Angiotensin II Administration to Atherosclerotic Mice Increases Macrophage Uptake of Oxidized LDL
A Possible Role for Interleukin-6

Shlomo Keidar, Ronit Heinrich, Marielle Kaplan, Tony Hayek, Michael Aviram

Abstract—The goal of the present study was to elucidate mechanisms for angiotensin II (Ang II) induction of oxidized low density lipoprotein (Ox-LDL) uptake by macrophages, the hallmark of early atherosclerosis. Compared with placebo treatment, Ang II injections (0.1 mL, $10^{-7}$ mol/L per day) for 2 weeks to apolipoprotein E–deficient mice significantly increased Ox-LDL degradation, CD36 mRNA expression, and CD36 protein expression by their peritoneal macrophages (MPMs). These effects were abolished by treatment with losartan (5 to 50 mg/kg per day) before Ang II administration. Because no such effect was obtained in vitro, the ex vivo effect of Ang II on macrophage uptake of Ox-LDL could be mediated by a factor that is not expressed at a significant level in vitro. Because Ang II stimulates cellular production of interleukin-6 (IL-6), we analyzed the possible role of IL-6 as a mediator of Ang II–mediated cellular uptake of Ox-LDL by using several approaches. First, incubations of IL-6 with MPM or IL-6 administration in mice increased macrophage Ox-LDL degradation and CD36 mRNA expression. Second, injection of IL-6 receptor antibodies in mice during Ang II treatment reduced macrophage Ox-LDL uptake and CD36 expression compared to treatment with Ang II alone. Finally, Ang II treatment of IL–6–deficient mice did not affect their MPM Ox-LDL uptake and CD36 protein levels. Thus, we conclude that a novel mechanism for Ang II atherogenicity, related to macrophage cholesterol accumulation and foam cell formation, may involve its stimulatory effect on macrophage uptake of Ox-LDL, a process mediated by IL-6.

Key Words: angiotensin ■ losartan ■ oxidized LDL ■ CD36 ■ interleukin-6 ■ macrophages ■ atherosclerosis

Oxidized LDL (Ox-LDL) plays a crucial role in the pathophysiology of atherosclerosis.1 Ox-LDL is taken up by macrophages at an increased rate via their scavenger receptors, leading to the accumulation of cellular cholesterol and oxysterols, the hallmark of early atherosclerosis.2,3 Macrophage receptors for Ox-LDL include the scavenger receptor-A and the CD36 molecules.4–6 The CD36 receptor is highly expressed in aortic foam cells7 and contributes to the enhanced macrophage uptake of Ox-LDL.

Angiotensin II (Ang II), a vasoconstrictor produced by the renin-angiotensin system, is implicated in atherosclerosis8–10 and has been identified in macrophages obtained from atherosclerotic lesions.11 Ang II significantly increases the atherosclerotic lesion area in atherosclerotic apoE-deficient (E0) mice,12 as related to the Ang II–induced macrophage cholesterol accumulation. We have previously shown that Ang II induces macrophage-mediated oxidation of LDL13 and macrophage cholesterol biosynthesis,12 both leading to macrophage cholesterol accumulation and foam cell formation. ACE inhibitors were shown to decrease the development of atherosclerosis in animal models,14 and we have demonstrated that ACE inhibitors attenuate the enhanced propensity of LDLs from hypertensive patients to lipid peroxidation.15 Moreover, the ACE inhibitors captopril, fosinopril, and ramipril inhibited LDL oxidation and attenuated the progression of atherosclerosis in E0 mice.16–18

Studies with Ang II receptor antagonists in macrophage cell lines showed that the Ang II type 1 receptor (AT1) antagonist, CV1197, significantly decreased oxidative stress induced by Ang II.19 In the mouse aorta, the AT1 antagonist losartan inhibited Ang II–induced upregulation of extracellular superoxide dismutase mRNA expression.20 Losartan also inhibited LDL oxidation and macrophage cholesterol biosynthesis12 and attenuated atherosclerosis in E0 mice.21

