British Journal of Pharmacology (1999) 127, 498–504
http://www.stockton-press.co.uk/bjp

Tacrolimus suppresses tumour necrosis factor-α and protects against splanchnic artery occlusion shock

*,[1]Francesco Squadrito, [1]Domenica Altavilla, [2]Giovanni Squadrito, [1]Marcella Ferlito, [1]Giuseppe M. Campo, [1]Mariarita Arlotta, [1]Serafino Grimaldi, [2]Cristina Quartarone, [2]Antonino Saitta, & [1]Achille P. Caputi

[1]Institute of Pharmacology, School of Medicine, University of Messina, Piazza XX Settembre, 4 98121 Messina, Italy and [2]Department of Internal Medicine, School of Medicine, University of Messina, Piazza XX Settembre, 4 98121 Messina, Italy

1 Tumour necrosis factor (TNF-α) is a pleiotropic cytokine which is deeply involved in the pathogenesis of splanchnic artery occlusion (SAO) shock. Tacrolimus, formerly known as FK506, is a macrolide antibiotic, that blocks the transcription of several proinflammatory cytokines including TNF-α.

2 Male anaesthetized rats were subjected to clamping of the splanchnic arteries for 45 min. This surgical procedure resulted in an irreversible state of shock (SAO shock). Sham operated animals were used as controls. SAO shocked rats had a decreased survival rate (0% at 4 h of reperfusion, while sham shocked rats survived more than 4 h), enhanced serum TNF-α concentrations (415 ± 12 U ml^{-1}), decreased mean arterial blood pressure (MAP), leukopenia and increased ileal leukocyte accumulation studied by means of myeloperoxidase activity (MPO $= 7.5 \pm 0.3$ U g^{-1} tissue). Moreover aortic rings from shocked rats showed a marked hyporeactivity to phenylephrine (PE, 1 nM – 10 μM), reduced responsiveness to acetylcholine (ACh, 10 nM – 10 μM) and increased staining for intercellular adhesion molecule-1 (ICAM-1). Furthermore increased mRNA for TNF-α was observed in peritoneal macrophages of SAO shocked rats.

3 Tacrolimus (100 μg kg^{-1}, 5 min after splanchnic arteries occlusion) increased survival rate (SAO + Tacrolimus = 100% at 4 h of reperfusion), reverted the marked hypotension, reduced serum TNF-α (15 ± 3 U ml^{-1}), ameliorated leukopenia, reduced ileal MPO (0.9 ± 0.01 U g^{-1} tissue), restored to control values the hyporeactivity to PE, improved the reduced responsiveness to ACh and blunted the enhanced immunostaining for ICAM-1 in the aorta. Finally tacrolimus suppressed cytokine mRNA levels in peritoneal macrophages.

4 The data suggest that tacrolimus may represent a new therapeutic approach in circulatory shock.

Keywords: Splanchnic artery occlusion shock; vascular dysfunction; tacrolimus; TNF-α

Abbreviations: ACh, acetylcholine; ICAM-1, intercellular adhesion molecule-1; PE, phenylephrine; SAO shock, splanchnic arterial occlusion; TNF-α, tumour necrosis factor-α

Introduction

Tacrolimus (formerly known as FK506) is a macrolide antibiotic produced from the fermentation broth of *Streptomyces tsukubaenis*. Although it bears no structural homology to the immunosuppressant cyclosporin (CsA), its mode of action closely parallels that of the latter drug. Tacrolimus differs from (CsA) in its potency, exhibiting similar *in vitro* effects at concentrations 100 times lower than those of CsA (Kino *et al*., 1987).

Tacrolimus appears to exert its effects principally by altering gene expression in target cells (Bierer *et al*., 1991). It binds to specific cellular proteins of the immunophilin class termed FK506-binding proteins (FKBPs), of which at least four have been described: FKBPs 12, 13, 25 and 59 (Fruman *et al*., 1994; Schreiber, 1991).

These proteins have peptidyl-prolyl-cis-trans isomers, or rotamase activity, and are postulated to accelerate protein folding. The complex tacrolimus-FKBP inhibits calcineurin phosphatase, thus blunting calcium-dependent events such as interleukin-2 gene transcription, nitric synthase activation, cell degranulation and apoptosis (Wiederrecht *et al*., 1993).

Immunophilins of the FKBP class have been identified associated with the glucocorticoid and progesteron receptors. Tacrolimus binds to the steroid receptor-associated heat-shock protein 56 (hsp56), thus protecting the glucocorticoid receptor from inactivation and degradation (Schmitt *et al*., 1993; Tai *et al*., 1992). This results in enhanced translocation of the glucocorticoid receptor to the nucleus, with subsequent potentiation of binding of the steroid receptor to the glucocorticoid receptor response element (Ning & Sanchez, 1993; Oyanagui, 1994). Thus transcription of several proinflammatory cytokines, including Interleukin-1 (IL-1) and Tumour Necrosis Factor (TNF-α) is inhibited (Ray *et al*., 1990; Guyre *et al*., 1988). Indeed tacrolimus has been shown to affect endotoxin-induced pathologies (Hattori & Nakanishi, 1995; Whitcup *et al*., 1998), nitric oxide generation as a predictive parameter of acute allograft rejection (Devlin *et al*., 1994), Intercellular adhesion molecule 1 (ICAM-1) expression (Arreaza *et al*., 1995; Hayashi *et al*., 1996) and nitric oxide generation in smooth muscle cells (Akita *et al*., 1994).

