Participation of tumour necrosis factor and nitric oxide in the mediation of vascular dysfunction in splanchnic artery occlusion shock

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1 Splanchnic artery occlusion (SAO) shock is characterized by irreversible circulatory failure. Tumour necrosis factor (TNF- α) may affect the L-arginine/nitric oxide (NO) pathway, thus contributing to the cardiovascular derangements of circulatory shock.

2 We investigated the contribution of both TNF- α and the L-arginine/nitric oxide pathway to the vascular dysfunction of SAO shock. Anaesthetized rats, subjected to total occlusion of the superior mesenteric artery and the coeliac trunk for 45 min developed a severe shock state (SAO shock) resulting in a fatal outcome within 75–90 min after the release of occlusion. Sham operated animals were used as controls. SAO shocked rats had also a marked hypotension and enhanced macrophage and serum levels of TNF- α . Furthermore, aortic rings from shocked rats showed a marked hyporeactivity to phenylephrine (PE 1 nM–10 μ M) and reduced responsiveness to acetylcholine (ACh 10 nM–10 μ M). Endothelium-denuded aortic rings had also a marked hyporeactivity to phenylephrine, which was restored to control values by *in vitro* administration of N^G nitro-L-arginine-methyl ester (L-NAME 10 μ M).

3 *In vivo* administration of cloricromene (2 mg kg⁻¹, i.v.), an inhibitor of TNF- α biosynthesis, increased survival, enhanced mean arterial blood pressure and reduced macrophage and serum levels of TNF- α . Furthermore, aortic rings from shocked rats treated with cloricromene exhibited a greater contractile response to phenylephrine and improved responsiveness to ACh when compared to aortic rings from vehicle-treated SAO shocked rats.

4 Our results suggest that TNF- α alters both endothelial and muscular L-arginine/nitric oxide pathways which in turn produce vascular dysfunction in SAO shock.

Keywords: Cytokine; impaired vascular reactivity; endothelium; L-arginine-NO pathway

Introduction

Splanchnic artery occlusion (SAO) shock is an experimental type of circulatory shock which is the consequence of a prolonged ischaemia of the splanchnic region (Sturniolo *et al.*, 1988). SAO shock is characterized by impaired activity of the reticuloendothelial system (Sturniolo *et al.*, 1989), elevated macrophage and plasma levels of thromboxane B₂ (Squadrito *et al.*, 1992c) increased plasma levels of platelet activating factor (Squadrito *et al.*, 1991b; Zingarelli *et al.*, 1992b) and enhanced macrophage and serum levels of tumour necrosis factor (TNF- α) (Squadrito *et al.*, 1992b). Furthermore, SAO shock produces an irreversible circulatory failure which leads to the death of the animals within 75–90 min after the release of the occlusion (Squadrito *et al.*, 1989).

Acute splanchnic ischaemia induced by SAO shock may, therefore, induce an impaired vascular reactivity, but the mechanisms underlying these cardiovascular derangements are not yet well known.

It has been proposed that the L-arginine/nitric oxide pathway may play a key role in the regulation of vascular tone (Vane *et al.*, 1990). The biosynthesis of nitric oxide (NO) from L-arginine is a pathway for the regulation of cell function and communication (Moncada *et al.*, 1991). In all cell types so far studied NO is generated following oxidation and cleavage of the terminal nitrogen atom(s) of L-arginine by an enzyme, NO synthase. At least two types of NO synthase have been identified (Palmer *et al.*, 1987). The enzyme found in the endothelial cells (Pollock *et al.*, 1991) and in the brain (Bredt *et al.*, 1990) is constitutive, Ca²⁺-dependent and releases picomolar amounts of NO for a short period follow-

ing receptor stimulation. In contrast, the enzyme found in the macrophages is induced following stimulation with cytokines or endotoxin, is Ca²⁺-independent, and releases nanomolar amounts of NO for a long period (Knowles *et al.*, 1990). Furthermore, the presence of a Ca²⁺-independent NO synthase in vascular smooth muscle cells has been shown (Busse *et al.*, 1990).

