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Angiotensin II Induces Leukocyte–Endothelial Cell Interactions In Vivo Via AT_1 and AT_2 Receptor–Mediated P-Selectin Upregulation

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Background—Angiotensin II (Ang II) plays a critical role in the development of vascular lesions in hypertension, atherosclerosis, and several renal diseases. Because Ang II may contribute to the leukocyte recruitment associated with these pathological states, the aim of the present study was to assess the role of Ang II in leukocyte–endothelial cell interactions in vivo.

Methods and Results—Intravital microscopy of the rat mesenteric postcapillary venules was used. Sixty minutes of superfusion with 1 nmol/L Ang II induced a significant increase in leukocyte rolling flux (83.8±20.7 versus 16.4±3.1 cells/min), adhesion (11.4±1.0 versus 0.8±0.5 cells/100 μm), and emigration (4.0±0.7 versus 0.2±0.2 cells/field) without any vasoconstrictor activity. These effects were not mediated by mast cell activation. Intravenous pretreatment with AT_1 (losartan) or AT_2 (PD123,319) receptor antagonists significantly reduced Ang II–induced responses. A combination of both receptor antagonists inhibited the leukocyte rolling flux, adhesion, and extravasation elicited by Ang II at 60 minutes. Pretreatment of animals with fucoidin or an adhesion-blocking anti–rat P-selectin monoclonal antibody abolished Ang II–induced leukocyte responses. Furthermore, rat platelet P-selectin expression was not affected by Ang II stimulation.

Conclusions—Ang II induces significant leukocyte rolling, adhesion, and emigration, which may contribute not only to hypertension but also to the onset and progression of the vascular damage associated with disease states in which plasma levels of this peptide are elevated. **(*Circulation*. 2000;102:2118-2123.)**

Key Words: angiotensin ■ endothelium ■ leukocytes ■ cell adhesion molecules ■ glycoproteins

Angiotensin II (Ang II), the main effector peptide of the renin-angiotensin system, may contribute to the development of vascular lesions in hypertension, atherosclerosis, and many glomerular diseases of the kidney.[1–3] In addition to its role as a potent vasoconstrictor and regulator of blood pressure and fluid homeostasis, Ang II seems to be involved in the activation of monocytes and polymorphonuclear leukocytes (PMNs). Ang II receptors have also been demonstrated on monocytes.[4] This may be directly relevant, because hypertension is associated with migration of monocytes through the vessel wall, a critical event leading to the development of the atherosclerotic lesion that can be attenuated by ACE inhibition.[5,6] Furthermore, Ang II can promote monocyte adhesion and activation in vitro[7–9] and stimulates the expression of monocyte chemoattractant protein-1 (MCP-1) and RANTES in several animal models in vivo.[5,10,11] In addition, Ang II releases a neutrophil chemoattractant factor from cultures of arterial endothelial cells.[12]

Leukocytes migrate from the blood to sites of extravascular injury in response to locally produced stimuli that activate specific cell surface receptors. Initial capture of leukocytes is dependent on P-selectin expression.[13] Preliminary studies suggest that elevated levels of vasoconstrictors such as vasopressin or endothelin-1 (ET-1) contribute to leukocyte–endothelial cell interactions via P-selectin surface expression but do not provoke cellular migration.[14,15] Ang II is the dominant vasoconstrictor in many vascular diseases, and it might constitute a stimulus for the subendothelial infiltration of leukocytes associated with these pathological conditions. Therefore, in the present study, we investigated the capacity and profile of Ang II and the molecular mechanisms by which it elicits in vivo leukocyte–endothelial cell interactions within the rat mesenteric microcirculation.