In human and rat vascular smooth muscle cells, Ang II stimulated interleukin-6 (IL-6) production, which was shown to be one of the mediators of the inflammatory effects of Ang II.22 Furthermore, IL–6 colocalized with Ang II in the atherosclerotic lesion in a macrophage-rich area.23

The goal of the present study was to analyze a possible mechanism for the atherosclerotic effect of Ang II, which contributes to macrophage cholesterol accumulation and foam cell formation.
Methods

Mice

E\(^{-}\) mice were created by gene targeting.\(^{24}\) In these mice, apoE deficiency causes severe hypercholesterolemia and advanced atherosclerosis by the age of 20 to 30 weeks.\(^{12-18}\) Ang II injections in E\(^{-}\) mice for 2 weeks did not affect their blood pressure (measured by tail cuff), which was 89±2 mm Hg before Ang II treatment and 93±4 mm Hg after Ang II injections.

IL-6–deficient mice (C57BL background) were obtained as a generous gift from Prof Helmut Drexler (Medizinische Hochschule, Hannover, Germany).

Ang II (Sigma Chemical Co), IL-6 (Perpo Tech Ltd), or saline (control) was injected intraperitoneally into E\(^{-}\) mice for 2 weeks. When needed, losartan (5 to 50 mg/kg per day, Merck) or anti-IL-6 receptor antibodies (1 \(\mu\)g per mouse per day, Biogenesis) were administered to the E\(^{-}\) mice 1 week before Ang II injections, followed by 2 more weeks of treatment with together Ang II.

MPM Isolation

Mouse peritoneal macrophages (MPMs) were harvested from the peritoneal fluid of E\(^{-}\) mice (2 months old) 3 days after intraperitoneal injection of 4% thioglycolate. The cells were then suspended in culture medium. 29 Degradation of Ox-LDL in the absence of cells was measured by determination of the trichloroacetic acid–soluble chloroform-insoluble radioactivity in the incubation medium. Ox-LDL protein was assayed by determination of the trichloroacetic acid–soluble chloroform-insoluble radioactivity in the incubation medium.

Lipoprotein Preparation

LDL was prepared from human plasma of fasted normolipidemic volunteers. LDL (density 1.019 to 1.063 g/mL) was prepared by discontinuous density gradient ultracentrifugation as previously described.\(^{29}\) Lipoprotein protein content was determined with the Folin phenol reagent,\(^{26}\) and LDL was radiolabeled by use of the iodine monochloride method.\(^{27}\) Ox-LDL was prepared by incubation with 10 \(\mu\)mol/L CuSO\(_4\) for 18 hours at 37°C.\(^{10}\) LDL oxidation was determined by using thiobarbituric acid reactive substances,\(^{28}\) and it ranged between 20 to 30 nmol malondialdehyde per milligram lipoprotein protein.

Macrophage Uptake of Lipoproteins

Cellular lipoprotein uptake can be assessed by the measurement of lipoprotein cellular degradation. Once the lipoproteins are taken up by macrophages, they are transferred to the lysosome, where their various components are hydrolyzed.\(^{129}\) Due to the acidic pH of the lysosome, specific lipoprotein fractions are released to the medium. This reaction represents lipoprotein influx into the macrophages. Cell-mediated degradation of Ox-LDL was measured after incubation of MPMs with [\(^{125}\)I-Ox-LDL (15 \(\mu\)g of protein per milliliter) for 4 hours at 37°C in serum-free DMEM. Cell-mediated hydrolysis of Ox-LDL protein was assayed by determination of the trichloroacetic acid–soluble chloroform-insoluble radioactivity in the incubation medium.\(^{29}\) Degradation of Ox-LDL in the absence of cells was minimal and was subtracted from the total Ox-LDL degradation. The cell layer was incubated overnight with 0.1N NaOH for determination of cell association and cell protein level.

Mouse Serum IL-6 Determination

Mouse serum was stored at –20°C until the assay. ELISA for mouse IL-6 was performed with the OptEIA Set (Pharmingen) according to the manufacturer’s instructions. The assay selectively recognizes IL-6, with a limit of detection of \(\sim 10\) pg/mL.