These findings, taken together, suggest that vascular tone results from a cooperative participation of both endothelial and smooth muscle L-arginine/nitric oxide pathways.

TNF- α is a pleiotropic cytokine that plays an important role in nonseptic ischaemic states (Squadrito *et al.*, 1993) and that causes a marked impairment in vascular reactivity (Takahashi *et al.*, 1992). Since TNF- α has been shown to play an important role in the pathogenesis of SAO shock, we investigated the contribution of TNF- α and L-arginine/nitric oxide pathway to the vascular dysfunction of SAO shock. To investigate the role of TNF- α we used a pharmacological tool and, more specifically cloricromene. This coumarin derivative inhibits both *in vivo* and *in vitro* TNF- α production, an effect that appears to be the consequence of an increase in intracellular adenosine 3',5'-cyclic monophosphate (cyclic AMP) (Squadrito *et al.*, 1991a).

Methods

Surgical procedures

Male Sprague-Dawley rats weighing 250–300 g were permitted access to food and water *ad libitum*. The rats were anaesthetized with urethane (1.3 g kg⁻¹, i.p.). During the

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experiment, no supplemental dose of anaesthetic was administered. After anaesthesia, catheters were placed in the carotid artery and jugular vein. Blood pressure was monitored continuously with a Statham pressure transducer. 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 given heparin (1,000 u kg⁻¹, i.v.) and were observed for a 30 min stabilization period prior to either splanchnic ischaemia or sham ischaemia. SAO shock was induced by clamping both the superior mesenteric artery and the coeliac trunk resulting in total occlusion of these arteries for 45 min. After this period of occlusion the clamps were removed. Following reperfusion the rats were observed for 240 min. Sham shocked rats were subjected to all the same surgical procedures as SAO shocked rats except that the arteries were not occluded.

Survival evaluation

Five minutes before the splanchnic artery occlusion shock procedures, treated rats received intravenously cloricromene (2 mg kg⁻¹) dissolved in NaCl saline solution and control rats received the vehicle. Survival was evaluated for 4 h.

Arterial blood pressure

Animals were anaesthetized with urethane (1.3 g kg⁻¹, i.p.) and a cannula (PE 50) was inserted into the left common carotid artery as described elsewhere (Caputi *et al.*, 1980). The arterial catheter was connected to a pressure transducer. The pressure pulse triggered a cardiometer, and arterial blood pressure was displayed on a polygraph. Arterial blood pressure is reported as mean arterial pressure (MAP) in mmHG. Rats were subjected to the same experimental protocol as described above.

Biological assay for tumour necrosis factor- α activity

Killing of L929 mouse tumour cells was used to measure TNF- α levels in serum and in the supernatants of peritoneal macrophages by use of a standard assay (Ruff & Gifford, 1980). Animals were killed 80 min after the onset of reperfusion. Peritoneal macrophages were obtained by peritoneal lavage with RPMI 1640 (Altavilla *et al.*, 1989), counted and diluted to 10⁶ ml⁻¹. The cells were then incubated in Petri dishes at 37°C in a humidified CO₂ atmosphere for 1 h. L929 cells in RPMI 1640 medium containing 5% foetal calf serum were seeded at 3 × 10⁴ cells per well in 96-well microdilution plates and incubated overnight at 37°C in an atmosphere of 5% CO₂ in air. Serial dilutions of serum and supernatants of peritoneal macrophages, collected as previously described (Squadrito *et al.*, 1991b) were made in a medium containing actinomycin D, 1.0 µg ml⁻¹, and 100 µl of each dilution was added to the wells. On the next day, cell survival was assessed by fixing and staining the cells with crystal violet (0.2% methanol) and 0.1 ml of 1% sodium dodecyl sulphate was added to each well to solubilize the stained cells. The absorbance of each well was read at 490 nm with a model BT-100 Microelisa Autoreader. The percentage of cytotoxicity was calculated as [1 - (490 of sample/A490 of control)] × 100. One unit of TNF- α was defined as the amount giving 50% cell cytotoxicity. TNF- α content in the sample was calculated by comparison with a calibration curve performed with recombinant murine TNF- α (Nuclear Laser Medicine, Italy). To verify if the cytotoxicity tested was due to the presence of TNF- α or to other factors, we preincubated our samples for 2 h at 37°C with an excess of rabbit anti-recombinant murine TNF- α polyclonal antibodies (Nuclear Laser Medicine, Milan, Italy) or with control rabbit serum. Our results showed that cytotoxicity against L929

cells was completely neutralized by rabbit anti-recombinant TNF- α polyclonal antibodies but not by control rabbit serum.

