

Effects of *n*-acetylcysteine in a rat model of ischemia and reperfusion injury

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Abstract

Objective: Splanchnic artery occlusion shock (SAO) causes an enhanced formation of reactive oxygen species (ROS), which contribute to the pathophysiology of shock. Here we have investigated the effects of *n*-acetylcysteine (NAC), a free radical scavenger, in rats subjected to SAO shock. **Methods and results:** Treatment of rats with NAC (applied at 20 mg/kg, 5 min prior to reperfusion, followed by an infusion of 20 mg/kg/h) attenuated the mean arterial blood and the migration of polymorphonuclear cells (PMNs) caused by SAO-shock. NAC also attenuated the ileum injury (histology) as well as the increase in the tissue levels of myeloperoxidase (MPO) and malondialdehyde (MDA) caused by SAO shock in the ileum. There was a marked increase in the oxidation of dihydrorhodamine 123 to rhodamine in the plasma of the SAO-shocked rats after reperfusion. Immunohistochemical analysis for nitrotyrosine and for poly (ADP-ribose) synthetase (PARS) revealed a positive staining in ileum from SAO-shocked rats. The degree of staining for nitrotyrosine and PARS were markedly reduced in tissue sections obtained from SAO-shocked rats which had received NAC. Reperfused ileum tissue sections from SAO-shocked rats showed positive staining for P-selectin, which was mainly localised in the vascular endothelial cells. Ileum tissue section obtained from SAO-shocked rats with anti-intercellular adhesion molecule (ICAM-1) antibody showed a diffuse staining. NAC treatment markedly reduced the intensity and degree of P-selectin and ICAM-1 in tissue section from SAO-shocked rats. 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). NAC treatment improved contractile responsiveness to noradrenaline, enhanced the endothelium-dependent relaxations and significantly improved survival. **Conclusion:** Taken together, our results clearly demonstrate that NAC treatment exert a protective effect and part of this effect may be due to inhibition of the expression of adhesion molecule and peroxynitrite-related pathways and subsequent reduction of neutrophil-mediated cellular injury. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Arteries; Free radicals; Shock

1. Introduction

Splanchnic artery occlusion shock (SAO) is an ex-

perimental type of circulatory shock, which is the consequence of a prolonged ischemia of the splanchnic region. This model of shock is characterised by a marked decrease in systemic blood pressure and leukopenia [1,2] as well as by disturbances in reticulo-endothelial system activity [3], increased macrophage and plasma levels of thromboxane B2 [4] and elevated plasma levels of platelet-activating factor [5]. Other important characteristics of the SAO

Abbreviations: ICAM-1, intercellular adhesion molecule; iNOS, inducible nitric oxide synthase; NAC, *n*-acetylcysteine; MDA, malonaldehyde; MPO, myeloperoxidase; NO, nitric oxide; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocyte; SAO, splanchnic artery occlusion

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shock are: local splanchnic release of lysosomal hydrolases, enhanced proteolysis, hemoconcentration, intestinal injury and the production of cardiotoxic substances [1,2,6,7]. Recently it has been shown that polymorphonuclear leukocytes (PMN) play a key role in the pathogenesis of SAO shock [8,9]. PMNs moving out of the circulation into inflamed tissue play a crucial role in the destruction of foreign antigens and in the breakdown and remodelling of injured tissue [8]. PMNs can also be directly injurious to tissue during ischemia-reperfusion [10]. Leukocyte-endothelial interaction involves a complex interplay among adhesion glycoproteins (i.e. integrins, immunoglobulin superfamily members and selectins). One member of the selectin family, P-selectin, is rapidly translocated from the Weibel-Palade bodies to the endothelial cell surface upon endothelial cell activation with thrombin, histamine, hypoxia-reoxygenation, or oxygen-derived free radicals [11,12]. Leukocyte rolling is the first step in leukocyte-endothelial interaction and facilitates PMNs activation and adherence [12,13]. However, leukocyte accumulation is a complex phenomenon, which also involves endothelium-based adhesion molecule. In fact, endothelial adhesion molecules are considered to play a pivotal role in the localisation and development of an inflammatory reaction [14]. Intercellular adhesion molecule (ICAM-1) is an adhesion molecule normally expressed at a low basal level, but its expression can be enhanced by various inflammatory mediators such as interleukin-1 (IL-1) and TNF- α [15].

Recent work has suggested that peroxynitrite (ONOO⁻), a toxic oxidant formed from the reaction of NO and superoxide, can be present in the reperfused heart [16,17], liver [18], kidney [19], intestine [9,20], brain [21], and lung [22]. The biological activity and decomposition of peroxynitrite is very much dependent on the cellular or chemical environment (presence of proteins, thiols, glucose, the ratio of NO and superoxide, carbon dioxide levels and other factors), and these factors influence its toxic potential [23].

Oxygen radical scavengers, administered before or at the onset of sepsis, were shown to improve the survival in animal models of sepsis [24]. NAC has antioxidant property [25] and as a sulfhydryl donor, may contribute to the regeneration of endothelium-derived relaxing factor and glutathione [26]. Increasing evidence indicates that the action of NAC is pertinent to microcirculatory blood flow and tissue oxygenation. NAC was shown to enhance oxygen consumption via increased oxygen extraction in patients 18 h after the onset of fulminant liver failure [26]. It was speculated that NAC could also exert beneficial effects on impaired nutritive blood flow in patients with severe sepsis [26].

In present study, we examined the protective effect of NAC against oxidative stress during reperfusion of the splanchnic region after induced ischemia using both biochemical and morphological parameters as follows: the levels of lipid peroxidation, the number of PMNs that

infiltrate the oxydatively damaged region of the intestine were detected.

2. Methods

2.1. Animals

Male Sprague-Dawley rats (300-350 g; Charles River; Milan; Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (D.M. 116192), as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

2.2. Surgical procedures

Male Sprague-Dawley rats weighing 250-300 g were allowed access to food and water ad libitum. Following anaesthesia, a catheter was placed in the jugular vein as described previously [27]. The rats were anaesthetised with sodium pentobarbital (45 mg/kg, i.p.). 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 celiac 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 stabilisation period before either splanchnic ischemia or sham ischemia. Clamping both the superior mesenteric artery and the celiac trunk, resulting in a total occlusion of these arteries for 45 min induced SAO shock. After this period of occlusion, the clamps were removed. In one study, the various groups of rats were sacrificed 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.

