BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **239,** 63–67 (1997)
ARTICLE NO. RC977428

Angiotensin II Injection into Mice Increases the Uptake of Oxidized LDL by Their Macrophages via a Proteoglycan-Mediated Pathway

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Received August 18, 1997

Angiotensin II (Ang-II) has been shown to possess several atherogenic properties including its ability to induce macrophage-mediated oxidation of LDL and to form Ang-II-modified LDL which is taken up by macrophages at enhanced rate. Oxidized-LDL (Ox-LDL) is also taken up by macrophages at enhanced rate via several scavenger receptors, leading to macrophage cholesterol accumulation. In the present study we examined the effect of Ang-II on the uptake of Ox-LDL by peritoneal macrophages derived from Balb/c mice (MPM). Intraperitoneal injection of Ang-II (10^{-7}M, once daily for a period of 2 days) to the mice resulted in an increased Ox-LDL uptake up to 60%, in comparison to macrophages from placebo-treated mice. Similar results were obtained when Ang-II (10^{-7}M) was injected to the mice twice a week for a period of three months. This Ox-LDL uptake was Ang-II dose-dependent. The cellular uptake of acetylated-LDL (Ac-LDL), another ligand for scavenger receptors, however, was not affected by Ang-II injection to the mice. Furthermore, preincubation of the MPM with the monoclonal antibody, anti CD36, reduced macrophage uptake of Ox-LDL in Ang-II-treated mice by only 11%. Ang-II administration to mice resulted in a 60% increase in the macrophage cellular proteoglycan content. Chondroitinase treatment of MPM decreased Ox-LDL cellular uptake by 20% and by 38% in placebo-treated and Ang-II-treated cells, respectively. We thus conclude that Ang-II administration to mice enhances their macrophage Ox-LDL uptake via its stimulating effect on cellular proteoglycan content and this process can lead to foam cell formation and atherosclerosis.

Oxidative modification of LDL by cells of the arterial wall, especially macrophages, has been suggested to be associated with increased atherogenicity of the lipoprotein, and the presence of oxidized LDL (Ox-LDL) has been demonstrated in areas of atherosclerotic lesion (1,2). Macrophages take up Ox-LDL at an increased rate, in comparison with native LDL, and thus can promote cellular cholesterol accumulation and foam cell formation (3,4). Ox-LDL uptake by macrophages is not regulated by the macrophage cholesterol content (5) and can occur by different pathways including different macrophage scavenger receptors. The scavenger receptor family includes the subclass A and B scavenger receptors. The subclass A scavenger receptor is responsible for Ox-LDL and Ac-LDL macrophage uptake (6-8). The subclass B, which includes the CD36 receptor that can bind and take up Ox-LDL (9,10), can also recognizes other non oxidized particles (11).

Hypertensive patients with elevated plasma levels of angiotensin II (Ang-II) show a five-fold increased incidence of myocardial infarction (MI), compared with hypertensives with normal or decreased levels of Ang-II (12). Ang-II may have important effects on atherosclerotic plaque development (13), on the propensity of the atherosclerotic plaque to rupture, and on the formation of intravascular thrombosis following plaque rupture (14,15). We have demonstrated several lipidic atherogenetic properties of Ang-II (16,17), including increased macrophage lipid peroxidation, which can lead to LDL oxidation by these cells and the formation of Ang-II-modified LDL which is taken up at enhanced rate by macrophages (16,17). It was recently shown that Ang-II can induce proteoglycan (PG) synthesis in smooth muscle cells (SMC) derived from spontaneously hypertensive rat (18) and stimulation of PG synthesis by human aortic smooth muscle cells by Ang-II was also demonstrated (19). Since evidence has been accumulated that cell-surface proteoglycans (PG) may also be involved in lipoprotein uptake by macrophages (20-22), our aim was to study whether Ang-II has an effect on Ox-LDL uptake by MPM isolated from angiotensin II-treated mice and if proteoglycans are involved.