Occlusion of the major splanchnic arteries followed by reperfusion in anaesthetized rats results in an irreversible circulatory failure and shock (splanchnic artery occlusion shock; SAO shock) (Squadrito *et al*., 1996). It has been demonstrated that in SAO shock a marked and composite vascular dysfunction is present in which TNF-α plays an important role (Squadrito *et al*., 1994a). Indeed aortic rings from shocked rats showed a marked hyporeactivity to

*Author for correspondence; E-mail: squadrito@csnet.it

phenylephrine and removal of endothelium did not restore the phenylephrine-induced contractile responses to the values of the sham animal, thus suggesting that smooth muscle cells are involved in the hyporesponsiveness to phenylephrine. This complex dysfunction is probably the result of an increase in the endogenous NO produced by the inducible NO synthase activated by TNF-α.

Therefore the aim of our study was to investigate whether tacrolimus exerts protective effects on the pathological sequelae associated with SAO shock, by reducing TNF-α and modulating vascular dysfunction. We found that tacrolimus inhibits the inflammatory cytokine reverts vascular failure and therefore it may represent a new treatment for acute circulatory shock.

Methods

Animal preparation

Male Sprague-Dawley rats weighing 200–250 g were permitted access to food and water *ad libitum*. The rats were anaesthetized with urethane (1.3 g kg^{-1}, i.p.). 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 a warmed 0.9% NaCl solution. Rats were given heparin (1000 U kg^{-1}, i.v.) and were observed for a 30 min stabilization period prior to either splanchnic ischaemia or sham ischaemia. Splanchnic artery occlusion shock (SAO) was induced by clamping both the superior mesenteric artery and the celiac trunk so as to produce a total occlusion of these arteries for 45 min. The clamps were then removed. Following reperfusion the rats were observed for 4 h. Sham-operated rats were subjected to the same surgical procedures as SAO except the arteries were not occluded.

Survival evaluation and arterial blood pressure monitoring

A first group of animals was used to study survival and arterial blood pressure. Tacrolimus (25; 50 and 100 μg kg^{-1} as an i.v. infusion of 0.5 ml kg^{-1} min^{-1}) or vehicle (NaCl 0.9%; 0.5 ml kg^{-1} min^{-1} for 15 min as an i.v. infusion) were injected 5 min following the onset of reperfusion. We chose this dosage regimen of tacrolimus in agreement with previous experiments in the rat (Matsuda *et al*., 1998; Wakita *et al*., 1998). Survival was evaluated for 4 h after the onset of reperfusion and expressed either as survival rate or survival time. A group of animals was also implanted with cannulae (PE 50) into the left common carotid artery, as described elsewhere (Squadrito *et al*., 1994a). The arterial catheter was connected to a pressure transducer. The pressure pulse triggered a cardiotachometer, 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

A third group of animals was used to measure TNF-α, myeloperoxidase activity, leukocyte count, vascular reactivity and intercellular adhesion molecule-1 (ICAM-1) expression on vascular endothelium.

Killing of L929 mouse tumour cells was used to measure TNF-α levels in serum and in peritoneal macrophage supernatants on the basis of a standard microelisa assay (Ruff & Gifford, 1980). L929 cells in RPMI 1640 medium containing 5% foetal calf serum 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 1 : 2 dilution of serum (drawn 70 min following the onset of reperfusion) and supernatants of peritoneal macrophages, harvested at the same time as the serum using a previously described method (Altavilla *et al*., 1989), were made in the above-described medium containing 1.0 μg of actinomycin D per ml and 100 μl volumes of each dilution were added to the wells. One TNF-α unit 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, Milan, Italy). To test whether the cytotoxicity was due to the presence of TNF-α or to other factor(s), 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.

Extraction of mRNA from peritoneal macrophages and polymerase chain reaction

Macrophages were obtained at different time points (0. 25 and 45 min of occlusion and 10 and 70 min following the release of occlusion) by peritoneal lavage with RPMI (1640) from animals subjected to either sham shock or SAO shock. Macrophages were counted and diluted to 10^6/ml. the cells were then incubated in Petri dishes at 37°C in a humidified 5% CO_2 atmosphere for 1 h. After adhesion of the cells to coverslips in Petri dishes for 1 h and removal of nonadherent cells by washing, macrophages were lysed directly in the culture dish by the addition of RNAzol^{TM}B (1 ml per 3.5 cm Petri dish or 10 cm^2). RNA was solubilized by passing the lysate few times through the pipette. One-hundred-nanogram samples of mRNA were transcribed into cDNA using 400-U reverse transcriptase (200 U μl^{-1}; BRL U.S.A.) and 0.5 μg oligo(dT) 12–18 primer (BRL) for 30 min at 42°C. The reaction mixture (50 μl) conatined (mM): Tris-HCl 25 (pH 8.3), KCl 37.5 dithiothreitol (DTT) 10 $MgCl_2$ 1.5 and dNTP 10 of each (Perkin Elmer U.S.A.). Polymerase chain reaction (PCR) primers for TNF-α and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were purchased from Celbio S.r.l. (Milan, Italy). G3PDH mRNA transcripts were monitored to determine constitutive expression. An aliquot containing one-tenth of the cDNA synthesized from mRNA was amplified in a 50-μl volume with the reagents supplied in the GeneAMP PCR kit (Perkin Elmer, U.S.A.). Using the HBTRE02 Hybaid Thermal Cycler (Celbio, Milan, Italy) amplification was initiated by 1 min denaturation at 94°C for one cycle and then followed by three temperature steps for PCR including 30 s of denaturation at 94°C, 30 s of annealing at 55°C and 1 min of polymerization at 72°C. This process was continued for 25, 30 or 35 cycles of amplification depending on the abundance of the cytokine message. After the last cycle of amplification, the samples were incubated for 7 min at 72°C. An aliquot of each amplified sample was electrophoresed through 2% agarose (UltraPure, molecular biology grade, Sigma) and stained with 0.5 μg ethidium bromide ml^{-1} in tris-Borate ethylene diaminetetracetic acid (EDTA) buffer. The resultant gel was illuminated in a darkroom with a fixed camera. The captured image, sent to an image analysis software (BIO-PROFIL Celbio, Milan, Italy), was subjected to a densitometric analysis and then printed on VPN-120 printer.