Methods

Intravital Microscopy

The details of the experimental preparation have been described previously.[15] In short, Sprague-Dawley rats (200 to 250 g) were

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fasted for 24 hours and anesthetized with pentobarbital sodium (50 mg/kg IP), and the trachea, right carotid artery, and jugular vein were cannulated. After a midline abdominal incision, a segment of the midjejunal mesentery was exteriorized and placed over an optically clear viewing pedestal maintained at 37°C, which permitted tissue transillumination. The exposed mesentery was superfused continuously with a warmed bicarbonate-buffered saline (pH 7.4). An orthostatic microscope (Nikon Optiphot-2, SMZ1) equipped with an ×20 objective lens (Nikon SLDW) and an ×10 eyepiece allowed tissue visualization. A video camera (Sony SSC-C350P) mounted on the microscope projected the image onto a color monitor (Sony Trinitron PVM-14N2E), and the images were video recorded (Sony SVT-S3000P) for playback analysis (final magnification of the video screen was ×1300). Single unbranched mesenteric venules were selected, and the diameters (20 to 40 μm) were measured online with a video caliper (Microcirculation Research Institute, Texas A&M University). The number of rolling, adherent, and emigrated leukocytes was determined offline during playback of videotaped images. Centerline red blood cell velocity was also measured online with an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University). Venular blood flow and wall shear rate were calculated as previously described.[15]

Experimental Protocol

Preparations were allowed to stabilize for 30 minutes before baseline (time 0) measurements of mean arterial blood pressure, red blood cell velocity, vessel diameter, shear rate, leukocyte rolling flux and velocity, and leukocyte adhesion and emigration were obtained. The superfusion buffer was then supplemented with Ang II (0.1 to 100 nmol/L), and subsequent recordings were performed for 5 minutes at 15-minute intervals over a 60-minute period. On the basis of these initial experiments, 1 nmol/L Ang II was used for the remainder of the experiments.

Involvement of mast cell activation was determined by pretreatment of a group of animals before the start of surgery with sodium cromoglycate (cromolyn, 20 mg/kg IV), a mast cell–stabilizing agent, 0.33 mg/mL of which was added to the superfusate containing Ang II, as previously described.[15]

To identify which Ang II receptor was implicated, selective antagonists of receptor subtype AT_1 (losartan, 10 mg/kg IV), subtype AT_2 (PD123,319, 10 mg/kg IV), or a combination of the 2 were administered 15 minutes before suffusion with Ang II 1 nmol/L. The doses of both antagonists were based on previous in vivo data.[16,17]

The adhesion molecules involved in these responses were determined by pretreatment of the animals 5 minutes before Ang II suffusion with fucoidin (25 mg/kg IV), a P- and L-selectin–binding carbohydrate, an adhesion-blocking monoclonal antibody (mAb) directed against rat P-selectin (RMP-1, IgG2a, 2.5 mg/kg IV), or the nonblocking anti–rat P-selectin mAb (RP-2, IgG1, 2.5 mg/kg IV).[15]

Flow Cytometry

All the analyses were performed with an EPICS XL-MCL Flow Cytometer (Coulter Electronics) as described previously.[15]

Determination of Surface Expression of CD11b/CD18 ($\alpha_M\beta_2$) Integrins and L-Selectin (CD62L) in Rat PMNs and Monocytes

Duplicated samples (100 μL) of rat citrated peripheral whole blood were incubated for 15 minutes at 25°C with vehicle, PAF (1000 nmol/L), or Ang II (10 to 10 000 nmol/L). Samples were then incubated in darkness and on ice for 20 minutes with saturating amounts (10 μL) of the corresponding FITC-conjugated mAb. Removal of red blood cells and fixation of leukocytes was performed through an automated lysing procedure with an EPICS Q-PREP system (Coulter Electronics).

Determination of Surface Expression of P-Selectin in Rat Platelets

Rat citrated peripheral whole blood with prostaglandin E_1 (PGE_1) (1 μmol/L) was diluted 1:10 in modified Tyrode's buffer, and the procedure followed was similar to that previously described.[15]

