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# Role of peroxynitrite and activation of poly (ADP-ribose) synthetase in the vascular failure induced by zymosan-activated plasma

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**1** Zymosan is a wall component of the yeast *Saccharomyces Cerevisiae*. Injection of zymosan into experimental animals is known to produce an intense inflammatory response. Recent studies demonstrated that the zymosan-induced inflammatory response *in vivo* can be ameliorated by inhibitors of nitric oxide (NO) biosynthesis. The cytotoxic effects of NO are, in part, mediated by the oxidant preoxynitrite and subsequent activation of the nuclear enzyme poly (ADP-ribose) synthetase (PARS). In the present *in vitro* study, we have investigated the cellular mechanisms of vascular failure elicited by zymosan-activated plasma and the contribution of peroxynitrite production and activation of PARS to the changes.

**2** Incubation of rat aortic smooth muscle cells with zymosan-activated plasma (ZAP) induced the production of nitrite, the breakdown product of NO, due to the expression of the inducible isoform of NO synthase (iNOS) over 6–24 h. In addition, ZAP triggered the production of peroxynitrite in these cells, as measured by the oxidation of the fluorescent dye dihydrorhodamine 123 and by nitrotyrosine Western blotting.

**3** Incubation of the smooth muscle cells with ZAP induced DNA single strand breakage and PARS activation. These effects were reduced by inhibition of NOS with $N^G$-methyl-L-arginine (L-NMA, 3 mM), and by glutathione (3 mM), a scavenger of peroxynitrite. The PARS inhibitor 3-aminobenzamide (1 mM) inhibited the ZAP-induced activation of PARS.

**4** Incubation of thoracic aortae with ZAP *in vitro* caused a reduction of the contractions of the blood vessels to noradrenaline (vascular hyporeactivity) and elicited a reduced responsiveness to the endothelium-dependent vasodilator acetylcholine (endothelial dysfunction).

**5** Preincubation of the thoracic aortae with L-NMA (1 mM), glutathione (3 mM) or by the PARS inhibitor 3-aminobenzamide (1 mM) prevented the development of vascular hyporeactivity in response to ZAP. Moreover, glutathione and 3-aminobenzamide treatment protected against the ZAP-induced development of endothelial dysfunction. The PARS-related loss of the vascular contractility was evident at 30 min after incubation in endothelium-intact, but not in endothelium-denuded vessels and also manifested at 6 h after incubation with ZAP in endothelium-denuded rings. The acute response is probably related, therefore, to peroxynitrite formation (involving the endothelial NO synthase), whereas the delayed response may be related to the expression of iNOS in the smooth muscle.

**6** The data obtained suggest that zymosan-activated plasma causes vascular dysfunction by inducing the simultaneous formation of superoxide and NO. These radicals combine to form peroxynitrite, which, in turn causes DNA injury and PARS activation. The protective effect of 3-aminobenzamide demonstrates that PARS activation contributes both to the development of vascular hyporeactivity and endothelial dysfunction during the vascular failure induced by ZAP.

**Keywords:** Inflammation; nitric oxide; superoxide; endothelium; shock

## Introduction

Pro-inflammatory cytokines and bacterial lipopolysaccharide (LPS) induce the expression of an inducible isoform of nitric oxide synthase (iNOS) in vascular smooth muscle cells and other cell types, which produces large amounts of NO from its substrate, L-arginine (Nathan, 1992; Palmer, 1993; Szabó, 1995). Pharmacological inhibition of NOS by L-arginine-based and non-L-arginine based inhibitors restores the vascular hyporeactivity (reduced contractile response to vasoconstrictor agents), which develops after *in vitro* or *in vivo* treatment with pro-inflammatory cytokines or LPS (Fleming *et al*., 1991; Szabó *et al*., 1993a; Wu *et al*., 1994). Based on these results, it has been suggested that the vascular hyporeactivity associated with immune hyperactivation or endotoxic shock is due to production of NO within the vasculature, which activates the soluble guanylyl cyclase in the smooth muscle.

Recent studies have challenged the prevailing point of view that NO, *per se*, is a highly cytotoxic species. Shock and inflammation is associated with the production of superoxide anion, and the simultaneous production of superoxide and NO favours the generation of a highly cytotoxic reaction product, the oxidant peroxynitrite ($ONOO^-$) (Beckman *et al*., 1990; Ischiropoulos *et al*., 1992; Pryor & Squadrito, 1995; Szabó, 1996a). In various forms of shock and inflammation, the production of peroxynitrite has been demonstrated, and peroxynitrite has been proposed to contribute importantly to the cellular metabolic failure and vascular injury (Szabó *et al*., 1995a,b; Szabó, 1996a; Zingarelli *et al*., 1997a).

We have recently demonstrated that incubation of vascular rings with peroxynitrite causes a delayed vascular hyporeactivity which resembles the hyporeactivity seen in endotoxic shock (Szabó *et al*., 1996a). In addition, we have

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identified a novel pathway or peroxynitrite-induced cellular injury (Szabó, 1996b). In this pathway, peroxynitrite causes DNA single strand breakage, which activates the nuclear enzyme poly (ADP-ribose) synthetase (PARS). PARS activation causes a massive depletion of $NAD^+$ and adenosine 5′-triphosphate (ATP) and eventually induces irreversible cytotoxicity and cell death (Szabó *et al*., 1996a,b; Zingarelli *et al*., 1996a). We have also demonstrated that activation of PARS importantly contributes to the vascular hyporeactivity in blood vessels exposed to exogenous peroxynitrite *in vitro* and also in blood vessels exposed to endogenous peroxynitrite, produced during endotoxic shock (Szabó *et al*., 1996a).

Zymosan is a wall component of the yeast *Saccharomyces Cerevisiae* that produces a severe inflammatory response (Lundberg & Arfors, 1983; Teixeira *et al*., 1993). Zymosan is widely used as a trigger of a non-septic model of multiple organ dysfunction (Mainous *et al*., 1993; Demling *et al*., 1994). Recent data have demonstrated the protective effects of NOS inhibitors in zymosan-induced shock, suggesting the involvement of NO, or a related species (such as peroxynitrite) in the zymosan-induced local or systemic (Teixeira *et al*., 1993; Cuzzocrea *et al*., 1996; 1997) inflammatory process. The cellular mechanisms responsible for the vascular failure elicited by zymosan have not been elucidated. Here we present results of our *in vitro* studies investigating the role of NO (derived either from constitutive, endothelial isoform of NO synthase (ecNOS) or from iNOS), peroxynitrite and PARS activation in the vascular failure elicited by zymosan-activated plasma (ZAP).

## Methods

### *Zymosan-activated plasma*

Heparin-treated rat plasma was incubated with zymosan for 60 min at 37°C in order to activate the complement system. Zymosan was then removed by centrifugation at 10,000 *g* for 5 min at room temperature (Lundberg & Arfors, 1983). For the treatment of cells or vascular rings, zymosan-activated plasma (ZAP) was prepared as described above and then 20 $\mu$l of stock solution was added into every 1 ml of culture medium or Krebs solution.

### *Cell culture*

Rat aortic smooth muscle (RASM) cells were cultured in RPMI medium, supplemented with L-glutamine (3.5 mM) and 10% foetal calf serum, as described previously (Szabó *et al*., 1996a). Cells were cultured in 96 well plates (200 $\mu$l medium per well) or in 12-well plates (3 ml medium per well) until confluence. At the beginning of the experiments, fresh medium was added. In some experiments, foetal calf serum-free medium was used. Cells were treated with zymosan-activated plasma (20 $\mu$l $ml^{-1}$) and incubated for 1–24 h. These experiments were performed in the presence of vehicle treatment, the NOS inhibitor $N^G$-methyl-L-arginine (M-NMA, 3 mM), the free radical and peroxynitrite scavenger glutathione (3 mM) the PARS inhibitor 3-aminobenzamide (1 mM) and the glucocorticoid dexamethasone (10 $\mu$M). These inhibitors were added 15 min before treatment with ZAP.

