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Beneficial effects of 3-aminobenzamide, an inhibitor of poly (ADP-ribose) synthetase in a rat model of splanchnic artery occlusion and reperfusion

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1 Peroxynitrite, a potent cytotoxic oxidant formed by the reaction of nitric oxide with superoxide anion, and hydroxyl radical, formed in the iron-catalysed Fenton reaction, are important mediators of reperfusion injury. In *in vitro* studies, DNA single strand breakage, triggered by peroxynitrite or by hydroxyl radical, activates the nuclear enzyme poly (ADP-ribose) synthetase (PARS), with consequent cytotoxic effects. Using 3-aminobenzamide, an inhibitor of PARS, we investigated the role of PARS in the pathogenesis of splanchnic artery occlusion shock.

2 Splanchnic artery occlusion and reperfusion shock (SAO/R) was induced in rats by clamping both the superior mesenteric artery and the coeliac trunk for 45 min, followed by release of the clamp (reperfusion). At 60 min after reperfusion, animals were killed for histological examination and biochemical studies.

3 SAO/R rats developed a significant fall in mean arterial blood pressure, significant increase of tissue myeloperoxidase activity and marked histological injury to the distal ileum. SAO/R was also associated with a significant mortality (0% survival at 2 h after reperfusion).

4 There was a marked increase in the oxidation of dihydrorhodamine 123 to rhodamine (a marker of peroxynitrite-induced oxidative processes) in the plasma of the SAO/R rats, starting early after reperfusion, but not during ischaemia alone. Immunohistochemical examination demonstrated a marked increase in the immunoreactivity to nitrotyrosine, a specific 'footprint' of peroxynitrite, in the necrotic ileum in shocked rats, as measured at 60 min after the start of reperfusion.

5 In addition, in *ex vivo* studies in aortic rings from shocked rats, we found reduced contractions to noradrenaline and reduced responsiveness to a relaxant effect to acetylcholine (vascular hyporeactivity and endothelial dysfunction, respectively).

6 In a separate set of studies, using a 4000 Dalton fluorescent dextran tracer, we investigated the changes in epithelial permeability associated with SAO/R. Ten minutes of reperfusion, after 30 min of splanchnic artery ischaemia, resulted in a marked increase in epithelial permeability.

7 There was a significant increase in PARS activity in the intestinal epithelial cells, as measured 10 min after reperfusion *ex vivo*. 3-Aminobenzamide, a pharmacological inhibitor of PARS (applied at 10 mg kg^{-1}, i.v., 5 min before reperfusion, followed by an infusion of 10 mg kg^{-1} h^{-1}), significantly reduced ischaemia/reperfusion injury in the bowel, as evaluated by histological examination. Also it significantly improved mean arterial blood pressure, improved contractile responsiveness to noradrenaline, enhanced the endothelium-dependent relaxations and reduced the reperfusion-induced increase in epithelial permeability.

8 3-Aminobenzamide also prevented the infiltration of neutrophils into the reperfused intestine, as evidenced by reduced myeloperoxidase activity. It improved the histological status of the reperfused tissues, reduced the production of peroxynitrite in the late phase of reperfusion and improved survival.

9 In conclusion, our study demonstrates that the PARS inhibitor 3-aminobenzamide exerts multiple protective effects in splanchnic artery occlusion/reperfusion shock. We suggest that peroxynitrite and/or hydroxyl radical, produced during the reperfusion phase, trigger DNA strand breakage, PARS activation and subsequent cellular dysfunction. The vascular endothelium is likely to represent an important cellular site of protection by 3-aminobenzamide in SAO shock.

Keywords: Nitric oxide; peroxynitrite; poly (ADP ribose) synthetase; reperfusion; shock

Introduction

Splanchnic artery occlusion shock (SAO) is a severe form of circulatory shock produced by ischaemia and reperfusion of the splanchnic organs. This type of shock is associated with a large number of pathophysiological alterations, the combination of which can lead to a fatal outcome. Reperfusion is typically associated with both local and systemic changes. Local functional alterations include polymorphonuclear neutrophil (PMN) adhesion and activation, intestinal hyperpermeability, changes in the vascular reactivity of the splanchnic vessels, as well as morphological changes, such as necrotic injury of the reperfused bowel (see for reviews: Lefer & Lefer, 1993; Zimmerman *et al*., 1993). Systemic alterations upon reperfusion include a progressive fall in the mean arterial blood pressure, the release of pro-inflammatory mediators from the reperfused intestinal tissue into the systemic circulation (Bittermann & Lefer, 1988; Bittermann *et al*., 1991; Zingarelli *et al*., 1992; Squadrito *et al*., 1994), alterations in the function of remote organs, such as the heart and lungs (Hinshaw *et al*., 1973;

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Koike *et al*., 1993; Jacinto & Jandhyala, 1994), and alterations in the *in vivo* and *ex vivo* reactivity of splanchnic and non-splanchnic blood vessels (Greenberg *et al*., 1981; Bittermann *et al*., 1988; Squadrito *et al*., 1994).

Simultaneous production of both oxygen and nitrogen-centred free radicals favours the production of a toxic reaction product, the oxidant peroxynitrite (Beckman *et al*., 1990; Pryor & Squadrito, 1995; Szabó *et al*., 1996a), as it has been recently demonstrated in various forms of inflammation and reperfusion injury (for reviews see: Crow & Beckman, 1995; Szabó, 1996a; Rubbo *et al*., 1996). Peroxynitrite and hydroxyl radical are potent triggers of DNA single strand breakage, with subsequent activation of the nuclear enzyme poly (ADP-ribose) synthetase (PARS) (Berger, 1991; Cochrane, 1991; Zhang *et al*., 1994; Szabó *et al*., 1996a; Zingarelli *et al*., 1996; Szabó, 1996b). Activation of PARS triggers a futile energy-consuming cycle, resulting in massive depletion of cellular NAD^+ and ATP and eventually induces irreversible cytotoxicity and cell death (see: Cochrane, 1991; and Szabó, 1996b for reviews).

Hydroxyl radical and peroxynitrite are two pathophysiologically relevant triggers of DNA single strand breakage. There is convincing evidence demonstrating the production of superoxide (and, hydroxyl radical, via the iron-catalysed Haber-Weiss reaction) during reperfusion injury (Bittermann *et al*., 1988; Zimmermann *et al*., 1993). However, the production of peroxynitrite has not been examined in the reperfused intestine. Thus, by use of nitrotyrosine immunohistochemistry, which detects a specific marker of peroxynitrite formation (Ischiropoulos *et al*., 1992) and measuring the oxidation of dihydrorhodamine 123 in the plasma (Szabó *et al*., 1995), we first performed experiments to detect the production of peroxynitrite in the intestine upon reperfusion. Then, in subsequent studies, we investigated whether pharmacological inhibition of PARS modifies the pathophysiological changes associated with splanchnic artery occlusion and reperfusion shock. Specifically, we investigated the effect of PARS inhibition on arterial blood pressure, tissue PMN immigration (assessed by the measurement of tissue myeloperoxidase (MPO) activity), changes in the contractile and endothelium-dependent relaxant responsiveness of the blood vessels (assessed by changes in the reactivity of thoracic aortic rings *ex vivo*), epithelial permeability, survival, and morphological changes in the bowel. The results of the current study implicate PARS activation in the pathophysiology of SAO shock and suggest that pharmacological inhibition of PARS may be a novel approach of therapeutic potential in ischaemia/reperfusion injury.

