British Journal of Pharmacology (2001) 132, 677–684 

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# Angiotensin II is involved in nitric oxide synthase and cyclo-oxygenase inhibition-induced leukocyte-endothelial cell interactions *in vivo*

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**1** Chronic inhibition of nitric oxide synthase (NOS) provokes a hypertensive state which has been shown to be angiotensin II (Ang-II) dependent. In addition to raising blood pressure, NOS inhibition also causes leukocyte adhesion. The present study was designed to define the role of Ang-II in hypertension and in the leukocyte-endothelial cell interactions induced by acute NOS or cyclo-oxygenase (COX) inhibition using intravital microscopy within the rat mesenteric microcirculation.

**2** While pretreatment with an Ang-II $AT_1$ receptor antagonist (losartan) reversed the prompt increase in mean arterial blood pressure (MABP) caused by indomethacin, it had no effect on the increase evoked by systemic L-NAME administration.

**3** Pretreatment with losartan inhibited the leukocyte rolling flux, adhesion and emigration which occurs after 60 min NOS inhibition by 83, 80 and 70% respectively, and returned leukocyte rolling velocity to basal levels.

**4** Losartan significantly reduced the leukocyte-endothelial cell interaction elicited by COX inhibition. In contrast, leukocyte recruitment induced by acute mast cell activation was not inhibited by losartan.

**5** $AT_1$ receptor blockade also prevented the drop in haemodynamic parameters such as mean red blood cell velocity ($V_{mean}$) and shear rate caused by NOS and COX inhibition.

**6** In this study, we have demonstrated a clear role for Ang-II in the leukocyte-endothelial cell interactions and haemodynamic changes which arise in the absence of NO or prostacyclin ($PGI_2$). This is of interest since leukocyte recruitment, which culminates in the vascular lesions that occur in hypertension, atherosclerosis and myocardial ischemia-reperfusion injury, might be prevented using $AT_1$ Ang-II receptor antagonists.

*British Journal of Pharmacology* (2001) **132,** 677–684

**Keywords:** Angiotensin II, nitric oxide, prostacyclin, cyclo-oxygenase, leukocyte, endothelium, intravital microscopy, mast cells.

**Abbreviations:** Ang-II, angiotensin-II; ACEI, angiotensin converting enzyme inhibitor; CMP 48/80, compound 48/80; Dv, venular diameter; ET-1, endothelin-1; ICAM-1, intercellular adhesion molecule-1; L-NAME, $N^G$-nitro-L-arginine methyl ester; MABP, mean arterial blood pressure; $PGI_2$, prostacyclin; TNFα, tumour necrosis factor-α; $V_{mean}$, mean red blood cell velocity; $V_{rbc}$, centreline red blood cell velocity; $V_{wbc}$, leukocyte rolling velocity

## Introduction

Leukocyte accumulation in the vessel wall is a hallmark of the early stages of atherosclerosis, acute myocardial infarction and several renal diseases of diverse etiology (Badimon *et al*., 1993; Ricevuti *et al*., 1990; Klahr *et al*., 1988) where angiotensin-II (Ang-II) seems to play a critical role (Alderman *et al*., 1991; Badimon *et al*., 1993; Lafayette *et al*., 1992, Thaiss *et al*., 1996). Ang-II is the main effector peptide of the renin-angiotensin system and we have recently revealed that it has proinflammatory activity at sub-vasoconstrictor doses *in vivo*. In particular, it induces leukocyte recruitment in the rat mesenteric microvasculature through endothelial P-selectin up-regulation in the vessel wall and this effect is primarily mediated *via* a subtype Ang-II $AT_1$ receptor interaction (Piqueras *et al*., 2000).

Inhibition of endogenous NO with the NO synthase (NOS) inhibitor, $N^G$-nitro-L-arginine methyl ester (L-NAME), evokes significant leukocyte-endothelial cell interactions followed by increased microvascular permeability, suggesting that constitutive NO production from microvascular endothelial cells may play a role in maintaining the functional integrity of microvascular endothelium (Kubes *et al*., 1991; Kubes & Granger, 1992; Ardnt *et al*., 1993). Likewise, during circulatory perturbations such as ischemia followed by reperfusion, traumatic shock or hypertension, the loss of endothelial nitric oxide (NO) is believed to underlie the development of inflammation, including enhanced leukocyte-endothelial cell interactions (Ma *et al*., 1993; Scalia *et al*., 1999).

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Like NO, prostacyclin ($PGI_2$) is an endothelium-derived vasodilator involved in maintaining the balance in systemic and regional haemodynamics by opposing endothelium-derived vasoconstrictive factors (Tolins *et al*., 1991). In this context, patients with ischemic heart disease have decreased prostacyclin plasma levels (Neri Serneri *et al*., 1982). In addition, it has been reported that stimulation of prostacyclin synthesis protects the reperfused myocardium from ischemic injury, thereby reducing neutrophil infiltration in the ischemic tissue (Hohfeld *et al*., 1993). Moreover, there is evidence that prostacyclin agonists reduce early atherosclerosis in hyperlipidemic hamsters by, among other actions, suppressing monocyte adhesion to vascular endothelium, monocyte chemotaxis and tumour necrosis factor-$\alpha$ (TNF$\alpha$) production (Kowala *et al*., 1993). Finally, indomethacin superfusion within the rat mesenteric microvasculature elicits leukocyte-endothelial cell interactions (Wallace *et al*., 1993).

