Angiotensin II mediates postischemic leukocyte-endothelial interactions: role of calcitonin gene-related peptide

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Yusof M, Kamada K, Gaskin FS, Korthuis RJ. Angiotensin II mediates postischemic leukocyte-endothelial interactions: role of calcitonin gene-related peptide. Am J Physiol Heart Circ Physiol 292: H3032–H3037, 2007. First published February 16, 2007; doi:10.1152/ajpheart.01210.2006.—Vascular inflammation and enhanced production of angiotensin II (ANG II) are involved in the pathogenesis of hypertension and diabetes, disease states that predispose the afflicted individuals to ischemic disorders. In light of these observations, we postulated that ANG II may play a role in promoting leukocyte rolling (LR) and adhesion (LA) in postcapillary venules after exposure of the small intestine to ischemia-reperfusion (I/R). Using an intravital microscopic approach in C57BL/6J mice, we showed that ANG II type I (AT1) or type II (AT2) receptor antagonism (with valsartan or PD-123319, respectively), inhibition of angiotensin-converting enzyme (ACE) with captopril, or calcitonin gene-related peptide (CGRP) receptor blockade (CGRP8-37) prevented postischemic LR but did not influence I/R-induced LA. However, both postischemic LR and LA were largely abolished by concomitant AT1 and AT2 receptor blockade or chymase inhibition (with Y-40079). Additionally, exogenously administered ANG II increased LR and LA, effects that were attenuated by pretreatment with a CGRP receptor antagonist or an NADPH oxidase inhibitor (apocynin). Our work suggests that ANG II, formed by the enzymatic activity of ACE and chymase, plays an important role in inducing postischemic LR and LA, effects that involve the engagement of both AT1 and AT2 receptors and may be mediated by CGRP and NADPH oxidase.

Angiotensin II (ANG II) is the main effector of the renin-angiotensin system, acting as a potent vasoconstrictor and key regulator of blood pressure and electrolyte homeostasis (6). An emerging body of evidence indicates that in addition to these well-known actions, ANG II also stimulates a wide variety of proinflammatory responses including increased leukocyte rolling and adhesion, production of oxidative stress, and induction of CXC chemokine expression (27). ANG II is formed by the enzymatic action of angiotensin-converting enzyme (ACE) and mast cell-derived chymase and mediates its effects by binding to specific cell surface receptors (9, 13). In the cardiovascular system, the main angiotensin receptor subtypes include type I (AT1) and type II (AT2) receptors (8). In contrast to the well-established physiological role of AT1 receptors in the cardiovascular effects of ANG II, the significance of AT2 receptor activation is not well characterized. However, exogenous ANG II has been shown to increase leukocyte rolling and adhesion via AT1 and AT2 receptor-mediated P-selectin expression (25). More recent work indicates that the increase in leukocyte-endothelial cell adhesive interactions induced by intestinal ischemia and reperfusion (I/R) can be prevented by treatment with an AT1 receptor antagonist (24).

While the aforementioned studies clearly establish a role for ANG II in postischemic leukosequestration in intestinal tissues, the source of this inflammatory octapeptide (ACE vs. chymase) and the relative contribution of AT1 vs. AT2 receptors to increased leukocyte rolling and adhesion in the small bowel are undefined. In addition, the downstream signaling mechanisms underlying postischemic ANG II-dependent leukocyte rolling and adherence are unclear. However, it is well established that ANG II increases vascular and neutrophilic superoxide production by a NADPH oxidase-dependent mechanism (11, 34). In addition, recent work indicates that ANG II increases circulating levels of calcitonin gene-related peptide (CGRP) (20, 26, 35). Since I/R-induced leukocyte adhesion occurs by an oxidant-dependent mechanism and because CGRP is a proadhesive neuropeptide that plays an important role in neurogenic inflammation, we hypothesized that ANG II derived from mast cell chymase induces inflammation in postischemic venules of the mouse small intestine via an AT1 and AT2 receptor-dependent mechanism that involves CGRP and NADPH oxidase.

