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Angiotensin II Type 1 Receptor Antagonists Inhibit Basal as well as LDL- and Platelet Activating Factor- Stimulated Human Monocyte Chemoattractant Protein-1

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Non-standard abbreviations

MCP-1, monocyte chemoattractant protein-1; Ang II, angiotensin II; AT1, angiotensin II Type 1; LDL, low density lipoprotein; AT2, angiotensin II Type 2; PAF, platelet activating factor; ROS, reactive oxygen species; ACE, angiotensin converting enzyme; HBSS, Hank's balanced salt solution; HIFCS, heat-inactivated fetal calf serum; HSA, human serum albumin; fMLP, N-formylmethionyl-leucyl-phenylalanine; VCAM-1, vascular cell-adhesion molecule 1; TNF-alpha, tumor necrosis factor-alpha; C5a, complement protein fragment 5a; TXA_2, thromboxane A_2; HUVEC, human umbilical vein endothelial cells; BSA, bovine serum albumin; NO, nitric oxide.

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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 the site of inflammation. Angiotensin II (Ang 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 200μg/ml LDL protein giving a two fold increase in MCP-1. Irbesartan and Losartan dose-dependently blocked LDL-stimulated MCP-1. An AT2 (type 2) receptor antagonist (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 ^{3}H-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.

Chemotactic cytokines or chemokines are structurally related small proteins involved in trafficking and activation of leukocytes (Baggiolini, 1998). The chemokine monocyte chemoattractant protein-1 (MCP-1) is highly expressed in human atherosclerotic lesions, and is thought to be important in monocyte recruitment into the arterial wall and developing lesions (Nelken et al., 1991;Yla-Herttuala et al., 1991). The development of atherosclerosis has been associated with numerous risk factors, including elevated levels of plasma cholesterol (particularly low density lipoprotein (LDL)), hypertension, diabetes and smoking (Ross, 1993). High concentrations of plasma LDL lead to higher concentrations in the sub-endothelial space where LDL can become oxidatively modified. Oxidised LDL may injure the endothelium and play a role in the migration of leukocytes into the vascular wall (Ross, 1993). Oxidised LDL exposure increased MCP-1 mRNA expression and MCP-1 protein levels in rabbit macrophages (Wang et al., 1997). LDL, minimally modified by oxidation, increased MCP-1 mRNA expression in human endothelial cells and smooth muscle cells (Cushing et al., 1990).

Monocyte/macrophages make up the bulk of infiltrated leukocytes in atherosclerotic plaque and are considered to be the main inflammatory mediators in atherosclerosis. Numerous studies suggest that MCP-1 has an important role in the infiltration of monocytes into lesions (Nelken et al., 1991; Yla-Herttuala et al., 1991;Yu et al., 1992). Mice lacking MCP-1 exhibited attenuated atherosclerosis and monocyte accumulation in the artery (Gu et al., 1998). Deletion of the receptor for MCP-1 resulted in decreased lesion formation (Boring et al., 1998).

Ang II has been implicated in the pathogenesis of atherosclerosis. Ang II can stimulate production of reactive oxygen species (ROS) and increase expression of proinflammatory gene products, and both oxidative stress and inflammation are thought to have a role in atherogenesis (Griendling and Alexander, 1997;Ross, 1999). Studies in experimental models

of atherosclerosis have shown that inhibition of ACE (angiotensin converting enzyme) or blockade of AT1 receptors decreases atherosclerosis (Keidar et al., 1997;Makaritsis et al., 1998;Schuh et al., 1993).

Ang II has been shown to increase MCP-1 mRNA expression in cultured monocytic 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.

PAF (platelet activating factor) is a phospholipid with proinflammatory and thrombogenic properties and PAF has been shown to stimulate MCP-1 production (Sugano et al., 2001). We studied the effect of the AT1 receptor antagonists, Irbesartan and Losartan, on ^{3}H-PAF binding and carbamyl-PAF stimulation of MCP-1 in human monocytes.

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.

METHODS

Materials

RPMI 1640 media, penicillin/streptomycin and fetal calf serum were purchased from GibcoBRL, Life Technologies, NY, USA. Media and other reagents were made up in sterile water (Baxter Healthcare, Australia). The fetal calf serum was heat-inactivated prior to use. Ficoll-Paque and PD 10 Sephadex G-25 columns were purchased from Amersham Pharmacia Biotech, Uppsala, Sweden. Dynabeads (M-450 CD2 (pan T)) were purchased from Dynal Australia. Carbamyl PAF (PAF agonist) was purchased from Biomol Research Labs, Sapphire Bioscience, Australia. Radioactively labelled PAF (1-O-[^{3}H]Octadecyl)[1-O-[^{3}H]Octadecyl-2-acetyl-sn-glycero-3-phosphocholine] (6.03 TBq/mmol) was purchased from Amersham Pharmacia Biotech UK. The AT2 receptor antagonist PD123319 was bought from Research Biochemicals International, MA, USA. WEB 2086 (PAF antagonist) was from Boehringer Ingelheim. Irbesartan was a gift from Bristol-Myers Squibb, NJ, USA. Losartan was a gift from Merck Sharp and Dohme, Australia. All other reagents were purchased from Sigma Chemical Co., MO, USA.

