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Platelets, neutrophils, and vasoconstriction after arterial injury by angioplasty in pigs: effects of MK-886, a leukotriene biosynthesis inhibitor

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1 Leukotrienes constitute a class of potent bioactive mediators known to play a pivotal role in inflammation. Since their biosynthesis has been shown to be enhanced by platelet-neutrophil interactions, leukotrienes may be involved in these interactions and the arterial response to injury. Therefore, we investigated the effects of the selective leukotriene biosynthesis inhibitor 3-[1-(4-chlorobenzyl)-3-*t*-butylthio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid (MK-886) on the acute thrombotic and vasomotor responses after arterial injury by angioplasty.

2 Carotid arterial injury was produced by balloon dilatation in control (molecusol vehicle; $n=10$) and treated (MK-886, 10 mg kg^{-1}, i.v.; $n=9$) pigs. The acute thrombotic reaction following deep arterial wall injury was quantified with ^{51}Cr labelled platelets and ^{111}In labelled neutrophils, and the vasomotor response was determined angiographically.

3 Platelet deposition at the site of deep arterial wall injury averaged $56.4 \pm 11.0 \times 10^6$ platelets cm^{-2} in the control group, and was significantly reduced to $18.2 \pm 3.8 \times 10^6$ platelets cm^{-2} ($P<0.005$) by treatment with MK-886. Neutrophil deposition was also decreased by MK-886, from 242.8 ± 36.8 to $120.9 \pm 20.3 \times 10^3$ neutrophils cm^{-2} ($P<0.01$). MK-886-treated animals had a significant decrease in the postangioplasty vasoconstrictive response at the site of endothelial injury distally, from $37.5 \pm 3.1\%$ in the control group to $13.5 \pm 2.5\%$ ($P<0.001$).

4 The effects of MK-886 were associated with a profound inhibition of *ex vivo* leukotriene B_4 (LTB_4) synthesis in blood stimulated by the calcium ionophore A23187 and a significant reduction of neutrophil aggregation in whole blood ($P<0.01$), whereas neutrophil superoxide anion production, serum thromboxane B_2 and platelet aggregation in whole blood were not influenced.

5 The relevant effects of MK-886 are primarily related to inhibition of neutrophil function and suggest an important modulatory role for leukotrienes in the pathophysiological response associated with platelet and neutrophil interactions following arterial injury *in vivo*.

Keywords: Balloon angioplasty; arterial injury; thrombosis; platelet; neutrophil; vasoconstriction; MK-886; leukotrienes; 5-lipoxygenase inhibitor

Introduction

Deep arterial wall injury induced by balloon angioplasty is characterized by rupture of the internal elastic lamina with exposure of the highly thrombogenic media to the circulation (Steele *et al*., 1985; Fuster *et al*., 1992). The subsequent pathophysiological responses such as mural thrombus formation and localized vasoconstriction may compromise the eventual success of the procedure. Although both have been shown to be influenced by platelet adhesion and activation (Steele *et al*., 1985; Merhi *et al*., 1995; 1997), recent evidence suggests an important role for neutrophils in these events (Merhi *et al*., 1994; 1995; Provost & Merhi, 1996). Neutrophils, which have been found to be activated after angioplasty *in vivo* (De Servi *et al*., 1990), may modulate platelet thrombosis and the vasomotor response of the injured artery through the release of proteolytic enzymes, such as cathepsin G (Selak *et al*., 1988) and elastase (Kornecki *et al*., 1986), oxygen-free radicals (Laurindo *et al*., 1991), platelet-activating factor (PAF) (Snyder, 1990) and leukotrienes (Samuelsson, 1983).

Leukotrienes are a group of mediators derived from the 5-lipoxygenase pathway of arachidonic acid metabolism (Samuelsson, 1983). In neutrophils, the intitial step in leukotriene biosynthesis involves the translocation of the cytosolic 5-lipoxygenase to membrane structures and the membrane-bound 18-kD 5-lipoxygenase activating protein (FLAP) (Dixon *et al*., 1990; Reid *et al*., 1990). The 5-lipoxygenase catalyses the conversion of arachidonate into leukotriene A_4 (LTA_4), which can be further metabolized by various blood cells and endothelial cells into LTB_4 (Borgeat & Samuelsson, 1979; McGee & Fitzpatrick, 1986; Claesson & Haeggström, 1988), a powerful activator of neutrophil function (Ford-Hutchinson *et al*., 1980; Ford-Hutchinson, 1990), and cysteinyl-leukotrienes (Feinmark & Cannon, 1986; Claesson & Haeggström, 1988; Maclouf & Murphy, 1988), which are potent vasoconstrictor agents (Michelassi *et al*., 1982; Greenwald *et al*., 1984; Letts *et al*., 1985) with platelet-stimulating properties (Letts *et al*., 1985; Mehta *et al*., 1986). Increased leukotriene production, indicative of 5-lipoxygenase activation *in vivo*, has been observed in patients during episodes of myocardial ischaemia (Carry *et al*., 1992) and after angioplasty (Brezinski *et al*., 1992). Brezinski *et al*. (1992) also showed that transcellular metabolic events between platelets and leukocytes can contribute to eicosanoid forma-

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tion *in vivo* following balloon angioplasty. Therefore, leukotrienes may play an important role in the acute thrombotic and vasomotor responses to arterial injury by angioplasty.

