Angiotensin II Increases Macrophage-mediated Modification of Low Density Lipoprotein via a Lipoxygenase-dependent Pathway*

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The molecular and cellular mechanisms by which hypertension enhances atherosclerosis are poorly understood. Angiotensin II (Ang II) has been implicated in the regulation of cellular lipoxygenases (LO), which are thought to play a role in atherogenesis by inducing oxidative modification of low density lipoprotein (LDL). We sought to test the hypothesis that Ang II would stimulate murine macrophage LO activity (which has both 12- and 15-LO activity). Competitive binding studies revealed the presence of Ang II AT1 receptors on mouse peritoneal macrophages (MPM) and J-774 cells, but not on the RAW cell line. Valsartan, a specific AT1 receptor antagonist inhibited Ang II binding, whereas PD 123319, an AT2 receptor antagonist did not. Incubation of MPM or J-774 cells with Ang II (10 pM to 1 μM) for 24 h led to a 2.5–3.5-fold increase in LO activity, measured as generated 13-HODE or 12(S)-HETE. This stimulation was inhibited by valsartan, but not by PD 123319. In contrast, Ang II did not stimulate LO activity in RAW macrophages. Semiquantitative reverse transcriptase-polymerase chain reaction showed a 2–3-fold increase in LO mRNA in MPM, but not in RAW cells after treatment with Ang II. Ang II also induced an increase in 12-LO protein. In addition, pretreatment of J-774 cells with Ang II increased in a dose-dependent manner the ability of the cells to modify LDL, resulting in greater chemoattractant activity for monocytes, typical of minimally modified LDL. This stimulation was inhibited by AT1 receptor blockade.

In summary, these data suggest that Ang II increases macrophage LO activity via AT1 receptor-mediated mechanisms and this further increases the ability of the cells to generate minimally oxidized LDL. These studies provide a link between hypertension and the associated increased atherosclerosis observed in hypertensive patients.

The importance of high blood pressure as a risk factor for developing cardiovascular disease is well established. However, it has been found that drug therapies that successfully reduce blood pressure and the incidence of stroke do not necessarily reduce the morbidity and mortality associated with coronary artery disease (1). These findings appear to indicate a multifactorial relationship between hypertension and atherosclerosis, suggesting that additional mechanisms, other than increased systemic blood pressure, contribute to the accelerated atherosclerosis associated with hypertension. Increased plasma or tissue levels of vasoactive hormones, such as Ang II, may play a role in such mechanisms, by inducing vascular cell growth in the vessel wall (2–4), stimulation of proto-oncogene expression (5, 6), and modulation of myocardial hypertrophy and fibrosis (7, 8).

For many years our laboratory has been interested in the “oxidation hypothesis” of atherosclerosis (9). Oxidation of LDL4 converts it into an atherogenic form contributing to the development of the atherosclerotic lesion. Foam cells, one of the hallmarks of atherosclerosis, develop when monocyte-derived macrophages or smooth muscle cells within the artery wall take up oxidized LDL via scavenger receptors. There is increasing evidence to suggest that certain cellular lipoxygenase (LO) enzymes are involved in this process by inducing oxidation of LDL (10, 11). Evidence supporting a causal role of human 15-LO in LDL modification during atherosclerosis includes co-localization of 15-LO protein and mRNA with epitopes of oxidized LDL in macrophage-rich areas of atherosclerotic lesions (12, 13), and the presence of stereospecific products of 15-LO activity in lesions, but not in normal arteries (14, 15). Additionally, fibroblasts transfected with human 15-LO cDNA showed an enhanced ability to seed hydroperoxides into LDL incubated with such cells (16, 17) and transfer of the 15-LO cDNA into arteries in vivo led to the appearance of oxidation-specific products that co-localized with the transduced 15-LO (18).

Ang II has been shown to up-regulate both lipoxygenase activity and expression in human smooth muscle cells (19). Murine macrophages and murine macrophage cell lines contain a “macrophage-type” lipoxygenase which possesses both 12- and 15-LO activity (20), which is highly homologous to human and rabbit 15-LO, the enzyme believed to play a role in the maturation of red blood cells and the oxidative modification of LDL (21). Accordingly, due to their dual specificity, these enzymes have been referred to as 12/15-LO (20). In contrast, not much is known about the role of vasoactive agents, such as Ang II, on macrophage function and metabolism. Although Ang II mediates some immunological responses, such as increased interferon-γ production and increased macrophage phagocytosis.