MATERIALS AND METHODS

Animals

Wild-type male C57BL/6J mice (6–7 wk of age) were obtained from Jackson Laboratories (Bar Harbor, ME). All mice were maintained on standard mouse chow and used at 8–12 wk of age. The experimental procedures described herein were performed according to the criteria outlined in the National Institutes of Health guidelines and were approved by the University of Missouri-Columbia Institutional Animal Care and Use Committee.

Surgical Procedures and Induction of I/R

The mice were anesthetized initially with a mixture of ketamine (150 mg/kg body wt ip) and xylazine (7.5 mg/kg body wt ip). The right carotid artery was cannulated, and systemic arterial pressure was measured with a Statham P23A pressure transducer (Gould) connected to the carotid artery catheter. Systemic blood pressure was recorded continuously with a personal computer (Power Macintosh 8600; Apple) equipped with an analog-to-digital converter (MP 100; Biopac Systems). Carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE; Molecular Probes, Eugene, OR) was dissolved in DMSO (CFDA-SE; Molecular Probes, Eugene, OR) was dissolved in DMSO

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at a stock concentration of 5 mg/ml, divided into 25-μl aliquots, and stored in light-tight containers at −20°C until further dilution immediately before intravenous injection. The left jugular vein was cannulated for administration of CFDA-SE. After these procedures, a midline abdominal incision was performed, and the superior mesenteric artery (SMA) was occluded with a microvascular clip for 0 (Sham) or 45 min. After the ischemic period, the clip was gently removed and leukocytes were labeled with CFDA-SE by intravenous administration of the fluorochrome solution (250 μg/ml saline) at 20 μl/min for 5 min. During the preparation, storage, and administration of CFDA-SE, care was taken to minimize light exposure. Leukocyte-endothelial cell adhesive interactions were quantitated over minutes 30–40 and 60–70 of reperfusion.

**Intravital Fluorescence Microscopy**

The mice were positioned on a 20 × 30-cm Plexiglas board in a manner that allowed a selected section of small intestine to be exteriorized and placed carefully and gently over a glass slide covering a 4 × 3-cm hole centered in the Plexiglas. The exposed small intestine was superfused with warmed (37°C) bicarbonate-buffered saline (BBS, pH 7.4) at 1.5 ml/min with a peristaltic pump (model M312; Gilson). The exteriorized region of the small bowel was covered with BBS-soaked gauze to minimize tissue dehydration, temperature changes, and the influence of respiratory movements. The superfusate was maintained at 37 ± 0.5°C by pumping the solution through a heat exchanger warmed by a constant-temperature circulated (model 1130; VWR). Body temperature of the mouse was maintained between 36.5 and 37.5°C by use of a thermostatically controlled heat lamp. The board was mounted on the stage of an inverted microscope (Diaphot TMD-EF; Nikon), and the intestinal microcirculation was observed through a ×20 objective lens. Intravital fluorescence images of the microcirculation (excitation wavelength 420–490 nm, emission wavelength 520 nm) were detected with a charge-coupled device (CCD) camera (XC-77; Hamamatsu Photonics), a CCD camera control unit (C2400; Hamamatsu Photonics), and an intensifier head (M4314; Hamamatsu Photonics) attached to the camera. Microfluorographs were projected on a television monitor (PVM-1953MD; Sony) and recorded on digital video with a digital video recorder (DMR-E50; Panasonic) for off-line quantification of measured variables during playback of the recorded image. A video time-date generator (WJ810; Panasonic) displayed the stopwatch function onto the monitor.

The intravitral microscopic measurements described below were obtained over minutes 30–40 and 60–70 of reperfusion or at equivalent time points in the control groups. The intestinal segment was scanned from the oral to the aboral section and 10 single, unbranched venules (20- to 50-μm diameter, 100-μm length) were observed, each scanned from the oral to the aboral section and 10 single, unbranched venules (20- to 50-μm diameter, 100-μm length) were observed, each for 30 s. Leukocyte-endothelial cell interactions (numbers of rolling and firmly adherent leukocytes) were quantified in each of the 10 venules, followed by calculation of the mean value, which was used in the statistical analysis of the data. Circulating leukocytes were considered to be firmly adherent if they did not move or detach from the venular wall for a period equal to or greater than 30 s. Rolling cells were defined as cells crossing an imaginary line in the microvessel at a velocity that is significantly lower than center line velocity; their numbers were expressed as rolling cells per minute. The numbers of rolling or adherent leukocytes were normalized by expressing each as the number of cells per square millimeter of vessel area.

