BRIEF COMMUNICATIONS

Bovine and Human Endothelial Cell Production of Neutrophil Chemoattractant Activity in Response to Components of the Angiotensin System

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SUMMARY. Although there is growing evidence that angiotensin II affects macrophage-mediated inflammatory responses, it is unclear whether it can affect neutrophil-mediated inflammatory responses. Because vascular endothelial cells are capable of releasing neutrophil chemoattractant activity, we attempted to determine whether components of the angiotensin system could affect neutrophil-mediated responses indirectly by stimulating endothelial cells to release neutrophil chemoattractant substances. Cultured bovine and human endothelial cells incubated with angiotensin II released neutrophil chemoattractant activity. This activity appeared within 1 minute of exposure to angiotensin II, and was blocked by saralasin, an angiotensin II antagonist. Angiotensin I also caused release of neutrophil chemoattractant activity, but its effect required conversion to angiotensin II. Bradykinin, another substrate for angiotensin-converting enzyme, did not stimulate appearance of chemoattractant. Chemoattractant generation was not inhibited by indomethacin but was blocked by diethylcarbamazine and 5,8,11,14-eicosatetraynoic acid. This study demonstrates that angiotensin II may influence neutrophil accumulation, via production of neutrophil chemoattractant activity by vascular endothelial cells. (Circ Res 57: 898-902, 1985)

THE importance of the renin-angiotensin system in maintaining blood pressure has been well established (Gavras et al., 1974). In addition, there is growing evidence that angiotensin II affects inflammatory responses. As indirect evidence for this, components of the angiotensin system—angiotensin I, angiotensin II, and angiotensin I-converting enzyme (ACE)—have been found in hepatic granulomas induced by schistosoma eggs in mice (Weinstock and Blum, 1983). Pretreatment of mice with captopril or MK 421, structurally different ACE inhibitors, decreased granuloma size and ACE activity and angiotensin II content in schistosoma-induced granulomas (Weinstock et al., 1981, Weinstock and Blum, 1983). Similarly, pretreatment with captopril decreased the size of granulomas induced by bacillus Calmette-Guerin (BCG) in mice (Shrier et al., 1982). Also, angiotensin II directly affects the function of mononuclear inflammatory cells. For example, angiotensin II receptors have been demonstrated on human mononuclear leukocytes (Shimada and Yazaki, 1978). Macrophages isolated from granulomas induced by schistosoma eggs bear receptors for angiotensin II which are functional, as evidenced by rises in levels of intracytoplasmic cyclic adenosine monophosphate (Weinstock and Kassab, 1984). Angiotensin II exerted a concentration-dependent effect on phagocytosis by rat peritoneal macrophages (Foris et al., 1983), inhibited migration of murine peritoneal macrophages (Weinstock and Blum, 1983), and stimulated chemotaxis of human mononuclear cells (Goetzl et al., 1980).

The effects of the angiotensin system on neutrophil-mediated responses are less well characterized. Exposure to angiotensin II caused a marginal chemotactic response of human neutrophils (Goetzl et al., 1980). Because vascular endothelial cells have been demonstrated to release neutrophil chemoattractant activity (O'Brien et al., 1984, Mercandetti et al., 1984), we determined whether components of the angiotensin system could affect neutrophil-mediated inflammatory responses indirectly by stimulating vascular endothelial cells to produce neutrophil chemoattractant activity.

