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Sequential release of tumour necrosis factor, platelet activating factor and eicosanoids during endotoxin shock in anaesthetized pigs; protective effects of indomethacin

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1 The effects of indomethacin were investigated on haemodynamics, haematological and blood glucose values, and the release of tumour necrosis factor (TNF), platelet activating factor (PAF) and eicosanoids in anaesthetized pigs receiving 5 $\mu g\,kg^{-1}$ *E. coli* lipopolysaccharide (LPS) over 60 min into the superior mesenteric artery. The animals were observed for an additional period of 2 h after the termination of LPS infusion.

2 Eight of the 17 animals infused with LPS and not treated with indomethacin died within 30 min after the beginning of LPS infusion (non-survivors), while the other 9 survived the experimental period of 3 h though in a state of shock (survivors).

3 No alterations were observed in plasma concentrations of PAF and eicosanoids (thromboxane B_2 (TXB_2), 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) and leukotriene B_4 (LTB_4)) in non-survivors. However, a marked increase was detected in TNF release. A significant, though transient, increase in concentrations of PAF, TNF and eicosanoids occurred in the survivors. The peak in the concentrations of PAF and TXB_2 preceded the maximum in TNF values in survivors.

4 Another group of 7 LPS-infused pigs was treated with indomethacin (2 $mg\,kg^{-1}$, i.v. bolus 60 min before the start of LPS infusion, followed by a continuous infusion of 3 $mg\,kg^{-1}\,h^{-1}$). This treatment prevented death and shock despite the high concentrations of circulating TNF and PAF. Concentrations of cyclo-oxygenase enzyme products were reduced, whereas LTB_4 release was not affected. The effect of indomethacin on haemodynamic changes occurred earlier than on cyclo-oxygenase products.

5 In another group of 6 pigs indomethacin (2 $mg\,kg^{-1}$, i.v.) was given 20–25 min after the start of LPS infusion at which time mean arterial blood pressure (MABP) had decreased below 40 mmHg indicating imminent death. This indomethacin treatment immediately reversed the hypotension, restored the organ perfusion, delayed the haemoconcentration and thrombocytopenia and prevented death. However, TNF and PAF concentrations remained elevated. Concentrations of cyclo-oxygenase products studied were reduced by the end of the observation period, whereas LTB_4 production was unaffected.

6 The decrease in MABP induced by exogenous PAF was temporarily prevented by indomethacin.

7 These data indicate that the beneficial effect of indomethacin in LPS-induced septic shock is related to cyclo-oxygenase inhibition as well as to a direct vasoconstrictor property of the drug.

Keywords: Endotoxin; shock; indomethacin; tumour necrosis factor (TNF); PAF; eicosanoids

Introduction

Specific treatment for the cardiopulmonary derangements characteristic of septic shock is lacking, although circulatory shock resulting from bacterial sepsis continues to be a major medical problem (Zimmermann, 1990). Endotoxin, the lipopolysaccharide (LPS) moiety of the microbial cell wall, initiates synthesis of inflammatory mediators, such as eicosanoids, platelet activating factor (PAF) and tumour necrosis factor (TNF) (Morrison & Ryan, 1987; Beutler & Cerami, 1988). Although the opposing actions and interactions of these substances are complex, their net effect in initiating the shock state appears to be very significant (Morrison & Ryan, 1987). Non-steroidal anti-inflammatory drugs (NSAIDs), including indomethacin, represent a rational approach to break up the chain of mediators, because the clinical effectiveness of NSAIDs is thought to be due to the inhibition of cyclo-oxygenase enzyme (Vane, 1971). Indomethacin is effective in improving survival with concomitant decrease in cyclo-oxygenase enzyme products in endotoxin-induced shock in rats (Ball *et al.*, 1986). Furthermore, indomethacin is able to prevent the initial rapid drop in arterial blood pressure after endotoxin challenge in cats (Parratt & Sturgess, 1974; 1975). Additionally, indomethacin has been found to reduce the hypotensive and toxic effect of TNF (Kettelhut *et al.*, 1987; Goto *et al.*, 1989). Recently, BN 52021, a PAF receptor antagonist, has been claimed to protect against septic shock by attenuating the eicosanoid release (Fletcher *et al.*, 1990). These data, taken together, suggest an important relationship between PAF, TNF and eicosanoids and, what is more, seem to indicate that some of the effects of TNF and PAF may be mediated via the cyclo-oxygenase pathway. However, effective blockade of eicosanoid production by BW755C, an inhibitor of both cyclo-oxygenase and 5-lipoxygenase, failed to improve survival in endotoxaemia (McKechnie *et al.*, 1985). Though indomethacin, given i.v. during other kind of shock improved the haemodynamics (Mózes *et al.*, 1989), it is unclear whether the beneficial action was actually due to inhibition of cyclo-oxygenase enzyme or to some other pharmacological property of indomethacin.

The purpose of the present experiments was to determine the relation between the protective effect of indomethacin in

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septic shock and its effect on haemodynamics, haematological and blood glucose changes and the release of TNF, PAF and eicosanoids during septic shock.

Methods

Experimental set-up

After an overnight fast, young female Yorkshire pigs (body weight: 20–24 kg, age: 12–16 weeks) were initially anaesthetized with i.m. injections of ketamine (20 mg kg^{-1}) and midazolam (0.25 mg kg^{-1}) as well as atropine (0.05 mg kg^{-1}). The anaesthesia was maintained throughout the experiment with i.v. sodium pentobarbitone (20 mg kg^{-1} bolus followed by 20 mg kg^{-1} h^{-1} infusion). A tracheostomy was performed and the animals were ventilated with intermittent positive pressure (Bear 2E adult volume ventilator, Lameris, The Netherlands). Respiratory rate, tidal volume and oxygen to air ratio were adjusted to keep arterial blood gases within normal ranges: pH 7.35–7.45, $90 < Po_2$ mmHg < 150, $35 < Pco_2$, mmHg < 45. The animals' temperature, measured with a thermometer (Philips, HP 5311, Japan) attached to the liver, was maintained at around 38°C by use of an electric blanket.

Catheters were placed via femoral vessels into the aorta and pulmonary artery (Swan-Ganz 7 F catheter). For injection of radioactive microspheres a catheter (7 or 8 F) was inserted via the left carotid artery into the left ventricle. A midline laparotomy was performed and the superior mesenteric artery was exposed. After gently clearing the surrounding fat, nerves and connective tissues, a needle (0.5 mm external diameter) connected to a suitable polyethylene tube was inserted directly into the superior mesenteric artery for infusion of LPS or normal saline. Another catheter was inserted into the superior mesenteric vein through a branch vein for collecting blood samples and measurement of mean portal venous pressure (MPVP). The abdomen was then closed and a Ringer-lactate infusion at a rate of 4 ml kg^{-1} h^{-1} was started. The animals were allowed to remain undisturbed for 30 min to ensure haemodynamic stability. Mean arterial blood pressure (MABP), and MPVP were continuously monitored by electromanometers with Statham P23dB strain gauges (Hato Rey, PR). Cardiac output (CO) was determined intermittently by thermodilution (WTI Computer, Holland). The determinations were performed in triplicate and the results averaged. Systemic vascular resistance was calculated by dividing MABP by cardiac output and was reported in arbitrary units (u).