**Experimental Protocols**

The general design of the experimental protocols for each group in the study is described below. Drug doses were selected based on reports in the literature (4, 25, 27).

**Group 1: Sham.** As a time control for the effects of experimental duration, the mesentery of each mouse in this group (n = 6) was superfused with BBS, which was also used as the route of administration for the pharmacological agents used in this study in groups 3–9 outlined below. The SMA was exposed but not subjected to occlusion, with leukocyte-endothelial cell adhesive interactions quantitated at time points comparable to those described for mice subjected to 45 min of intestinal ischemia followed by 70-min reperfusion (group 2, below).

**Group 2: I/R alone.** Mice in this group (n = 6) were treated as described for group 1 above except that I/R was induced by occlusion of the SMA for 45 min followed by reperfusion for 70 min. Leukocyte rolling and adhesion were quantified during minutes 30–40 and 60–70 of reperfusion.

**Group 3: I/R + valsartan.** To determine whether AT1 receptor blockade would prevent I/R-induced leukocyte rolling and adhesion, mice in this group (n = 6) were treated as described for group 2 except that valsartan (10 mM; Novartis, East Hanover, NJ) was added to the superfusate 10 min before reperfusion.

**Group 4: I/R + PD-123319.** To determine the effects of AT2 receptor blockade on I/R-induced leukocyte rolling and adhesion, mice in this group (n = 6) were treated as described for group 2 except that PD-123319 (Sigma-Aldrich, St. Louis, MO; 10 mM) was added to the superfusate 10 min before reperfusion.

**Group 5: I/R + valsartan + PD-123319.** The effects of combined AT1 and AT2 receptor blockade on I/R-induced leukocyte rolling and adhesion were examined in this group (n = 6) by superfusing the mesentery with both valsartan (10 mM) and PD-123319 (10 mM) beginning 10 min before reperfusion.

**Group 6: I/R + captopril.** To determine the role of ACE in I/R-induced leukocyte rolling and adhesion, mice in this group (n = 6) were treated as described for group 2 except that captopril (10 mM; Sigma-Aldrich) was added to the superfusate 10 min before reperfusion.

**Group 7: I/R + Y-40079.** To evaluate the role of chymase in I/R-induced leukocyte rolling and adhesion, mice in this group (n = 6) were treated as described for group 2 except that Y-40079 (100 μM; a generous gift from Mitsubishi Pharma) (2) was added to the superfusate 10 min before reperfusion.

**Group 8: I/R + CGRP8-37.** To determine the role of CGRP in I/R-induced leukocyte rolling and adhesion, mice in this group (n = 6) were treated as described for group 2 except that the CGRP receptor antagonist CGRP8-37 (10 mM; Sigma-Aldrich) was added to the superfusate 10 min before reperfusion.

**Group 9: I/R + apocynin.** The role of NADPH oxidase in postischemic leukocyte rolling and adhesion was interrogated by treating mice in this group (n = 3) as described for group 2 except that apocynin (7.2 mM) was added to the superfusate 10 min before reperfusion.

To further explore the mechanisms underlying I/R-induced, ANG II-mediated leukocyte rolling and adhesion, mice were treated with exogenous ANG II (100 nM, Sigma, St. Louis, MO) by intraperitoneal injection 2 h before assessment of leukocyte rolling and adhesion, in the absence [group 10: ANG II (n = 6)] and presence of CGRP receptor blockade [group 11: ANG II + CGRP8-37 (n = 6)] or NADPH oxidase inhibition [group 12: ANG II + apocynin (n = 3)]. CGRP8-37 and apocynin were administered via the superfusate, as described for groups 8 and 9, respectively.