2.3. Experimental groups

In the treated group of animals, NAC was given as a intravenous bolus 5 min before reperfusion (20 mg/kg) followed by infusion of 20 mg/kg/h during the period of reperfusion (SAO+NAC group). In a vehicle-treated group of rats, vehicle (saline) was given instead of NAC (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 NAC (dose as above) (Sham+NAC).

2.4. Measurement of nitrite/nitrate in the plasma

Nitrite/nitrate production, an indicator of NO synthesis, was measured in plasma samples from sham or SAO-shocked rats at 60 min after reperfusion as previously described [9]. First, nitrate in the plasma was reduced to nitrite by incubation with nitrate reductase (670 mU/ml) and NADPH (160 μ M) at room temperature for 3 h. After 3 h, nitrite concentration in the samples was measured by the Griess reaction, by adding 100 μ l of Griess reagent (0.1% naphthalethylenediamine dihydrochloride in H₂O and 1% sulphanilamide in 5% concentrated H₃PO₄; 1:1, v/v) to 100 μ l samples. The optical density at 550 nm (OD₅₅₀) was measured using a Spectramax 250 microplate reader (Molecular Devices Sunnyvale, CA). Nitrate concentrations were calculated by comparison with OD₅₅₀ of standard solutions of sodium nitrate prepared in saline solution.

2.5. Measurement of oxidation of dihydrorhodamine 123 to rhodamine 123

The oxidation of dihydrorhodamine 123 to rhodamine is partially peroxynitrite-dependent as previously described [9,22]. In separate groups, animals were injected with dihydrorhodamine 123 (2 μ mol/kg in 0.3 ml saline, i.v.) 25 min after ischemia or 40 min after reperfusion. Twenty minutes later, rats were sacrificed and plasma samples taken for rhodamine fluorescence evaluation using 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 rate of rhodamine formation, an index of peroxynitrite production, was calculated using a standard curve obtained with authentic rhodamine (1–30 nM) prepared in plasma obtained from untreated rats. Background plasma fluorescence was subtracted from all samples.

2.6. Immunofluorescence localisation for nitrotyrosine and for PARS

Indirect immunofluorescence staining was performed on 7- μ m thick sections of unfixed rat ileum. Sections were cut in with a Slee & London cryostat at -30°C , transferred onto clean glass slides and dried overnight at RT. Sections were permeabilized with acetone at -20°C for 10 min and rehydrated in PBS (phosphate-buffered saline, 150 mM NaCl, 20 mM sodium phosphate pH 7.2) at RT for 45 min. Sections were co-incubated overnight with anti-nitrotyrosine rabbit polyclonal antibody (1:500 in PBS, v/v) or with anti-poly (ADP-Ribose) goat polyclonal antibody rat (1:500 in PBS, v/v) (DBA, Milan, Italy). Sections were

washed with PBS, and co-incubated with secondary antibody (TRITC-conjugated anti-goat and with FITC-conjugated anti-rabbit (Jackson, West Grove, PA) antibody (1:80 in PBS, v/v) for 2 h at RT. Sections were washed as before, mounted with 90% glycerol in PBS, and observed with a Nikon RCM8000 confocal microscope equipped with a 40 \times oil objective.

2.7. Immunofluorescence localisation for P-selectin and for ICAM-1

Indirect immunofluorescence staining was performed on 7- μ m thick sections of unfixed rat ileum. Sections were cut in with a Slee & London cryostat at -30°C , transferred onto clean glass slides and dried overnight at RT. Sections were permeabilized with acetone at -20°C for 10 min and rehydrated in PBS (phosphate-buffered saline, 150 mM NaCl, 20 mM sodium phosphate pH 7.2) at RT for 45 min. Sections were co-incubated overnight with rabbit anti-human polyclonal antibody directed at P-selectin (CD62 P) which react with rat and with mouse anti-rat antibody directed at ICAM-1 (CD54) (1:500 in PBS, v/v) (DBA, Milan, Italy). Sections were washed with PBS, and co-incubated with secondary antibody (TRITC-conjugated anti-rabbit and with FITC-conjugated anti-mouse (Jackson, West Grove, PA) antibody (1:80 in PBS, v/v) for 2 h at RT. Sections were washed as before, mounted with 90% glycerol in PBS, and observed with a Nikon RCM8000 confocal microscope equipped with a 40 \times oil objective.

2.8. Measurement of vascular reactivity *ex vivo*

Animals were sacrificed under anaesthesia at 60 min after the start of reperfusion. Thoracic descending aortas were immediately excised, cut into rings and mounted in organ baths (5 ml) filled with warmer (37°C), oxygenated (95% O₂/5% CO₂) Krebs' solution (pH 7.4) consisting of (mM): NaCl 118, KCl 4.7, KH₂PO₄ 1.2, CaCl₂ 2.5, MgSO₄ 1.2, NaHCO₃ 25, glucose 11.7 in presence of indomethacin (10 μ M). Isometric force was measured with isometric transducers (Kent Scientific Corp.) digitised using 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. 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 μ M) 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 μ M) in aortic rings precontracted with noradrenaline (1 μ M). Relaxation was calculated as % of precontractile vascular tone.

2.9. Myeloperoxidase activity

Myeloperoxidase activity, an index of PMN accumulation, was determined as previously described [28]. Intestinal tissues, collected 60 min after reperfusion, were homogenised in a solution containing 0.5% hexa-decyltrimethyl-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-methylbenzidine (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 peroxide min at 37°C and was expressed in microunits per gram weight of wet tissue.

2.10. Leukocyte count

Tail vein blood samples for leukocyte count were taken at 60 min after reperfusion. The number of leukocytes ($\text{WBC}\times 10^3/\text{mm}^3$) is shown as mean \pm S.D.

2.11. Malonaldehyde (MDA) measurement

Levels of malonaldehyde (MDA) in the intestinal tissues was determined as an index of lipid peroxidation, as described by Okhawa et al. [29]. Intestinal tissues, collected 60 min after reperfusion, were homogenised in 1.15% KCl solution. An aliquot (100 μl) of the homogenate was added to a reaction mixture containing 200 μl of 8.1% SDS, 1500 μl of 20% acetic acid (pH 3.5), 1500 μl of 0.8% thiobarbituric acid and 700 μl distilled water. Samples were then boiled for 1 h at 95°C and centrifuged at $3000\times g$ for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 650 nm.

