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The involvement of tumour necrosis factor-α in the protective effects of 17β oestradiol in splanchnic ischaemia-reperfusion injury

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1 Tumour necrosis factor-α (TNF-α) is a cytokine that is implicated in the pathogenesis of ischaemic states and atherosclerosis. We tested the hypothesis that the vasoprotective effects of the oestrogens may be mediated *in vivo* by inhibition of the formation of TNF-α.

2 Anaesthetized rats, subjected to total occlusion of the superior mesenteric artery and the coeliac trunk for 45 min developed a severe shock state resulting in a fatal outcome within 75–90 min after the release of occlusion. Sham-operated animals were used as controls.

3 Splanchnic artery occlusion (SAO) shocked rats had a marked hypotension, enhanced levels of TNF-α in serum and macrophages, leukopenia and increased ileal leukocyte accumulation, studied by means of myeloperoxidase activity (MPO). Furthermore, aortae from SAO rats showed a marked hyporeactivity to phenylephrine (PE, 1 nM–10 μM), reduced responsiveness to acetylcholine (ACh, 10 nM–10 μM) and an increased staining for intercellular adhesion molecule-1 (ICAM-1).

4 *In vivo* administration of 17β oestradiol (500 μg kg^{-1}, i.m., three hours before the induction of SAO) increased survival rate (100%, 4 h after SAO), enhanced mean arterial blood pressure; reduced serum TNF-α (25 ± 5 u ml^{-1} vs 379 ± 16 u ml^{-1}), ameliorated leukopaenia and reduced ileal MPO (0.7 ± 0.02 u 10^{-3} g^{-1} tissue vs 4.2 ± 0.4 u 10^{-3} g^{-1} tissue). Furthermore aortae from SAO rats treated with 17β oestradiol exhibited a greater contractile response to phenylephrine, improved responsiveness to ACh and a blunted staining of ICAM-1. Finally 17β oestradiol, added *in vitro* to peritoneal macrophages collected from untreated SAO rats, significantly reduced TNF-α production.

5 Our results suggest that inhibition of TNF-α *in vivo* may explain, at least in part, the vasoprotective effects of oestrogens.

Keywords: 17β Oestradiol; ischaemia-reperfusion injury; tumour necrosis factor-α (TNF-α)

Introduction

Postmenopausal women on oestrogen replacement therapy develop less severe coronary artery disease and have a lower cardiovascular mortality than those not receiving hormonal treatment (Barrett-Connor & Bush, 1991). Although the possible prevention of cardiovascular diseases has become an important argument in favour of hormone replacement therapy during the later years (Stampfer & Colditz, 1991), the mechanisms of the vasoprotective properties of oestrogens are still controversial.

Previous clinical and epidemiological surveys have preferentially analysed the hormonal influences on cardiovascular risk factors, insulin secretion and blood pressure (McAde & Berra, 1993), but recent experimental data indicate a variety of additional direct vascular actions of ovarian sex steroids (Riedel *et al*., 1993).

In animals, oestrogens have pronounced effects on the vascular tone and have been shown to induce relaxation of coronary and systemic arteries within a few minutes (Raddino *et al*., 1986; Magness & Rosenfeld, 1989; Jiang *et al*., 1991a; Mugge *et al*., 1993). Several studies support the idea that oestrogen-induced vasoprotective effect may be due to the release of nitric oxide (NO) from the vascular endothelium. Compared with aortic rings of male rabbits, the basal release of NO from rings of female rabbits was increased and correlated with circulating oestradiol levels (Hayashi *et al*., 1992). Furthermore, acetylcholine infusion caused constriction of coronary arteries in ovariectomized atherosclerotic monkeys and this vasoconstrictor effect of acetylcholine was reversed to vasodilatation by continuous treatment with transdermal oestrogen (Williams *et al*., 1992).

A hypothesis that has not been tested sufficiently is that oestrogen-induced vascular protection is mediated by oestrogen-induced inhibition of tumour necrosis factor-α (TNF-α) synthesis.

Besides its involvement in the pathogenesis of septic shock (Mathison *et al*., 1988), TNF-α has been shown to play an important role in the pathogenesis of ischaemic states. Increased serum levels of this inflammatory cytokine can be found in patients with chronic heart failure (McMurray *et al*., 1991) and coronary atherosclerosis (Arbustini *et al*., 1991). TNFα also primes leukocyte-endothelial interaction by inducing the expression of endothelial adhesion molecules (Mantovani & Dejana, 1989): this latter phenomenon has a key role in the pathogenesis of experimental and human myocardial infarction (Squadrito *et al*., 1996). Furthermore, exogenous human recombinant TNF-α, administered systemically, produces a severe hypotension in dogs (Kilbourn *et al*., 1990) and a decrease in vascular responsiveness to contractile agents in rats (Takahashi *et al*., 1992).