Cells

Human peripheral blood mononuclear cells were prepared by centrifuging blood (containing 1mg/ml EDTA) from healthy volunteers, on Ficoll-Paque at 500*g* for 30min at 20^{o}C. Cells in the resultant interface layer were further purified by washing with centrifuged plasma and then with Hank's balanced salt solution (HBSS) without calcium or magnesium, centrifuging at 100*g* for 10 min at 4^{o}C to remove platelets. The cells were counted and CD2 Dynabeads added to remove T-cells, according to the manufacturer's instructions. This resulted in a cell population enriched in monocytes (60% CD14+) and limited possible pre-experimental activation of cells if adherence had been used for purification. The cells were counted and

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viability assessed with trypan blue. Cells were resuspended in HBSS or in RPMI 1640 containing 2% penicillin-streptomycin and 1% heat-inactivated fetal calf serum (HIFCS), prior to plating in Falcon 24-well culture plates (Becton-Dickinson, NJ, USA) at 1-2x10^6 per well (0.8-1.0ml per well). Reagents were added and the cells incubated overnight at 37^{o}C in 5% CO_2. The following day, the medium was removed, centrifuged to pellet any cells, the supernatant stored at –80^{o}C and cell viability assessed using trypan blue.

LDL preparation

Blood from healthy volunteers was collected into EDTA (1mg/ml). LDL was isolated immediately from fresh plasma by density gradient ultracentrifugation as previously described (Croft et al., 1991). Briefly, plasma density was increased to 1.07 by addition of NaCl and then a four-step gradient was constructed over the plasma using the following densities (kg/l): 1.8ml 1.063 NaCl, 1.8ml 1.04 NaCl, 1.8ml 1.02 NaCl and 2.1ml water. Samples were ultracentrifuged at 205,000*g* (average) for 20 h using a Centrikon T-1190 Ultracentrifuge (Kontron Instruments, Milano, Italy). The LDL band was collected by aspiration and passed through a Pharmacia PD10 Sephadex column to remove the excess salt and the majority of the EDTA. The LDL was stored in the dark at 4^{o}C. LDL protein was determined by a modification of the Lowry method (Markwell et al., 1978) using Sigma BSA protein standard. Just prior to use, isolated LDL was passed through a second PD10 column to remove the remaining EDTA.

Assay for MCP-1

Human MCP-1 OptEIA (Pharmingen, CA, USA) and Costar EIA/RIA ½ area flat bottom 96 well plates (Corning Inc, NY, USA) were used to measure MCP-1 levels in cell supernatants. The sensitivity of the assay was 30pg/ml, the intra-assay coefficient of variation was 10% and the inter-assay coefficient of variation was 12%.

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PAF binding assay

Cells were washed twice with cold Hepes-Tyrode's buffer (Ishii et al., 1997) containing 0.25%w/v fatty acid-free human serum albumin (HSA) and kept on ice. Cells (1-$2x10^6$/200µl/tube) were pre-incubated for 5 min with (non-specific binding) or without (total binding) unlabelled PAF (10µM) or test reagents prior to addition of ^{3}H-PAF (4nM final) and incubation on ice for 10min. Cells were then washed three times with the cold buffer and solubilized with 1% TritonX-100. Radioactivity associated with the cells was measured with liquid scintillation counting. Separate experiments were carried out using varying concentrations of ^{3}H-PAF and saturation binding data analyzed with nonlinear regression using GraphPad Prism 3. Competitive binding experiments were carried out measuring the binding of 4nM ^{3}H-PAF in the presence of varying concentrations of irbesartan and unlabelled PAF and IC_{50} (inhibitory concentration 50%) concentrations determined for each ligand using GraphPad Prism3.

Searching GenBank

GenBank was searched using human AT1 receptor GenBank Accession Number AAB34644.

Statistics

Results are expressed as mean ± standard error of the mean (SEM) and data analyzed using the paired samples t-test. Data for figure 8 was expressed as percent of control (100%) values prior to 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^{o}C and cell supernatants 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 pre-incubated for 20 min with or without Irbesartan (50μM) 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 to 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 15μM Irbesartan, MCP-1 was reduced by more than 60% ($p<0.02$), while at 50μM Irbesartan, MCP-1 inhibition was greater than 95% ($p=0.001$). A similar effect was seen with Losartan, another AT1 antagonist, at concentrations two times higher than Irbesartan (Fig.3B).

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

Irbesartan $p<0.03$) (Fig.3A). Losartan dose-dependently reduced MCP-1 levels stimulated with 200μg/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. Fig.4 shows that the AT2 antagonist had no significant effect on basal MCP-1 levels, while 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. This data suggests 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 ^{3}H-fMLP to the fMLP receptor, which was found to have 25-30% structural homology with the AT1 receptor. The platelet activating factor (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 pre-incubated with Irbesartan, Losartan, WEB 2086 (PAF receptor antagonist) or unlabelled PAF (non-specific binding) prior to addition of ^{3}H-PAF (Fig. 5). Irbesartan and Losartan dose-dependently inhibited ^{3}H-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 non-specific binding to the lipid bilayer of plasma membranes (Chao and Olson, 1993), which may account for some of the uninhibitable ^{3}H-PAF binding.

Competitive binding

Varying concentrations of ^{3}H-PAF were added to human monocytes to determine binding parameters (Fig. 6). Data was analyzed using nonlinear regression in GraphPad Prism 3 to obtain B_{max} (36280) and K_D (41nM) for ^{3}H-PAF.

Competitive binding experiments were carried out using Irbesartan and unlabelled PAF (Fig. 7), and IC_{50} (inhibitory concentration 50%) determined. K_i (affinity of PAF receptor) was calculated from K_D and IC_{50} using Prism. Using five different monocyte preparations, unlabelled PAF gave mean IC_{50} 6.4±1.6 x10^{-8}M and K_i 5.8±1.5 x10^{-8}M. Irbesartan gave mean IC_{50} 49.0±4.3 x10^{-6}M and Ki 43.1±3.9 x10^{-6}M. These results indicated that Irbesartan had 700-800 times lower affinity for the PAF receptor than unlabelled PAF.