2.12. Light microscopy

For histopathological examination, biopsies of small intestine were taken 60 min after reperfusion. The tissue were fixed in Dietric solution (14.25% ethanol, 1.85% formaldehyde, 1% acetic acid) for 1 week at room-temperature, dehydrated by graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ). From each biopsy, 7- μm thick were obtained and stained with hematoxylin and eosin to evaluate intestine morphology. The tissue slices were observed with a Dialux 22 Leitz microscope.

2.13. 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.

2.14. Materials

Zambon Italia, Bresso (MI), Italy supplied *n*-acetylcysteine. Biotin-blocking kit, biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex were obtained from Vector Laboratories (Burlingame, CA, USA). Primary anti-nitrotyrosine antibody was purchased from Upstate Biotech (Saranac Lake, NY). Primary P-selectin (CD62 P) and ICAM-1 (CD54) were purchased from Pharmingen (DBA, Milan, Italy). Dihydrorhodamine 123 and rhodamine 123 were purchased from Molecular Probes (Eugene OR). All other reagents and compounds used were purchased from Sigma Chemical Company (St. Louis, MO).

2.15. Data analysis

All values in the figures and text are expressed as mean \pm standard error of the mean of *n* observations, where *n* represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments performed on different experimental days. Data sets were examined by one- and two-way analysis of variance and individual group means were then compared with Student's unpaired *t*-test. Non-parametric data were analysed with the Fisher's exact test. A *P*-value less than 0.05 was considered significant.

3. Results

3.1. Arterial blood pressure

Occlusion of the splanchnic arteries for 45 min did not induce a marked change in mean arterial blood pressure (Fig. 1). Upon release of the occlusion, there was a gradual fall in mean arterial blood pressure in vehicle-treated rats (Fig. 1). Administration of NAC alone did not change arterial blood pressure in sham rats (Fig. 1). The administration of NAC, however, attenuated the SAO and reperfusion-induced fall in mean arterial blood pressure (Fig. 1).

3.2. NO and peroxynitrite production in splanchnic artery occlusion shock

There was no change in the plasma levels of nitrate/nitrite during occlusion or 60 min of the reperfusion period (Fig. 2A), in agreement with previous observations suggesting that the current protocol of ischemia and reperfusion does not trigger the expression of the inducible isoform of NOS (iNOS) [9,20]. The NAC treatment did not affect baseline nitrite/nitrate levels (Fig. 2A).

In agreement with previous observations [9,20], SAO shock caused a significant increase in the rhodamine

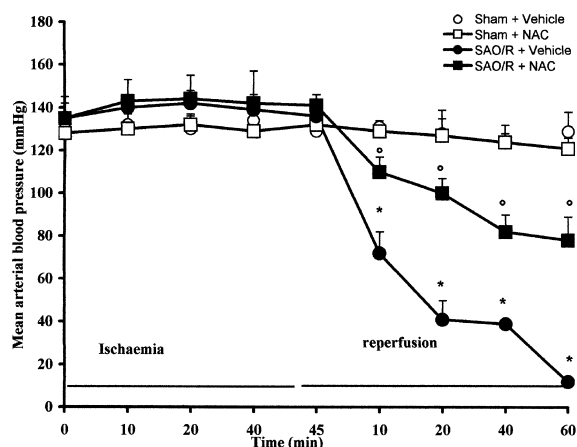


Fig. 1. Effect of *n*-acetylcysteine on mean arterial blood pressure in rats subjected to splanchnic artery occlusion (SAO) shock. Upon release of the occlusion, there was a gradual fall in mean arterial blood pressure in vehicle-treated rats. NAC treatment ameliorated the SAO and reperfusion-induced fall in mean arterial blood pressure. Values are means \pm S.E.M. for $n=8$ animals. * $P<0.01$ vs. sham; $^{\circ}P<0.01$ vs. SAO.

fluorescence of plasma, partially indicative of peroxynitrite-induced oxidation of dihydrorhodamine during the reperfusion phase, but not during the occlusion period (Fig. 2B). In vivo treatment with NAC reduced the oxidation of dihydrorhodamine 123 during reperfusion (Fig. 2B).

At 60 min after reperfusion ileum sections were taken from sham or shocked rats in order to determine the immunohistological staining for nitrotyrosine and for PARS. While there was negligible staining in the intestinal sections of control animals (data not shown), immunohistochemical analysis, using a specific anti-nitrotyrosine antibody, and a specific anti-PARS antibody revealed a positive staining in the vascular wall (large arrow) and in the inflammatory cells (small arrows) located in the mucosae of the ileum from SAO-shocked rats (Fig. 3A,B). As is possible see in the Fig. 3C (staining combination of panel A and B) the positive staining for nitrotyrosine and for PARS are co-localised in the vascular wall and in the inflammatory cells. NAC treatment reduced the degree of immunostaining for nitrotyrosine (Fig. 3D) and for PARS (Fig. 3E) in the reperfused intestine.

3.3. Malonaldehyde and myeloperoxidase activities in the reperfused intestine

At 60 min after reperfusion, small intestine was investigated for MPO activity, indicative of neutrophil infiltration, and MDA levels, indicative of lipid peroxidation. As shown in Fig. 4, MPO activity and MDA levels were significantly increased in the ileum of SAO-shocked rats ($P<0.01$). In vivo treatment with NAC reduced the MPO activity and MDA levels (Fig. 4).