Splanchnic ischaemia-reperfusion injury (SAO) in rats is an experimental model of circulatory failure in which TNF-α seems to be importantly involved: in fact, passive immunization with specific antibodies raised against this inflammatory cytokine induces protection against the lethality of this experimental procedure (Squadrito *et al*., 1992).

In light of these findings, we studied whether the vasoprotective effects of oestrogens may be mediated *in vivo* by TNF-α inhibition and, hence, we investigated the effects of 17β oestradiol on the pathological sequelae associated with the occlusion and reperfusion of the splanchnic region.

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Methods

Animal preparation

Female ovariectomized Sprague-Dawley rats weighing 200–250 g were allowed 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 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 before either splanchnic ischaemia or sham ischaemia. Splanchnic artery ischaemia-reperfusion injury (SAO) was induced by clamping both the superior mesenteric artery and the coeliac 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 rats except the arteries were not occluded.

Survival evaluation and arterial blood pressure monitoring

The first group of animals was used to study survival ($n=80$) and arterial blood pressure ($n=24$). Three hours before the initiation of the surgical procedures, treated rats received 17β oestradiol (125, 250 and 500 μg kg^{-1}, i.m.) progesterone (1 mg kg^{-1}), 17β oestradiol (500 μg kg^{-1}) + tamoxifen (2 mg kg^{-1}) or vehicle (1 ml kg^{-1}). 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 (Caputi *et al*., 1980). 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 presented 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 ($n=28$) 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 by a previously described method (Altavilla *et al*., 1989), were made in the above-described medium containing 1.0 μg of actinomycin D ml^{-1} and 100 μl of each dilution were added to the wells. For the *in vitro* studies macrophages collected from untreated SAO and sham-operated rats were incubated for 3 h either with RPMI or several doses of 17β oestradiol (250, 500 and 1000 pg ml^{-1}) or progesterone (300 pg ml^{-1}). In another set of experiments, cytokine production was evaluated in oestrogen-treated macrophages before and after lipopolysaccharide stimulation (50 μg ml^{-1} for 4 h). One TNF-α unit was defined as the amount giving 50% cell cytotoxicity. The TNFα 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.

Myeloperoxidase activity and leukocyte count

Leukocyte accumulation was investigated by measuring the activity of myeloperoxidase (MPO). MPO activity was determined in intestinal mucosa, as previously described (Mullane *et al*., 1985). The samples of intestinal mucosa were obtained at 0 min before the splanchnic arteries were occluded and at 70 min following the onset of reperfusion. The samples were first homogenized in a solution containing 20 mM 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) and then centrifuged for 30 min at 20,000 *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 being thawed, 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} 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 *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^{-1} at 25°C and was expressed in milliunits g^{-1} weight (u 10^{-3} g^{-1} tissue). Tail vein blood samples for the leukocyte count were taken at 0 min before the initiation of reperfusion and at 70 min after the onset of reperfusion. The number of leukocytes (WBC $\times10^3\times mm^3$) is presented 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, Na HCO_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 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 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 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 % 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, air dried 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 before 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 as control. Biotinylated, species-specific second layer reagents were then applied, followed by avidin-biotin-horse radish peroxidase complex as a chromogenic substrate, as previously described (Hse *et al*., 1981). The experiments were carried out by two observers (P.C., G.F.) who were unaware of the experimental protocol. The microscope image was sent to a computer assisted image analyser that analysed the changed in staining. Densitometric analysis of the captured image was performed on a PC computer by use of image analysis software.

Drugs

Acetylcholine chloride, phenylephrine hydrochloride, indomethacin, 17β oestradiol, tamoxifen and progesterone were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Statistical analysis

Data are expressed as means $\pm$ 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 ischaemia-reperfusion injury or sham ischaemia. 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 = 75 ± 10 min). Progesterone was ineffective in protecting against splanchnic ischaemia-reperfusion injury. Administration of 17β oestradiol increased in a dose-dependent manner survival rate and time in SAO rats. The most effective dose was 500 μg kg^{-1} and therefore we used it in the further studies. Furthermore rats surviving at 4 h were still alive 24 h after the surgical procedures. Tamoxifen (3 mg kg^{-1}) completely abated the protective effects of 17β oestradiol.

In intact female rats SAO shock also markedly decreased survival time (87 ± 4 min). Tamoxifen (3 mg kg^{-1}) administration reduced the resistance of intact female rats to surgical procedures of SAO shock (survival time = 50 ± 5 min; $P < 0.05$).

Oestrogen treatment was effective only when the hormone was administered 3 h before the splanchnic ischaemia-reperfusion injury procedures. In contrast no protective effect was observed when 17β oestradiol was given at the onset of reperfusion.