In the present study, the selective leukotriene biosynthesis inhibitor MK-886 (3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid) was used to investigate the involvement of leukotrienes in mural platelet and neutrophil deposition, and vascular tone modulation in a porcine model of arterial injury by angioplasty. MK-886 has been shown to block the membrane association of 5-lipoxygenase by specifically interacting with the membrane-bound protein FLAP (Gillard *et al*., 1989; Rouzer *et al*., 1990), which is necessary for cellular leukotriene synthesis. Here, we describe the efficacy of MK-886 at inhibiting platelet and neutrophil interactions with the balloon-injured arterial wall, and preventing postangioplasty vasoconstriction. The results suggest an important role for leukotrienes in these pathophysiological events.

Methods

Animal preparation

Nineteen, 6–8 week-old, normal cross-breed Yorkshire pigs of either sex weighing 19.4 ± 0.8 kg were prepared in accordance with the guidelines of the Canadian Council on Animal Care regulations. The animals were sedated by intramuscular injection of 225 mg ketamine and 125 mg azaperone, intubated, and mechanically ventilated with ambient air. Anaesthesia was maintained with 0.5% halothane. The haematological parameters of each animal were determined, and the electrocardiogram and arterial blood pressure were monitored.

Isolation and labelling of platelets and neutrophils

Ninety ml of autologous blood anticoagulated with acid-citrate dextrose was collected from the cranial vena cava and used to obtain a platelet-rich plasma by differential centrifugation as previously described (Merhi *et al*., 1994; 1995; Provost *et al*., 1994). This platelet suspension was incubated with 400 μCi ^{51}Cr (Merck Frosst Canada Inc., Pointe-Claire, Quebec, Canada) for 40 min. The suspension was centrifuged to remove unbound ^{51}Cr and the radiolabelled platelets were then resuspended in plasma and reinjected into the animal. In the vehicle and MK-886-treated groups, the labelling efficiency of ^{51}Cr platelets averaged $57.1 \pm 3.3\%$ and $57.6 \pm 4.1\%$, the amount of ^{51}Cr activity injected was 223 ± 14 μCi and 227 ± 17 μCi, the specific activity of ^{51}Cr in blood averaged $1.06 \pm 0.14 \times 10^5$ and $0.87 \pm 0.16 \times 10^5$ platelets per c.p.m., and free circulating ^{51}Cr was only $3.0 \pm 0.3\%$ and $3.0 \pm 0.6\%$, respectively. The radiolabelled platelets were allowed to circulate for approximately 4 h (236 ± 11 min) before the angioplasty procedure.

Neutrophil isolation was performed with the pellet obtained after the first centrifugation of the blood according to a method previously described (Merhi *et al*., 1993; 1997; Provost *et al*., 1994; Provost & Merhi, 1996). The neutrophil suspension was incubated with 450 μCi of [^{111}In]-tropolone (Merck Frosst Canada Inc.) for 30 min. The suspension was centrifuged to remove unbound ^{111}In, and the radiolabelled neutrophils were then resuspended in plasma and reinjected into the animal. This procedure yielded a neutrophil preparation that is over 95% pure and 90% viable, as assessed by the trypan blue exclusion test. In the vehicle and MK-886-treated groups, the labelling efficiency of ^{111}In neutrophils averaged $93.6 \pm 1.8\%$ and $92.6 \pm 3.0\%$, the amount of ^{111}In activity injected was 412 ± 15 μCi and 363 ± 20 μCi, the specific activity of ^{111}In in blood averaged 138 ± 17 and 120 ± 16 neutrophils per c.p.m., and free circulating ^{111}In was only $7.6 \pm 1.6\%$ and $5.4 \pm 1.0\%$, respectively. The radiolabelled neutrophils were allowed to circulate for approximately 3.5 h (206 ± 10 min) before the angioplasty procedure.