Methods

Bovine endothelial cells were obtained from calf aorta, primary cultures being prepared according to the method of Ryan et al. (1978). Endothelial cells were obtained by lightly scraping the luminal surface of longitudinally opened aortas. The cells were suspended in minimal essential medium (MEM; Flow Laboratories) containing 20% heat-inactivated neonatal calf serum (Biocell) and initially seeded in 25 cm² flasks (Falcon Plastics). Human endothelial cells were obtained from umbilical veins according to the method of Jaffe (Jaffe et al., 1973). Umbilical vein endothelial cells were harvested by collagenase treatment (0.2%; Worthington) for 20 minutes at 37°C, pooled in MEM with 20% heat-inactivated neonatal calf serum, 100
U/ml heparin (Sigma), and 50 μg/ml endothelial cell growth factor (Collaborative Research) and seeded in 16-mm, 24-well tissue culture plates (Costar). Cultures were incubated at 37°C in 95% air: 5% CO₂ and passed following treatment with viocose (0.25%, Gibco). Endothelial cell purity was assessed by phase microscopic "cobblestone appearance," the presence of factor VIII antigen and angiotensin-converting enzyme activity (Ryan et al., 1978). Experiments were performed with bovine endothelial cells of passage 3–12 from several different primary cell lines and bovine endothelial cells passed once.

Bovine endothelial cells were passed and grown to confluence in 35-mm plastic dishes (Costar). Confluence was assessed by phase microscopy and occurred 2–4 days after the dishes were seeded. The confluent monolayers, containing approximately 10⁴ cells, were washed with phosphate-buffered saline (PBS, pH 7.35) and incubated with 1.5 ml of MEM alone (control) or with MEM containing angiotensin I (10⁻⁵ M; Sigma) for 4 hours at 37°C in 95% air; 5% CO₂. Experimental conditions followed the same protocol except that the cells were grown to confluence in 16-mm tissue culture wells, containing approximately 10⁵ cells, and were incubated with 0.5 ml of MEM or with MEM containing 10⁻⁵ M angiotensin I. No serum was added during the 4-hour experimental period except where noted. After 4 hours, culture media were collected and centrifuged at 5000 rpm for 15 minutes. The supernatant was frozen at −70°C until chemotaxis assays were performed.

Neutrophil migration was assessed by a modified Boyden chamber assay utilizing Ficol-Hypaque (Pharmacia Fine Chemicals) enriched human neutrophils suspended at a concentration of 5–10 × 10⁵ cells/ml in Medium 199 (M-199, MA Bioproducts) containing 0.2% ovalbumin (Center et al., 1979). The target cells were separated from the putative chemoattractant substances by a 3-μm nitrocellulose filter (Sartorius) in a 48-well microwell chamber (Neuro Probe). The upper wells were filled with 50 μl of the neutrophil suspension; the lower wells with 25 μl of buffer (M-199) or endothelial cell supernatant. After 1 hour of incubation at 37°C in 95% air: 5% CO₂, the filters were fixed, stained, and mounted on glass slides. Neutrophil migration was quantified by computerized image analyzer (Optimax) by counting the number of neutrophils appearing, adherent cell counts, and chromium-51 release. Adherent cell counts from experimental monolayers were considered significant when the ratio X 100 (%) of counts in the supernatant divided by counts in the supernatant plus cell lysates (O'Brien et al., 1984). To control for the effect of angiotensin II on neutrophils, bovine endothelial cell monolayers were incubated with MEM alone, the supernatant was collected, and angiotensin II (10⁻⁵ M) was added just prior to chemotaxis assay. In other experiments, bovine and human endothelial cell monolayers were incubated with MEM alone, angiotensin II (10⁻⁵ M), or angiotensin II plus saralasin (10⁻³ M). To control for any affect on neutrophils, saralasin (10⁻³ M) was added to supernatants from angiotensin II-stimulated bovine endothelial cells before the chemotaxis assay. In addition, bovine endothelial cell monolayers were incubated with MEM alone or with MEM containing various concentrations of angiotensin II (10⁻¹ to 10⁻⁸ M). The time course of appearance of neutrophil chemoattractant activity was determined by incubating angiotensin II (10⁻³ M) with bovine endothelial cell monolayers for varying times (1, 15, 30, 60, and 240 minutes) and assaying culture supernatants for chemoattractant activity.