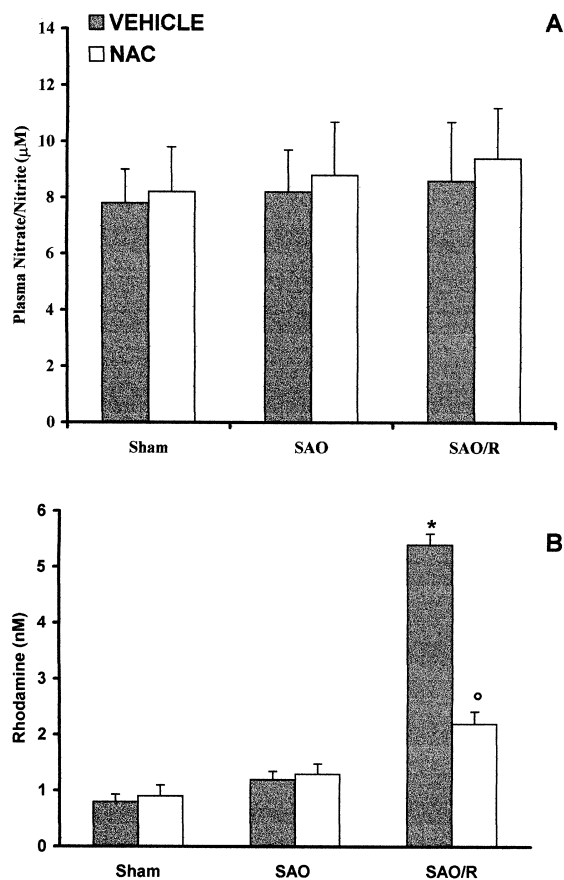


Fig. 2. Plasma nitrate/nitrite (NO_x) levels and peroxynitrite production at 45 min after ischemia and at 60 min after reperfusion in splanchnic artery occlusion (SAO). Plasma NO_x levels (A); plasma peroxynitrite production assessed by oxidation of dihydrorhodamine 123 to rhodamine (B). There was no change in the plasma levels of nitrate/nitrite during occlusion or 60 min of reperfusion period. Peroxynitrite production in the SAO-shocked rats was significantly increased versus sham group. NAC-treated rats show a significant reduction of the SAO-induced elevation of the plasma peroxynitrite production. Values are the means \pm S.E.M. of ten rats for each group. * $P<0.01$ versus vehicle. $^{\circ}P<0.01$ versus SAO.

3.4. Leukocytes count

The administration of NAC did not modify the leukocyte count in sham-shocked rats. In contrast, SAO shock produced a marked leukopenia. Our data show that the leukocyte count was markedly decreased at 60 min after reperfusion. The administration of NAC significantly ameliorated leukopenia (Table 1).

3.5. Immunohistochemical localisation of P-selectin and ICAM-1 in the reperfused intestine

Staining of ileum tissue sections obtained from sham-operated rats with anti-ICAM-1 antibody showed a specific staining along vessels (arrows), demonstrating that ICAM-1 is constitutively expressed (Fig. 5A). After 1 h of reperfusion, the staining intensity substantially increased

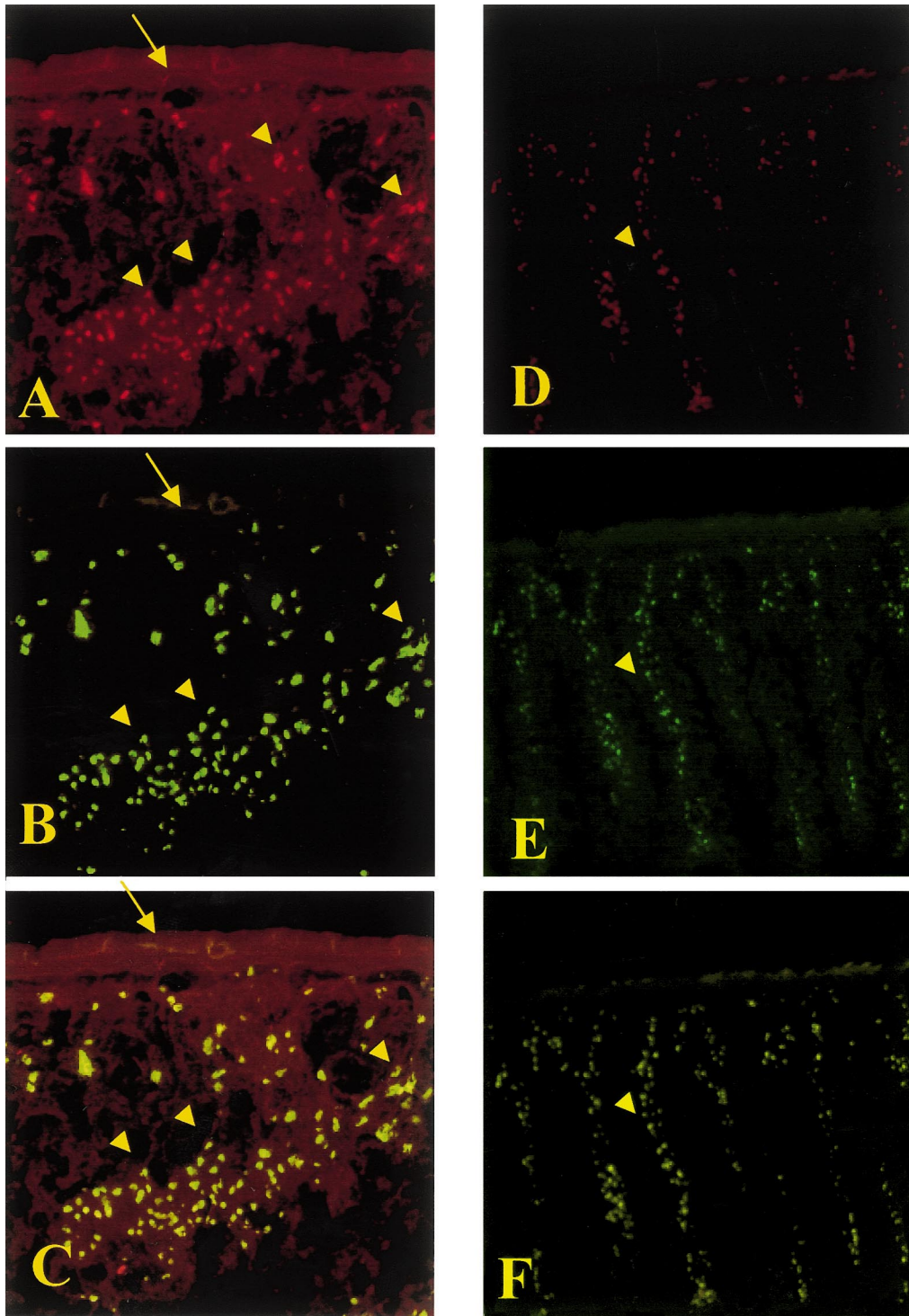


Fig. 3. Immunohistochemical localisation for nitrotyrosine (A) and for PARS (B) show positive staining localised in the vascular wall (large arrow) and in the inflammatory cells (small arrows) in the injured area from a SAO-shocked rats. The intensity of the positive staining for nitrotyrosine (D) and for PARS (E) was significantly reduced in the ileum from NAC-treated rats. C and D represent the staining combination of panel A-B and C-D, respectively. Original magnification: $\times 100$. Figure is representative of at least three experiments performed on different experimental days.