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The abbreviations used are: LDL, low density lipoprotein; MPM, mouse peritoneal macrophage(s); Ang II, angiotensin II; RAS, renin angiotensin system; BSA, bovine serum albumin; FMLP, N-formyl-L-methionyl-L-leucyl-L-phenylalanine; ETYA, eicosatetraynoic acid; 12-LO, 12-lipoxygenase; 15-LO, 15-lipoxygenase; 13-HODE, 13-(S)-hydroxyoctadecadienoic acid; 12(S)-HETE, 12-hydroxyeicosatetraenoic acid; TBARS, thiobarbituric acid-reactive substances; RT-PCR, reverse transcriptase-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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Angiotensin II-mediated LDL Modification

EXPERIMENTAL PROCEDURES

Materials—Ang II (human), FMLP, indomethacin, Tyrode’s salts, and glutaraldehyde were purchased from Sigma. [Sar1,Ile8]Ang II was from Bachem Fine Chemicals (Torrance, CA). Valsartan was a generous gift from Dr. M. de Gasparo (Novartis, Basel, Switzerland) and PD 123319 and PD 146176 were kindly provided by Pfizer-Davis (An Arbor, MI). [14C]Linoleic acid (53.0 mCi/mmol), [125I-Sar1,Ile8]Ang II (0.2 nm, specific activity, 2200 Ci/mmol), and [3H]thymidine (35 Ci/mol) were from KRN Life Science Products (Boston, MA). 13/S-Hydroxyoctadecadienoic acid (13-HODE) and eicosatetraynoic acid (ETYA) were purchased from Cayman Chemical Co. (Ann Arbor, MI). HPLC grade water and methanol were obtained from Fisher (Fair Lawn, NJ). SDS gels were obtained from Novex (San Diego, CA), the enhanced chemiluminescence reagents were from Amersham, and the polyvinylidene fluoride blotting membranes were from Millipore (Bedford, MA). The peroxidase-conjugated rabbit anti-sheep was purchased from Cappel (Aurora, OH). The sheep antibody against rabbit reticulocyte 15-LO and authentic 15-LO protein were a generous gift from Dr. J. Cornicelli.

Measurement of 15-LO Activity—Resident mouse peritoneal macrophages were harvested from C57Bl/6J mice by lavage with ice-cold phosphate-buffered saline. Cells were plated in 6-well plates (Costar, Cambridge, MA) and maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. Cells of the murine macrophage cell lines J-774 A.1 and RAW 264.7 (noted as J-774 and RAW, respectively) were maintained in 10% heat-inactivated fetal bovine serum, 100 units/ml penicilline, 100 μg/ml streptomycin, 2 mM l-glutamine. The medium for the murine leukocyte 12-LO (35). They were encoded across several exons to exclude the possibility of DNA contamination. The resulting 505-bp PCR product, except for a 102-base pair deletion (kindly supplied by Dr. H. Lukhaup, Heidelberg). The primers were chosen based on the published sequence designed with PCR buffer (50 mM Tris-HCl, pH 8.2, 10 mM dithiotreitol, 100 mM NaCl), 2.5 mM of dNTP, 10 ng/ml BSA, 30 μM RNase inhibitor (RNasin), 3 μM oligo(DT)12, and Moloney murine leukemia virus reverse transcriptase (Superscript II 200 U, Life Technologies, Inc.) in a final volume of 50 μl. The samples were reverse transcribed in a thermal cycler at 72 °C for 4 min, 42 °C for 60 min, and 94 °C for 10 min. Murine 12-LO cdNA amplification was carried out by mixing 0.1 μg of cdNA in a 25-μl volume with 0.24 μl each of the 5′- and 3′-primer for the 12-LO and 0.05 μl of Taq polymerase (Boehringer Mannheim). The primers were chosen based on the published sequence of the murine leukocyte 12-LO (35). They were encoded across several exons to exclude the possibility of DNA contamination. The resulting PCR product for 12-LO resulted in a single band of the predicted size (195 kilobases). As internal standard, GAPDH cdNA was coamplified by the addition of primers for murine GAPDH. To ensure that GAPDH amplification would not reach the plateau phase earlier than the target gene, addition of GAPDH primers was delayed, as described previously (36). GAPDH primers (0.24 μl each of 5′- and 3′-primer, 5′-CTGCACTGTCCTGGGACAGC-3′ and 3′-GGGACGGCGGG-5′) were added after completion of the fifth extension phase. PCR was carried out with a denaturing step at 94 °C for 1 min, annealing at 66 °C for 1 min, and extension at 72 °C for 1 min for 30–35 cycles. Similar PCR conditions were used for the quantitative determination of GAPDH expression by competitive PCR, which confirmed that GAPDH expression in cells was constant and not affected by Ang II. The competitor cdNA was identical to the GAPDH PCR product, except for a 102-base pair deletion (kindly supplied by Dr. H. Lukhaup, Heidelberg). Six macrophage concentrations were performed in the presence of subtype-specific antagonists. Dissociation constant (Kd) and maximum binding (Bmax) were determined using non-linear least regression curve analysis according to the equation: b = c × Bmax/e(c + Kd), where b is bound radioligand, c is the radioligand concentration, Bmax is maximum binding, and Ke is the dissociation constant.