Methods

Surgical procedures

Male Sprague-Dawley rats weighing 250–300 g were allowed access to food and water *ad libitum*. The rats were anaesthetized with sodium pentobarbitone (45 mg kg^{-1}, i.p.). Following anaesthesia, catheters were placed in the carotid artery and jugular vein as described previously (Caputi *et al*., 1980). Blood pressure was monitored continuously by a Maclab A/D converter (AD Instruments) and stored and displayed on a Macintosh personal computer. After midline laparotomy, the coeliac and superior mesenteric arteries were isolated near their aortic origins. During this procedure, the intestinal tract was maintained at 37°C by placing it between gauze pads soaked with warmed 0.9% NaCl solution.

Rats were observed for a 30 min stabilization period before either splanchnic ischaemia or sham ischaemia. SAO shock was induced by clamping both the superior mesenteric artery and the coeliac trunk, resulting in a total occlusion of these arteries for 45 min. After this period of occlusion, the clamps were removed. In one study, the various groups of rats were killed at 60 min for histological examination of the bowel and for biochemical studies, as described below. In another sets of studies, following reperfusion, the various groups of rats were observed for 240 min in order to determine survival differences.

Experimental groups

In the treated group of animals, 3-aminobenzamide, an inhibitor of PARS, was given as a intravenous bolus 5 min before reperfusion (10 mg kg^{-1}) followed by infusion of 10 mg kg^{-1} h^{-1} during the period of reperfusion (SAO + 3-aminobenzamide group). In a vehicle-treated group of rats, vehicle (saline) was given instead of 3-aminobenzamide (SAO group). In separate groups of rats, surgery was performed in its every aspect identical to the one in the SAO group, except that the blood vessels were not occluded (time-controlled sham group; Sham). In an additional group of animals, sham surgery was combined with the administration of 3-aminobenzamide (dose as above) (Sham + 3-aminobenzamide).

Measurement of nitrite/nitrate in the plasma

Nitrite/nitrate production, an indicator of NO synthesis, was measured in plasma samples from sham or SAO rats at 45 min after ischaemia or 60 min after reperfusion as previously described (Zingarelli *et al*., 1996). First, nitrate in the plasma was reduced to nitrite by incubation with nitrate reductase (670 mu ml^{-1}) and NADPH (160 μM) at room temperature for 3 h. After 3 h, nitrite concentration in the samples was measured by the Griess reaction, by adding 100 μl of Griess reagent (0.1% naphthalethylenediamine dihydrochloride in H_2O and 1% sulphanilamide in 5% concentrated H_3PO_4; vol. 1:1) to 100 μl samples. The optical density at 550 nm (OD_{550}) was measured with a Spectramax 250 microplate reader (Molecular Devices Sunnyvale, CA). Nitrate concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrate prepared in saline solution.

Measurement of peroxynitrite production

The formation of peroxynitrite was estimated by the peroxynitrite-dependent oxidation of dihydrorhodamine 123 to rhodamine 123, by a previously described method (Szabó *et al*., 1995). In separate groups, animals were injected with dihydrorhodamine 123 (2 μmol kg^{-1} in 0.3 ml saline, i.v.) 25 min after ischaemia, 35 min after ischaemia or 40 min after reperfusion. Twenty minutes later, (i.e. immediately before reperfusion, 10 min after reperfusion and 60 min after reperfusion, respectively), rats were killed and plasma samples taken for rhodamine fluorescence evaluation 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). The plasma concentration of rhodamine 123, an index of peroxynitrite production, was calculated by use of a standard curve obtained with authentic rhodamine 123 (1–30 nM) prepared in plasma obtained from untreated rats. Background plasma fluorescence was subtracted from all samples.

Histological examination

For histopathological examination, biopsies of small intestine were taken 60 min after reperfusion. The tissue slices were fixed in 10% neutral-buffered formaldehyde for 5 days, embedded in paraffin and sectioned. The sections were stained with haematoxylin and eosin.

Immunohistochemical localization of nitrotyrosine

Tyrosine nitration, a specific 'footprint' of peroxynitrite formation, was detected in ileal sections by immunohistochemistry. After reperfusion, tissues were fixed in 10% buffered formalin and 8 μm sections were prepared from paraffin embedded tissues. After the paraffin had been removed endo-

genous 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 2% normal goat serum in phosphate buffered saline for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin (biotin blocking kit, Vector Laboratories). The sections were then incubated overnight with 1:1000 dilution of primary anti-nitrotyrosine antibody (Upstate Biotech, Saranac Lake, NY) or with 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).

Myeloperoxidase activity

Myeloperoxidase activity, an index of PMN accumulation, was determined as previously described (Mullane *et al*., 1985). Intestinal tissues, collected 60 min after reperfusion, were homogenized in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at $20{,}000 \times g$ at 4°C. An aliquot of the supernatant was then allowed to react with a solution of tetra-methyl-benzidine (1.6 mM) and 0.1 mM H_2O_2. The rate of change in absorbance was measured by a spectrophotometer at 650 nm. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol of hydrogen peroxide min^{-1} at 37°C and was expressed in u g^{-1} weight of wet tissue.

Measurement of PARS activity in intestinal epithelial cells ex vivo

Rats were subjected to sham surgery or splanchnic ischaemia for 45 min followed by 10 min reperfusion as described above. After reperfusion, a 7 to 8 cm section of small intestine was excised and the mucosal surface exposed. This surface was then rinsed with sterile saline and the epithelium scraped with a number 10 scalpel blade. The resultant cell/tissue pellet was divided into four equal parts and each placed into a microfuge tube containing 500 μl PARS buffer (mM: HEPES 56, KCl 28, NaCl 28, $MgCl_2$ 2, 0.01% digitonin and NAD^+ 0.125 μM). Two out of the four tubes also contained the PARS inhibitor 3-aminobenzamide (1 mM). PARS was then measured by a modification of the previously described protocol (Szabó *et al*., 1996a). First, to each tube 0.25 μCi [^{3}H]-NAD^+ was added and the tubes incubated in a 37°C water bath for 10 min. After incubation, 200 μl ice-cold 50% trichloroacetic acid (TCA) was added to each tube and the tubes placed on ice. Samples were then transferred to 4°C for an additional period of 3 h. After precipitation, the samples were centrifuged at 10,000 *g* for 10 min and the supernatant removed. The pellets were washed twice with 5% TCA and solubilized in 1.0 ml 2% sodium dodecyl sulphate (SDS): 0.1 N NaOH at 37°C for 96 h. An aliquot of the solubilized samples was taken for protein determination by the Bradford assay. The remaining sample was placed in 5.3 ml scintillation cocktail and radioactivity determined by a Wallac 1409 beta scintillation counter. Values for PARS activity are expressed as c.p.m. mg^{-1} protein.