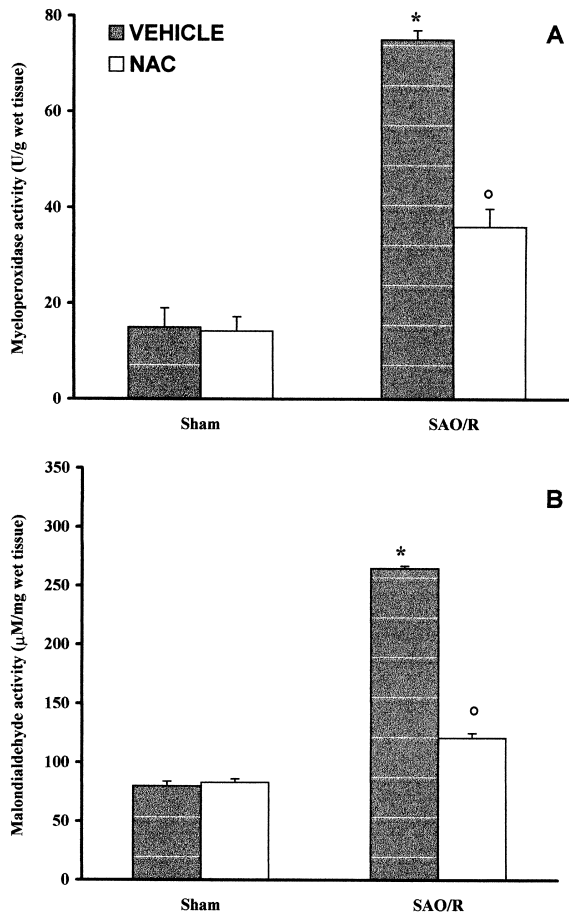


Fig. 4. SAO-induced neutrophil infiltration and lipid peroxidation. Myeloperoxidase (MPO) activity (A) and malondialdehyde (MDA) (B) in the reperfused intestine of SAO-shocked rats sacrificed at 60 min after reperfusion. MPO activity and MDA levels were significantly increased in SAO-shocked rats in comparison to sham. NAC-treated rats show a significant reduction of MPO activity and MDA levels. Values are the means \pm S.E.M. for $n=10$. * $P<0.01$ versus vehicle. ° $P<0.01$ versus SAO.

along the vessels (Fig. 4D; see arrows). Section from NAC-treated rats did not reveal any upregulation of the constitutive ICAM-1, which was normally expressed in the endothelium along the vascular wall (Fig. 5G, see arrows). Ileum tissue section obtained from at SAO-shocked rats

Table 1
Effect of vehicle or *n*-acetylcysteine (NAC) on white cell blood count (WBC) of rats subjected to splanchnic artery occlusion (SAO) shock^a

	Time (min)	
	Basal 0	Reperfusion 60
Sham+vehicle	7.8 \pm 1.5	7.6 \pm 1.5
Sham+NAC	6.5 \pm 0.9	8.2 \pm 1.3
SAO+vehicle	8 \pm 1	3.6 \pm 0.6
SAO+NAC	7.5 \pm 0.8	7.1 \pm 0.5*

^a Animals received *n*-acetylcysteine (20 mg/kg i.v. 5 min before reperfusion followed by 20 mg/kg/h for all the reperfusion period) and an equal volume of vehicle (0.9% NaCl solution).

* $P<0.01$ vs. SAO+vehicle.

undergoing 45 min of ischemia followed by 1 h reperfusion showed positive staining for P-selectin localised in the vascular endothelial cells of vessels (Fig. 5E, see arrows). No staining was observed in sham-operated rats (Fig. 5B). In tissue obtained at 1 h after reperfusion from NAC-treated rats, less staining for P-selectin was found (Fig. 5H, see arrows).

As is possible see in Fig. 5F (staining combination of panel D and E) the positive staining for P-selectin and for ICAM-1 are co-localised in the endothelium along the vascular wall.

3.6. Histological change

At histological examinations of small intestine at 60 min of reperfusion (see representative sections in Fig. 6) reveal pathologic changes. No morphological modification was observed in the sham-operated rats (Fig. 6A). Ileum section from SAO-shocked rats showed significant damage of the villus (Fig. 6B). NAC-treated rats show a significant reduction in organ injury (Fig. 6C).

3.7. Vascular reactivity

In order to investigate whether SAO shocked 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 animals, contractile responses to noradrenaline were significantly reduced, when compared to that observed in aortic rings from sham animals (Fig. 7). The maximum force of contraction induced by 10 μ M noradrenaline in aortic ring from sham rats was 2.0 \pm 0.12 g, whereas it was 1.12 \pm 0.20 g in ring from SAO shocked rats ($n=8$, Fig. 7).

An impairment of endothelium-dependent dilatation was also observed in aortic rings from SAO shocked rats, as evidenced by a reduced relaxant effect of the acetylcholine (10 nM–10 μ M) (Fig. 8).

In vivo pre-treatment with NAC during SAO and reperfusion improved the vascular responsiveness to noradrenaline and caused a partial improvement of the degree of the endothelial dysfunction (Figs. 6 and 7). Treatment with NAC did not alter the contractions to noradrenaline or the dilations to acetylcholine response in aortic rings from sham rats (Figs. 7 and 8).