### *Measurement of nitrite production*

At various time points after ZAP exposure (3, 6, 12, 18 and 24 h), the production of nitrite, a breakdown product of NO, was measured by adding 100 $\mu$l of Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 100 $\mu$l samples of medium. The optical density at 550 nm ($OD_{550}$) was measured with a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA). Nitrite concentrations were calculated by comparison with the $OD_{550}$ of standard solutions of sodium nitrite prepared in culture medium (Szabó *et al*., 1996a).

### *Measurement of peroxynitrite-induced oxidation of dihydrorhodamine 1,2,3*

At various time points after ZAP exposure (3, 6, 12, 18 and 24 h), the formation of peroxynitrite was measured by the peroxynitrite-dependent oxidation of dihydrorhodamine 123 to rhodamine 123, as previously described (Zingarelli *et al*., 1996). Cells were rinsed with phosphate-buffered saline and then medium was replaced with phosphate-buffered saline containing 5 $\mu$M dihydrorhodamine 123. After a 60 min incubation at 37°C, the fluorescence of rhodamine 123 was measured with a Perkin-Elmer fluorimeter (Model LS50B; Perkin-Elmer, Norwalk, CT) at an excitation wavelength of 500 nm, emission wavelength of 536 nm (slit widths 2.5 and 3.0 nm, respectively).

### *Determination of DNA single-strand breaks*

At various time points after ZAP exposure (3, 6, 12, 18 and 24 h), the formation of DNA strand breaks in double-stranded DNA was determined by the alkaline unwinding methods as previously described (Schraufstatter *et al*., 1986; Zingarelli *et al*., 1996; Szabó *et al*., 1996b). Cells in 12-well plates were scraped into 0.2 ml of solution A buffer (myoinositol 250 mM, $NaH_2PO_3$ 10 mM, $MgCl_2$ 1 mM, pH 7.2). The cell lysate was then transferred into plastic tubes designated T (maximum fluorescence), P (fluorescence in sample used to estimate extent of DNA unwinding), or B (background fluorescence). To each tube, 0.2 ml of solution B (alkaline lysis solution: NaOH 10 mM, urea 9 M, ethylenediaminetetraacetic acid 2.5 mM, sodium dodecyl sulphate 0.1%) was added and incubated at 4°C for 10 min to allow cell lysis and chromatin disruption; 0.1 ml each of solutions C (0.45 volume solution B in 0.2 N NaOH) and D (0.4 volume solution B in 0.2 N NaOH) was then added to the P and B tubes; 0.1 ml of solution E (neutralising solution: glucose 1 M, mercaptoethanol 14 mM) was added to the T tubes before solutions C and D were added. From this point incubations were carried out in the dark. A 30 min incubation period at 0°C was then allowed during which the alkali diffused into the viscous lysate. Since the neutralising solution, solution E, was added to the T tubes before addition of the alkaline solutions C and D, the DNA in the T tubes was never exposed to a denaturing pH. At the end of the 30 min incubation, the contents of the B tubes were sonicated for 30 s to ensure rapid denaturation of DNA in the alkaline solution. All tubes were then incubated at 15°C for 10 min. Denaturation was stopped by chilling to 0°C and adding 0.4 ml of solution E to the P and B tubes, 1.5 ml of solution F (ethidium bromide 6.7 $\mu$g $ml^{-1}$ in 13.3 mM NaOH) was added to all the tubes. Throughout the experiments, at each step of addition of reagents or buffers, where not specified, equivalent volumes of control buffer solutions were added to the remaining tubes, to control for volume differences.

Fluorescence (excitation: 520 nm, emission: 590 nm) was measured by a Perkin-Elmer fluorimeter. Under the conditions used, in which ethidium bromide binds preferentially to double stranded DNA, the percentage of double stranded DNA (D) may be determined from the equation: $\%D = 100 \times [F(P) - F(B)]/[F(T) - F(B)]$; where F(P) is the fluorescence of the sample, F(B) the background fluorescence, i.e. fluorescence due to all cell components other than double stranded DNA, and F(T) the maximum fluorescence.

### *Nitrotyrosine Western blotting in rat aortic smooth muscle cells*

Cells cultured in 6-well plates were treated with ZAP for 24 h. Cells were then washed with 1.0 ml cold PBS and scraped in

1.0 ml cold phosphate-buffered saline (PBS) and transferred to microfuge tubes. Tubes were spun in Eppendorf microfuge for 20 s. PBS was then removed and 150 $\mu$l RIPA (1 × PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 $\mu$g ml$^{-1}$ leupeptin, 10 $\mu$g ml$^{-1}$ pepstatin A, 0.5 mM PMSF) were added to each tube and DNA sheared by repeated passage through a 22 gauge needle. Cells were incubated on ice for 30 min, then centrifuged at 14,000 *g* for 20 min at 4°C. Thirty micrograms of each sample was diluted in an equal volume of treatment buffer and heated to 95°C for 3 min. Samples were then loaded into 8–16% Tris-glycine. Gels were run at 120 V for 2 h, then transferred to 0.45 $\mu$m nitro-cellulose at 70 V for 60 min by use of a 1/2 X Towbin buffer system (1.45 g Tris, 7.2 g glycine, 800 ml di $H_2O$ and 200 ml MeOH). Membrane was blocked in 1% BSA: 1% non-fat-milk in PBS-T for 1 h then probed with rabbit anti-nitrotyrosine (Upstate Biotechnology, Saranac Lake, NY), 1 $\mu$g ml$^{-1}$ in PBS-T (phosphate-buffered saline with 0.05% Tween 20), overnight at 4°C. The blot was washed 3 times with PBS-T, once with $H_2O$, the incubated for 1.5 h with secondary antibody, goat anti-rabbit-HRP (1:3000). The blot was washed 3 times with PBS-T, once with di $H_2O$, then 1.5 ml mixed ECL chemiluminescence reagent (Amersham) was added for 1 min. The blot was then exposed to X-ray film for 60 s.

### *Measurement of PARS activation in rat aortic smooth muscle cells in culture*

Cells were treated with ZAP for 24 h. The culture medium in 12-well plates was then replated with 0.5 ml of 56 mM HEPES buffer, pH 7.5 containing 28 mM KCl, 28 mM NaCl, 2 mM $MgCl_2$, 0.01% digitonin and 125 nmol $NAD^+$ spiked with 0.25 $\mu$Ci [$^3$H]-$NAD^+$. PARS activity was then measured as described previously (Zingarelli *et al*., 1996; Szabó *et al*., 1996b). Digitonin was used to permeabilize plasma membranes. The permeabilized cells were incubated for 10 min at 37°C, and the ADP-ribosylated protein was precipitated with 200 $\mu$l of 50% TCA. After two washes with TCA, the protein pellet was solubilized in 2% SDS in 0.1 M NaOH, incubated at 37°C overnight and the radioactivity determined by scintillation counting. Parallel experiments were performed in the presence and in the absence of the PARS inhibitor 3-aminobenzamide (1 mM).