Epidemiological evidence suggests that high blood pressure may have a direct role in enhancing atherosclerotic lesion formation (Chobanian & Alexander, 1996). Atherosclerosis is three times more common in patients with hypertension, and there is a positive, although not linear, correlation between hypertension and atherosclerosis (Doyle, 1990). There is a body of evidence which shows that inhibition of NO synthesis results in hypertension and that inhibiting Ang-II with Ang-II selective antagonists or angiotensin converting enzyme inhibitors (ACEI) can blunt, if not prevent this response (Pollock *et al*., 1993; Michel *et al*., 1996; Takemoto *et al*., 1997). The primary aim of the present study was, therefore, to investigate if Ang-II is involved in the acute and systemic increase in mean arterial blood pressure (MABP) induced by L-NAME and indomethacin. To determine whether there is a relation between the acute hypertension and leukocyte recruitment witnessed after endothelial disruption by lack of vasodilators, we next investigated the possible involvement of Ang-II in the leukocyte-endothelial cell interactions caused as a result of acute NOS inhibition by L-NAME. In addition, since NO and $PGI_2$ are the most important mediators which preserve endothelial integrity within the vasculature, this study also aimed to determine the role of Ang-II in the leukocyte-endothelial cell interactions provoked by indomethacin-induced-COX inhibition. Furthermore, it has been shown that L-NAME activates mast cells, and that human chymase, a serin protease expressed by mast cells (Urata *et al*., 1996), is an important alternative pathway for the local generation of Ang-II within the human heart and blood vessels. Thus, the possible participation of Ang-II in the leukocyte recruitment evoked by the acute mast cell degranulation elicited by compound 48/80 (CMP 48/80) was also investigated in the present study.

## Methods

### *Systemic studies*

Sprague-Dawley rats (200–250 g) were anaesthetized with pentobarbital sodium (50 mg $kg^{-1}$, i.p.). The right carotid artery and jugular vein were cannulated 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 permit the intravenous administration of anaesthetic and drugs respectively. After a 10 min stabilization period animals received either saline or losartan (10 mg $kg^{-1}$) intravenously. After a further 10 min, animals received either indomethacin (20 mg $kg^{-1}$) or L-NAME (10 mg $kg^{-1}$) as a bolus intravenous injection. MABP was recorded every 20 min for 60 min and expressed as a percentage of basal values. The doses chosen for indomethacin and L-NAME in this study have previously been reported to inhibit prostaglandin synthesis and to increase systemic blood pressure respectively (Wallace *et al*., 1993; Sigmon & Beierwaltes, 1993). The dose of 10 mg $kg^{-1}$ i.v. of losartan has been shown to produce maximum reductions in MABP in spontaneously hypertensive rats and to inhibit the pressor response to Ang-II. Indeed, higher doses of losartan (e.g., 30 mg $kg^{-1}$) do not result in further reductions of MABP (Wong *et al*., 1990).

### *Intravital microscopy*

The experimental preparation used in this study was similar to that described previously (Sanz *et al*., 1999). Male Sprague-Dawley rats (200–250 g) were fasted for 24 h and anaesthetized with pentobarbital sodium (50 mg $kg^{-1}$, i.p.). A tracheostomy was performed to maintain a patent airway throughout the experiment. A polyethylene catheter was inserted in the right carotid artery to monitor mean arterial blood pressure (MABP) through a pressure transducer (Spectramed Stathan P-23XL) connected to a recorder (GRASS RPS7C8B, Quincy, MA, U.S.A.), and a second catheter was placed in the contralateral jugular vein to permit the intravenous administration of additional reagents (anaesthetic or drug). A midline abdominal incision was made and the rats were then placed in a supine position on an adjustable Plexiglass microscope stage. A segment of the midjejunum was exteriorized and draped over an optically clear viewing pedestal which allowed transillumination of a 2 $cm^2$ segment of the tissue. The temperature of the pedestal was maintained at 37°C and the exposed tissue was covered with saline-soaked gauze to minimize tissue dehydration. The exposed mesentery was suffused continuously at a rate of 1 ml $min^{-1}$ with a warmed bicarbonate-buffered salt solution (pH 7.4).

The mesenteric preparation was then observed using an intravital orthostatic microscope (Nikon Optiphot-2, SMZ1, Badhoevedorp, The Netherlands) equipped with a 20× objective lens (Nikon SLDW, Badhoevedorp, The Netherlands) and a 10× eyepiece. A video camera (Sony SSC-C350P, Cologne, Germany) mounted on the microscope projected the image onto a colour monitor (Sony Trinitron PVM-14N2E, Cologne, Germany) and the images were video recorded (Sony SVT-S3000P, Cologne, Germany) for playback analysis. The final magnification of the video screen was 1300×. Animal temperature, monitored using a rectal electrothermometer, was maintained at 37°C with an infrared heat lamp.

Single unbranched mesenteric venules (20–40 $\mu$m in diameter) were selected for study and their diameters were measured on-line using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, Texas, U.S.A.). Centreline red blood cell velocity ($V_{rbc}$) was also measured on-line using an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M Univer-

sity). Venular blood flow was calculated from the product of mean red blood cell velocity ($V_{mean} = V_{rbc}\ 1.6^{-1}$) and cross sectional area, assuming cylindrical geometry. Venular wall shear rate ($\gamma$) was calculated based on the Newtonian definition: $\gamma = 8 \times (V_{mean}\ D_v^{-1})\ s^{-1}$, in which $D_v$ is venular diameter (House & Lipowsky, 1987).