Serum and macrophage TNF-α

Serum and macrophage levels of TNF-α were undetectable in sham-operated rats treated either with vehicle or 17β oestradiol. TNF-α was significantly increased in both serum and macrophages collected from SAO rats at the end of the reperfusion period (Table 2). The administration of 17β oestradiol significantly blunted the macrophage and serum levels of this cytokine. Macrophages harvested from untreated SAO rats showed a marked TNF-α production that was reduced by adding 17β oestradiol *in vitro* (Table 3). This result suggests a direct inhibitory effect on the cytokine production. In contrast progesterone did not change macrophage TNF-α (Table 3).

Macrophages, harvested from control normal rats, were pretreated with either 17β oestradiol or progesterone and then TNF-α production was evaluated before or after lipopolysaccharide stimulation (LPS; 50 μg ml^{-1}). Both 17β oestradiol and progesterone had no effect on cytokine production in the absence of LPS stimulation (Table 3). In contrast, 17β oestradiol significantly reduced macrophage TNF-α primed by LPS, while progesterone did not exert any effect (Table 3).

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 (4.2 ± 0.4 u 10^{-3} g^{-1} tissue) at 70 min after reperfusion (Table 4 in SAO rats treated with the vehicle.

Administration of 17β oestradiol (500 μg kg^{-1}, i.m.) before the induction of surgical procedures significantly lowered the increase in ileal (0.7 ± 0.02 u 10^{-3} g^{-1} tissue) MPO activity (Table 4).

Table 1 Effects of 17β oestradiol or progesterone on survival rate, percentage survival and survival time in splanchnic ischaemia reperfusion injury

	Hours following the onset of reperfusion				
	2		*4*		
Treatment	*Surviving*	*(%)*	*Surviving*	*(%)*	*Survival time (min)*
Sham + vehicle (1 ml kg^{-1})	10/10	100	10/10	100	>240
Sham + 17β oestradiol (500 μg kg^{-1})	10/10	100	10/10	100	>240
SAO + vehicle (1 ml kg^{-1})	0/10	0	0/10	0	75 ± 10
SAO + progesterone (1 mg kg^{-1})	0/10	0	0/10	0	72 ± 13
SAO + 17β estradiol (125 μg kg^{-1})	2/10	20	0/10	0	89 ± 12
SAO + 17β estradiol (250 μg kg^{-1})	7/10	70*	5/10	50*	182 ± 13*
SAO + 17β estradiol (500 μg kg^{-1})	10/10	100**	10/10	100**	<240**
SAO + 17β estradiol (500 μg kg^{-1}) + tamoxifen (3 mg kg^{-1})	3/10	30	0/10	0	93 ± 12

Animals received 17β oestradiol, progesterone, tamoxifen or vehicle 3 h before the surgical procedures. Survival was monitored for 4 h. *$P < 0.05$, **$P < 0.001$, vs SAO + vehicle.

Table 2 Effects of 17β oestradiol on serum and macrophage tumour necrosis factor-α (TNF-α) in splanchnic ischaemia-reperfusion injury (SAO)

Treatment	*Serum TNF-α* (u ml^{-1})	*Macrophage TNF-α* (u ml^{-1})
Sham + vehicle	ND	ND
Sham + 17β oestradiol	ND	ND
SAO + vehicle	379 ± 16	198 ± 14
SAO + 17β oestradiol	25 ± 5*	10 ± 5*

Serum and macrophages were collected 70 min following the onset of reperfusion. Each point represents mean ± s.e.mean from six experiments. Animals received 17β oestradiol (500 μg kg^{-1}) or vehicle (1 ml kg^{-1}) 3 h before the surgical procedures. *$P<0.001$ vs SAO + vehicle. ND = not detectable.

Table 3 *In vitro* effects of 17β oestradiol and progesterone on tumour necrosis factor (TNF-α) production by peritoneal macrophages collected from either rats subjected to splanchnic ischaemia-reperfusion injury (SAO) or normal control rats (Control)

SAO	*Macrophage TNF-α* (u ml^{-1})
RPMI (1 ml)	279 ± 19
17β Oestradiol (250 pg)	100 ± 11*
17β Oestradiol (500 pg)	36 ± 12**
17β Oestradiol (1000 pg)	123 ± 10*
Progesterone (300 pg)	234 ± 21

Control	*Before LPS*	*After LPS*
RPMI (1 ml)	ND	250 ± 11
17β Oestradiol (250 pg)	ND	97 ± 14°
17β Oestradiol (500 pg)	ND	21 ± 10#
17β Oestradiol (1000 pg)	ND	78 ± 13°
Progesterone (300 pg)	ND	234 ± 9

Peritoneal macrophages were collected from untreated rats subjected to splanchnic ischaemia-reperfusion injury 70 min following the onset of reperfusion. Macrophages were then incubated for 3 h with several doses of 17β oestradiol, progesterone or with the vehicle in which oestrogen and progesterone were dissolved. *$P<0.05$, **$P<0.001$, vs RPMI. Macrophages were also harvested from normal rats, pretreated with 17β oestradiol or progesterone for 3 h and TNF-α was measured before and after LPS stimulation (50 μg ml^{-1}). °$P<0.05$, #$P<0.01$ vs control LPS. Each point represents the mean ± s.e.mean of seven experiments. ND = not detectable.