Chemotaxis Assay—The chemotactic response of human monocytes to FMLP, Ang II, and/or LDL-containing media conditioned by exposure to cells was assessed in a 48-well chemotactic chamber (Neuroprobe, Cabin John, MD). Human mononuclear cells were isolated by Ficoll-Paque density centrifugation (29). LDL (d = 1.019–1.063 g/ml) was isolated by density gradient ultracentrifugation from pooled human plasma (29). J-774, RPMI 1640, and RAW264.7 cells were seeded in a 96-well plate. After incubation in the absence or presence of Ang II for 24 h, cells were carefully washed and incubated in the absence or presence of 250 μg/ml LDL in Ham’s F-10 for another 24 h. Superнатants of the treated cells, in the absence or presence of LDL, with and without pretreatment with Ang II, as well as native LDL alone, were plated in a lower chamber of a chemotaxis assay in the upper well. The migrates from the upper compartment by a 5-μm pore-sized polycarbonate membrane (Poretics, Livermore, CA). 5 × 105 monocytes resuspended in 0.1% BSA/Tyrode per chamber were loaded in the upper wells. The cells were then incubated for 90 min at 37 °C (5% CO2, 100% humidity). The filters were washed and cells remaining on the upper surface of the filters were removed mechanically. After fixation in 1% glutaraldehyde and staining of the filters in 0.1% crystal violet, the number of migrated cells was counted microscopically. Each experimental condition was carried out in quadruplicate, and 6 fields were examined for each well encompassing 50–1000 cells. In additional experiments Ang II antagonists, lipoxigenase and cyclooxygenase antagonists were incubated with macrophages for 6 h prior to addition of Ang II. FMLP and FMLP’s salt buffer served as positive and 0.1% BSA/Tyrode’s as negative control.

The role of secreted low-molecular weight proteins into the medium (e.g. MCP-1) induced by minimally modified LDL (31) was assessed after filtration of the cell supernatants. Cell supernatants were filtered through a 25,000 M, cut-off filter cone (Amicon, Beverly, MA) at 1000 x g for 30 min. The 25,000 molecular mass cut-off was chosen to exclude low molecular mass compounds, such as MCP-1 which has a mass of 14 kDa (32). The LDL fraction was diluted to the original LDL concentration. The filtrate and the remainder in the cone were assessed for chemotactic activity and compared with an aliquot of the supernatant prior to filtration.

Measurements of LDL Modification—The mobility of LDL was determined by agarose gel electrophoresis (33). The degree of LDL modification was also determined by the amount of thioarbituric acid-reactive substances (TBARS) (34).

RT-PCR of 12-LO mRNA—Total RNA was extracted from cultured J-774, RAW, and freshly isolated MPM treated with Ang II (RNAzol B, Tel-Test Inc., Friendswood, TX). 2.0–3.0 μg of RNA were reverse transcribed with PCR buffer (50 mM Tris-HCl, pH 8.2, 6 mM MgCl2, 10 mM dithiotreitol, 100 mM NaCl, 2.5 mM of dNTP, 10 ng/ml BSA, 30 μM RNase inhibitor (RNasin), 3 μM oligo(DT)12, and Moloney murine leukemia virus reverse transcriptase (Superscript II 200 U, Life Technologies, Inc.) in a final volume of 50 μl. The samples were reverse transcribed in a thermal cycler at 72 °C for 4 min, 42 °C for 60 min, and 94 °C for 10 min. Murine 12-LO cdNA amplification was carried out by mixing 0.1 μg of cdNA in a 25-μl volume with 0.24 μl each of the 5′- and 3′-primer for 12-LO. The 12-LO was separated from the upper compartment by a 5-μm pore-sized polycarbonate membrane (Poretics, Livermore, CA). 5 × 105 monocytes resuspended in 0.1% BSA/Tyrode per chamber were loaded in the upper wells.
compared with GAPDH as internal control (37). Each image analysis was performed twice by the same observer. Gene expression was determined in triplicate. The identity of the amplified product from MPM and J-774 was confirmed by sequence analysis of the 12-LO product purified from agarose gels (data not shown).

**Western Blot Analysis of 15-LO—** Cultured MPM were washed with phosphate-buffered saline and lysed in lysis buffer. Lysates were subjected to SDS-PAGE on 8% gels and separated proteins were transferred to polyvinylidene fluoride membranes. Blots were blocked with 5% dry milk, incubated with sheep anti-15-LO antibody, and then incubated with peroxidase-conjugated rabbit anti-sheep antibody. Immunopositive bands were visualized by enhanced chemiluminescence. Purified reticulocyte 15-LO was included as positive control; non-immune sheep IgG was used as negative control. This antibody has previously been shown to cross-react with both human and mouse 12/15-LO (38).