3.8. Survival rate

Table 2 summarises survival rate, percentage survival, and survival time for the groups of the 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 characterised by a 100% lethality: all rats died within 2 h (mean survival time 73 \pm 5

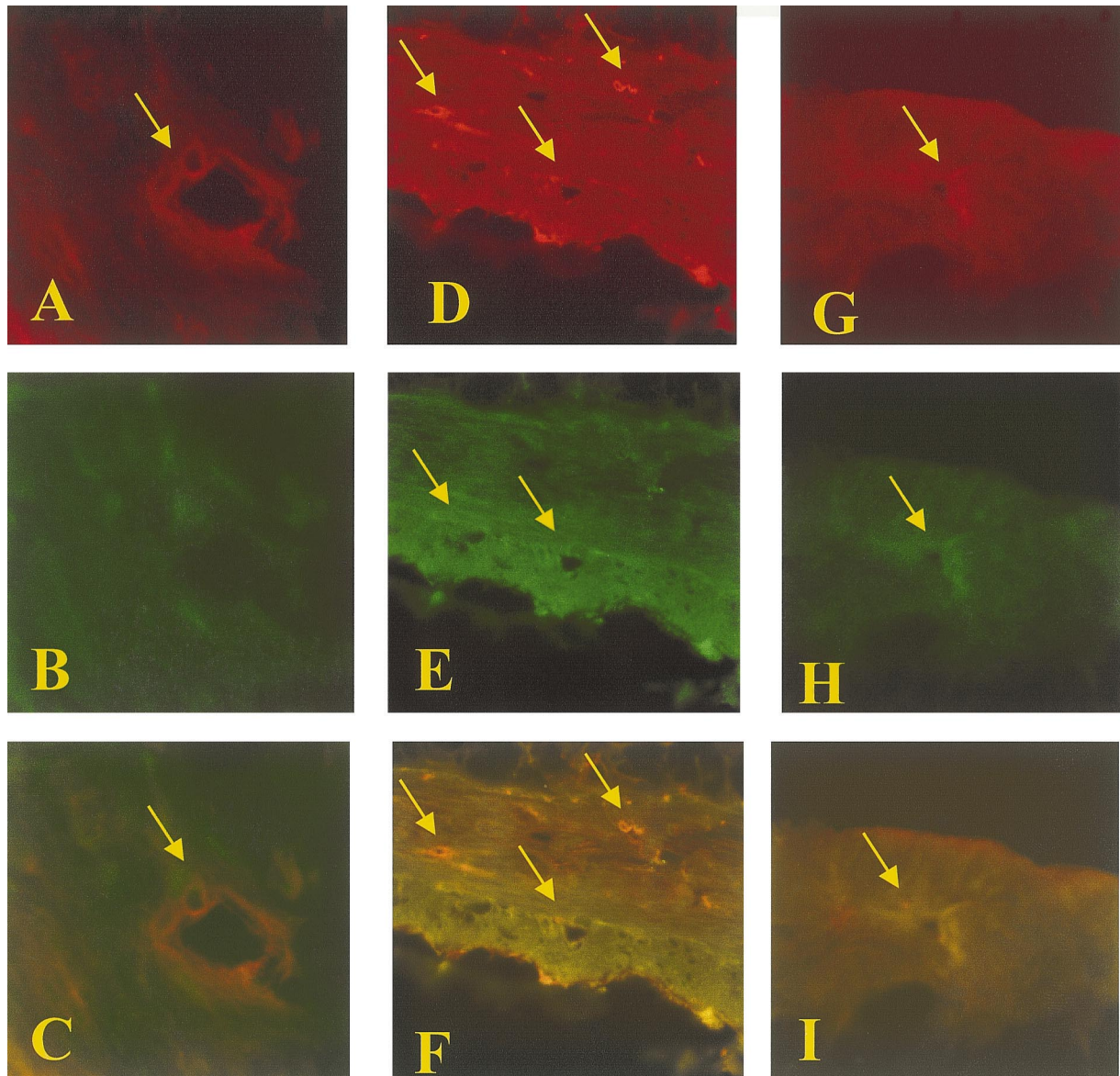


Fig. 5. Immunohistochemical localisation for P-selectin and for ICAM-1 in the reperused intestine. Staining of ileum tissue sections obtained from sham-operated rats with anti-ICAM-1 antibody showed a specific staining along vessels (arrow), demonstrating that ICAM-1 is constitutively expressed (A). Ileum section from sham-operated rats revealed no positive for P-selectin staining (B). Section obtained from SAO shocked-rats showed intense positive staining (see arrows) for ICAM-1 (D) and for P-selectin (E) on endothelial cells. The degree of endothelial staining for ICAM-1 (G) and for P-selectin (H) was markedly reduced in tissue section obtained from NAC-treated rats. C, F and I represent the staining combination of panel A-B, D-E and G-H, respectively. Original magnification: $\times 145$. Figure is representative of at least three experiments performed on different experimental days.

min; Table 2). All NAC-treated rats survived at SAO-shock (Table 2).

4. Discussion

Errando et al. [30], described a patient with occlusion of the superior mesenteric artery, resulting in acute renal failure, intestinal ischemia, necrosis of both legs and multiple organ failure leading to death. Several animal models have been described in order to understand the pathophysiologic mechanism. Among them splanchnic

artery occlusion shock (SAO) is a severe form of circulatory shock produced by ischemia and reperfusion of the splanchnic organs. This type of shock is characterised by a decrease in systemic blood pressure upon release of the splanchnic arteries, which leads to a fatal outcome [1,2]. Other important characteristics of the SAO shock are: local splanchnic release of lysosomal hydrolases, enhanced proteolysis, hemoconcentration, intestinal injury and the production of cardiotoxic substances [1,2,6,7].

Our data demonstrate that NAC treatment exert an important protective effect against SAO shock. In fact, the present study provide evidence that (1) NAC reduces the