The number of rolling, adherent and emigrated leukocytes was determined off-line during playback of videotaped images. Rolling leukocytes were defined as white blood cells moving at a slower velocity than erythrocytes. Leukocyte rolling velocity ($V_{wbc}$) was determined as the time required for a leukocyte to move along 100 $\mu$m length of the microvessel and is expressed as $\mu m\ s^{-1}$. Rolling leukocyte flux was defined as those cells crossing a defined reference point in the vessel. The same reference point was used throughout the experiment as leukocytes may roll for only a section of the vessel before rejoining the blood flow or becoming firmly adherent. A leukocyte was defined as adherent to venular endothelium if it was stationary for at least 30 s. Leukocyte adhesion was expressed as the number per 100 $\mu$m length of venule. Leukocyte emigration was expressed as the number of white blood cells per microscopic field surrounding the venule.

### *Experimental protocol*

After stabilization of the mesentery for 30 min, a baseline recording was taken to establish basal values (time 0) of mean arterial blood pressure (MABP), $V_{rbc}$, $D_v$, shear rate and leukocyte rolling flux, velocity, adhesion and emigration. The superfusion buffer was then supplemented with L-NAME (100 $\mu$M), since previous studies have demonstrated that this dose causes the maximum and most consistent increase in leukocyte rolling and adhesion after 60 min superfusion (Arndt *et al*., 1993). Recordings were performed for 5 min at 15 min intervals over a 60 min period and the aforementioned leukocyte and haemodynamic parameters were measured at each stage. To determine if Ang-II is involved in L-NAME-mediated events, a second group of animals was pretreated 10 min before starting L-NAME superfusion with a selective antagonist of subtype $AT_1$ Ang-II receptor, losartan (10 mg $kg^{-1}$, i.v.).

To further investigate if Ang-II is implicated in the leukocyte-endothelial cell interactions provoked by a lack of prostaglandins, we performed another set of experiments in which 25 $\mu g\ ml^{-1}$ dose of indomethacin was suffused onto the exposed mesentery. The dose chosen for this study was based on previously reported data where a pronounced peak of leukocyte adhesion was observed within 30 min of indomethacin superfusion and blood levels of $PGE_2$ were reduced by more than 90% (Wallace *et al*., 1993). Similarly, a second group of animals was administered with a bolus injection of losartan (10 mg $kg^{-1}$, i.v.) 10 min prior to indomethacin superfusion. Video recordings were again made at the same time points as those previously described for L-NAME.

Finally, as Ang-II can be locally generated *via* mast cell chymase, its possible role in the leukocyte recruitment evoked by acute mast cell degranulation was also investigated in the present study. The mesentery was suffused for 60 min with CMP 48/80 at 1 $\mu g\ ml^{-1}$, a dose previously found to achieve maximal responses (Gaboury *et al*., 1995). Responses were determined throughout 1 h at the same time points as previously described for the other agents under investigation. Similarly, losartan was administered at the same dose and time point as in the aforementioned protocols.

### *Statistical analysis*

All data are expressed as mean $\pm$ s.e.mean. The data within groups were compared using an analysis of variance (1 way-ANOVA) with a Bonferroni *post hoc* correction for multiple comparisons. A $P$ value $<0.05$ was considered to be statistically significant.

### *Materials*

L-NAME, indomethacin and CMP 48/80 were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Losartan was kindly donated by Merck Sharp & Dohme, Spain.

## Results

Figure 1 shows that a bolus injection of L-NAME (10 mg $kg^{-1}$) induced a significant increase in MABP which lasted the remainder of the 60 min experimental period (Figure 1a). Pretreatment with losartan did not prevent L-NAME-induced MABP elevation. Interestingly, when animals were administered with a bolus dose of indomethacin (20 mg $kg^{-1}$), a significant increase in MABP was observed during the 30 min after NSAID injection, while a return to basal levels was witnessed after this time period. Losartan pretreatment prevented the increase detected in MABP by COX inhibition and normalized it to control values (Figure 1b).

Figure 2 illustrates the time course of changes in leukocyte rolling flux, adhesion and emigration induced by superfusion of the rat mesentery with L-NAME. Significant increases in leukocyte rolling ($85.4 \pm 16.0$ vs $19.7 \pm 2.1$ cells $min^{-1}$), adhesion ($10.4 \pm 2.8$ vs $0.3 \pm 0.2$ cells 100 $\mu m^{-1}$) and emigration ($4.0 \pm 1.7$ vs $0.0 \pm 0.0$ cells $field^{-1}$) were observed at 60 min with a 100 $\mu$M dose of L-NAME vs buffer. Concomitant significant decreases in leukocyte rolling velocity were also detected ($46.5 \pm 5.0$ vs $97.3 \pm 5.2$ $\mu m\ s^{-1}$ at 60 min). Administration of losartan inhibited L-NAME-induced leukocyte rolling flux and adhesion by 83 and 80% respectively at 60 min (Figure 2). In addition, losartan significantly decreased L-NAME-induced leukocyte extravasation by 70% at the same time point and returned leukocyte rolling velocity to basal levels ($113.4 \pm 15$ vs $97.3 \pm 5.2$ $\mu m\ s^{-1}$ at 60 min).

Table 1 summarizes the results obtained for different haemodynamic parameters prior to (0 min) and 60 min following L-NAME superfusion. As expected, local L-NAME induced no significant changes in MABP, however, a significant decrease in $V_{rbc}$, $V_{mean}$ and shear rate in single mesenteric venules was observed 60 min after L-NAME superfusion. The venular diameter did not change. Losartan pretreatment significantly attenuated the effect of L-NAME superfusion on $V_{rbc}$, $V_{mean}$ and shear rate.

