 12 h without (circles) or with (squares) 2 mM SNAP. Viability was determined as described in section 2.

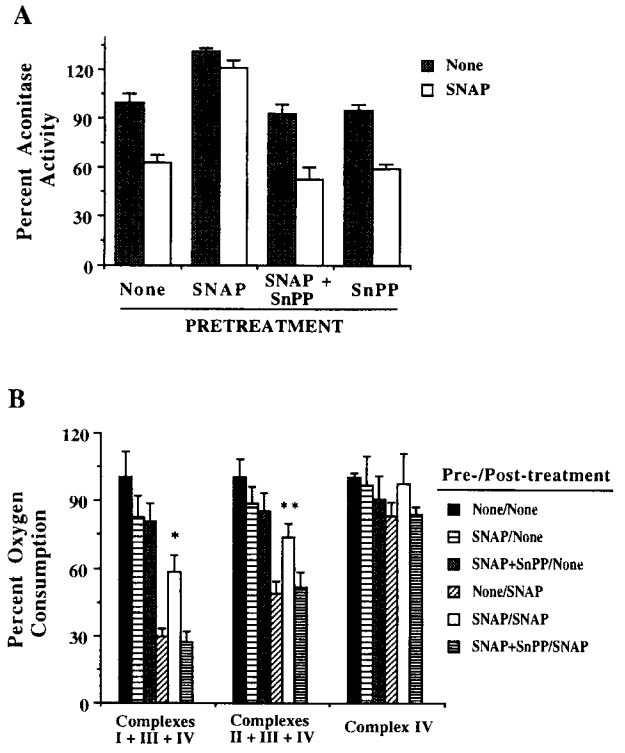

Fig. 3. Effect of low concentration SNAP pretreatment on subsequent high-concentration SNAP-induced inhibition of mitochondrial aconitase activity (A) and mitochondrial respiration (B). Hepatocytes were pretreated 14 h as described in section 2 without or with 100 μM SNAP, 25 μM and then posttreated without or with 2 mM SNAP for 4 h. Mitochondrial aconitase and respiration were measured as described in section 2.

[10,25,26]. As we expected, no addition during the pretreatment followed by high-level (2 mM) SNAP for 12 h results in significant decrease in viability and also the appearance, after 4 h, of this signal. Pretreatment with SNAP alone (no SNAP during the second incubation) does not alter viability and the g = 2.04 signal was undetectable. However, concomitant with substantial protection against killing the pretreatment also significantly increases the intensity of the EPR signal from DNIC. Pretreatment with the parent compound (NAP) results in no resistance to SNAP-induced viability nor increase in the intensity of the g = 2.04 signal, again demonstrating that the resistance inductive effect is due to nitrogen oxide formation from SNAP. Addition of the translational inhibitor cycloheximide (CHX, 10 μg/ml) prevents the low SNAP-induced protection and increased DNIC formation. This effect is not due to increased non-specific toxicity from CHX alone, since there is no increase in toxicity or effect on the g = 2.04 signal when CHX is present during the preincubation without SNAP. This result, along with the relatively slow time frame of the resistance induction effect (Fig. 2B), indicates that this effect involves an increase in expression of protective protein(s). The autoinductive protective effect of low-level SNAP against toxicity and also increase in the g = 2.04 signal are abrogated by SnPP. As with CHX, the effect of SnPP is not due to increased non-specific toxicity. As also shown in Table 1, pretreatment with low-level SNAP induces protection against oxidative toxicity from H_2O_2, which is not exhibited by the parent compound and prevented

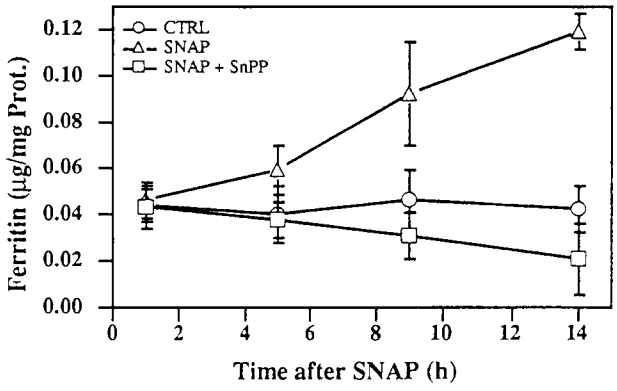

Fig. 4. Effect of SNAP (100 μM) without or with SnPP (25 μM) on hepatocyte ferritin levels. Cells were treated with SNAP and at various times intracellular ferritin levels were determined as described in section 2.

by CHX and by SnPP. This indicates that nitrogen oxide exposure induces resistance to injury from both reactive nitrogen and reactive oxygen species.