**Statistical Analysis—** Data are analyzed using analysis of variance (ANOVA). Student’s two-tailed, unpaired t test was used to identify the groups between which the differences were significant. Data are represented as mean ± S.E., and p < 0.05 was considered statistically significant.

**RESULTS**

**Angiotensin II Binding—** The presence of Ang II receptors and/or its receptor subtype have not been formally established on MPM or on macrophage cell lines. Therefore, we determined the parameters of Ang II binding to MPM, J-774, and RAW cell lines. Specific binding of \([^{125}\text{I}-\text{Sar}_1,\text{Ile}_8]\text{Ang II}\) to intact MPM or J-774 cells was saturable. Fig. 1A shows representative saturation isotherms in MPM and in J-774 yielding an apparent K_d value of 0.18 nM and a B_max of 0.80 fmol/mg protein in MPM and a K_d of 0.50 nM and B_max 1.20 fmol/mg protein in J-774, respectively. No specific binding could be found on RAW cells (data not shown). Ang II-specific binding on J-774 and on MPM could be dose-dependently competed by valsartan, an AT_1 receptor antagonist with an IC_{50} of 1 nM, whereas PD 123319, an AT_2 receptor blocker was not able to inhibit \([^{125}\text{I}-\text{Sar}_1,\text{Ile}_8]\text{Ang II}\) binding even at millimolar concentrations (Fig. 1B).

**Effect of Ang II on 15/12-LO Activity—** Incubation of MPM or J-774 cell lines with Ang II for 24 h induced a dose-dependent increase in 15-LO activity, as measured by the generation of 13-HODE from linoleic acid. An EC_{50} was apparent at 10 μM with a maximum response with 100 μM. Higher concentrations did not increase 15-LO activity any further (Fig. 2). In contrast, Ang II did not augment 15-LO activity in RAW cells (data not shown). The Ang II-induced increase was inhibited by 76% by 2 nM concentration of the Ang II AT_1 antagonist valsartan (Table I), similarly to its inhibition of Ang II binding (Fig. 1B). In contrast, the AT_2 receptor antagonists PD 123319 did not have any inhibitory effect (Table I). The antagonists alone had no effect on basal 15-LO activity and were not different from control (data not shown). None of the antagonists were toxic to cells in the concentration used, as evaluated by cell protein determination and trypan blue exclusion. Similarly, Ang II increased macrophage 12-LO activity. Treatment with Ang II at concentrations of 10^{-11} and 10^{-7} M for 1 h significantly stimulated the release of 12(S)-HETE, as measured by radioimmunoassay in the J-774 cell line, in a dose-dependent manner (control, 282 ± 31; Ang II, 10 ps 488 ± 85; Ang II, 100 nM, 955 ± 26 pg/mg protein, respectively).

**Effect of Ang II on 15-LO mRNA Expression in MPM, J-774, and RAW—** To determine if Ang II treatment of macrophages altered the level of 12/15-LO mRNA in MPM and in the J-774 macrophage cell line, semiquantitative RT-PCR was used to amplify the murine “macrophage-type” 12/15-LO (20). In the absence of Ang II, both murine macrophage cell lines, J-774 and RAW, as well as MPM contained the predicted 195-base pair sized product, consistent with the macrophage-type of 12/15-LO. The amplified product was confirmed to be of murine 12-LO origin by sequence analysis (data not shown). In re-
Angiotensin II (Ang II) has been shown to increase the expression of 12- and 15-LO in macrophages and fibroblasts, which are key players in the development of atherosclerosis. In this study, we aimed to investigate the effect of Ang II on 12-LO protein levels and activity in mouse peritoneal macrophages (MPM).

We observed a significant increase in 12-LO protein expression starting at 2.8-fold after a 24-h incubation with 1 nM Ang II (Fig. 3). This finding is consistent with previous studies showing that Ang II up-regulates 12-LO expression in fibroblasts transfected with human 15-LO.

12-LO activity was measured by determining the generation of 13-HODE from linoleic acid through reverse transcription polymerase chain reaction (RT-PCR) analysis (Fig. 2). 15-LO activity was assessed by the generation of 13-HODE from linoleic acid in MPM.

We found that Ang II (1 nM) increased 12-LO mRNA expression in MPM by approximately 10 pM (Fig. 5). This increase was observed after 4, 8, and 24 h of incubation, with a maximum seen after 24 h of incubation. Incubation with Ang II for 48 h, however, did not further increase LO protein levels.

Effect of Ang II on 12-LO Protein in MPM—To determine whether Ang II increased 12/15-LO activity in MPM, we measured the generation of 13-HODE from linoleic acid in MPM. Data are mean ± S.E. (n = four to nine separate experiments each performed in duplicate, all points, p < 0.001 compared with control).