Regional blood flows

To measure organ perfusion five times during an experiment, microspheres (15 ± 1 μm labelled with ^{141}Ce, ^{113}Sn, ^{46}Sc, ^{103}Ru and ^{95}Nb; Dupont de Nemours, NEN Division, Dreieich, F.R.G.) were used as described elsewhere in detail (Saxena *et al.*, 1980). Briefly, approximately 1×10^6 microspheres, suspended in 1 ml of normal saline containing a drop of Tween 80, were injected into the left ventricular catheter over 10 s and the catheter flushed with an additional 3 ml of normal saline. A reference arterial blood sample, starting 10 s before the injection of microspheres and continuing for a total duration of 75 s, was withdrawn at a rate of 10.5 ml min^{-1} via the aortic catheter by use of a pump (B. Braun, Melsungen, FRG). At the end of the experiment, the animals were killed with an overdose of sodium pentobarbitone and heart, kidneys, liver and small intestine were dissected out, weighed and placed into vials. The radioactivity in the vials containing the tissues and blood samples was counted for 5–10 min in a gamma scintillation counter (Packard, Minaxi Autogamma 5000) using suitable windows for discrimination of the different isotopes used. The microsphere data were processed by a PDP-11/70 computer; a set of programmes were used that had been especially developed for the microsphere techniques (Saxena *et al.*, 1980).

Assay for serum levels of tumour necrosis factor

Blood was collected in sterile tubes (Costar, Cambridge, MA). Serum was separated by centrifugation and aliquots were stored at −20°C until assay. TNF activity was determined by measuring the cytostatic effect of TNFα on the murine transformed fibroblast cell line L929 (a gift from Dr W. Fiers, State University of Ghent, Belgium) (Hay & Cohen, 1989; Meager *et al.*, 1989). L929 cells were plated in a 96 well flat-bottom microtiter plate (NUNC, Roskilde, Denmark) at a density of 1×10^4 cells per well in 25 μl of RPMI 1640 culture medium. Medium (25 μl control), human recombinant TNF standard solutions (10, 100, 1000 u ml^{-1}) (Roche Research, Ghent, Belgium) or serum were added (final dilution 1 : 10) to quadruplicate wells. After incubation for 24 h in a humidified atmosphere (7.5% CO_2) at 37°C, 50 μl of a 10 μCi ml^{-1} [^{3}H]-thymidine (Amersham Laboratories, Amersham, England) solution was added. After 2 h of incubation, L929 cells were harvested on glass fibre filtermats (Shatron Inc., Sterling, VA, U.S.A.). The uptake of [^{3}H]-thymidine was measured by liquid scintillation spectroscopy. A standard curve of cytostasis by human recombinant TNF was obtained yielding progressive cytostasis ranging from 10–1000 u ml^{-1} of TNF. The bioactivity of TNF in experimental samples was determined in quadruplicate and compared against the standard curve.

Assay for blood levels of platelet activating factor

Blood (5 ml) was collected into polypropylene tubes containing 45 ml of ice-cold methanol, [^{3}H]-PAF (94000 d.p.m.; 80 Ci mmol^{-1}; radiochemical purity, 99.2%; Amersham, UK) was added and lipids were extracted as described by Filep *et al.*, (1989) and Mózes *et al.*, (1989). Briefly, samples were mixed by slow rotation for 60 min, and the methanolic extract was separated by centrifugation. The pellet was extracted once more with 5 vol of methanol. Supernatants were combined and chloroform and water were added to effect phase separation (methanol-chloroform-water 1 : 1 : 0.9 vol : vol : vol). The lower chloroform-rich phase contained all PAF activity. Chloroform was evaporated under a stream of nitrogen. The samples were redissolved in chloroform-methanol (1 : 1 vol : vol), spotted on silica gel plates (Merck, Darmstadt, FRG) and developed with methanol-chloroform-water (65 : 35 : 6 vol : vol : vol). The area corresponding to authenic PAF (R_F from 0.20 to 0.28) was scraped off and lipids were eluted with 2 ml of methanol. The clear solvent layer was carefully removed and considered to be PAF extract.

Bioassayable PAF activity was determined by platelet aggregation assay (Pinckard *et al.*, 1984) in which washed rabbit platelets were used (Ardlie *et al.*, 1970). Platelet aggregation was determined at 37°C in an aggregometer (Payton Aggregation Module, Payton Ltd., Scarborough, Ontario, Canada) with 200 μl of platelet suspension (200 G l^{-1}) containing 10 μM indomethacin and 0.4 u ml^{-1} apyrase (Sigma). A standard curve for platelet aggregation by authentic PAF was obtained, yielding progressive platelet aggregation ranging from 10^{-10} to 10^{-7} M PAF. PAF bioactivity of experimental samples (after extraction and purification) was determined in duplicate and compared against the standard curve. Values were corrected for individual recovery (ranging from 46 to 56%).

Assay for plasma levels of thromboxane B_2, 6-keto prostaglandin $F_{1\alpha}$, and leukotriene B_4

Blood samples for eicosanoid determinations were obtained from the superior mesenteric vein. The samples were collected into ice-cooled polypropylene tubes containing 20 μl of heparin (5000 u ml^{-1}, Thromboquine, Organon, Oss, The Netherlands) and 50 μl of indomethacin (0.1 mg ml^{-1} in 0.1 M phosphate buffer pH 8). After centrifugation plasma was decanted and stored at −20°C until extraction of eicosanoids, while the red blood cells were suspended in 0.9% saline and

were injected back after each blood collection. Details of the radioimmunoassay (RIA) for eicosanoid determinations have been described reported (Zijlstra & Vincent, 1984).

Determination of blood chemistry values

Plasma glucose concentrations were determined by Glucoquant kit (Boehringer, Mannheim, F.R.G.). Laboratory values of blood haemoglobin, haematocrit as well as leucocyte and platelet counts were measured by haematology analyzers (Sysmex CC-108 and Pl-100, Kobe, Japan, as appropriate).

Experimental protocols

Group I: Sham-operated Three animals were prepared as described above, except that instead of LPS, normal saline was infused into the superior mesenteric artery. Ten ml of blood was collected from the superior mesenteric vein for eicosanoid determination. Five ml of blood was taken from pulmonary artery for laboratory measurements.

Group II: LPS-induced shock Seventeen pigs were infused with $5\,\mu g\,kg^{-1}$ *Escherichia coli* LPS (O111, B4, Serva) into the superior mesenteric artery over a 60 min period and the animals were observed for an additional 120 min period. Blood was collected from the superior mesenteric vein 10 min before and 15, 30 and 60 min after starting LPS infusion and 60 and 120 min after LPS infusion. Radioactive microspheres were injected in 8 of the 17 pigs 2 min before each blood collection, except at 60 min after LPS infusion.

Group III: Indomethacin pretreated Seven pigs were pretreated with $2\,mg\,kg^{-1}$ i.v. of indomethacin 60 min before the start of LPS infusion followed by an infusion of $3\,mg\,kg^{-1}\,h^{-1}$ over the entire experimental period of 4 h. Blood was collected immediately before (−60 min), 15, 30, 60 min after starting indomethacin treatment (−45, −30, 0 min before and 15, 30 and 60 min after the start of LPS infusion and 60 and 120 min after the end of LPS infusion). Radioactive microspheres were injected in 3 of 7 pigs 2 min before each blood collection, except at −45, −30, 15 and 120 min.