Carotid arterial injury

Carotid injury was performed with a 7F-polyethylene balloon dilatation catheter (size, 8 mm × 3 cm, Meditech Inc., Watertown, MA, U.S.A.), as previously described (Merhi *et al*., 1994; 1995; 1997; Provost & Merhi, 1996). A 9F-introducer sheath was inserted into the right femoral artery for introduction of the balloon dilatation catheter, and an 8F-introducer sheath was inserted into the femoral vein for blood sampling and drug infusion. One hour before the angioplasty procedure, animals received either MK-886 (10 mg kg^{-1}, i.v., $n=9$) or molecusol 25% vehicle (i.v., $n=10$). Molecusol (2-hydroxypropyl-β-cyclodextrin) was recommended by Merck Frosst for the intravenous formulation of MK-886. After a single bolus of heparin (100 iu kg^{-1}, i.v.), the balloon dilatation catheter was inserted into the right femoral artery and advanced under fluoroscopic control into the left and right common carotid arterial segments between the fifth and fourth cervical vertebrae. Five inflations were performed at 6 atmosphere pressure, each for 30 s with a 60 s interval between each inflation. The vasoconstrictive response, localized at the site of the distal tapering end of the balloon in the area denuded of endothelial cells without rupture of the internal elastic lamina, as determined by histological analyses and scanning electron microscopy observations, was quantified angiographically as detailed previously (Merhi *et al*., 1994; 1995; Provost & Merhi, 1996). In all pigs, angiograms of the common carotid arteries were obtained before and during, angioplasty and approximately 30 s after the last inflation with selective intra-arterial injection of 5.0 ml Contray 30. The degree of vasoconstriction was calculated by measuring the lumen diameter at baseline and after balloon dilatation at the site of the greatest narrowing at the distal tapering end of the balloon. The vasoconstriction was defined as the lumen diameter (measured with an electronic calliper to the nearest 0.1 mm) on the postdilatation angiogram, and expressed as percentage of the lumen diameter on the predilatation angiogram. The angiograms obtained before and during balloon dilatation were used to determine the balloon-to-artery ratio for each artery.

Quantification of platelet and neutrophil deposition

One hour after angioplasty, the animals were killed and the carotid arteries were perfusion-fixed *in situ* with a buffered solution of 2% glutaraldehyde and 1% paraformaldehyde (Merhi *et al*., 1994; 1995; Provost & Merhi, 1996). The fixed carotid arteries were then removed and cleaned of all adventitial tissue. The dilated portion was divided into three segments, and the internal diameter and length of each segment were measured with an electronic calliper to determine the surface area (cm^2). After surface measurements, the radioactivity of each segment as well as that of reference blood samples was counted for 5 min in a gamma counter (Minaxi 5000, Packard Instruments Co., Downers Grove, IL, U.S.A.), equipped with a computer and a multinuclide analysis

programme. As the blood platelet and neutrophil counts and the radioactivity of each radionuclide in blood and on the arterial segments were known, platelet ($\times 10^6$) and neutrophil ($\times 10^3$) deposition per cm^2 on the deeply injured carotid arteries was calculated, as previously described (Merhi *et al*., 1993).

Histological analysis

After radioactivity counting, representative 2- to 3-mm sections from each dilated arterial segment were processed and embedded in paraffin. Cross-sections (4 μm) were stained with Movat pentachrome stain, which produces intense staining of the internal and external elastic lamina. All specimens were evaluated for the presence of deep arterial wall injury, which is characterized by the presence of tears through the internal elastic lamina with the exposure of the arterial media.

Morphometric analysis was performed on each section of the deeply injured arteries in order to quantify the extent of injury (Provost *et al*., 1997). The number of internal elastic laminal tears and the arc length of the internal elastic laminal fracture (fracture length), traced from one dissected laminal end to the other, were used as a measure of the extent of injury. The circumferences demarcated by the external and internal elastic lamina were also measured, and the ratios of fracture length-to-external elastic lamina and of fracture length-to-internal elastic lamina were calculated to correct for vessel size.

Platelet and neutrophil aggregation studies

Aggregation studies were performed with an impedance aggregometer (Type 500, Chronolog Corp., Harvestown, PA, U.S.A.) (Merhi *et al*., 1993; 1994; Provost & Merhi, 1996) on fresh arterial blood samples obtained before and 1 hour after the administration of MK-886. Aggregation was induced by adding to 450 μl of blood, either 50 μl of the platelet agonist ADP 10 μM, or 50 μl of the neutrophil agonist formyl-methionyleucyl phenylalanine (FMLP) 200 nM. All studies were performed within the first minute after blood sampling. The extent of platelet and neutrophil aggregation was automatically quantified (amplitude, ohms) with AGGRO/LINK software (Chronolog Corp).

Assessment of LTB_4 synthesis in whole blood

Fresh arterial blood samples, anticoagulated with heparin (20 iu ml^{-1}), were obtained from the animals before, 1 h and 2 h after the administraton of MK-886 or its vehicle. One-milliliter aliquots of blood were dispensed into 5 ml polystyrene test tubes and treated with the calcium ionophore A23187 (stock solution in DMSO, final concentration 50 μM) or DMSO 10 min after blood collection. After 30 min of incubation at 37°C, blood samples were cooled in an ice-water bath and centrifuged at 1900 *g* for 10 min at 4°C. Plasma was collected and stored at −70°C until analysis. LTB_4 levels in plasma samples were determined by reverse-phase high-performance liquid chromatography (r.p.-h.p.l.c.) analysis, as described previously (Surette *et al*., 1994). Briefly, 200 μl plasma samples were denatured with 2.5 ml of acetonitrile containing 12.5 ng of PGB_2 as internal standard. Samples were centrifuged to remove the precipitated material and the supernatants were evaporated under reduced pressure. The residues were dissolved with 500 μl of methanol, diluted to a final volume of 2 ml and directly analysed by r.p.-h.p.l.c. Elution of compounds was monitored by u.v. photometry at 280 nm.