**Statistical Analysis**

The data were analyzed with standard statistical analysis, i.e., ANOVA with Fisher (post hoc) test for multiple comparisons. All values are expressed as means ± SE. Statistical significance was defined at P < 0.05.

**RESULTS**

Figure 1 illustrates the average numbers of rolling (top) and adherent (bottom) leukocytes in postcapillary venules of the
murine small intestine exposed to I/R alone (I/R, group 2) or I/R coincident with AT1 receptor blockade (I/R + valsartan, group 3) or AT2 receptor blockade (I/R + PD-123319, group 4) alone or combined AT1 + AT2 receptor blockade (I/R + valsartan + PD-123319, group 5) relative to nonischemic controls (Sham, group 1). I/R induced marked increases in the numbers of rolling and adherent leukocytes after 30 and 60 min of reperfusion. It is important to note that postischemic leukocyte rolling and adhesion appeared to be exclusively confined to postcapillary venules because leukocyte-endothelial cell adhesive interactions were not observed in arterioles in any experiment. The postischemic increase in leukocyte rolling was abolished by AT1 receptor or AT2 receptor blockade alone or coincident administration of AT1 + AT2 receptor antagonists. However, I/R-induced leukocyte adhesion was not affected by treatment with either an AT1 or an AT2 receptor antagonist alone but was largely abolished by concomitant AT1 + AT2 receptor blockade. These results indicate that I/R-induced leukosequestration occurs by an ANG II-dependent mechanism.

To determine the source of ANG II that mediates postischemic leukocyte rolling and adhesion, we evaluated the effect of mesenteric superfusion with inhibitors of ACE (I/R + captopril, group 6) or chymase (I/R + Y-40079, group 7). Like AT1 or AT2 receptor blockade alone, ACE inhibition abrogated I/R-induced leukocyte rolling (Fig. 2, top) but did not prevent postischemic leukocyte adhesion (Fig. 2, bottom). However, chymase inhibition effectively reduced both leukocyte rolling and adhesion after I/R (Fig. 2).

Figure 3 illustrates the effect of CGRP receptor blockade (I/R + CGRP8-37, group 8) or NADPH oxidase inhibition (I/R + apocynin, group 9) on I/R-induced leukocyte rolling (Fig. 3, top) and adhesion (Fig. 3, bottom). Treatment with CGRP8-37 prevented postischemic leukocyte rolling but did not abrogate the development of stationary leukocyte adhesive responses to I/R. Interestingly, we used the same dose of CGRP8-37 to prevent the antiadhesive effects of preconditioning with exogenous CGRP or ethanol administered 24 h before I/R (16), suggesting that the dose of the receptor antagonist was sufficient to effectively block the effects of the neuropeptide. Apocynin treatment also prevented the increase in leukocyte rolling induced by I/R and attenuated the postischemic rise in leukocyte adhesion (Fig. 3).

The data depicted in Fig. 4 demonstrate that exogenous administration of ANG II by intraperitoneal injection (ANG II, group 10), in lieu of I/R, induces significant increases in leukocyte rolling and adhesion. In contrast to the pattern noted after I/R, blockade of CGRP receptors with CGRP8-37...
Our results contribute to a growing body of evidence indicating that the renin-angiotensin system contributes to I/R-induced inflammatory responses (3, 4, 7, 10, 24, 25, 27). For example, Riaz et al. (27) reported that large increases in circulating ANG II concentrations, colonic CXC chemokine expression, and ACE mRNA levels occur after 2 h of reperfusion following a 30-min occlusion of the SMA. Petnehazy et al. (24) showed that SMA occlusion for 45 min induces AT1 receptor expression, leukocyte rolling, and stationary leukocyte adhesion in small intestinal venules, when assessed 4 h after reperfusion is initiated. Interestingly, a similar pattern of response was noted in studies conducted in chimeric mice that express AT1 receptors on the vessel wall, but not circulating cells. This latter result suggests that engagement of AT1 receptors on circulating cells vs. those expressed in postcapillary venules plays a more dominant role in eliciting the proinflammatory state induced by intestinal I/R. The role of AT2 receptors was not evaluated in either study.

