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Cyclic AMP elevating agents and nitric oxide modulate angiotensin II-induced leukocyte-endothelial cell interactions *in vivo*

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1 Angiotensin (Ang-II) is a key molecule in the development of cardiac ischaemic disorders and displays proinflammatory activity *in vivo*. Since intracellular cyclic nucleotides elevating agents have proved to be effective modulators of leukocyte recruitment, we have evaluated their effect on Ang-II-induced leukocyte-endothelial cell interactions *in vivo* using intravital microscopy within the rat mesenteric microcirculation.

2 Pretreatment with iloprost significantly inhibited (1 nM) Ang-II-induced increase in leukocyte rolling flux, adhesion and emigration at 60 min by 96, 92 and 90% respectively, and returned leukocyte rolling velocity to basal levels. Pretreatment with salbutamol or co-superfusion with forskolin exerted similar effects.

3 When theophylline was administered, leukocyte rolling flux, adhesion and emigration elicited by Ang-II were significantly attenuated by 81, 89 and 71% respectively. Rolipram administration caused similar reduction of Ang-II-induced leukocyte responses.

4 Co-superfusion of Ang-II with the NO-donor, spermine-NO, or 8-Br-cyclic GMP, or pretreatment with a transdermal nytroglycerin patch, resulted in a significant reduction of the leukocyte-endothelial cell interactions elicited by Ang-II.

5 Salbutamol preadministration did not modify leukocyte-endothelial cell interactions elicited by either L-NAME or L-NAME + Ang-II, indicating that the inhibitory leukocyte effects caused by cyclic AMP-elevating agents are mediated through NO release.

6 In conclusion, we have provided evidence that cyclic AMP elevating agents and NO donors, are potent inhibitors of Ang-II-induced leukocyte-endothelial cell interactions. Thus, they could constitute a powerful therapeutical tool in the control of the leukocyte recruitment characteristic of the vascular lesions that occur in cardiovascular disease states where Ang-II plays a critical role. *British Journal of Pharmacology* (2001) **133,** 485–494

Keywords: Angiotensin II; nitric oxide; cyclic AMP; cyclic GMP; leukocyte; endothelium; intravital microscopy

Abbreviations: CAM, cell adhesion molecule; cyclic AMP, adenosine 3′ : 5′-cyclic monophosphate; cyclic GMP, guanosine 3′ : 5′-cyclic monophosphate; DMSO, dimethylsulphoxide; Dv, venular diameter; HLMVECs, human lung microvascular endothelial cells; HUVECs, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; L-NAME , N^G-nitro-L-arginine methyl ester, PDE, phosphodiesterase; PGI_2, prostacyclin; PKA, proteinkinase A; TNFα, tumour necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1; V_{mean}, mean red blood cell velocity; V_{rbc}, centreline red blood cell velocity; V_{wbc}, leukocyte rolling velocity

Introduction

Local leukocyte recruitment to the vessel wall is a hallmark of the early stages of atherosclerosis, acute myocardial infarction and several renal diseases of diverse aetiology (Badimon *et al*., 1993; Ricevuti *et al*., 1990; Klahr *et al*., 1988). Clinical observations suggest a link between augmented renin-angiotensin system activity and the development of cardiac ischemic disorders (Brunner *et al*., 1972; Cambien *et al*., 1992). In this context, we have recently revealed that angiotensin II (Ang-II) shows proinflammatory activity *in vivo* at sub-vasoconstrictor doses. In particular, it induces leukocyte trafficking into the rat mesenteric microvasculature through endothelial P-selectin up-regulation in the vessel wall (Piqueras *et al*., 2000). These findings indicate that inappropriate leukocyte-endothelial cell interactions may occur prior to hypertension and that Ang-II contributes to the onset and progression of the vascular damage associated with cardiovascular disease states such as hypertension, atherosclerosis and myocardial ischaemia-reperfusion injury.

Recent interest has focused on the intracellular signalling mechanisms involved in the regulation of cell adhesion molecule (CAM) expression as a tool for modulating inflammation. In a wide range of cells and tissues, adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP) has proved to be an important target. Increased levels of this second messenger within the cells activates proteinkinase A (PKA) which, in turn, phosphorylates other substrates and has been shown to have anti-inflammatory effects (Giembycz & Raeburn, 1991; Teixeira *et al*., 1997).

In this way, prostacyclin (PGI_2) exerts its action through activation of adenylate cyclase and the consequent increase in

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cyclic AMP levels. Similarly, interaction with β_2-adrenoceptors is a transcriptional effect mediated through elevation of this second intracellular messenger. There is evidence that both exert anti-inflammatory effects by, among other actions, suppressing tumour necrosis factor-α (TNFα) production, leukocyte recruitment and CAM expression (Kowala *et al*., 1993; Severn *et al*., 1992; Teixeira & Hellewell, 1997; Derian *et al*., 1995). On the other hand, phosphodiesterases (PDEs) are enzymes responsible for the breakdown of cyclic nucleotides within cells (Nicholson *et al*., 1991) and inhibition of PDE enzymes has gained strong support as a possible mechanism of theophylline action (Kuehl *et al*., 1987). Moreover, inflammatory cells contain mainly PDE type IV (PDE–IV) isoenzyme (cyclic AMP-specific) which accounts for most of the metabolism of cyclic AMP in these cells and is selectively inhibited by rollipram. Indeed, theophylline and rolipram have been shown to decrease leukocyte recruitment and function and cause downregulation of both leukocyte and endothelial CAMs expression (Spoelstra *et al*., 1998; Braun *et al*., 1997; Okubo *et al*., 1997; Santamaria *et al*., 1997; Teixeira *et al*., 1997; Blease *et al*., 1998). Finally, production and release of NO stimulates guanylyl cyclase, increasing guanosine 3′:5′-cyclic monophosphate (cyclic GMP) formation, therefore regulating blood pressure, blood flow, platelet aggregation and leukocyte adhesion (Moncada *et al*., 1991; Radomski *et al*., 1987; Kubes *et al*., 1991).

Since agents which elevate cyclic AMP and cyclic GMP are effective modulators of leukocyte recruitment *in vivo*, the present study was undertaken to evaluate whether Ang-II-induced leukocyte-endothelial cell interactions can be reduced or inhibited by cyclic AMP or cyclic GMP elevating agents. To test this hypothesis we used intravital microscopy within the rat mesenteric microcirculation and examined the effect of various agents known to elevate cyclic nucleotides through different mechanisms of action and evaluated their effect on the leukocyte responses elicited by Ang-II. This is of relevance as new therapeutical strategies based on reduced leukocyte recruitment could be used in the control of vascular disease disorders where Ang-II plays a critical role.