CD36 mRNA Expression by Semiquantitative RT-PCR Analysis

Total RNA was extracted from MPMs with Tri-reagent (Molecular Research Center, Inc). cDNA was generated from 1 \(\mu\)g of total RNA by reverse transcriptase (RT, Boehringer-Mannheim). RT products were subjected to radioactive polymerase chain reaction (PCR) amplification by using \([\(^{32}\)P]dCTP (Amersham). The forward primer used for CD36 was 5'-TGC-GGA-CTG-GCT-CAT-TGA-TAT-TTT-C-3', and the reverse primer was 5'-CCT-CGG-GGT-CCT-GAG-TTA-TAT-TTT-C-3'. Primer sequences were generously recom-
mended by A.C. Nicholson (written communication, 2000). PCR was carried out for 32 cycles (95°C for 1 minute, 55°C for 1 minute, and 72°C for 1.5 minutes). Similar conditions were used to amplify the housekeeping gene GAPDH by using the forward primer (5'-CTG-CCA-TTT-GCA-GTG-GAA-AGG-TGG-3') and the reverse primer (5'-TTG-TCA-TGG-ATC-ACC-TTG-GCC-AGG-3'). Specific PCR products obtained for CD36 (314 bp) and GAPDH (439 bp) were separated on 6% polyacrylamide gel.

CD36 Protein Quantification by FACS

MPMs were incubated with 2.5 \(\mu\)L of CD36 antibody (rabbit anti-mouse CD36, Santa Cruz) for 40 minutes at room temperature and further incubated with fluorescein isothiocyanate (FITC) goat anti-rabbit IgG (Jackson Immunoresearch Laboratories) for 20 minutes at room temperature. The cells were then washed with PBS, incubated with 150 \(\mu\)L of 0.5% formaldehyde, and kept in the dark. Fluorescence-activated cell sorting (FACS) analysis was performed with a flow cytometer (FACSCalibur, Becton Dickinson). Data were calculated by subtracting the cell autofluorescence from the fluorescence of the treated samples.

Statistical Analysis

Statistical analysis of the data were performed by using the Student \(t\) test when 2 treatments were compared and ANOVA when \(>2\) treatments were evaluated. Results are expressed as mean±SD.

Results

Effect of Ang II Injection in E\(^{0}\) Mice on Ox-LDL Uptake and CD36 mRNA Expression in Their Harvested MPMs

Injection of Ang II (0.1 mL, \(10^{-7}\) mol/L per mouse per day) for 2 weeks in E\(^{-}\) mice (2 months old) resulted in a significant increase of 28% and 33% in Ox-LDL degradation and cell association, respectively, by the MPMs compared with MPMs from placebo-treated mice (Figure 1A and 1B). Pretreatment of E\(^{-}\) mice with losartan (5 to 50 mg/kg per day) for 1 week, followed by Ang II (\(10^{-7}\) mol/L per day) injections for an additional 2 weeks, significantly reduced Ox-LDL degradation and cell association (by up to 41% and 50%, respectively) by their MPMs compared with MPMs from E\(^{-}\) mice injected only with Ang II (Figure 1A and 1B). A significant increase (60%) in CD36 mRNA levels was observed in macrophages from Ang II–injected mice compared with control mice as analyzed by densitometry (Figure 1C). Moreover, CD36 mRNA expression significantly decreased (by up to 60%) in MPMs from mice administered Ang II together with losartan (5 to 50 mg/kg per day) compared with MPMs from mice injected with Ang II alone (Figure 1C). Ox-LDL degradation, cell association, and CD36 mRNA expression obtained in E\(^{-}\) mice treated with Ang II and losartan were even lower than the basal values obtained in the control placebo-treated mice (Figure 1).

Injections of increasing concentrations of Ang II (\(10^{-10}\) to \(10^{-7}\) mol/L) led to a significant dose-dependent increase in Ox-LDL degradation and cell association by MPMs by up to 35% and 50%, respectively, compared with placebo treatment (Table). This effect was associated with a dose-dependent increase in the CD36 protein expression in MPMs from the Ang II-treated mice by up to 34%, as measured by FACS (Table).