Group IV: Indomethacin revived In 6 pigs just after the mean arterial blood pressure had decreased below 40 mmHg at 20–25 min after starting LPS infusion, $2\,mg\,kg^{-1}$ of indomethacin was given i.v. Blood was collected from the superior mesenteric vein and aorta at the same times as in group II. Radioactive microspheres were also injected, identical to group II in four animals.

Group V: PAF injections In 4 pigs $0.02\,\mu g\,kg^{-1}$ PAF (Sigma, Chemicals, Deisenhofen, F.R.G.) was injected into the superior mesenteric vein. Effectiveness of indomethacin treatment on mean arterial blood pressure was tested in four animals by administering PAF into the superiod mesenteric vein 60 min before and 5, 30 and 60 min after i.v. injection of indomethacin ($2\,mg\,kh^{-1}$).

Statistical analysis

All values are expressed as means ± s.e.mean. The data were evaluated by a non-parametric two-way analysis of variance (Friedman test) followed by the Wilcoxon-Wilcox' test to identify differences between measurements performed during the control period and at different times during and after LPS infusion. The Mann-Whitney U test was performed to evaluate the differences between the groups. A P value of 0.05 or less was considered statistically significant for all tests.

Results

Sham-operated animals

In sham-operated animals, systemic haemodynamic variables, organ blood flows, blood chemistry values and eicosanoid levels were stable during the experiment (Table 1).

Animal survival

A total number of 30 pigs received LPS infusion for 60 min (group II, III and IV). Group II consisted of 17 animals which received LPS only. Eight of these 17 animals died at or close to 30 min after the start of LPS infusion. The remaining 9 animals survived the experimental period of 3 h (i.e. 2 h after termination of the LPS infusion), though in a state of profound shock (survivors). None of the animals receiving indomethacin (group III, $n = 7$: indomethacin $2\,mg\,kg^{-1}$ 60 min before the start of LPS infusion and then $3\,mg\,kg^{-1}\,h^{-1}$; group IV, $n = 6$: indomethacin $2\,mg\,kg^{-1}$ 20–25 min after

Table 1 Systemic haemodynamic variables, organ blood flows, laboratory values and eicosanoid concentrations in sham-operated animals

Variables	*Control*	*60 min*	*120 min*	*180 min*
Systemic haemodynamics				
MABP (mmHg)	99 ± 8	116 ± 8	121 ± 8	121 ± 8
MPVP (mmHg)	6.0 ± 0.1	5.7 ± 0.9	5.0 ± 0.5	5.3 ± 0.6
CO ($l\,min^{-1}$)	2.7 ± 0.4	2.8 ± 0.1	2.2 ± 0.1	2.4 ± 0.3
SVR (u)	38 ± 5	42 ± 5	52 ± 4	51 ± 9
Regional haemodynamics ($ml\,min^{-1}\,100\,g^{-1}$)				
Heart	132 ± 44	115 ± 18	NM	100 ± 11
Kidneys	296 ± 22	280 ± 9	NM	291 ± 38
Liver	20 ± 7	18 ± 7	NM	27 ± 11
Small intestines	47 ± 1	46 ± 4	NM	42 ± 2
Laboratory values				
Hb ($mM\,l^{-1}$)	5.7 ± 0.4	6.0 ± 0.4	6.1 ± 0.5	6.3 ± 0.4
WBC count ($G\,l^{-1}$)	13.5 ± 2.3	20.0 ± 5.0	20.0 ± 5.0	20.0 ± 6.0
Platelet count ($G\,l^{-1}$)	350 ± 49	340 ± 48	330 ± 49	330 ± 52
Blood glucose ($mM\,l^{-1}$)	6.0 ± 0.2	6.3 ± 0.3	5.8 ± 0.2	5.8 ± 0.4
Eicosanoid concentration ($pg\,ml^{-1}$)				
TxB_2	180 ± 8	130 ± 14	120 ± 17	110 ± 37
6-ketoPGF$_{1\alpha}$	150 ± 10	160 ± 8	160 ± 10	150 ± 10
LTB_4	180 ± 27	170 ± 73	180 ± 19	160 ± 17

Values are means ± s.e.mean. TXB_2, thromboxane B_2; 6-keto $PGF_{1\alpha}$, 6-keto prostaglandin $F_{1\alpha}$; LTB_4, leukotriene B_4; Hb, haemoglobin; WBC, while blood cells, SVR, systemic vascular resistance (u).

starting LPS infusion) died during the observation period of 3 h.

Systemic haemodynamics (Figure 1)

In group II pigs not surviving the LPS infusion, MABP dropped to 28 ± 2 mmHg, MPVP increased to 12 ± 1 mmHg and CO decreased to 1.0 ± 0.1 l min^{-1}, shortly before death (30 min after the start of LPS infusion). In pigs surviving the LPS infusion, MABP gradually decreased to 55 ± 5 mmHg at the end of the 3 h observation period (2 h after the end of LPS infusion), MPVP first increased to peak values at 30 min before decreasing slightly but remaining higher than baseline values; CO first decreased rapidly (15–30 min after the start of LPS infusion) and then gradually to about 36% of the baseline value (Figure 1; a).

In group III treated with indomethacin before the start of LPS infusion, MABP increased immediately after indomethacin administration and then, at the start of LPS infusion, it gradually decreased but remained still higher than in indomethacin-untreated LPS survivors: 87 ± 6 mmHg (n = 7) vs. 55 ± 5 mmHg (n = 8), respectively, at the end of the 3 h observation period; $P < 0.05$. The early increase but not the late increase in MPVP levels as well as the early decrease but not the late decrease in CO levels were attenuated in this group (Figure 1; b).

In animals revived with indomethacin (group IV), MABP increased dramatically immediately after indomethacin and remained high up to the end of the observation period: 79 ± 8 mmHg (n = 6) vs. 55 ± 5 mmHg (n = 9) in the group surviving the LPS infusion; $P < 0.05$. MPVP increased shortly after the indomethacin administration in this group and then decreased to levels observed in the untreated LPS-infused survivors and in the indomethacin pretreated group. CO was transiently elevated and then decreased to the same level as in the untreated LPS-infused survivors: (1.0 ± 0.2 l min^{-1} vs. 1.0 ± 0.1 l min^{-1}) (Figure 1c). A typical record of the immediate reversing effect induced by i.v. administration of indomethacin on arterial blood pressure is shown in Figure 2.