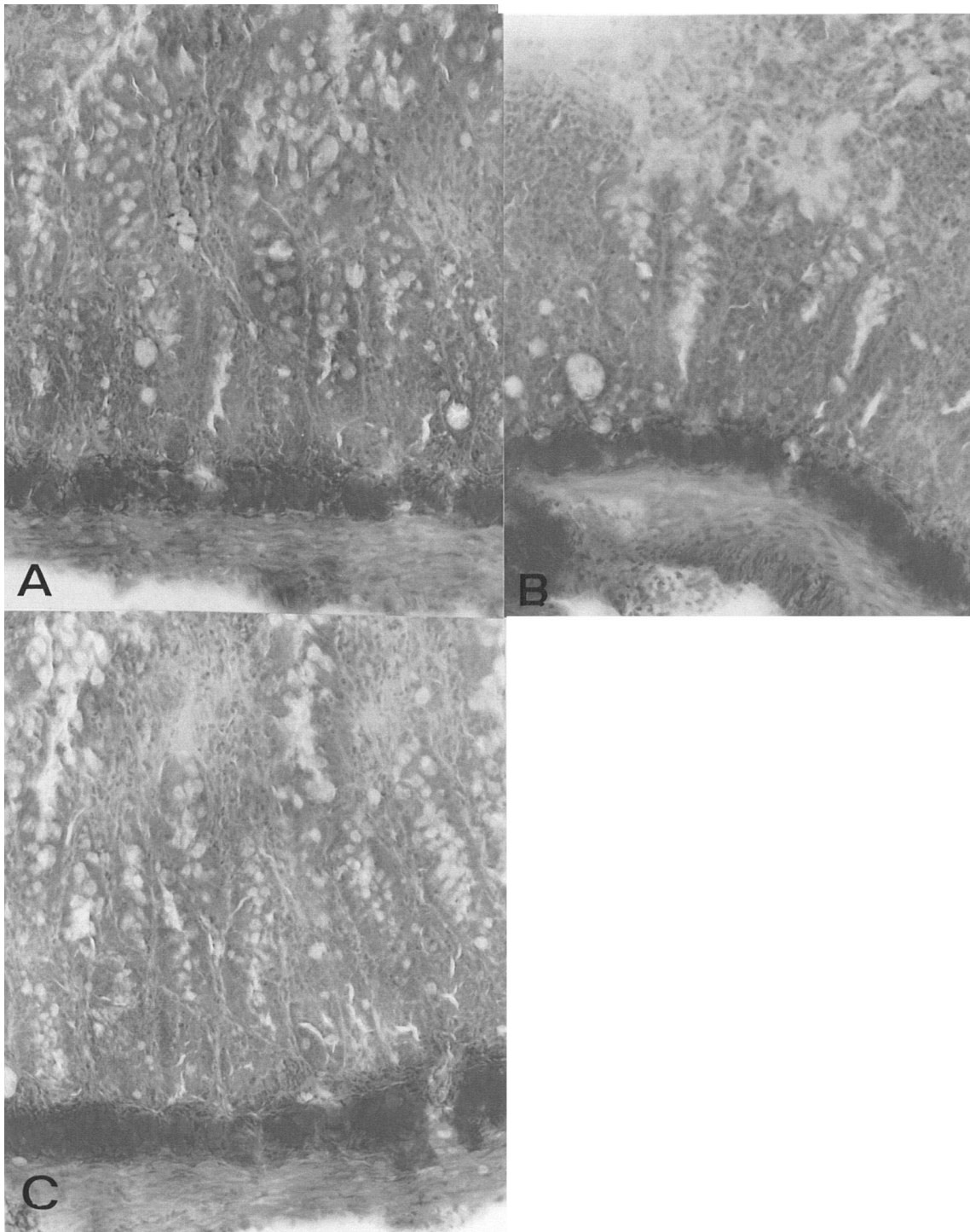


Fig. 6. SAO-induced intestinal injury. No morphological modification was observed in the sham-operated rats (A). Distal ileum section from a SAO shocked-rats showed oedema of the distal portion of the villus and necrosis of the epithelium at the villus tips (B). Distal ileum from NAC-treated rats shows reduced SAO-induced organ injury (C) Original magnification: $\times 100$.

development of SAO-induced shock, (2) NAC reduces morphological injury and neutrophil infiltration in SAO shock, (3) NAC reduces lipid peroxidation, (4) in NAC-treated rats subjected to occlusion and reperfusion of the splanchnic artery, the upregulation of P-selectin and ICAM-1 in the ileum was completely abolish, and (5)

NAC reduced cardiovascular derangement associated with SAO-shock. What then, is the mechanism by which NAC protects the ileum of the rat against injury and dysfunction? NAC exerts its effect both as a source of sulfhydryl groups (repletion of intracellular reduced glutathione) and through a direct reaction with hydroxyl radical [31].

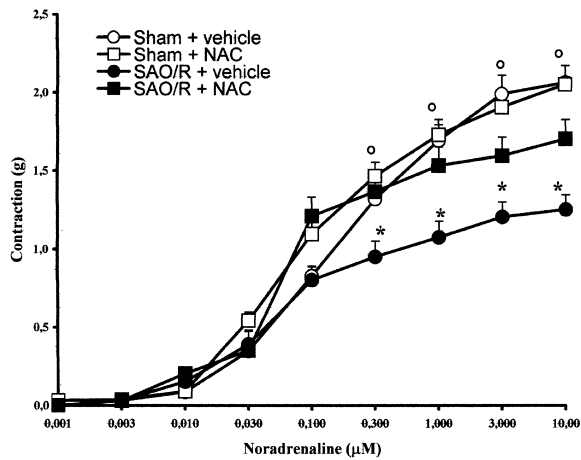


Fig. 7. Cumulative dose-response curves in response to noradrenaline in aortic rings from sham-operated rats and splanchnic artery occlusion (SAO)-shocked rats treated with vehicle, or with NAC. Each point is the mean \pm S.E.M. for $n=8$ animals. * $P<0.01$ vs. sham; $^{\circ}P<0.01$ vs. SAO.

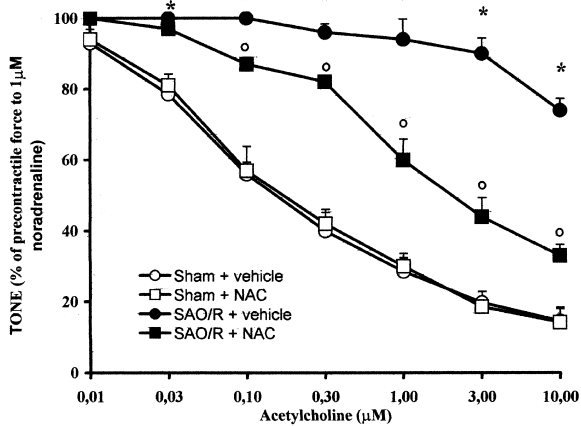


Fig. 8. Relaxant effect of acetylcholine in aortic rings precontracted with noradrenaline (1 μ M) of sham-operated rats and splanchnic artery occlusion (SAO)-shocked rats treated with vehicle, or with NAC. Each point is the mean \pm S.E.M. for $n=8$ animals. * $P<.01$ vs. sham; $^{\circ}P<.01$ vs. SAO.