In Vitro Effects of Ang II on Ox-LDL Degradation by MPMs

MPMs from untreated E\(^{-}\) mice were incubated with increasing Ang II concentrations (\(10^{-12}\) to \(10^{-7}\) mol/L) for 24 hours...
at 37°C, and Ang II did not significantly affect Ox-LDL macrophage degradation. Ox-LDL cellular degradation of 3146 ± 681, 3127 ± 697, 3182 ± 162, 3372 ± 227, and 3345 ± 182 ng/mg cell protein was obtained by MPMs incubated with Ang II at 0, 10⁻¹², 10⁻¹⁰, 10⁻⁸, and 10⁻⁷ mol/L, respectively.

**Effect of Ang II on IL-6 Levels in E₀ Mice Serum and in Cultured Macrophage Media**

Because no stimulatory effect of Ang II on Ox-LDL cellular uptake was obtained in vitro, we searched for a possible mediator contributing to the in vivo effect of Ang II. In view of the role played by IL-6 in the Ang II inflammatory process,22,23 we analyzed serum IL-6 levels in mice after administration of Ang II. As seen in Figure 2A, administration of Ang II at increasing concentrations (10⁻¹² to 10⁻⁷ mol/L) to E₀ mice induced a dose-dependent increase, by up to 93%, in the serum IL-6 levels compared with levels in placebo-treated mice (Figure 2A). Moreover, pretreatment of E₀ mice with losartan (5 to 50 mg/kg per day) for 1 week before Ang II administration totally abolished the stimulatory effect of Ang II on serum IL-6 levels. Mice treated with a combination of losartan plus Ang II exhibited low serum levels of IL-6 similar to those levels observed for the control placebo-treated mice (Figure 2B).

![Figure 1. Effect of Ang II combined with losartan on Ox-LDL degradation, cell association, and CD36 mRNA expression in their MPMs. E₀ mice were injected with Ang II (0.1 mL, 10⁻⁷ mol/L per day) 1 week after pretreatment without or with losartan (5 to 50 mg/kg per day), and the combined treatment was continued for an additional 2 weeks. MPMs from E₀ mice were incubated for 4 hours at 37°C with 125I-Ox-LDL (15 μg protein/mL) before cellular degradation (A) and cell association (B) assay of Ox-LDL. Total RNA extracted from E₀ MPMs was subjected to RT-PCR. Band intensity for CD36 mRNA was analyzed by PhosphorImager (Molecular Dynamics) and normalized to that of GAPDH (C). *P<0.01 vs control; **P<0.01 vs Ang II treatment (n=3).](http://atvb.ahajournals.org/)

![Figure 2. Serum IL-6 levels in E₀ mice administered Ang II. Serum from E₀ mice injected with increasing concentrations (10⁻¹² to 10⁻⁷ mol/L) of Ang II (A) or injected with Ang II (0.1 mL, 10⁻⁷ mol/L per day) 1 week after pretreatment without or with losartan at 5 to 50 mg/kg per day (B) were assayed for serum IL-6 levels by ELISA. *P<0.01 vs control; **P<0.01 vs Ang II treatment (n=3).](http://atvb.ahajournals.org/)
We also analyzed the in vitro effect of Ang II on macrophage IL-6 release to the culture medium. Incubation of increasing concentrations of Ang II (10⁻¹⁰ to 10⁻⁷ mol/L) for 18 hours with macrophages induced the release of IL-6 to the culture medium but only up to a concentration of 0.5 ng/mL. IL-6 concentrations of 0.20±0.02, 0.22±0.01, 0.27±0.03, and 0.50±0.10 ng/mL were released from macrophages treated with Ang II at 10⁻¹⁰, 10⁻⁹, 10⁻⁸, and 10⁻⁷ mol/L, respectively.

Involvement of IL-6 in Ang II Stimulation of Ox-LDL Uptake by Macrophages

Effect of IL-6 on Ox-LDL Degradation and on CD36 mRNA Expression in Macrophages

To examine the effect of IL-6 on in vitro Ox-LDL degradation, MPMs were incubated with increasing concentrations of IL-6 (2 to 100 ng/mL) for 48 hours. Ox-LDL cellular degradation rates in macrophages demonstrated a significant dose-dependent increment, up to 57%, compared with rates in control cells (Figure 3A). CD36 mRNA expression in MPMs incubated with IL-6 (10 to 50 ng/mL) increased by up to 30% compared with expression in control cells (Figure 3B and 3C).