Measurement of serum TXB_2

For determination of serum thromboxane B_2 (TXB_2), blood samples were obtained before and 1 h after the administration of MK-886 or its vehicle, and allowed to clot for 30 min at room temperature. After centrifugation, the serum was stored at −70°C until analysis. After solid-phase extraction using C2 reverse phase column, TXB_2 concentration was measured by a specific RIA kit (Amersham, Buckinghamshire, U.K.).

Cytochrome c reduction assay

Cytochrome c reduction assay was performed before and 1 h after administration of MK-886, as previously described (Provost & Merhi, 1996). Briefly, 3.1 mg ml^{-1} of cytochrome c type IV and 1 mg ml^{-1} of opsonized zymosan were incubated in HBSS-HEPES buffer, pH 7.4 for 5 min at 37°C. One hundred microlitres of heparin-treated blood was then added, and the reaction allowed to proceed for 10 min. The reaction was interrupted with ice-cold HBSS-HEPES buffer containing 325 u ml^{-1} superoxide dismutase (SOD). The mixture was then centrifuged and the supernatant was read spectrophotometrically at 550 and 468 nm. Each blood sample was analysed in the absence (test tube) and the presence (reference tube) of exogenous SOD.

Drugs

Ketamine hydrochloride (Rogarsetic, 100 mg ml^{-1}) was purchased from Rogar/STB Inc. (Montreal, Quebec, Canada), azaperone (Stresnil, 40 mg ml^{-1}) from Janssen Pharmaceutica (Mississauga, Ontario, Canada) and halothane (Fluothane) from Ayerst (Montreal, Quebec, Canada). MK-886 was generously donated by Merk Frosst Center for Therapeutic Research (Pointe-Claire, Quebec, Canada). Molecusol 25% was obtained from Pharmatec Inc. (Alashua, FL, U.S.A.). Heparin (10,000 iu ml^{-1}) was purchased from Leo Laboratories (Ajax, Ontario, Canada) and Conray 30 (iothalamate meglumine 30%) from Mallinckrodt Medical Inc. (Montreal, Quebec, Canada). ADP, FMLP, calcium ionophore A23187, cytochrome c and SOD were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Statistical analysis

Results are expressed as mean ± s.e. Intergroup analyses were performed with Student's unpaired *t* test and intragroup comparisons were assessed by Student's paired *t* test. Differences were considered statistically significant at the 95% confidence level ($P < 0.05$).

Results

Haematological parameters of the vehicle and MK-886-treated groups are shown in Table 1. Haematocrit, erythrocyte and haemoglobin concentrations, blood platelet and leukocyte counts, and the percentage of neutrophils were similar between the vehicle and MK-886-treated animals. Heart rate and arterial blood pressure were not significantly influenced by the administration of MK-886 or its vehicle and were similar between the two groups.

Histological analysis

Deep arterial injury was found in 17 of 20 dilated arteries in the vehicle-treated pigs, and in 16 of 18 dilated arteries in the MK-886-treated animals. Results from the quantitative morphometric analysis of the deeply injured carotid arteries are shown in Table 2. Rupture of the internal elastic lamina was found in 94.9% and 94.7% of the arterial segments analysed in the vehicle and MK-886-treated groups, respectively. The number of internal elastic laminal tears per segment and the fracture length of the internal elastic lamina were also similar between the two groups. In addition, the fracture length-to-external elastic lamina and the fracture length-to-internal elastic lamina ratios were not different. These data indicate that the extent of deep arterial injury was similar between the vehicle and MK-886-treated groups.

Platelet and neutrophil deposition

One hour after angioplasty, mural platelet deposition on the deeply injured carotid arteries was significantly reduced by more than 65% by treatment with MK-886, as compared to controls (Figure 1). The leukotriene biosynthesis inhibitor also significantly decreased neutrophil deposition on the deeply injured arteries by more than 50% ($P<0.001$) (Figure 1).

Table 1 Haematological parameters of vehicle and MK-886-treated animals

Parameters	*Vehicle*	*MK-886*
Haematocrit (%)	22.6±1.0	22.3±1.4
Erythrocytes ($\times 10^9$ ml $^{-1}$)	3.94±0.17	4.04±0.28
Haemoglobin (g l^{-1})	88±3	85±4
Platelets ($\times 10^6$ ml^{-1})	664±59	627±68
Leukocytes ($\times 10^6$ ml^{-1})	14.7±2.2	13.7±1.1
Neutrophils (%)	54±4	53±3

Values are mean±s.e.mean; $n=10$ (vehicle), $n=9$ (MK-886-treated).

