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Protective Effects of the New Lazaroid "U-83836E" in Splanchnic Artery Occlusion (SAO) Shock

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We studied the effects of the new antioxidant drug U-83836E during splanchnic artery occlusion (SAO) shock in the rat. Serum tumor necrosis factor (TNF-α), white blood cell (WBC) count, mean arterial blood pressure (MAP), survival rate and the responsiveness to acetylcholine of aortic rings were investigated.

SAO shock produced a marked increase in serum TNF-α (241.4 ± 18.2 U/ml vs Not Detectable in basal), reduced MAP (51.4 ± 4 mmHg vs 85.1 ± 5 mmHg), survival time (80 ± 10 min vs > 240 min), WBC count ($2.8 \pm 0.4 \times 10^3/mm^3$ cells vs $11.7 \pm 0.9 \times 10^3/mm^3$ cells) and blunted the responsiveness to ACh of aortic rings ($60 \pm 3\%$ tension vs $23 \pm 4\%$ tension).

The analogue of vitamin E, U-84836E, administered at onset of reperfusion, lowered serum TNF-α (38.4 ± 6.5 U/ml; $p < 0.001$), improved MAP (67.5 ± 3.8 mmHg; $p < 0.001$), WBC count ($8.9 \pm 0.6 \times 10^3/mm^3$; $p < 0.001$), and survival time (235 ± 15 min; $p < 0.001$), and restored the responsiveness to ACh of aortic rings ($32 \pm 3.7\%$ tension; $p < 0.001$).

These preliminary data suggest that this new compound could be a promising drug in shock therapy.

Keywords: Antioxidants, free radicals, lipid peroxidation, vitamin E, splanchnic artery occlusion shock

INTRODUCTION

Circulatory shock is characterized by a reduced blood flow in the tissue. The consequence of this inadequate perfusion is an altered cell membrane permeability and cellular dysfunction. Several reports have suggested that oxygen-derived free radicals may have a role in this cascade of pathological events.[1,2] In fact the initial release of highly reactive species during the reperfusion is thought to induce a cascade of secondary tissue injury due mainly to lipid peroxidation.[3] Polyunsaturated fatty acids, found most prevalently incorporated in lipids forming cellular membranes, are the most vulnerable to free radical attack.[4] Associated with reperfusion of the ischemic tissue is a cellular reaction characterized by the accumulation of inflammatory cells and production of inflammatory mediators in the

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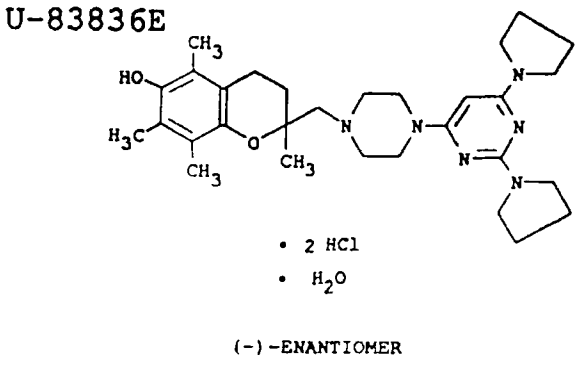

FIGURE 1 Chemical structure of U-83836E.

damaged tissue.[5] Following free radical attack the concentration of endogenous antioxidants has been shown to be severely depleted.[6] SAO shock is an experimental type of shock produced by prolonged ischemia of the splanchnic region followed by reperfusion.[7] It has been suggested that oxygen free radicals are involved in the pathogenesis of SAO shock.[8] However the phenomenon of lipid peroxidation has not been fully investigated in SAO shock. The new compound U-83836E (−)-2-((4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl-1-piperazinyl)methyl)-3,4-dihydro-2,5,7,8-tetramethyl-2h-1-benzopyran-6-dihydrochloride) (Figure 1) is one of the second-generation lazaroids with a structure based on a ring portion of α-tocopherol linked with various active groups. The aim of the present study was to investigate the possible action of this drug in limiting the damage after SAO shock.

