Angiotensin II Induces Neutrophil Accumulation In Vivo Through Generation and Release of CXC Chemokines

Yafa Naim Abu Nabah, BPharm*; Teresa Mateo, BPharm*; Rossana Estellés, BPharm, PhD; Manuel Mata, BSc, PhD; John Zagorski, BSc, PhD; Henry Sarau, BSc, PhD; Julio Cortijo, BPharm, PhD; Esteban J. Morcillo, MD, PhD; Peter J. Jose, BSc, PhD; Maria-Jesus Sanz, BPharm, PhD

Background—Angiotensin II (Ang II) is implicated in the development of cardiac ischemic disorders in which prominent neutrophil accumulation occurs. Ang II can be generated intravascularly by the renin-angiotensin system or extravascularly by mast cell chymase. In this study, we characterized the ability of Ang II to induce neutrophil accumulation.

Methods and Results—Intraperitoneal administration of Ang II (1 nmol/L) induced significant neutrophil recruitment within 4 hours (13.3 ± 2.3 × 10⁶ neutrophils per rat versus 0.7 ± 0.5 × 10⁶ in control animals), which disappeared by 24 hours. Maximal levels of CXC chemokines were detected 1 hour after Ang II injection (577 ± 224 pmol/L cytokine-inducible neutrophil chemoattractant [CINC]/keratinocyte-derived chemokine [KC] versus 5 ± 3, and 281 ± 120 pmol/L macrophage inflammatory protein [MIP-2] versus 14 ± 6). Intravital microscopy within the rat mesenteric microcirculation showed that the short-term (30 to 60 minutes) leukocyte–endothelial cell interactions induced by Ang II were attenuated by an anti-rat CINC/KC antibody and nearly abolished by the CXCR2 antagonist SB-517785-M. In human umbilical vein endothelial cells (HUVECs) or human pulmonary artery media in culture, Ang II induced interleukin (IL)-8 mRNA expression at 1, 4, and 24 hours and the release of IL-8 at 4 hours through interaction with Ang II type 1 receptors. When HUVECs were pretreated with IL-1 for 24 hours to promote IL-8 storage in Weibel-Palade bodies, the Ang II–induced IL-8 release was more rapid and of greater magnitude.

Conclusions—Ang II provokes rapid neutrophil recruitment, mediated through the release of CXC chemokines such as CINC/KC and MIP-2 in rats and IL-8 in humans, and may contribute to the infiltration of neutrophils observed in acute myocardial infarction. (Circulation. 2004;110:3581-3586.)

Key Words: angiotensin ■ interleukins ■ cells ■ endothelium ■ inflammation
cell types can synthesize IL-8, endothelial cells have the additional capacity to store this chemokine in Weibel-Palade bodies, together with von Willebrand factor and P-selectin. We have previously demonstrated that Ang II induces leukocyte recruitment in postcapillary venules and that this response is dependent on the increased expression of P-selectin on the endothelial surface.

Despite these studies, there have been no direct investigations into the ability of Ang II to induce neutrophil accumulation in vivo. Therefore, the present study focused on the potential of Ang II to mediate neutrophil accumulation in vivo and the mechanisms involved in this response.

**Methods**

**Materials**

Ang II, histamine, cycloheximide, and PD123,319 were purchased from Sigma Chemical Co; endothelial basal medium (EBM)-2 supplemented with endothelial growth media (EGM)-2 was from In VitroGenetics; N,N’-[12-ethanediylbis(oxy-2,1-phenylene)]bis[2-[(acetyloxy)methyl]-2-oxoethyl] ester (Glycine: BAPTA-AM) was from Molecular Probes; human IL-8 and IL-1β, rat cytokine-inducible neutrophil chemotactic protein (CINC)/keratinocyte-derived chemokine (KC) and macrophage inflammatory protein (MIP)-2, and antibodies to rat CINC/KC and MIP-2 were from PeproTech; the antibody pair for the human IL-8 ELISA was from R&D Systems; neutrophin–horseradish peroxidase was from Perbio Science; the K-Blue substrate was from Neogen; Dulbecco’s modified Eagle’s medium and TRIZol were from Life Technologies; and the TaqMan predevelopment and reverse transcription (RT) reagents were from PE Biosystems. Losartan was donated by Merck Sharp & Dohme, Madrid, Spain. The neutralizing anti-rat CINC/KC antibody was obtained as previously described. SB-517785-M was donated by GlaxoSmithKline.