Table 2 Results of histological analysis of sections from deeply injured carotid arteries of pigs in the vehicle and MK-886-treated groups

	Vehicle	*MK-886*
Number of arteries analysed	17	16
Number of segments analysed	98	94
Number of segments with deep injury (%)	93 (94.9%)	89 (94.7%)
Number of internal elastic laminal tears per segment	1.35±0.10	1.30±0.13
Fracture length (mm)	1.68±0.08	1.59±0.08
Fracture length-to-external elastic lamina ratio	0.168±0.008	0.176±0.009
Fracture length-to-internal elastic lamina ratio	0.191±0.009	0.204±0.010

Values are mean±s.e.mean.

Postangioplasty vasoconstriction

As shown in Table 3, the lumen diameter of the carotid arteries before angioplasty and the balloon-to-artery ratio were similar between the vehicle and MK-886-treated groups. Immediately after the last inflation (≈30 s), a localized vasoconstrictive response was observed at the distal edge of the dilated portion, in the area denuded of endothelial cells without rupture of the internal elastic lamina. This postangioplasty vasoconstriction was reduced by almost 65% by the leukotriene biosynthesis inhibitor MK-886 ($P<0.001$ versus vehicle); this relevant effect resulted in a significantly greater carotid arterial diameter in the MK-886-treated group, as compared to controls.

Ex vivo *platelet and neutrophil aggregation*

We examined the effects of the leukotriene biosynthesis inhibitor MK-886 on *ex vivo* platelet and neutrophil aggregation induced by specific agonists in whole blood by use of impedance aggregometry. Neutrophil aggregation induced by the neutrophil agonist FMLP was significantly reduced by MK-886 by nearly 50% from 18.9±2.3 to 9.9±2.2 Ω ($n=7$, $P<0.01$), whereas platelet aggregation in response to the platelet agonist ADP was not influenced (from 18.8±1.6 to 18.5±2.0 Ω; $n=6$, NS).

Assessment of LTB_4 synthesis in whole blood

Figure 2 shows profiles from r.p.-h.p.l.c. analysis of plasma samples. Stimulation of porcine blood with A23187, before the infusion of MK-886 or its vehicle, resulted in the formation of

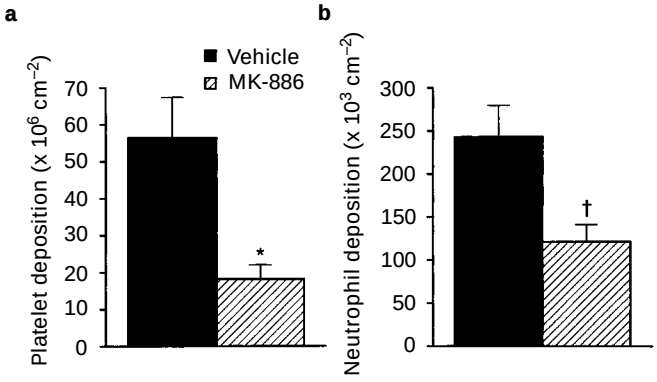

Figure 1 The effects of MK-886 on platelet and neutrophil deposition on deeply injured carotid arteries by angioplasty. Treatment with MK-886 ($n=16$ arteries) significantly reduced platelet (a) and neutrophil (b) deposition on the injured artery, as compared with the vehicle-treated group ($n=17$ arteries). Results are shown as mean±s.e. *$P<0.005$ and †$P<0.01$ versus vehicle.

Table 3 The balloon-to-artery ratio, the diameter of the carotid arteries before and after balloon dilation, and the degree of vasoconstriction in the vehicle and MK-886-treated groups

Group	*Balloon-to-artery ratio*	*Carotid arterial diameter* (mm) *Before*	*After*	*Vasoconstriction* (%)
Vehicle	1.33±0.05	3.9±0.1	2.5±0.2*	37.5±3.1
MK-886	1.36±0.04	3.7±0.1	3.2±0.2*†	13.5±2.5‡

Values are mean±s.e.mean; $n=17$ and 16 arteries for vehicle and MK-886-treated groups, respectively. *$P<0.001$ versus before dilation; †$P<0.05$ versus vehicle; ‡$P<0.001$ versus vehicle.

