Angiotensin II Type 1 Receptor Antagonists Inhibit Basal As Well As Low-Density Lipoprotein and Platelet-Activating Factor-Stimulated Human Monocyte Chemoattractant Protein-1

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Received December 19, 2002; accepted February 24, 2003

ABSTRACT

Monocyte chemoattractant protein-1 (MCP-1) is a potent chemotactic agent for monocytes and other cells and is thought to be involved in atherosclerosis, recruiting monocytes to the subendothelial space or to the site of inflammation. Angiotensin II has been demonstrated, at least in animal models, to stimulate MCP-1 expression. We investigated the effect of the angiotensin II type 1 (AT1) receptor antagonists irbesartan and losartan on MCP-1 production by freshly isolated human monocytes. Irbesartan and losartan inhibited basal MCP-1 production in a dose-dependent manner. Low-density lipoprotein (LDL) stimulated MCP-1 in a concentration-dependent manner, with 200 µg/ml LDL protein giving a 2-fold increase in MCP-1. Irbesartan and losartan dose dependently blocked LDL-stimulated MCP-1. An angiotensin II type 2 receptor antagonist, S-(+)-1-[(4-(dimethylamino)-3-methylphenyl)methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo(4,5-c)pyridine-6-carboxylic acid (PD123319), had no significant effect on basal MCP-1 levels or LDL-stimulated MCP-1. After noting homology between the AT1 receptor and the platelet-activating factor (PAF) receptor, we showed that irbesartan inhibited both [3H]PAF binding to human monocytes and carbamyl-PAF stimulation of MCP-1. However, irbesartan affinity for the PAF receptor was 700 times less than PAF, suggesting that there may be another mechanism for irbesartan inhibition of PAF-stimulated MCP-1. This is the first report showing that AT1 receptor antagonists inhibit basal as well as LDL- and PAF-stimulated MCP-1 production in freshly isolated human monocytes.

ABBRIVIATIONS: MCP-1, monocyte chemoattractant protein-1; LDL, low-density lipoprotein; Ang II, angiotensin II; AT1, angiotensin II type 1; PAF, platelet-activating factor; AT2, angiotensin II type 2; c-PAF, carbamyl-PAF; HBSS, Hanks’ balanced salt solution; HIFCS, heat-inactivated fetal calf serum; BSA, bovine serum albumin; HSA, human serum albumin; DMSO, dimethyl sulfoxide; fMLP, N-formylmethionyl-leucyl-phenylalanine; TXA2, thromboxane A2; HUVEC, human umbilical vein endothelial cell; PD123319, S-(+)-1-[(4-(dimethylamino)-3-methylphenyl)methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo(4,5-c)pyridine-6-carboxylic acid; WEB 2086, 3-[4-[2-chlorophenyl]-9-methyl-6H-thienol-[3,2-f][1,2,4]-triazolo-[4,3-a][1,4]-diazepine-2-yl]-1-[4-morpholynil]-1-propionate; CV11974, 2-ethoxy-1-[[2'-(1H-tetrazol-5-yl)benzimidazole-4-yl)methyl]-1H-benzoimidazole-7-carboxylic acid.
Ang II has been implicated in the pathogenesis of atherosclerosis. Ang II can stimulate production of reactive oxygen species and increase expression of proinflammatory gene products, and both oxidative stress and inflammation are thought to have a role in atherosclerosis (Griendling and Alexander, 1997; Ross, 1999). Studies in experimental models of atherosclerosis have shown that inhibition of angiotensin-converting enzyme or blockade of AT1 receptors decreases atherosclerosis (Schuh et al., 1993; Keidar et al., 1997; Makaritsis et al., 1998).

Ang II has been shown to increase MCP-1 mRNA expression in cultured mononuclear U937 cells and in rat thoracic aortic vascular smooth muscle cells (Hernandez-Presa et al., 1997). Ang II can also activate MCP-1 gene transcription and stimulates MCP-1 mRNA in rat aortic smooth muscle cells (Chen et al., 1998). The increase in MCP-1 mRNA can be prevented by the AT1 receptor antagonist losartan (Chen et al., 1998).

We investigated the effect of Ang II and the AT1 receptor antagonists irbesartan and losartan on the production of MCP-1 by human monocytes. Ang II had no effect on MCP-1 levels in our study, perhaps due to its metabolism during incubation with monocytes. Irbesartan and losartan inhibited both basal and stimulated release of MCP-1 possibly through a non-AT1 receptor-related mechanism.