MATERIALS AND METHODS

Surgical Procedures

Male Sprague–Dawley rats (250–300 g body weight) were permitted access to food and water *ad libitum*. The rats were anaesthetized with urethane (1.3 g/kg, i.p.). After anesthesia, polyethylene catheters were placed in the carotid artery and jugular vein. For monitoring blood pressure a cannula (PE 50) was inserted into the left common carotid artery as described elsewhere.[9] The arterial catheter was connected to a pressure transducer (Mac Lab/4E transducer module, AD Instruments, Hastings, UK) and the arterial blood pressure was displayed on a computer monitor. Arterial blood pressure is reported as mean arterial pressure (MAP) in mmHg. 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 given heparin (1000 U/kg, 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 celiac 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 and survival was expressed either as survival time or survival rate. Sham shocked rats were subjected to all the same surgical procedures as SAO shocked rats except that arteries were not occluded.

Tumor Necrosis Factor Measurement

Serum tumor necrosis factor was evaluated in serum obtained before occlusion and at end of reperfusion by using a biological assay,[10] killing of L929 mouse tumor cells in RPMI 1640 medium containing 5% fetal calf serum. Cells were seeded at 3×10^4 cells per well in 96-well microdilution plates and incubated overnight at 37°C in an atmosphere of 5% CO_2 in air. Serial dilutions of serum (0.3 ml, drawn at different time intervals) were made in a medium containing actinomycin D (1 μg/ml) 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 (Microspectrophotometer, mod.340 ATTC, S.L.T. Lab Instruments, Austria). The percentage of cytotoxicity was calculated as [1-(Abs490 of sample/Abs490 of control)] × 100. One unit of TNF-α was defined as the amount giving 50% cell cytotoxicity. The TNF-α content in the sample was calculated by comparison with a calibration curve obtained with recombinant murine TNF-α (Nuclear Laser Medicine, Milano, Italy). To test whether 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, Milano, 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.

Leukocyte Count

Tail vein blood samples for the leukocyte count (Haemochrome autoanalyzer, Cobas Micros, mod. Argos 5 DIFF, Roche Diagnostic Systems, Switzerland) were taken before occlusion and at the end of reperfusion. The number of leukocytes (WBC × $10^3/mm^3$) is reported as mean ± s.e.m.

Isolated Aortic Rings

Animals were killed 80 min after the start of reperfusion. Thoracic aortae were removed and placed in cold Krebs solution of the following composition (mM):[11] NaCl 118.4, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 11.7. The aortae were cleaned of adherent connective and fat tissue, then cut into rings of approximately 2 mm in length. Cold Krebs solution was only used to prepare aortae before mounting them in the organ bath. The rings were then placed under 1 g tension in an organ bath containing 30 ml of Krebs solution at 37°C and bubbled with 95% O_2 and 5% CO_2 (pH = 7.4). All experiments were carried out in the presence of indomethacin (10 μM) in order to exclude the involvement of eicosanoids and their metabolites. Developed tension was measured with an isometric force transducer (BM 9000 IDAS, Biomedica Mangoni, Pisa, Italy) and data were amplified (BM 614/2 amplifier, Biomedica Mangoni, Pisa, Italy), acquired and elaborated by a computer software program (Biomedica Mangoni, Pisa, Italy). After a 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 three times over 30 min. Endothelium-dependent relaxation was evaluated with cumulative concentrations of ACh (10 nM–1 μM) in aortic rings precontracted with PE (100 nM). Endothelium-independent relaxation was investigated with cumulative concentrations of sodium nitroprusside (15–30 nM) precontracted with PE (100 nM). Relaxation of the rings was calculated as percentage decrease of contractile force.