### *Measurement of isometric tension in vascular rings*

Thoracic aortae from rats were cleared of adhering periadventitial fat and cut into rings of 3–4 mm width. Endothelium was removed from some of the rings by gently rubbing the intimal surface. Lack of significant acetylcholine-induced relaxation (less than 5% of the precontractile tone) was taken as evidence that endothelial cells had been removed. The rings were mounted in organ baths (5 ml) filled with warmed (37°C), oxygenated (95% $O_2$/5% $CO_2$) Krebs solution (pH 7.4) consisting of (mM): NaCl 118, KCl 4.7, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11.7, in the presence of indomethacin (10 $\mu$M). Isometric force was measured as described (Szabó *et al*., 1996a) with isometric transducers (Kent Scientific Corp. Litchfield, CT, U.S.A.), digitalized by a Maclab A/D converter (AD Instruments, Milford, MA, U.S.A.) and stored and displayed on a Macintosh personal computer. A tension of 1 g was applied and the rings were equilibrated for 60 min. Fresh Krebs solution was provided at 15 min intervals. Concentration-response curves to noradrenaline ($10^{-9}$ $10^{-5}$ M) were obtained in aortic rings from control animals and in rings following a 30 min incubation with ZAP (20 $\mu$l ml$^{-1}$). Various groups of rings were incubated with ZAP alone, or in the presence of 3-aminobenzamide (1 mM), glutathione (3 mM) or L-NMA (3 mM).

In a separate study, concentration-response curves to acetylcholine ($10^{-8}$–$10^{-5}$ M) were evaluated in noradrenaline-precontracted ($10^{-6}$ M) aortic rings from control animals and in rings following a 30 min incubation with ZAP (20 $\mu$l ml$^{-1}$). Various groups of rings were incubated with ZAP alone, or in the presence of 3-aminobenzamide (1 mM) or glutathione (3 mM).

In a separate study, noradrenaline-precontracted ($10^{-6}$ M) aortic rings (without endothelium) were exposed to ZAP (20 $\mu$l ml$^{-1}$) for 6 h and the gradual loss of vascular tone was monitored over 6 h. In these experiments, some groups of rings were pretreated with L-NMA (3 mM), 3-aminobenzamide (1 mM) or glutathione (3 mM).

### *Measurement of PARS activity in vascular tissue*

Endothelium-intact or denuded rings were incubated in oxygenated Krebs solution for 30 min or 6 h in the presence or absence of ZAP (concentration as above). Then, PARS activity in the vascular tissue was measured as previously described (Szabó *et al*., 1996a). Briefly, vascular rings were placed in 0.5 ml of 56 mM HEPES buffer (pH 7.5) containing 28 mM KCl, 28 nM NaCl, 2 mM $MgCl_2$, 0.01% digitonin, and 125 nM $NAD^+$ spiked with 0.25 $\mu$Ci [$^3$H]-$NAD^+$. ADP-ribosylated protein precipitated in 200 $\mu$l of 50% TCA. Aortic rings were washed twice and incubated overnight in 2 N HCl, followed by neutralization in NaOH. The protein pellet was solubilized in 2% SDS in 0.1 M NaOH and the [$^3$H]-$NAD^+$ incorporation determined by scintillation counting.

### *Immunohistochemical localization of nitrotyrosine in vascular tissue*

Thoracic descending aortae were immediately excised and placed in Krebs solution. The solution was aerated with 95% $O_2$ and 5% $CO_2$, and pH was maintained at 7.4. The aortae were suspended in organ chambers containing 5 ml of Krebs at 37°C. After 30 min incubation with zymosan-activated plasma aortae were then perfused for 15 min with 10% buffered formalin and sections were prepared from the fixed aorta tissue. Endogenous peroxidase was quenched with 0.3% $H_2O_2$ in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 20 min. Non-specific adsorption was minimized by incubating the section in 3% normal goat serum in phosphate-buffered saline for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min each with avidin and biotin (biotin blocking kit, Vector Laboratories, Burlingame, CA). The sections were then incubated overnight with primary anti-nitrotyrosine polyclonal rabbit (Upstate Biotech, Saranac Lake, NY) serum or control solutions. Controls included buffer alone or non-specific purified rabbit IgG. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA).

### *Data analysis*

All values in the figures and text are expressed as mean ± s.e.mean of *n* observations, where *n* represents the number of wells or vascular rings studied (6–9 wells or rings from 2–3 independent experiments). Data sets were examined by one- and two-way analysis of variance and individual group means were then compared with Bonferroni's *t* test. A *P* value less than 0.05 was considered significant. In the experiments with Western blots or immunohistochemistry, the figures shown are representative of at least 3 experiments performed on different experimental days.

## Results

### *Production of NO and peroxynitrite in rat aortic smooth muscle cells incubated with ZAP*

Stimulation of rat aortic smooth muscle cells with ZAP resulted in the production of nitrite and peroxynitrite. A signif-

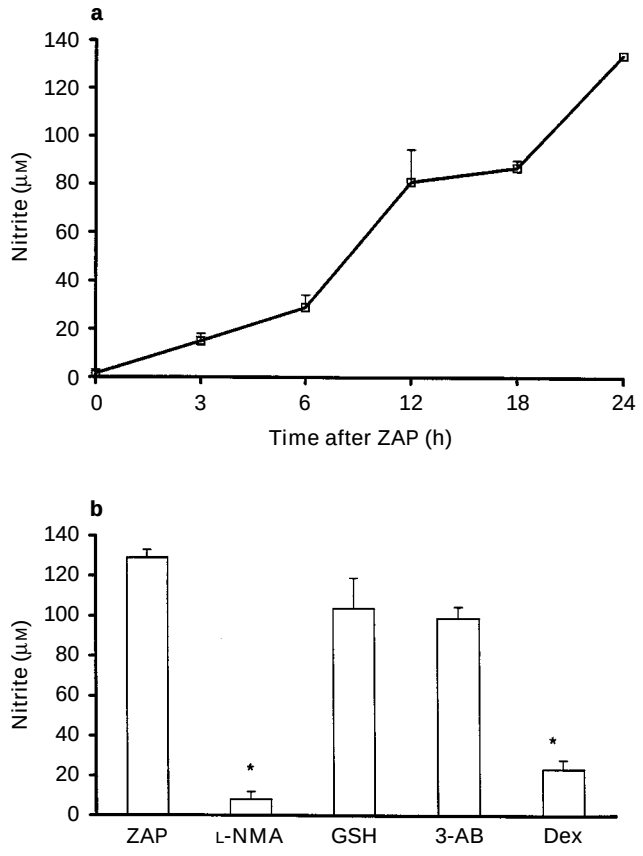


**Figure 1** (a) Time course of nitrite production in rat aortic smooth muscle cells exposed to zymosan-activated plasma (20 $\mu$l ml$^{-1}$). (b) Effect of N$^{G}$-methyl-L-arginine (L-NMA, 3 mM), glutathione (GSH, 3 mM), 3-aminobenzamide (3-AB, 1 mM) and dexamethasone (Dex, 10 $\mu$M) on the production of nitrite, as measured at 24 h. Data represent means, and vertical lines show s.e.mean, of $n=9$ wells *$P<0.01$ represents a significant inhibitory effect of the pharmacological agents used on nitrite production at 24 h.