The aforementioned observations support a role for ANG II, acting via AT1 receptors, in I/R-induced stationary leukocyte adhesion but not in the weaker adhesive interactions associated with leukocyte rolling in the small and large intestine. In stark contrast, our work indicates that two mechanistically distinct approaches that interfere with the actions of ANG II (chymase inhibition and combined AT1 + AT2 receptor blockade) prevented the increases in both leukocyte rolling and stationary adhesion induced by I/R. On the other hand, we report that ACE inhibition and independent blockade of AT1 or AT2 receptors largely abolished posts ischemic leukocyte rolling without influencing the increase in stationary leukocyte adhesion induced by I/R. Our findings are consistent with the observations of Piqueras et al. (25), who showed that 1-h exposure of the mesentery to exogenous ANG II at subpressor levels induces leukocyte rolling and adhesion via AT1 and AT2 receptor-dependent expression of P-selectin.

Before the present work, the enzymatic source for ANG II in the small intestine and the possible contribution of AT2 receptors in the development of this proinflammatory phenotype were largely unknown. While ACE is a major generator of ANG II under normal conditions and in many hypertensive

**DISCUSSION**

The results of this study provide two lines of evidence implicating the renin-angiotensin system in the proinflammatory response to intestinal I/R. First, the postischemic increases in leukocyte rolling and adhesion were largely abolished by concomitant treatment with AT1 (valsartan) + AT2 (PD-123319) receptor antagonists. Second, inhibition of chymase, an important enzymatic source of ANG II especially in injured vasculature, also abrogated I/R-induced leukocyte rolling and adhesion. ACE inhibition or independent blockade of AT1 or AT2 receptors was also effective in preventing postischemic leukocyte rolling but failed to modify stationary adhesive responses to I/R. Other significant new findings pertain to evidence supporting a role for CGRP and NADPH oxidase in I/R-induced, ANG II-mediated leukocyte-endothelial cell adhesive interactions.
states, a number of recent studies have pointed to the potential involvement of mast cell chymase as another enzymatic source of this peptide in the vessel wall, especially at sites of vascular injury (9, 13). In normal tissues, chymase is stored in mast cells in an inactive form and ACE plays a dominant role in ANG II formation. However, chymase acquires the ability to form ANG II after secretion from degranulating mast cells and can become the predominant source of ANG II in injured tissues. Importantly, this enzyme can account for as much as 80–90% of ANG II formation in the heart and is a significant source of the peptide in damaged arterial vessels (32). Because mast cell density is very high in gastrointestinal tissues and these perivascular cells play a prominent role in the inflammatory response to I/R in the small bowel (17), we sought to determine the relative contribution of chymase vs. ACE as potential sources of ANG II in posts ischemic intestine. Our results indicate that ACE inhibition with captopril completely prevented the posts ischemic increase in leukocyte rolling, without influencing stationary adhesive interactions induced by I/R. In contrast, chymase inhibition largely abolished both posts ischemic leukocyte rolling and adhesion. These results suggest that chymase-generated ANG II may play a more dominant role in inducing the cellular changes that promote stationary leukocyte adhesion, while both ACE- and chymase-dependent generation of ANG II are important for I/R-induced leukocyte rolling. It is possible that ACE may generate ANG II that is more readily accessible to circulating leukocytes while chymase-derived ANG II distributes preferentially to the vascular wall. The latter postulate may also explain the apparent discrepancies noted in the studies above (Refs. 24 and 27 vs. Ref. 25 and present study), because ACE was targeted for inhibition and only AT1 receptors were blocked in some studies (24, 27), without examination of the effect of chymase or combined AT1 + AT2 receptor blockade (present study). Differences in time of reperfusion or ANG II exposure (2 and 4 h in data from Refs. 24 and 27 vs. 1 h in Ref. 25 and present study) over which the responses were evaluated may also explain the divergent results.