**Neutrophil Migration Into the Peritoneal Cavity**

Sprague-Dawley rats (200 g to 250 g; Charles River, Barcelona, Spain) were sedated with ether and injected intraperitoneally with 5 mL phosphate-buffered saline (PBS) or 1 nmol/L Ang II. After 1, 4, 8, or 24 hours, the rats were killed with an overdose of anesthetic, and the peritoneal cavity was first lavaged with 5 mL PBS and then with 30 mL heparinized (10 U/mL) PBS. The exudates were centrifuged separately to obtain cell pellets and supernatant fluids. The supernatant from the first (5-mL) lavage was used for determination in vivo. Therefore, the present study focused on the potential of Ang II to mediate neutrophil accumulation in vivo and the mechanisms involved in this response.

Experimental Protocol

All preparations were left to stabilize for 30 minutes, and baseline (time 0) measurements of leukocyte rolling flux and velocity, leukocyte adhesion, leukocyte emigration, mean arterial blood pressure, centerline red blood cell velocity, shear rate, and venular diameter were obtained. The superfusion buffer was either continued or supplemented with 1 nmol/L Ang II. Recordings were performed for 5 minutes at 15-minute intervals for 60 minutes, and the aforementioned leukocyte and hemodynamic parameters were measured. Some animals were pretreated with a polyclonal antibody against rat CINC/KC (10 mg/kg IV at −15 minutes) or with a selective CXCR2 receptor antagonist (SB-517785-M, 25 mg/kg PO at −60 minutes) before Ang II superfusion.

**Cell Culture**

Human umbilical endothelial cells (HUVECs) were isolated by collagenase treatment and maintained in human endothelial cell–specific EBM-2 supplemented with EGM-2 and 10% fetal calf serum (FCS). HUVECs up to passage 2 were grown to confluence on 24-well culture plates. Before every experiment, cells were incubated for 16 hours in medium containing 1% FCS and then returned to the 10% FCS medium for all experimental incubations. Human pulmonary arteries were obtained from patients undergoing lobectomy or pneumonectomy from bronchial carcinoma. Studies were approved by the institutional ethics committee. The arteries were opened; the endothelial surface was removed by gentle scraping with a scalpel; the surrounding adventitia was carefully dissected away; and the tunica media, cut into ∼3- to 5-mm sections, was placed on 24-well culture plates in Dulbecco’s modified Eagle with 10% FCS. Cells or human pulmonary artery media (HPAM) was stimulated with 1 to 1000 nmol/L Ang II for 1, 4, 24, or 48 hours. Selective antagonists of Ang II type 1 (AT1; losartan, 10 μmol/L) or type 2 (PD123,319, 10 μmol/L) receptors or a combination of both was added to some wells 1 hour before Ang II (100 nmol/L). Where stated, HUVECs were preincubated with IL-1β (1000 U/mL) for 24 hours to induce synthesis and storage of IL-8 in Weibel-Palade bodies. washed twice, and incubated for 1 hour in fresh medium with or without 1 to 1000 nmol/L Ang II or 100 μmol/L histamine as the positive control. Cycloheximide (0.1 mg/mL) or BAPTA-AM (100 μmol/L) was added to some wells 1 hour before Ang II (100 nmol/L). At the end of the experiment, cell-free supernatants were stored at −20°C for IL-8 ELISA, and HUVECs were washed before digestion in 0.5 mol/L NaOH for determination of protein content by the Lowry procedure or for weighing of the arterial media.

Quantitative RT-PCR

IL-8 mRNA was determined by real-time quantitative RT–polymerase chain reaction (PCR). HUVECs or HPAM was incubated with medium or 100 nmol/L Ang II for 1, 4, or 24 hours, and total RNA was extracted with TRIZol. Quantitative data of relative gene expression were determined by the comparative Ct method (ΔΔCt), as described by the manufacturer (PE-ABI PRISM 7700 sequence detection system) and previously reported. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the endogenous control gene. TaqMan predevelopment assay reagents were used to determine IL-8 mRNA, and TaqMan RT reagents were used to generate cDNA.