Methods

Animal preparation

Male Sprague-Dawley rats (200–250 g) were deprived of food but not water for 20–24 h before each experiment. The animals were anaesthetized with pentobarbital sodium (65 mg kg^{-1}, i.p.) and a tracheotomy was performed to facilitate spontaneous breathing. A polyethylene tube was inserted into the right carotid artery and jugular vein to measure systemic arterial blood pressure (MABP) through a pressure transducer (Spectramed Stathan P-23XL) connected to a recorder (GRASS RPS7C8B, Quincy, MA, U.S.A.) and to enable intravenous administration of additional reagents (anaesthetic or drug) respectively.

Intravital microscopy

The experimental preparation used was similar to that previously described (Sanz *et al*., 1999). The abdominal cavity was opened *via* a midline incision and a loop of the mid-jejunal mesentery was gently exteriorized and placed carefully on a heated transparent pedestal to allow transillumination of a 3 cm^2 segment of the tissue. Pedestal and animal temperature were maintained at 37°C. The exposed mesentery was superfused continuously at a rate of 1–2 ml min^{-1} with a warmed bicarbonate-buffered saline solution (pH 7.4). All exposed tissue was covered with saline-soaked gauze to prevent evaporation. An orthostatic microscope (Nikon Optiphot-2, SMZ1, Badhoevedorp, The Netherlands) with a 20× objective lens and a 10× eyepiece (Nikon SLDW, Badhoevedorp, The Netherlands) was used to observe the mesenteric microcirculation. A video camera (Sony SSC-C350P, Koeln, Germany) mounted on the microscope projected the image onto a color monitor (Sony Trinitron PVM-14N2E, Koeln, Germany) and the images were video recorded (Sony SVT-S3000P, Koeln, Germany) for play back analysis. The final magnification of the video screen was 1300×.

Single unbranched mesenteric venules with diameters of 25–40 μm were selected for study. Venular diameter (Dv) was measured on-line with a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, Texas, U.S.A.). Centerline red blood cell velocity (V_{rbc}) was also measured on-line by using an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, College Station, Texas, U.S.A.). Venular blood flow was calculated from the product of mean red blood cell velocity ($V_{mean} = V_{rbc}/1.6$) and microvascular cross-sectional area, assuming cylindrical geometry. Venular wall shear rate (γ) was calculated based on the Newtonian definition: $\gamma = 8 \times (V_{mean}/D_v)$ s^{-1} (House & Lipowsky, 1987).

The number of rolling, adherent and emigrated leukocytes as determined off-line during playback analysis of videotaped images. Rolling leukocytes were defined as those white blood cells moving more slowly than erythrocytes in the same vessel and were determined by counting the number of rolling leukocytes min^{-1} passing a reference point in the microvessel. The same reference point was used throughout the experiment because leukocytes may roll for only a section of the vessel before rejoining the blood flow or becoming firmly adherent. Leukocyte rolling velocity (V_{wbc}) was determined from the time required for a leukocyte to transverse a 100 μm length of the venule and was expressed as μm s^{-1}. A leukocyte was considered to be adherent to venular endothelium if it remained stationary for 30 s or longer. Adherent cells were expressed as the number per 100 μm length of venule. Leukocyte emigration was expressed as the number of white blood cells per microscopic field. The rate of emigration was determined from the difference between the number of any interstitial leukocytes present at the beginning of the experiment and those present at the end of the experiment.

Experimental protocol

After a 30 min stabilization period, baseline measurements (time 0) of mean arterial blood pressure (MABP), V_{rbc}, vessel diameter, shear rate, leukocyte rolling flux and velocity and leukocyte adhesion and emigration were taken. The superfusion buffer was then supplemented with Ang-II (1 nM) and recordings were performed for 5 min at 15 min intervals over a 60 min period during which the aforementioned leukocyte and haemodynamic parameters were measured.

In the first group of experiments, animals were pretreated with the membrane receptor-dependent prostacyclin analogue iloprost (3 μg kg^{-1}, i.v.), 5 min before and 30 min after Ang-II (1 nM) suffusion, to maintain adequate plasma levels of the drug. A second group of animals was pretreated with the β_2-adrenoceptor agonist salbutamol (1 mg kg^{-1}, i.v.), 10 min before Ang-II (1 nM) superfusion. In a further group of rats, a direct adenylate cyclase activator, forskolin (1 μM) was applied topically 10 min prior to Ang-II suffusion. Forskolin was co-superfused with Ang-II to avoid the toxic effects which develop after systemic administration of this drug. The doses used for the different treatments were based on those applied in previous *in vivo* studies (Harada *et al*., 1999; Altenburg *et al*., 1994; Frisbee *et al*., 1999).

To determine the effect of PDE enzymes inhibition on leukocyte infiltration elicited by Ang-II, two PDE inhibitors were used. In the first set of experiments, animals were pretreated 10 min before Ang-II suffusion with the non-specific PDE inhibitor theophylline (5 mg kg^{-1}, i.v.). In the second group of rats, selective PDE-IV inhibitor rolipram (8 mg kg^{-1}, i.p.) was administered 30 min prior to Ang-II suffusion to achieve appropriate plasma levels of its active metabolite. Rolipram was dissolved in dimethylsulphoxide (DMSO) and diluted further in saline as previously described (Teixeira *et al*., 1994). The same percentage of DMSO was given i.p. to the rolipram control group, 30 min before Ang-II superfusion. Similarly, the doses administered were those used in previous *in vivo* studies (Teixeira *et al*., 1994; Elwood *et al*., 1995).

To investigate the ability of nitric oxide to modulate Ang-II-induced leukocyte-endothelial cell interactions, the nitric oxide donor spermine-NO was used. This compound (100 μM) was co-superfused with Ang-II, which allowed a slow release of NO and, through very local administration, complications due to alterations in haemodynamic parameters were avoided. As many actions of NO are due to the activation of soluble guanylate cyclase, a series of experiments were carried out to examine the role of cyclic GMP in leukocyte responses induced by Ang-II. The cyclic GMP analog 8-Br-cyclic GMP (100 μM) was also co-superfused with Ang-II and the effects determined. The doses administered in this experiment are the same used to inhibit leukocyte-endothelial cell interactions induced by CINC/gro in the rat mesenteric microcirculation (Johnston *et al*., 1999).

To determine the possibility of using nitroglycerin to control the leukocyte recruitment elicited by Ang-II, the effect of a transdermal nitroglycerin patch was assayed in this system. The patch was applied to the dorso-cervical area of the body which had been shaved under light ether anaesthesia the day before. Patches were cut to release a nitroglycerin dose of 160 ng min^{-1} rat^{-1} which has previously been proved to inhibit leukocyte-endothelial cell interactions induced by indomethacin (Calatayud *et al*., 1999). Control animals were treated with placebo patches of equal size. Both treatments were applied before beginning surgical procedures and were maintained in place until the end of the experiment.