Isolated aortic rings

Animals were killed 80 min after the onset of reperfusion. Thoracic aortae were removed and placed in cold Krebs solution of the following composition (mM): NaCl 118.4, KCl 4.7, MgSO₄ 1.2, CaCl₂ 2.5, KH₂PO₄ 1.2, NaHCO₃ 25.0 and glucose 11.7. Then aortae were cleaned of adherent connective and fat tissue and cut into rings of approximately 2 mm in length. In some rings the vascular endothelium was removed mechanically by gently rubbing the luminal surface with a thin wooden stick. Rings were then placed under 1 g of tension in an organ bath containing 10 ml Krebs solution at 37°C and bubbled with 95% O₂ and 5% CO₂ (pH 7.4). All experiments were carried out in the presence of indomethacin (10 µM) in order to exclude the involvement of prostaglandins and their metabolites. Developed tension was measured with an isometric force transducer and recorded on a polygraph (Ugo Basile, Torino, Italy). After an equilibration period of 60 min during which time the rings were washed with fresh Krebs solution at 15–20 min intervals and basal tension was readjusted to 1 g, the tissue was exposed to phenylephrine (PE, 100 nM). When the contraction was stable, the functional integrity of endothelium was assessed by a relaxant response to acetylcholine (ACh, 100 nM). The tissues were then washed occasionally for 30 min.

Endothelium-dependent relaxation was evaluated with cumulative concentrations of ACh (10 nM–1 µM) in aortic rings precontracted with PE (100 nM). Relaxation of the rings was calculated as the percentage decrease in contractile force.

Concentration-response curves were obtained by cumulative concentrations of PE (1 nM–10 µM) in intact or endothelium-denuded aortic rings.

In some experiments L-NAME (10 µM), L-arginine (300 µM) or their dextro-isoforms, D-NAME (10 µM) and D-arginine (300 µM) were added to the organ bath 1 h before the rings were contracted with cumulative concentrations of PE. The results (mean ± s.d.) are expressed as g of tension per mg of tissue.

Drugs

Acetylcholine chloride, phenylephrine hydrochloride, indomethacin, L-arginine hydrochloride, D-arginine hydrochloride, N^G-nitro-L-arginine methyl ester hydrochloride and N^G-nitro-D-arginine methyl ester hydrochloride were obtained from Sigma. Cloricromene (8-monochloro-3- β -diethylaminoethyl-4-methyl-7-ethoxycarbonylmethoxycoumarin) was obtained from Fidia Research Laboratories. We used the dose of 2 mg kg⁻¹ in keeping with previous studies that have shown that this dose is the most effective for *in vivo* studies (Squadrito *et al.*, 1991a).

Statistical analysis

The difference between the means of two groups was evaluated with an ANOVA followed by Bonferroni's test and was considered significant when $P < 0.05$.

Results

Relaxant response to acetylcholine

Addition of PE (100 nM) to the organ bath contracted intact aortic rings (80–90% of the maximum response). These rings were relaxed in a concentration-dependent manner by ACh (10 nM–10 µM). The relaxant effect of ACh in aortic rings from shocked rats was significantly lower in SAO shocked rats than in Sham shocked rats. The maximum relaxation

induced by $1\text{ }\mu\text{M}$ ACh was $36 \pm 2\%$ and $90 \pm 2\%$ respectively ($P < 0.001$; Figure 1).