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0006-291X/97 $25.00

METHODS

1. MPM isolation and Ang-II injection. Ang-II (10^{-7}M) or saline was injected once daily into the peritoneal cavity of Balb/c mice for a period of 2 days. Mouse peritoneal macrophages (MPM) were harvested from the peritoneal fluid of Balb/c mice (weight 15-25 g), three days after intraperitoneal injection into each mouse of 3 ml 24 g/l thioglycolate in saline. The cells (20×10^6 per mouse) were pooled, treated with 3 mg 8.3 g/l Nacl (pH 7.4), and incubated for 5 minutes at 37°C to remove red blood cell contamination. The cells were then washed, suspended in DMEM containing 10% horse serum, and plated into 16 mm or 35 mm plastic dishes (23).

2. Lipoprotein preparation. Low density lipoprotein (LDL) was prepared from human plasma (drawn into 1 mM of Na_2 EDTA) from fasted normolipidemic volunteers. LDL (d=1.019-1.063 g/ml) was prepared by discontinuous density gradient ultracentrifugation as described previously (24). Then, LDL was washed at d=1063 g/ml and dialyzed against 150 mM Nacl, 1 mM Na_2 EDTA, pH 7.4. The protein content of the lipoprotein was determined by the method of Lowry et al (25). LDL was iodinated by the method of McFarlane as modified for lipoprotein (26). Ox-LDL was prepared by the incubation of the lipoprotein in the presence of 10 μM $CuSO_4$ at 37°C for 24 hours. Oxidation was terminated by refrigeration and the addition of 0.1 mmol/l EDTA. LDL oxidation was determined by malondialdehyde (MDA) analysis using the thiobarbituric acid reactive substances (TBARS) assay (27). LDL was acetylated by repeated addition of acetic anhydride to 5 mg of protein/ml of LDL diluted 1:1 (vol/vol) with saturated ammonium acetate at 4°C (28). Acetic anhydride was added at a 40-fold molar excess with regard to total lysines in LDL, and the modification was confirmed by electrophoresis on cellulose acetate at pH 8.6 in barbital (29).

3. Macrophage uptake of lipoproteins. Cellular degradation of ^{125}I lipoproteins was measured following their incubation with MPM for 4 hours at 37°C. The hydrolysis of the lipoprotein protein was assayed in the incubation medium by measurement of trichloroacetic acid soluble, noniodide radioactivity (30). (Cell-free lipoprotein degradation was minimal and was subtracted from total degradation). The cell layer was washed three times with phosphate buffered saline (PBS) incubated with 0.1N NaOH for 1 hour at room temperature, and aliquots were taken for cell protein level determination and cell-associated radioactivity. Ox-LDL binding was analyzed at 4°C after 2 hours of Ox-LDL incubation with the cells (31).

4. Glycosaminoglycan (GAG) macrophage content. MPM were scraped into PBS and homogenized. The GAG content in the homogenized macrophages was then analyzed using the 1,9-dimethylmethylene blue (DMMB) spectrophotometric assay for sulfated glycosaminoglycans (32).

5. Materials. Dulbecco's modified Eagle's medium (DMEM), Horse calf serum (HCS) and phosphate buffered saline (PBS) were purchased from Biological Industries (Beth Haemek, Israel). Angiotensin II, heparinase (Heparinase III), chondroitinase (ABC) were obtained from Sigma Chemical Co., St. Louis MO USA. Anti CD36 was purchased from Immuno Quality Products, Groninger, the Netherlands.

Statistics. The non paired student's t-test was used. Results are given as mean ± SD.