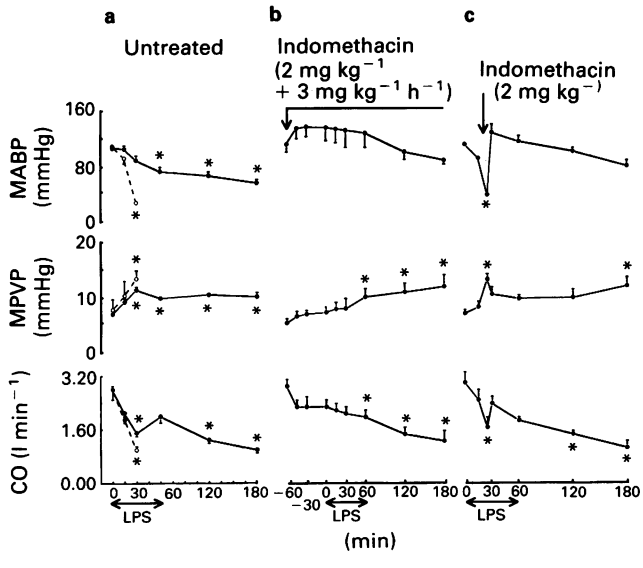

Figure 1 Effect of indomethacin treatments on systemic haemodynamics in pigs receiving endotoxin (LPS) infusion into the superior mesenteric artery. Arrows indicate the time for administration of either LPS or indomethacin, as appropriate. In the untreated group, survivors (●—●) and non-survivors (o----o) indicate, respectively, the animals that survived or died during the observation period of 3 h after the beginning of LPS infusion. Values are means with s.e.mean shown by vertical bars. Abbreviations; MABP, mean arterial blood pressure; MPVP, mean portal venous pressure; CO, cardiac output. *$P < 0.05$ value was obtained by 2-way analysis of variance (Friedman test) followed by Wilcoxon-Wilcox' test for multiple comparisons, control (t = 0 min) versus 15 min, control versus 30 min, etc.

Systemic vascular resistance (MABP/CO ratio) increased transiently in the untreated LPS-infused survivors from 15–30 min after the start of LPS infusion (from 43 ± 4 u to 66 ± 5 u and 68 ± 4 u at 15 and 30 min respectively), and then fell sharply to approximately the baseline value: 50 ± 5 u at 180 min. No changes in systemic vascular resistance were observed in LPS-infused non-surviving pigs: 38 ± 1 u at 30 min vs. 40 ± 3 u at baseline. Systemic vascular resistance increased slowly in the indomethacin pretreated group; at 30 min after the start of indomethacin administration the value was 64 ± 11 u vs. 37 ± 3 u at baseline. This increase was not affected by the LPS infusion and remained high up to the end of the observation period: 74 ± 23 u. Increase in the systemic vascular resistance was also observed in the animals revived with indomethacin.

Organ blood flows (Figure 3)

In LPS-infused non-survivors, LPS significantly decreased blood flow to all organs studied. The flow to the heart, kidneys and small intestine was reduced by about 40% and to the liver by more than 60% at 15 min after starting LPS infusion. In survivors, blood flow to the heart and kidneys was stable during LPS infusion, but by the end of the experiment (2 h after stopping LPS infusion) blood flow to the heart and kidneys was markedly decreased. Perfusion of the liver and small intestine was sharply reduced at 15 min after the start of LPS infusion. Subsequently the liver flow increased above the baseline levels and the flow to the small intestine returned to about the baseline levels at 30–60 min of LPS infusion. After stoppage of LPS infusion, perfusion of the liver and small intestine decreased again during the prolonged hypotension.

Indomethacin pretreatment in itself did not affect organ perfusion. The drug, however, attenuated the decreases in coronary and hepatic blood flow found at the end of the observation period. The decreases in the renal and small intestinal blood flow were not affected by indomethacin pretreatment.

The seriously reduced flow to the heart returned to baseline within 10 min in animals revived with indomethacin. Despite the gradual decline, the cardiac flow stabilized at a higher level than that observed in untreated survivors: 112 ± 21 ml min^{-1} 100 g^{-1} vs. 62 ± 7 ml min^{-1} 100 g^{-1}, $P < 0.05$. Similar changes were observed in the flow to the

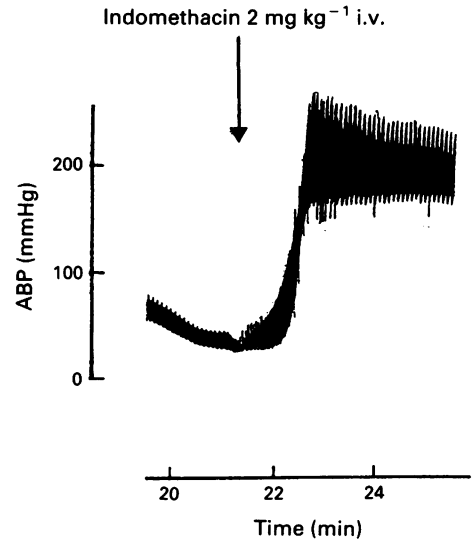

Figure 2 Effect of i.v. treatment with indomethacin on arterial blood pressure (ABP) in an anaesthetized pig during endotoxin shock. The arrow indicates the start of indomethacin administration. Time scale shows time after the start of LPS infusion.

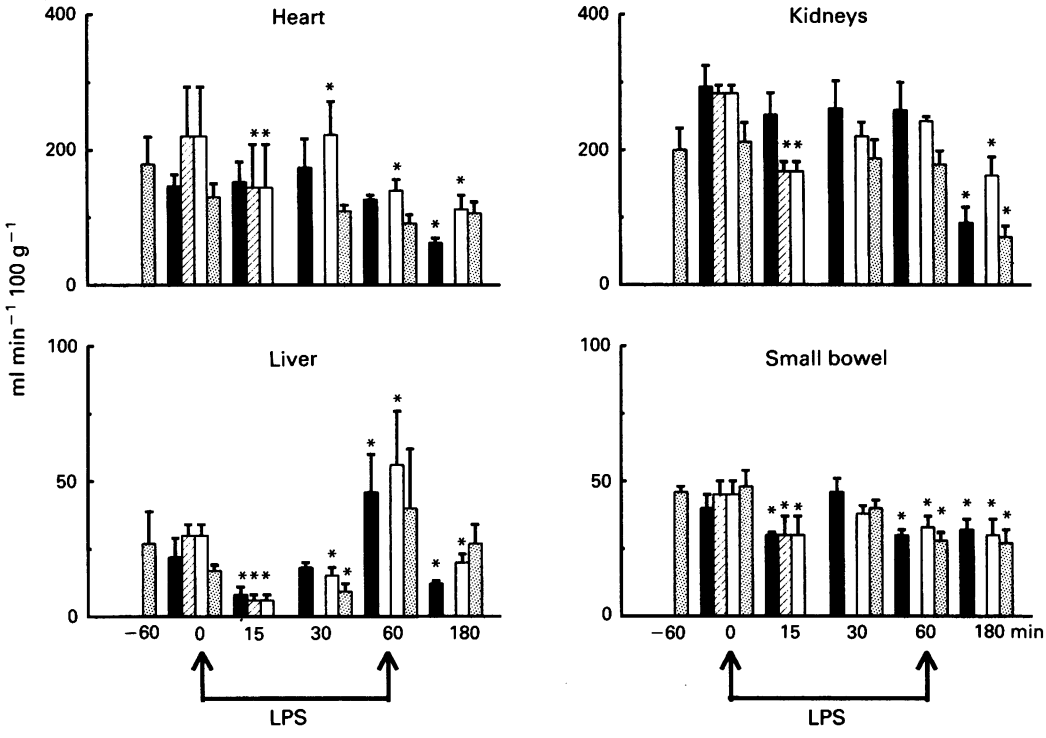

Figure 3 Effect of indomethacin treatment on organ blood flows in pigs receiving endotoxin (LPS) infusion into the superior mesenteric artery: solid columns, untreated survivors; hatched columns, untreated non-survivors; stippled columns, indomethacin ($2\,mg\,kg^{-1} + 3\,mg\,kg^{-1}\,h^{1}$) pretreated group; open columns, indomethacin ($2\,mg\,kg^{-1}$) revived group. $^{*}P < 0.05$ vs. control (t = 0 min).

kidneys and liver. The diminished flow to the small intestine was transiently elevated. This improvement did not last up to the end of the experiment.