Irbesartan inhibits PAF-stimulated MCP-1 production

Human monocytes were pre-incubated with Irbesartan, Losartan or the PAF antagonist WEB 2086, then incubated overnight with the stable PAF agonist, carbamyl-PAF, and supernatant MCP-1 levels 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 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 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 20μM or 50μM 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 1μM $p<0.04$). Fig. 8B shows that in RPMI containing1%HIFCS, Irbesartan and Losartan inhibited c-PAF MCP-1 stimulation, similar to the PAF antagonist WEB 2086 (c-PAF 1μM plus Irbesartan $p<0.04$; plus Losartan $p<0.09$; plus WEB2086 $p<0.04$) (c-PAF 0.6μM plus Irbesartan $p<0.05$; plus Losartan $p<0.04$; plus WEB2086 $p<0.07$). 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 WEB2086 $p<0.002$). 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

Figure 9 shows that increasing concentrations of WEB 2086 resulted in increasing inhibition of basal MCP-1 production. At 10μM WEB 2086 there was a 20% reduction ($p<0.05$) while 50μM WEB 2086 resulted in a 60% decrease ($p<0.009$) in basal MCP-1. Irbesartan was more effective, with 10μM Irbesartan reducing basal MCP-1 levels by 40% ($p<0.05$), 20μM giving 75% reduction ($p<0.02$) and 50μM reducing levels by greater than 95% ($p<0.008$). LDL (200μg/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 20μM and by 50% ($p<0.02$) at 50μM. Irbesartan was again more effective, reducing LDL-stimulated MCP-1 by 50% ($p<0.02$) at 10μM, 70% ($p<0.001$) at 20μM and greater than 95% ($p<0.002$) at a concentration of 50μM.

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 VCAM-1, TNF-alpha 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 (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, since reactive oxygen species (ROS), 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

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no effect on MCP-1 protein levels while 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 hour 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 (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, C5a 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. We showed that Irbesartan and Losartan inhibited both binding of ^{3}H-PAF to monocytes and PAF stimulation of MCP-1 in monocytes. When Irbesartan binding to the human monocyte PAF receptor was examined more closely, it was found that the affinity of the PAF receptor for Irbesartan (43μM) was about 700 times less than affinity for PAF (0.06μM). This suggests that the inhibition of PAF-stimulated MCP-1 by Irbesartan may be partially independent of Irbesartan binding to the PAF receptor.

PAF stimulates thromboxane A_2 (TXA_2) production (Ishizuka et al., 1994), a TXA_2 analog stimulates MCP-1 production and TXA_2 receptor antagonists inhibit PAF-induced MCP-1 in human umbilical vein endothelial cells (HUVEC) (Ishizuka et al., 2000). These studies suggest that the TXA_2 receptor may be involved in PAF-stimulated MCP-1 in HUVEC. Irbesartan inhibited TXA_2-induced vasoconstriction in canine coronary arteries and human

platelet aggregation and high concentrations of Irbesartan significantly inhibited TXA_2 receptor antagonist binding (Li et al., 2000). However the affinity of the TXA_2 receptor for Irbesartan (10μM) was 1400 times less than the affinity for the TXA_2 analog (7nM) (Li et al., 2000). These findings suggest that inhibition of PAF-stimulated MCP-1 by Irbesartan may be partially independent of Irbesartan binding to the TXA_2 receptor. Irbesartan was 2-fold more potent than Losartan in the inhibition of TXA_2 analog induced vasoconstriction (Li et al., 2000) as it was in the inhibition of MCP-1 production in monocytes in the current study. Other AT1 antagonists may behave differently to Irbesartan and Losartan depending on their binding properties.

A 300mg dose of Irbesartan results in human plasma concentrations of around 10μM Irbesartan (Pool et al., 1998). Our results suggest that this concentration of Irbesartan inhibits basal and LDL-stimulated MCP-1 production from monocytes (Fig. 3A and Fig. 9). Sugano et al (Sugano et al., 2001) showed that 10nM carbamyl-PAF stimulated MCP-1 in cultured human uterine cervical fibroblasts and this stimulation of MCP-1 was abolished by co-incubation with 10μM (1000-fold excess) WEB 2170, a PAF receptor antagonist, in medium containing 0.1% BSA. We showed that 1μM carbamyl-PAF stimulated MCP-1 production in human monocytes. This stimulation was inhibited by 50μM (50-fold excess) WEB 2086, a PAF receptor antagonist, and by 50μM 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 appear to be due to blocking the PAF receptor. Similarly, in our experiments, the inhibitory effects of Irbesartan, Losartan and

WEB 2086 on carbamyl-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 oxidised phospholipids have been shown to activate monocytes via the PAF receptor (Tokumura et al., 1996;Heery et al., 1995;Lehr et al., 1997). Hayek et al (Hayek et al., 2000) have shown that the AT1 antagonist, Losartan, significantly reduced human monocyte-derived macrophage uptake of oxidised LDL and also decreased CD36 (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 since 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 carbamyl-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 HUVEC. Increased nitric oxide release in the presence of AT1 receptor antagonists may decrease MCP-1 levels since NO has been shown to inhibit MCP-1 production (Zeiher et al., 1995;Tsao et al., 1997). Li et al (Li et al., 2000) found that a nitric oxide synthase inhibitor had no effect on inhibition of TXA_2-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.