RESULTS

1. Ox-LDL Uptake by Ang-II-Treated Mouse Peritoneal Macrophages

Ox-LDL metabolism by macrophages obtained from Balb/c mice after two consecutive intraperitoneal injections of angiotensin II (10^{-7}M) was studied. Ox-LDL cellular binding was increased 2-fold in macrophages from angiotensin II-treated mice in comparison with placebo-treated mice in all concentrations studied (Fig. 1A). Ox-LDL-cell association (Fig. 1B) and Ox-LDL macrophage cellular degradation (Fig. 1C) were increased by up to 60% in angiotensin II-treated mice compared with placebo-treated mice. Similar results were obtained when Ang-II was injected to the mice twice a week for a period of 3 months (Ox-LDL [10 μg/ml] degradation was 720±92 and 1971±260 by MPM obtained from placebo and Ang-II-treated mice, respectively). This effect was angiotensin II dose-dependent. Ox-LDL (20 μg of protein/ml) degradation by macro-

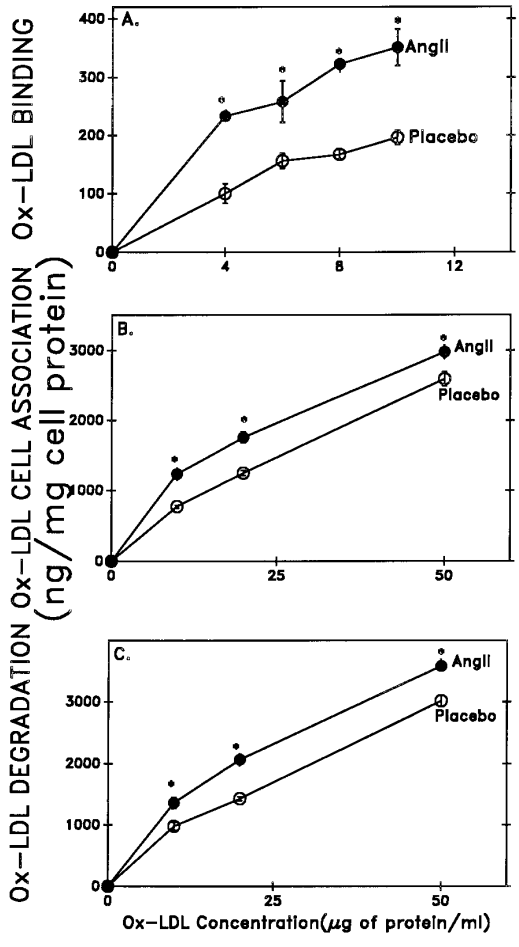

FIG. 1. The effect of Ang-II injection to mice on Ox-LDL metabolism by their harvested MPM. A. Ang-II- and placebo-treated MPM were incubated for 2 hours at 4°C with increasing concentrations of Ox-LDL. Then the cells were extensively washed in PBS and dissolved in NaOH (0.1N). The data represent the mean ± SD of 3 different experiments, each performed in triplicate ($p<0.01$). B and C show Ox-LDL cell association and degradation in Ang-II and placebo-treated cells. The cells were incubated for 4 hours at 37°C with increasing Ox-LDL concentrations. At the end of the incubation, ^{125}I-Ox-LDL degradation and cell association were determined (1B, 1C). The results represent the mean ± SD of 5 different experiments, each performed in triplicate ($p<0.01$).