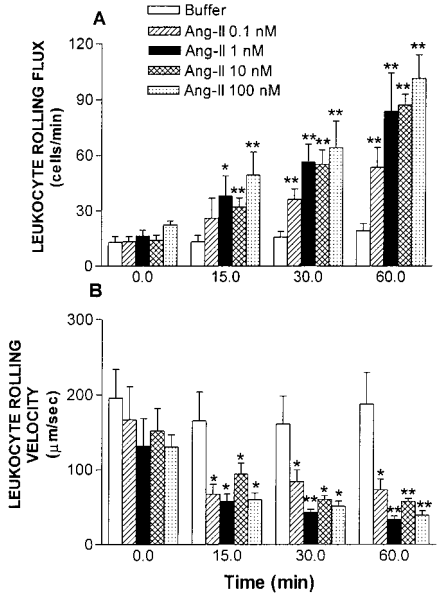

Figure 1. Dose-response and time-dependent effects of Ang II superfusion on leukocyte rolling flux (A) and rolling velocity (B) in rat mesenteric postcapillary venules. Animals were divided into 5 groups: buffer (n=6) or Ang II 0.1 nmol/L, n=4; 1 nmol/L, n=5; 10 nmol/L, n=4; and 100 nmol/L, n=5, and parameters were measured at 0, 15, 30, and 60 minutes. Results are mean±SEM. *$P<0.05$, **$P<0.01$ vs control value (0 minutes) for animals within each group.

Statistical Analysis

All data are expressed as mean±SEM. The data within groups were compared by a paired Student's *t* test. An unpaired Student's *t* test was used to compare groups. In both cases, the Student's *t* test was performed with a Bonferroni correction for multiple comparisons. A value of $P<0.05$ was considered statistically significant.

Materials

Ang II, Cromolyn, Thrombin, PGE_1, Mouse IgG_1, Goat Anti-Mouse IgG_1-FITC, and PD123,319 were purchased from Sigma Chemical Co. Losartan was kindly donated by Merck Sharp & Dohme. Antibodies RMP-1 and RP-2 were acquired as previously stated.[18] Conjugated mAb anti–rat-CD11b-FITC (OX-42) was purchased from Immunotech. Anti–rat L-selectin (HRL-3) and anti–hamster IgG FITC were supplied by LabClinics SA.

Results

The time- and concentration-dependent effects of Ang II on leukocyte rolling flux and rolling velocity are shown in Figure 1. Significant increases in leukocyte rolling and significant concomitant decreases in the leukocyte rolling velocity were observed at each stage for all concentrations with optimal changes at 1 nmol/L of Ang II (83.8±20.7 versus 16.4±3.1 cells/min and 37.3±3.5 versus 143.1±42.9 μm/s at 60 minutes, respectively). Similarly, Ang II induced a time- and concentration-dependent increase in leukocyte adhesion and emigration at 30 and 60 minutes, as shown in Figure 2 (11.4±1.0 versus 0.8±0.5 cells/100 μm and 4.0±0.7 versus 0.2±0.2 cells/field at 60 minutes, respectively, with the 1-nmol/L dose of Ang II).

Ang II 0.1 to 10 nmol/L induced no significant changes in arteriolar or venular diameter or mean arterial blood pressure (data not shown). However, 100 nmol/L Ang II, despite causing similar leukocyte responses, provoked a significant

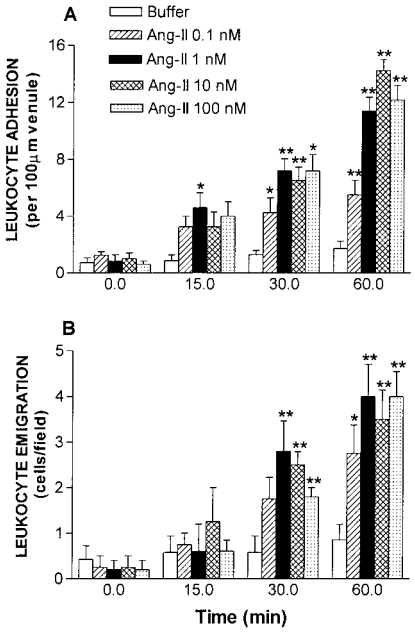

Figure 2. Dose-response and time-dependent effects of Ang II superfusion on leukocyte adhesion (A) and emigration (B) in rat mesenteric postcapillary venules. Parameters were determined at 0, 15, 30, and 60 minutes after superfusion with Ang II 0.1 nmol/L, n=4; 1 nmol/L, n=5; 10 nmol/L, n=4; or 100 nmol/L, n=5 or without Ang II (n=6). Results are mean±SEM. *$P<0.05$, **$P<0.01$ vs control value (0 minutes) for animals within each group.