Because inhibitors of lipoxygenase activity prevented the release of neutrophil chemoattractant activity from cultured endothelial cells exposed to other stimuli (O'Brien et al., 1984), bovine and human endothelial cell monolayers were incubated with MEM alone, angiotensin II (10⁻⁵ M), or angiotensin II plus either the cycooxygenase inhibitor indomethacin (10⁻³ M; Sigma) or one of two lipoxygenase inhibitors, 5,8,11,14-eicosatetraynoic acid (ETYA, 10⁻⁴ M; courtesy of Hoffman LaRoche) or diethylcarbamazine (DEC, 10⁻³ M; Sigma). As controls for the effects of these agents on neutrophils, either DEC (10⁻⁴ M) or ETYA (10⁻⁴ M) was added to supernatants from angiotensin II-stimulated bovine endothelial cells before the chemotaxis assay. To ensure that the dose of indomethacin that we used inhibited prostaglandin synthesis, radioimmunoassay of 6-keto prostaglandin F₁α (6-keto-PGF₁α), the stable metabolite of prostacyclin (PGI₂), was performed according to methods previously described (Goldstein and Polgar, 1982).

Data were expressed as mean ± SEM. One-way analysis of variance, followed by Student Newman-Keuls multiple comparison test were used to compare means. Differences were considered significant when P < 0.05.

Results

Supernatants from bovine and human endothelial cell monolayers incubated with angiotensin I (10⁻⁵ M) increased neutrophil migration compared to supernatants from endothelial cells incubated with MEM alone (Table 1). Addition of either captopril (10⁻⁴ M) or saralasin (10⁻³ M) to endothelial cell monolayers blocked production of angiotensin I-stimulated neutrophil chemoattractant activity. This was not due to an effect of captopril on neutrophils,
compared to control monolayers, by phase microscopic appearance, adherent cell counts (angiotensin II: $9.3 \pm 0.8 \times 10^5$ cells, $n=5$; control: $9.0 \pm 1.1 \times 10^5$ cells, $n=5$) and chromium-51 release (angiotensin II: $7.8 \pm 1.6\%$, $n=3$; control: $8.7 \pm 0.7\%$, $n=8$). The effect of angiotensin II was not a direct effect on neutrophils, because the addition of angiotensin II ($10^{-8} \text{M}$) to control supernatants prior to chemotaxis assay did not stimulate neutrophil migration (Table 1).

Incubation of bovine and human endothelial cell monolayers with the angiotensin II antagonist, saralasin, blocked the appearance of neutrophil chemoattractant activity (Table 1). This was not due to an effect on neutrophils, as saralasin did not inhibit neutrophil migration when added to angiotensin II-stimulated supernatants just before chemotaxis assay (Table 1). Supernatants from bovine aortic endothelial cells incubated with angiotensin II at concentrations as low as $10^{-9} \text{M}$ caused an increase in neutrophil migration compared to supernatants from endothelial cells incubated with MEM alone (Fig. 1). Neutrophil chemoattractant activity was present in the supernatants of bovine endothelial cells after 1 minute of incubation with angiotensin II ($10^{-5} \text{M}$); maximal activity was present after 60 minutes (Fig. 2).

Despite inhibition of prostaglandin synthesis (6-keto-PGF$_{1\alpha}$ production: control $22.8 \pm 0.9$ ng/ml; All ($10^{-5} \text{M}$) and indomethacin ($10^{-5} \text{M}$) $1.9 \pm 0.1$ ng/ml, $n=3$), incubation of bovine and human endothelial cell monolayers with indomethacin had no effect on angiotensin II-induced appearance of neutrophil chemoattractant activity (Table 3). Incu-

![Figure 1](image-url)
bation of the monolayers with either lipooxygenase inhibitor, DEC or ETYA, blocked its appearance. This was not due to an effect on neutrophils, as neither agent inhibited neutrophil migration when added to angiotensin II-stimulated supernatants just before the chemotaxis assay (Table 3).