**Enzyme-Linked Immunosorbent Assays**

Rat CINC/KC and MIP-2 levels were determined by conventional sandwich ELISAs. Results are expressed as picomoles chemokine in the supernatant from the 5-mL lavage. No cross-reactions were
detected in the CINC/KC and MIP-2 assays with any rat chemokines tested: regulated on activation normal T expressed and secreted, monocyte chemoattractant protein-1, and MIP-2 or KC (<0.01%). Human IL-8 was measured in culture supernatants. The blocking step was omitted as unnecessary in samples containing 10% FCS and diluted in PBS/0.5% bovine serum albumin.

**Statistical Analysis**

All values are mean±SEM. Data between groups were compared by 1-way ANOVA with a Newman-Keuls post hoc correction for multiple comparisons. Statistical significance was set at P<0.05.

**Results**

Intraperitoneal injection of 5 mL of 1 nmol/L Ang II in rats induced a significant neutrophil recruitment that was maximal at 4 hours and had resolved by 24 hours (Figure 1A).

Total protein content in the peritoneal exudate was not modified by Ang II administration at any of the times studied (Figure 1B). CINC/KC and MIP-2 levels were elevated after Ang II injection, peaking at 1 hour (Figure 1C and 1D) before significant neutrophil infiltration was seen. Significant CXC chemokine levels were still evident at 4 hours but had declined to basal levels by 8 hours. This time course is consistent with a contribution of the CXC chemokines to neutrophil recruitment.

To investigate the mechanisms involved, Ang II–induced responses were studied for 1 hour by intravital microscopy to evaluate leukocyte rolling, adherence to the endothelium, and emigration into the mesentery, events that would be expected to precede leukocyte accumulation in the peritoneal cavity.

**Figure 1.** Time course of Ang II–induced neutrophil accumulation (A), protein extravasation (B), and CINC/KC (C) and MIP-2 (D) generation. Rats were injected intraperitoneally with 5 mL PBS or 1 nmol/L Ang II. Results are mean±SEM for n=4 or 5 animals per group.

*P<0.05, **P<0.01 relative to values in PBS-injected group. Abbreviations are as defined in text.

**Figure 2.** Effect of anti-rat CINC/KC antibody and CXCR2 antagonist on Ang II–induced leukocyte rolling flux (A), rolling velocity (B), adhesion (C), and emigration (D) in rat mesenteric postcapillary venules. Parameters were measured 0, 15, 30, and 60 minutes after superfusion with buffer (n=5) or with 1 nmol/L Ang II in animals untreated (n=7) or pretreated with anti rat CINC/KC antibody (10 mg/kg IV, n=9) or with CXCR2 antagonist (25 mg/kg PO, n=5). Results are mean±SEM. *P<0.05, **P<0.01 relative to values in buffer group. +P<0.05, ++P<0.01 relative to untreated Ang II group. Abbreviations are as defined in text.
As shown in Figure 2, superfusion of the mesentery with 1 nmol/L Ang II induced a decrease in leukocyte rolling velocity and increases in leukocyte rolling flux, adhesion, and emigration within 30 minutes. Administration of a neutralizing anti-rat CINC/KC antibody inhibited the Ang II–induced responses at 30 and 60 minutes, the inhibition of leukocyte rolling flux, adhesion, and emigration at 60 minutes being 66%, 89%, and 67%, respectively (Figure 2). Because the peritoneal exudate fluids contained MIP-2 in addition to CINC/KC (Figure 1), we next used a CXCR2 antagonist that is known to inhibit rat neutrophil responses to both chemokines.20 Blockade of CXCR2 with SB-517785-M was more effective than the antibody treatment, the inhibition of Ang II–induced leukocyte rolling flux, adhesion, and emigration responses at 60 minutes being 100%, 91%, and 100%, respectively (Figure 2). Likewise, SB-517785-M treatment returned the Ang II–induced decrease in leukocyte rolling velocity to basal levels (Figure 2B). Neither the Ang II superfusion nor the systemic anti-CINC/KC or SB-517785-M pretreatments affected circulating leukocyte counts, mean arterial blood pressure, or shear rate (Table).