Measurement of vascular reactivity ex vivo

Animals were killed under anaesthesia at 60 min after the start of reperfusion. Thoracic descending aortae were immediately excised, cut into rings and 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, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.7, in the presence of indomethacin (10 μM). Isometric force was measured with isometric transducers (Kent Scientific Corp.) digitized by a Maclab A/D converter (AD Instruments), and stored and displayed on a Macintosh personal computer. A tension of 1 g was applied and the rings were equilibrated for 60 min. During the equilibration period, fresh Krebs solution was provided at 15 min intervals.

Cumulative concentration-response curves to noradrenaline were obtained by adding increasing concentrations of noradrenaline (1 nM to 10 μM) to the organ baths surrounding the aortic rings.

In a separate study, endothelium-dependent relaxations were evaluated with concentration-response curves to acetylcholine (10 nM to 10 μM) in aortic rings precontracted with noradrenaline (1 μM). Relaxation was calculated as % of precontractile vascular tone.

Measurement of intestinal permeability

After a midline abdominal incision, 3 ml warmed saline was poured into the abdominal cavity. Then, a segment of the terminal ileum (approx. 800 mg) supplied by 3 blood vessel arcades was isolated from the remaining part of the intestine by incising the mesentery and transsecting the bowel wall between two aneurysm clips. Proximal and distal to the clips, the gut lumen was closed by placing purse string sutures. The segment used for permeability measurements was cannulated at both ends and its luminal content was gently flushed with warmed (37°C) saline (10 ml) from oral to aboral direction and the distal end was closed with a suture. Then, the coeliac and superior mesenteric arteries were exposed and occluded as described above, by placing small aneurysm clips near to their aortic origin. The abdomen was filled up with warmed saline solution and closed.

Intestinal permeability (lumen to plasma) was measured with a 4000 Dalton fluorescent dextran (FD4) according to previously described methods (Otamiri *et al*., 1988; Otamiri & Tagesson, 1989). After cannulation of the femoral vessels, a continuous infusion of saline was started at a rate of 2.5 ml h^{-1}. At $T = -30$, the splanchnic vessels were occluded for 30 min. Five minutes before the occlusion was released, the renal vessels were ligated and the segment was filled up with 0.5 ml warmed (37°C) FD4 solution (25 mg ml^{-1}). At 10 min of reperfusion, 0.3 ml blood samples were taken for fluorescein concentration measurements in the plasma. At the end of the experiment, the bowel segment was removed and weighed.

Blood samples were stored on ice in the dark and centrifuged at $100 \times g$ for 10 min. After the plasma and luminal solution had been diluted (1:200 and 1:90000, respectively), the concentration of FD4 was determined with the Perkin Elmer luminescence spectrophotometer (excitation wavelength: 492 nm; emission wavelength: 515 nm). In order to calculate epithelial permeability, the following equation was used:

Percentage of FD4 measured in the plasma = arterial FD4 concentration (ng ml^{-1})/luminal FD4 concentration (ng ml^{-1}).

The ischaemia-reperfusion protocol was, in its every aspect identical to the protocols used for the other studies, except for the additional surgery involved and the fact that the ischaemic period was reduced from 45 min to 30 min. This reduction in the length of ischaemia was necessary to achieve a severity of shock comparable with that of the other sets of studies, since the additional surgery and ligation of the renal vessels involved in this model increased the mortality of the animals in the reperfusion phase (data not shown).

Evaluation of survival

The various groups of rats were monitored for 4 h after SAO and reperfusion, and survival rates and survival times were evaluated.

Reagents

Biotin blocking kit, biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex were obtained from

Vector Laboratories (Burlingame, CA, U.S.A.). Primary anti-nitrotyrosine antibody was from Upstate Biotech (Saranac Lake, NY, U.S.A.). Dihydrorhodamine 123 and rhodamine 123 were from Molecular Probes (Eugene, OR, U.S.A.). All other reagents and compounds used were obtained from Sigma Chemical Company (Sigma, St. Louis, MO, U.S.A.).

Statistical analysis

Data are expressed as mean ± s.e.mean in all figures. ANOVA test was used to compare means of the various experimental groups, followed by Bonferroni's test for multiple comparison. Survival rates were compared by the Chi-square test. Differences were considered significant when P value was less than 0.05.

Results

NO and peroxynitrite production in splanchnic artery occlusion shock

There was no change in the plasma levels of nitrate/nitrite during occlusion or during 60 min of the reperfusion period (Figure 1), in agreement with previous observations suggesting that the current protocol of ischaemia and reperfusion does not trigger the expression of the inducible isoform of NOS (iNOS) (Kanwar *et al*., 1994). The PARS inhibitor 3-aminobenzamide did not affect baseline nitrite/nitrate levels (Figure 1).

In agreement with previous observations (Szabó *et al*., 1995), SAO shock (but not ischaemia alone) caused a significant increase in the rhodamine fluorescence of plasma, indicative of peroxynitrite-induced oxidation of dihydrorhodamine during the reperfusion phase, but not during the occlusion period (Figure 2). Already at 0–10 min after reperfusion, there was a significant increase in rhodamine fluorescence, and a significant further increase at 40–60 min (Figure 2). *In vivo* treatment with 3-aminobenzamide markedly reduced the oxidation of dihydrorhodamine 123 during the late phase, but not the early phase of reperfusion (Figure 2).

At 60 min after reperfusion ileal sections were taken from sham or shocked rats in order to determine the immunohistological staining for nitrotyrosine. While there was negligible staining in the intestinal sections of control animals (Figure 3a), immunohistochemical analysis, with a specific anti-nitrotyrosine antibody, revealed a positive staining in ileum from shocked rats, which mainly appeared to be localized in mononuclear cells (Figure 3b).

In agreement with its effect on plasma dihydrorhodamine oxidation (Figure 2), 3-aminobenzamide treatment reduced the degree of immunostaining for nitrotyrosine in the reperfused intestine (Figure 3c).