Figure 3 shows the effect of leukocyte responses elicited by indomethacin superfusion. Indomethacin induced a significant increase in leukocyte rolling flux, adhesion and

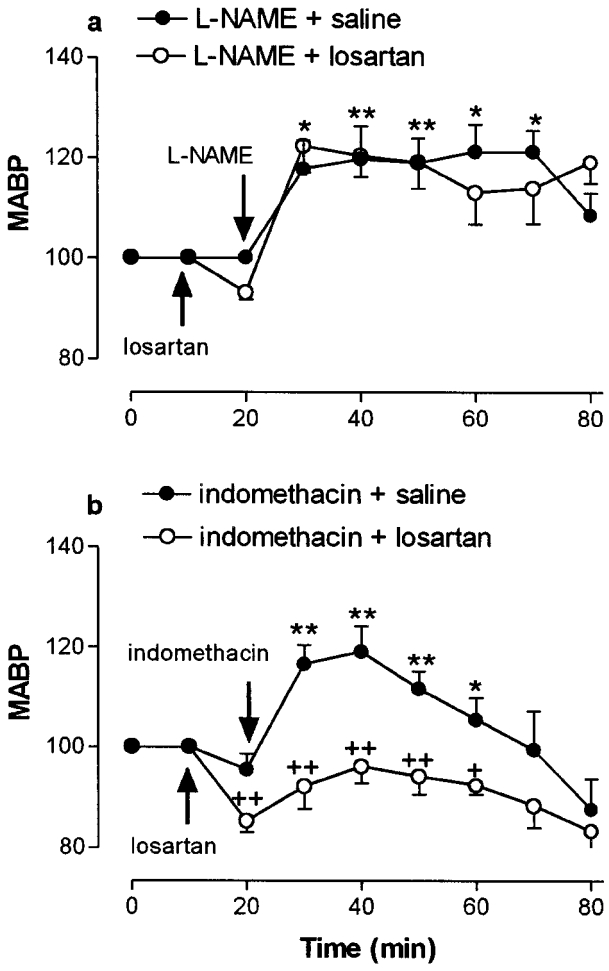


**Figure 1** Effects of losartan (10 mg kg$^{-1}$, i.v.) on mean arterial blood pressure (MABP) in anaesthetized rats treated with L-NAME (10 mg kg$^{-1}$, i.v.) (a) or with indomethacin (20 mg kg$^{-1}$, i.v.) (b). Results are expressed as percentage of basal values. Each point and bar represent the mean±s.e.mean of $n=4-6$ animals per group. *$P<0.05$ 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.

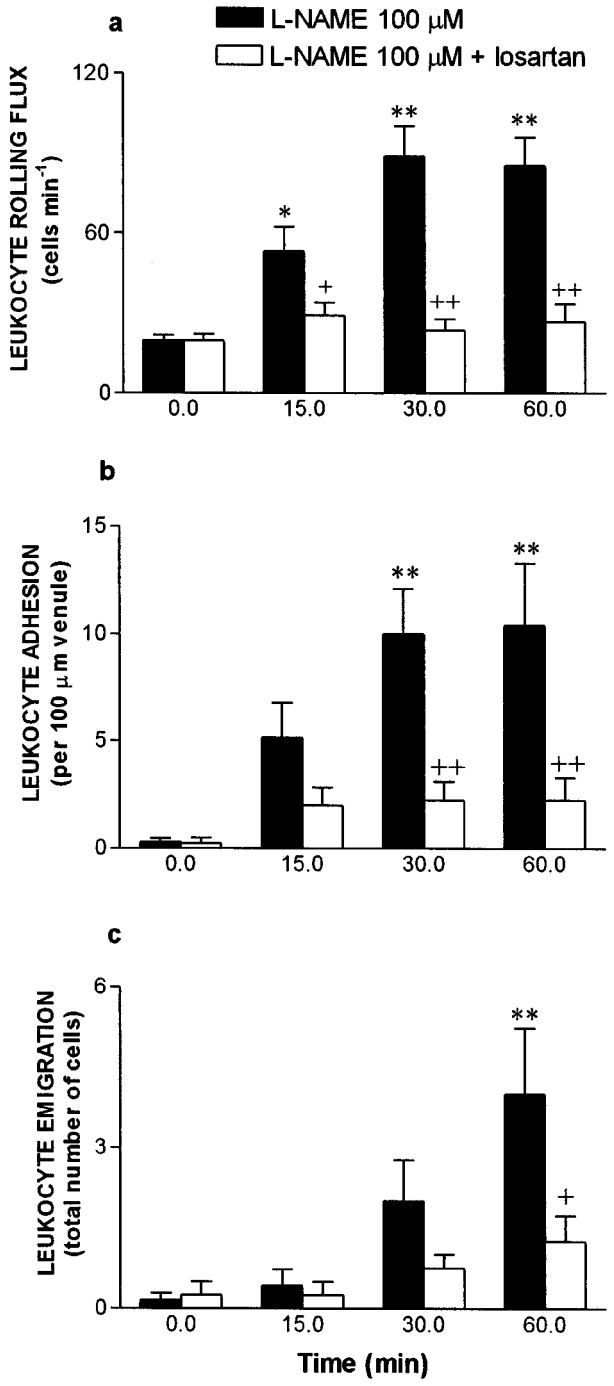


**Figure 2** Effect of losartan pretreatment on L-NAME-induced leukocyte rolling flux (a), leukocyte adhesion (b) and leukocyte emigration (c) in the rat mesenteric postcapillary venules. The mesentery was superfused with bicarbonate-buffered saline. Baseline parameters (0 min) were determined after a 30 min stabilization period. The superfusion buffer was then supplemented with L-NAME (100 $\mu$M). Parameters were measured 15, 30 and 60 min after superfusion with L-NAME in animals untreated ($n=5$) or pretreated with losartan (10 mg kg$^{-1}$, $n=5$). 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. $^{+}P<0.05$ or $^{++}P<0.01$ relative to the untreated group.