Leukocyte count

The administration of vehicle did not modify the white blood cell count in sham-operated rats (Table 4). In contrast, splanchnic ischaemia-reperfusion injury produced a marked leukopenia. Leukocyte count was markedly decreased at the end of reperfusion (70 min). The administration of 17*β* oestradiol significantly ameliorated this leukopenia (Table 4).

ICAM-1 staining on vascular endothelium

The presence of ICAM-1 was studied in thoracic aortae 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 1). In contrast, samples obtained from SAO rats had an increase in ICAM-1 staining. Aortic and mesenteric endothelium obtained from SAO rats treated with 17*β* oestradiol showed a marked reduction in ICAM-1 immunostaining (Figure 1).

Table 4 Effects of 17β oestradiol on myeloperoxidase (MPO) activity of ileum and on white blood cell count (WBC) of rats subjected to splanchnic ischaemia-reperfusion injury (SAO)

	Time (min)	
	Basal 0	*Reperfusion* 70
MPO activity in ileum (u 10^{-3} g^{-1}tissue)		
Sham + vehicle	0.5 ± 0.02	0.5 ± 0.04
Sham + 17β oestradiol	0.9 ± 0.08	0.4 ± 0.01
SAO + vehicle	0.3 ± 0.02	4.2 ± 0.4
SAO + 17β oestradiol	0.8 ± 0.09	0.7 ± 0.02*
White blood cell count (cells × 10^3 per mm^3)		
Sham + vehicle	12.7 ± 1.8	13.6 ± 1.4
Sham + 17β oestradiol	11.4 ± 2.1	12.8 ± 1.3
SAO + vehicle	12.9 ± 2.2	6.1 ± 1.1
SAO + 17β oestradiol	13.2 ± 1.7	10.2 ± 1.5*

Each point represents the mean ± s.e.mean of seven experiments. *$P<0.001$ vs SAO + vehicle. Animals received 17β oestradiol (500 μg kg^{-1}) or its vehicle (1 ml kg^{-1}) 3 h before the surgical procedures.

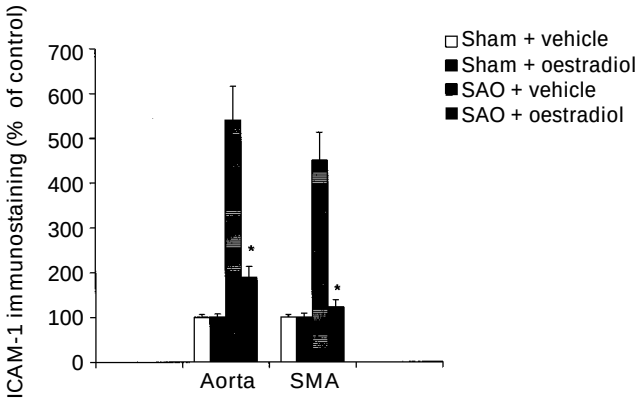

Figure 1 Effects of vehicle (1 ml kg^{-1}, i.m., 3 h before the surgical procedures) of 17*β* oestradiol (500 *μ*g kg^{-1}, i.m., 3 h before the surgical procedures) on immunohistochemical staining for ICAM-1 in aortic (Aorta) and superior mesenteric artery (SMA) endothelium from rats subjected to splanchnic ischaemia-reperfusion injury (SAO). Each column represents the mean ± s.e.mean of seven experiments. *$P<0.01$ vs SAO + vehicle.

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 *μ*M). The relaxant effect of ACh was significantly smaller in aortic rings obtained from SAO rats than from sham-operated rats (Figure 2). Administration of 17*β* oestradiol significantly improved the responsiveness of aortic rings obtained from SAO rats to ACh (Figure 2).

In intact aortic rings prepared from SAO rats, the contractile response to PE (1 nM – 10 *μ*M) was significantly reduced. The maximum force of contraction induced by 10 *μ*M PE in aortic rings from sham rats was 1.8 ± 0.6 g mg^{-1} tissue, whereas it was 0.9 ± 0.3 g mg^{-1} 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 either SAO rats or sham operated animals (Figure 3). However, the contractile response to PE in endothelium-denuded aortic rings was also significantly smaller in SAO rats than in sham operated animals. Administration of 17*β* oestradiol improved the impaired contractile response to PE in SAO rats (Figure 3).