Platelet-activating factor (PAF) is a phospholipid with proinflammatory and thrombogenic properties, and PAF has been shown to stimulate MCP-1 production in cultured human monocytes (Sugano et al., 1994; Nakamura et al., 1995; Brennan et al., 1997). PAF and its analogs stimulate MCP-1 production in rat aortic smooth muscle cells (Chen et al., 1998). Our results suggest a mechanism for inhibition of atherosclerosis by AT1 receptor antagonists involving inhibition of basal MCP-1 levels and blockade of the effects of PAF and perhaps other structurally related molecules, resulting in decreased MCP-1 production and subsequent cell migration.

Materials and Methods

Materials. RPMI 1640 medium, penicillin/streptomycin, and fetal calf serum were purchased from Invitrogen (Carlsbad, CA). Media and other reagents were made up in sterile water (Baxter Healthcare, Old Toongabbie, NSW, Australia). The fetal calf serum was heat-inactivated before use. Ficoll-Paque and PD 10 Sephadex G-25 columns were purchased from Amersham Biosciences AB (Uppsala, Sweden). Dynabeads [M-450 CD2 (pan T)] were purchased from Dynal (Victoria, Australia). Carbamyl PAF (PAF agonist) was purchased from BIOMOL Research Laboratories (Sapphire Bioscience, St. Crows Nest, NSW, Australia). Radioactively labeled 1-

Statistics. Results are expressed as mean ± S.E.M., and data were analyzed using the paired samples t test. Data for Fig. 8 was expressed as percentage of control (100%) values before the t test because of large variability in MCP-1 levels between monocyte populations.
Results

Time Course of MCP-1 Production. Isolated human monocytes were incubated at 37°C, and cell supernatants were collected at varying time points. MCP-1 production increased over time (Fig. 1), so future incubations were carried out overnight.

Effect of Ang II and Irbesartan on MCP-1 Production by Human Monocytes. Human monocytes in HBSS were preincubated for 20 min with or without irbesartan (50 μM) and then incubated overnight with or without Ang II (10⁻⁷ M) (Fig. 2). Levels of MCP-1 produced by cells incubated with DMSO (vehicle for irbesartan) were not different from control cells incubated with medium alone. There was no significant effect of Ang II on basal MCP-1 production. Irbesartan reduced basal MCP-1 by more than 90% under these conditions.

AT1 Receptor Antagonists Dose Dependently Inhibit Basal and LDL-Stimulated MCP-1 Production. Fig. 3A shows that increasing concentrations of irbesartan resulted in increasing inhibition of basal MCP-1 production by human monocytes. At 15 μM irbesartan, MCP-1 was reduced by more than 60% (p < 0.02), whereas at 50 μM irbesartan, MCP-1 inhibition was greater than 95% (p < 0.001). A similar effect was seen with losartan, another AT1 antagonist, at concentrations 2 times higher than irbesartan (Fig. 3B).

Addition of increasing concentrations of LDL before the overnight incubation resulted in dose-dependent increases in MCP-1, with 200 μg/ml LDL protein resulting in a 2-fold increase in MCP-1 levels compared with control cells (p < 0.04) (Fig. 3A). LDL-stimulated MCP-1 levels were reduced by irbesartan in a dose-dependent manner (200 μg/ml LDL...
plus 50 μM irbesartan, p < 0.02; 100 μg/ml LDL plus 50 μM irbesartan, p < 0.02; 50 μg/ml LDL plus 50 μM irbesartan, p < 0.03) (Fig. 3A). Losartan dose dependently reduced MCP-1 levels stimulated with 200 μg/ml LDL (Fig. 3B).

**Specificity of AT1 Antagonist.** To determine whether this result was specific for AT1 receptor antagonists, the effect of an AT2 receptor antagonist, PD123319, was examined. Figure 4 shows that the AT2 antagonist had no significant effect on basal MCP-1 levels, whereas the same concentration of AT1 antagonist almost completely blocks MCP-1 production. The LDL-stimulated increase in MCP-1 was blocked by the AT1 antagonist, but the same concentration of AT2 antagonist had no significant effect. These data suggest that the effect on both basal and LDL-stimulated MCP-1 is due mainly to the AT1 type receptor antagonist.

**Possible Binding of Irbesartan to Other Receptors.** The inhibition of basal MCP-1 production by irbesartan and losartan suggested the possibility that these reagents are able to bind to cell receptors other than AT1. A previous study (Raiden et al., 1997) reported that losartan blocked the binding of [3H]fMLP to the fMLP receptor, which was found to have 25 to 30% structural homology with the AT1 receptor. The PAF receptor is in the same family of chemoattractant receptors as the fMLP receptor. A search conducted using GenBank found 22% sequence similarities between the angiotensin receptor and the PAF receptor. This raised the possibility that irbesartan and losartan may bind to the PAF receptor.