Contractile response to phenylephrine

Figure 2 shows the contractile force induced by PE (1 nM – $10\text{ }\mu\text{M}$) in intact aortic rings from Sham and SAO shocked rats. SAO shock markedly reduced the contractile response to PE (1 nM – $10\text{ }\mu\text{M}$) of intact aortae. The maximum force of contraction (g tension per mg tissue) induced $10\text{ }\mu\text{M}$ PE in aortic rings from Sham rats was 1.8 ± 0.08 whereas it was 1.06 ± 0.04 in rings from SAO shocked rats ($P < 0.001$; Figure 2).

Removal of endothelium increased slightly but not significantly the vasoconstrictor potency of PE in the aortae from sham-shocked rats (Figure 3). In contrast, when PE was tested in denuded aortae from shocked rats, it produced overlapping effects in aortic rings with (Figure 2) or without endothelium (Figure 3). Therefore, removal of endothelium in aortae from SAO shocked animals did not restore the PE-induced contractile response to the values of the Sham

group. These results indicated that smooth muscle cells are involved in the hyporesponsiveness to PE. In endothelium-denuded aortic rings from shocked rats, N^{G} -nitro-L-arginine-methyl ester (L-NAME $10\text{ }\mu\text{M}$), an inhibitor of NO synthase,

Figure 3 Effect of N^{G} -nitro-L-arginine methyl ester (L-NAME, $10\text{ }\mu\text{M}$) on contractile response to cumulative concentrations of phenylephrine in aortic rings without endothelium from sham or splanchnic artery occlusion (SAO) shocked rats. L-NAME was added to the bath 1 h before phenylephrine. (○) Sham + vehicle; (■) SAO + vehicle; (□) SAO + L-NAME. Each point represents the mean $\pm$ s.d. from six experiments. * $P < 0.05$ vs SAO + vehicle; ** $P < 0.001$ vs SAO shocked rats.

Figure 1 Relaxant effect of acetylcholine in aortic rings (contracted with phenylephrine, 100 nM) of sham-shocked rats and splanchnic artery occlusion (SAO) shocked rats treated either with vehicle (1 ml kg^{-1} , i.v. of a 0.9% NaCl saline solution) or cloricromene (2 mg kg^{-1} , i.v., 5 min before SAO). Each point represents the mean $\pm$ s.d. from seven experiments. * $P < 0.05$ vs SAO + vehicle; ** $P < 0.001$ vs SAO + vehicle.

Figure 2 Contractile response to cumulative concentrations of phenylephrine in aortic rings from sham-shocked rats and splanchnic artery occlusion (SAO) shocked rats treated either with vehicle (1 ml kg^{-1} , i.v. of a 0.9% NaCl saline solution) or cloricromene (2 mg kg^{-1} , i.v., 5 min before SAO). Each point represents the mean $\pm$ s.d. from seven experiments. (○) Sham + vehicle; (■) SAO + vehicle; (□) SAO + cloricromene. * $P < 0.05$ vs SAO + vehicle; ** $P < 0.001$ vs SAO + vehicle.

Figure 4 (a) Effects of N^{G} -nitro-L-arginine methyl ester (L-NAME, $10\text{ }\mu\text{M}$) or D-NAME ($10\text{ }\mu\text{M}$) on maximal contractile response to phenylephrine in aortic rings without endothelium from sham and splanchnic artery occlusion (SAO) shocked rats. Each point represents the mean $\pm$ s.d. from six experiments. * $P < 0.001$ vs shock + vehicle. (b) Effects of L-arginine ($300\text{ }\mu\text{M}$) or D-arginine ($300\text{ }\mu\text{M}$) on maximal contractile response to phenylephrine in aortic rings without endothelium from sham and SAO-shocked rats. Each point represents the mean $\pm$ s.d. from six experiments. * $P < 0.05$ vs shock + vehicle.