emigration after 60 min superfusion vs buffer (122.4±29.1 vs 17.0±5.4 cells min$^{-1}$, 8.6±1.9 vs 0.2±0.2 cells 100 $\mu$m$^{-1}$ and 1.4±0.2 vs 0.0±0.0 cells field$^{-1}$) which was accompanied by a significant decrease in leukocyte rolling velocity (76.6±12.5 vs 141.3±15.3 $\mu$m s$^{-1}$). Pretreatment with losartan significantly reduced indomethacin-induced increase in leukocyte rolling flux and adhesion by 98 and 88% respectively at this time point (Figure 3) and again returned leukocyte rolling velocity to basal levels (130.6±18.7 vs 144.7±8.2 $\mu$m s$^{-1}$). Although the decrease in indometacin-induced leukocyte emigration in the losartan pretreated group was not statistically significant (Figure 3), it should be noted that the leukocyte emigration evoked by indomethacin was also very subtle.

Local indomethacin suffusion induced no significant changes in $D_v$, $V_{rbc}$ or MABP, however, it caused a significant diminution of $V_{mean}$ and shear rate after 60 min superfusion. Pretreatment with losartan inhibited the decrease observed in both haemodynamic parameters elicited by indomethacin (Table 2).

As shown in Figure 4, CMP 48/80 elicited a significant increase in leukocyte rolling flux (104.8±8.7 vs 26.8±1.0

**Table 1** Haemodynamic parameters in untreated and losartan (10 mg kg$^{-1}$) treated animals before (0 min) and after (60 min) L-NAME superfusion (100 $\mu$M)

| | *Untreated animals* | | *Losartan-treated animals* | |
|---|---|---|---|---|
| | *0 min* | *60 min* | *0 min* | *60 min* |
| $D_V$ ($\mu$m) | 25.4±0.8 | 26.4±0.7 | 25.0±1.0 | 25.8±2.5 |
| $V_{rbc}$ (mm s$^{-1}$) | 3.0±0.2 | 2.0±0.3* | 2.4±0.4 | 2.3±0.6 |
| $V_{mean}$ (mm s$^{-1}$) | 1.9±0.2 | 0.8±0.3** | 1.5±0.2 | 1.4±0.4 |
| Shear rate (s$^{-1}$) | 557.8±61.3 | 360.2±61.5* | 470.4±64.4 | 441.4±107.3 |
| MABP (mm Hg) | 119.4±11.2 | 123.9±10.2 | 106.7±6.7 | 90.0±10.0 |

*$P<0.05$ relative to control value (0 min). All values are mean±s.e.mean ($n=5$ animals per group).

cells min$^{-1}$ at 60 min), adhesion (2.8±0.8 vs 0.0±0.0 cells 100 $\mu$m$^{-1}$ at 60 min) and emigration (0.8±0.3 vs 0.0±0.0 cells field$^{-1}$ at 60 min) versus buffer which was accompanied by a significant reduction in leukocyte rolling velocity (66.5±16.9 vs 122.8±8.4 $\mu$m s$^{-1}$ at 60 min). However, pretreatment with losartan did not provoke any reduction in the leukocyte-endothelial cell interactions induced by CMP 48/80 (Figure 4). In addition, haemodynamic parameters were neither decreased after CMP 48/80 superfusion nor affected by losartan pretreatment (Table 3).

## Discussion

Chronic NOS inhibition results in hypertension, which is known to be Ang-II-dependent (Pollock *et al*., 1993; Michel *et al*., 1996; Takemoto *et al*., 1997). In our study, losartan showed no effect on the increased blood pressure evoked by acute and systemic L-NAME administration. This is in agreement with the results obtained by Sigmond & Beierwaltes (1993), which found that losartan had no effect on MABP elevation induced during acute NOS inhibition. Conversely, Ang-II seems to be involved in the MABP elevation elicited by systemic COX inhibition. In chronic NOS inhibition an enhancement of angiotensin converting enzyme (ACE) activity and Ang-II receptors during the first week of treatment with L-NAME has been demonstrated (Takemoto *et al*., 1997). In contrast, in our study, 1 h of NOS inhibition was probably not enough to provoke an increase in ACE activity, and therefore, increase in MABP by acute L-NAME administration could not be effectively blocked by losartan pretreatment. Thus, the difference in ACE activity may explain the discrepancy between our results and those obtained in chronic NOS inhibition.