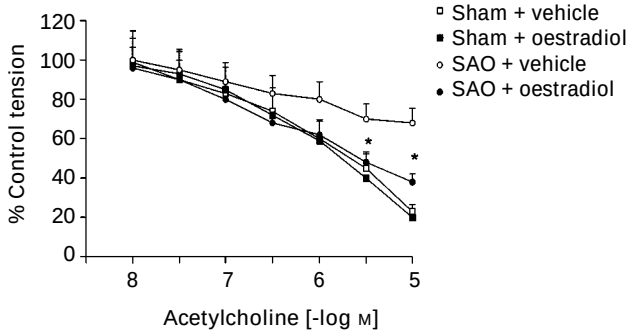

Figure 2 Relaxant effects of acetylcholine (ACh) in aortic rings (contracted with phenylephrine, 100 nM) of sham-operated rats and rats subjected to splanchnic ischaemia-reperfusion injury (SAO) treated with vehicle (1 ml kg^{-1}, i.m. 3 h before the surgical procedures) or 17β oestradiol (500 μg kg^{-1} i.m. 3 h before the surgical procedures). Each point represents the mean, and vertical lines shows s.e.mean, from six experiments. *$P<0.01$ vs SAO + vehicle.

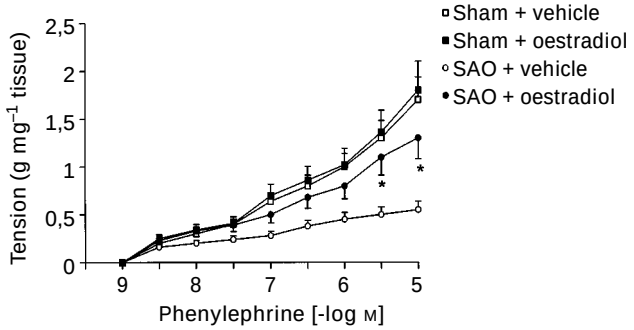

Figure 3 Contractile response to cumulative doses of phenylephrine (PE) in endothelium-denuded aortic rings from sham-operated rats and rats subjected to splanchnic ischaemia-reperfusion injury (SAO) treated with vehicle (1 ml kg^{-1}, i.m., 3 h before the surgical procedure) or 17β oestradiol (500 μg kg^{-1}, i.m., 3 h before the surgical procedures. Each point represents the mean, and vertical lines show s.e.mean, of seven experiments. *$P<0.02$ vs SAO + vehicle.

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 4). The administration of 17β oestradiol significantly blunted the reduction in mean arterial blood pressure (Figure 4).

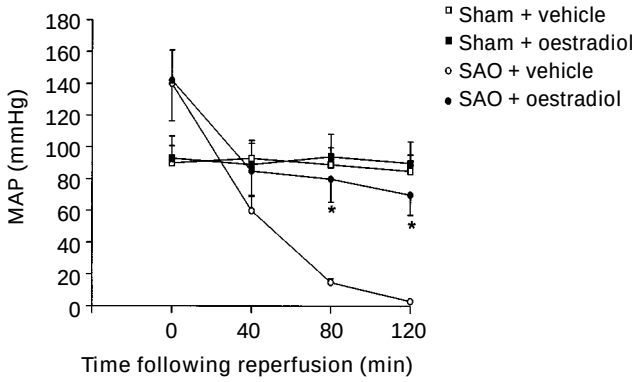

Figure 4 Effects of vehicle (1 ml kg^{-1}, i.m. 3 h before the surgical procedures) or 17β oestradiol (500 μg kg^{-1}, i.m., 3 h before the surgical procedures) on mean arterial blood pressure (MAP) of sham operated rats and rats subjected to the splanchnic ischaemia-reperfusion injury procedures (SAO). Each point represents the mean, and vertical lines show s.e.mean, of six experiments. *$P<0.01$ vs SAO + vehicle.

Discussion

Oestrogen administration in postmenopausal women is associated with nearly a 50% reduction in the development of clinical manifestations of atherosclerosis (Stampfer & Colditz, 1991). However, the mechanism of the vasoprotective effect of oestrogen has not been completely defined. Although oestrogen has been shown to alter favourably the lipid profile (Wahl *et al*., 1983) and inhibit endothelial hyperplasia (Fisher *et al*., 1981), these effects do not fully account for the degree of clinical benefit attributed to oestrogen therapy.