Drug

U-83836E was supplied by Upjohn S.p.A. Caponago (MI), Italy. The compound diluted in citrate buffer, was administered intravenously at onset of reperfusion in a volume of 1 ml/kg body weight.

Statistical Analysis

Data are expressed as mean ± s.e.m. The difference between the means of two groups was evaluated with an ANOVA followed by Bonferroni's test and was considered significant when $p < 0.05$.

Statement of Animal Care

The studies reported in this manuscript have been carried out in accordance with the

declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals.

RESULTS

Serum TNF-α

Serum levels of TNF-α were undetectable, before occlusion, in all experimental groups of animals (Table I). TNF-α was also undetectable during the occlusion period. In contrast, serum TNF-α was significantly enhanced at the end of the reperfusion in SAO vehicle group (241.4 ± 18.2 U/ml). Treatment with the drug substantially decreased TNF-α levels during reperfusion (Table I).

Leukocyte Count

The leukocyte number did not change in each group before occlusion (Table II). Instead, SAO shock produced a marked leukopenia. In fact the leukocyte number was significantly decreased at the end of the reperfusion in SAO vehicle animals ($2.8 \pm 0.4 \times 10^3/mm^3$). The administration of U-83836E improved the leukocyte count with all used doses (Table II). No variations were found in sham rats.

TABLE I Effects of U-83836E on serum TNF-α activity (U/ml) after splanchnic artery occlusion shock

Experimental group	Basal	Reperfusion (80 min)
Sham + vehicle ($n = 7$)	N.D.	N.D.
Sham + U-83836E (30 mg/kg) ($n = 7$)	N.D.	N.D.
SAO + vehicle ($n = 10$)	N.D.	241.4 ± 18.2 *
SAO + U-83836E (7.5 mg/kg) ($n = 10$)	N.D.	174.5 ± 12.2 **
SAO + U-83836E (15 mg/kg) ($n = 10$)	N.D.	89.6 ± 8.7 **
SAO + U-83836E (30 mg/kg) ($n = 10$)	N.D.	38.4 ± 6.5 **

The total number of animals in each group is indicated in parentheses, values are mean ± s.e.m. N.D. = not detectable; *$p < 0.001$ vs basal; **$p < 0.001$ vs SAO + vehicle.

TABLE II Effects of U-83836E on circulating white cells ($\times 10^3/mm^3$) after splanchnic artery occlusion shock

Experimental group	Basal	Reperfusion (80 min)
Sham + vehicle ($n = 7$)	11.3 ± 1.2	11.4 ± 1.3
Sham + U-83836E (30 mg/kg) ($n = 7$)	11.4 ± 1.3	11.2 ± 1.1
SAO + vehicle ($n = 10$)	11.7 ± 0.9	2.8 ± 0.4 *
SAO + U-83836E (7.5 mg/kg) ($n = 10$)	11.5 ± 1.4	4.2 ± 0.5 **
SAO + U-83836E (15 mg/kg) ($n = 10$)	11.6 ± 1.1	6.6 ± 0.8 **
SAO + U-83836E (30 mg/kg) ($n = 10$)	11.5 ± 1.2	8.9 ± 0.6 **

The total number of animals in each group is indicated in parentheses, values are mean ± s.e.m.; *$p < 0.001$ vs basal; **$p < 0.001$ vs SAO + vehicle.

Survival Rate

Sham-shocked rats, treated either with carrier vehicle or with U-83836E, survived the entire 4 h observation period (Table III). In contrast, in rats treated with vehicle, occlusion and reperfusion of the splanchnic arteries produced a profound shock state characterized by a high lethality and no rats survived at 2 h (survival time 80 ± 10 min). The administration of the drug significantly protected the rats from death (Table III).

Arterial Blood Pressure

Occlusion of the splanchnic arteries produced a marked increase in MAP (Figure 2). Upon release of the occlusion pressure decreased substantially and progressively until death. Administration of the compound significantly blunted the reduction in MAP (Figure 2). No significant variations were observed in sham groups.