Table I

<table>
<thead>
<tr>
<th>Treatment</th>
<th>12-LO activity, 13-HODE (nmol/mg cell protein)</th>
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<tbody>
<tr>
<td>Control</td>
<td>73.5 ± 25.3</td>
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<tr>
<td>Ang II</td>
<td>162.6 ± 54.0*</td>
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<tr>
<td>Ang II + antagonist</td>
<td></td>
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<tr>
<td>Valsartan (2 nM)</td>
<td>94.9 ± 36.0*</td>
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<tr>
<td>Valsartan (200 nM)</td>
<td>91.9 ± 12.5*</td>
</tr>
<tr>
<td>PD 123319 (2 nM)</td>
<td>140.3 ± 54.1</td>
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<tr>
<td>PD 123319 (200 nM)</td>
<td>288.2 ± 256.9</td>
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</table>

* p < 0.01 compared to control.

**p < 0.05 compared to Ang II alone.

GAPDH

Ang II (1 nM) increased 12-LO mRNA expression in MPM starting at the 2-h incubation time, with a maximum increase of 2.8-fold after a 24-h incubation (Fig. 3). In contrast, Ang II did not have any effect on 12-LO mRNA expression in RAW cells (data not shown).

Effect of Ang II on 12-LO Protein in MPM—To determine whether Ang II increased 12/15-LO activity in MPM through up-regulation of 12-LO protein, cells were treated for increasing periods of time with Ang II. Cell lysates were then prepared for SDS-PAGE, electroblotted onto polyvinylidene fluoride membranes, and subjected to Western blot analysis using an antibody to rabbit reticulocyte 15-LO. Membranes were then probed with antibody to rabbit reticulocyte 12-LO. Membranes were then probed with antibody to rabbit reticulocyte 12-LO.

Effect of Ang II on LDL Modification by Macrophages—We have previously shown that fibroblasts transfected with human 15-LO had an increased ability to modify LDL (16). Accordingly, we determined the Ang II-induced effect on macrophage-mediated LDL modification by two different parameters. One type involves physical properties of the conditioned LDL and the other involves altered biological behavior. For the physical properties we determined the appearance of lipid peroxidation decomposition products as measured by the TBARS assay and the effect on LDL mobility in agarose gel.

LDL incubated with J-774 showed an increase in TBARS compared with non-cell control or native LDL, but no significant difference in TBARS from LDL exposed to J-774 alone or compared with cells that had been pretreated with Ang II could be found (Table II). Of all the lipoxygenase and cyclooxygenase inhibitors used, only ETYA, an unspecific LO inhibitor (39), and PD 146167, a lipoxygenase inhibitor lacking significant antioxidant properties (40), inhibited the cell-mediated increase in TBARS, whereas the cyclooxygenase inhibitor indo-methacin had no effect (Table II).

LDL incubated with J-774 showed greater mobility than LDL incubated in the absence of cells or native LDL (data not shown). However, LDL incubated with Ang II-pretreated cells did not show any greater increase in mobility than J-774-conditioned LDL (data not shown).

Ang II-mediated Increase in Chemotaxis—It is well established that although minimally modified forms of LDL have few discernable changes in physical properties, they have altered biological characteristics (41). Therefore, we assayed for the chemotactic activity of conditioned LDL. Native LDL or LDL conditioned with and without cells for 24 h, elicited a small chemotactic response. However, pretreatment of J-774 and MPM with Ang II (10 pM to 100 nM) prior to the incubation with LDL, increased in a dose-dependent manner the chemotactic ability of the conditioned LDL. The EC50 was approximately 10 pM (Fig. 5A). In contrast, LDL from media conditioned by Ang II-treated RAW cells, did not elicit an increased chemotactic response (data not shown).

Because LDL was not reisolated in these experiments, it was possible that some or all of the chemotactic activity was due to components (e.g., low molecular weight proteins, such as

**Fig. 3. RT-PCR analysis of the effect of Ang II on 12-LO mRNA in MPM.** Cells were treated with 10 pM Ang II for different times (0, 2, 6, and 24 h). RNA was extracted using the guanidinium thiocyanate-phenol-chloroform method and reverse transcribed, as described under "Experimental Procedures." cDNA samples were amplified for 35 cycles with murine macrophage-specific 12-LO primers together with murine GAPDH primers for 29 cycles as internal standard. Data are normalized to GAPDH. The amplified 12/15-LO product is 195 kilobases, while the GAPDH product is 438 kilobases. bp, base pair.