Myeloperoxidase activity and leukocyte count

Leukocyte accumulation was investigated using the activity of myeloperoxidase (MPO). MPO activity was determined in intestinal mucosa, as previously reported (Mullane *et al*., 1985). The samples of intestinal mucosa were obtained at 0 min before occluding the splanchnic arteries and at 70 min following the onset of reperfusion. The samples were first homogenized in a solution containing 20 mM of potassium phosphate buffer (pH 7.4), 0.01 M EDTA, 50 U ml^{-1} of a protease inhibitor (aprotinin) in proportions of 1 : 10 (w v^{-1}) and then centrifuged for 30 min at $20{,}000 \times g$ at 4°C. The supernatant of each sample was then discarded and the pellet was immediately frozen on dry ice. The samples were kept at a temperature of 0°C for 14 h before sonication. After thawing, the resulting pellet was added to a buffer solution consisting of 0.5% hexacyltrimethylammonium bromide (Sigma Chemical Co., St. Louis, MO, U.S.A.) dissolved in 50 mM potassium phosphate buffer (pH 6) containing 30 U ml^{-1} of protease inhibitor. Each sample was then sonicated (intensity 2) for 1 min at a temperature of 4°C. After sonication the samples were allowed to chill on ice for approximately 30 min, and then they were centrifuged for 30 min at $40{,}000 \times g$ at 4°C. An aliquot of the supernatant was then allowed to react with 0.167 mg ml^{-1} o-dianisidine dihydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.) and 0.001% H_2O_2 and the rate of change in absorbance was measured at 405 nm in a microtitre plate reader. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide min at 25°C and was expressed in units g weight (U g^{-1} of tissue). Tail vein blood samples for the leukocyte count were taken at 0 min before initiating reperfusion and at 70 min after the onset of reperfusion. The number of leukocytes ($WBC \times 10^3 \times mm^3$) is reported as mean ± s.e.mean.

Isolated aortic rings

Thoracic aortae were removed 70 min after reperfusion and placed in cold Krebs' solution of the following composition (nM): 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; 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 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 prostaglandins and their metabolites. Developed tension was measured with an isometric force transducer and recorded on a polygraph (Ugo Basile, Varese, Italy). After an equilibrium 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 tissue was 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 a per cent decrease of contractile force. Concentration-response curves were obtained by cumulative concentrations of PE (1 nM – 10 μM) to intact or endothelium denuded aortic rings.

Immunohistochemistry

ICAM-1 expression was studied in thoracic aortae and in superior mesenteric arteries collected 70 min following the release of occlusion. Immunohistochemical evaluation was accomplished by staining 5 μm-thick cryostat sections according to the avidin-biotin-peroxidase complex procedure (Hsu *et al*., 1981). An average of seven sections per immunohistochemical stain was cut from each sample, airdried for 30 min and then fixed in cold acetone for 10 min. Endogenous peroxidases were blocked with horse serum for 15 min at room temperature prior to incubation with primary antibodies. Monoclonal antibodies consisted of mouse monoclonal antibodies raised against rat ICAM-1 (clone: IA 29, subclass IgG_1) and were obtained from British Bio-technology Products Ltd (Abingdon, U.K.). A monoclonal mouse IgG_1 antibody was used for controls. Biotinylated, species-specific second layer reagents were then applied, followed by avidin-biotin-horse radish peroxidase complex as a chromogenic substrate, as previously reported (Squadrito *et al*., 1992). The experiments were carried out by two observers (PC, GF) who were unaware of the experimental protocol. The microscope image was sent to a computer assisted image analyzer that analysed the changes in staining. Densitometric analysis of the captured image was performed on a PC computer using an image analysis software.

Drugs

Acetylcholine chloride, phenylephrine hydrochloride and indomethacin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Tacrolimus was obtained from APHOGEPHA Arzneimittel GmbH, Dresden, Germany.

Statistical analysis

Data are expressed as means ± s.e.mean and were analysed by analysis of variance for multiple comparison of results; Duncan's multiple range test was used to compare group means. In all cases, a probability error of less than 0.05 was selected as criterion for statistical significance. For survival data, statistical analysis was done with Fisher's exact probability test.

Results

Survival

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 rats survived the entire 4 h observation period. In contrast in rats treated with the vehicle, occlusion and reperfusion of the splanchnic region produced a profound shock state characterized by a high lethality; no rat survived at 2 h of reperfusion (survival 73 ± 11 min). Administration of tacrolimus increased in a dose-dependent manner survival rate and time in SAO rats. Surviving animals were still alive 24 h following the induction of splanchnic artery occlusion shock.