Another proposed mechanism of the vasoprotective effect of oestrogen is a favourable modulation of vasoreactivity. This is suggested by studies that have demonstrated the ability of oestrogen to block endothelin-1 and calcium-mediated vasoconstriction in isolated coronary arteries (Jiang *et al*., 1991b; 1992), and to stimulate prostacyclin production (Chang *et al*., 1980; Makila *et al*., 1982) and modulate the production of nitric oxide (Van Buren *et al*., 1992), an endothelium-derived vasoactive substance that induces vascular smooth muscle relaxation and vasodilatation and inhibits vascular smooth muscle cell proliferation and mitogenesis (Garg & Hassid, 1989).

It has also been suggested that the pleiotropic cytokine TNF-α plays an important role in the pathogenesis of ischaemic states (Squadrito *et al*., 1993). 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.

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. In addition, administration of recombinant human TNF-α in conscious rats has been shown 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 probably due to TNF-α-induced stimulation of a 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 ischaemia-reperfusion injury is an experimental model characterized by the presence of adhesion mechanism for leukocyte accumulation (Squadrito *et al*., 1994a) and by a marked vascular dysfunction (Squadrito *et al*., 1994b). We have shown that these two important pathological aspects of this type of experimental model of ischaemia-reperfusion injury are due to increased production of TNF-α. Therefore in order to test the hypothesis that the vasoprotective effects of oestrogens may be mediated *in vivo* by inhibition of this inflammatory cytokine, we investigated the effects of 17β oestradiol in splanchnic ischaemia-reperfusion injury.

Our results showed that 17β oestradiol reduced the enhanced macrophage and serum levels of TNF-α. This effect is a consequence of direct inhibition of this inflammatory cytokine: in fact 17β oestradiol added *in vitro* to macrophages collected from untreated rats subjected to splanchnic ischaemia-reperfusion injury caused a marked inhibition in the production of TNF-α. This finding is in agreement with previous findings showing that oestrogens may down-regulate the production of

this cytokine, via inhibition of the mRNA for TNF-α (Shanker *et al*., 1994). Data in the literature are not uniform with respect to the effects of oestrogen on TNF-α production: in fact cytokine release is finely regulated by relatively small changes in hormone concentrations and elevated hormone levels may also have opposite effects on cytokine production (Chao *et al*., 1995). It has been suggested that *in vivo* oestrogen administration may enhance the formation of TNF-α (Zuckerman *et al*., 1995). However, the dose of 17β oestradiol used in the study by Zuckerman *et al*. was 1 mg kg^{-1}, a dose that in mice largely exceeds the physiological range. Furthermore the effects of TNF-α required several days of administration (5 days) and the time course of these effects showed that 17β oestradiol decreased TNF-α during the first 2 days of treatment. In contrast, it has been suggested that physiological doses of the hormone have marked inhibitory effects on TNF-α production (Ralston *et al*., 1990). In agreement with this hypothesis our data indicate that 17β oestradiol over a physiological dose range does not alter the basal release of TNF-α in normal macrophages, but strongly reduced LPS-stimulated cytokine production. Macrophages have classical oestrogen complexes for binding of genomic oestrogen response elements (ERE) (Gulshan *et al*., 1990). ERE have been found in the 5′ flanking region of the TNF-α gene: this suggests that 17β oestradiol reduces TNF-α production by altering gene transcription (Shanker *et al*., 1994).

The administration of 17β oestradiol reduced ICAM-1 expression, ameliorated leukopenia and decreased MPO activity, an index of leukocyte accumulation. Since leukocyte-endothelial interaction (more specifically the ICAM-1 dependent leukocyte adhesion) is primed by TNF-α, it is proposed that 17β oestradiol, by inhibiting this inflammatory cytokine, limits leukocyte accumulation at the ischaemic sites and finally protects against SAO shock.

In addition, other mechanisms, such as inhibition of lipid peroxidation, increase in prostacyclin production and enhancement of NO production by the constitutive NOS isoform, might also be involved in the protective effects of 17β oestradiol in splanchnic ischaemia reperfusion injury.

Aortic rings from rats subjected to splanchnic-ischaemia reperfusion injury had a markedly reduced responsiveness to vasorelaxant effects of ACh: this finding indicates the presence of reduced NO production in this type of experimental ischaemia-reperfusion injury. However, our results also showed reduced vascular sensitivity to vasoconstrictor stimuli. This impaired vascular reactivity, as suggested for other models of experimental shock (Thiemermann *et al*., 1993), is a consequence of an overproduction of NO by the inducible NO synthase (iNOS) (Squadrito *et al*., 1994b). Therefore all these data, taken together, suggest that in splanchnic ischaemia-reperfusion injury: (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*., 1994b) as this inflammatory cytokine either inhibits eNOS and stimulates iNOS. This hypothesis is confirmed by evidence that an inhibitor of TNF-α synthesis is able to revert this complex vascular dysfunction (Squadrito *et al*., 1994b). In the present study, aortic rings collected from rats subjected to ischaemia-reperfusion injury and treated with 17β oestradiol exhibited a greater contractile response to PE and improved responsiveness to ACh when compared to vehicle-treated rats. Thus, it is hypothesized that oestrogens improve vascular dysfunction by inhibiting the detrimental vascular effects of TNF-α.