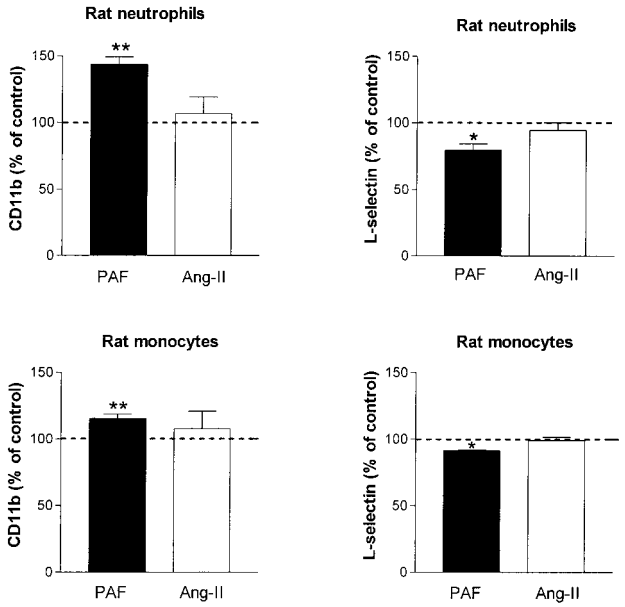

Figure 3. Effect of stimulation with PAF 1000 nmol/L or Ang II 1000 nmol/L on surface expression of CD11b/CD18 integrin and L-selectin on both rat neutrophils and monocytes. FITC fluorescence values are expressed as percentage of mean fluorescence intensities of control cells (dotted line). Data are mean±SEM of 4 experiments. *$P<0.05$, **$P<0.01$ vs absolute mean fluorescence intensity between control samples and agonist-treated samples.

vasoconstriction in both venular and arteriolar diameter, a significant decrease in shear rate, and the collapse of 40% of the vessels investigated. Therefore, a single dose of 1 nmol/L Ang II, 100-fold less than a dose that caused vasoconstriction, was selected to investigate further the mechanisms of Ang II–induced leukocyte–endothelial cell interactions.

Flow cytometry analysis showed that whereas PAF induced L-selectin shedding and increased expression of CD11b/CD18 integrins in both leukocyte subtypes, Ang II at 10 to 10 000 nmol/L had no effect on expression of either of these leukocyte surface adhesion molecules (Figure 3).

Stabilization of mast cells by cromolyn administration had no influence on Ang II–elicited effects (Figure 4). In contrast, as shown in Figure 5, both losartan and PD123,319 pretreatment significantly reduced leukocyte rolling flux, adhesion, and emigration after Ang II suffusion. However, neither antagonist completely inhibited these responses. This was obtained only after simultaneous administration of both receptor antagonists. Furthermore, fucoidin pretreatment abolished the leukocyte–endothelial cell interactions induced by Ang II (Figure 6). Although pretreatment with the P-selectin–blocking antibody RMP-1 inhibited the leukocyte rolling, adhesion, and emigration associated with Ang II superfusion, administration of a binding, nonblocking control antibody (RP-2) had little or no effect on the Ang II–induced responses (Figure 7). Finally, flow cytometry analysis on rat platelets revealed the lack of effect of Ang II on platelet P-selectin expression (Figure 8).

Discussion

Leukocyte accumulation in the vessel wall is a hallmark of early stages of atherosclerosis, acute myocardial infarction, and renal diseases of diverse causes in which Ang II seems to play a critical role.[2,10,19] In this study, we show that subvasoconstrictor and physiologically relevant doses of Ang II (0.1 to 1 nmol/L) induce a significant increase in leukocyte–endothelial cell interactions. This observation suggests that in various diseases, inappropriate leukocyte–endothelial cell interactions occur before hypertension and in response to elevated Ang II levels. Indeed, this is one of the most striking observations of this study, because it indicates that disruption of the vascular balance between vasodilators and vasoconstrictors may expose the vascular endothelium to the deleterious action of the latter. Ang II therefore could trigger the initial leukocyte recruitment that leads to the subsequent vascular damage observed in hypertension, atherosclerosis, and myocardial ischemia-reperfusion injury, constituting a prominent role for this molecule in the pathogenesis of these cardiovascular disease states.