Serum and macrophage TNF-α

Serum and macrophage levels of TNF-α were undetectable in sham operated rats treated either with vehicle or tacrolimus. TNF-α was significantly increased in both serum and macrophages collected from SAO rats at the end of the

Table 1 Effects of tacrolimus on survival rate, percentage survival and survival time in splanchnic artery occlusion (SAO) shock

	Hours following the onset of reperfusion				
	2		*4*		
Treatment	*Surviving*	(%)	*Surviving*	(%)	*Survival time* (min)
Sham + vehicle	10/10	100	10/10	100	>240
Sham + tacrolimus (100 μg kg^{-1})	10/10	100	10/10	100	>240
SAO + vehicle	0/10	0	0/10	0	73±11
SAO + tacrolimus (25 μg kg^{-1})	0/10	0	0/10	0	76±9
SAO + tacrolimus (50 μ kg^{-1})	6/10	60*	4/10	40	164±13*
SAO + tacrolimus (100 μg kg^{-1})	10/10	100**	10/10	100*	>240

Tacrolimus (25, 50 and 100 μg kg^{-1} as an i.v. infusion of 0.5 ml kg^{-1} min^{-1}) or vehicle (NaCl 0.9%; 0.5 ml kg^{-1} min^{-1} for 15 min as an i.v. infusion) were injected 5 min following the onset of reperfusion. Survival was monitored for 4 h. *$P<0.05$ vs SAO + vehicle; **$P<0.001$ vs SAO + vehicle.

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

Treatment	*Serum TNF-α* (U ml^{-1})	*Macrophage TNF-α* (U ml^{-1})
Sham + vehicle	N.D.	N.D.
Sham + tacrolimus	N.D.	N.D.
SAO + vehicle	415±12	201±11
SAO + tacrolimus	15±3*	9±4*

Serum and macrophages were collected 70 min following the onset of reperfusion. Each point represents mean±s.e.mean from six experiments. Animals received tacrolimus (100 μg kg^{-1} as an i.v. infusion of 0.5 ml kg^{-1} min^{-1}) or vehicle (NaCl 0.9%; 0.5 ml kg^{-1} min^{-1} for 15 min as an i.v. infusion) 5 min following the onset of reperfusion. *$P<0.001$ vs SAO + vehicle. N.D., not detectable.

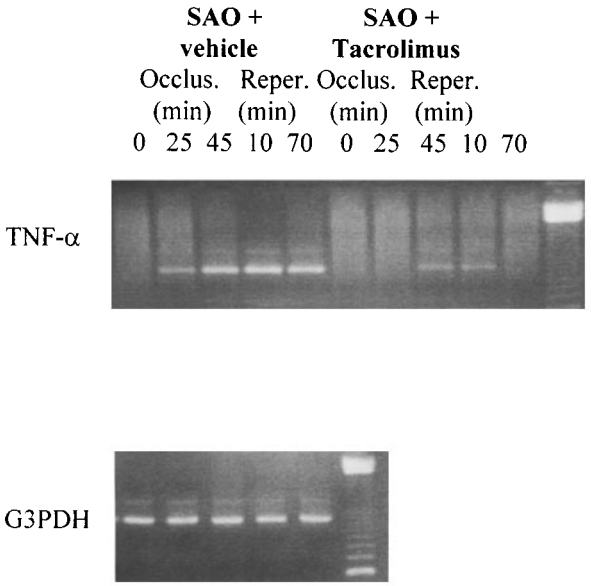

Figure 1 Macrophage TNF-α mRNA expression in peritoneal macrophages harvested at different time points in rats subjected to splanchnic artery occlusion (SAO) shock and treated with tacrolimus (100 μg kg^{-1} as an i.v. infusion of 0.5 ml kg^{-1}min^{-1}) or vehicle (NaCl 0.9%; 0.5 ml kg^{-1} min^{-1} for 15 min as an i.v. infusion), both injected 5 min following the onset of reperfusion.

reperfusion period (Table 2). The administration of tacrolimus significantly blunted the macrophage and serum levels of this cytokine.

TNF-α mRNA expression in peritoneal macrophages

Figure 1 shows representative autoradiograms highlighting mRNA expression for TNF-α in peritoneal macrophage of SAO shocked rats treated with vehicle or tacrolimus. Increased macrophage mRNA levels of TNF-α were found in SAO shocked rats treated with vehicle 25 min after occlusion of arteries and were significantly increased during reperfusion. The administration of tacrolimus markedly suppressed macrophage TNF-α expression (Figure 1).

Leukocyte infiltration

Leukocyte infiltration was determined by measurement of the myeloperoxidase (MPO) activity in rats at different times: 0 min before occlusion (basal; at the beginning of the experiment) and 70 min following the onset of reperfusion. MPO levels were significantly increased in the ileum (7.5±0.3 U g^{-1} tissue) at 70 min after reperfusion (Table 3) in SAO shocked rats treated with the vehicle.

Administration of tacrolimus significantly lowered the increase in ileal (0.9±0.01 U g^{-1} tissue) MPO activity (Table 3).

Leukocyte count

The administration of vehicle or tacrolimus did not modify the white blood cell count in sham-operated rats (Table 3). In contrast, splanchnic occlusion shock produced a marked leukopenia. Leukocyte count was markedly decreased at the end of reperfusion (70 min). The administration of tacrolimus significantly ameliorated this leukopenia (Table 3).

ICAM-1 staining on vascular endothelium

ICAM-1 presence was studied in thoracic aorta and in superior mesenteric arteries collected 70 min following the release of occlusion. Immunohistochemical evaluation indicated that a very low constitutive staining of ICAM-1 was present in sham operated animals (Figure 2). By contrast samples obtained from SAO shocked rats had an increase in ICAM-1 staining. Aortic and mesenteric endothelium obtained from SAO shocked rats treated with tacrolimus showed a marked reduction in ICAM-1 immunostaining (Figure 2).

Vascular reactivity of aortic rings

Addition of phenylephrine (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 was significantly smaller in aortic rings obtained

from SAO shocked rats than from sham-operated rats (Figure 3). Administration of tacrolimus significantly improved the responsiveness of aortic rings obtained from SAO shocked rats to ACh (Figure 3).