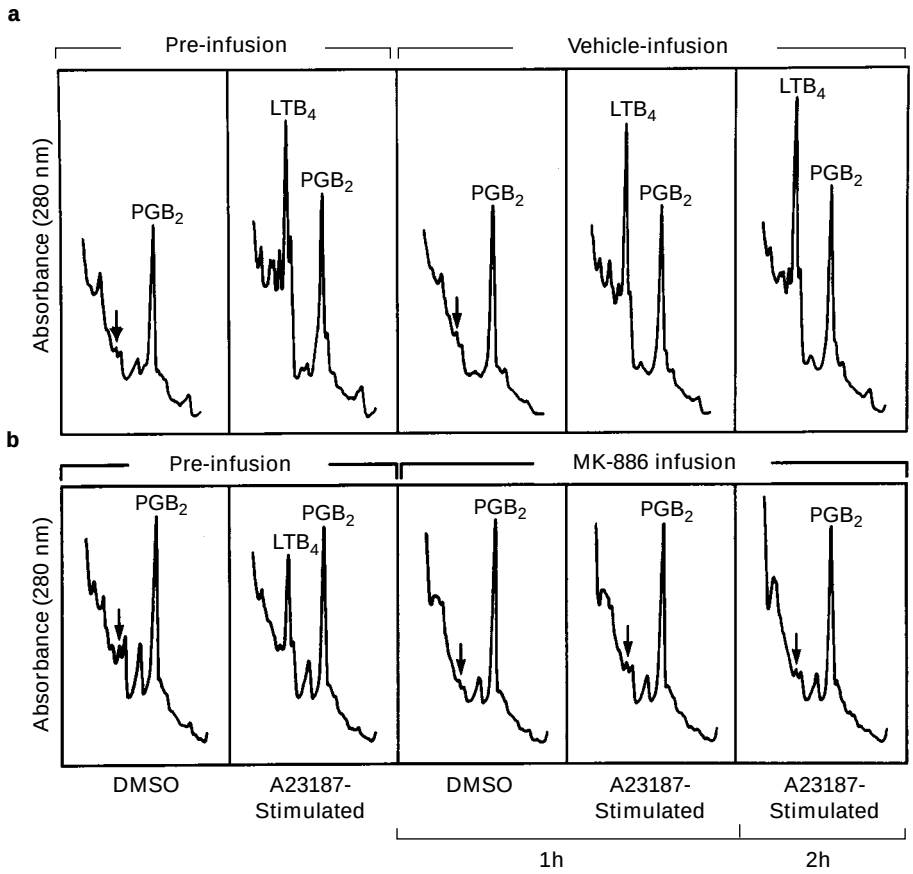

Figure 2 Reverse phase-h.p.l.c. chromatograms of plasma samples (200 μl) from heparin-treated porcine blood stimulated *ex vivo* with 50 μM A23187 (30 min at 37°C) or DMSO (diluent). Blood samples were obtained before and 1 h or 2 h after administration of MK-886 (10 mg kg^{-1}, i.v.) (b) or its vehicle (molecusol) (a). PGB_2 (12.5 ng) was used as an internal standard; as assessed with calibrated standards, the amount of LTB_4 in plasma from A23187-stimulated blood before MK-886 infusion was ~10 ng. Retention times for PGB_2 and LTB_4 were 15.5 and 17 min, respectively. Attenuation of the u.v. photometer was 0.01 absorbance unit at full scale. The data shown are from one experiment representative of five.

detectable amounts of LTB_4. Treatment of the blood with the diluent only (DMSO) had no stimulating effect and only traces of LTB_4 were observed. Treatment of animals with MK-886, but not with the vehicle, 1 h or 2 h before collection of blood samples and *ex vivo* stimulation with A23187 resulted in profound inhibition of LTB_4 synthesis; in five experiments, the levels of LTB_4 synthesis were at $14 \pm 4\%$ and $15 \pm 4.9\%$ of control (100%), at 1 h and 2 h after beginning of MK-886 infusion, respectively. Only traces of cysteinyl-leukotrienes were formed in these experimental conditions (data not shown), the synthesis of which was also inhibited in blood from MK-886-treated animals.

Serum TXB_2 levels

The selectivity of the leukotriene synthesis inhibitor MK-886 for the 5-lipoxygenase pathway was assessed by measuring serum TXB_2, an index of cyclo-oxygenase activity. Serum TXB_2 levels were not significantly altered by MK-886 (from 757 ± 174 to 827 ± 108 pg TXB_2 ml^{-1}; $n = 5$, NS) or its vehicle (from 858 ± 139 to 823 ± 142 pg TXB_2 ml^{-1}; $n = 8$, NS).

Superoxide anion production

We performed a cytochrome c reduction assay in order to ascertain that MK-886 did not interfere with neutrophil superoxide anion production. Results show that administration of MK-886 did not modify superoxide anion generation from neutrophils challenged with opsonized zymosan in whole blood (from 10.9 ± 0.8 to 11.2 ± 0.3 nmol superoxide anion 10 min^{-1} per 10^6 neutrophils; $n = 4$, NS).

Discussion

Many compounds known for their ability to inhibit leukotriene synthesis act on 5-lipoxygenase through redox mechanisms, are nonspecific and affect the activity of cyclo-oxygenase and other lipoxygenases (Duniec *et al.*, 1983). The lack of specificity of these agents has somehow hampered the elucidation of the exact role of leukotrienes in various pathophysiological disorders, particularly those involving cyclo-oxygenase activation and oxygen-free radical generation, such as arterial thrombosis and myocardial ischaemia and reperfusion injury. Recently, a new class of leukotriene inhibitors known as FLAP antagonists, and exemplified by MK-886 (Gillard *et al.*, 1989), has been shown to inhibit selectively leukotriene biosynthesis by blocking the translocation of cytosolic 5-lipoxygenase to the membrane-bound FLAP (Rouzer *et al.*, 1990).