PARS activation in splanchnic artery occlusion shock

There was a significant increase in PARS activity, as measured by incorporation of tritium-labelled NAD^+ into the intestinal epithelial cells, at 10 min of reperfusion following SAO *ex vivo* (Figure 4). The increase in PARS activity was blocked by *in vitro* treatment of the epithelial cells with 3-aminobenzamide (1 mM). There was a significant degree of incorporation of tritium-labelled NAD^+ into proteins in control intestinal epithelial cells (not subjected to SAO/R). However, this was unaffected by 3-aminobenzamide (Figure 4). This latter finding suggests that the incorporation of tritium-labelled NAD^+ in epithelial cells from control animals is not related to specific PARS activity.

Histological changes and myeloperoxidase activities in the reperfused intestine

At 60 min after reperfusion, tissue damage was evaluated by histological examination and PMN infiltration was assessed by the measurement of tissue myeloperoxidase activity. As shown in Figure 5, myeloperoxidase (MPO) activity significantly ($P<0.01$) increased in the ileum of shocked rats. Histological examination of the small intestine (see representative sections in Figure 6) revealed pathological changes that closely correlated with the increase in MPO activity. There was a significant oedema in the space bounded by the villus and the basement membrane (Figure 6b).

In vivo treatment with 3-aminobenzamide significantly reduced the SAO-induced increase in MPO activity ($P<0.01$; Figure 5) and reduced organ injury, as determined by histological examination (Figure 6c).

Arterial blood pressure

Occlusion of the splanchnic arteries for 45 min did not induce a marked change in mean arterial blood pressure (Figure 7). Upon release of the occlusion, there was a gradual fall in mean

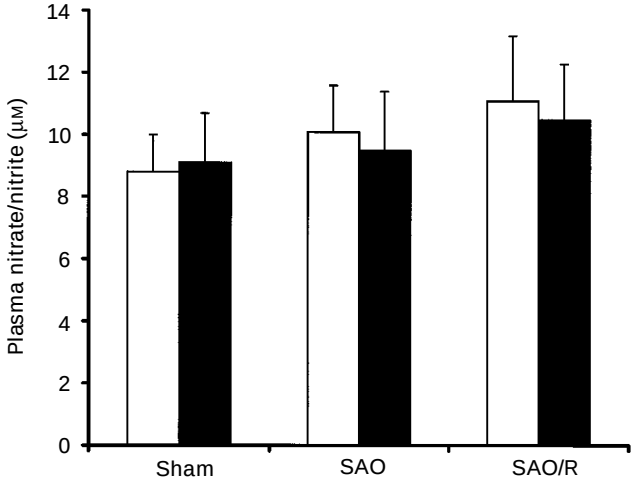

Figure 1 Plasma levels of nitrate and nitrite at 45 min after ischaemia and at 60 min after reperfusion in splanchnic artery occlusion (SAO). Effect of vehicle (open columns) and 3-aminobenzamide (solid columns). There was no change in the plasma levels of nitrate/nitrite during occlusion or 60 min of reperfusion period. Each value represents the mean ± s.e.mean for $n=8$ animals.

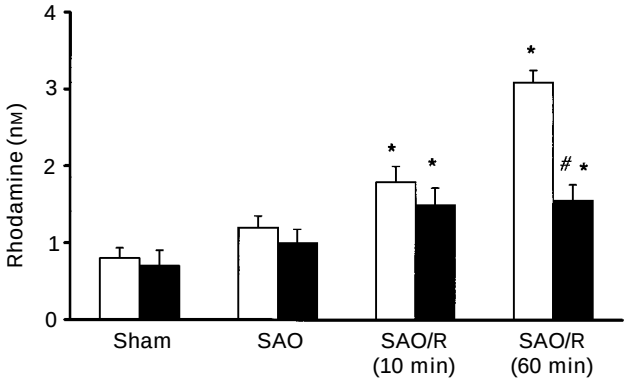

Figure 2 Plasma levels of rhodamine in splanchnic artery occlusion (SAO) and reperfusion (SAO/R): effect of vehicle (open columns) and 3-aminobenzamide (solid columns). SAO/R, but not SAO alone caused an increase in the rhodamine fluorescence of plasma at 0–10 min and 40–60 min of reperfusion period. *Represents significant increase in rhodamine fluorescence during reperfusion. ($P<0.01$); #represents significant inhibitory effect of the PARS inhibitor at the same time point. ($P<0.01$). Each value represents the mean ± s.e. mean for $n=8$ animals.

arterial blood pressure in vehicle-treated rats (Figure 7). Administration of 3-aminobenzamide alone did not change arterial blood pressure in sham rats (Figure 7). However, the administration of 3-aminobenzamide, ameliorated the SAO and reperfusion-induced fall in mean arterial blood pressure (Figure 7).

Vascular reactivity

In order to investigate whether SAO shock was associated with alterations in vascular reactivity *ex vivo*, a separate group of *ex vivo* experiments was carried out. In aortic rings from SAO shocked and reperfused animals (SAO/R), contractile responses to noradrenaline were significantly reduced, when compared to that observed in aortic rings from sham animals (Figure 8). The maximum force of contraction induced by 10 μM noradrenaline in aortic ring decreased by approximately 40% in the SAO/R rats when compared to sham rats (Figure 8).

An impairment of endothelium-dependent dilatation was also observed in aortic rings from SAO/R rats, as evidenced by a reduced relaxant effect of the acetylcholine (10 nM – 10 μM) (Figures 9 and 10).

In vivo pretreatment with 3-aminobenzamide during SAO/R improved the vascular responsiveness to noradrenaline and caused a partial improvement of the degree of the endothelial