In intact aortic rings prepared from SAO shocked rats, the contractile response to PE (1 nM – 10 μM) was significantly reduced. The maximum force of contraction induced by 10 μM PE in aortic rings from sham rats was 1.7 ± 0.8 g mg^{-1} tissue, whereas it was 0.9 ± 0.3 g mg^{-1} tissue in rings from SAO shocked rats. Removal of the endothelium did not increase the constrictor response elicited by PE in rat aortic rings obtained from both SAO shocked rats and sham operated animals (Figure 4). However, the contractile response to PE in endothelium denuded aortic rings was also significantly smaller in SAO shocked rats than in sham operated animals. Administration of tacrolimus improved the impaired contractile response to PE in SAO rats (Figure 4).

Mean arterial blood pressure

Occlusion of the splanchnic arteries produced a marked increase in mean arterial blood pressure. Subsequently mean arterial blood pressure decreased upon the release of the occlusion (Figure 5). The administration of tacrolimus significantly blunted the reduction in mean arterial blood pressure (Figure 5).

Discussion

It has been suggested that the pleiotropic cytokine TNF-α plays an important role in the pathogenesis of splanchnic artery occlusion shock (Squadrito *et al*., 1992). In fact TNF-α may induce vascular dysfunction and cause leukocytes to adhere to the vascular endothelium where they discharge deleterious mediators (i.e. oxygen free radicals, leukotrienes, cytokines etc) able to amplify the vascular damage. This latter phenomenon involves the interaction between several adhesive receptors (adhesion molecules) present on both the endothelial and leukocyte surfaces.

Table 3 Effects of tacrolimus on myeloperoxidase (MPO) activity of ileum and on white blood cell count (WBC) of rats subjected to splanchnic artery occlusion (SAO) shock

	Time (min)	
	Basal	*Reperfusion*
	0	*70*
MPO activity in ileum (Ug^{-1} tissue)		
Sham + vehicle	0.3 ± 0.01	0.4 ± 0.02
Sham + tacrolimus	0.6 ± 0.06	0.5 ± 0.04
SAO + vehicle	0.5 ± 0.03	7.5 ± 0.3
SAO + tacrolimus	0.7 ± 0.05	0.9 ± 0.01*
White blood cell count $WBC^{-1} \times 10^3$ mm^3		
Sham + vehicle	11.7 ± 1.5	12.6 ± 1.8
Sham + tacrolimus	11.1 ± 3.1	11.9 ± 1.5
SAO + vehicle	13.9 ± 3.2	5.8 ± 2.1
SAO + tacrolimus	12.8 ± 1.6	11.6 ± 3.8*

Each point represents the mean ± s.e.mean of seven experiments. *$P < 0.001$ vs SAO + vehicle. Animals received tacrolimus (100 μg kg^{-1} as an i.v. infusion of 0.5 ml kg^{-1} min^{-1}) or vehicle (NaCl 0.9%; 0.5 ml kg^{-1} min^{-1} for 15 min as an i.v. infusion) 5 min following the onset of reperfusion.

As far as vascular dysfunction is concerned, it has been suggested that TNF-α may impair the release of NO from endothelial cells (Aoki *et al*., 1990), thus leading to a reduced production of endothelial-derived relaxing factor. However, administration of recombinant human TNF-α 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). This phenomenon is likely due to a TNF-α-induced stimulation of the Ca^{2+} independent NO synthase in vascular smooth muscle (Busse & Mulsch, 1990).

Recent evidence has suggested that adhesion mechanisms supporting leukocyte adhesion and accumulation to the endothelium are present in ischaemic states (Simmons *et al*., 1988; Ioculano *et al*., 1994).

Splanchnic artery occlusion shock is an experimental model characterized by the presence of adhesion mechanism for leukocyte accumulation (Squadrito *et al*., 1994b) and by a marked vascular dysfunction (Squadrito *et al*., 1994a). We have shown that these two important pathological aspects of this type of experimental model of circulatory shock are due to

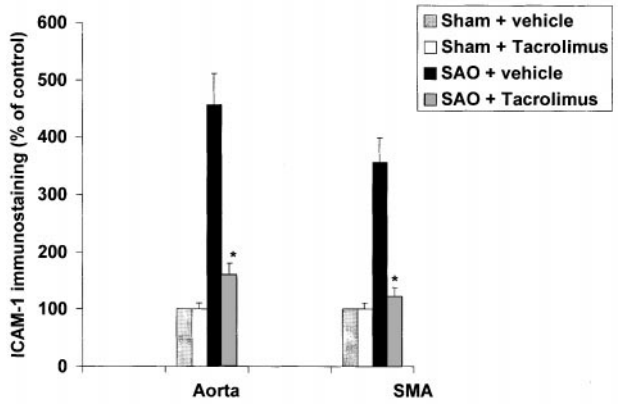

Figure 2 Effects of tacrolimus (100 μg kg^{-1} as an i.v. infusion of 0.5 ml kg^{-1} min^{-1}) or vehicle (NaCl 0.9%; 0.5 ml kg^{-1} min^{-1} for 15 min as an i.v. infusion), both injected 5 min following the onset of reperfusion, on immunohistochemical staining for ICAM-1 in aortic (Aorta) and superior mesenteric artery (SMA) endothelium from rats subjected to splanchnic artery occlusion (SAO) shock. Each point represents the mean ± s.e.mean of seven experiments. *$P < 0.01$ vs SAO + vehicle.