In addition to catalyzing the formation of ANG II, ACE can also contribute to the formation of bradykinin, a neuropeptide that exhibits both pro- and anti-inflammatory properties, depending on its concentration (29). Since posts ischemic leukocyte rolling was inhibited by AT1 and/or AT2 receptor blockade, as well as by ACE inhibition, it is most likely that the beneficial actions of ACE inhibition are related to prevention of ANG II, rather than bradykinin formation. A significant new finding of the present study is that AT2 receptor blockade was as effective as AT1 receptor antagonism in attenuating posts ischemic leukocyte rolling, while combined blockade of both AT1 and AT2 receptors was required to prevent I/R-induced leukocyte adhesion. This observation provides the first evidence supporting a role for AT2 receptor engagement in provoking I/R-induced leukocyte-endothelial cell adhesive interactions. Our observations are consistent with the fact that exogenously applied ANG II increases leukocyte rolling and adhesion in the colon and mesentery in the absence of I/R by a mechanism that involves AT1 and AT2 receptor-dependent P-selectin expression (25, 27).

The demonstration of a proadhesive role for AT2 receptor engagement in I/R was somewhat surprising in light of several studies implicating antagonistic effects of AT1 and AT2 in vascular inflammation (4, 5, 14, 15, 18, 22). AT2 receptor activation may induce leukocyte adhesion in intestinal I/R by three possible mechanisms. The first involves the potential generation of proinflammatory reactive nitrogen oxide species formed secondary to nitric oxide (NO)-superoxide interactions initiated by AT2-dependent endothelial NO synthase activation coincident with AT1 receptor-mediated stimulation of NADPH oxidase (5, 12, 28). Another potential mechanism is suggested by the fact that neutrophils isolated from hypercholesterolemic patients and exposed to ANG II demonstrate an oxidative response that is more dependent on AT2 receptors, while those isolated from normal patients depend on AT1 receptor activation (23). Thus I/R may induce a phenotypic change in circulating neutrophils such that AT2 receptor activation contributes to the oxidative burst in these cells. A third, and perhaps more likely, mechanism is suggested by the observations that both oxidants and NO can activate capsaicin-sensitive neurons to release CGRP, a proadhesive peptide well known for its role in neurogenic inflammation (1, 31). In this regard, it is important to note that ANG II increases circulating CGRP levels and CGRP receptor expression (20, 35). Moreover, perivascular nerves, some of which may be capsaicin sensitive, express AT2 receptors (33). Our results indicating that CGRP receptor blockade attenuates leukocyte rolling and adhesion induced by I/R or exogenously applied ANG II suggest that this inflammatory peptide plays a critical role in producing these adhesive interactions.

The proinflammatory effects of AT1 receptor engagement are well known and appear to involve activation of NADPH oxidases expressed by endothelial cells, vascular smooth muscle, adventitial fibroblasts, and leukocytes during early reperfusion (30). Our work is consistent with this concept in that treatment with a specific NADPH oxidase inhibitor (apocynin) attenuated posts ischemic leukocyte rolling and adhesion, a finding that corroborates our earlier work demonstrating a similar effect with another NADPH oxidase inhibitor, PR-39 (19). In addition, AT1 receptor activation is associated with a reduction in extracellular superoxide dismutase (5), an effect that would serve to increase the cytotoxicity of oxidants produced at the cell surface. However, AT1 receptor-stimulated leukocyte adhesion occurs by a superoxide-independent mechanism when assessed after 4 h of reperfusion (24).

It is of interest to note that several groups have reported that ANG II-induced leukocyte adhesion is not confined to postcapillary venules but also occurs in arterioles when assessed after 4 h of exposure to exogenous ANG II, but not when evaluated after 1 h of exposure (3, 21). Our results are consistent with the latter observations and suggest that arteriolar adhesion requires a more prolonged exposure to ANG II than the 1-h exposure protocol used in the present study.

In summary, the results of this study demonstrate that ANG II derived from the enzymatic activity of chymase, as well as ACE, contributes to the proadhesive phenotype demonstrated by postcapillary venules after intestinal I/R. The increased numbers of rolling and adherent leukocytes that occur secondary to I/R-induced ANG II formation appear to be mediated by an AT1 and AT2 receptor-dependent mechanism that involves release of CGRP and generation of oxidants derived from NADPH oxidase.
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