Compared with placebo treatment, injection of IL-6 (50 to 1000 pg per mouse per day for 2 weeks) to E0 mice resulted in a dose-dependent increase (up to 26%) in Ox-LDL degradation by their MPMs. After administration of 1000 pg IL-6 per mouse per day, 3427±261 ng Ox-LDL protein per milligram cell protein was degraded by MPMs from control placebo-treated mice versus 4314±290 ng Ox-LDL protein per milligram cell protein that was degraded by MPMs from IL-6–treated mice. IL-6 administration to E0 mice significantly increased, by up to 28%, the ratio of CD36/GAPDH mRNA band intensity, and values of 0.83±0.01 optical density were noted in MPMs from control placebo-treated mice versus 1.06±0.02 optical density in MPMs from mice treated with IL-6 at 1000 pg per mouse per day.

Effect of IL-6 Receptor Antibody Injection to E0 Mice

Repeated injections of IL-6 receptor antibodies (1 µg per mouse per day) to E0 mice 1 week before and during Ang II administration (10⁻⁷ mol/L per day, for 2 weeks) reduced Ox-LDL degradation by MPMs by 15% compared with that in mice that were treated with Ang II only (Figure 4A). Similarly, compared with MPMs from mice treated with Ang II only, MPMs from mice treated with IL-6 receptor antibodies plus Ang II exhibited a significant decrease (by 20%) in CD36 expression. The ratio of CD36/GAPDH mRNA band intensity measured by densitometry decreased from
The importance of IL-6 in the effect of Ang II on Ox-LDL cellular uptake was further assessed by using IL-6–deficient mice. Injections of Ang II (10⁻⁷ mol/L per day) for 2 weeks to IL-6–deficient mice did not affect Ox-LDL uptake by their MPMs (Figure 4B), whereas when control littermate mice (C57BL) were treated with Ang II, MPM Ox-LDL uptake significantly increased (by 55%) compared with that in placebo-treated C57BL mice (Figure 4B), which was similar to the results obtained with E⁰ mice (Figure 1).

Similarly, macrophage CD36 expression was not affected by Ang II treatment compared with treatment with placebo in IL-6–deficient mice (CD36 protein expression of 75.5% and 76.4% of FITC-labeled cells in MPMs from IL-6–deficient mice treated with Ang II and untreated mice, respectively). In contrast, macrophage CD36 expression in their control littermate mice (C57BL) treated with Ang II increased by 26% compared with expression in placebo-treated C57BL mice (CD36 protein expression of 70.7% and 56.1% of FITC-labeled cells in MPMs from C57BL mice treated with Ang II and untreated mice, respectively).

**Discussion**

The present study demonstrates that Ang II injection to E⁰ mice increased Ox-LDL uptake by their peritoneal macrophages, which could partially be the result of increased cellular CD36 mRNA expression. These effects were shown to be mediated in vivo by Ang II–induced release of IL-6 and could contribute to the accelerated atherosclerosis observed in E⁰ mice.

We have recently shown that Ang II injection to E⁰ mice substantially accelerates the buildup of their atherosclerotic lesion in association with the formation of cholesterol-loaded foam cells. The present study demonstrated that Ang II increases macrophage uptake of Ox-LDL and that this phenomenon was associated with an increase in the expression of the macrophage CD36 mRNA. Ang II–induced enhanced macrophage uptake of Ox-LDL can affect macrophage cholesterol and lead to oxysterol accumulation, the hallmark of early atherogenesis.

The effect of Ang II was dose dependent and specific, inasmuch as it was abolished when the mice were pretreated with losartan, a selective AT₁ antagonist. Ox-LDL degradation, cell association, and CD36 mRNA expression obtained in E⁰ mice treated with Ang II plus losartan were even lower than the basal values obtained in the control placebo-treated mice. These results can reveal the blocking effect of losartan not only on the added Ang II but also on endogenous Ang II.