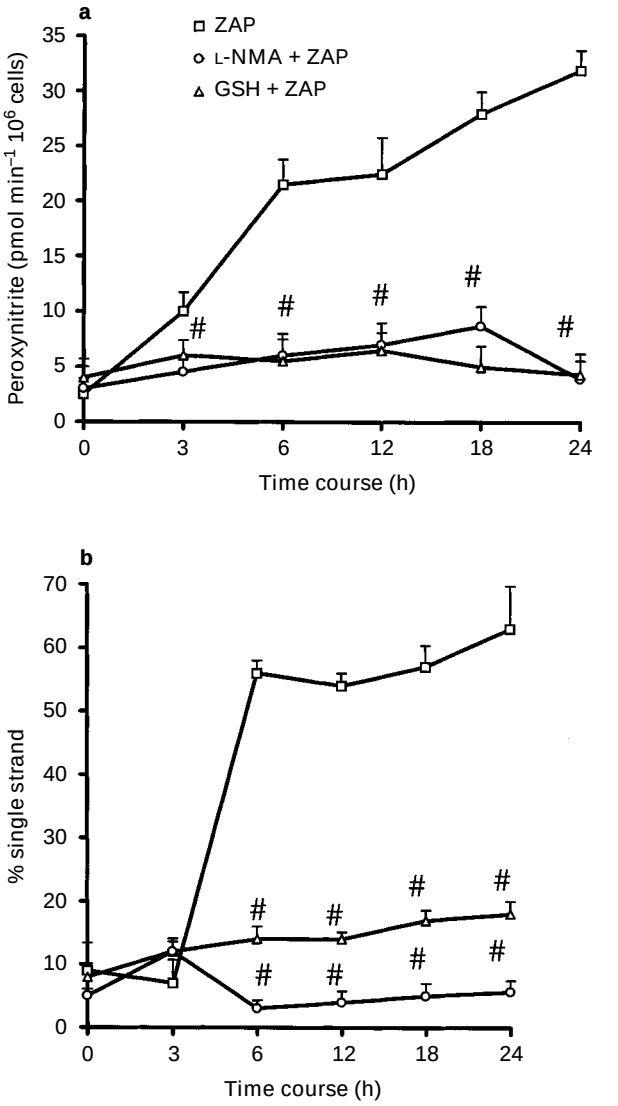


**Figure 2** Peroxynitrite production, as measured by the production of dihydrorhodamine 123 (a), a development of DNA single strand breakage (b) in rat aortic smooth muscle cells at various time points (3–24 h) after exposure to zymosan-activated plasma (ZAP, 20 $\mu$l ml$^{-1}$). Values shown represent peroxynitrite production (a) and DNA single strand breakage (b) in cells exposed to zymosan-activated plasma (20 $\mu$l ml$^{-1}$) alone and to zymosan-activated plasma in the presence of L-NMA or glutathione. Data represent means of $n=8$ wells; vertical lines show s.e.mean. #$P<0.01$ represents a significant inhibitory effect of the pharmacological agents used.

icant nitrite production was already detectable at 3 h ($15\pm1.6$ $\mu$M) after stimulation with ZAP and reached its maximum at 24 h ($134\pm10$ $\mu$M; $P<0.01$, $n=9$; Figure 1a). A rapid and sustained production of peroxynitrite was also observed after stimulation with ZAP ($n=8$; Figure 2a). By use of Western blotting, some nitrotyrosine immunoreactivity was detected in unstimulated cells (Figure 3). There was a marked increase in the nitrotyrosine immunoreactivity 24 h after ZAP treatment, which was prevented by glutathione or by inhibition of NOS with L-NMA (Figure 3). ZAP induced the nitration of several proteins, most notably a protein of approximately 60 kD, another one of approximately 135 kD, and several proteins in the range of 110 kDa (Figure 3).

L-NMA and dexamethasone inhibited the production of NO and formation of peroxynitrite throughout the incubation period (Figures 1b and 2a). The peroxynitrite scavenger glutathione at 3 mM reduced the ZAP-induced increase in dihydrorhodamine 123 oxidation (Figure 2a) and tyrosine nitration (Figure 3), without altering the amount of nitrite produced (Figure 2a). While L-NMA and glutathione appeared to be similarly potent and caused a complete reduction of dihydrorhodamine 123 oxidation in cells exposed to ZAP (Figure 2), L-NMA appeared to be more potent than glutathione in reducing tyrosine nitration (Figure 3). In fact, L-NMA reduced tyrosine nitration below baseline levels (compare band 1 and band 4 in Figure 3).

In unstimulated smooth muscle cells, in the presence of serum (but not in the absence of serum), the accumulation of a small, but significant amount of nitrite was detected over 24 h ($1.11\pm0.15$ $\mu$M, $n=9$), which was reduced by pretreatment with 3 mM L-NMA ($0.52\pm0.04$ $\mu$M, $n=9$, $P<0.05$). In the absence of serum, baseline tyrosine nitration in extracts of unstimulated RASM cells were lower than in cells incubated in the presence of serum for 24 h (data not shown).

### *Development of DNA single-strand breaks and activation of PARS in smooth muscle cells exposed to ZAP*

There was a marked increase in DNA strand breakage in RASM cells exposed to ZAP (Figure 2b). Treatment with L-NMA and glutathione prevented the ZAP-induced DNA single strand breakage (Figure 2b). A significant increase in the PARS activity in cells exposed to ZAP was also observed, as measured at 24 h, which was inhibited by 3-aminobenzamide (Figure 4). In agreement with previous observations (Szabó *et al*., 1996a), there were low levels of basal PARS activity in unstimulated rat aortic smooth muscle cells (Figure 4).

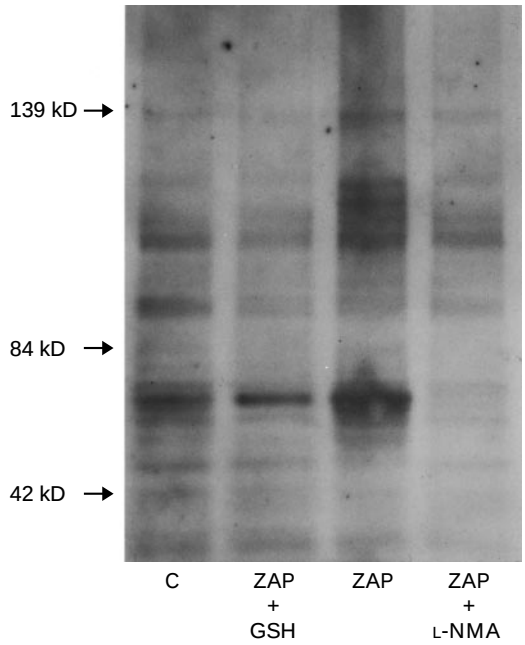


**Figure 3** Tyrosine nitration, a marker of peroxynitrite production in cellular proteins of rat aortic smooth muscle cells at 24 h after exposure to vehicle (control, C), after exposure to zymosan-activated plasma (ZAP, 20 $\mu$l ml$^{-1}$); the effect of N$^{G}$-methyl-L-arginine (L-NMA, 3 mM) and glutathione (GSH, 3 mM) pretreatment in the presence of ZAP is also shown.

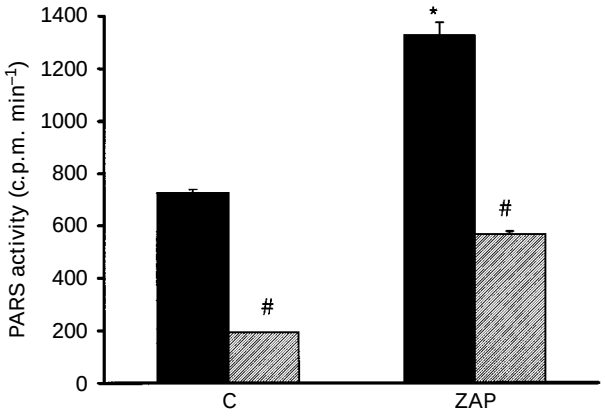


**Figure 4** PARS activity in rat aortic smooth muscle cells at 24 h after exposure to vehicle (C) or exposure to zymosan-activated plasma (20 $\mu$l ml$^{-1}$) for 24 h (ZAP); effect of 3-aminobenzamide (3-AB, 1 mM). Solid columns represent PARS activity in the absence of 3-AB, hatched columns represent PARS activity in the presence of 3-AB. Data represent means $\pm$ s.e.mean of $n=9$ wells. $*P<0.05$ represents a significant increase in PARS activity in response to zymosan-activated plasma; $\#P<0.01$ represents a significant inhibitory effect of 3-aminobenzamide on PARS activity.