**Discussion**

These studies demonstrate that cultured bovine and human vascular endothelial cells incubated with angiotensin II release neutrophil chemotactic activity. Although this release occurs at a concentration of angiotensin II higher than circulating levels in vivo (Jones et al., 1984; Nussberger et al., 1984), it is possible that local concentrations of angiotensin II might attain this level. Neutrophil chemotactic activity appears within 1 minute of exposure to angiotensin II, and is blocked by saralasin, an angiotensin II antagonist. Angiotensin I also causes release of neutrophil chemotactic activity, but its effect requires conversion to angiotensin II, since the effect of angiotensin I is blocked by saralasin or by the angiotensin-converting enzyme inhibitor, captopril. Bradykinin, another substrate for ACE, did not stimulate the appearance of neutrophil chemoattractant activity when incubated with bovine endothelial cells.

We have not identified the neutrophil chemoattractant activity induced by angiotensin II. However, coincubation of bovine and human endothelial cells with angiotensin II and indomethacin in doses sufficient to inhibit cyclooxygenase activity failed to inhibit the generation of neutrophil chemoattractant activity. The lipooxygenase inhibitors, DEC and ETYA, both blocked its elaboration. Although the action of these inhibitors may not be specific, these findings suggest that the neutrophil chemoattractant activity may involve a lipooxygenase pathway of fatty acid metabolism, or another pathway affected by other agents.

This study demonstrates that angiotensin II can influence neutrophil accumulation via production of neutrophil chemoattractant activity by vascular endothelial cells, and supports the concept that endothelial cells are capable of modulating neutrophil function. The in vivo significance of this finding is unclear, but may be relevant to some of the vascular alterations found in hypertension. Granulocytes have been observed adherent to the luminal surface of the aortic endothelium and in the subendothelial space (Chobanian et al., 1984).

![Figure 2](http://circres.ahajournals.org/)

**Figure 2.** Bovine aortic endothelial cells were incubated with angiotensin II (10^{-8} M) at 37°C for varying times (1 min, 15 min, 30 min, 60 min, and 240 min). No serum was added during the experimental period. After 4 hours, culture media were collected and centrifuged at 5000 rpm for 15 minutes. The supernatant was frozen at -70°C until chemotaxis assay was performed. Twenty-five microliters of the supernatant were assessed, and the result was expressed as a percentage of the neutrophil response to buffer (M-199) alone (mean of two to four monolayers tested).

**Table 3**

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Added prior to chemotaxis assay</th>
<th>Neutrophil migration (% buffer control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM alone</td>
<td>BAEC</td>
<td>HUVEC</td>
</tr>
<tr>
<td>All</td>
<td>106 ± 9 (6)</td>
<td>118 ± 13 (4)</td>
</tr>
<tr>
<td>All + Indo</td>
<td>256 ± 19* (6)</td>
<td>306 ± 27* (4)</td>
</tr>
<tr>
<td>All + DEC</td>
<td>323 ± 67* (6)</td>
<td>328 ± 35* (4)</td>
</tr>
<tr>
<td>All + ETYA</td>
<td>78 ± 10 (6)</td>
<td>143 ± 35 (4)</td>
</tr>
<tr>
<td>All + DEC</td>
<td>96 ± 9 (6)</td>
<td>146 ± 29 (4)</td>
</tr>
<tr>
<td>All + ETYA</td>
<td>281 ± 14* (3)</td>
<td>BAEC</td>
</tr>
<tr>
<td>All</td>
<td>233 ± 21* (4)</td>
<td>BAEC</td>
</tr>
</tbody>
</table>

Data are mean ± SEM; Numbers in parentheses = number of endothelial cell monolayers tested.

*P < 0.05 compared to MEM alone. BAEC = bovine aortic endothelial cells; HUVEC = human umbilical vein endothelial cells; MEM = minimum essential media; All = angiotensin II (10^{-8} M); Indo = indomethacin (10^{-6} M); DEC = diethylcarbamazine (10^{-6} M); ETYA = 5,8,11,14-eicosatetraynoic acid (10^{-6} M).

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INDEX TERMS: cultured endothelial cells • angiotensin II • chemotaxis • neutrophils

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