CD36 expression was shown to be modulated by oxidative stress, as induced by Ox-LDL. Because Ang II has previously been shown to induce oxidative stress mainly by activating NADPH oxidase, the pro-oxidative effects of Ang II could mediate its effect on CD36.

We have previously shown that Ang II increases Ox-LDL uptake by MPMs derived from BALB/c mice, mainly via increment in cellular proteoglycan content. In the present study, however, the effect of Ang II on the ability of E⁰ MPMs to take up Ox-LDL is associated with increased macrophage CD36 mRNA expression. This pronounced effect of Ang II on CD36 in E⁰ mice, but not in BALB/c mice, may be related to a 50% higher AT₁ mRNA expression in E⁰ mice compared with BALB/c mice (authors’ unpublished data, 2001). This latter observation is in agreement with a significant increment in AT₁ receptor mRNA expression found in aortas from hypercholesterolemic rabbits.

Serum IL-6 levels in E⁰ mice injected with Ang II rose up to 93% compared with levels in control mice. In agreement with this observation, Ang II was demonstrated to mediate transcriptional expression of IL-6 that functions as an autocrine growth factor in rat and in human vascular smooth muscle cells, as well as in cultured mouse mesangial cells. The possible involvement of IL-6 in the in vivo effect of Ang II may be supported by the finding of elevated serum IL-6 levels in patients with acute coronary syndrome and high plasma renin activity. Furthermore, in E⁰ mice, recombinant IL-6 treatment increased their lesion size 5-fold. IL-6 might play a key role in the development of coronary disease through the release of adhesion molecules by the endothelium, as well as by enhancing procoagulant activity. In the present study, IL-6 dose-dependently increased macrophage Ox-LDL degradation and CD36 mRNA expression in vitro, similar to the effects shown by Ang II. Furthermore, exposure of immortalized human brain capillary endothelial cells to IL-6 resulted in a significant increase in the cellular CD36 protein expression.

The involvement of IL-6 in the stimulatory effects of Ang II on macrophage CD36 mRNA expression and its subsequent enhanced cellular uptake of Ox-LDL were further supported, inasmuch as blocking of Ang II interaction with its receptor by the AT₁ antagonist significantly decreased IL-6 production and, in parallel, decreased CD36 mRNA expression and cellular Ox-LDL degradation. The possible involvement of IL-6 in the effect of Ang II on macrophage and CD 36 mRNA expression was also assessed by using antibodies against the IL-6 receptor or by studying the effect of Ang II injection in IL-6–deficient mice. Multiple injections of anti–IL-6 receptor antibodies to E⁰ mice, before the injection of Ang II, significantly inhibited the stimulatory effect of Ang II on MPM CD36 mRNA expression, suggesting a cause and effect relationship between Ang II and IL-6. Furthermore, Ang II treatment of IL-6–deficient mice, unlike control or E⁰ mice,
did not affect the uptake of Ox-LDL by their MPMs nor did it affect MPM CD36 protein expression.

The present study suggests that antiatherogenicity of ACE inhibitors and of Ang II receptor antagonists can be attributed, at least in part, to their reduction of macrophage cholesterol accumulation, secondary to the decrease in Ox-LDL cellular uptake. Because IL-6 mediates the above effects, inhibition of IL-6 may be an additional target for future investigation aiming at attenuation of atherosclerosis.

Acknowledgments

This study was supported by a grant from the “Niedersachsen Ministerium for Science and Arts Research Program.” The authors would like to express their deep thanks to Dr Dale Franck for his technical advice.

References

Angiotensin II Administration to Atherosclerotic Mice Increases Macrophage Uptake of Oxidized LDL: A Possible Role for Interleukin-6

Shlomo Keidar, Ronit Heinrich, Marielle Kaplan, Tony Hayek and Michael Aviram

*Arterioscler Thromb Vasc Biol.* 2001;21:1464-1469
doi: 10.1161/hq0901.095547

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/21/9/1464

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Arteriosclerosis, Thrombosis, and Vascular Biology* 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 *Arteriosclerosis, Thrombosis, and Vascular Biology* is online at:
http://atvb.ahajournals.org//subscriptions/