### *Role of peroxynitrite and PARS in the changes in vascular reactivity of rat aortic rings exposed to ZAP* in vitro

*In vitro* exposure of intact rat aortic rings to ZAP caused a reduction in contractile response to noradrenaline, when compared to the control response (Figure 5a,b). An impairment of endothelium-dependent relaxation was also observed in aortic rings exposed to ZAP (Figure 6a,b). However, in endothelium-denuded rings, exposure of the rings to ZAP for 30 min did not significantly affect the

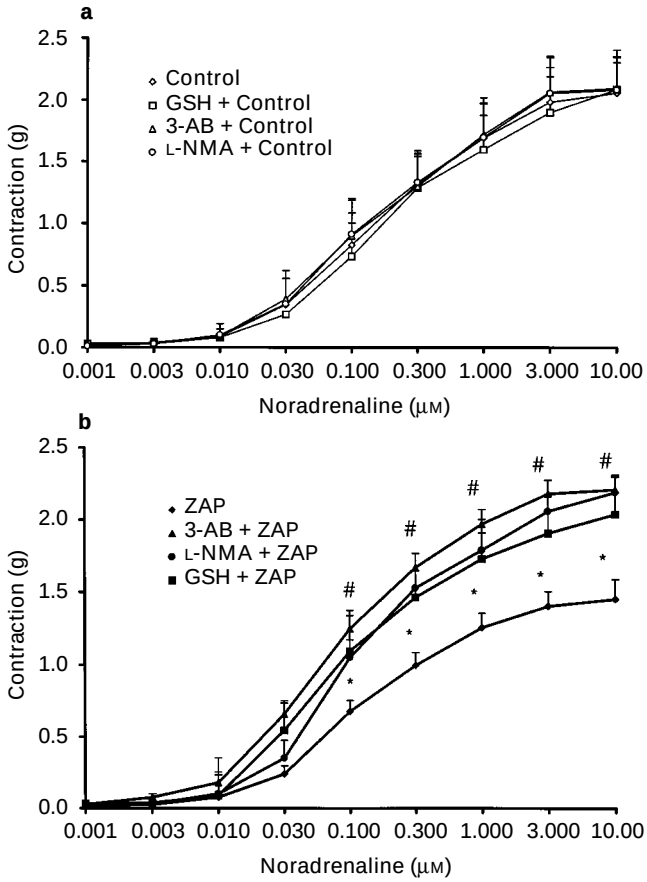


**Figure 5** Effect of exposure to zymosan-activated plasma (20 $\mu$l ml$^{-1}$) for 30 min on the contractile activity to noradrenaline (1 nM – 10 $\mu$M) in thoracic aortic rings *in vitro*. In (a) responses represent contractions in sham-treated control rings (control) and control responses in the presence of L-NMA, glutathione (GSH) or 3-aminobenzamide (3-AB). In (b), responses represent contractions in rings exposed to zymosan-activated plasma (20 $\mu$l ml$^{-1}$) (ZAP) and responses to zymosan-activated plasma in the presence of L-NMA, glutathione or 3-aminobenzamide. Data represent means of $n=9$ rings; vertical lines show s.e.mean. $*P<0.05$ represents a significant effect of zymosan-activated plasma, when compared to control; $\#P<0.05$ represents a significant protection by the various treatments.

contractions (Figure 7). In the endothelium-intact rings, there was a significant increase in PARS activity at 30 min after ZAP exposure (to 163 $\pm$ 7% of control, $P<0.01$, $n=4$), whereas there was no significant increase in endothelium-denuded rings (107 $\pm$ 12%, $n=4$).

Pretreatment with glutathione, 3-aminobenzamide or L-NMA prevented the ZAP-induced suppression of the vascular responsiveness to noradrenaline in intact rings (Figure 5b). Glutathione and 3-aminobenzamide also reduced the degree of impairment of the endothelium-dependent relaxations in response to ZAP (Figure 6b), without altering the relaxations in control rings (Figure 6a).

In a separate study, precontracted, endothelium-denuded rings were exposed to ZAP and the gradual loss of tone was monitored over 6 h (Figure 8). A similar, gradual loss of the vascular tone has previously been described in vascular rings incubated with endotoxin, and the loss of the tone has been linked to the expression of iNOS in the vascular smooth muscle (Rees *et al*., 1990). The loss of tone was, in part, prevented by pretreatment of the rings with glutathione, 3-aminobenzamide and L-NMA (Figure 8). In these rings, at 6 h after exposure to ZAP, a significant increase in PARS activity was found (121 $\pm$ 13%, $P<0.01$, $n=4$).

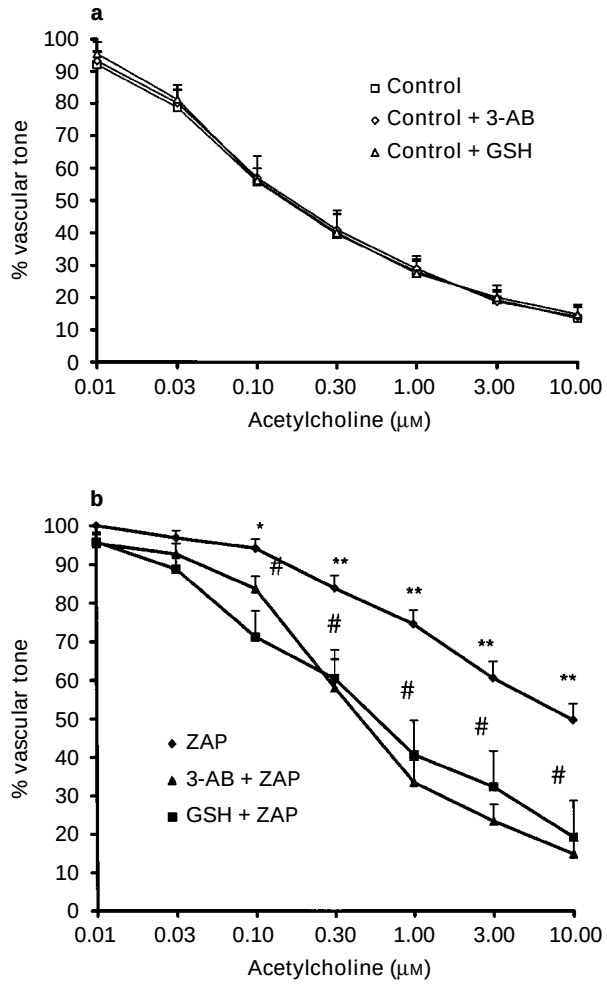


**Figure 6** Effect of zymosan-activated plasma (ZAP, 20 $\mu$l ml$^{-1}$) on the relaxant responses to activity to acetylcholine (10 nM – 10 $\mu$M) in thoracic aortic rings *in vitro*. In (a), responses represent relaxations in sham-treated control rings (control) and control responses in the presence of glutathione (GSH) or 3-aminobenzamide (3-AB). In (b), responses represent relaxations in rings exposed to zymosan-activated plasma (20 $\mu$l ml$^{-1}$) in the absence and presence of glutathione or 3-aminobenzamide. Data represent means of $n=9$ rings; vertical lines show s.e.mean. $^*P<0.05$ and $^{**}P<0.01$ represent significant impairments of the relaxations in response to zymosan-activated plasma; $\#P<0.05$ represents a significant protection by the various treatments.