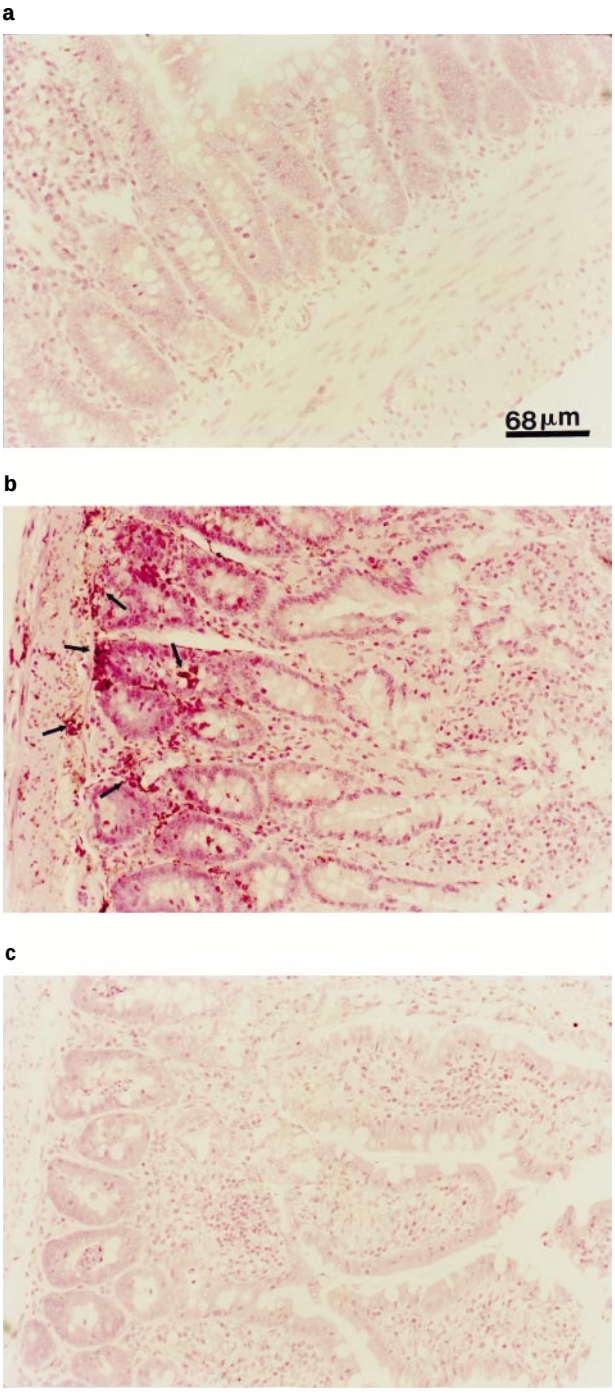

Figure 3 Immunohistochemical localization of nitrotyrosine in the distal ileum, (a) Lack of staining in control tissues; (b) SAO-shocked rat 60 min after reperfusion. Nitrotyrosine staining (dark brown staining, indicated by arrows) was localized in mononuclear cells and villus wall. (c) Immunostaining of distal ileum of a SAO-shocked rat treated with 3-aminobenzamide 60 min after reperfusion; there was a reduced staining for nitrotyrosine.

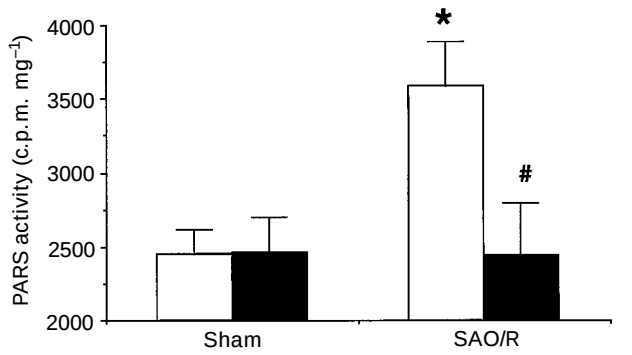

Figure 4 PARS activity in intestinal epithelial cells *ex vivo* in sham-treated animals and in rats subjected to SAO and 10 min of reperfusion: effect of *in vitro* treatment with vehicle (open columns) or 3-aminobenzamide (1 mM) (solid columns). SAO/R shock caused an increase in the PARS activity, which was inhibited by 3-aminobenzamide. *Represents significant increase in PARS activity during reperfusion. ($P<0.01$); #represents significant inhibitory effect of the PARS inhibitor at the same time point. ($P<0.01$). Each value represents the mean ± s.e.mean for $n=6$ animals.

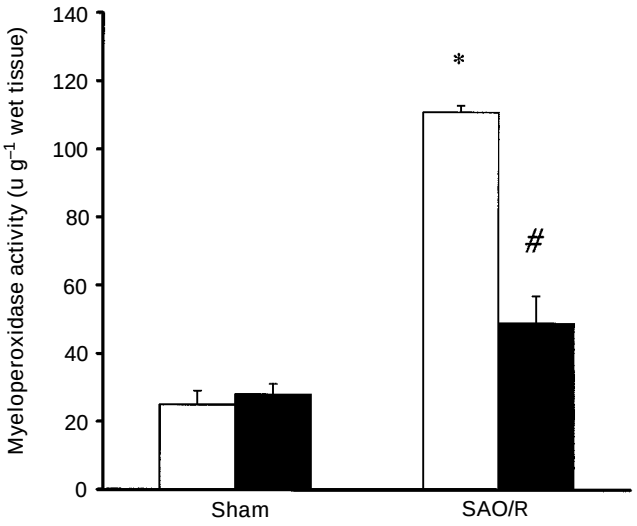

Figure 5 Myeloperoxidase (MPO) activity in the reperfused intestine of SAO shocked-rats killed at 60 min after reperfusion: effect of vehicle (open columns) and 3-aminobenzamide (solid columns). MPO activity was significantly increased in SAO shocked and reperfused rats (SAO/R) treated with vehicle. 3-Aminobenzamide treatment prevented the increase in MPO activity. *Represents significant increase in MPO activity in response to SAO/R. ($P<0.01$); #represents significant inhibitory effect of the PARS inhibitor, ($P<0.01$). Each value represent the mean ± s.e.mean for $n=8$ animals.

dysfunction (Figure 8–10). Treatment with 3-aminobenzamide did not alter the contractions to noradrenaline or the dilatation responses to acetylcholine in aortic rings from sham rats (Figures 8 and 9).

Epithelial permeability

There was a massive increase in the intestinal epithelial permeability within 10 min of reperfusion, as evidenced by a marked increase in the lumen to plasma flux of the fluorescent dye FD 4 (Figure 11). Treatment with 3-aminobenzamide reduced the increase in the epithelial permeability during reperfusion (Figure 11).

Survival rate

Table 1 presents a summary of survival rate, percentage survival and survival time for the groups of rats subjected to splanchnic artery occlusion shock or sham shock. All sham rats survived the entire 4 h observation period. In contrast, in rats treated with vehicle, splanchnic artery occlusion produced a profound shock state characterized by 100% death: no rats survived after 2 h (mean survival time 65 ± 9 min; Table 1). Treatment with 3-aminobenzamide during the reperfusion period significantly increased survival rate in SAO shocked animals (Table 1).