Despite these findings, in our study, we have clearly demonstrated a role for Ang-II in acute L-NAME and indomethacin-induced leukocyte–endothelial cell interactions. Losartan pretreatment inhibited the leukocyte rolling flux, adhesion and emigration which occurs after 60 min superfusion with L-NAME and returned leukocyte rolling velocity to basal levels. Similarly, losartan significantly reduced the leukocyte rolling and adhesion provoked by indomethacin superfusion at the same time point. In contrast, it showed no effect on the leukocyte recruitment which results from the acute mast cell activation evoked by CMP 48/80 superfusion. Therefore, these results indicate that the vasoconstrictor and proinflammatory effects of Ang-II are somehow disassociated. This is of considerable interest, since

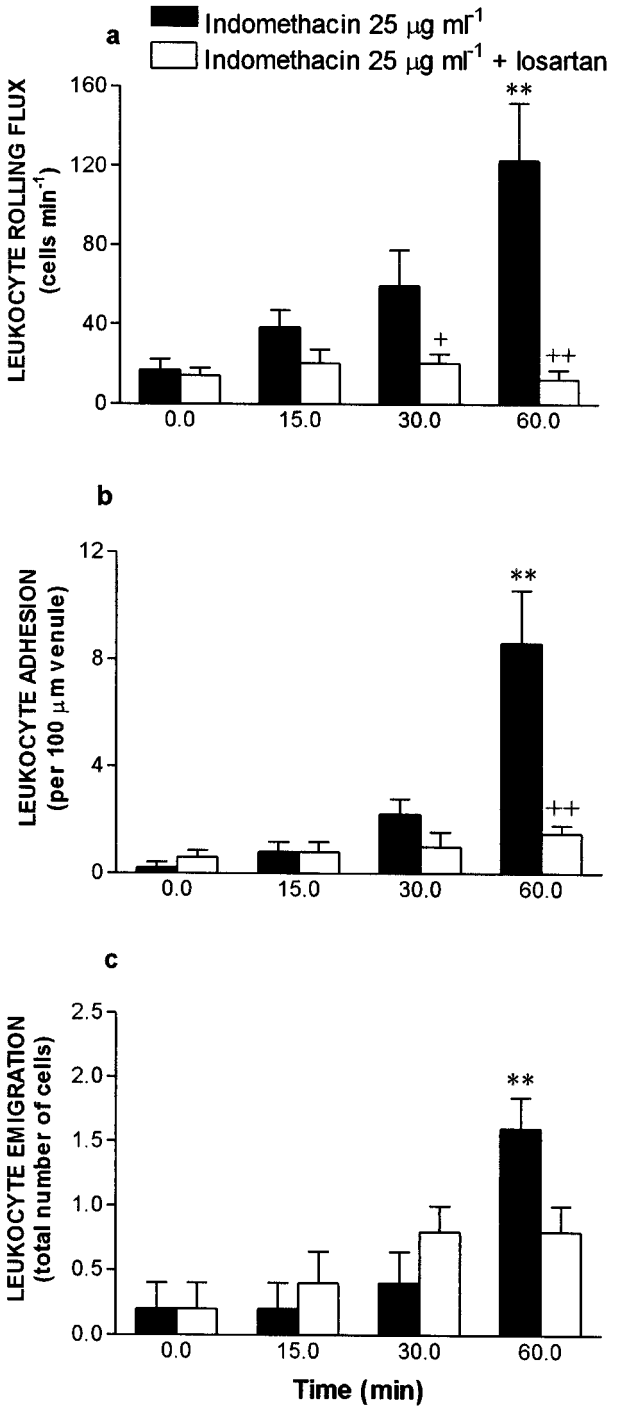


**Figure 3** Effect of losartan pretreatment on indomethacin-induced leukocyte rolling flux (a), leukocyte adhesion (b) and leukocyte emigration (c) in the rat mesenteric postcapillary venules. Parameters were determined at 0, 15, 30 and 60 min after indomethacin (25 $\mu$g ml$^{-1}$) superfusion in animals untreated ($n=5$) or pretreated with losartan (10 mg kg$^{-1}$, i.v., $n=5$). 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. $^{+}P<0.05$ or $^{++}P<0.01$ relative to the untreated group.

it indicates that lack of NO or $PGI_2$ may result in the exposure of the vascular endothelium to the deleterious actions of vasoconstrictors. These vasoconstrictors could then trigger the subsequent leukocyte recruitment that may cause

**Table 2** Haemodynamic parameters in untreated and losartan (10 mg $kg^{-1}$) treated animals before (0 min) and after (60 min) indomethacin superfusion (25 $\mu g\ ml^{-1}$)

| | *Untreated animals* | | *Losartan-treated animals* | |
|---|---|---|---|---|
| | *0 min* | *60 min* | *0 min* | *60 min* |
| $D_V$ ($\mu m$) | 28.4±1.7 | 28.0±1.8 | 33.4±1.8 | 33.8±1.8 |
| $V_{rbc}$ (mm $s^{-1}$) | 2.7±0.3 | 1.9±0.2 | 3.6±0.3 | 3.4±0.4 |
| $V_{mean}$ (mm $s^{-1}$) | 1.7±0.2 | 1.1±0.1* | 2.1±0.2 | 2.1±0.2 |
| Shear rate ($s^{-1}$) | 511.5±51.5 | 326.1±21.9* | 495.2±31.6 | 513.1±88.2 |
| MABP (mm Hg) | 120.0±4.5 | 118.3±3.1 | 126.3±8.5 | 109.2±12.8 |

*$P<0.05$ relative to control value (0 min). All values are mean±s.e.mean ($n=5$ animals per group).

the development of the vascular lesion detected in various disease states such as hypertension, atherosclerosis and myocardial ischemia-reperfusion injury (Mervaala *et al*., 1999; Hernandez-Presa *et al*., 1997; Ma *et al*., 1993). In this context, it is well established that vasoconstrictors such as endothelin-1 (ET-1) or Ang-II can elicit leukocyte-endothelial cell interactions within the rat mesenteric microvasculature regardless of their vasoconstrictor activity (Sanz *et al*., 1999; Piqueras *et al*., 2000).