Table 1 Effect of cloricromene on survival rate, percentage survival and survival time in splanchnic artery occlusion (SAO) shocked rats

Treatment	Hours after SAO				Survival time (min)
	Surviving ²	(%)	Surviving ⁴	(%)	
Sham + vehicle	10/10	100	10/10	100	> 240
Sham + cloricromene	10/10	100	10/10	100	> 240
SAO + vehicle	0/10	0	0/10	0	83 ± 15
SAO + cloricromene	10/10*	100*	8/10*	80*	235 ± 12*

Animals received cloricromene (2 mg kg⁻¹) or vehicle (1 ml kg⁻¹ of a 0.9% NaCl solution) as a bolus 5 min before inducing SAO shock. $P < 0.001$ vs SAO + vehicle. Survival rate was monitored for 4 h.

Table 2 Effects of cloricromene on serum and macrophage tumour necrosis factor- α (TNF- α) in splanchnic artery occlusion (SAO) shocked rats

Treatment	Serum TNF- α (u ml ⁻¹)	Macrophage TNF- α (u ml ⁻¹)
Sham + vehicle	ND	ND
Sham + cloricromene	ND	ND
SAO + vehicle	345 ± 21	110 ± 12
SAO + cloricromene	18 ± 1*	9 ± 2

Serum and macrophages were collected 80 min after clamp removal. Each point represents mean $\pm$ s.d. from six experiments. Animals received cloricromene (2 mg kg⁻¹) or vehicle (1 ml kg⁻¹ of a 0.9% NaCl solution) as a bolus 5 min before inducing SAO shock. $P < 0.001$ vs SAO + vehicle. ND = not detectable.

Figure 5 Effects of vehicle (1 ml kg⁻¹, i.v. of a 0.9% NaCl saline solution) or cloricromene (2 mg kg⁻¹, i.v., 5 min before splanchnic artery occlusion (SAO) shock) on mean arterial blood pressure (MAP) in rats subjected to SAO shock. Each point represents the mean $\pm$ s.d. from eight experiments. (○) Sham + vehicle; (●) Sham + cloricromene; (□) SAO + vehicle; (■) SAO + Cloricromene. * $P < 0.001$ vs SAO + vehicle.

restored PE-sensitivity to control values (Figure 3). The effect was stereospecific because D-NAME (10 μ M) was without effect (Figure 4a). L-Arginine (300 μ M), but not D-arginine, significantly increased the vascular hyporeactivity of aortic rings from SAO shocked rats, whilst it had no effect in rings from sham-shocked rats (Figure 4b).

Effect of in vivo cloricromene pretreatment

Survival rate Table 1 summarizes survival rate, percentage survival and survival time for the groups of rats subjected to splanchnic artery occlusion shock or sham shock. All sham shocked rats survived the entire 4 h observation period. In contrast, in rats treated with vehicle, SAO shock produced a profound shock state characterized by a high lethality: no rat survived at 2 h (survival time 83 $\pm$ 15 min). Treatment with

cloricromene significantly increased survival rate and time in shocked rats when compared with rats treated with vehicle (Table 1).

Macrophage and serum TNF- α Macrophage and serum levels of TNF- α were undetectable in sham shocked rats treated with vehicle or cloricromene. TNF- α was significantly increased in the serum and in the supernatants of peritoneal macrophages collected from SAO shocked rats (Table 2). Treatment with cloricromene decreased the macrophage and serum TNF- α levels in SAO shocked rats (Table 2).

Mean arterial blood pressure SAO shock caused a sustained increase in mean arterial blood pressure. Subsequently pressure decreased sharply upon release of the clamps (Figure 5). Administration of cloricromene significantly improved these cardiovascular changes (Figure 5).

Relaxant response to ACh and contractile response to PE

Administration of cloricromene, an inhibitor of TNF- α production, significantly improved responsiveness to ACh (Figure 1; 80 $\pm$ 5%). The treatment with the drug also succeeded in partially restoring the contractile response to PE (Figure 2; 1.63 $\pm$ 0.2 g mg⁻¹ tissue).

Discussion

Acute splanchnic ischaemia results in an irreversible circulatory failure and death (Squadrito *et al.*, 1991b; 1992c). The mechanisms underlying these cardiovascular derangements of SAO shock are not yet well known and therefore we carried out experiments to investigate the various components which together produce the severe vascular dysfunction of SAO shock.