We have previously shown that endogenous or exogenous nitrogen oxide exposure induces modest but significant increase in cytosolic non-heme iron [20,21]. Increased cytosolic iron is known to upregulate the synthesis of ferritin, which may be an important protective response against oxidative injury by sequestering iron and thus preventing the formation of hydroxyl radical via the Fenton reaction [27]. As shown in Fig. 4, compared with control hepatocytes treatment with 100 μM SNAP induces increased (~3-fold) ferritin expression over a time frame similar to the induction of resistance (Fig. 2) and this increase is prevented by SnPP. Interestingly, in the presence of SnPP the levels of ferritin decrease substantially, perhaps indicative of nitrogen oxide-mediated downregulation of ferritin expression via activation of the iron-responsive element mechanism, as documented previously [18,19]. This result suggests that in hepatocytes iron liberation from heme by the action of heme oxygenase indeed upregulates ferritin expression.

4. Discussion

We have shown previously that •NO upregulates hsp32 (heme oxygenase-1; HO-1) via liberation of intracellular heme [20] and suggested that this may be a protective response [21]. Welsh and Sandler have shown induction of HO mRNA and protection against •NO toxicity by addition of heme [28], although exogenous heme addition could protect by directly scavenging •NO. HO-1 has been shown to be upregulated under conditions of oxidative stress [29] and may also be involved in the •NO-induced protective response observed here since addition of the inhibitor SnPP abrogates the installation of resistance by •NO pretreatment. The mechanism of protection could involve the production of billirubin (a potent antioxidant [30]) after enzymatic reduction of billiverdin, a product of HO-1 activity. In addition, we show here that low-dose •NO upregulates ferritin, which could be due to iron release from HO-mediated heme breakdown [20]. Ferritin has been proposed to be protective against oxidative stress by sequestering 'loosely bound' iron which may otherwise participate in hydroxyl radical formation via the Fenton reaction [27,31].

It is also possible that •NO-induced increased cellular cGMP levels may increase protection (through unknown mechanisms), since SnPP is also an inhibitor of soluble guanylyl cyclase [23]. In addition to •NO, GC activity is also stimulated by oxidants and based on this effect such a protective role for cGMP has been hypothesized previously [32]. It is also possible that •NO-induced heme loss increases cGMP levels since both heme [33] and CO (a product of HO activity) [34] stimulate GC activity. There is also evidence that exposure of hemoproteins to reactive oxygen species is capable of inducing heme liberation, which could also result in HO-1 increase [35,36]. However, although it has been shown that •NO-cGMP effects can be protective against ischemia/reperfusion [37] and allograft survival posttransplant [38] through increased vasodilatation and decreased thrombosis, we know of no reports of a role for cGMP in protection against cellular oxidative injury in vitro.

In addition to decreased cytotoxicity, low-SNAP pretreatment decreases the degree of inhibition of mitochondrial electron transfer caused by high-SNAP treatment and also increases the intensity of the EPR signal from cellular DNIC. We have shown a similar increase in DNIC formation in cells expressing increased levels of metallothionein which also induces resistance to SNAP toxicity and DNA damage [39], although mitochondrial function was not measured. However, at present we do not know the identity of the thiol ligands in the DNIC and whether these complexes play a causal role in protection. It is worth noting in this regard that Kuo and Slivka have shown that endogenously produced ·NO is capable of modulation of intracellular glutathione levels in hepatocytes suggesting the possibility that the changes in the intensity of the DNIC signal may reflect changes in thiol levels [40]. The lesser amount of mitochondrial activity loss by low-SNAP pretreatment could be due to several factors, including increased scavenging of •NO (perhaps reflected by the increased DNIC signal) and/or increased recovery/repair of activity [17,41].

Finally, we emphasize that all these treatments were performed aerobically, which means that the effects (both induction of resistance and cellular damage) may be due to multiple reactive nitrogen oxide species in addition to •NO [42]. Protection may involve a general response to oxidative injury, which could explain the cross-resistance imparted to H_2O_2 toxicity. Also, although SNAP can spontaneously liberate ·NO the mechanism of this liberation is complex, involving metal ions [43], oxygen [44] or cellular metabolism [45]. As an *S*-nitrosothiol, some of the actions of SNAP could also be due to transnitrosation (transfer of nitrosonium, NO^+), as has been suggested previously for several important actions of •NO [46].

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