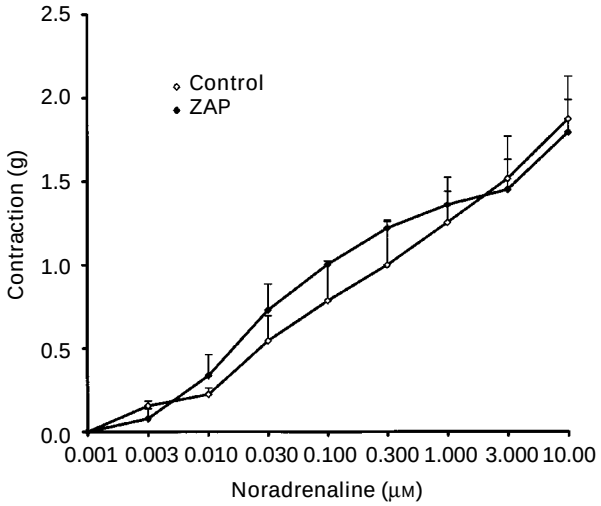


**Figure 7** Lack of effect of zymosan-activated plasma (ZAP, 20 $\mu$l ml$^{-1}$) on the contractile activity to noradrenaline (1 nM – 10 $\mu$M) in endothelium-denuded thoracic aortic rings *in vitro*. Data represent means of $n=8$ rings; vertical lines show s.e.mean.

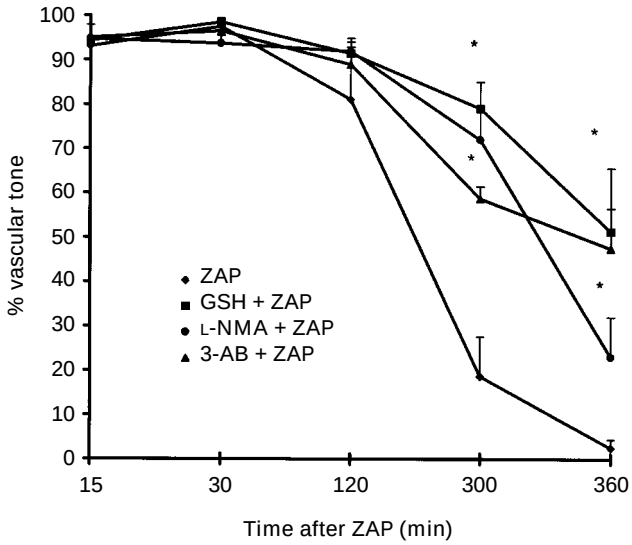


**Figure 8** Effect of zymosan-activated plasma (ZAP, 20 $\mu$l ml$^{-1}$) on the vascular tone over time (0 – 6 h) in noradrenaline (1 $\mu$M) precontracted, endothelium-denuded vascular rings. Data shown represent responses in rings exposed to zymosan-activated plasma in the absence and presence of L-NMA, glutathione (GSH) or 3-aminobenzamide (3-AB). Data represent means of $n=8$ rings; vertical lines show s.e.mean. $^*P<0.05$ represents a significant protection against the loss of the tone by the various treatments.

### *Zymosan-activated plasma induces peroxynitrite production in vascular rings*

Endothelium-intact aortic rings were also obtained after a 30 min incubation with ZAP, and the presence of nitrotyrosine, as marker for peroxynitrite formation was examined. Staining was absent in control tissue (Figure 9a). Immunohistochemical analysis, with a specific anti-nitrotyrosine antibody, revealed a positive nitrotyrosine staining in aortae incubated with ZAP (Figure 9b).

## Discussion

### *Zymosan-activated plasma induces iNOS expression and peroxynitrite production in vascular smooth muscle cells* in vitro

Administration of zymosan or zymosan-activated plasma (ZAP) is used as a non-septic model of circulatory shock and multiple organ failure in experimental animals (Goris *et al*., 1986). The role of the production of pro-inflammatory cytokines, such as tumour necrosis factor (Von Asmuth *et al*., 1990), and pro-inflammatory lipid mediators, such as platelet-activating factor (Damas *et al*., 1993), and of prostaglandin metabolites (Doherty *et al*., 1990; Mainous *et al*., 1993; Rao *et al*., 1993) is well established in pathophysiology of zymosan-induced shock. The role of oxygen-derived free radicals (Doherty *et al*., 1990; Van Bebber *et al*., 1992; Mainous *et al*., 1993; Demling *et al*., 1995) and, more recently, the role of NO (Cuzzocrea *et al*., 1996; 1997) has also been demonstrated in various models of zymosan-induced shock and inflammation. Since vascular failure is one of the prominent features of various forms of shock, the present study was designed to investigate the cellular mechanisms of zymosan-induced vascular failure.

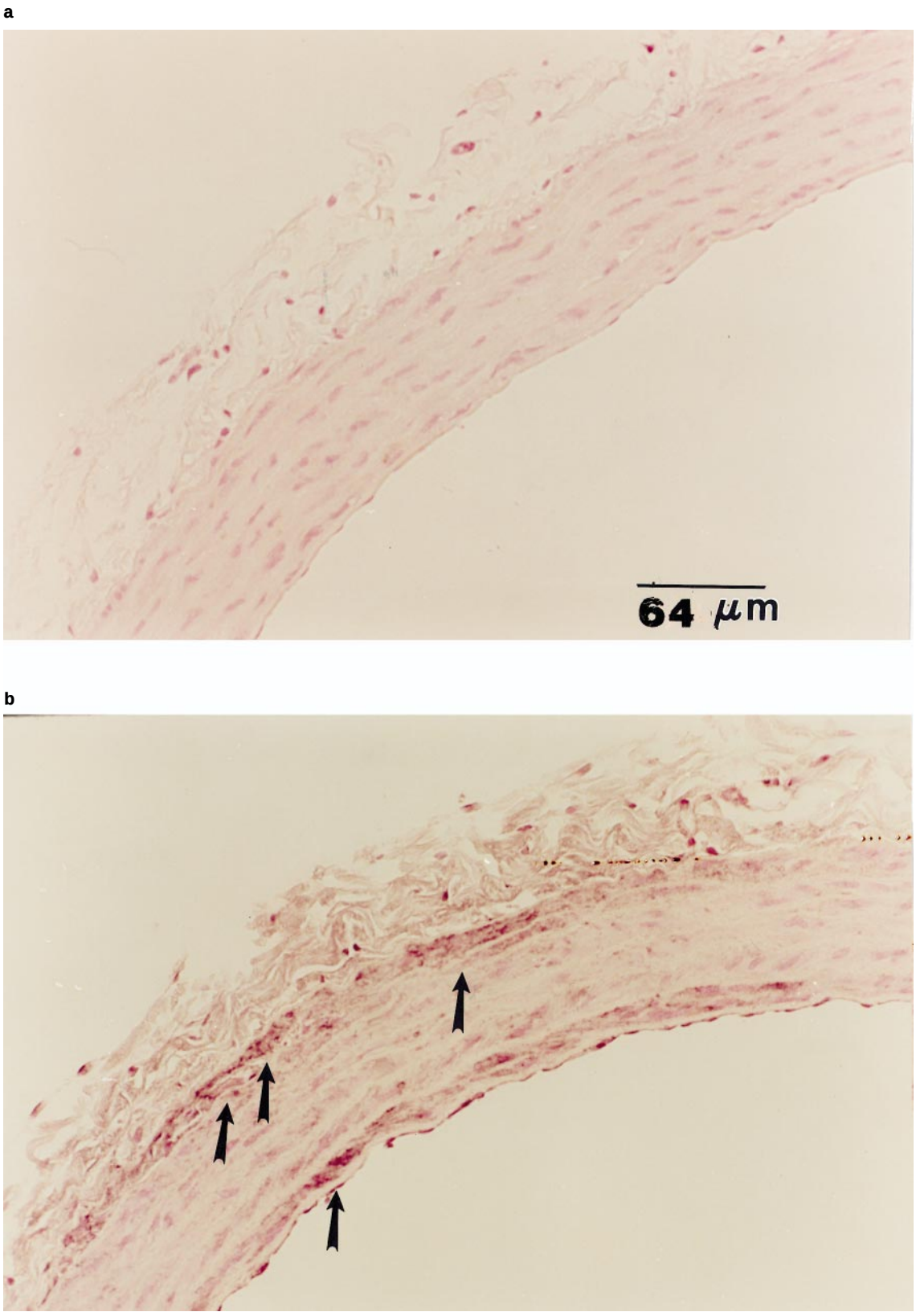


**Figure 9** Immunohistochemical localization of nitrotyrosine in the rat thoracic aorta at 30 min after exposure to zymosan-activated plasma (20 $\mu$l ml$^{-1}$). (a) Staining was negligible in control tissue, (b) zymosan-activated plasma induced significant nitrotyrosine staining. Arrows in (b) represent typical areas of nitrotyrosine staining.