**Fig. 4. Western blot analysis of the effect of Ang II on 12-LO protein in MPM.** Cells were treated with 1 nM Ang II for different times (0, 4, 8, and 24 h). Protein from cell lysates were subjected to SDS-PAGE under reducing conditions on 8% gels, and transferred to polyvinylidene fluoride membranes. Membranes were then probed with antibody to rabbit reticulocyte 12-LO. Lane 1, rabbit reticulocyte 15-LO; lane 2, control cells; lanes 3–5, cells were incubated with 1 nM Ang II for 4, 8, and 24 h, respectively. Lanes 6–9 show protein samples from a separate experiment. Lane 6, control cells; lanes 7–9, cells were treated with 1 nM Ang II for 4, 8, and 24 h, respectively. 0.5 mg of protein were loaded in lanes 2–5 and 1 mg in lanes 6–9.
MCP-1) in the conditioned media not associated with LDL. Therefore, the conditioned supernatants were filtered through 25,000 Mₙ cut-off filters and the filtrate and the LDL fraction assessed for chemotactic activity. 85 ± 1.0% of the recovered chemotactic activity was associated with the LDL fraction, whereas 15 ± 1.1% was found in the filtrate (n = 3, p = 0.0009). Since the filtrate was only marginally chemotactic compared with the LDL in the filter or unfiltered supernatants, it is not likely that low molecular factors contributed markedly to the chemotactic activity.

The Ang II-induced increase in chemotactic response to LDL conditioned by J-774 and MPM could be inhibited up to 77% by valsartan, the Ang II AT₁ receptor antagonist, whereas the AT₂ receptor antagonist PD 123319. Data are mean ± S.E. (n = four separate experiments each performed in quadruplicate) and are expressed as % control (10 nM FMLP in 0.1% BSA/Tyrode’s salt buffer). Statistics refer to control of LDL-containing media conditioned by macrophage cells (data are mean (% control) ± S.E. (n = 4, *, p < 0.05; **, p < 0.01). B, inhibition of the Ang II-induced effect on chemotaxis by Ang II receptor antagonists. Ang II-induced (1 nM) increase in chemotaxis is mediated via the Ang II AT₁ receptor. AT₁, AT₁ receptor antagonist valsartan; AT₂, AT₂ antagonist PD 123319. Data are mean ± S.E. from six experiments performed in quadruplicate. ***, p < 0.001 compared with control cells. #, p < 0.05; and ###, p < 0.001 compared with Ang II.

**DISCUSSION**

These studies show that Ang II stimulates macrophage-mediated modification of LDL, both in MPM and in the murine macrophage cell line J-774 through up-regulation of 12-LO mRNA, LO protein, and enzymatic activity. Both cells showed the presence of AT₁ receptors in binding studies. This ability of Ang II to increase 12/15-LO activity was shown to be mediated primarily through the AT₁ receptor, as valsartan, an AT₁ receptor antagonist substantially inhibited the increase in 12/15-LO activity and inhibited the Ang II-induced effect on LDL modification, as measured by enhanced monocyte chemotaxis, whereas PD 123319, an AT₂ antagonist, did not. Further evidence that Ang II was specifically acting through Ang II receptors to stimulate 12/15-LO activity and subsequent cell-mediated modification of LDL was provided by studies with RAW cells. These cells, which failed to show specific Ang II binding, also failed to have an increase in 15-LO activity or to modify LDL, when preincubated with Ang II, despite expressing basal levels of 12/15-LO mRNA. The concentrations of Ang II used in our studies (10⁻¹¹ to 10⁻⁷ M) are comparable to physiological plasma levels of Ang II in humans, which lie in the pico- to nanomolar range (42).

It has previously been shown that Ang II stimulates 12-LO activity, mRNA expression, and protein levels in porcine and human smooth muscle cells (19, 43). A recent study provided

**TABLE II**

<table>
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<tr>
<th>Condition</th>
<th>TBARS nmol MDA/mg LDL protein</th>
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<tr>
<td>Native LDL</td>
<td>0.66 ± 0.28</td>
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<tr>
<td>LDL without cells</td>
<td>2.93 ± 0.18</td>
</tr>
<tr>
<td>LDL + cells</td>
<td>13.67 ± 0.58</td>
</tr>
<tr>
<td>LDL + cells + Ang II</td>
<td>14.21 ± 1.29</td>
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<tr>
<td>+ ETYA 10 nM</td>
<td>14.54 ± 2.12</td>
</tr>
<tr>
<td>+ ETYA 1 µM</td>
<td>16.87 ± 1.36</td>
</tr>
<tr>
<td>+ ETYA 100 µM</td>
<td>4.31 ± 0.50</td>
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<tr>
<td>LDL + cells + Ang II</td>
<td>12.60 ± 2.07</td>
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<tr>
<td>+ PD 146176 10 nM</td>
<td>10.75 ± 4.11</td>
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<tr>
<td>+ PD 146176 100 µM</td>
<td>2.12 ± 0.30</td>
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<tr>
<td>LDL + cells + Ang II</td>
<td>13.24 ± 0.77</td>
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<tr>
<td>+ indomethacin 10 nM</td>
<td>13.84 ± 1.02</td>
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*p < 0.001 compared with cell-conditioned LDL.