**Irbesartan Inhibits PAF Binding to Human Monocytes.** To determine whether irbesartan binds to the PAF receptor, human monocytes were preincubated with irbesartan, losartan, WEB 2086 (PAF receptor antagonist), or unlabeled PAF (nonspecific binding) before addition of [3H]PAF (Fig. 5). Irbesartan and losartan dose dependently inhibited [3H]PAF binding, indicating that they bind to the PAF receptor. Their efficacy was similar to the PAF antagonist WEB 2086 in our study. PAF is very hydrophobic and exhibits high nonspecific binding to the lipid bilayer of plasma membranes (Chao and Olson, 1993), which may account for some of the uninhibitable [3H]PAF binding.

**Competitive Binding.** Varying concentrations of [3H]PAF were added to human monocytes to determine binding parameters (Fig. 6). Data were analyzed using nonlinear regression in GraphPad Prism 3 to obtain B max (36,280) and K D (41 nM) values for [3H]PAF.

Competitive binding experiments were carried out using...
Irbesartan and unlabeled PAF (Fig. 7), and the IC$_{50}$ value (inhibitory concentration 50%) determined. $K_i$ (affinity of PAF receptor) was calculated from $K_D$ and IC$_{50}$ values using Prism. Using five different monocyte preparations, unlabeled PAF gave a mean IC$_{50}$ value of 49.0 ± 2.1 μM and a $K_i$ value of 43.1 ± 3.9 × 10$^{-6}$ M. Irbesartan gave a mean IC$_{50}$ value of 5.8 ± 1.5 × 10$^{-8}$ M. These results indicated that irbesartan had 700 to 800 times lower affinity for the PAF receptor than unlabeled PAF.

Irbesartan Inhibits PAF-Stimulated MCP-1 Production. Human monocytes were preincubated with irbesartan, losartan, or the PAF antagonist WEB 2086, and then incubated overnight with the stable PAF agonist carbamyl-PAF, and supernatant MCP-1 levels were measured. The stable metabolite of PAF was required for stimulation of MCP-1, probably because native PAF is degraded during incubation with cells. This experiment was carried out in HBSS (Fig. 8A) and RPMI 1640 medium containing 1% HIFCS, a more physiological medium (Fig. 8B). In serum-free HBSS (Fig. 8A), irbesartan dose dependently inhibited basal (control plus irbesartan, $p < 0.002$ for all irbesartan concentrations) and carbamyl-PAF (c-PAF)-stimulated (control plus c-PAF, $p < 0.03$; c-PAF plus irbesartan, $p < 0.002$ for all irbesartan concentrations) MCP-1 production. WEB 2086 also dose dependently inhibited basal (control plus WEB 2086, $p < 0.05$ for all WEB 2086 concentrations) and c-PAF-stimulated (c-PAF plus 20 or 50 μM WEB 2086, $p < 0.009$) MCP-1 release, but was less effective than irbesartan.

Carbamyl-PAF stimulated monocyte MCP-1 production in a dose-dependent manner (Fig. 8B) (control plus c-PAF 1 μM, $p < 0.04$). Figure 8B shows that in RPMI 1640 medium containing 1% HIFCS, irbesartan, and losartan inhibited c-PAF MCP-1 stimulation, similar to the PAF antagonist WEB 2086 (c-PAF 1 μM plus irbesartan, $p < 0.04$; plus losartan, $p < 0.09$; plus WEB 2086, $p < 0.04$) (c-PAF 0.6 μM plus irbesartan, $p < 0.05$; plus losartan, $p < 0.04$; plus WEB 2086, $p < 0.07$). Basal MCP-1 levels were significantly reduced in the presence of irbesartan, losartan, and WEB 2086 (control plus irbesartan, $p < 0.001$; plus losartan, $p < 0.001$; plus WEB 2086, $p < 0.002$). Thus, the receptor or mechanism involved in the inhibition of human monocyte MCP-1 is sensitive to LDL, PAF, AT1 antagonists, and PAF antagonists.