To investigate Ang II–induced chemokine release at the cellular level, we used cultures of HUVECs and HPAM. IL-8 mRNA was increased within 1 hour after stimulation with 100 nmol/L Ang II (Figure 3). IL-8 protein secretion in both HUVECs and HPAM was unaffected in the first hour of incubation with Ang II (data not shown) but was significantly increased after 4 hours (Figure 4A HUVECs, 179±41 and 241±65 nmol/mg cellular protein at 100 and 1000 nmol/L Ang II, respectively, versus 129±21 in unstimulated cells; Figure 4B HPAM, 4.49±1.19 and 6.81±1.59 nmol/mg tissue at 100 and 1000 nmol/L Ang II, respectively, versus 1.33±0.38 in controls). These effects appear to be mediated through interaction of Ang II with its AT1 receptor, because losartan but not the type 2 receptor antagonist PD123,319 inhibited 100 nmol/L Ang II–induced IL-8 release (Figure 4). IL-8 protein concentration was no higher at 24 and 48 hours than at 4 hours. In contrast to many endothelial cells, HUVECs have little preformed IL-8 stored in Weibel-Palade bodies.14 To investigate the ability of Ang II to induce IL-8 release, HUVECs were pretreated with IL-1 to promote IL-8 storage14 and then incubated for 1 hour in fresh medium with or without Ang II but without IL-1. Under these conditions, Ang II caused a dose-dependent release of IL-8 that was markedly higher (2689±234 and 3327±477 nmol/mg cellular protein with 100 and 1000 nmol/L Ang II, respectively) than that seen without pretreatment; again, these effects were AT1 receptor mediated (Figure 5). Pretreatment with the protein synthesis inhibitor cycloheximide had no effect on the amount of IL-8 released in response to Ang II. In contrast, inhibition of Weibel-Palade body degranulation by pretreatment with BAPTA-AM resulted in complete inhibition of Ang II–induced IL-8 release (Figure 5).

**Discussion**

Activation of the renin-angiotensin system, including the generation of Ang II, has been clearly demonstrated in several...
inflammatory conditions, in particular in the heart. In the present study, we show for the first time that Ang II can induce rapid neutrophil infiltration in vivo, a finding that might be relevant in acute myocardial infarction. To investigate this possibility, we chose to inject Ang II into the peritoneal cavity for 2 reasons. First, the neutrophils could be unequivocally identified and measured by microscopy without resort to measuring secondary markers such as myeloperoxidase activity or the use of labeled cells. Second, the preceding events of rolling and adhesion to the endothelium could be investigated in detail by intravital microscopy of the mesenteric microcirculation. We established that Ang II induces neutrophil accumulation that is preceded by the generation of the neutrophil chemoattractant CXC chemokines, CINC/KC and MIP-2. Accordingly, the early leukocyte–endothelial cell interactions were substantially inhibited by neutralization of the activity of CINC/KC and almost totally inhibited by blockade of CXCR2, the only high-affinity receptor on rat neutrophils for CXC chemokines.20 Thus, although rat neutrophils possess Ang II receptors,6 our results suggest an indirect mechanism by which the release of CINC/KC, MIP-2, and possibly other CXC chemokines in response to Ang II accounts for the majority of neutrophil accumulation in this model.

The major CXC chemokine involved in human myocardial inflammation is thought to be IL-8.13 Many cell types are capable of producing this potent neutrophil chemoattractant. We used HUVECs and fragments of HPAM, mainly comprising smooth muscle cells, and showed that Ang II induced IL-8 mRNA synthesis within 1 hour and secretion of the chemokine within 4 hours. Release of IL-8 in response to Ang II is expressed as percentage of that in medium control, mean±SEM of n=4 or 5 experiments. *P<0.05, **P<0.01 relative to values in medium control group. +P<0.05, ++P<0.01 relative to 100 nmol/L Ang II. Abbreviations are as defined in text.