Finally, to investigate whether the effects of cyclic AMP elevation on leukocyte responses are mediated through endothelial NO release, another set of experiments were carried out. In the first group of animals, after the initial 30 min stabilization period, the superfusion buffer was changed to one containing L-NAME (100 μM). Previous

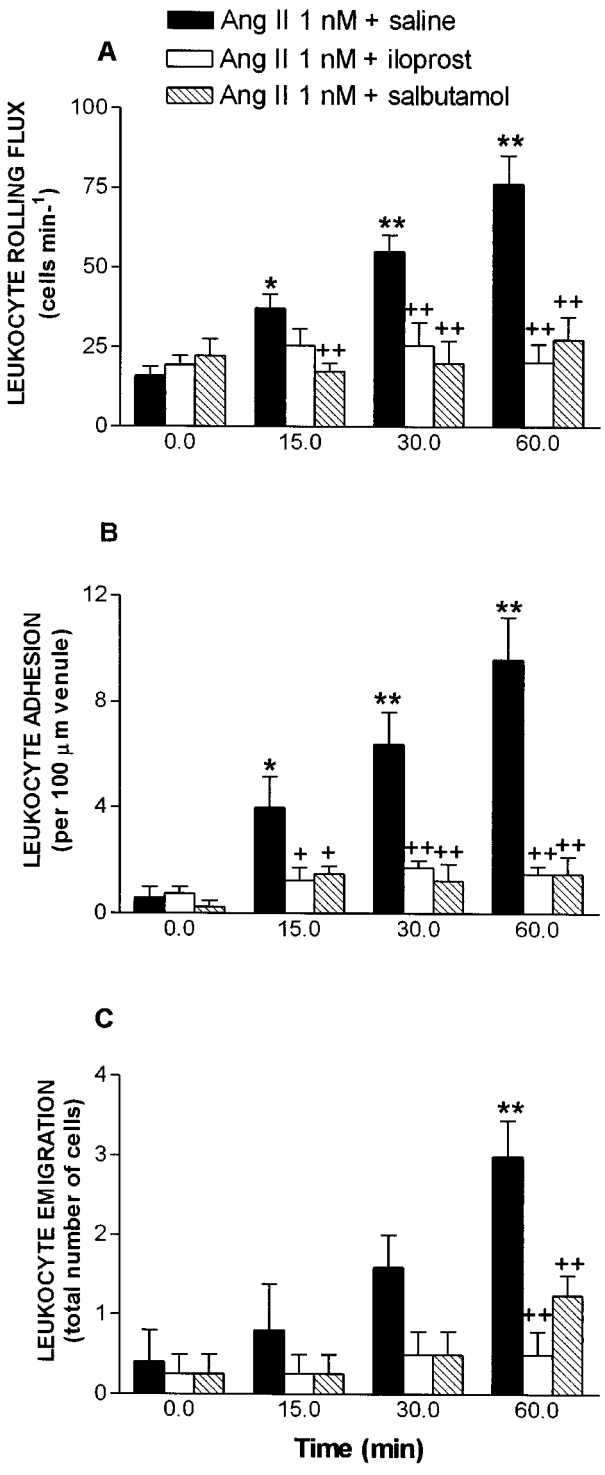

Figure 1 Effect of the prostacyclin analogue iloprost and the β_2-receptor agonist salbutamol on Ang-II-induced leukocyte rolling flux (A), leukocyte adhesion (B) and leukocyte emigration (C) in rat mesenteric postcapillary venules. Parameters were measured 0, 15, 30 and 60 min after superfusion with Ang-II (1 nM) in animals untreated ($n=5$) or pretreated with iloprost (3 μg kg^{-1}, i.v., $n=5$) or with salbutamol (1 mg kg^{-1}, i.v., $n=5$). Results are represented as mean $\pm$ s.e.mean. $^*P<0.05$ or $^{**}P<0.01$ relative to the control value (0 min) in the untreated group. $+P<0.05$ or $++P<0.01$ relative to the untreated group.

studies have demonstrated that this dose causes a consistent increase in leukocyte rolling and adhesion over a 60 min time course (Arndt *et al*., 1993). A second group of animals was pretreated with the β_2-adrenoceptor agonist salbutamol (1 mg kg^{-1}, i.v.) 10 min before L-NAME superfusion, and a third group of animals pretreated with salbutamol at the same conditions, was then co-superfused with L-NAME and Ang-II (1 nM).

Statistical analysis

All data are expressed as mean ± s.e.mean. The data within groups were compared using an analysis of variance (one way-ANOVA) with a Newman-Keuls *post hoc* correction for multiple comparisons. A P value <0.05 was considered to be statistically significant.

Materials

Ang-II, salbutamol, forskolin, theophylline, rolipram, spermine-NO, 8-Br-cyclic GMP, L-NAME and DMSO were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.. Placebo and nitroglycerin patches (Nitro-Dur® 15) and iloprost (Ilomedin®) were generously donated by Schering-Plough Laboratories, Spain.

Results

Figure 1 illustrates the effect of Ang-II-induced leukocyte responses. Significant increases in leukocyte rolling flux (76.4 ± 8.9 vs 16.0 ± 2.9 cells min^{-1}), adhesion (9.6 ± 1.6 vs 0.6 ± 0.4 cells 100 μm^{-1}) and emigration (3.0 ± 0.4 vs 0.4 ± 0.4 cells field^{-1}) were observed at 60 min in animals subjected to 1 nM Ang-II superfusion vs buffer. Pretreatment with iloprost or salbutamol attenuated the Ang-II induced leukocyte rolling flux, adhesion and emigration and were inhibited by 96, 92 and 90% respectively when animals were pretreated with iloprost and by 83, 86 and 62% respectively, in the group pretreated with salbutamol after 60 min Ang-II suffusion. In addition, the decrease in leukocyte rolling velocity induced by Ang-II at 60 min was reversed by the administration of either of the agents under investigation (Table 1).

Figure 2 presents the effects of the direct adenylate cyclase activator forskolin on the leukocyte-endothelial cell interactions elicited by mesenteric exposure to 1 nM Ang-II. At

Table 1 Leukocyte rolling velocity in animals untreated and treated with cyclic AMP elevating agents before (0 min) and after (60 min) Ang-II superfusion (1 nM)

Treatment	*0 min*	*60 min*
Untreated animals	94.9 ± 13.2	36.0 ± 4.8**
Iloprost	105.3 ± 1.0	92.9 ± 15.1
Salbutamol	159.3 ± 35.3	128.8 ± 58.8
Forskolin	122.4 ± 8.7	105.0 ± 12.6
Theophylline	99.5 ± 3.8	114.8 ± 21.3
Untreated (rolipram vehicle)	138.1 ± 19.0	79.5 ± 15.8*
Rolipram	143.7 ± 17.4	142.6 ± 40.8

*$P<0.05$ or **$P<0.01$ relative to the control group (0 min). All values are mean ± s.e.mean.