**PAF Receptor Antagonist Inhibits Basal and LDL-Stimulated MCP-1 Production.** Fig. 9 shows that increasing concentrations of WEB 2086 resulted in increasing inhibition of basal MCP-1 production. At 10 μM WEB 2086, there was a 20% reduction ($p < 0.05$), whereas 50 μM WEB 2086
resulted in a 60% decrease ($p < 0.009$) in basal MCP-1. Irbesartan was more effective, with 10 µM irbesartan reducing basal MCP-1 levels by 40% ($p < 0.05$), 20 µM giving 75% reduction ($p < 0.02$), and 50 µM reducing levels by greater than 95% ($p < 0.008$). LDL (200 µg/ml) significantly increased MCP-1 production 2-fold ($p < 0.01$). WEB 2086 inhibited LDL-stimulated MCP-1 levels by 30% ($p < 0.02$) at 20 µM and by 50% ($p < 0.02$) at 50 µM. Irbesartan was again more effective, reducing LDL-stimulated MCP-1 by 50% ($p < 0.02$) at 10 µM, 70% ($p < 0.001$) at 20 µM, and greater than 95% ($p < 0.002$) at a concentration of 50 µM.

**Discussion**

Recent studies suggest that AT1 receptor antagonists used in the treatment of hypertension may also be beneficial in the treatment of atherosclerosis. Lesion development in Apo E-deficient mice was significantly reduced by treatment with the AT1 antagonists irbesartan (Dol et al., 2001) and losartan (Keidar et al., 1997). Irbesartan-treated mice had less macrophages in the lesion area, suggesting irbesartan inhibits monocyte/macrophage influx into the vessel wall. Irbesartan treatment also decreased MCP-1 mRNA levels and MCP-1 immunostaining in the lesion area (Dol et al., 2001). Treatment of patients with coronary artery disease with irbesartan reduced levels of the inflammatory markers vascular cell adhesion molecule-1, tumor necrosis factor-α, and superoxide (Navalkar et al., 2001). Lipid peroxidation, superoxide levels, and monocyte-binding capacity were reduced in subjects with coronary artery disease receiving irbesartan (Khan et al., 2001).

Our in vitro studies with freshly isolated human monocytes suggest that AT1 receptor antagonists may inhibit the inflammatory component of atherosclerosis. We found that irbesartan and losartan inhibited basal production of the inflammatory marker MCP-1 by human monocytes in the absence of any stimulant. Yanagitani et al. (1999) showed that the AT1 antagonist CV11974 decreased basal levels of peroxide production in macrophages. This may be related to our findings, because reactive oxygen species, which include peroxide and superoxide, are involved in MCP-1 production (De Keulenaer et al., 2000).

Ang II stimulated MCP-1 mRNA in the cultured monocytic cell line U937 and in cultured rat thoracic aortic vascular smooth muscle cells (Hernandez-Presa et al., 1997). In rat aortic smooth muscle cells, Ang II stimulated MCP-1 mRNA and the increase was prevented by losartan (Chen et al., 1998). We found that in freshly isolated human monocytes, Ang II had no effect on MCP-1 protein levels, whereas the AT1 receptor antagonists irbesartan and losartan inhibited basal MCP-1. It is possible that the absence of an effect of Ang II was caused by its degradation during the 20-h incubation with cells. It may be necessary to use a stable derivative of Ang II to see an effect under these conditions. It is also possible that there was basal release of Ang II by the cells and that MCP-1 stimulation by basal Ang II was inhibited by irbesartan and losartan. These hypotheses were not examined in our study. Our results suggested the possibility that inhibition of basal MCP-1 was independent of the AT1 receptor. Raiden et al. (1997) reported that the AT1 receptor antagonist losartan inhibited neutrophil recruitment and activation by fMLP, by inhibiting neutrophil binding of FMLP through a mechanism independent of losartan binding to AT1 receptors. The AT1 receptor and the high-affinity receptor for fMLP share 25 to 30% sequence identity.

The receptors for Ang II, fMLP, PAF, complement protein fragment 5a, and the chemokines belong to the family of seven-transmembrane-domain rhodopsin-like G protein-coupled receptors (Murphy, 1994). On searching GenBank, we found that the AT1 receptor has 22% homology with the PAF receptor. Raiden et al. (1997) reported that the AT1 receptor inhibitor losartan inhibited neutrophil recruitment and activation by fMLP, by inhibiting neutrophil binding of FMLP through a mechanism independent of losartan binding to AT1 receptors. The AT1 receptor and the high-affinity receptor for fMLP share 25 to 30% sequence identity.