**Fig. 5. Human monocyte chemotactic response to Ang II-treated J-774-conditioned LDL.** A, J-774 were pretreated with increasing concentrations of Ang II, washed three times with Ham’s F-10 medium, and incubated with LDL 250 µg/ml in F-10 for 24 h. Conditioned media were tested for chemotactic activity as described under “Experimental Procedures.” Data are mean ± S.E. (n = four separate experiments each performed in quadruplicate) and are expressed as % of control (10 nM FMLP in 0.1% BSA/Tyrode’s salt buffer). Statistics refer to control of LDL-containing media conditioned by macrophage cells (data are mean (% control) ± S.E. (n = 4, *, p < 0.05; **, p < 0.01). B, inhibition of the Ang II-induced effect on chemotaxis by Ang II receptor antagonists. Ang II-induced (1 nM) increase in chemotaxis is mediated via the Ang II AT₁ receptor. AT₁, AT₁ receptor antagonist valsartan; AT₂, AT₂ antagonist PD 123319. Data are mean ± S.E. from six experiments performed in quadruplicate. ***, p < 0.001 compared with control cells. #, p < 0.05; and ###, p < 0.001 compared with Ang II.
evidence that Ang II stimulated the ability of macrophages to oxidatively modify LDL, as measured by enhanced degrees of lipid peroxidation in the LDL (44). Since phenidone, presumably a 15-LO inhibitor, inhibited the Ang II-mediated stimulation of LDL-lipid peroxidation, it was concluded that 15-LO may be involved. However, this study did not demonstrate specific binding sites on macrophages for Ang II, nor did it demonstrate a direct action of Ang II on 12/15-LO enzyme activity, on mRNA expression or on 12/15-LO protein induction in macrophages. Additionally, very high concentrations of Ang II were used (100 nM) to stimulate the macrophages and high concentration of saralasin (100 μM), a nonspecific Ang II receptor antagonist, were required to test receptor specificity of the Ang II-induced macrophage-mediated oxidative modification of LDL (44). Our data demonstrate a dose-dependent increase in 12/15-LO activity with Ang II concentrations in the physiological range and the increase required binding of Ang II to the AT1 receptors on macrophages, as only valsoartan significantly inhibited this increased activity. RT-PCR analysis demonstrated that Ang II stimulated an increase in 12-LO mRNA, consistent with regulation at the transcriptional level, although additional post-transcriptional regulation cannot be ruled out as well. Furthermore, we show that Ang II induces LO protein in MPM. In our studies we did not find enhanced levels of lipid peroxidation in the LDL incubated with macrophages preincubated with Ang II, in contrast to the report of Keidar et al. (45). This difference could be due to the fact that we used doses of Ang II in the physiological range. Alternatively, our studies also differ in that in the studies of Keidar et al. (44) Ang II and LDL were coincubated with the macrophages, a potentially confounding variable, since the same authors have shown that Ang II-LDL complexes are more readily bound by macrophages (45). Despite these differences, our finding that Ang II, by binding to the AT1 receptor, increases macrophage-mediated modification of LDL via a 12/15-LO-dependent pathway, extends the findings of Keidar et al. (44) and proves a novel role for Ang II on macrophage metabolism.

The recent discovery of selective, high affinity nonpeptide antagonists has resulted in the identification of at least two major Ang II receptor subtypes with differential tissue distri-

![Fig. 6. Effect of Ang II on LDL modification is mediated by a lipoxygenase-dependent pathway. Cells pretreated with Ang II were incubated with 250 μg/ml LDL in the presence of various lipox-
genase inhibitors and cyclooxygenase inhibitor. Chemotaxis assay was assessed in a modified Boyden chamber, as described under “Experimental Procedures.” Each value is the mean ± S.D. from two separate experiments performed in quadruplicate (***, p < 0.001).]

ution and functional responses, namely the AT1 and the AT2 receptor (46, 47). While most of the known physiological and pathophysiological actions of Ang II (hemodynamics, aldosterone release, growth of vascular cells) have been attributed to the AT1 receptor, the role of the AT2 subtype is less clear (48). We document the presence of Ang II AT1 receptors on MPM and the J-774 cell line with an apparent Kd of 0.18 and 0.50 nM, respectively, which is in agreement with studies on macrophages isolated from murine liver granulomas following Schistosoma mansoni infection (49). The fact that only an AT1 receptor antagonist was able to compete for the Ang II binding on MPM and J-774, suggests that AT1-binding sites are present and that binding of Ang II to its receptor is required for the Ang II-induced effects. Additional support comes from the finding that RAW cells lacking the AT1 receptor, did not respond to Ang II treatment in any of the parameters measured. Because Ang II has the ability to affect many metabolic and cellular processes, these data suggest that Ang II may have an important effect on other macrophage functions as well.