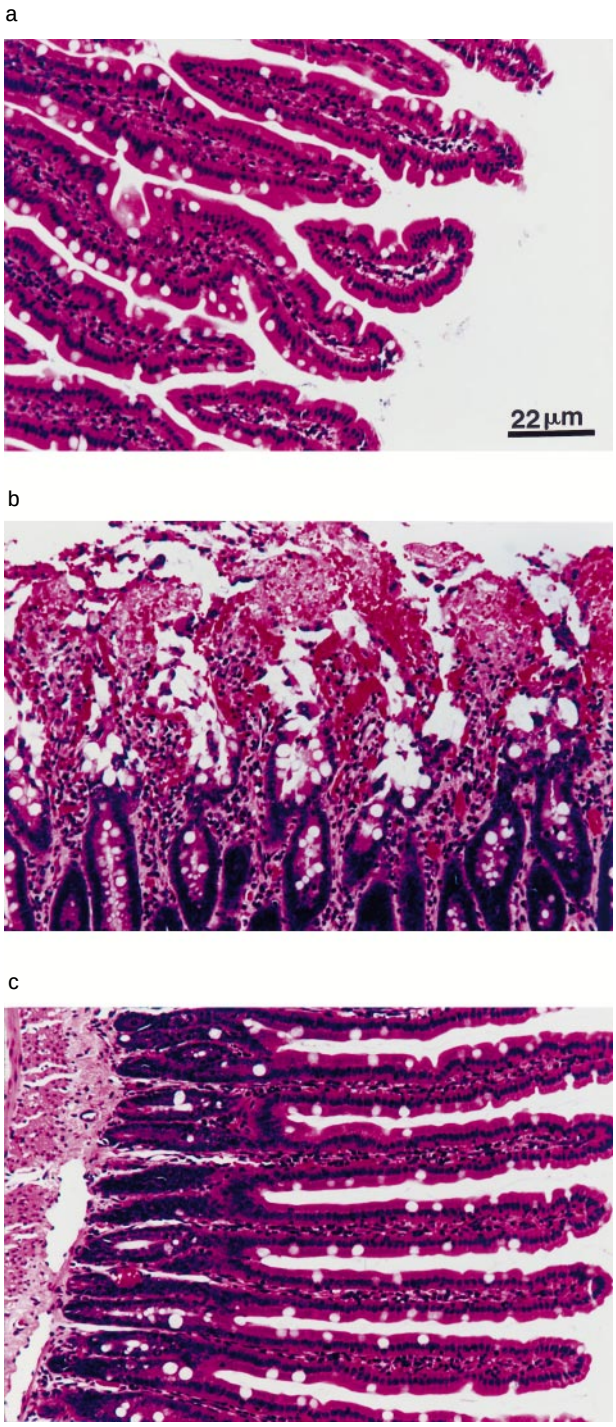

Figure 6 (a) Distal ileum section from a sham rats demonstrating the normal architecture of the intestinal epithelium and wall. (b) Distal ileum from a SAO shocked-rat reperfused for 60 min (SAO/R) demonstrating oedema of the distal portion of the villi and necrosis of the epithelium at the villus tips. (c) Distal ileum from a SAO/R rat treated with 3-aminobenzamide. Treatment with 3-aminobenzamide reduced the degree of mucosal injury.

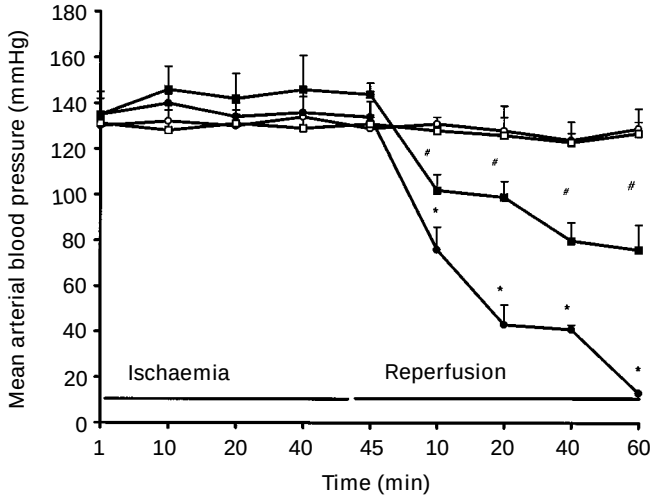

Figure 7 Effect of 3-aminobenzamide on mean arterial blood pressure (MAP, mmHg) in sham-operated rats (□) and in rats subjected to splanchnic artery occlusion (SAO) and reperfusion (■). *Represents significant fall in MAP in response to SAO/R ($P<0.01$); #represents significant protection against the fall in MAP by the PARS inhibitor ($P<0.01$). Each point represents the mean and vertical lines s.e.mean for $n=8$ animals. (○) sham + vehicle; (●) SAO/R + vehicle.

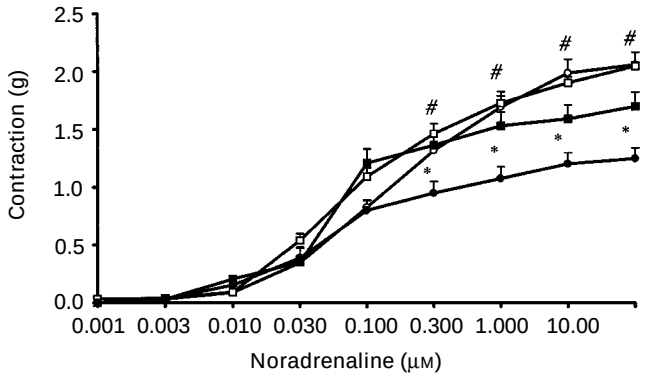

Figure 8 Cumulative dose-response curves in response to noradrenaline in aortic rings from sham-operated rats (open symbols) and splanchnic artery occlusion (SAO) shocked and reperfused rats (solid symbols) treated with vehicle (○, ●) or with 3-aminobenzamide (□, ■). *Represents significant reduction in contractility in response to SAO/R. ($P<0.01$); #represents significant protection against the vascular hyporeactivity by the PARS inhibitor ($P<0.01$). Each point represents the mean and vertical lines s.e.mean for $n=8$ rings.

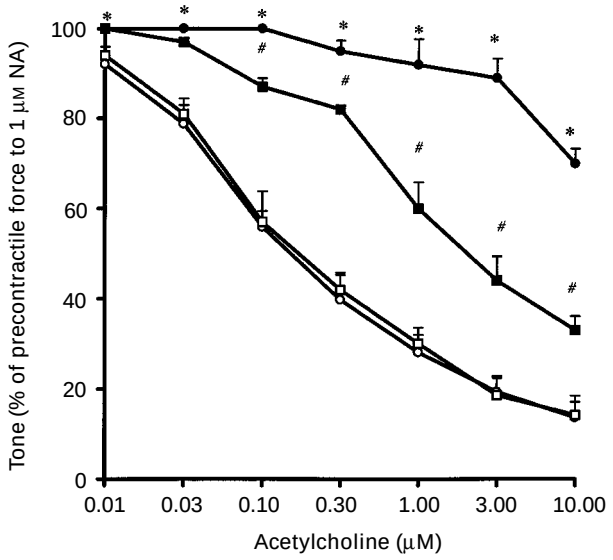

Figure 9 Relaxant effect of acetylcholine in aortic rings precontracted with noradrenaline (NA, 1 μM) of sham-operated rats (open symbols) and splanchnic artery occlusion (SAO) shocked rats (solid symbols) treated with vehicle (○, ●) or with 3-aminobenzamide (□, ■). *Represents significant impairment of the relaxations in response to SAO/R. ($P<0.01$); #represents significant protection against the endothelial dysfunction by the PARS inhibitor ($P<0.01$). Each point represents the mean and vertical lines s.e.mean for $n=8$ rings.