Since 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 AngII, 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 atherogenic 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.

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References

Baggiolini M (1998) Chemokines and leukocyte traffic. *Nature* **392**:565-568.

Boring L, Gosling J, Cleary M, and Charo IF (1998) Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature* **394**:894-897.

Chao W and Olson MS (1993) Platelet-activating factor: receptors and signal transduction. *Biochem.J.* **292 (Pt 3)**:617-629.

Chen XL, Tummala PE, Olbrych MT, Alexander RW, and Medford RM (1998) Angiotensin II induces monocyte chemoattractant protein-1 gene expression in rat vascular smooth muscle cells. *Circ.Res.* **83**:952-959.

Croft KD, Proudfoot J, Moulton C, and Beilin LJ (1991) The effect of lipoproteins on the release of some eicosanoids by stimulated human leukocytes. A possible role in atherogenesis. *Eicosanoids* **4**:75-81.

Cushing SD, Berliner JA, Valente AJ, Territo MC, Navab M, Parhami F, Gerrity R, Schwartz CJ, and Fogelman AM (1990) Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc.Natl.Acad.Sci.U.S.A* **87**:5134-5138.

De Keulenaer GW, Ushio-Fukai M, Yin Q, Chung AB, Lyons PR, Ishizaka N, Rengarajan K, Taylor WR, Alexander RW, and Griendling KK (2000) Convergence of redox-sensitive and mitogen-activated protein kinase signaling pathways in tumor necrosis factor-alpha-mediated monocyte chemoattractant protein-1 induction in vascular smooth muscle cells. *Arterioscler.Thromb.Vasc.Biol.* **20**:385-391.

Dol F, Martin G, Staels B, Mares AM, Cazaubon C, Nisato D, Bidouard JP, Janiak P, Schaeffer P, and Herbert JM (2001) Angiotensin AT1 receptor antagonist irbesartan decreases lesion size, chemokine expression, and macrophage accumulation in apolipoprotein E-deficient mice. *J.Cardiovasc.Pharmacol.* **38**:395-405.

Griendling KK and Alexander RW (1997) Oxidative stress and cardiovascular disease. *Circulation* **96**:3264-3265.

Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, and Rollins BJ (1998) Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol.Cell* **2**:275-281.

Hayek T, Aviram M, Heinrich R, Sakhnini E, and Keidar S (2000) Losartan inhibits cellular uptake of oxidized LDL by monocyte- macrophages from hypercholesterolemic patients. *Biochem.Biophys.Res.Commun.* **273**:417-420.

Heery JM, Kozak M, Stafforini DM, Jones DA, Zimmerman GA, McIntyre TM, and Prescott SM (1995) Oxidatively modified LDL contains phospholipids with platelet- activating factor-like activity and stimulates the growth of smooth muscle cells. *J.Clin.Invest* **96**:2322-2330.

Hernandez-Presa M, Bustos C, Ortego M, Tunon J, Renedo G, Ruiz-Ortega M, and Egido J (1997) Angiotensin-converting enzyme inhibition prevents arterial nuclear factor-kappa B activation, monocyte chemoattractant protein-1 expression, and macrophage infiltration in a rabbit model of early accelerated atherosclerosis. *Circulation* **95**:1532-1541.

Ishii I, Izumi T, Tsukamoto H, Umeyama H, Ui M, and Shimizu T (1997) Alanine exchanges of polar amino acids in the transmembrane domains of a platelet-activating factor receptor generate both constitutively active and inactive mutants. *J.Biol.Chem.* **272**:7846-7854.

Ishizuka T, Sawada S, Sugama K, and Kurita A (2000) Thromboxane A2 (TXA2) receptor blockade suppresses monocyte chemoattractant protein-1 (MCP-1) expression by stimulated vascular endothelial cells. *Clin.Exp.Immunol.* **120**:71-78.

Ishizuka T, Suzuki K, Kawakami M, Kawaguchi Y, Hidaka T, Matsuki Y, and Nakamura H (1994) DP-1904, a specific inhibitor of thromboxane A2 synthesizing enzyme, suppresses ICAM-1 expression by stimulated vascular endothelial cells. *Eur.J.Pharmacol.* **262**:113-123.

Kalinowski L, Matys T, Chabielska E, Buczko W, and Malinski T (2002) Angiotensin II AT1 receptor antagonists inhibit platelet adhesion and aggregation by nitric oxide release. *Hypertension* **40**:521-527.

Keidar S, Attias J, Smith J, Breslow JL, and Hayek T (1997) The angiotensin-II receptor antagonist, losartan, inhibits LDL lipid peroxidation and atherosclerosis in apolipoprotein E-deficient mice. *Biochem.Biophys.Res.Commun.* **236**:622-625.

Khan BV, Navalkar S, Khan QA, Rahman ST, and Parthasarathy S (2001) Irbesartan, an angiotensin type 1 receptor inhibitor, regulates the vascular oxidative state in patients with coronary artery disease. *J.Am.Coll.Cardiol.* **38**:1662-1667.

Lehr HA, Weyrich AS, Saetzler RK, Jurek A, Arfors KE, Zimmerman GA, Prescott SM, and McIntyre TM (1997) Vitamin C blocks inflammatory platelet-activating factor mimetics created by cigarette smoking. *J.Clin.Invest* **99**:2358-2364.

Li P, Fukuhara M, Diz DI, Ferrario CM, and Brosnihan KB (2000) Novel angiotensin II AT(1) receptor antagonist irbesartan prevents thromboxane A(2)-induced vasoconstriction in canine coronary arteries and human platelet aggregation. *J.Pharmacol.Exp.Ther.* **292**:238-246.