To further assess whether the Ang II-induced effect on macrophages chemotaxis was due to a more extensively oxidized LDL particle or a minimally modified LDL, we used both physicochemical and biological parameters. The increase of TBARS and the electrophoretic mobility were not enhanced and suggest that the effect of Ang II on LO only induced “minimal” further modification of LDL (41) than was already induced by the macrophages themselves. The finding that the Ang II-induced stimulation of 12/15-LO resulted in no further measures of oxidative modification of the LDL, as measured by TBARS and agarose gel electrophoresis, yet resulted in LDL with enhanced chemotactic properties, is similar to recent studies from this laboratory, showing that fibroblasts overexpressing human 15-LO induce only minimal changes in lipid peroxidation in LDL, yet these LDL are bioactive (50). Further evidence that the Ang II-induced macrophage-mediated modification of LDL was due to stimulation of 12/15-LO was provided by the experiments using PD 146176, ETYA, and indomethacin. This suggests that the chemotactant activity is mediated via a lipoxygenase-dependent pathway, rather than cyclooxygenase-dependent pathway, since only PD 146176, supposedly a specific 12/15-LO inhibitor (40) and ETYA were able to reduce the Ang II-induced ability of macrophages to modify LDL chemotactant activity. The fact that Ang II stimulation of RAW cells also failed to increase LDL’s chemotactic activity further supports this hypothesis.

The precise mechanisms linking the Ang II-induced stimulation of 12/15-LO activity to the increased modification of LDL, rendering it more chemotactic for human monocytes, has not been established in these studies. One possibility is that macrophage 12/15-LO acts on endogenous lipids in the cell resulting in fatty acid hydroperoxides which are subsequently transferred to extracellular LDL. In turn, the LDL seeded with hydroperoxides may undergo subtle degrees of lipid peroxidation, giving rise to products that have chemotactic activity (16, 17, 41). Another possibility is that 12/15-LO, despite being an intracellular enzyme, acts directly on LDL making contact with the cell’s surface or that its activation is linked with the increased generation of reactive oxygen species, as suggested by Cathcart and co-workers (51). Additionally, minimally modified LDL has been shown to induce secretion of MCP-1 (31). Although the majority of the chemotactic activity was associated with the LDL fraction, it cannot be excluded that chemotactic proteins, such as MCP-1, could be bound to LDL. This possibility, however, is unlikely as MCP-1 is very hydrophilic and thus not likely to associate with LDL. Whatever the exact mechanism, it appears that activation of the 12/15-LO pathway
increases LDL’s chemotactic properties out of proportion to the extent of lipid peroxidation, at least as measured by the TBARS assay.

Despite the large body of literature accumulated around the hypothesis that lipoxigenases are involved in oxidative modification of LDL (10–13, 16, 17), the role of these enzymes in atherosclerosis in vivo are less clear. Sendobry et al. (40) have reported that administration of PD 141176 to cholesterol-fed rabbits significantly inhibited the progression of atherosclerosis. On the other hand, a recently published report suggests that macrophage-specific overexpression of human 15-LO in rabbits significantly inhibited the progression of atherosclerosis (53), but also promote atherosclerosis by stimulation of macrophage metabolism and an important link between its known role in hypertension and atherosclerosis. Thus, increased local Ang II concentrations, may exert important autocrine and paracrine functions. Consequently, it has been proposed that local synthesis of Ang II on macrophage metabolism and an important role for Ang II on macrophage overexpression of human 15-LO in rabbits does not increase atherosclerosis, and possibly even protects from lipid deposition in the arterial wall (52). Nevertheless, the finding that Ang II stimulates macrophage-mediated LDL modification in vitro via a 12/15-LO pathway and consequently enhances monocyte chemotaxis, suggests a novel role for Ang II on macrophage metabolism and an important link between its known role in hypertension and atherosclerosis. Obviously more studies in this area will be needed.

Although very little is known about local concentrations of Ang II within the vascular wall, it has been suggested that locally generated Ang II, whose levels may exceed the circulating levels, may exert important autocrine and paracrine functions. Consequently, it has been proposed that local synthesis of Ang II in cardiovascular organs, such as the vasculature may play an important role in the development of hypertension and cardiovascular diseases. Thus, increased local Ang II concentrations in the arterial wall may not only promote hypertension (53), but also promote atherosclerosis by stimulation of macrophage lipoperoxidase activity and subsequent enhanced oxidative modification of LDL, rendering it potentially more atherogenic and resulting in enhanced monocyte recruitment into the arterial wall.

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