In the present study, *ex vivo* stimulation of blood samples from MK-886-treated animals clearly indicates that the *in vivo* administration of the drug efficiently inhibited leukotriene synthesis by blood leukocytes. In addition, neither platelet cyclo-oxygenase activity, as estimated by serum TXB_2, nor neutrophil superoxide anion generation, as evaluated by SOD-

dependent reduction of cytochrome c, were modified by MK-886. Furthermore, MK-886 has been shown to have no effect on the formation of 12- (Gillard *et al*., 1989) or 15-lipoxygenase products (Ménard *et al*., 1990). The selective character of MK-886 for the 5-lipoxygenase pathway was an absolute requirement for elucidating the exact implication of leukotrienes in our arterial injury model.

The implication of platelets and derived products in thrombosis and vasospasm has been extensively studied during the past decade. Pharmacological studies have demonstrated the antithrombotic efficacy of various platelet inhibitors in porcine carotid angioplasty models, such as aspirin (Merhi *et al*., 1997), heparin (Heras *et al*., 1992), hirudin (Heras *et al*., 1990) and SIN-1, a NO donor (Groves *et al*., 1993). Platelets may also play a role in vasospasm, since aggregating platelets have been shown to induce contraction of isolated arteries denuded of endothelium through the release of TXA_2 and 5-hydroxytryptamine (5-HT) in organ chamber experiments (Houston *et al*., 1986). The fact that postangioplasty vasoconstriction is inhibited by aspirin (Merhi *et al*., 1997) is also in agreement with the importance of platelets and platelet-derived products in vascular tone modulation.

The presence of neutrophils in developing thrombi, as described by Henry in 1965, has suggested a potential modulatory role for neutrophils. Recently, we have shown that, in addition to platelets, neutrophils are involved in mural thrombus formation and vasoconstriction related to arterial injury by angioplasty (Merhi *et al*., 1994; 1995; Provost & Merhi, 1996; Provost *et al*., 1997). Neutrophils have been shown to be activated after angioplasty (De Servi *et al*., 1990) and to interact with the balloon-injured arterial wall. More recently, we have shown that the beneficial effects of the dual lipoxygenase/cyclo-oxygenase inhibitor BW755C and the NO donor SIN-1 in our porcine model of balloon angioplasty were predominantly related to inhibition of neutrophil function (Provost & Merhi, 1996; Provost *et al*., 1997). This has led us to examine, more specifically, the potential implication of leukotrienes with the use of MK-886, a more selective leukotriene inhibitor.

In the present study, MK-886 significantly reduced platelet and neutrophil interactions with the deeply injured arteries, and prevented postangioplasty vasoconstriction, suggesting an important contribution of leukotrienes to these events. No inhibitory effect of MK-886 was observed on platelet aggregation induced by the specific platelet agonist ADP. In addition, MK-886, which has been shown not to influence the synthesis of 12-hydroxyeicosatetraenoic acid or TXA_2 in isolated platelet suspensions (Gillard *et al*., 1989), did not modify serum TXB_2 levels. In fact, it is unlikely that MK-886 will directly inhibit platelet function, knowing that platelets lack 5-lipoxygenase activity. These findings suggest that the antithrombotic efficacy of MK-886 *in vivo* may rather imply an alternative modulatory pathway, which is more likely to involve neutrophil-derived leukotrienes.

Our results show that *in vivo* treatment with MK-886 profoundly inhibited LTB_4 synthesis by blood leukocytes, in association with a significant reduction of neutrophil aggregation in blood. These findings are in agreement with the potent neutrophil activating and aggregating properties of LTB_4, which also possesses potent chemotactic and chemokinetic activities (Maclouf & Murphy, 1988; Ford-Hutchinson, 1990). LTB_4, which has no documented vascular effect (Lewis & Austen, 1984), also stimulates a number of neutrophil functions, including aggregation (Ford-Hutchinson *et al*., 1980), calcium mobilization, degranulation and adherence (Ford-Hutchinson, 1990). Neutrophil degranulation may lead to the release of proteolytic enzymes, such as cathepsin G and elastase, which may contribute to neutrophil-mediated platelet activation (Kornecki *et al*., 1986; Selak *et al*., 1988). When activated, platelets have been shown to promote LTB_4 synthesis in stimulated neutrophils (Palmantier & Borgeat, 1991) which, in turn, increase platelet thromboxane formation (Maugeri *et al*., 1992), through biochemical cooperations involving the transfer of unmetabolized arachidonic acid. Activated neutrophils also synthesize the phospholipid platelet-activating factor (PAF), a strong activator of platelet and neutrophil function (Snyder, 1990). Interestingly, the formation of PAF has been shown to be enhanced by LTB_4 (Billah *et al*., 1985). In turn exogenous PAF (Lin *et al*., 1982) as well as endogenous PAF and LTB_4 (McDonald *et al*., 1994) may enhance leukotriene synthesis in neutrophils. These autocrine mechanisms may potentially amplify leukotriene synthesis and neutrophil activation *in vivo*.