Makaritsis KP, Gavras H, Du Y, Chobanian AV, and Brecher P (1998) Alpha1-adrenergic plus angiotensin receptor blockade reduces atherosclerosis in apolipoprotein E-deficient mice. *Hypertension* **32**:1044-1048.

Markwell MA, Haas SM, Bieber LL, and Tolbert NE (1978) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal.Biochem.* **87**:206-210.

Murphy PM (1994) The molecular biology of leukocyte chemoattractant receptors. *Annu.Rev.Immunol.* **12**:593-633.

Navalkar S, Parthasarathy S, Santanam N, and Khan BV (2001) Irbesartan, an angiotensin type 1 receptor inhibitor, regulates markers of inflammation in patients with premature atherosclerosis. *J.Am.Coll.Cardiol.* **37**:440-444.

Nelken NA, Coughlin SR, Gordon D, and Wilcox JN (1991) Monocyte chemoattractant protein-1 in human atheromatous plaques. *J.Clin.Invest* **88**:1121-1127.

Pool JL, Guthrie RM, Littlejohn TW, III, Raskin P, Shephard AM, Weber MA, Weir MR, Wilson TW, Wright J, Kassler-Taub KB, and Reeves RA (1998) Dose-related antihypertensive effects of irbesartan in patients with mild-to-moderate hypertension. *Am.J.Hypertens.* **11**:462-470.

Raiden S, Giordano M, Andonegui G, Trevani AS, Lopez DH, Nahmod V, and Geffner JR (1997) Losartan, a selective inhibitor of subtype AT1 receptors for angiotensin II, inhibits the binding of N-formylmethionyl-leucyl- phenylalanine to neutrophil receptors. *J.Pharmacol.Exp.Ther.* **281**:624-628.

Ross R (1993) Rous-Whipple Award Lecture. Atherosclerosis: a defense mechanism gone awry. *Am.J.Pathol.* **143**:987-1002.

Ross R (1999) Atherosclerosis--an inflammatory disease. *N.Engl.J.Med.* **340**:115-126.

Schuh JR, Blehm DJ, Frierdich GE, McMahon EG, and Blaine EH (1993) Differential effects of renin-angiotensin system blockade on atherogenesis in cholesterol-fed rabbits. *J.Clin.Invest* **91**:1453-1458.

Subbanagounder G, Leitinger N, Shih PT, Faull KF, and Berliner JA (1999) Evidence that phospholipid oxidation products and/or platelet- activating factor play an important role in early atherogenesis : in vitro and In vivo inhibition by WEB 2086. *Circ.Res*. **85**:311-318.

Sugano T, Narahara H, Nasu K, Arima K, Fujisawa K, and Miyakawa I (2001) Effects of platelet-activating factor on cytokine production by human uterine cervical fibroblasts. *Mol.Hum.Reprod.* **7**:475-481.

Tokumura A, Toujima M, Yoshioka Y, and Fukuzawa K (1996) Lipid peroxidation in low density lipoproteins from human plasma and egg yolk promotes accumulation of 1-acyl analogues of platelet- activating factor-like lipids. *Lipids* **31**:1251-1258.

Tsao PS, Wang B, Buitrago R, Shyy JY, and Cooke JP (1997) Nitric oxide regulates monocyte chemotactic protein-1. *Circulation* **96**:934-940.

Wang GP, Deng ZD, Ni J, and Qu ZL (1997) Oxidized low density lipoprotein and very low density lipoprotein enhance expression of monocyte chemoattractant protein-1 in rabbit peritoneal exudate macrophages. *Atherosclerosis* **133**:31-36.

Yanagitani Y, Rakugi H, Okamura A, Moriguchi K, Takiuchi S, Ohishi M, Suzuki K, Higaki J, and Ogihara T (1999) Angiotensin II type 1 receptor-mediated peroxide production in human macrophages. *Hypertension* **33**:335-339.

Yla-Herttuala S, Lipton BA, Rosenfeld ME, Sarkioja T, Yoshimura T, Leonard EJ, Witztum JL, and Steinberg D (1991) Expression of monocyte chemoattractant protein 1 in macrophage-rich areas of human and rabbit atherosclerotic lesions. *Proc.Natl.Acad.Sci.U.S.A* **88**:5252-5256.

Yu X, Dluz S, Graves DT, Zhang L, Antoniades HN, Hollander W, Prusty S, Valente AJ, Schwartz CJ, and Sonenshein GE (1992) Elevated expression of monocyte chemoattractant protein 1 by vascular smooth muscle cells in hypercholesterolemic primates. *Proc.Natl.Acad.Sci.U.S.A* **89**:6953-6957.

Zeiher AM, Fisslthaler B, Schray-Utz B, and Busse R (1995) Nitric oxide modulates the expression of monocyte chemoattractant protein 1 in cultured human endothelial cells. *Circ.Res.* **76**:980-986.

Footnotes

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FIGURE LEGENDS

Figure 1 Time course of MCP-1 production in human monocyte supernatants.

Human peripheral blood mononuclear cells were isolated as described in the Methods and resuspended in HBSS. The cells were incubated at 37^{o}C and cell supernatants collected after 2, 4, 8 and 20 hours incubation. Results are the mean of 2 experiments.

Figure 2 Inhibition of MCP-1 in human monocytes by the AT1 receptor antagonist Irbesartan (IRB).