In addition, losartan administration significantly diminished the effect of L-NAME superfusion on haemodynamic parameters such as $V_{mean}$ and shear rate in the rat mesenteric microvessels. Likewise, it significantly reversed the effect of indomethacin superfusion on $V_{mean}$ and shear rate. This confirms previous reports in which it has been demonstrated that acute NOS inhibition increases blood pressure and decreases blood flow in visceral organs, and, that losartan has a tendency to attenuate the haemodynamic responses to L-NAME in all organ beds, including the intestine, without affecting the pressor response to L-NAME (Sigmon & Beierwaltes, 1993). These results, raise the question of whether leukocyte-endothelial cell interactions induced by COX or NOS inhibition are due to shear rate reductions, and whether restoration of vascular haemodynamics by losartan can explain the inhibition of leukocyte responses in these situations. Previous reports have shown that reduction of shear rate accounts for only a small fraction of the leukocyte adhesive response associated with inhibition of NO production (Kubes *et al*., 1991). Moreover, 40% reduction in wall shear rate has been shown not to produce significant leukocyte adhesion (Perry & Granger, 1991). In our study, 60 min superfusion with either L-NAME or indomethacin caused significant reductions in shear rate of 35.5 and 36.2% respectively, while significant leukocyte-endothelial cell interactions were detected after 30 min superfusion. Therefore, we feel that the effects provoked by losartan on COX and NOS inhibition-induced leukocyte endothelial cell interactions are due to a direct effect on adhesion molecule expression, although a partial contribution of restoration of local haemodynamics cannot be discarded.

Among the different adhesion molecules involved in L-NAME-induced leukocyte-endothelial cell interactions, P-selectin seems to be the first to express itself on the vascular endothelium after acute NOS inhibition (Davenpeck *et al*., 1994; Lefer *et al*., 1999; Scalia *et al*., 1999). Similarly, indomethacin superfusion or systemic administration causes significant vascular P-selectin expression within 1 h (Wallace

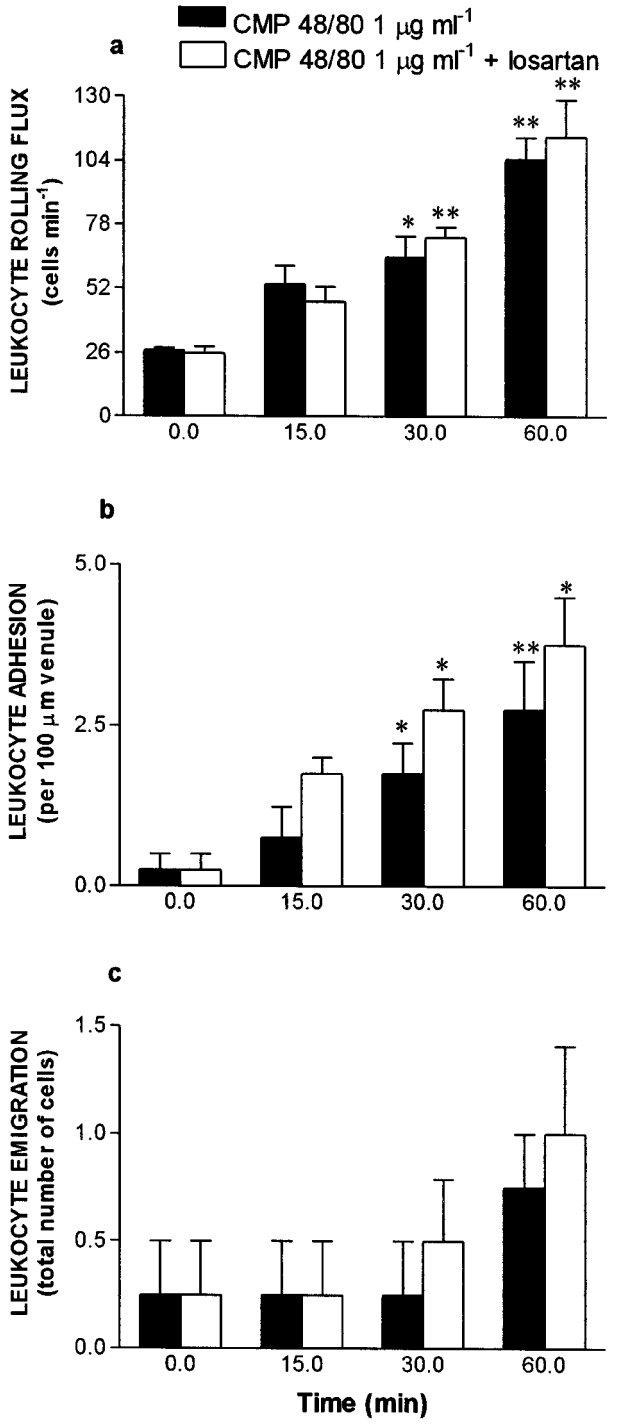


**Figure 4** Effect of losartan treatment on CMP 48/80-induced leukocyte rolling flux (a), leukocyte adhesion (b) and leukocyte emigration (c) in rat mesenteric postcapillary venules. After the 30 min stabilization period, baseline values were determined (0 min). Parameters were measured 15, 30 and 60 min after superfusion with CMP 48/80 (1 $\mu g\ ml^{-1}$) in animals untreated ($n=4$) or pretreated with losartan (10 mg $kg^{-1}$ i.v., $n=4$). 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.