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

TABLE III Effects of U-83836E on survival time after splanchnic artery occlusion shock

Experimental group (%)	Survival time (min)	Surviving animals	Survival (%)
Sham + vehicle	> 240	7/7	100
Sham + U-83836E (30 mg/kg)	> 240	7/7	100
SAO + vehicle ($n = 10$)	80 ± 10*	0/10	0
SAO + U-83836E (7.5 mg/kg)	135 ± 16**	5/10	50
SAO + U-83836E (15 mg/kg)	187 ± 13**	6/10	60
SAO + U-83836E (30 mg/kg)	235 ± 15**	8/10	80

The total number of animals in each group is indicated in parentheses. Statistical analysis was performed by using Fisher's exact probability test. Values are mean ± s.e.m.; *$p < 0.001$ vs basal; **$p < 0.001$ vs SAO + vehicle.

in a concentration-dependent manner by ACh (10 nM–1 μM). The relaxant effect following the 1 μM ACh in aortic rings from SAO shocked rats was significantly lower (40.1 ± 3.2%) in SAO-shocked rats than in Sham rats (sham + vehicle = 77.3 ± 2.7%; sham + U-83836E = 76.1 ± 3.2%). The administration of U-83836E significantly improved the responsiveness to ACh (Figure 3).

Endothelium-independent relaxation was studied also by evaluating the effect of sodium nitroprusside (15–30 nM) in aortic rings precontracted with PE (100 nM) did not show any difference between sham animals and SAO-shocked rats. The relaxant effect following 30 nM sodium nitroprusside in aortic rings was 83 ± 3.2% in sham rats and 81 ± 2.3% in SAO-shocked rats.