IRB inhibits and Ang II has no effect on basal MCP-1. Cells were isolated as described in the Methods and resuspended in HBSS. After pre-incubation for 20min with IRB (50μM; AT1 antagonist), DMSO (D) (0.05%; vehicle for IRB) or medium (control, C), Ang II (AII; 10^{-7}M) was added and the cells incubated overnight. Results are the mean ± SE from 6 experiments. **p=0.001 vs control.

Figure 3 AT1 receptor antagonists inhibit basal and LDL-stimulated MCP-1.

(A) Monocytes were isolated as described in the Methods, resuspended in HBSS and pre-incubated for 20min with 0, 5, 15 or 50μM IRB. Varying concentrations of LDL were added and the cells were incubated overnight. Results are the mean ± SE from 2 to 4 experiments.

(B) Isolated cells were pre-incubated for 20min with 0, 25, 50 or 100μM Losartan. Medium or LDL (final concentration 200μg/ml) was added and the cells incubated overnight. Results are the mean of 2 experiments.

Figure 4 Specificity of AT1 receptor antagonists.

Cells were isolated as described in the Methods, resuspended in HBSS and pre-incubated for 20 min with IRB (50μM; AT1 antagonist), PD123319 (PD) (50μM; AT2 antagonist), DMSO (D) (0.05%; vehicle) or medium (control, C). Medium or LDL (final concentration 200 μg/ml protein) was added and the cells incubated overnight. Results are the mean ± SE from 7 experiments. **p<0.005 vs control, ## p=0.001 vs C+LDL.

Figure 5 AT1 receptor antagonists inhibit ^{3}H-PAF binding to human monocytes.

Cells were isolated as described in the Methods, resuspended in Hepes-Tyrode's +0.25% HSA and pre-incubated with unlabelled PAF (10μM; non-specific binding), varying concentrations of Irbesartan (IRB), Losartan (LOS), or WEB 2086 (WEB), or medium prior to addition of ^{3}H-PAF as described in the Methods. Bound ^{3}H-PAF cpm was calculated by subtracting non-specific counts (bound in the presence of unlabelled PAF) from total ^{3}H-PAF counts bound. Results are the mean ± SE from 3 or 4 experiments. *p<0.05 vs control.

Figure 6 ^{3}H-PAF monocyte binding parameters.

Cells were isolated as described in the Methods, resuspended in Hepes-Tyrode's +0.25% HSA and varying concentrations of ^{3}H-PAF were added as described in the Methods. Bound ^{3}H-PAF cpm was calculated as described in Figure 5. Results are the mean ± SE from 5 experiments. Saturation binding data was analysed with non-linear regression using GraphPad Prism 3 and the data displayed as a Scatchard plot (inset).

Figure 7 Competitive binding curves for Irbesartan and PAF.

Cells were isolated as described in the Methods, resuspended in Hepes-Tyrode's +0.25% HSA and pre-incubated with unlabelled PAF (10μM; non-specific binding), varying concentrations of Irbesartan or unlabelled PAF or medium prior to addition of ^{3}H-PAF as described in the Methods. Bound ^{3}H-PAF cpm was measured and IC_{50} (inhibitory concentration 50%) determined for each ligand using GraphPad Prism3. Shown are two examples of competition binding curves.

Figure 8 AT1 receptor antagonists inhibit carbamyl-PAF-stimulated MCP-1.

Cells were isolated as described in the Methods and resuspended in HBSS **(A).** Cells were pre-incubated for 20min with varying concentrations of Irbesartan or WEB 2086 or medium (control), then carbamyl-PAF (c-PAF; 0.5μM) was added and the cells incubated overnight. Results are expressed as % of control levels and are the mean±SE from 3 to 5 experiments. **(B)** Cells were resuspended in RPMI + 1% HIFCS and pre-incubated for 20min with Irbesartan (50μM), Losartan (100μM), WEB 2086 (50μM) or medium (control). Varying concentrations of carbamyl-PAF (c-PAF) were added and the cells incubated overnight. Results are expressed as % of control levels (mean=4015±2276 pg/ml) and are the mean±SE from 2 to 5 experiments.

Figure 9 PAF receptor antagonist inhibits basal and LDL-stimulated MCP-1.

Monocytes were isolated as described in the Methods, resuspended in HBSS and pre-incubated for 20min with 0, 10, 20 or 50μM Irbesartan or WEB 2086. Medium or LDL (final concentration 200μg/ml) was added and the cells incubated overnight. Results are mean ± SE from 3 experiments.

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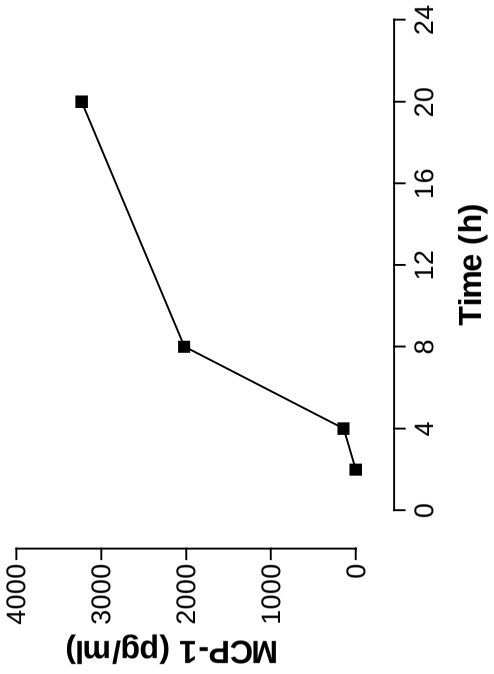