Blood chemistry values (Figure 4)

LPS infusion was followed by a rapid increase in haemoglobin values, evident from 30 min after the start of LPS infusion. This haemoconcentration was apparent towards the end of the observation period in survivors and did not improve after the LPS infusion was stopped. The increase in haemoglobin concentration seemed attenuated in both the indomethacin-pretreated and indomethacin-revived groups.

Platelet counts dropped 30 min after LPS infusion was started in both survivors and non-survivors. There were no significant differences between groups at 30 min. After LPS infusion was stopped, platelet counts in the survivors remained significantly lower than at the baseline. The appearance of thrombocytopenia observed in endotoxic shock was not much affected by indomethacin.

In untreated LPS-infused non-survivors a clear hyperglycaemia developed, while in survivors a severe hypoglycaemia was observed. In the indomethacin pretreated group, hyperglycaemia failed to develop but hypoglycaemia was similar to that observed in endotoxic shock ($3.5 \pm 1.1\,mM\,l^{-1}$ vs. $2.8 \pm 0.4\,mM\,l^{-1}$). Hyperglycaemia decreased after revival with indomethacin, but hypoglycaemia was also not affected in this group.

Mediator release

In untreated LPS-infused non-survivors no changes were found in either eicosanoid ($n = 4$, data not shown) or PAF release ($n = 3$), but a marked increase was detected in serum TNF concentration ($n = 5$), which reached a maximum level at the time of death i.e., at 30 min after the start of LPS infusion (Figure 5). In contrast, blood concentration of TXB_2 in superior mesenteric vein increased markedly, but only for a short time, during LPS infusion in survivors ($n = 5$). Similarly a transient, but less marked increase was detected in 6-keto $PGF_{1\alpha}$ concentrations of superior mesenteric vein in survivors ($n = 5$). The increase in TXB_2 concentration started 30 min earlier than 6-keto $PGF_{1\alpha}$ elevation. In untreated survivors, the plasma concentration of LTB, also showed an increase in superior mesenteric vein ($n = 5$). LTB_4 production was elevated when the concentrations of TXB_2 and 6-keto $PGF_{1\alpha}$ had returned to baseline. In contrast to prostanoid release, the LTB_4 production seems to be continuous during the observation period. A significant, though transient increase was also measured in concentrations of PAF ($n = 2$) and TNF ($n = 4$) in survivors. The maximum release of PAF and thromboxane occurred at the same time and both preceded the peak in TNF concentration, which was lower in survivors than in non-survivors ($4000 \times 10^2\,u\,ml^{-1}$ $n = 5$, vs. $2000 \times 10^2\,u\,ml^{-1}$ $n = 4$) (Figure 5a).

Indomethacin pretreatment (Figure 5b)) did not modify the release of TNF ($n = 5$) and PAF ($n = 3$). Both concentrations increased to the same levels with a similar time course to that observed in animals that survived LPS infusion without treatment. Indomethacin infusion was followed by a significant decrease in plasma concentrations of TXB_2 and 6-keto $PGF_{1\alpha}$ evident from 30 min after the start of indomethacin treatment. This fall was apparent toward the end of the experiment ($n = 7$ each). However, LTB_4 production remained unaffected during indomethacin treatment ($n = 6$).

The markedly elevated TNF levels decreased in animals revived with indomethacin ($n = 4$). At the end of the experiment TNF concentrations were just detectable (Figure 6). In non-survivors a lack of PAF release was observed but after indomethacin injection PAF increased in the same manner as observed in LPS-infused survivors, but the peak occurred 30 min later ($n = 3$). The time course of PAF release in this group of animals was identical to that observed in untreated animals surviving LPS infusion as well as in the indomethacin pretreated group. At the end of the experiment the concentrations of TXB_2 and 6-keto $PGF_{1\alpha}$ were reduced significantly, however, LTB_4 production remained unchanged ($n = 4$, each).

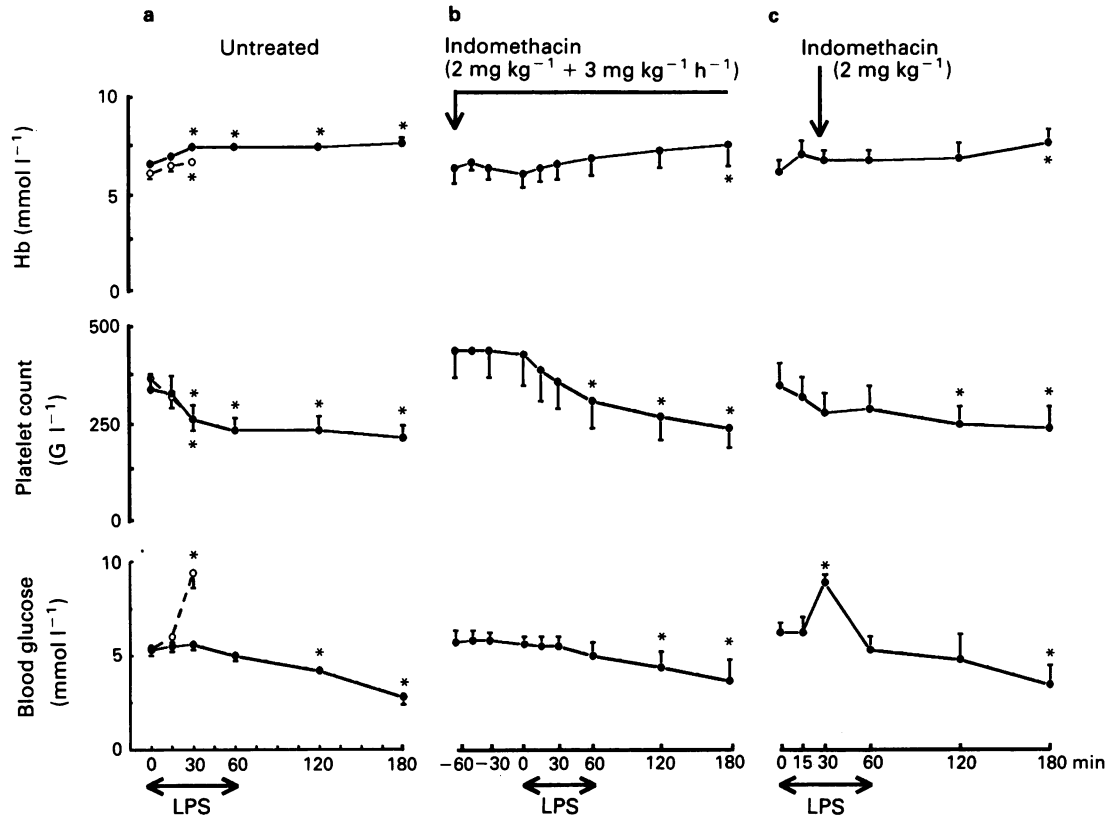

Figure 4 Effect of indomethacin treatments on haematological and blood sugar values in pigs receiving endotoxin (LPS) infusion into the superior mesenteric artery. In the untreated group, survivors (●—●) and non-survivors (o---o) indicate, respectively, the animals that survived or died during the observation period of 3 h after the beginning of LPS infusion. Hb, haemoglobin; $*P < 0.05$ vs. control (t = 0 min).