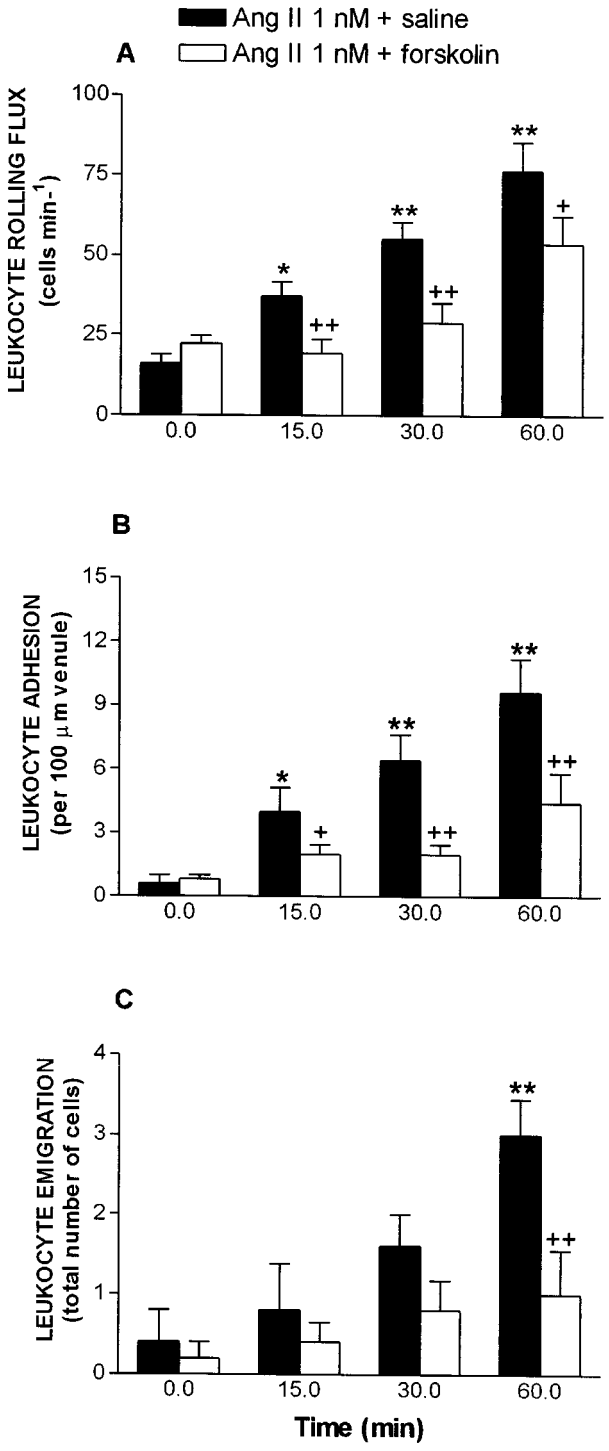

Figure 2 Effect of the adenylyl cyclase activator forskolin on Ang-II-induced leukocyte rolling flux (A), leukocyte adhesion (B) and leukocyte emigration (C) in rat mesenteric postcapillary venules. Parameters were measured 0, 15, 30 and 60 min after superfusion with Ang-II (1 nM) in animals untreated ($n=5$) or treated with forskolin (0.1 μM, $n=5$). Results are represented as mean ± s.e.mean. *$P<0.05$ or **$P<0.01$ relative to the control value (0 min) in the untreated group. +$P<0.05$ or ++$P<0.01$ relative to the untreated group.

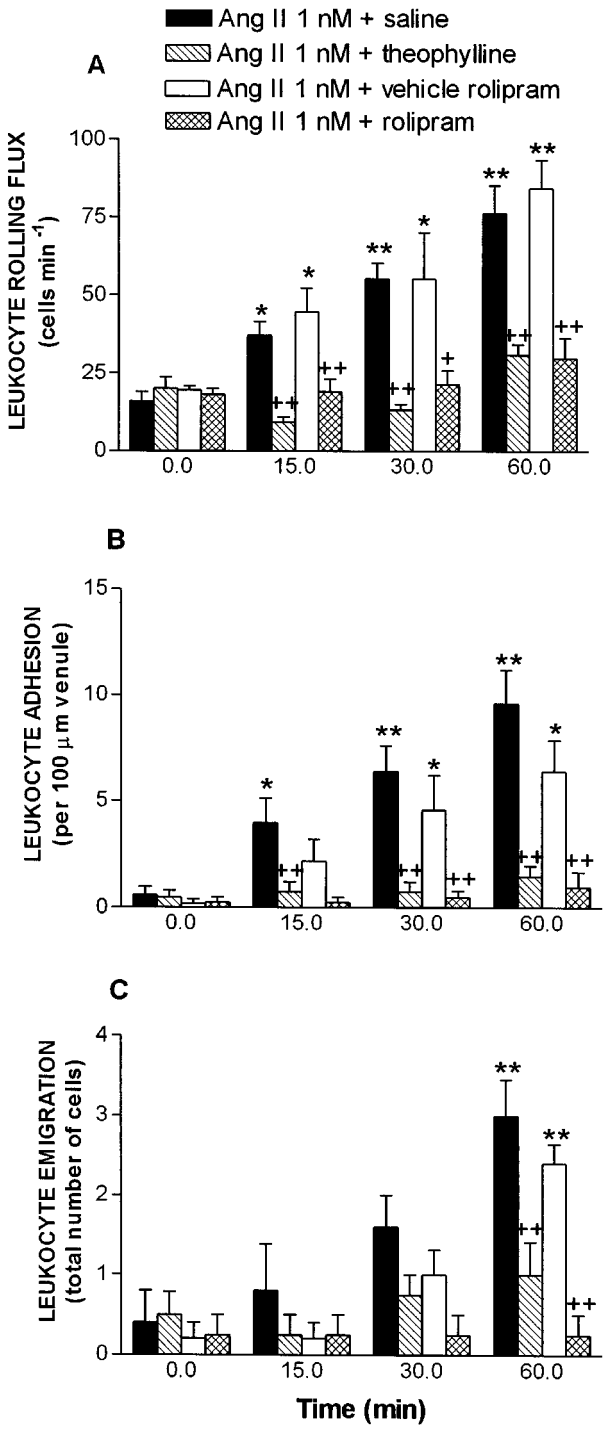

Figure 3 Effect of the PDE IV inhibitor rolipram and the non-specific PDE inhibitor theophylline on Ang-II-induced leukocyte rolling flux (A), leukocyte adhesion (B) and leukocyte emigration (C) in rat mesenteric postcapillary venules. Parameters were measured 0, 15, 30 and 60 min after superfusion with Ang-II (1 nM) in animals untreated (rolipram vehicle, $n=5$) or pretreated with rolipram (8 mg kg^{-1}, i.p., $n=5$) and in animals untreated ($n=5$) or pretreated with theophylline (5 mg kg^{-1}, i.v., $n=5$). Results are represented as mean ± s.e.mean. *$P<0.05$ or **$P<0.01$ relative to the control value (0 min) in the untreated group. +$P<0.05$ or ++$P<0.01$ relative to the untreated group.