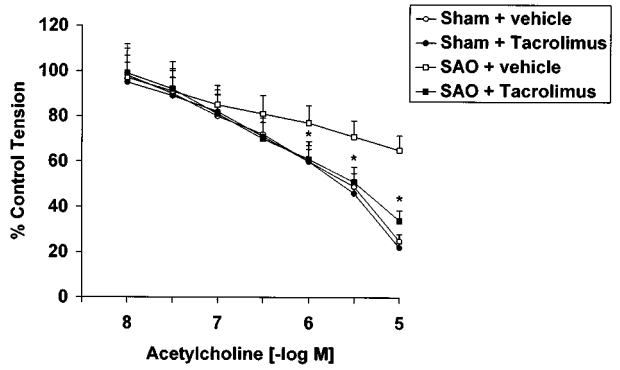

Figure 3 Relaxant effects of acetylcholine (ACh) in aortic rings (contracted with phenylephrine, 100 nM) of sham operated rats and rats subjected to splanchnic artery occlusion (SAO) shock treated with tacrolimus (100 μg kg^{-1} as an i.v. infusion of 0.5 ml kg^{-1} min^{-1}) or vehicle (NaCl 0.9%; 0.5 ml kg^{-1} min^{-1} for 15 min as an i.v. infusion), both injected 5 min following the onset of reperfusion. Each point represents the mean ± s.e.mean from six experiments. *$P < 0.01$ vs SAO + vehicle.

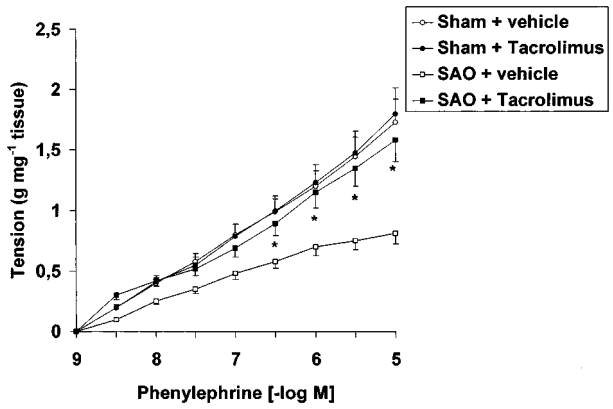

Figure 4 Contractile response to cumulative doses of phenylephrine (PE) in endothelium denuded aortic rings from sham-operated rats and rats subjected to splanchnic artery occlusion (SAO) shock treated with tacrolimus (100 μg kg^{-1} as an i.v. infusion of 0.5 ml kg^{-1} min^{-1}) or vehicle (NaCl 0.9%; 0.5 ml kg^{-1} min^{-1} for 15 min as an i.v. infusion), both injected 5 min following the onset of reperfusion. Each point represents the mean$\pm$s.e.mean of seven experiments. $P<0.02$ vs SAO + vehicle.

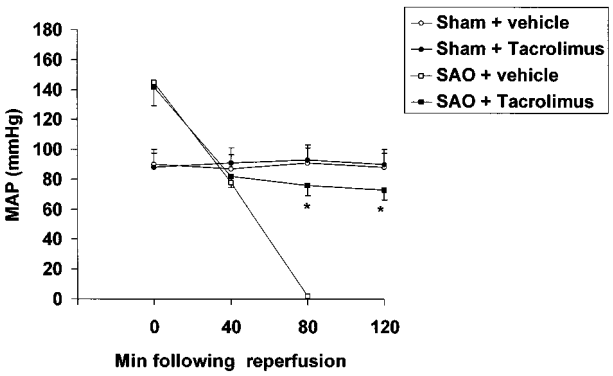

Figure 5 Effects of tacrolimus (100 μg kg^{-1} as an i.v. infusion of 0.5 ml kg^{-1} min^{-1}) or vehicle (NaCl 0.9%; 0.5 ml kg^{-1} min^{-1} for 15 min as an i.v. infusion), both injected 5 min following the onset of reperfusion, on mean arterial blood pressure (MAP) of sham operated rats and rats subjected to the splanchnic artery occlusion (SAO) shock. Each point represents the mean$\pm$s.e.mean of six experiments. *$P<0.01$ vs SAO + vehicle.

increased production of TNF-α. Tacrolimus has been shown to suppress several cytokines including TNF-α. Therefore we test the hypothesis that tacrolimus may exert anti-shock effect *in vivo* by an inhibition of this inflammatory cytokine, and we investigated the effects of tacrolimus in splanchnic artery occlusion shock. We in fact decided to study the antishock effect of Tacrolimus in SAO shock, primarily because TNF-α plays a pivotal role in pathogenesis of this type of experimental shock (Squadrito *et al.*, 1992).

Our results indicate that tacrolimus reduced the enhanced macrophage and serum levels of TNF-α. This effect is a consequence of a direct inhibition of this inflammatory cytokine: in fact macrophages harvested at several time points from splanchnic artery occlusion shocked rats showed a marked increase in mRNA levels for TNF-α. Indeed peritoneal macrophages represent one of the most important sources of TNF-α in SAO shock (Squadrito *et al.*, 1992). Tacrolimus blunted the message for the inflammatory cytokine. This finding is in agreement with previous findings showing that tacrolimus may down regulate the production of this cytokine, via an inhibition of the mRNA for TNF-α (Guyre *et al.*, 1988).

The administration of tacrolimus reduced ICAM-1 expression, ameliorated leukopenia and decreased MPO activity, an index of leukocyte administration. Since leukocyte-endothelial interaction (more specifically the ICAM-1 dependent leukocyte adhesion) is primed by TNF-α, it can be proposed that tacrolimus, by inhibiting this inflammatory cytokine, limits leukocyte accumulation at the ischaemic sites and finally protects against SAO shock.