Also significant is the fact that although Ang II elicits leukocyte adhesion and emigration, flow cytometry analysis revealed that it has no direct effect on leukocyte chemotaxis. Several explanations may account for the proadhesive effects observed. It is likely that Ang II activates the endothelium and induces leukocyte rolling, which leads to firm adhesion of leukocytes to the endothelium, resulting in their subsequent emigration. Indeed, we discovered that the initial leukocyte–endothelial cell interactions induced by Ang II are due to P-selectin expression on the endothelium. The firm adhesion could also be due to the release of endogenously generated chemotactic mediators elicited by Ang II. In this context, there is some evidence that Ang II can evoke leukocyte recruitment through increased expression and in-

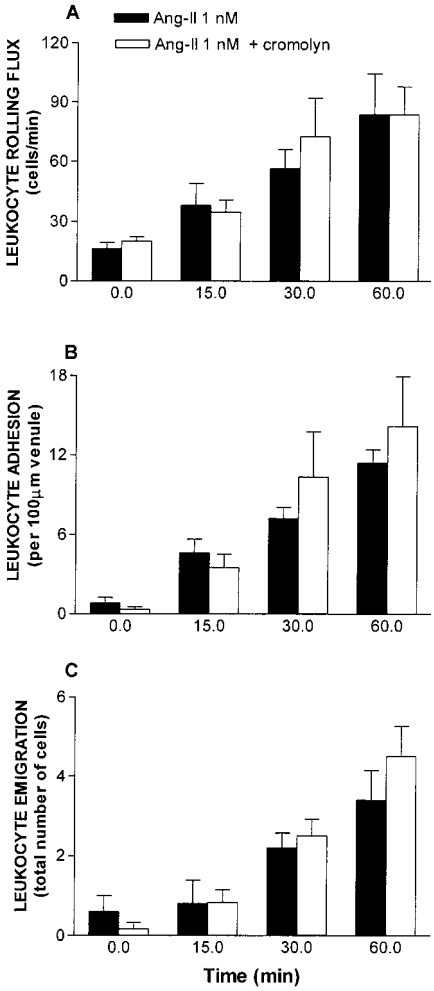

Figure 4. Effect of cromolyn treatment on Ang II–induced leukocyte rolling flux (A), adhesion (B), and emigration (C) in rat mesenteric postcapillary venules. Parameters were measured 0, 15, 30, and 60 minutes after superfusion with Ang II 1 nmol/L in animals untreated (n=5) or pretreated with cromolyn (n=6). Results are mean±SEM.

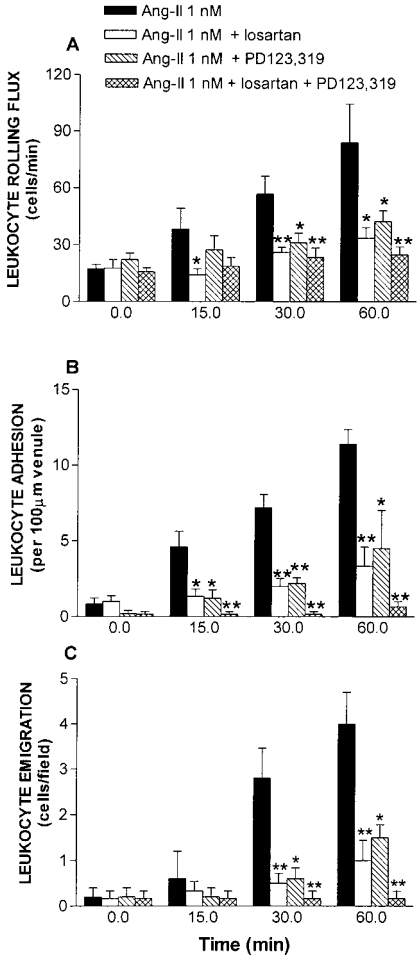

Figure 5. Effect of selective AT_1 (losartan) and AT_2 (PD123,319) receptor antagonists on Ang II–induced rolling flux (A), adhesion (B), and emigration (C) in rat mesenteric postcapillary venules. Parameters were measured 0, 15, 30, and 60 minutes after superfusion with Ang II 1 nmol/L in animals untreated (n=5) or pretreated with losartan (n=6), PD123,319 (n=5), or a combination of both antagonists (n=6). Results are mean±SEM. *$P<0.05$, **$P<0.01$ vs untreated group.

duction of chemotactic mediators such as IL-8, MCP-1, or RANTES.[5,10,12] In fact, IL-8 was recently shown to be costored with P-selectin in Weibel-Palade bodies.[20] In addition, Mangat et al[21] have showed a role for Ang II in cytosolic phospholipase A_2 activation, which is critical for the synthesis and release of potent chemotactic mediators such as PAF or leukotriene B_4. This is relevant because the release of these inflammatory mediators may constitute an amplifying mechanism for further leukocyte recruitment after Ang II stimulation.