Since recent studies point towards the importance of peroxynitrite in the cell and tissue injury in various forms of shock and inflammation (see: Introduction), here we investigated the production and the role of peroxynitrite in our *in vitro* models in response to ZAP challenge. The experimental models we have chosen are reductionist models of vascular injury, which only involve the target cells or tissues and zymosan-activated plasma, but do not involve zymosan itself or other cell types, such as neutrophils. The main findings of the present study were the following: (1) ZAP induced a time-dependent pro-

duction of nitrite in cultured smooth muscle cells; (2) ZAP induced peroxynitrite formation in cultured smooth muscle cells and in aortic rings; (3) ZAP induced DNA injury and PARS activation in smooth muscle cells; (4) ZAP rapidly induced the simultaneous development of endothelial dysfunction and vascular hypocontractility in vascular rings and (5) the ZAP-induced vascular failure was prevented by glutathione and by the PARS inhibitor 3-aminobenzamide.

Induction of iNOS in vascular smooth muscle cells and many other cell types has been described in response to pro-inflammatory stimuli, such as cytokines (tumour necrosis factor, interleukin-1, interferon-$\gamma$), and in response to gram positive and gram negative cell wall components (lipopolysaccharides, Lipid A, lipoteichoic acid, peptidoglycan, etc.) (Hattori *et al*., 1995; Szabó, 1995). Moreover, recent studies have demonstrated that iNOS in various cell types, including smooth muscle cells, can be induced by non-immunological stimuli, for example by agents that increase intracellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels (Boese *et al*., 1996). There are data demonstrating that zymosan can enhance the expression of iNOS in macrophages and in microglia (Corradin & Mauël, 1991; Corradin *et al*., 1993; Assreuy *et al*., 1994). However, in the current experiments, zymosan was removed from the plasma after activation (Lundberg & Arfors, 1983). Despite the fact that zymosan was removed, we found that ZAP induced the rapid expression of iNOS in smooth muscle cells. Similar to the results of our present study, expression iNOS in response to ZAP has been demonstrated in other cell types, such as pulmonary alveolar epithelial cells *in vitro* (Gutierrez *et al*., 1995). Platelet-activating factor and complement factors are examples of mediators, the production of which is induced by zymosan, and which, in turn, are able to induce iNOS or enhance its induction in response to other stimuli (Szabó *et al*., 1993b; Leu *et al*., 1993; Mustafa *et al*., 1996). The nature of the stimuli leading to the expression of iNOS in smooth muscle cells (and other cell types) in response to ZAP exposure remains to be clarified.

It is well established that treatment of plasma with zymosan activates complement and generates $C_{5a}$, which is a potent chemotactic factor for neutrophils (Synderman *et al*., 1970; Fernandez *et al*., 1978). Since zymosan, ZAP and complement can trigger the production of oxygen-derived free radicals in various cell types (Mehta *et al*., 1991; Murohara *et al*, 1993), it is not unexpected that we found that incubation of cultured smooth muscle cells with ZAP results in the formation of peroxynitrite in our experiments.

The increase in the production of peroxynitrite in cells challenged with ZAP preceded the maximal production of NO. In this respect, it is noteworthy that the oxidant potential of peroxynitrite is determined by the ratio of superoxide and NO, rather than the net amount of these two precursors (Szabó *et al*., 1995a; Szabó, 1996; Miles *et al*., 1996; Rubbo *et al*., 1996). In our experiments, a small, but significant and L-NMA inhibitable production of nitrite was already present in the unstimulated smooth muscle cells. This finding is similar to findings by other groups (Boese *et al*., 1996). Since markedly reduced nitrite production and nitrotyrosine staining were observed in the absence of foetal calf serum in the tissue culture medium, we speculate that the small basal production of nitrite may be related to a small degree of immune activation due to serum factors. Taken together, it is conceivable that low-levels of NO and superoxide production trigger a basal, low-level tyrosine nitration in unstimulated cells in the presence of foetal calf serum. In response to ZAP, early production of superoxide combines with low levels of NO, produced basally, resulting in the production of peroxynitrite. It is also possible that at later time points, excess NO limits the peroxynitrite-induced oxidation of dihydrorhotamine 123 (Szabó *et al*., 1995a).

### *Zymosan-activated plasma induces DNA strand breakage and PARS activation in vascular smooth muscle cells*

There are two major triggers of DNA single strand breakage under inflammatory conditions: hydroxyl radical and peroxynitrite (Berger, 1991; Cochrane, 1991; Szabó, 1996b), in the latter case activated peroxynitrous acid being the likely oxidant species (Szabó, 1996b). The increase in the DNA strand breakage in ZAP-treated smooth muscle cells paralleled the production of peroxynitrite (in both cases, the most pronounced increase seen between 3 and 6 h). This temporal correlation and the suppression of DNA single strand breakage by inhibition of NOS, strongly indicates that, in this system, peroxynitrite (and not other oxidants, such as hydroxyl radical) may be primarily responsible for the DNA strand breakage.

The cytotoxic effects of peroxynitrite are multiple and include protein oxidation, lipid peroxidation, inhibition of cellular metabolic pathways and signal transduction processes (Szabó, 1996a,b). A novel, distinct pathway of peroxynitrite-induced cytotoxicity involves DNA single strand breakage and activation of the nuclear enzyme PARS with consequent energetic depletion (see: Introduction). In the current study, we presented evidence that exposure of cultured smooth muscle cells to ZAP triggers PARS activation. This is not unexpected, considering the fact that single stranded DNA is an obligatory trigger for the activation of PARS (Berger, 1991; Cochrane, 1991; Szabó, 1996b).

Glutathione is known as a scavenger of both oxyradicals and of peroxynitrite (Pryor & Squadrito, 1995; Szabó, 1996a). Since glutathione has a limited cellular uptake, it is conceivable that part of the effects of glutathione are related to extracellular neutralization of the peroxynitrite produced. In addition, it is conceivable that glutathione may also exert its effect, at least in part, via neutralization of other oxyradicals, generated extracellularly.

### *Zymosan-activated plasma induces vascular hyporeactivity and endothelial dysfunction in vascular rings, which may be related to peroxynitrite production and subsequent PARS activation*

Peroxynitrite causes cellular and tissue injury in a variety of inflammatory states (Ischiropoulos *et al*., 1992; Wizemann *et al*., 1994; Villa *et al*., 1994; Pryor & Squadrito, 1995; Szabó *et al*., 1995a,b). The data obtained from rat thoracic aortic rings in the present study suggest that peroxynitrite, generated within the vasculature in response to ZAP challenge, leads to an impairment of vascular function. Similar simultaneous impairment of the contractile and endothelium-dependent responses is a prominent feature of various forms of shock (endotoxic shock, haemorrhagic shock) (Szabó, 1995) and also the shock induced by zymosan (Cuzzocrea *et al*., 1996; 1997).