It has been suggested that during SAO shock endothelial NO is reduced and administration of NO donors in this type of experimental shock increased short term survival and improves endothelial dysfunction (Carey *et al.*, 1992). In concurrence with this hypothesis our present data show that aortic rings from SAO shocked rats had a marked reduced

responsiveness to vasorelaxant effects of ACh. Indeed our present data and those of Carey *et al.* (1992) would indicate that in SAO shock a reduced NO production might be present and therefore these findings would justify the use of NO enhancing drugs in circulatory shock.

However, these results are in disagreement with data from both our laboratory (Zingarelli *et al.*, 1992a; 1994) and other research groups (Rees *et al.*, 1990; Thiemermann *et al.*, 1993) that, in contrast, claim an overproduction of NO as responsible for the vascular dysfunction observed in other types of circulatory shock such as haemorrhagic or septic shock. Previous studies on vascular reactivity in SAO shock (Aoki *et al.*, 1990; Carey *et al.*, 1992) have not been complete and conclusive; for example, in those studies a dose-response curve to vasoconstrictor agents in vessels with or without endothelium was not performed.

We therefore investigated the contractile response to phenylephrine. Our results demonstrate that a reduced vascular sensitivity to phenylephrine also occurs in aortic rings from SAO shocked rats. In addition, no difference between the aortae with or without endothelium was observed in the responses to phenylephrine.

The mechanism responsible for vascular dysfunction seems to be due to an overproduction of the potent vasodilator NO. In fact L-NAME, an inhibitor of NO synthase, restored the contractile responses to phenylephrine in aortic rings from SAO-shocked rats to control values. The stereospecificity of this effect was shown by the lack of effect of the dextro-enantiomer D-NAME which is not able to affect NO syntheses (Palmer *et al.*, 1988). Furthermore, our findings suggest that, even in the absence of endothelium, a continuous production of NO persists, probably due to the expression of the inducible NO synthase in the vascular smooth muscle (Busse *et al.*, 1990).

Therefore all these data, taken together, would suggest that both endothelial (reduced production of NO) and muscular (overproduction of NO) L-arginine/nitric oxide pathways are significantly, even if differently, affected by SAO shock.

The subsequent question that we raised was: is TNF- α , a pleiotropic cytokine, involved in mediating the vascular dys-

function of SAO shock? As far as this aspect is concerned it has been suggested that TNF- α may impair the release of NO from endothelial cells (Aoki *et al.*, 1989), thus leading to decreased production of endothelial-derived relaxing factor. On the other hand, TNF- α administration in conscious rats has been reported to induce a decrease in mean arterial blood pressure and to produce vascular hyporesponsiveness to contractile agents that is reversed by inhibitors of NO synthesis (Takahashi *et al.*, 1992). Furthermore, it has been shown that TNF- α is able to induce production of the Ca²⁺-independent NO synthase in vascular smooth muscle cells (Busse *et al.*, 1990). These apparently conflicting results may be interpreted as follows: TNF- α might either inhibit the constitutive and endothelial NO synthase or stimulate the inducible form of NO synthase at the smooth muscle level and, finally, vascular dysfunction produced by this pleiotropic cytokine would result from the sum of these events.

To test this hypothesis we performed experiments using cloricromene, a previously reported inhibitor of TNF- α synthesis (Squadrito *et al.*, 1991a; 1992a). Our results indicate that cloricromene reduced macrophage and serum TNF- α , significantly increased survival and mean arterial blood pressure, and succeeded in restoring both the contractile response to phenylephrine and the vasorelaxant effects of ACh.

Therefore these findings, taken together, strongly suggest that TNF- α is deeply involved in the pathogenesis of vascular dysfunction that occurs in SAO shock. However (an)other factor(s) might also contribute to producing the irreversible circulatory failure of SAO shock.

In conclusion, our data suggest that in SAO shock: (a) endogenous NO generated by the constitutive NO synthase is blunted; (b) endogenous NO produced by the inducible NO synthase is increased; (c) these opposite effects on the two forms of NO synthase are probably induced by TNF- α .

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