We have also demonstrated the role of both Ang II receptor subtypes, AT_1 and AT_2, on Ang II–induced effects within the rat mesenteric microcirculation, because a combination of both receptor blockers returned all parameters to basal levels. Notably, the present findings are supported by in vitro data, albeit under static conditions, in which the involvement of both receptor subtypes in the adhesion of human monocytes to endothelial cells after incubation with Ang II has been demonstrated.[8] We have also discarded the possibility of a direct activation of mast cells on the release of mediators by Ang II and demonstrated a direct effect on the endothelium via endothelial P-selectin upregulation.

There is some evidence to suggest that Ang II induces adhesion molecule expression, but these findings are controversial. Some authors have found no role for E-selectin, vascular cell adhesion molecule-1 (VCAM-1), or intercellular cell adhesion molecule-1 (ICAM-1) in Ang II–induced monocyte adhesion to cultured endothelial cells.[8] Others have found that treatment with ACE inhibitors downregulates the expression of VCAM-1 and ICAM-1 in animal models.[6,22] Similarly, enalaprilat treatment in septic patients decreases the plasma levels of soluble adhesion molecules such as E-selectin or ICAM-1.[23] Furthermore, in an in vitro study, Gräfe et al[9] found a clear effect for Ang II in inducing E-selectin expression on coronary endothelial cells.

In this way, our data clearly demonstrate an implication of P-selectin in Ang II–mediated responses. First, pretreatment of animals with fucoidin totally inhibited the leukocyte responses elicited by Ang II. Second, administration of a blocking anti–rat P-selectin mAb (RMP-1) abolished all Ang

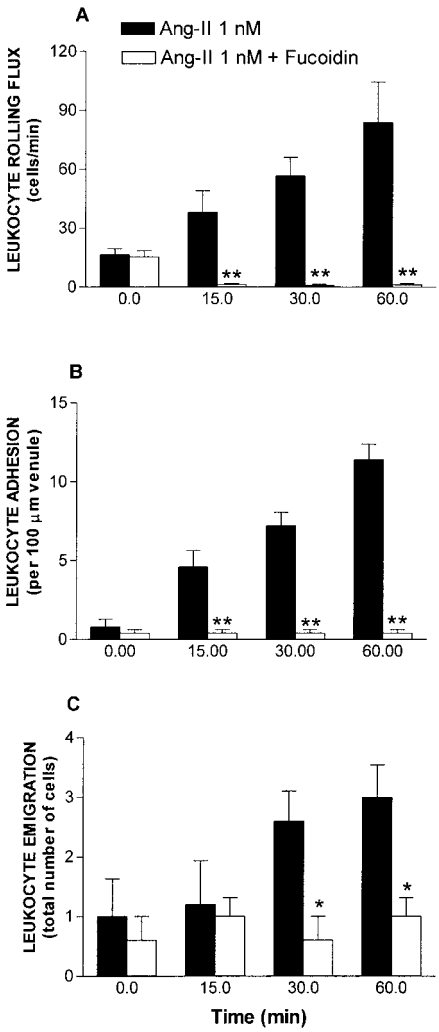

Figure 6. Effect of fucoidin pretreatment on Ang II–induced leukocyte rolling flux (A), adhesion (B), and emigration (C) in rat mesenteric postcapillary venules. Parameters were determined at 0, 15, 30, and 60 minutes after Ang II 1 nmol/L superfusion in animals untreated (n=5) or pretreated with fucoidin (n=5). Results are mean±SEM. *$P<0.05$, **$P<0.01$ vs untreated group.