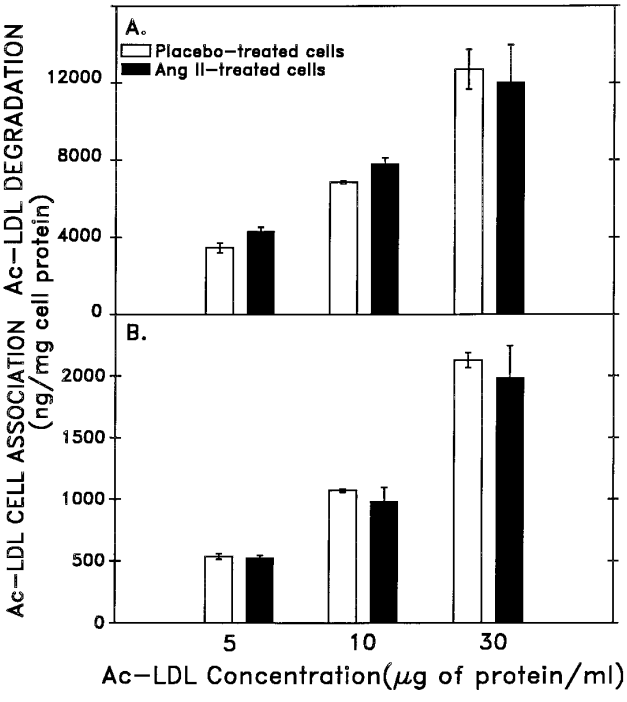

FIG. 2. The effect of Ang-II injection to mice on Ac-LDL uptake by their harvested MPM. Ang-II- and placebo-treated MPM were incubated for 4 hours with increasing concentrations of Ac-LDL. At the end of the incubation, ^{125}I-Ac-LDL degradation and cell association were determined. The results represent the mean ± SD of 3 different experiments, each performed in triplicate.

phages was 1077±55 ng/mg protein in placebo-treated mice in comparison with 1026±90, 2271±229 and 2883±407 ng/mg protein in macrophages from mice that were injected with 10^{-8}M, 10^{-7}M and 10^{-6}M Ang-II, respectively.

2. The Involvement of Proteoglycans in Ox-LDL Uptake by Mouse Peritoneal Macrophages in Angiotensin II-Treated Mice

In order to determine which receptors for Ox-LDL (SR-A and CD36) were involved in the stimulatory effect of Ang-II on Ox-LDL uptake by macrophages, cellular uptake of acetylated LDL (Ac-LDL), another ligand for SR-A, as well as Ox-LDL uptake in the presence of anti CD36 antibodies were determined. Acetylated-LDL macrophage uptake, measured as Ac-LDL degradation or cell association in mice treated with angiotensin II was similar to that obtained with cells from placebo-treated mice (Fig. 2). Next, we studied the possible involvement of the CD36 receptor in Ox-LDL cellular uptake following MPM harvesting from angiotensin II-treated mice. Preincubation (for 4 hours at 37°C) of the MPM with 10 μg/ml of anti CD36 led to only 11% decrease in Ox-LDL (10μg/ml) cellular degradation in both placebo-treated and Ang-II-treated mice (data not shown).

Finally, a possible role of the cellular proteoglycan content in Ox-LDL uptake by macrophages from Ang-II-treated mice was analyzed. Ang-II (10^{-7}M) injection into the mice led to a 60% increment in macrophage proteoglycan content in comparison with macrophages from placebo-treated mice (Fig. 3A). Upon hydrolysis of cellular glycosaminoglycans, the enzyme chondroitinase led to a 38% or 20% reduction in Ox-LDL degradation by macrophages from Ang-II- or from placebo-treated macrophages, respectively. Heparinase reduced Ox-LDL degradation by the harvested MPM by 19% in both placebo-injected and angiotensin II-treated mice (Fig. 3).

DISCUSSION

In the present study we demonstrate that Ang-II injection into mice enhances Ox-LDL uptake by their mouse peritoneal macrophages, in comparison with