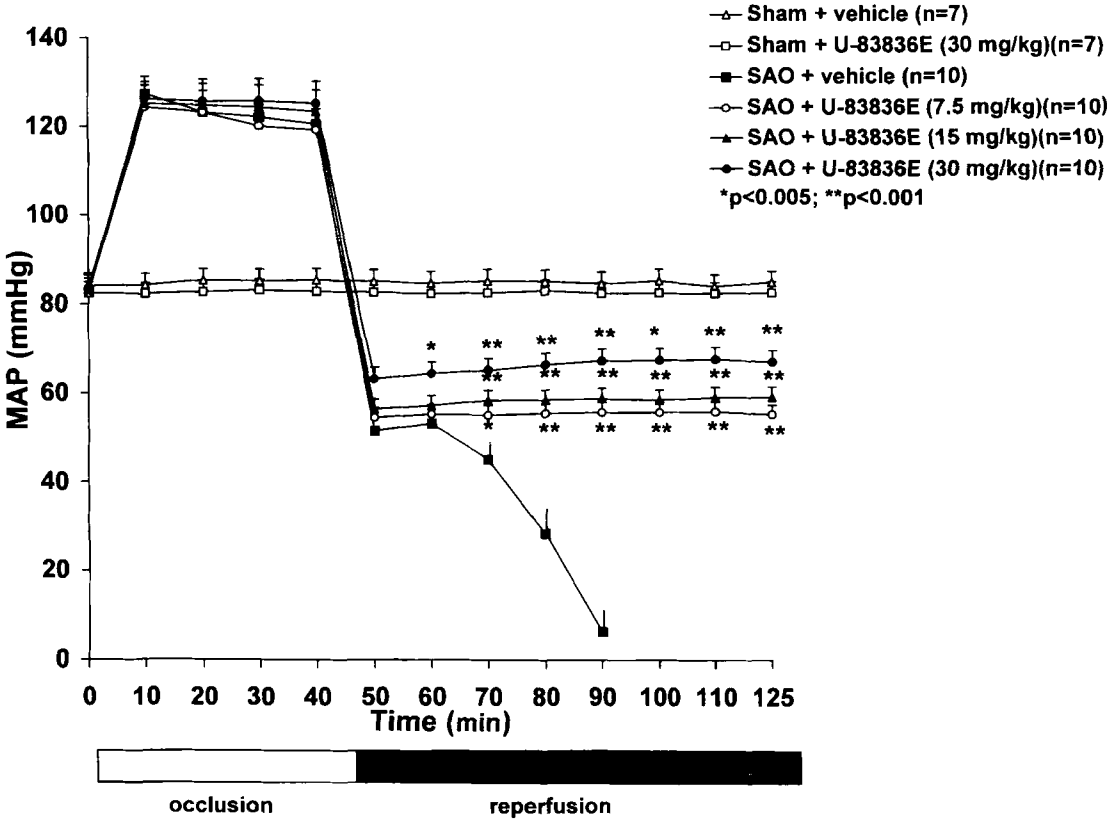

FIGURE 2 Effect of U-83836E or vehicle in mean arterial blood pressure (MAP) in rats subjected to splanchnic artery occlusion shock. Each point represents the mean ± s.e.m. The total number of animals is reported in parentheses.

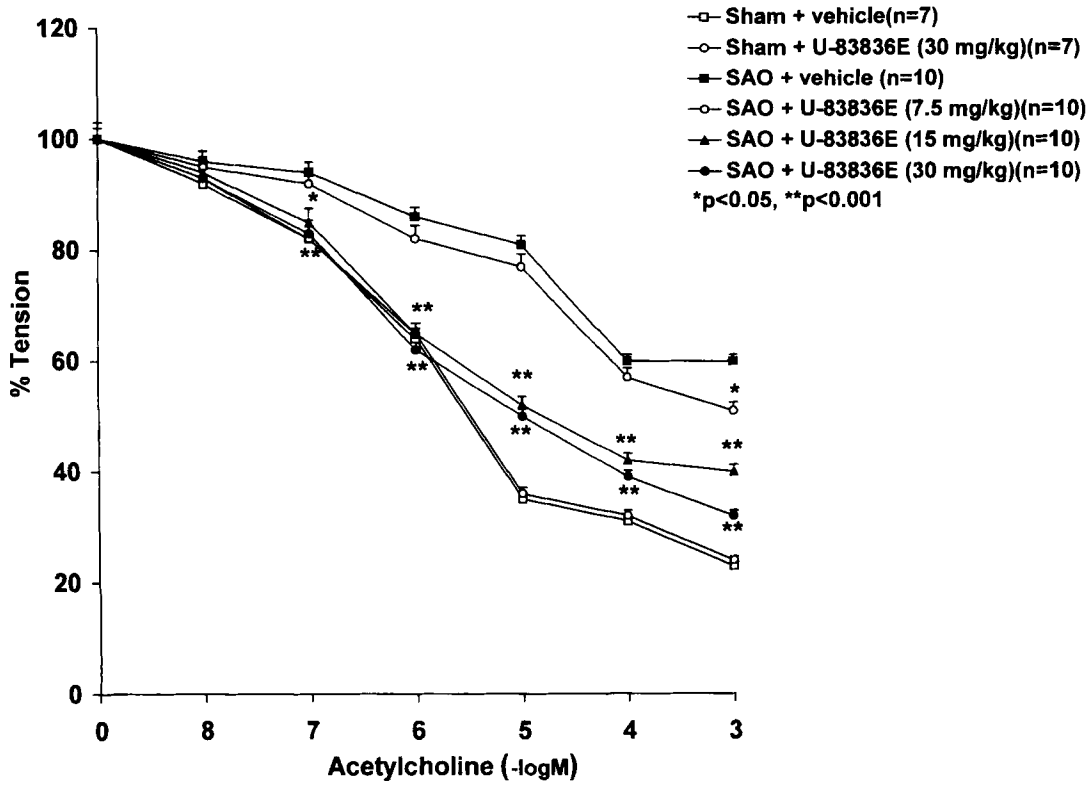

FIGURE 3 Relaxant effect of acetylcholine in aortic rings (pre-contracted with phenylephrine, 100 nM) of splanchnic artery occlusion shocked rats given either U-83836E or vehicle. Each point represents mean ± s.e.m. The total number of animals is reported in parentheses.