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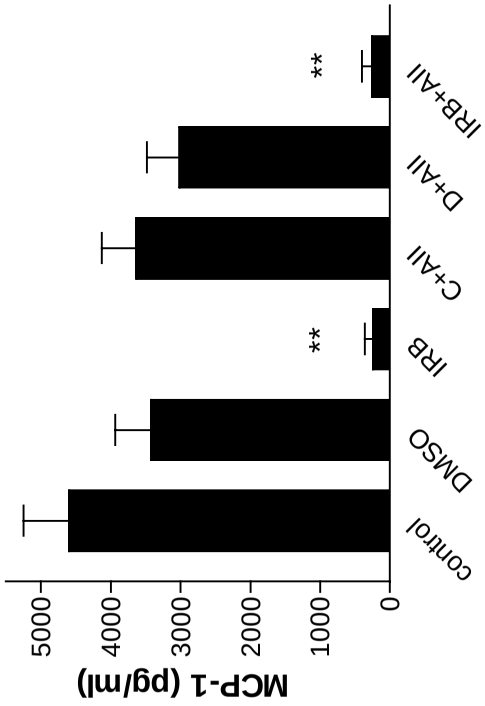

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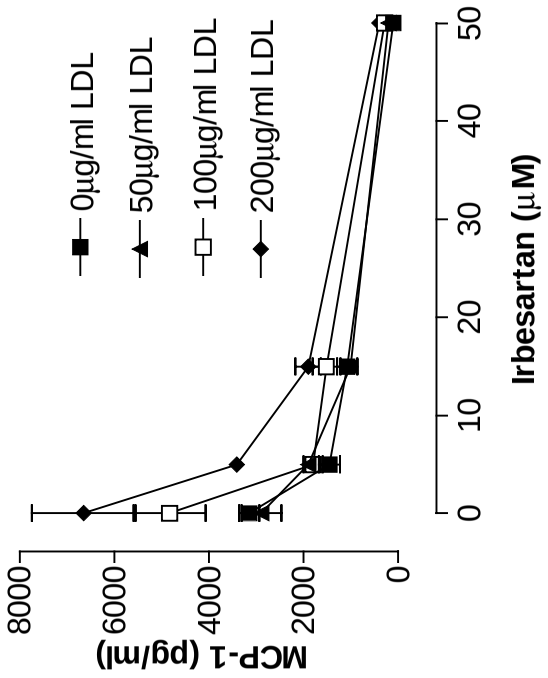

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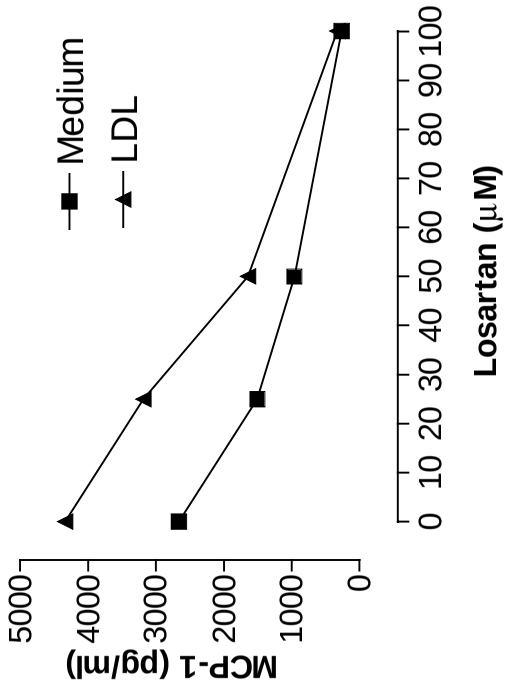

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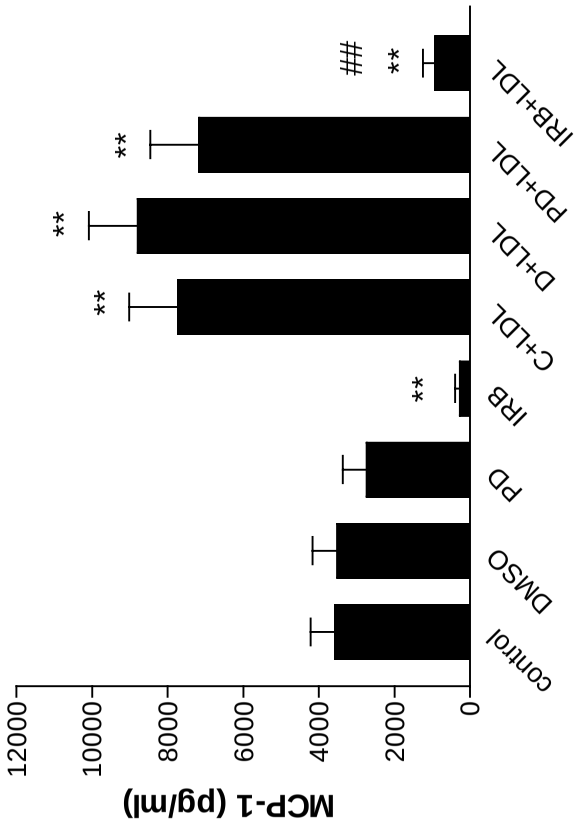