Figure 4. Effect of Ang II on IL-8 release in HUVECs (A) and HPAM (B). HUVECs or HPAM was stimulated with Ang II (1 to 1000 nmol/L), 100 nmol/L Ang II+10 μmol/L losartan, +10 μmol/L PD123,319, or combination of both antagonists for 4 hours. Release of IL-8 in response to Ang II is expressed as percentage of that in medium control, mean±SEM of n=4 or 5 experiments. *P<0.05, **P<0.01 relative to values in medium control group. Abbreviations are as defined in text.
strongly suggests the release of IL-8 from preformed stores such as endothelial Weibel-Palade bodies. Accordingly, inhibition of Weibel-Palade body degranulation with a calciobal chelator abolished the Ang II–induced IL-8 release. Thus, the generation of Ang II at sites of inflammation could lead to CXC chemokine release involving both a posttranslational event, contributing to the initial phase of neutrophil infiltration, and a transcriptional event that may contribute to a more sustained neutrophil recruitment.

Expression of IL-8 on the endothelial surface leads to rapid neutrophil–endothelial cell interactions. Such interactions are multistep processes that include P-selectin expression, leading to rolling of leukocytes on the endothelium.\(^2\) Like IL-8, P-selectin is presynthesized and stored in endothelial cell Weibel-Palade bodies\(^14\) and is rapidly mobilized to the cell surface after exposure to Ang II.\(^15\) Because CXC chemokines induce P-selectin upregulation,\(^24\) it is possible that the release of IL-8 in response to Ang II augments the P-selectin upregulation. Therefore, in addition to their role in Ang II–induced neutrophil adhesion and migration, CXC chemokines may also contribute to the rolling response elicited by this peptide hormone. All of the responses described in this study were inhibited by losartan but not PD123,319, suggesting that the effects were mediated through interaction of Ang II with its AT\(_1\) receptor. The Ang II concentrations required to stimulate cells in culture were physiologically relevant, at least for the arterial media, and similar to that used in the in vivo studies. The rapid and transient effects of Ang II in vivo, when compared with those detected in cell culture, may be explained by the rapid metabolism of Ang II in vivo.

In conclusion, in this study we have demonstrated that Ang II induces neutrophil accumulation in vivo via the secretion of CXC chemokines, the source of which may include endothelial cell Weibel-Palade bodies and vascular smooth muscle cells, and this effect is mediated through interaction of Ang II with its AT\(_1\) receptor. Ang II is formed from the decapeptide Ang I by the action of a carboxydipeptidase, angiotensin-converting enzyme, found on the endothelial cell surface and in the plasma. An alternative source of Ang II generation in the heart is mast cell chymase,\(^4\) which generates Ang II directly from Ang I.\(^4\) Therefore, Ang II should be considered a potential inflammatory mediator of neutrophil infiltration observed in acute myocardial infarction through the release of CXC chemokines, and CXC receptor antagonists may become a powerful tool in the control of inflammation associated with acute myocardial infarction.

Acknowledgments

This study was supported by grants SAF 2002-01482, SAF2002-04667, and SAF-2003-07206-C02-01 from CICYT, the Spanish Ministry of Science and Technology, and research groups grant 03/166 from Generalitat Valenciana and awarded with the 2002 prize of the Spanish Pharmacological Society and Almirall-Prodesfarma Laboratories. Y.N.A.N., M.M., and P.J.J. were supported by a grant from the Spanish Ministry of Education, Culture and Sports. P.J.J. is primarily supported by Asthma, UK, and T.M. with a grant from Conselleria of Education, Culture and Sports (Generalitat Valenciana).

References

Angiotensin II Induces Neutrophil Accumulation In Vivo Through Generation and Release of CXC Chemokines
Yafa Naim Abu Nabah, Teresa Mateo, Rossana Estellés, Manuel Mata, John Zagorski, Henry Sarau, Julio Cortijo, Esteban J. Morcillo, Peter J. Jose and Maria-Jesus Sanz

_Circulation_. 2004;110:3581-3586; originally published online November 29, 2004; doi: 10.1161/01.CIR.0000148824.93600.F3

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/110/23/3581

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/