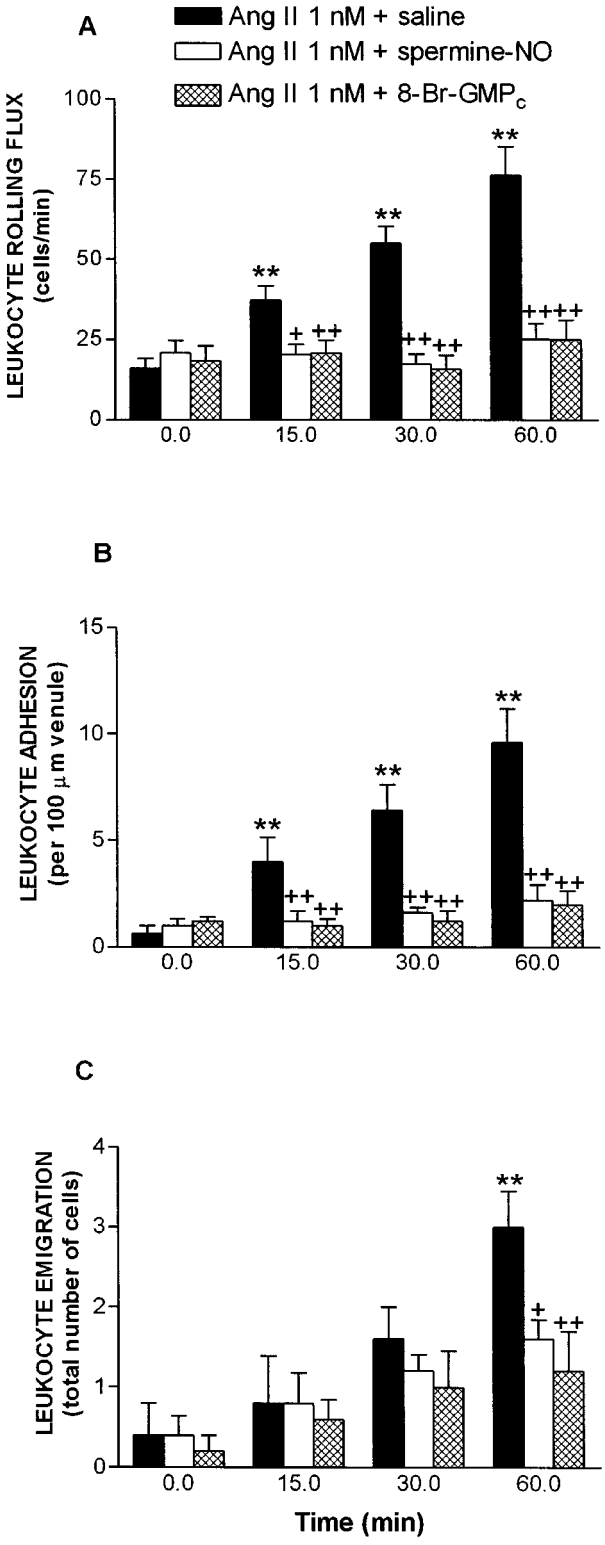

Figure 4 Effect of the NO donor spermine-NO and the cyclic GMP analog 8-Br-cyclic GMP on Ang-II-induced leukocyte rolling flux (A), leukocyte adhesion (B) and leukocyte emigration (C) in rat mesenteric postcapillary venules. Parameters were measured 0, 15, 30 and 60 min after superfusion with Ang-II (1 nM) in animals untreated ($n=5$) or co-superfused with spermine-NO (100 μM, $n=5$) or with 8-Br-cyclic GMP (100 μM, $n=5$). Results are represented as mean ± s.e.mean. *$P<0.05$ or **$P<0.01$ relative to the control value (0 min) in the untreated group. +$P<0.05$ or ++$P<0.01$ relative to the untreated group.

60 min, forskolin significantly reduced Ang-II-induced leukocyte rolling flux, adhesion and emigration by 48, 60 and 69% respectively. As previously found with iloprost and salbutamol, forskolin maintained leukocyte rolling velocity at basal levels when co-suffused with Ang-II (Table 1).

Figure 3 shows the effects of theophylline and rolipram on Ang-II induced leukocyte endothelial cell interactions. Theophylline pretreatment significantly reduced Ang-II-induced leukocyte rolling flux, adhesion and emigration by 81, 89 and 71% respectively after 60 min Ang-II superfusion. In animals pretreated with rolipram vehicle, similar values of leukocyte rolling flux (84.4 ± 9.1 vs 19.6 ± 1.3 cells min^{-1}), adhesion (6.4 ± 1.5 vs 0.2 ± 0.2 cells 100 μm^{-1}) and emigration (2.4 ± 0.3 vs 0.2 ± 0.2 cells $field^{-1}$) to those obtained in the Ang-II untreated group were detected. Rolipram pretreatment provoked significant reductions in leukocyte responses elicited after 60 min Ang-II suffusion, inhibiting these parameters by 80, 84 and 90% respectively. Again, decreases in leukocyte rolling velocity elicited by Ang-II were totally reversed by administration of theophylline and rolipram (Table 1).

Figure 4 summarizes the effect of the NO donor, spermine-NO and the cyclic GMP analogue, 8-Br-cyclic GMP on leukocyte recruitment caused by Ang-II. Co-superfusion of Ang-II with spermine-NO resulted in a significant reduction of the leukocyte rolling flux (83.4%), adhesion (82.2%) and emigration (57.3%) provoked by 60 min suffusion with Ang-II. Similarly, when Ang-II was co-superfused with 8-Br-cyclic GMP, these three parameters were inhibited by 83.3, 91.1 and 61.5% respectively. In addition, Table 2 shows that the diminution in leukocyte rolling velocity provoked by 60 min superfusion with Ang-II, was reversed to basal values when it was co-superfused with either spermine-NO or 8-Br-cyclic GMP.