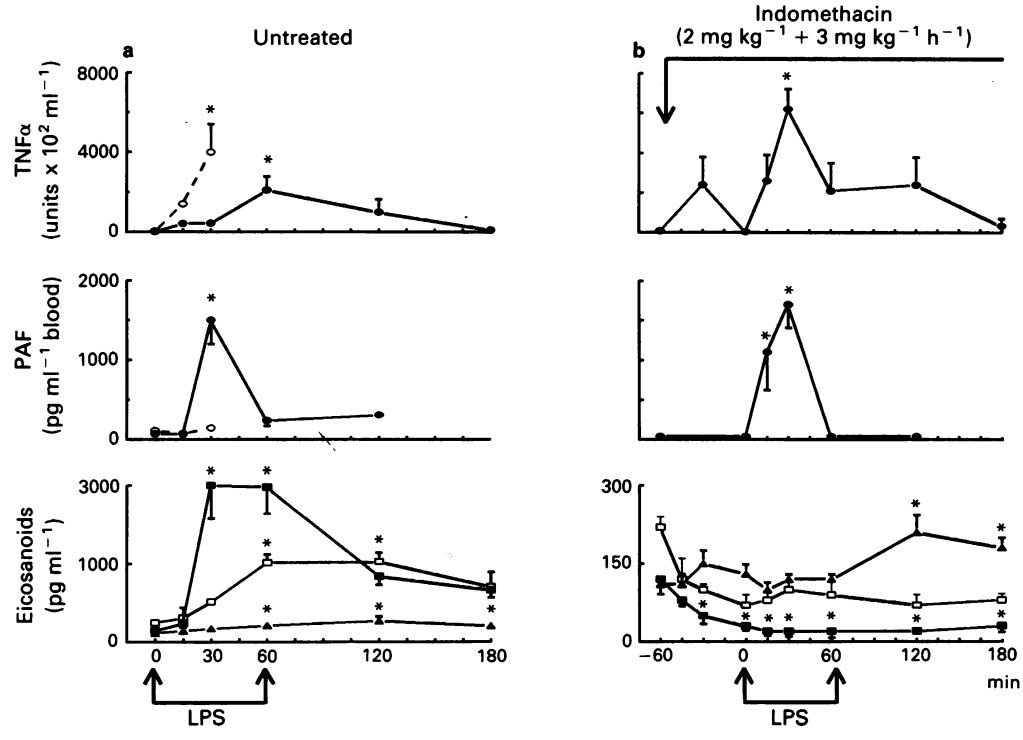

Figure 5 Effect of indomethacin pretreatment on blood levels of tumour necrosis factor (TNFα), platelet activating factor (PAF) and eicosanoids in pigs receiving endotoxin (LPS) infusion into the superior mesenteric artery. In the untreated group, suvivors (●—●) and non-survivors (o---o) indicate, respectively, the animals that survived or died during the observation period of 3 h after the beginning of LPS infusion. Bottom panel represents eicosanoid release in the survivors: thromboxane B_2(■); 6-keto prostaglandin $F_{1\alpha}$(□); leukotriene B_4 (▲). $*P < 0.05$ vs. control (t = 0 min).

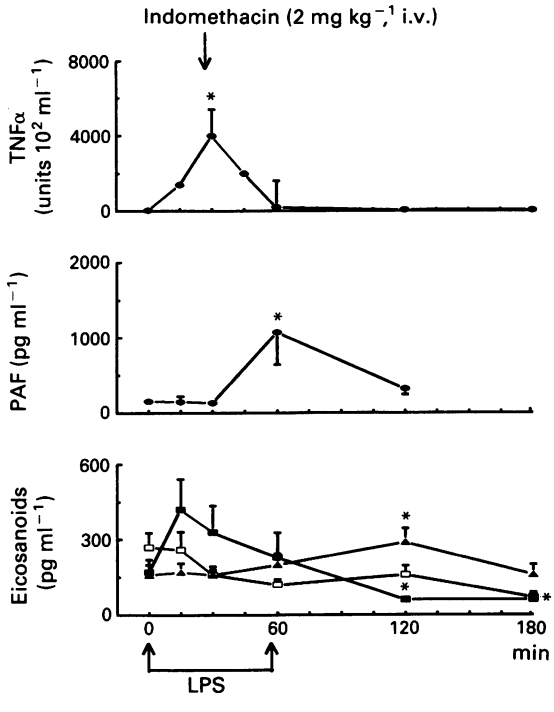

Figure 6 Effects of revival with indomethacin on blood levels of tumour necrosis factor (TNFα), platelet activating factor (PAF) and eicosanoids. Symbols as in Figure 5. *$P < 0.05$ vs. control (t = 0 min).

Effect of indomethacin on the hypotensive response to exogenous platelet activating factor

Administration of 0.02 μg kg^{-1} of PAF into the superior mesenteric vein reduced MABP by 74 ± 4% ($n = 4$) 60 min before and by 5 ± 5%, 13 ± 4% and 26 ± 1% at 5, 30 and 60 min, respectively, after 2 mg kg^{-1} of indomethacin (Figure 7, $P < 0.05$, $n = 4$ each). At 120 min after administration of indomethacin, MABP decreased again by 70 ± 5% after PAF injection. MABP increased after indomethacin injection from 95 ± 4 mmHg to 138 mmHg in 1 min. However, at 30 min after indomethacin injection MAPB had returned to the baseline (99 ± 5 mmHg) and remained at this level throughout the remaining period.

Discussion

The main findings in the present study are: (a) the non-survivors following LPS infusion showed marked haemodynamic alterations, no changes in release of PAF and eicosanoids but marked increase in TNF production; (b) the animals surviving LPS infusion remained in a severe shock state up to the end of the 3 hours observation period (2 hours after termination of LPS infusion); they presented a sequential release of PAF, TNF and eicosanoids but the increase in TNF was much less than in the non-surviving animals; (c) indomethacin administration, starting either before LPS (indomethacin pretreated group) or shortly before presumed death, improved circulatory derangements, reduced release of cyclo-oxygenase products without affecting leukotriene production; and (d) the release of TNF and PAF was not affected in the indomethacin pretreated group but the markedly elevated TNF levels decreased in animals revived with indomethacin.