The restoration of the contractility by 3-aminobenzamide suggests that PARS activation, in response to peroxynitrite, contributes to the development of vascular failure. Regarding the impairment of the vascular contractions, we have recently obtained similar data with authentic peroxynitrite, demonstrating a suppression of the vascular contractility, an effect which was partially prevented by 3-aminobenzamide, suggesting the role of PARS activation in the process (Szabó *et al*., 1996a). Moreover, in vascular rings obtained from endotoxin shock, the development of vascular hyporeactivity was prevented by treatment of the animals with 3-aminobenzamide (Szabó *et al*., 1996a). The current data with ZAP suggest that, similar to the endotoxin-induced hyporeactivity, peroxynitrite and PARS activation may play a role in the vascular hyporeactivity in the zymosan-induced vascular failure as well.

The endothelial dysfunction is another prominent feature of various forms of shock (Szabó, 1995), and has previously been described in coronary artery rings incubated with ZAP (Stahl *et al*., 1995). The ability of peroxynitrite to cause endothelial

dysfunction has also been demonstrated by Villa and coworkers (1994) and, recently, by Elliott (1996). The current data suggest that, similar to the mechanism of vascular hyporeactivity, peroxynitrite and PARS activation may also be an important mechanism in the endothelial dysfunction in ZAP-treated vascular rings. This hypothesis is in line with current data from our laboratory, showing that the endothelial dysfunction elicited by authentic peroxynitrite in thoracic aortic rings and the peroxynitrite-induced suppression of mitochondrial respiration in human cultured umbilical vein endothelial cells, can also be ameliorated by the PARS inhibitor 3-aminobenzamide (Szabó *et al*., 1997).

The gradual loss of vascular tone in the precontracted rings incubated with ZAP for 6 h resembles the situation where RASM cells were incubated with ZAP for 24 h. In both of these systems iNOS is likely to be expressed and the iNOS-derived NO then combines with superoxide to form peroxynitrite. However, in the vascular studies (as opposed to the *in vitro* experiments in the RASM cells), there was also a significant degree of hyporeactivity after ZAP exposure at an early time point (30 min). Since this hyporeactivity was also NO-dependent and was absent in endothelium-denuded rings, it is logical to assume, that in this acute setting, ecNOS is the source of NO for the formation of peroxynitrite. In this respect, it is noteworthy that we have described the early occurrence of an L-NMA inhibitable oxidation of the fluorescent dye dihydrorhodamine 1,2,3 in endotoxic shock, haemorrhagic shock and splanchnic artery reperfusion shock (Szabó *et al*., 1995a), consistent with the hypothesis that the early production of peroxynitrite is due to the reaction of superoxide with NO formed by constitutive NOS isoforms.

Taken together, the present results indicate that peroxynitrite production and consequent activation of the PARS pathway contributes to the vascular failure in response to ZAP exposure. However, the current data do not support the view that PARS activation is the sole mechanism of the vascular alterations. For instance, it is noteworthy that our data did not present a direct correlation between PARS activation and vascular relaxation. For instance, a relatively high degree of PARS activation after 30 min exposure to ZAP was associated with a modest loss of contractility (Figures 4 and 5), whereas a smaller degree of PARS activation was associated with a massive loss of contractile tone at 6 h after ZAP exposure (Figure 8). Clearly, the molecular mechanisms of the PARS-related loss of vascular contractility and the interactions of the PARS pathway with other, NO- or peroxynitrite-related pathways of vasorelaxation remains to be further clarified. One issue to be clarified in follow-up studies is related to the issue of PARS-dependent versus cyclic GMP-dependent pathways of vasorelaxation. For instance, peroxynitrite can react with various cellular components to produce NO-releasing adducts (Moro *et al*, 1995): NO, in turn, can activate the guanylyl cyclase- cyclic GMP system (Tarpey *et al*., 1996). However, this pathway is unlikely to lead to PARS activation, since the trigger of PARS activation is DNA single strand breakage and this type of DNA injury develops in response peroxynitrite and hydroxyl-radical, but not NO (see: Szabó, 1996b). Another cellular pathway which has been implicated in the LPS-induced vascular hyporeactivity is related to activation of potassium channels (Taguchi *et al*., 1996; Hall *et al*., 1996). It is conceivable that a PARS-related reduction in cellular ATP may influence the activation of these channels. Moreover, it is also conceivable that the effect of glutathione in our studies may be, at least in part, related to a potassium-channel related effect: glutathione has been demonstrated to affect potassium channel activation in the vascular smooth muscle (Yuan *et al*., 1994). The delineation of the interactions of the PARS pathway with other pathways of vascular relaxation in immunostimulated vascular smooth muscle remains a subject of further studies.

The conclusions of the present study have been reached, in significant part, by the use of 3-aminobenzamide, a pharmacological inhibitor of PARS (Banasik *et al*., 1992). Although this inhibitor has widely been used for inhibition of PARS in studies investigating oxidant-related cellular injury, we acknowledge that this agent may have PARS-independent, additional effects, such as scavenging. The inhibitor has been proposed as a hydroxyl radical scavenger, although its potency is rather low (Wilson *et al*., 1984). On the other hand, 3-aminobenzamide, up to 3 mM, did not affect peroxynitrite-induced oxidations in three different systems and it does not appear to be a scavenger of peroxynitrite (Zingarelli *et al*., 1997b). Moreover, 3-aminobenzamide does not inhibit NOS activity (Zingarelli *et al*., 1997b). We realise that, based on our studies with 3-aminobenzamide alone, we cannot exclude the possibility of PARS-independent, additional actions of this agent in the present study. Nevertheless, it is noteworthy that we have recently examined a range of PARS inhibitors against peroxynitrite-induced injury in endothelial cells in culture and found that nicotinamide, 3-aminobenzamide and (at lower concentrations) the more potent PARS inhibitor, 5-iodo-6-amino-1,2-benzopyrone, all protected against peroxynitrite-induced endothelial injury, whereas 3-aminobenzoic acid, a structurally related analogue with no inhibitory effect on PARS, did not (Szabó *et al*., 1997). Moreover, several recent studies demonstrate that cells from the PARS knockout mice are protected against various forms of oxidant injury, including peroxynitrite exposure (Heller *et al*., 1995; Szabó *et al*., 1997).

We have recently demonstrated that pharmacology inhibition of PARS improves vascular contractility, blood pressure and survival in rodent models of endotoxic shock, suggesting the central role of this pathway in the cardiovascular derangements associated with endotoxaemia (Szabó *et al*., 1996a). Whether PARS inhibition can also protect against the vascular and multiple organ failure in non-septic shock models (such as the shock induced by zymosan or ZAP) requires further study. However, the present study and other current observations (Szabó *et al*., 1996a,b; Zingarelli *et al*., 1996) suggest that pharmacological inactivation of PARS represents a novel strategy which may be useful to limit the energetic failure and vascular dysfunction associated with various forms of circulatory shock. While, in principle, there are several available approaches to reduce NO or peroxynitrite-related cytotoxicity (NOS inhibitors, peroxynitrite scavengers, oxyradical scavengers, to name a few), inhibition of PARS may be a viable strategy for several reasons: (1) inhibition of PARS is not likely to interfere with the physiological roles of NO; (2) inhibition of PARS affects multiple aspects of the inflammatory response in shock, such as endothelial and epithelial dysfunction, vascular hypocontractility and cellular energetic failure and (3) since invading prokaryotes do not contain PARS, pharmacological inhibition of PARS is unlikely to compromise NO- or peroxynitrite-mediated host defence systems. The viability of this strategy is also strengthened by the recent observation demonstrating that the presence of PARS is not obligatory for normal DNA repair (Wang *et al*., 1995), because of the simultaneous existence of multiple efficient DNA repair pathways. The present observations should encourage further studies on the use of PARS inhibitors (alone or in combination with other approaches) for the experimental therapy of various forms of shock and inflammation.

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