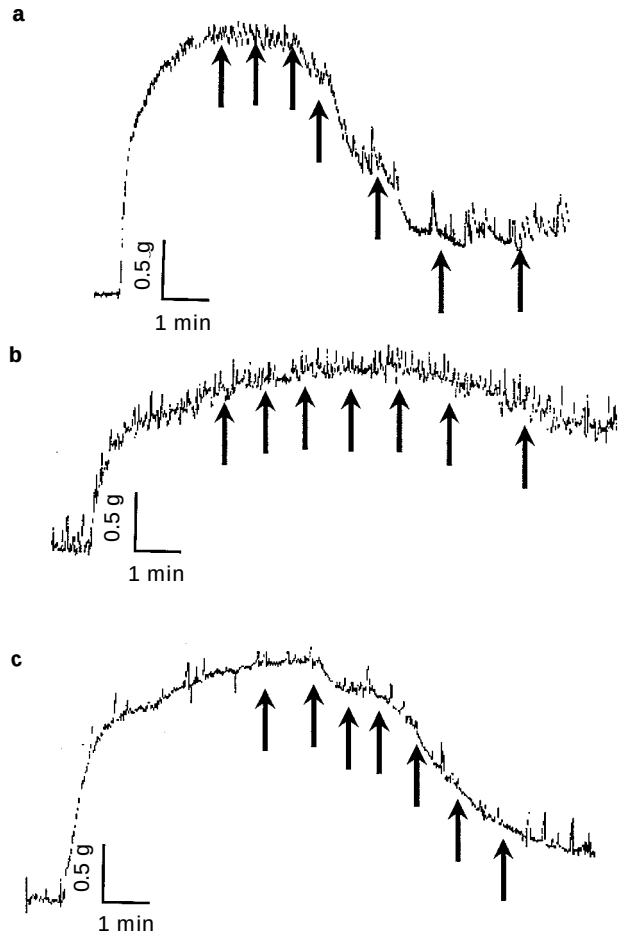

Figure 10 Typical recordings of the vasorelaxant responses of aortic rings (precontracted with noradrenaline, 1 μM) to acetylcholine (10 nM to 10 μM). Arrows represent additions of the following concentrations of acetylcholine (from left to right): 10 nM, 30 mM, 100 nM, 300 nM, 1 μM, 3 μM and 10 μM, respectively. (a) Sham + vehicle; (b) SAO + vehicle; (c) SAO + 3-aminobenzamide.

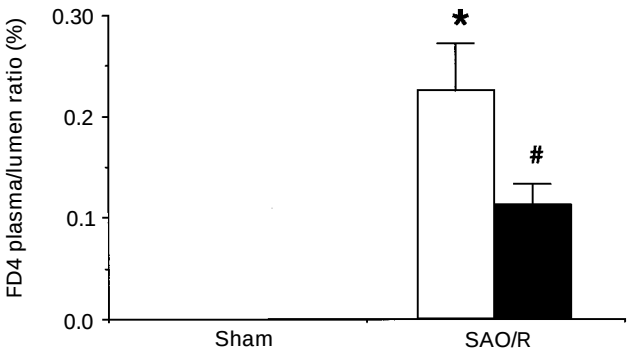

Figure 11 Plasma/lumen ratio of FD4, an indicator of intestinal epithelial permeability, in the reperfused intestine of SAO shocked-rats killed 10 min after reperfusion. Epithelial permeability significantly increased in SAO shocked-rats treated with vehicle (open column). 3-aminobenzamide treatment (solid column) reduced the increase in permeability. *Represents significant increase in permeability in response to SAO/R. ($P<0.01$); #represents significant protection against the intestinal hyperpermeability by the PARS inhibitor ($P<0.01$). Each value is the mean ± s.e.mean for $n=6-8$ animals.

Discussion

Peroxynitrite production in SAO shock

In ischaemia and reperfusion injury, superoxide, produced in the reperfusion phase, rapidly reacts with NO and forms peroxynitrite. Multiple lines of experimental data strongly imply the production of peroxynitrite in the reperfused heart (Matheis *et al*., 1995; Schulz & Wambolt, 1995; Naseem *et al*., 1995; Wang & Zweier, 1996), liver (Ma *et al*., 1995), intestine (Szabó *et al*., 1995) and lung (Ischiropoulos *et al*., 1995; Kooy *et al*., 1995). In these conditions, prevention of peroxynitrite generation markedly reduces reperfusion injury. The present results, demonstrating the increase in dihydrorhodamine 123 oxidation in the plasma and the increased nitrotyrosine immunoreactivity in the reperfused intestine, suggest that peroxynitrite is also produced in the reperfusion phase of splanchnic artery occlusion shock. The lack of oxidation of dihydrorhodamine 123 during ischaemia, and a marked increase in the oxidation after reperfusion suggests that the majority of free radical and oxidant production occurs during the reperfusion.

Peroxynitrite is formed from NO and superoxide. During intestinal ischaemia and reperfusion, mucosal xanthine oxidase, and NADPH oxidase in infiltrating PMNs are sources of superoxide production (Granger *et al*., 1981; Fantone & Ward, 1982; Zimmermann *et al*., 1993). The sources of NO, under the present conditions, must be the constitutive NO synthases, since we did not find evidence for the expression of inducible NOS (iNOS) and related overproduction of NO, during the relatively short time of reperfusion (1 h). An alternative source of NO may be tissue nitrite, which can be reduced to NO in acidotic tissues (Zweier *et al*., 1995).

Since hydroxyl radical and peroxynitrite are potent triggers of DNA strand breakage and subsequent activation of PARS (see: Introduction), in the present study we investigated the effect of the PARS inhibitor 3-aminobenzamide on the course of the pathophysiological alterations triggered by SAO shock. 3-Aminobenzamide is not an inhibitor of NOS and does not scavenge peroxynitrite or superoxide (Zingarelli *et al*., 1996). We have demonstrated a number of pathophysiological alterations in our model of SAO shock, including hypotension, vascular hypocontractility, endothelial dysfunction, epithelial hyperpermeability, massive morphological changes of the reperfused intestine and significant death. Further, we have demonstrated an increase in PARS activity in the reperfusion phase of SAO shock in the intestinal epithelium and showed that 3-aminobenzamide provides a marked protection against

Table 1 Effect of vehicle or 3-AB on survival rate, percentage survival, and survival time in sham shocked rats or splanchnic artery occlusion (SAO) shocked rats

	Time after reperfusion (h)				
	2		*4*		*Survival*
Treatment	*Surviving*	*%*	*Surviving*	*%*	*time* (min)
Sham + vehicle	10/10	100	10/10	100	> 240
Sham + 3-AB	10/10	100	10/10	100	> 240
SAO + vehicle	0/10	0	0/10	0	65 ± 9
SAO + 3-AB	10/10	100	10/10	100*	> 240*

Animals received 3-aminobenzamide (3-AB, 10 mg kg^{-1}, i.v., 10 min before reperfusion followed by 10 mg kg^{-1} h^{-1} for all the reperfusion period) or an equal volume of vehicle (0.9% NaCl solution). *$P < 0.01$ vs SAO + vehicle.

the pathophysiological alterations associated with SAO/R. These data suggest that PARS activation due to oxidant-related DNA single strand breakage contributes to the pathophysiology of SAO shock.