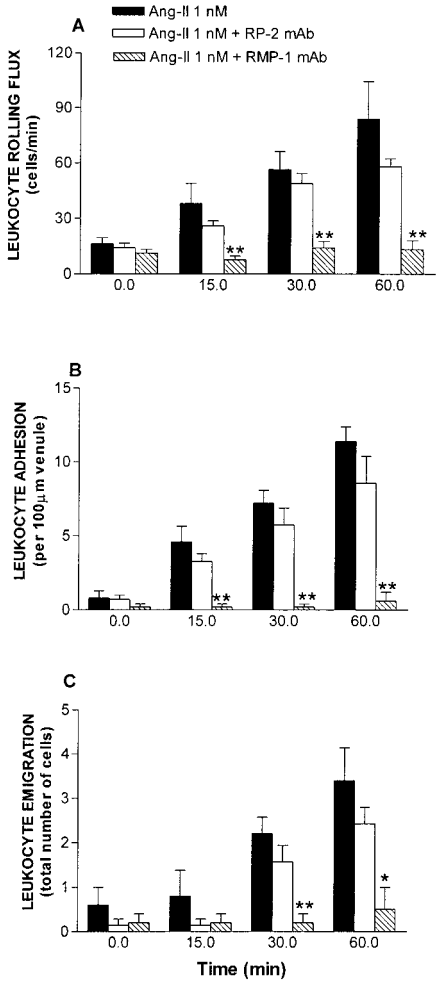

Figure 7. Effect of anti–P-selectin treatment on Ang II–induced leukocyte rolling flux (A), adhesion (B), and emigration (C) in rat mesenteric postcapillary venules. Animals were divided into 3 groups: untreated (n=5) and treated with a nonblocking (RP-2, n=5) or blocking (RMP-1, n=7) anti–P-selectin mAb. Ang II 1 nmol/L superfusion was begun 5 minutes later, and parameters were measured at 15, 30, and 60 minutes. Results are mean±SEM. *$P<0.05$, **$P<0.01$ vs untreated group.

II–induced leukocyte–endothelial cell interactions. Hence, we believe that responses induced by exogenous Ang II in our experiments are mediated through P-selectin upregulation on the endothelial cell surface. This conclusion is supported by the fact that no platelet-platelet or platelet–endothelial cell interactions were detected in this in vivo system and that flow cytometry analysis revealed no changes in rat platelet P-selectin expression after Ang II stimulation.

An explanation for all these findings is that at early stages of elevated plasma levels of Ang II, the leukocyte–endothelial cell interactions elicited by this peptide are mediated primarily through P-selectin upregulation. In chronic disorders, however, inducible adhesion molecules such as E-selectin are synthesized and may contribute to further leukocyte recruitment. Indeed, 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.[24,25] Furthermore, it was recently shown that in a mouse model of atherosclerosis, there is a clear involvement of both P- and E-selectins in the development of the atherosclerotic lesion at both early and advanced stages.[26]

To summarize, we have demonstrated for the first time in vivo that Ang II elicits leukocyte–endothelial cell interactions within the rat mesenteric microcirculation at subvasoconstrictor doses. This effect is both AT_1 and AT_2 receptor–mediated and totally dependent on endothelial P-selectin expression. Thus, Ang II may play a critical role in the leukocyte attachment to and emigration through the vascular endothelium and could contribute to the vascular damage present in pathological conditions in which plasma levels of this peptide are elevated.

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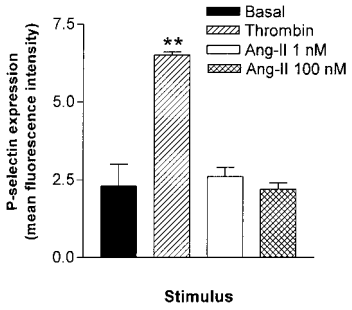

Figure 8. Effect of thrombin or Ang II stimulation on surface expression of P-selectin on rat platelets. Blood samples were incubated with vehicle, 1 U/mL thrombin, or 1 or 100 nmol/L Ang II. Anti–rat P-selectin mAb was then added, stained with FITC-conjugated mAb, and analyzed by flow cytometry. Values are expressed as mean fluorescence intensities. Data are mean±SEM of n=4 experiments. $^{**}P<0.01$ vs mean fluorescence intensity in control samples.

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