In conclusion, we have shown that 17β oestradiol inhibits TNF-α both *in vitro* and *in vivo*. The 17β oestradiol-induced inhibition of this inflammatory cytokine, at least in splanchnic ischaemia-reperfusion injury, 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 oestrogens.

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References

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.

ARBUSTINI, E., GRASSO, M., DIEGOLI, M., PUCCI, A., BRAMERIO, M., ARDISSINO, D., ANGOLI, L., DE SERVI, S., BRANUCCI, E., MUSSINI, A., MINZIONI, G., VIGANO M. & SPECCHIA, G. (1991). Coronary atherosclerotic plaques with and without thrombus in ischemic heart syndromes: a study. *Am. J. Cardiol*., **68,** 368 – 373.

BARRETT-CONNOR, H. & BUSH, R.L. (1991). Estrogen and coronary heart disease in women. *J. Am. Med. Assoc*., **265,** 1861 – 1867.

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

CAPUTI, A.P., ROSSI, F., CARNEY, K. & BREZENOFF, H.E. (1980). Modulatory effect of brain acetylcholine on reflex-induced bradycardia and tachycardia in conscious rats. *J. Pharmacol. Exp. Ther*., **215,** 309 – 316.

CHANG, W.C., NAKAO, J., ORIMO, J. & MUROTA, S.I. (1980). Stimulation of prostacyclin biosynthetic activity by estradiol in rat aortic smooth muscle cells in culture. *Biochem. Biophys. Acta*, **619,** 107 – 118.

CHAO, T.C., VAN ALTEN, P.J., GREAGER, J.A. & WALTER, R.J. (1995). Steroid sex hormones regulate the release of tumor necrosis factor by macrophages. *Cell Immunol*., **160,** 43 – 49.

FISHER, G.M., CHERIAN, K. & SWAIN, M.L. (1981). Increased synthesis of aortic collagen and elastin in experimental atherosclerosis: inhibition by contraceptive steroids. *Atherosclerosis*, **39,** 463 – 467.

GARG, U.C. & HASSID, A. (1989). Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J. Clin. Invest*., **83,** 1774 – 1777.

GULSHAN, S., MCCRUDEN, A.B. & STIMSON, W.H. (1990). Oestrogen receptors in macrophages. *Scand. J. Immunol*., **31,** 691 – 695.

HAYASHI, T., FUKUTO, J.M., IGNARRO, L.J. & CHAUDHURI, G. (1992). Basal release of nitric oxide from aortic rings to greater in female rabbits than in male rabbits: implications for atherosclerosis. *Proc. Natl. Acad. Sci. U.S.A*., **89,** 11259 – 11263.

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. Pathol*., **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.

JIANG, C., SARREL, P.M., LINDSAY, D.C., POOLE-WILSON, P.A. & COLLINS, P. (1991a). Endothelium-independent relaxation of rabbit coronary artery by 17β-oestradiol *in vitro*. *Br. J. Pharmacol*., **104,** 1033 – 1037.

JIANG, C., SARREL, P.M., LINDSAY, D.C., POOLE-WILSON, P.A. & COLLINS, P. (1991b). 17β-Estradiol has calcium antagonistic properties in the rabbit coronary artery *in vitro*. *Circulation*, **84,** (suppl 2): II-272 abstract.

JIANG, C., SARREL, P.M., POOLE-WILSON, P.A. & COLLINS, P. (1992). Acute effect of 17β-estradiol on rabbit coronary artery contractile responses to endothelin-1. *Am. J. Physiol*., **263,** H271 – H275.

KILBOURN, R.G., GROSS, S.S., JUBRANS, A., ADAMS, J., GIFFONS, O.W., LEVI, R. & LODATO, R.F. (1990). N^G-methyl-L-arginine inhibits tumor necrosis factor-induced hypotension:implications for the involvement of nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.*, **87,** 3629–3632.

MAGNESS, R.H. & ROSENFELD, C.R. (1989). Local and systemic estradiol-17β-: effects of uterine and systemic vasodilation. *Am. J. Physiol.*, **256,** H536–H542.

MAKILA, U.M., WAHLBERG, L., VIINIKKA, L. & YLIKORKALA, O. (1982). Regulation of prostacyclin and thromboxane production by human umbilical vessels: the effect of estradiol and progesterone in a superfusion model. *Prostaglandins Leukotrienes Medicine*, **18,** 115–124.