Furthermore, as shown in Figure 5, systemic pretreatment with the transdermal nitroglycerin patch (160 ng min^{-1} rat^{-1}) evoked significant changes in leukocyte rolling flux, even in the basal value when compared with that detected in the placebo treated group (7.8 ± 1.5 cells min^{-1} vs 19.6 ± 4.4 cells min^{-1} respectively). In the group pretreated with the placebo patch, Ang-II superfusion induced a significant increase in leukocyte rolling flux (80.8 ± 6.1 vs 19.6 ± 4.4 cells min^{-1}), adhesion (12.0 ± 1.6 vs 1.4 ± 0.4 cells 100 μm^{-1}) and emigration (3.2 ± 0.6 vs 0.2 ± 0.2 cells $field^{-1}$) vs buffer after 60 min superfusion, while showing no differences with respect to the effect of Ang-II superfusion in untreated animals. Treatment with the nitroglycerin patch induced a significant inhibition in all three parameters by 94,

Table 2 Leukocyte rolling velocity in animals untreated and treated with cyclic GMP elevating agents before (0 min) and after (60 min) Ang-II superfusion (1 nM)

Treatment	*0 min*	*60 min*
Untreated animals	94.9 ± 13.2	36.0 ± 4.8**
Spermine-NO	102.9 ± 7.1	105.0 ± 13.2
8-Br-cyclic GMP	100.5 ± 2.6	120.6 ± 8.1
Untreated (placebo patch)	100.1 ± 5.4	45.0 ± 4.0**
Nitroglycerin patch	156.3 ± 25.8	161.0 ± 67.0

*$P<0.05$ or **$P<0.01$ relative to the control group (0 min). All values are mean ± s.e.mean.

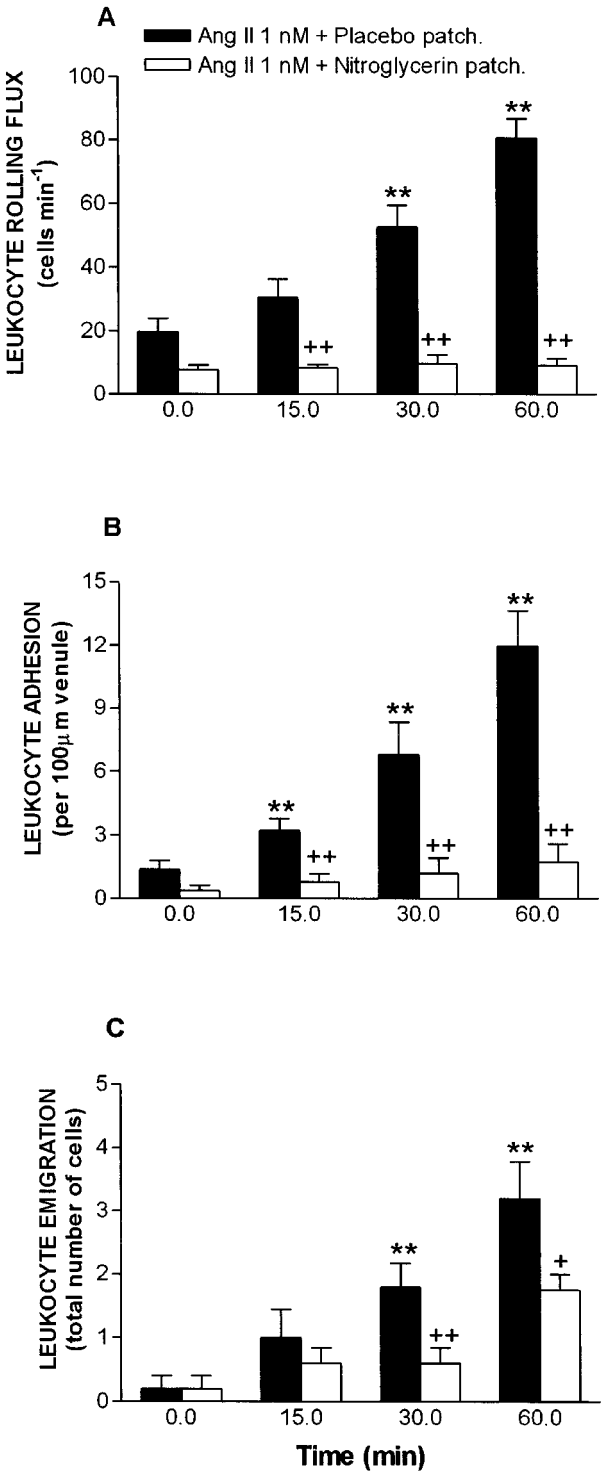

Figure 5 Effect of the nitric oxide donor nitroglycerin on Ang-II-induced leukocyte rolling flux (A), leukocyte adhesion (B) and leukocyte emigration (C) in rat mesenteric postcapillary venules. Parameters were measured 0, 15, 30 and 60 min after superfusion with Ang-II (1 nM) in animals pretreated with placebo patch ($n=5$) or with nitroglycerin patch (160 ng min^{-1} rat^{-1}, $n=5$). Results are represented as mean ± s.e.mean. *$P<0.05$ or **$P<0.01$ relative to the control value (0 min) in the untreated group. +$P<0.05$ or ++$P<0.01$ relative to the untreated group.

85 and 50% respectively after 60 min Ang-II suffusion, returning leukocyte rolling flux and adhesion to levels detected in basal conditions. In addition, treatment with the nitroglycerin patch also inhibited the Ang-II induced decrease in leukocyte rolling velocity (Table 2).

Tables 3 and 4 summarize the results obtained for MABP and shear rate prior to (0 min) and 60 min following Ang-II superfusion in animals untreated and pretreated with different cyclic AMP or cyclic GMP elevating agents. MABP was unaffected by most of the agents under investigation; only salbutamol pretreatment caused a significant reduction in this hemodynamic parameter, while no changes in blood flow were detected. Similarly, the shear rate remained unchanged throughout Ang-II suffusion in most of the untreated and treated groups; only forskolin co-superfusion provoked a significant decrease in this response at the 60 min time point. Yet, despite this effect, Ang-II-induced leukocyte-endothelial cell interactions were effectively inhibited.

Finally, Figure 6 shows the effect of salbutamol on L-NAME and L-NAME + Ang-II-induced leukocyte-endothelial cell interactions. Interestingly, β_2-adrenoceptor agonist pretreatment did not modify the leukocyte recruitment caused by nitric oxide synthase (NOS) inhibition. In addition, the inhibitory effect of salbutamol on Ang-II-induced leukocyte-endothelial cell interactions was prevented when NOS was inhibited with L-NAME. As previously found, salbutamol pretreatment caused a significant decrease in MABP without affecting the decrease in shear rate provoked by L-NAME (Table 5).