**Fig. 9.** PAF receptor antagonist inhibits basal and LDL-stimulated MCP-1. Monocytes were isolated as described under Materials and Methods, resuspended in HBSS, and preincubated for 20 min with 0, 10, 20, or 50 µM irbesartan or WEB 2086. Medium or LDL (200 µg/ml final concentration) was added, and the cells were incubated overnight. Results are mean ± S.E. from three experiments.
concentrations of around 10 μM irbesartan (Pool et al., 1998). Our results suggest that this concentration of irbesartan inhibits basal and LDL-stimulated MCP-1 production from monocytes (Figs. 3A and 9). Sugano et al. (2001) showed that 10 nM carboxyl-PAF stimulated MCP-1 in cultured human uterine cervical fibroblasts, and this stimulation of MCP-1 was abolished by coinubcation with 10 μM (1000-fold excess) WEB 2170, a PAF receptor antagonist, in medium containing 0.1% BSA. We showed that 1 μM carboxyl-PAF stimulated MCP-1 production in human monocytes. This stimulation was inhibited by 50 μM (50-fold excess) WEB 2086, a PAF receptor antagonist, and by 50 μM of the AT1 receptor antagonist irbesartan (Fig. 8B), in medium containing 1% serum, a more physiological environment with more potentially complicating factors than 0.1% BSA.

The involvement of PAF in atherosclerosis was suggested in a study where the PAF receptor antagonist WEB 2086 inhibited fatty streak development in LDL receptor null mice fed a western diet (Subbanagounder et al., 1999). They found that the in vitro inhibitory effects of WEB 2086 on monocyte binding to endothelial cells did not seem to be due to blocking the PAF receptor. Similarly, in our experiments, the inhibitory effects of losartan, and WEB 2086 on carboxyl-PAF stimulation of MCP-1 may not be due entirely to blocking the PAF receptor.

Ether- and ester-containing PAF-like lipids are generated during oxidation of LDL (Tokumura et al., 1996), and PAF and PAF-like oxidized phospholipids have been shown to activate monocytes via the PAF receptor (Heery et al., 1995; Tokumura et al., 1996; Lehr et al., 1997). Hayek et al. (2000) have shown that the AT1 antagonist losartan significantly reduced human monocyte-derived macrophage uptake of oxidized LDL and also decreased CD63 (an ox LDL receptor) mRNA expression. These reports raise the possibility that LDL incubated overnight with monocytes, as in our study, becomes oxidized and PAF-like lipids are generated and stimulate MCP-1 production. LDL may not be working exclusively through the PAF receptor because the PAF receptor antagonist did not completely inhibit LDL stimulated MCP-1 (Fig. 9), although it blocked PAF-stimulated MCP-1 (Fig. 8A). It is possible that other PAF receptor antagonists with different binding characteristics may show greater inhibition.

Our study shows that the AT1 receptor antagonists irbesartan and losartan decreased basal MCP-1 levels in human monocytes possibly through a mechanism independent of binding to the AT1 receptor. LDL-stimulated and carboxyl-PAF-stimulated MCP-1 levels in human monocytes were reduced by these AT1 receptor antagonists (Figs. 3, 8, and 9) through a mechanism partially independent of binding to the PAF receptor. Another possible mechanism of action is reduction of MCP-1 levels by nitric oxide. It has been shown (Kalinowski et al., 2002) that AT1 receptor antagonists stimulate nitric oxide release in rat platelets and HUVECs. Increased nitric oxide release in the presence of AT1 receptor antagonists may decrease MCP-1 levels because nitric oxide has been shown to inhibit MCP-1 production (Zeier et al., 1995; Tsao et al., 1997). Li et al. (2000) found that a nitric-oxide synthase inhibitor had no effect on inhibition of TXA2-induced vasoconstriction by irbesartan. However, the effect of a nitric-oxide synthase inhibitor on the reduction of monocyte MCP-1 production by irbesartan has not been studied.

Because PAF and Ang II may both be involved in atherosclerosis, AT1 receptor antagonists may inhibit atherosclerosis through more than one pathway; blocking AT1 receptors and the effects of Ang II, as well as inhibiting the effects of PAF or PAF-like lipids through some as yet unidentified mechanism. We have shown that the AT1 receptor antagonists irbesartan and losartan inhibit basal as well as LDL- and PAF-stimulated MCP-1 production by human monocytes, important inflammatory mediators in atherosclerosis. At athereogenic sites, LDL and/or LDL oxidation products as well as PAF are likely to be present. AT1 receptor antagonists may reduce the levels of MCP-1 at these sites by inhibiting basal release of MCP-1 and by blocking the stimulation of MCP-1 production by these molecules. Lower levels of MCP-1 may result in less circulating monocytes entering the vessel wall. In this way, AT1 receptor antagonists may inhibit the inflammatory component of atherosclerosis. Further work is required to determine the mechanism of the anti-inflammatory effect of AT1 receptor antagonists.

Acknowledgments

We thank Kitya Dufall for excellent technical assistance.

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