MANTOVANI, A. & DEJANA, E. (1989). Cytokines as communication signals between leukocytes and endothelial cells. *Immunol. Today*, **10,** 370–375.

MATHISON, J.C., WOLFSON, E. & ULEVITCH, R.J. (1988). Participation of tumor necrosis factor in the mediation of gram negative bacterial lipopolysaccharide-induced injury in rabbits. *J. Clin. Invest.*, **81,** 1925–1937.

MCADE, T.W. & BERRA, A. (1992). Hormone replacement therapy and cardiovascular disease. *Br. Med. Bull.*, **48,** 276–308.

MCMURRAY, J., ABDULLAH, I., DARGIE, H.J. & SHAPIRO, D. (1991). Increased concentrations of tumor necrosis factor in "cachectic" patients with severe chronic heart failure. *Br. Heart. J.*, **66,** 356–361.

MUGGE, A., RIEDEL, M., BARTON, M., KUHN, M. & LICHTLEN, P.R. (1993). Endothelium-independent relaxation of human coronary arteries by 17β-oestradiol *in vitro*. *Cardiovasc. Res.*, **27,** 1939–1942.

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

RADDINO, R., MANCA, C., POLLI, E., BOLOGNESI, R. & VISIOLI, O. (1986). Effects of 17β-estradiol on the isolated rabbit heart. *Arch. Int. Pharmacodyn. Ther.*, **281,** 57–65.

RALSTON, S.H., RUSSELL, R.G. & GOWEN, M. (1990). Estrogen inhibits release of tumor necrosis factor from peripheral blood mononuclear cell in postmenopausal women. *J. Bone Miner. Res.*, **5,** 983–988.

RIEDEL, M., RAFFLENBEUL, W. & LICHTLEN, P.R. (1993). Ovarian sex steroids and atherosclerosis *J. Clin. Invest.*, **71,** 406–12.

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

SHANKER, G., SORCI-THOMAS, M. & ADAMS, M.R. (1994). Estrogen modulates the expression of tumor necrosis factor alpha mRNA in phorbol ester-stimulated human monocytic THP-1 cells. *Lymph. Cytok. Res.*, **13,** 377–382.

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., IOCULANO, M., CALAPAI, G., ZINGARELLI, B., SAITTA, A., CAMPO, G.M., RIZZO, A. & CAPUTI, A.P. (1992). Passive immunication 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., ZINGARELLI, B., IOCULANO, M., CALAPAI, G., CAMPO, G.M. & CAPUTI, A.P. (1993). Tumor necrosis factor involvement in myocardial ischaemia-reperfusion injury. *Eur. J. Pharmacol.*, **273,** 223–230.

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

SQUADRITO, F., ALTAVILLA, D., CANALE, P., IOCULANO, M., CAMPO, G.M., AMMENDOLIA, L., FERLITO, M., ZINGARELLI, B., SQUADRITO, G., SAITTA, A. & CAPUTI, A.P. (1994b). 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., SAITTA, A., ALTAVILLA, D., IOCULANO, M., CANALE, P., CAMPO, G.M., SQUADRITO, G., DI TANO, G., MAZZU', A. & CAPUTI, A.P. (1996). Thrombolytic therapy with urokinase reduces increased circulating endothelial adhesion molecules in acute myocardial infarction. *Inflamm. Res.*, **45,** 14–19.

STAMPFER, M.J. & COLDITTZ, G.A. (1991). Estrogen replacement therapy and coronary heart disease: a quantitative assessment of epidemiologic evidence. *Preventative Med.*, **20,** 47–63.

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 hemorrhagic shock is mediated by nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.*, **90,** 267–271.

VAN BUREN, G.A., YANG, D. & CLARK, K.E. (1992). Estrogen induced uterine vasodilatation is antagonized by L-nitroarginine methyl ester, an inhibitor of nitric oxide synthesis. *Am. J. Obstet. Gynecol.*, **167,** 828–833.

WAHL, P., WALDEN, C., KNOPP, R., HOOVER, J., WALLACE, R., HEISS, G. & RIFKIND, B. (1983). Effect of estrogen/progestin potency on lipid/lipoprotein cholesterol. *N. Engl. J. Med.*, **308,** 862–867.

WILLIAMS, J.K., ADAMS, M.R., HERRINGTON, D.M. & CILARKSON, T.B. (1992). Short-term administration of estrogen and vascular responses of atherosclerotic coronary arteries. *J. Am. Coll. Cardiol.*, **20,** 52–457.

ZUCKERMAN, S.H., BRYAN-POOL, N., EVANS, G.F., SHORT, L. & GLASEBROOK, A.L. (1995). In vivo modulation of murine serum tumour necrosis factor and interleukin-6 levels during endotoxemia by oestrogen agonist and antagonist. *Immunology*, **86,** 18–24.

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