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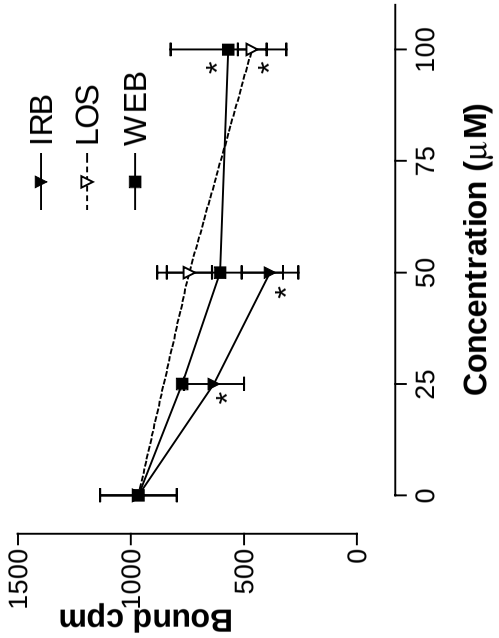

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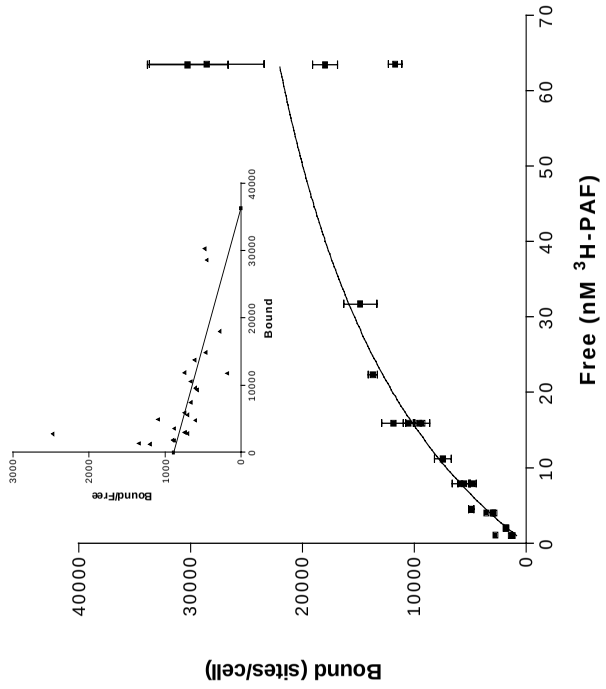

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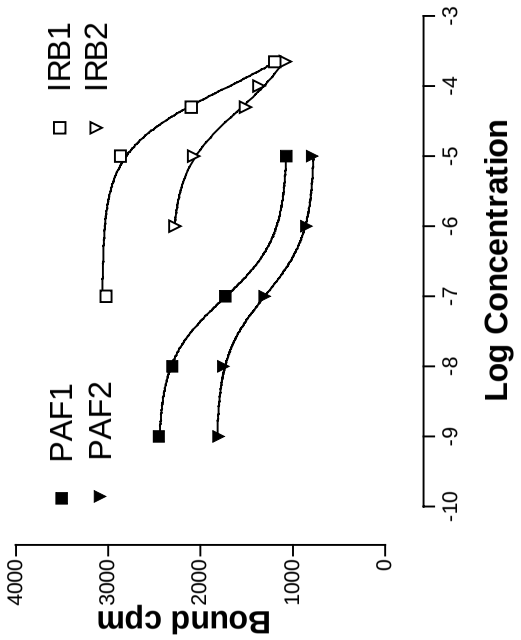

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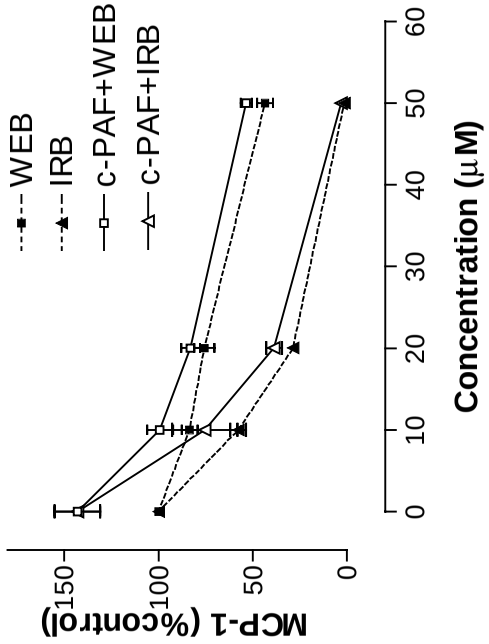

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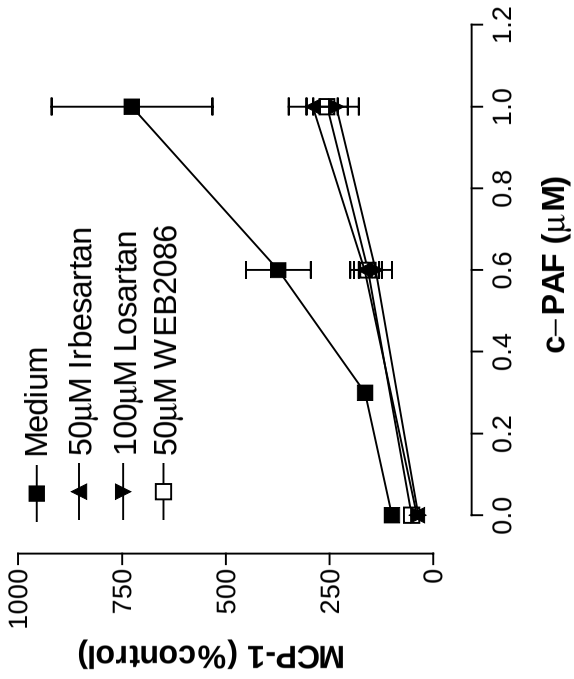

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