Table 2

Effect of vehicle or *n*-acetylcysteine (NAC) on survival rate, percentage survival, and survival time in sham-shocked rats or splanchnic artery occlusion (SAO)-shocked rats^a

Treatment	Time after reperfusion (h)				Survival time (min)
	2		4		
	Surviving	%	Surviving	%	
Sham+vehicle	10/10	100	10/10	100	>240
Sham+NAC	10/10	100	10/10	100	>240
SAO+vehicle	0/10	0	0/10	0	79 \pm 3.6
SAO+NAC	10/10	100	10/10	100*	>240*

^a Animals received NAC (20 mg/kg i.v. 5 min before reperfusion followed by 20 mg/kg/h for all the reperfusion period) and an equal volume of vehicle (0.9% NaCl solution).

* $P<0.01$ vs. SAO+vehicle.

Recently, a number of study in animals suggest benefits from acetylcysteine in the context of systemic inflammatory response syndrome caused by severe sepsis model. In a pig gram-negative sepsis model, an infusion of acetylcysteine reduced pulmonary capillary leak without reducing mortality [32]. Acetylcysteine also beneficially modulates inflammatory cell function in animals. Endotoxin-induced neutrophil activation in sheep lung is reduced [32].

Additional protective effects of NAC may lie within the ability of this compound to reduce oxyradical-related oxidant processes by either directly interfering with the oxidants, or up-regulating antioxidant systems such as superoxide dismutase [31] or enhancing the catalytic activity of glutathione peroxidase [33]. Therefore, oxygen radical scavengers, administered before or at the onset of sepsis, were shown to improve the survival in animal models of sepsis [24]. NAC has antioxidant property [25] and as a sulfhydryl donor, may contribute to the regeneration of endothelium-derived relaxing factor and glutathione [26]. Increasing evidence indicates that the action of NAC is pertinent to microcirculatory blood flow and tissue oxygenation. NAC was shown to enhance oxygen consumption via increased oxygen extraction in patients 18 h after the onset of fulminant liver failure [26]. It was speculated that NAC could also exert beneficial effects on impaired nutritive blood flow in patients with severe sepsis [26].

In the present report in which 1 h of reperfusion followed 45 min of ischemia, the number of PMNs was elevated over that of the control ileum with the effect being greatly reduced by NAC. Endothelial cells appear to be major regulators of the neutrophil traffic, regulating the process of neutrophil chemotraction, adhesion and emigration from the vasculature to the tissue. During the early phase of reperfusion, P-selectin is rapidly released to the cell surface from performed storage pools after exposure to certain stimuli, such as hydrogen peroxide, trombin, histamine, or complement, and allows the leukocytes to roll along the endothelium [34–36]. ICAM-1 constitutively expressed on the surface of endothelial cells, is then involved in the neutrophil adhesion [34,37]. Hypoxic endothelial cells synthesise pro-inflammatory cytokines, which can up regulate endothelial expression of the constitutive adhesion molecule ICAM-1 in autocrine fashion [38,39]. Significant expression of ICAM-1 in microvessels of previously ischemic tissues occurs within 1 h after reperfusion [40,41]. The expression of P-selectin and ICAM-1 corresponds with the induction of neutrophil recruitment, which is maximal within the first hour of reperfusion, and persists, at lower rate, in the late phase of reperfusion [42–44]. In accordance with these findings, we observed that a 45-min occlusion of splanchnic artery followed 1-h reperfusion induced the appearance of P-selectin on the endothelial vascular wall and unregulated the surface expression of ICAM-1 on endothelial cells.

NAC treatment abolish the expression of P-selectin and the upregulation of ICAM-1 (Fig. 5), while maintaining unaffected the constitutive levels of ICAM-1 on endothelial cells (data not shown). These results demonstrate that NAC treatment can interrupt the interaction neutrophils and endothelial cells both at early rolling phase mediated by P-selectin and at late firm adhesion phase mediated by ICAM. The absence of an increased expression of the adhesion molecule in the ileum tissue of NAC-treated rats correlated with the reduction of leukocyte infiltration as assessed by the specific granulocyte enzyme myeloperoxidase and with the moderation of the postreperfusion tissue damage as evaluated by histological examination. It is noteworthy, however, that tissue myeloperoxidase activity was not completely abolished. This results is consistent with previous studies demonstrating that constitutive levels of ICAM-1 appear to be sufficient to support a lower degree of CD11/CD18-dependent transendothelial migration of activated neutrophils [45,46].

In the present study, the levels of MDA, which is the products of lipid peroxidation, was significantly increased by ischemia-reperfusion. This observation is in agreement with previous studies where elevated levels of lipid peroxidation products [47,48]. NAC treatment abolishes the increase in lipid peroxidation products, probably in part by scavenging the very reactive $\cdot\text{OH}$ and $\text{ROO}\cdot$.

Reduction of lipid peroxidation was also paralleled with the inhibition of nitrotyrosine immunoreactivity. Nitrotyrosine formation, along with its detection by immunostaining, was initially proposed as a relatively specific means for detection of the “footprint” of peroxynitrite [49]. Recent evidence indicate, however, that certain other reactions can also induce tyrosine nitration; e.g. the reaction of nitrite with hypochlorous acid and the reaction of myeloperoxidase with hydrogen peroxide can lead to the formation of nitrotyrosine [50]. Increased nitrotyrosine staining is considered, therefore, as an indication of “increased nitrosative stress” rather than specific marker of peroxynitrite.

ROS and peroxynitrite produce cellular injury and necrosis via several mechanisms including peroxidation of membrane lipids, protein denaturation and DNA damage. ROS produce strand breaks in DNA which triggers energy-consuming DNA repair mechanisms and activates the nuclear enzyme PARS resulting in the depletion of its substrate NAD in vitro and a reduction in the rate of glycolysis. As NAD functions as a cofactor in glycolysis and the tricarboxylic acid cycle, NAD depletion leads to a rapid fall in intracellular ATP. This process has been termed ‘the PARS suicide hypothesis’. There is recent evidence that the activation of PARS may also play an important role in ischemia and reperfusion injury [9,16,17]. We demonstrate here that NAC attenuates the increase in PARS activity caused by SAO-shock. Thus, we propose that the effects of NAC reported here are – at least in part – due to the prevention of the activation of PARS.

Taken together, the results of the present study, support the view that NAC can exert a protective effect against SAO shock and put forward the hypothesis that NAC may represent potentially important therapeutic target in circulatory shock.

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