Discussion

Elevated cyclic AMP levels can be attained by either activating adenylate cyclase to increase synthesis or by inhibiting metabolism *via* PDE. In the present study we have demonstrated that cyclic AMP elevating agents which activate adenylate cyclase such as iloprost, salbutamol or forskolin, can significantly reduce Ang-II-induced leukocyte rolling, adhesion and emigration *in vivo*. Similarly, when a non-selective inhibitor of PDEs such as theophylline or a selective PDE-IV inhibitor such as rolipram is administered, leukocyte responses elicited by Ang-II are also dramatically attenuated. Therefore, the ability of these compounds to exert an inhibitory effect on leukocyte-endothelial cell interactions caused by Ang-II occurs regardless of the mechanism used to elevate intracellular cyclic AMP. In addition, we have also shown that treatment of animals with the NO donor, spermine-NO, or the cyclic GMP analogue, 8-Br-GMP or systemic pretreatment with a transdermal nitroglycerin patch, almost abolished Ang-II-induced leukocyte rolling and adhesion. We have also demonstrated that the anti-inflammatory effect exerted by salbutamol disappears when NO synthesis is inhibited. Finally, none of these treatments affect the different haemodynamic parameters, with the exception of salbutamol and forskolin. The former significantly reduce MABP and the latter reduce shear rate after 60 min superfusion with Ang-II. Despite these effects, both significantly reduce leukocyte-endothelial cell interactions elicited by Ang-II.

Among the different drugs used in the present study, iloprost, salbutamol, rolipram, spermine NO, 8-Br-GMP and nitrolglycerin patch treatments exerted similarly powerful inhibitory effects on the leukocyte recruitment elicited by Ang-II. In this context, it has been shown that stimulation of prostacyclin synthesis and the use of prostacyclin agonists or NO donors can impede the leukocyte recruitment associated with cardiac ischaemic injury and the development of atherosclerotic lesions (Hohfeld *et al*., 1993; Kowala *et al*., 1993; Johnson *et al*., 1991; Massoudy *et al*., 1999). In

Table 3 Haemodynamic parameters in animals untreated and treated with cyclic AMP elevating agents before (0 min) and after (60 min) Ang-II superfusion (1 nM)

	MABP (mmHg)		*Shear rate* (s^{-1})	
Treatment	*0 min*	*60 min*	*0 min*	*60 min*
Untreated animals	114.6±11.3	104.6±10.5	600.2±27.1	540.5±117.2
Iloprost	106.7±9.2	102.8±16.4	697.2±38.8	646.6±77.8
Salbutamol	124.4±5.3	102.0±8.6*	655.8±40.9	585.3±35.9
Forskolin	123.3±1.7	130.0±5.0	641.3±53.1	544.6±48.3**
Theophylline	112.1±7.5	114.6±7.5	601.6±47.6	624.8±36.1
Untreated (rolipram vehicle)	125.0±1.7	125.0±1.7	562.7±77.4	572.6±57.9
Rolipram	94.4±5.9	85.0±8.2	572.2±66.6	638.3±102.6

*$P<0.05$ or **$P<0.01$ relative to the control group (0 min). All values are mean±s.e.mean.

Table 4 Haemodynamic parameters in animals untreated and treated with cyclic GMP elevating agents before (0 min) and after (60 min) Ang-II superfusion (1 nM)

	MABP (mmHg)		*Shear rate* (s^{-1})	
Treatment	*0 min*	*60 min*	*0 min*	*60 min*
Untreated animals	114.6±11.3	104.6±10.5	600.2±27.1	540.5±117.2
Spermine-NO	117.9±6.7	125.0±9.6	694.9±68.0	650.7±74.0
8-Br-cyclicGMP	126.0±5.1	128.7±7.4	642.0±51.4	571.6±61.6
Untreated (placebo patch)	117.7±5.4	121.0±7.7	675.6±98.8	573.4±158.7
Nitroglycerin patch	122.2±8.7	108.3±2.9	665.1±54.9	725.8±92.6

*$P<0.05$ or **$P<0.01$ relative to the control group (0 min). All values are mean±s.e.mean.

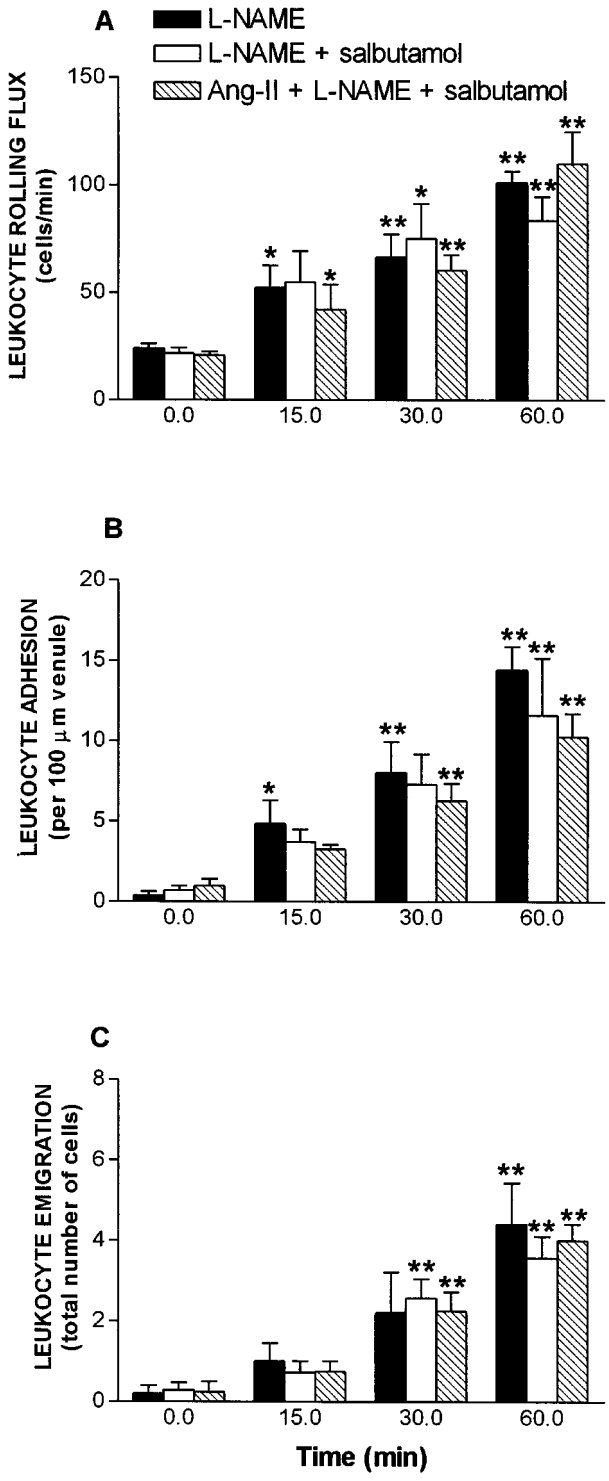

Figure 6 Effect of salbutamol pretreatment on L-NAME and L-NAME + Ang-II-induced leukocyte rolling flux (A), leukocyte adhesion (B) and leukocyte emigration (C) in the rat mesenteric postcapillary venules. Parameters were measured 0, 15, 30 and 60 min after superfusion with L-NAME (100 μM) or L-NAME + Ang-II (1 nM) in animals untreated ($n=5$) or pretreated with salbutamol (1 mg kg^{-1}, $n=5$ in both groups). Results are presented as mean ± s.e.mean. $^{*}P<0.05$ or $^{**}P<0.01$ relative to the control value (0 min) in the untreated group.