DISCUSSION

The data so far accumulated on SAO shock suggest that reperfusion is the main mechanism for the high death rate in this type of experimental shock: in fact death of the animals invariably occurs within 75–90 min after the release of occlusion.[12] Free radicals, particularly oxygen-derived such as superoxide radical, hydrogen peroxide, and hydroxyl radicals are implicated in the cellular damage that follows ischaemia and reperfusion: their toxicity is mainly due to their ability to induce lipid peroxidation.[13,14] There is a growing body of evidence which indicates that reperfusion of ischemic tissue leads to an acute inflammatory response in which neutrophils play a central role. Neutrophils may promote cellular damage by releasing superoxide radicals, proteolytic enzymes and cytokines.[16] These mediators can induce tissue damage and thought to contribute to the inflammatory process. Experimental strategies which have involved prevention of activation or depletion of neutrophils have been shown to reduce reperfusion injury.[15] Anti-inflammatory drugs that reduce white cell activation, or monoclonal antibodies directed against the cytokine tumor necrosis factor-α have been shown to significantly reduce necrosis extension in rats.[18,19] The product of degradation of lipid peroxidation may be one among the potential sources of the localized generation of chemotactic activity which induces neutrophil activation and accumulation during tissue ischemia/reperfusion. To confirm such as hypothesis we performed experiments with U-83836E, an analogue

of vitamin E and potent inhibitor of lipid peroxidation. It has been suggested that TNF-α might also promote the adherence of leukocytes to the endothelium.[20] Our shocked rats had increased serum levels of TNF-α and the administration of U-83836E reduced the increased concentrations of this inflammatory cytokine and reverted leukopenia. Therefore the inhibition of TNF-α serum levels might also contribute to the reduction in leukocyte accumulation. Besides TNF-α, these data, taken together, strongly suggest that the phenomenon of lipid peroxidation significantly contributes, *in vivo*, to the mechanisms of leukocyte accumulation.

The involvement of the L-arginine/nitric oxide (NO) pathway in the vascular dysfunction that occurs in experimental shock has been proposed.[21] Vane *et al.*[22] proposed that the L-arginine/nitric oxide pathway may play a key role in the regulation of vascular tone. In all cell types so far studied NO is generated following oxidation and cleavage of the terminal nitrogen atom (s) of L-arginine by the enzyme NO synthase. The constitutive enzyme is Ca^{2+}-dependent and releases picomolar amounts of NO for a short period following receptor stimulation. In contrast the enzyme found in the macrophages is induced following stimulation with cytokines or endotoxin, is Ca^{2+}-independent and releases nanomolar amounts of NO for a long period.[23] So far as SAO shock is concerned, experimental evidence has shown that NO donors exert beneficial effects in feline SAO shock,[24] thus suggesting that a dysfunction in the L-arginine/nitric oxide (i.e. a decrease in NO production derived by the constitutive and endothelial NO synthase) pathway might also be present in this type of experimental shock. In accordance with this hypothesis, our present data show that aortic rings from SAO-shocked rats had a marked reduced responsiveness to vasorelaxant effects of ACh. This result indicates that NO generated by the endothelial and constitutive NO synthase is reduced in SAO shocked rats. The administration of U-83836E improved the responsiveness to ACh of aortic rings from SAO-shocked rats. These data led us to hypothesize that free radicals and lipid peroxidation may induce, at least in part, endothelial dysfunction in SAO shock.

In conclusion, our study suggests that the lazaroid U-83836E possesses antishock and endothelial protective properties and it may have the potential for protective use in shock therapy.

Acknowledgements

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