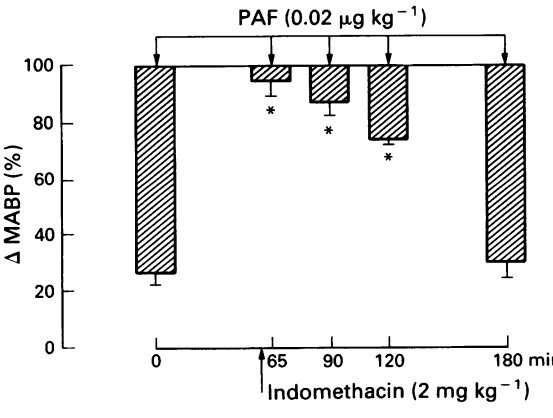

Figure 7 Effect of platelet activating factor (PAF; 0.02 μg kg^{-1}) injection into the superior mesenteric vein over 1 min on mean arterial blood pressure (MABP) in absence and presence of indomethacin. Arrows indicate the time of administration of either PAF or indomethacin as appropriate. *$P < 0.05$ vs. control ($t = 0$ min).

Endotoxic shock model

Infusion of a low amount of endotoxin is a recommended way to induce standardized shock which may more closely resemble clinical septic shock than bolus administration of LPS (Fink *et al.*, 1989; Naess *et al.*, 1989). In the present study with LPS infusion into the superior mesenteric artery, half of the animals died within 30 min of administration of endotoxin. The main changes observed in the pigs not surviving the LPS infusion were a marked decrease in blood pressure, rapid increase in portal pressure contributing to a reduced cardiac preload, reduction in CO without any compensatory increase in systemic vacular resistance, significant decrease in the blood flow to various organs, marked reduction in platelet counts, hyperglycaemia and marked increase in TNF release. These changes may have led to early death because of serious reduction of blood flow to essential organs and because of toxic properties of TNF. It seems that eicosanoids and PAF do not play an important role in causing death of the LPS-infused pigs because their levels remained unchanged in non-survivors. It might be that TNF, released in large quantities during rapid death, has a destructive effect on the cells able to synthesize PAF and eicosanoids.

The state of shock in the animals surviving the LPS infusion was manifested by systemic hypotension, elevated portal pressure, a transient increase in systemic vascular resistance, a damaged organ perfusion, haemoconcentration, leucocytopenia, thrombocytopenia, hypoglycaemia and a sequential release of eicosanoids. The survival following LPS infusion might be due to an increase in systemic vascular resistance which aids in maintenance of MABP, thus improving the supply of blood to essential organs. After exhausting this compensatory vasoconstriction the flow to organs markedly increased and, consequently, the systemic vacular resistance, cardiac output and arterial blood pressure were reduced. Moreover, the increase in TNF levels induced by LPS, was less in survivors than in non-survivors.

Indomethacin treatment

Indomethacin pretreatment prevented the circulatory collapse seen after LPS infusion despite the high concentration of TNF and PAF. The flow to heart and liver was preserved whereas renal and intestinal circulation diminished. Furthermore, an increase in portal pressure occurred reducing cardiac preload. This event may be responsible for the decreased cardiac output observed in the indomethacin pretreated group despite the preserved coronary circulation as demonstrated here. Haemoconcentration and thrombocytopenia were shifted to the end of the experiment after indomethacin pretreatment. Hypoglycaemia seen during endotoxic shock was not affected by indomethacin pretreatment but hyperglycaemia failed to develop.

Animals in group IV were treated with indomethacin (2 mg kg^{-1}, i.v.) at the time of the peak LPS-induced hypotensive effect, which, in the untreated animals, was followed by

death within 5–10 min. This indomethacin treatment was highly effective in preventing the imminent death and an immediate reversal of hypotension was observed with a concomitant improvement in cardiac output and organ blood flows. However, this beneficial effect on cardiac output and organ circulations was transient; by the end of the observation period both cardiac output and organ flows tended to decline again. Nevertheless, the flow to most of the organs studied was higher in animals revived with indomethacin than in untreated animals surviving LPS infusion. This may be due to the elevated systemic vascular resistance seen after indomethacin treatment. Importantly, renal blood flow was better preserved in the group revived with indomethacin than in the indomethacin pretreated group. The exact reason for this difference is not clear, but it may be due to the difference in the duration and dose of indomethacin treatment.

Indomethacin was effective in blocking cyclo-oxygenase as indicated by the lowered concentrations of TXB_2 and 6-keto $PGF_{1\alpha}$ in both types of indomethacin treatment. Since indomethacin effectively inhibited prostanoid production, it is reasonable to assume that its beneficial effects are due, at least in part, to the inhibition of prostanoid synthesis (Fletcher & Ramwell, 1977; Fletcher, 1982; Ball *et al.*, 1986; Lefer, 1989). However, the effect of indomethacin on blood pressure was rapid in onset, reaching its maximum within 60 s, implying that vasoconstriction seen after indomethacin administration may be independent of cyclo-oxygenase inhibition. Moreover, in the animals revived with indomethacin, no changes in prostanoid release were noticed before or immediately after indomethacin, yet the drug produced a marked vasoconstrictor response (see Figures 6 and 2, respectively). Treatment with indomethacin during mesenteric ischaemia/reperfusion-induced shock in dogs also restored the circulation in a similar way, namely, vasoconstriction occurred in 30 s while the decrease in prostanoid concentration occurred later on (Mózes *et al.*, 1989). Although indirect vasoconstriction via suppression of local prostacyclin generation in a particular vascular bed, associated with a slower change in the circulating 6-keto $PGF_{1\alpha}$, cannot be entirely excluded, this seems not very likely because both aspirin and ibuprofen, despite being potent cyclo-oxygenase inhibitors, are not capable of eliciting a rapid vasoconstrictor response (Fletcher & Ramwell, 1977; Balk *et al.*, 1988). The protective effect of ibuprofen against endotoxic shock in dogs develops with a delay of 45 min (Balk *et al.*, 1988) and the improvement in the survival during endotoxic shock in dogs by aspirin occurs without any attenuation of the haemodynamic effects of LPS (Fletcher & Ramwell, 1977).

Regardless of the precise mechanism of action, indomethacin has been shown to have a beneficial effect during different shock states in various animal species including endotoxic shock (Parratt & Sturgess, 1974; 1975; Fletcher & Ramwell, 1977; Fletcher, 1982; Mózes *et al.*, 1989). However, one should keep in mind, that this beneficial effect was transient in the present study. By the end of the experiment the decreased portal pressure increased again and the increased cardiac output improved diminishing organ blood flows in both types of indomethacin treatment. Despite these events the arterial blood pressure remained compensated probably due to elevated systemic vascular resistance. However, in these animals LPS infusion did cause hypoglycaemia, and only delayed the occurrence of thrombocytopenia and haemoconcentration. This indicates that mediators other than cyclo-oxygenase products may be involved in endotoxic shock. Indeed, the LTB_4 concentration remained elevated in animals revived with indomethacin reflecting an increased 5-lipoxygenase activity. Consequently, high concentrations of other lipoxygenase products (i.e. peptido-leukotrienes) can be expected. These peptido-leukotrienes (LTC_4 and LTD_4) are active on vascular smooth muscle and enhance leakiness (Lefer, 1989). Thus leukotrienes can contribute to the elevated portal pressure and haemoconcentration seen at the end of the experiment in the indomethacin-treated animals independently of the type of the treatment. Since leukotrienes have no effect on platelet aggregation (Lefer, 1989), the drop in platelet counts is probably due to the presence of increased release of PAF. Though indomethacin given i.v. markedly reduced the effect of PAF on blood pressure over more than 1 h, it failed to prevent the thrombocytopenia seen after LPS infusion. Since thrombocytopenia occurred later in indomethacin pretreated animals than in LPS infused animals, other mediators besides PAF may also be involved in this process, for example thromboxane.