*et al*., 1993; Kurose *et al*., 1996; Morise *et al*., 1998; 1999). Interestingly, we have recently demonstrated that leukocyte responses elicited by ET-1 and Ang-II are primarily mediated

**Table 3** Haemodynamic parameters in untreated and losartan (10 mg $kg^{-1}$) treated animals before (0 min) and after (60 min) CMP 48/80 superfusion (1 $\mu g\ ml^{-1}$)

| | *Untreated animals* | | *Losartan-treated animals* | |
|---|---|---|---|---|
| | *0 min* | *60 min* | *0 min* | *60 min* |
| $D_V$ ($\mu$m) | 26.0±0.6 | 26.0±0.6 | 30.0±2.9 | 29.0±3.3 |
| $V_{rbc}$ (mm $s^{-1}$) | 2.5±0.2 | 2.3±0.1 | 2.5±0.2 | 2.4±0.2 |
| $V_{mean}$ (mm $s^{-1}$) | 1.6±0.1 | 1.4±0.1 | 0.9±0.3 | 0.8±0.3 |
| Shear rate ($s^{-1}$) | 482.1±44.5 | 438.2±28.6 | 420.4±23.2 | 399.3±22.4 |
| MABP (mm Hg) | 111.7±4.1 | 117.9±5.9 | 108.9±5.9 | 90.8±3.8 |

*$P<0.05$ relative to control value (0 min). All values are mean±s.e.mean ($n=4$ animals per group).

through increased endothelial P-selectin expression and that Ang-II causes subendothelial leukocyte infiltration which is Ang-II subtype $AT_1$ receptor dependent (Sanz *et al*., 1999; Piqueras *et al*., 2000). Indeed, the upregulation of P-selectin is understood to be a vital early step in leukocyte recruitment. Therefore, the degree of P-selectin expression determines the abundance of rolling leukocytes that may eventually adhere to the endothelium and extravasate into the tissue. Considered together, all these findings suggest that endothelial barrier dysfunction produced by lack of NO or $PGI_2$ results in leukocyte recruitment, which seems to be mediated through P-selectin upregulation. Therefore, it is likely that the attenuation of P-selectin expression caused by $AT_1$ Ang-II receptor blockade accounts for the potential inhibitory effect of losartan on both L-NAME and indomethacin-induced leukocyte-endothelial cell interactions.

On the other hand, previous reports have found that NOS inhibitors can cause mast cell degranulation (Kubes *et al*., 1993), and, as we found that L-NAME-induced leukocyte-endothelial cell interactions were inhibited by losartan, we hypothesized that the $AT_1$ receptor antagonist may also inhibit the leukocyte responses elicited by acute mast cell activation. Moreover, there is evidence that perivascular mast cells store and release chymotrypsin-like protease (chymase) which, in turn, promotes the conversion of Ang-I to Ang-II (Urata *et al*., 1996) which is considered to constitute an alternative Ang-II generating-system. In our study, we found no role for Ang-II in the leukocyte-endothelial cell interactions provoked by acute mast cell degranulation. Several explanations may account for the effects observed. Upon stimulation, mast cells are known to release a variety of fast acting mediators such as histamine, leukotrienes and PAF. Indeed, leukocyte rolling and adhesion evoked by CMP 48/80 can be inhibited by pretreatment with an $H_1$ and with PAF receptor antagonists respectively or by antibodies directed against P-selectin or $\beta_2$ integrin function (Gaboury *et al*., 1995). Therefore, upon acute mast cell activation, it is likely that preformed mediators such as histamine or PAF may constitute a much more powerful stimulus for the observed leukocyte accumulation than the newly synthesized Ang-II. Alternatively, it has recently been reported that chymase activity varies depending on the subclass of the mast cells and their tissue distribution within the same species (Akasu *et al*., 1998). Therefore, the absence of effect of losartan on leukocyte responses elicited by CMP 48/80 superfusion could also be explained by the low chymase activity in rat peritoneal mast cells compared to that found in mast cells from other organs such as the lung, heart or aorta or that encountered in mast cells under pathophysiological conditions.

In conclusion, the most striking observation in the present study is the fact that leukocyte-endothelial cell interactions elicited by both NOS and COX inhibition can be dramatically attenuated by pretreatment with an Ang-II antagonist directed against its $AT_1$ receptor subtype. Therefore, it is possible that losartan modulates leukocyte–endothelial cell interactions regardless of its anti-hypertensive activity. Consistent with this concept, we have previously found that leukocyte recruitment elicited by Ang-II occurs at doses that are devoid of vasoconstrictor activity (Piqueras *et al*., 2000). Thus, it can be postulated that the pro-adhesive and vasoconstrictor actions induced by Ang-II are not associated and that, therefore, vascular lesions occur at early stages when the hypertensive process has not yet fully developed. Hence, our data strongly suggest that the beneficial effects exerted by systemic administration of losartan when vascular balance is disrupted by reduced levels of vasodilators are due to a significant inhibition of leukocyte-endothelial cell interaction and restoration of vascular haemodynamics. This may be a key mechanism by which losartan contributes to the amelioration of the consequences of an endothelial disruption and may represent a new strategy for modulating the pathophysiology of leukocyte-induced endothelial dysfunction in circulatory disorders.

The present study has been supported by grants PM 98 205, SAF 98 0118 and IFD 97 1029 from CICYT, Spanish Ministerio de Educación y Ciencia.

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*(Received July 14, 2000*
*Revised November 1, 2000*
*Accepted November 27, 2000)*