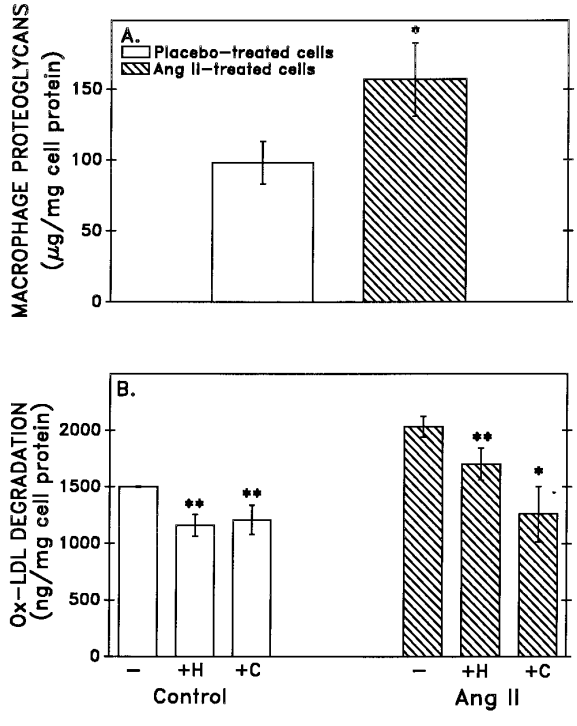

FIG. 3. PG involved in Ox-LDL uptake by MPM harvested from Ang-II-treated mice. A. PG content was determined in MPM homogenate from placebo and Ang-II injected mice. The PG content was determined as described in the Methods section. The results are expressed as the total content of PG/mg of cell protein and represent the mean ± SD of 2 different experiments, each performed in triplicate. B. The cells were preincubated for 1 hour with 0.1U/ml chondroitinase (ABC) or heparinase (III). Then ^{125}I-Ox-LDL was added for 4 hours. At the end of the incubation, ^{125}I-Ox-LDL degradation was determined. The results represent the mean ± SD of 2 different experiments, each performed in triplicate. H represents heparinase and C represents chondroitinase. – represents non treated cells (A. – $p<0.01$; B. – $p<0.05$).

MPM from placebo-treated mice. As Ac-LDL uptake by MPM from Ang-II-treated mice was not affected, it is suggested that the effect of angiotensin II in this system is specific to Ox-LDL receptors and not a general phenomenon of Ang-II on macrophage scavenger receptors. The existence of several binding sites on the surface of macrophages for Ox-LDL alone and for both Ac-LDL and Ox-LDL was demonstrated (6,11). Class A (collagen-like) scavenger receptors (SR-As), represent a binding site for Ox-LDL and Ac-LDL, whereas, class B scavenger receptors (SR-Bs) including the CD36 binding site, recognize only Ox-LDL (33). Recently, cellular proteoglycans were also shown to be involved in the uptake of the lipoprotein (23). Our results suggest that the increased Ox-LDL uptake by Ang-II-treated MPM does not occur via the subclass A scavenger receptors. Furthermore, the use of anti CD36 which blocked Ox-LDL uptake via the CD36 receptor did not significantly affect macrophage Ox-LDL uptake in both Ang-II- and placebo-treated mice. Our results, however, do not demonstrate differences between Ang-II- and placebo-treated MPM, suggesting that the CD36 receptor is not involved in the increased uptake of Ox-LDL by MPM from Ang-II-treated cells. A significant (38%) decrease in Ox-LDL degradation following 1 hour of chondroitinase incubation with MPM from Ang-II-treated mice was obtained. Similar treatment with heparinase resulted in a smaller effect (-20%). Our results thus suggest that chondroitin sulfate may have a major role in the uptake of Ox-LDL by MPM from Ang-II-treated mice. Bailey et al (18) demonstrated that chondroitin and dermatan sulfate proteoglycan increased production by Ang-II. One of the differences between our study and Bailey et al's (18) is that we work with MPM and Bailey's group works with smooth muscle cells. Recently, it was shown that Ang-II stimulates proteoglycan synthesis by human aortic SMC and produces a proteoglycan variant which increases binding ability to low density lipoprotein (19). Overall, these studies show an effect of Ang-II on cellular proteoglycan content (18,19,34).

It is possible that Ox-LDL can bind to glycosaminoglycans. This binding could occur through a positively charged domain that remains after LDL oxidation and apolipoprotein B fragmentation and that otherwise might repel the positively charged binding domain of SR-As (35). The glycosaminoglycans in Ox-LDL binding may concentrate Ox-LDL on the cell surface for presentation to the receptors. These bindings may also alter the Ox-LDL conformation to enhance receptor binding and lipoprotein uptake (35).

We conclude that Ang-II injection into mice led to increased cellular Ox-LDL uptake by their peritoneal macrophages via proteoglycan macrophage stimulation pathway. This can lead to cellular accumulation of cholesterol which leads to foam cell formation and accelerated atherosclerosis.

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