Vascular failure in SAO shock: role of PARS

Splanchnic artery occlusion shock leads to the development of hypotension and failure of the vasculature to respond to vasoconstrictor stimuli (Carey *et al*., 1992). The reduction in the contractile ability, as demonstrated in the thoracic aorta, is related to the production of NO, since normal contractility of the vessels can be restored with inhibition of NOS (Squadrito *et al*., 1994). In agreement with direct measurements by Kanwar *et al*., (1994), no increase in plasma nitrite/nitrate concentrations was found at 1 h reperfusion after SAO. Therefore, and similar to other models of early reperfusion injury (Ischiropoulos *et al*., 1995; Wang & Zweier, 1996), constitutive sources must provide the NO for the generation of peroxynitrite in our experiments. The current vascular changes may be similar to the changes occurring in thoracic aortic rings exposed to peroxynitrite *in vitro*, where the development of vascular hyporeactivity within 30 min – 1 h can be ameliorated by 3-aminobenzamide (Szabó *et al*., 1996b).

Another important component of splanchnic artery occlusion (SAO) shock is endothelial dysfunction (Altura *et al*., 1985; Carey *et al*., 1992; Zingarelli *et al*., 1992; Lefer & Lefer, 1993). The development of endothelial dysfunction was originally attributed to oxygen-derived free radicals released from both the reperfused endothelium (Ratych *et al*., 1987; Lefer & Lefer, 1993) and from activated adherent PMNs (Granger *et al*., 1981; McCord, 1981; Mullane *et al*., 1988; Bitterman *et al*., 1988). The endothelial dysfunction (i.e. a deficit of endothelial NO production) predisposes to vasospasm, platelet deposition and increased neutrophil adherence (thereby leading to a positive feedback cycle), which exacerbates the shock state. Recent evidence suggests that the ischaemia-reperfusion induced endothelial dysfunction may be, in fact, related to peroxynitrite production (Villa *et al*., 1994; Az-ma *et al*., 1996; Szabó, 1996a; Zingarelli *et al*., 1997a). PARS inhibitors have been shown to protect endothelial cells against hydrogen peroxide (Junod *et al*., 1989; Kirkland, 1991; Thies & Autor, 1991; Aalto & Raivio, 1993) and peroxynitrite-treated (Szabó *et al*., 1997) induced endothelial injury. The present data suggest that DNA injury and activation of PARS plays a role in the endothelial injury associated with ischaemia-reperfusion.

Epithelial hyperpermeability in SAO: role of PARS

It has been recently demonstrated that large amounts of NO induce an increase in paracellular permeability in intestinal epithelial cells *in vitro* (Salzman *et al*., 1995). Moreover, in immunostimulated intestinal epithelial cells, the increase in epithelial permeability was diminished by inhibitors of NOS (Unno *et al*., 1996). We have recently demonstrated PARS activation and consequent hyperpermeability in peroxynitrite-treated A549 human pulmonary epithelial cells (Szabó *et al*., 1996c). PARS inhibitors also protect against the acute cellular injury in intestinal epithelial cells exposed to hydrogen peroxide (Watson *et al*., 1995). Our present data extend these previous observations by demonstrating that SAO/R increases PARS activity in intestinal epithelial cells *ex vivo* and that the reperfusion injury-associated increase in intestinal epithelial permeability is attenuated by pharmacological inhibition of PARS. Based on the present data, we suggest that inhibition of PARS represents a novel pharmacological way for the prevention of oxidant-induced epithelial dysfunction *in vivo*.

Neutrophil infiltration and histological changes in SAO: role of PARS

Activation and accumulation of polymorphonuclear cells (PMNs) is one of the initial events of tissue injury which triggers the release of oxygen free radicals, arachidonic acid metabolites and lysosomal proteases, with subsequent tissue injury (Fantone & Ward, 1982). In our study, increased activity of myeloperoxidase, an enzyme specific to granulocyte lysosomes, (a parameter directly related to the absolute number of PMN cells), correlated well with morphological alterations in the small intestine at histological examination. Inhibition of PARS by 3-aminobenzamide prevented the PMN infiltration into the small intestine, as demonstrated by a significant reduction in MPO activity. This effect of 3-aminobenzamide was also observed histologically, since leukocyte infiltration into the tissues was reduced. Since NO is a potent inhibitor of both neutrophil aggregation and adherence (Kanwar & Kubes, 1995), we propose that the improvement of endothelial function by 3-aminobenzamide (see above) reduced the infiltration of neutrophils during reperfusion, thus, resulting in reduced tissue injury. Thus, the following positive feedback cycle may be present in SAO shock: early hydroxyl radical and peroxynitrite production >> PARS-related endothelial injury >> PMN infiltration >> more hydroxyl and peroxynitrite production. Inhibition of PARS would intercept this cycle at the level of endothelial injury. This hypothesis (which remains to be confirmed by direct measurements) would explain the reduction by 3-aminobenzamide of the dihydrorhodamine oxidation during the delayed, but not the early phase of reperfusion.

PARS activation: a novel pathway of shock and inflammation

In conclusion, the present study demonstrated marked protective effects with the PARS inhibitor 3-aminobenzamide in a SAO model of shock. The present data, coupled with other recent *in vitro* and *in vivo* observations (Szabó *et al*., 1996a, b; Thiemermann *et al*., 1997; Zingarelli *et al*., 1997b) support the view that activation of PARS, triggered by DNA single strand breakage, is an important novel mechanism of cellular injury during various forms of shock and reperfusion injury. Treatment with PARS inhibitors may represent a novel therapeutic strategy in the treatment of shock and reperfusion injury. The viability of this potential therapeutic strategy is also strengthened by recent findings in $PARS^{-/-}$ mice, which appear normal, and suggest that the presence of PARS is not obligatory for normal DNA repair (Wang *et al*., 1995).

Moreover, PARS inhibition is unlikely to interfere with the important antimicrobial effects of NO (Green & Nacy, 1993), since invading bacteria do not contain PARS. We propose that the PARS pathway may be an expendable pathway which could be targeted with pharmacological inhibitors for the experimental therapy of various forms of circulatory shock.

Abbreviations

NO, nitric oxide; NOS, nitric oxide synthase; ecNOS, constitutive endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; SAO, splanchnic artery occlusion; SAO/R, splanchnic artery occlusion and reperfusion; PARS, poly (ADP-ribose) synthetase; MPO, myeloperoxidase; PMN, polymorphonuclear cells.

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