contrast, little is known about the effectiveness of β_2-adrenoceptor agonists or PDE-IV inhibition treatments in the control of inflammatory responses associated with cardiovascular diseases. With regard to this, in the present study, we have observed the effect displayed by salbutamol, which in addition to its inhibitory effect on leukocyte-endothelial cell interactions elicited by Ang-II, also reduced arterial blood pressure. Therefore, salbutamol or β_2-adrenoceptor agonist therapy may be specially useful in hypertensive states where there exists the risk of development of an atherosclerotic lesion.

The exact mechanisms by which cyclic AMP inhibits leukocyte adhesion to the vascular endothelium is not known, however, one probable mechanism is the modulation of CAM expression. In this context, most of the studies which explore regarding this possibility have used *in vitro* models and contradictory findings have been encountered based on either the origin of the endothelial cells used or the stimulus employed to provoke leukocyte activation. For example, Pober *et al*. (1993) demonstrated that combination treatment with forskolin and the non-specific PDE inhibitor isobutyl methylxanthine, suppressed the induction by cytokines of E-selectin and vascular cell adhesion molecule-1 (VCAM-1). In contrast, Morandini *et al*. (1996) showed that, while rolipram significantly suppressed the expression and release of E-selectin in TNF-α-stimulated human umbilical vein endothelial cells (HUVECs), when combined with forskolin, it had no effect on VCAM-1 expression. Similarly, Blease *et al*. (1998), using human lung microvascular endothelial cells (HLMVECs), found a significant reduction in TNFα-induced E-selectin expression with a combination of rolipram and salbutamol, whereas no effect on intercellular adhesion molecule-1 (ICAM-1) and VCAM-1 expression was detected. In addition, cyclic AMP elevating agents also prevented mediator-induced upregulation of β_2 integrins on the leukocyte surface (Derian *et al*., 1995; Teixeira *et al*., 1996; Berends *et al*., 1997; Santamaria *et al*., 1997).

Despite these findings, our results strongly suggest that effects displayed *in vitro* should not necessarily correlate with those detected in *in vivo* studies. In fact, we have shown strong inhibitory responses in Ang-II-induced leukocyte-endothelial cell interactions, using an adenylate cyclase activator, a PDE inhibitor, a guanylate cyclase activator or different NO donors, without the need of combining any of these different cyclic nucleotides elevating agents. Additionally, it seems likely that, *in vivo*, downregulation of adhesion molecules other than E-selectin or VCAM-1 are involved in the inhibitory responses observed in the present study. In this context, one possible candidate is P-selectin. Indeed, we have recently demonstrated that Ang-II-induced leukocyte-endothelial cell interactions *in vivo* occur *via* endothelial P-selectin upregulation (Piqueras *et al*., 2000). Moreover, other studies have also shown that cyclic AMP elevating agents or NO donors can reduce P-selectin expression and release in stimulated platelets (Konstantopoulos *et al*., 1998; Salas *et al*., 1994). Furthermore, as witnessed in platelets, prostacyclin treatment in animals subjected to protamine administration provokes a decrease in E and P-selectin levels and a more marked preservation of left ventricular function (Katircioglu *et al*., 1999). Similarly, treatment with NO donors has been found to attenuate homocysteine-induced P-selectin expression in rat mesenteric venules (Pruefer *et al*., 1999).

Table 5 Haemodynamic parameters in animals untreated and treated with salbutamol (1 mg kg^{-1}) before (0 min) and after (60 min) L-NAME (100 μM) or L-NAME (100 μM) + Ang-II (1 nM) superfusion

	MABP (mmHg)		*Shear rate* (s^{-1})	
Treatment	*0 min*	*60 min*	*0 min*	*60 min*
L-NAME	115.5 ± 9.6	119.4 ± 10.6	763.2 ± 55.0	392.1 ± 58.6**
Salbutamol + L-NAME	116.4 ± 5.0	99.8 ± 7.6*	653.1 ± 77.1	408.0 ± 103.9*
Salbutamol + L-NAME + Ang-II	109.6 ± 9.7	97.4 ± 11.6*	588.2 ± 20.9	283.3 ± 55.6**

*$P<0.05$ or **$P<0.01$ relative to the control group (0 min). All values are mean ± s.e.mean.

This is of interest, since increased circulating levels of P-selectin, and to a lesser extent E-selectin, can be found in essential, renovascular and malignant hypertension and in hypercholesterolemic patients (Verhaar *et al*., 1998; Davi *et al*., 1998). In addition, it has recently been shown that there is a clear involvement of both P- and E-selectins in the development of the atherosclerotic lesion at both early and advanced stages in a mouse model of atherosclerosis (Dong *et al*., 1998).

Finally, we have also demonstrated that the inhibitory leukocyte responses elicited by salbutamol are mediated through NO release. These results echo those of previous studies in which vasorelaxation induced by β_2-adrenoceptor agonist was prevented by NOS inhibition or endothelium removal, an effect found to be cyclic AMP dependent (Ferro *et al*., 1999; Xu *et al*., 2000). Apart from extending these findings, our results show that cyclic AMP elevating agents can reduce CAM expression through endothelial NO release.

In conclusion, in the present study we have provided evidence that cyclic AMP elevating agents and NO donors are potent *in vivo* inhibitors of Ang-II-induced leukocyte-endothelial cell interactions, regardless of the mechanism employed to increase intracellular cyclic nucleotides. The effects observed seem to be mediated through P-selectin downregulation, since Ang-II-elicited leukocyte responses are primarily mediated *via* increased endothelial expression of this adhesion molecule. As the degree of P-selectin expression determines the abundance of rolling leukocytes which may eventually adhere to the endothelium and extravasate into the tissue, these agents have the ability to impair the leukocyte infiltration associated with the vascular damage detected in different circulatory disorders. Therefore, they could constitute a powerful and alternative therapeutical tool in the control of inappropriate inflammatory responses which occur in the vasculature in cardiovascular disease states where Ang-II plays a critical role.

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