Aortic rings from rats subjected to splanchnic artery occlusion shock had a marked reduced responsiveness to vasorelaxant effects of ACh: this finding would indicate the presence of a reduced NO production in this type of experimental shock. However our results also showed the presence of a reduced vascular sensitivity to vasoconstrictor stimuli. This impaired vascular reactivity, as suggested for other models of experimental shock (Szabo & Thiemermann, 1994; Thiemermann *et al.*, 1993; Moncada *et al.*, 1991), is a consequence of an overproduction of NO by the inducible NO synthase (iNOS) (Squadrito *et al.*, 1994a). Therefore all these data, taken together, suggest that in splanchnic artery occlusion shock (i) NO generated by the endothelial NO synthase (eNOS) is blunted, while (ii) NO produced by the iNOS is increased. These opposite effects in splanchnic ischaemia-reperfusion injury are induced by TNF-α (Squadrito *et al.*, 1994a). This inflammatory cytokine in fact either inhibits eNOS and stimulates iNOS. This hypothesis is confirmed by the evidence that an inhibitor of TNF-α synthesis is able to revert this complex vascular dysfunction (Squadrito *et al.*, 1994a). In the present paper aortic rings collected from rats subjected to SAO shock and treated with tacrolimus exhibited a greater contractile response to PE and improved responsiveness to ACh when compared to vehicle treated rats. Thus it can be hypothesized that tacrolimus improve dysfunction by inhibiting the detrimental vascular effects of TNF-α.

In conclusion we have shown that tacrolimus inhibits TNF-α: the tacrolimus-induced inhibition of this inflammatory cytokine, at least in splanchnic artery occlusion shock, increases survival, reduces ICAM-1 expression and leukocyte infiltration in the ileum and improves vascular dysfunction. These findings would suggest that TNF-α inhibition may contribute, at least in part, to the acute vasoprotective effects of tacrolimus.

References

AKITA, K., DUSTING, G.J. & HICKEY, H. (1994). Suppression of nitric oxide production by cyclosporin A and FK506A in rat vascular smooth muscle cells. *Clin. Exp. Pharmacol. Physiol.*, **21,** 231–233.

ALTAVILLA, D., BERLINGHIERI, M.C., SEMINARA, S., IANNELLO, D., FOCA, A. & MASTROENI, P. (1989). Different effects of bacterial lipopolysaccharide on superoxide anion production by macrophages from normal and tumor bearing rats. *Immunopharmacology,* **17,** 99–105.

AOKI, N., JOHNSON III G. & LEFER, A.M. (1990). Beneficial effects of two forms of NO administration in feline splanchnic artery occlusion shock. *Am. J. Physiol.*, **321,** G275–G281.

ARREAZA, G., YOSHIKAWA, N., MUKUTA, T., RETKOVA, E., BARSUK, A., NISHIKAWA, M., MUALLIM, C., MILLER, N., JAMIESON, C. & VOLPE, R. (1995). Expression of intercellular adhesion molecule-1 on human thyroid cells from patients with autoimmune thyroid disease: study of thyroid xenografts in nude and severe combined immunodeficient mice and treatment with FK-506. *J. Clin. Endocrinol. Metab.,* **80,** 3724–3731.

BIERERBE, JIN, Y.J., FRUMAN, D.A., CALVO, V & BURAKOFF, S.J. (1991). FK506 and rapamycin: molecular probes of T-lymphocyte activation. *Transplant. Proc.,* **23,** 2850–2855.

BUSSE, R. & MULSCH, A. (1990). Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. *FEBS Lett.,* **275,** 87–90.

DEVLIN, J., PALMER, R.M., GONDE, C.E., O'GRADY, J., HEATON, N., TAN, K.C., MARTIN, J.F., MONCADA, S & WILLIAMS, R. (1994). Nitric oxide generation. A predictive parameter of acute allograft injection. *Transplantation,* **58(5),** 592–595.

FRUMAN, D.A., BURAKOFF, S.J. & BIERER, B.E. (1994). Molecular actions of cyclosporin A, FK 506 and rapamycin. In: Thomson, A.W. & Starzl, T.F. eds. *Immunosuppressive drugs: developments in anti-rejection therapy*. pp. 15–35. London: Edward Arnold.

GUYRE, P.M., GIRARD, M.T., MORGANELLI, P.M. & MORGANIELLO, P.D. (1988). Glucocorticoid effects on the production and actions of immune cytokines. *J. Steroid. Biochem.,* **30,** 89–93.

HATTORI, Y. & NAKANISHI, N. (1995). Effects of cyclosporin A and FK506 on nitric oxide and tetrahydrobiopeterin synthesis in bacterial lipopolysaccharide-treated J774 macrophages. *Cell Immunol.,* **165,** 7–11.

HAYASHI, K., NAGAMATSU, T., ITO, M. & SUZUKI, K. (1996). Suppression of experimental crescentic-type anti-glomerual basement membrane (GBM) nephritis by FK506 (tacrolimus hydrate) in rats. *Jpn. J. Pharmacol.,* **70,** 43–54.

HSU, S.M., RAINE, L & FANGER, H. (1981). A comparative study of the peroxidase-antiperoxidase method and an avidin-biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies. *Am. J. Clin. Path.,* **75,** 734–737.

IOCULANO, M., SQUADRITO, F., ALTAVILLA, D., CANALE, P., CAMPO, G.M., SAITTA, A. & CAPUTI, A.P. (1994). Antibodies against intercellular adhesion molecule-1 protect against myocardial ischaemia-reperfusion injury in the rat. *Eur. J. Pharmacol.,* **264,** 143–149.

KINO, T., HATANAKA, H. & MYATA, S. (1987). FK-506, a novel immunosuppressant isolated from a streptomyces II. Immunosuppressive effect effect of FK-506 in vitro. *J. Antibiot.,* **40,** 1256–1265.