The synthesis of cysteinyl-leukotrienes, which was of a relatively small intensity compared to that of LTB_4 in *ex vivo* stimulated blood, was also inhibited by the leukotriene biosynthesis inhibitor MK-886. This observation suggests that inhibition of cysteinyl-leukotriene formation may also account for the *in vivo* beneficial effects observed with MK-886. Thus, through a biochemical cooperation, LTA_4 synthesized by neutrophils may be transferred to platelets (Maclouf & Murphy, 1988), where it is transformed into the platelet-stimulating (Letts *et al*., 1985; Mehta *et al*., 1986) and vasoconstrictive (Greenwald *et al*., 1984; Letts *et al*., 1985) LTC_4 which is further metabolized into LTD_4 and LTE_4. In addition, a marked synergism between the vascular effects of LTC_4 and TXA_2 (Nichols *et al*., 1988), and between cysteinyl-leukotrienes (LTC_4, LTD_4 and LTE_4) and thrombin in potentiating platelet aggregation *in vitro* (Mehta *et al*., 1986), has been found. The potent stimulating effects of LTC_4 and LTD_4 on endothelial P-selectin (Datta *et al*., 1995) and P-selectin, which is also expressed following platelet activation (Berman *et al*., 1986), may have important implications in thrombosis and haemostasis, since P-selectin is known to mediate neutrophil adhesion to surface adherent activated platelets in a model of vessel wall injury (Yeo *et al*., 1994) and to favour transcellular metabolism of arachidonic acid between platelets and neutrophils (Maugeri *et al*., 1994).

Recent studies have shown that both platelets and neutrophils, which accumulate at the site of arterial injury induced by balloon angioplasty, may modulate postangioplasty vasoconstriction (Merhi *et al*., 1994; 1995; 1997; Provost & Merhi, 1996; Provost *et al*., 1997). Close proximity between platelets and neutrophils at the site of injury may facilitate modulation of each activity and favour biochemical cooperation or exchange in the generation of vasoactive mediators. Inhibition of platelet and neutrophil interactions with the deeply injured arteries may therefore account, at least in part, for the decreased vasoconstrictive response observed in MK-886-treated animals. MK-886 may further reduce this vasoconstriction by inhibiting the formation and release of leukotrienes, known to be potent vasoconstrictors, since the beneficial vascular effects of MK-886 parallelled a sustained inhibition of leukotriene biosynthesis.

In addition to the thrombotic process and vasospasm, restenosis remains a major unsolved limitation of angioplasty. Many approaches to reduce postangioplasty restenosis have focused on retarding smooth muscle cell growth, lowering cholesterol and administering antiplatelet agents, anticoagulants, NO donors, fish oils, calcium channel blockers, angiotensin-converting enzyme inhibitors and antiproliferative agents. However, clinical studies have generally proved

unsuccessful. It is possible that this lack of success stems from the fact that the target has often been hyperplasia resulting from smooth muscle proliferation, which may not be the primary mechanism by which restenosis occurs. Given the complexity of the pathophysiology of restenosis, it seems unlikely that any single agent substantially prevents it. The now well-recognized multifactorial nature of the restenotic lesions implicates a homotypic, as well as a heterotypic, interaction between platelets, leukocytes and the damaged arterial wall. Arterial injury produced by percutaneous transluminal coronary angioplasty (PTCA) could induce activation and migration of neutrophils to the injured artery stenotic site. Activated neutrophils produce potent vasoactive substances, such as oxygen free radicals, leukotrienes, proteolytic enzymes and PAF that potentiate platelet activity and inhibit endothelial function. Neutrophil activation after coronary angioplasty has been demonstrated previously (De Servi *et al*., 1990; Ikeda *et al*., 1994). Pietersma *et al*., (1995) found that activated blood granulocytes prevent luminal renarrowing after coronary angioplasty, while activated blood monocytes promote late lumen loss. More recently, Mickelson *et al*., (1996) have shown that despite standard aspirin and heparin therapy, monocytes and neutrophil activation and leukocyte-platelet complexes after coronary angioplasty were higher in patients experiencing late clinical events including restenosis, myocardial infarction and unstable angina. These findings highlight the importance of leukocytes in association or not with platelet adhesion, not only in the acute events but also in restenosis. However, further experimental studies are required to clarify the involvement of neutrophils and/or their products in late luminal loss after angioplasty.

In summary, our use of the selective leukotriene biosynthesis inhibitor MK-886 has allowed us to demonstrate a key role for leukotrienes in mediating mural thrombus formation and the acute vasomotor response of the injured artery *in vivo*. Inhibition of leukotriene biosynthesis and/or actions may provide a new approach toward pharmacological inhibition of mural thrombosis and vasoconstriction associated with angioplasty injury.

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