Furthermore, it is worth mentioning that LTB_4 concentration in indomethacin-treated animals reached similar levels as measured in pigs treated with LPS alone. Thus a shift of arachidonic acid metabolism to the 5-lipoxygenase pathway in the presence of cyclo-oxygenase enzyme blockade was not observed in the present experiments.

In summary, indomethacin given either 60 min before or 20–25 min after starting LPS infusion (5–10 min before imminent death) attenuated or reversed most of the haemodynamic (hypotension, low cardiac output and reduced organ perfusion) and laboratory (haemoconcentration, thrombocytopenia and hyperglycaemia) changes in the early stage of endotoxic shock. Late changes, except hypoglycaemia, thrombocytopenia and portal hypertension, were less but significantly affected by indomethacin. The sequential release of different mediators during endotoxic shock indicates an important relationship between TNF, PAF and eicosanoids, though present results do not verify that effects of PAF and TNF are mediated via the cyclo-oxygenase pathway (Kettelhut *et al.*, 1986; Fletcher *et al.*, 1990). It should be noted that the effect of indomethacin on haemodynamics precedes the effective blockade of cyclo-oxygenase enzyme. Our data indicate that the protective effect of indomethacin in preventing septic shock death is probably due to the combined vasoconstrictor and cyclo-oxygenase blocking properties of the drug, which may be of value in rescuing patients in septic shock.

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References

ARDLIE, N.G., PACKAH, M.A. & MUSTRAD, J.F. (1970). Adenosine diphosphate-induced platelet aggregation in suspension of washed rabbit platelets. *Br. J. Haematol.*, **19**, 7–17.

BALK, R.A., JACOBS, R.F., TRYKA, F., WALLS, R.C. & BONE, R.C. (1988). Low ibuprofen reverses the haemodynamic alterations of canine endotoxin shock. *Crit. Care Med.*, **16**, 1128–1131.

BALL, H.A., COOK, J.A., WISE, W.C. & HALUSHKA, P.V. (1986). Role of thromboxane, prostaglandins and leukotrienes in endotoxic and septic shock. *Intensive Care Med.*, **12**, 116–126.

BEUTLER, B. & CERAMI, A. (1988). Tumor necrosis, cachexia, shock, and inflammation: A common mediator. *Annu. Rev. Biochem.*, **57**, 505–518.

FILEP, J., HERMAN, F., BRAQUET, P. & MÓZES, T. (1989). Increased levels of platelet-activating factor in blood following intestinal ischaemia in the dog. *Biochem. Biophys. Res. Commun.*, **158**, 353–359.

FINK, M.P., ROTHCHILD, H.R., DENIZ, Y.F. & COHN, S.M. (1989). Complement depletion with Naje naje cobra venom factor limits prostaglandin release and improve visceral perfusion in procine endotoxic shock. *J. Trauma*, **29**, 1076–1085.

FLETCHER, J.R. (1982). The role of prostaglandins in sepsis. *Scand. J. Infect. Dis.*, Suppl., **31**, 55–60.

FLETCHER, J.R. & RAMWELL, P.W. (1977). Modification, by aspirin and indomethacin, of haemodynamic and prostaglandin releasing

effects of *E. coli* endotoxin in the dog. *Br. J. Pharmacol.*, **61**, 175–181.

FLETCHER, J.R., DISIMONE, A.G. & ERNEST, M.A. (1990). Platelet activating factor receptor antagonist improves survival and attenuates eicosanoid release in severe endotoxemia. *Ann. Surg.*, **211**, 312–316.

GOTO, F., WATANABE, E., MARUYAMA, N. & FUJITA, T. (1989). Prevention of the toxic action of tumor necrosis factor by cyclo-oxygenase inhibitor and leukopenia. *Circ. Shock*, **29**, 175–180.

HAY, H. & COHEN, J. (1989). Studies on the specificity of the L929 cell bioassay for the measurement of tumour necrosis factor. *J. Clin. Lab. Immunol.*, **29**, 151–155.

KETTELHUT, I.C., FIERS, W. & GOLDBERG, A.L. (1987). The toxic effect of tumor necrosis factor in vivo and their prevention by cyclo-oxygenase inhibitors. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 4273–4277.

LEFER, A.M. (1989). Significance of lipid mediators in shock states. *Circ. Shock*, **27**, 3–12.

McKECHNIE, K., FURMAN, B.L. & PARRATT, J.R. (1985). Metabolic and cardiovascular effects of endotoxin infusion in conscious rats: effects of methylprednisolone and BW755C. *Circ. Shock*, **15**, 205–215.

MEAGER, A., LEUNG, H. & WOOLLEY, J. (1989). Assay for tumour records factor and related cytokines. *J. Immunol. Methods*, **116**, 1–17.

MORRISON, D.C. & RYAN, J.L. (1987). Endotoxins and disease mechanisms. *Annu. Rev. Med.*, **38**, 417–432.

MÓZES, T., WIESZT, E. & ZAHAJSZKY, T. (1989). Role of $PGF_{2\alpha}$ in the superior mesenteric artery induced-shock. *J. Surg. Res.*, **47**, 476–481.

NAESS, F., ROEISE, O., PILLGRAM-LARSEN, J., RUUD, T.E., STADAAS, J.O. & AASWN, A.O. (1989). Plasma proteolysis and circulatory cells in relation to varying endotoxin concentrations in porcine endotoxemia. *Circ. Shock*, **28**, 89–100.

PARRATT, J.R. & STURGESS, R.M. (1974). The effect of indomethacin on the cardiovascular and metabolic responses to *E. coli* endotoxin in cat. *Br. J. Pharmacol.*, **50**, 177–183.

PARRATT, J.R. & STURGESS, R.M. (1975). *E. coli* endotoxin shock in the cat; treatment with indomethacin. *Br. J. Pharmacol.*, **53**, 485–488.

PINCKARD, R.N., McMANUS, L.M. & HANAHAN, D.J. (1984). Molecular heterogeneity of platelet activating-factor produced by stimulated human polymorphonuclear leukocytes. *Biochem. Biophys. Res. Commun.*, **122**, 325–332.

SAXENA, P.R., SCHAMHARDT, H.C., FORSYTH, R.P. & LOEVE, J. (1980). Computer programs for the radioactive microsphere techniques: Determination of regional blood flows and other haemodynamic variables in different experimental circumstances. *Comp. Prog. Biomed.*, **12**, 63–84.

VANE, J.R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature (New Biol.)*, **231**, 232–235.

ZIJLSTRA, F.J. & VINCENT, J.E. (1984). Determinations of leukotrienes and prostaglandins in ^{14}C arachidonic acid labelled human lung tissue by high-performance liquid chromatography and radioimmunoassay. *J. Chromatogr.*, **311**, 39–50.

ZIMMERMAN, J.J. (1990). Therapy for overwhelming sepsis – Clues for treating disease and not just the symptoms. *Crit. Care Med.*, **18**, 118–119.

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