MATSUDA, T., YAMAGUCHI, Y., MATSUMURA, F., AKIZUKI, E., OKABE, K., LIANG, J., OHSHIRO, H., ICHIGUCHI, O., YAMADA, S., MORI, K. & OGAWA, M. (1998). Immunosuppressants decrease neutrophil chemoattractant and attenuate ischemia/reperfusion injury of the liver in rats. *J. Trauma,* **44,** 475–484.

MONCADA, S., PLAMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.,* **43,** 109–142.

MULLANE, K.M., KRAEMER, M.R. & SMITH, B. (1985). Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium. *J. Pharmacol. Meth.,* **14,** 156–157.

NING, Y.M. & SANCHEZ, E.R. (1993). Potentiation of glucocorticoid receptor-mediated gene expression by the immunophilin ligands FK506 and rapamycin. *J. Biol. Chem.,* **268,** 6073–6076.

OYANAGUI, Y. (1994). Nitric oxide and hydrogen peroxide-mediated gene expression by glucocorticoids and FK506 in histamine paw edema of mice. *Life Sci.,* **55,** PL177–PL185.

RAY, A., LAFORGE, K.S. & SCHGAL, P.B. (1990). On the mechanism for efficient repression of the interleukin-6 promoter by glucocorticoids: enhancer, TATA box, and RNA start site (1nr motif) occlusion. *Mol. Cell. Biol.,* **10,** 5736–5746.

RUFF, M.R. & GIFFORD, G.E. (1980). Purification and physicochemical characterization of rabbit tumor necrosis factor. *J. Immunol.,* **125,** 1671–1675.

SCHMITT, J., POHL, J. & STUNNENBERG, H.G. (1993). Cloning and expression of a mouse cDNA encoding p59, an immunophilin that associates with the glucocorticoid receptor. *Gene,* **132,** 267–271.

SCHREIBER, S.L. (1991). Chemistry and biology of the immunophillins and their immunosuppressive ligands. *Science,* **251,** 283–287.

SIMMONS, D., MAKGOBA, M.W. & SEED, B. (1988). ICAM, an adhesion ligand of LFA-1, is homologous to the neural cell adhesion molecule NCAM. *Nature,* **331,** 624–627.

SQUADRITO, F., ALTAVILLA, D., CANALE, P., IOCULANO, M., CAMPO, G.M., AMMENDOLIA, L., FERLITO, M., ZINGARELLI, B., SQUADRITO, G., SAITTA, A. & CAPUTI, A.P. (1994a). Participation of tumor necrosis factor and nitric oxide in the mediation of vascular dysfunction in splanchnic artery occlusion shock. *Br. J. Pharmacol.,* **113,** 1153–1158.

SQUADRITO, F., ALTAVILLA, D., CANALE, P., IOCULANO, M., CAMPO, G.M., AMMENDOLIA, L., SQUADRITO, G., SAITTA, A., CALAPAI, G. & CAPUTI, A.P. (1994b). Contribution of intercellular adhesion molecule-1 (ICAM-1) in the pathogenesis of splanchnic artery occlusion shock in the rat. *Br. J. Pharmacy,* **113,** 912–916.

SQUADRITO, F., ALTAVILLA, D., IOCULANO, M., CALAPAI, G., ZINGARELLI, B., SAITTA, A., CAMPO, G.M., RIZZO, A. & CAPUTI, A.P. (1992). Passive immunization with antibodies against tumor necrosis factor TNF-α protects from the lethality of splanchnic artery occlusion shock. *Circ. Shock.,* **37,** 236–244.

SQUADRITO, F., ALTAVILLA, D., SQUADRITO, G., CAMPO, G.M., IOCULANO, M., CANALE, P., ROSSI, F., SAITTA, A. & CAPUTI, A.P. (1996). Effects of S-ethylisothiourea, a potent inhibitor of nitric oxide synthase, alone or in combination with a nitric oxide donor in splanchnic artery occlusion shock. *Br. J. Pharmacol.,* **119,** 23–28.

SZABO', C., & THIEMERMANN, C. (1994). Invited opinion: role of nitric oxide in haemorrhagic, traumatic and anaphylactic shock and thermal injury. *Shock,* **2,** 145–155.

TAI, P.K., ALBERS, M.W., CHANG, H., FABER, L.E. & SCHREIBER, S.L. (1992). Association of a 59-Kilodalton immunophilin with the glucocorticoid receptor complex. *Science,* **256,** 1315–1318.

TAKAHASHI, K., ANDO, K., ANO, A., SHIMOSAWA, T., OGATA, E. & FUJITA, T. (1992). Tumor necrosis factor-α induces vascular hyporesponsiveness in Sprague-Dawley rats. *Life Sci.,* **50,** 1437–1444.

THIEMERMANN, C., SZABO', C., MITCHELL, J.A. & VANE, J.R. (1993). Vascular hyporeactivity to vasoconstrictor agents and hemodynamic decompensation in haemorrhagic shock is mediated by nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.,* **90,** 267–271.

WAKITA, H., TOMIMOTO, H., AKIGUCHI, I. & KIMURA, J. (1998). Dose-dependent, protective effect of FK506 against white matter changes in the rat brain after chronic-cerebral ischemia. *Brain Res.,* **792,** 105–113.

WHITCUP, S.M., PLEYER, U., LAI, J.C., MOCHIZUKI, M. & CHAN, C.C. (1998). Topical liposome-encapsulated FK506 for the treatment of endotoxin uveitis. *Ocul. Immunol. Inflamm.,* **6,** 51–56.

WIEDERRECHT, G., LAM, E., HUNG, S., MARTIN, M. & SIGNAL, N. (1993). The mechanisms of action of FK506 and cyclosporin A. *Ann. N.Y. Acad. Sci.,* **699,** 9–11.

(Received November 9, 1998
Revised January 25, 1999
Accepted February 10, 1999)