Inhibitory Effect of Troglitazone on Tumor Necrosis Factor Alpha–Induced Expression of Monocyte Chemoattractant Protein-1 in Human Mesangial Cells

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Insulin resistance is one of the risk factors for the progression of atherosclerosis and glomerulosclerosis. Recently, the new oral insulin-sensitizing agent troglitazone has been thought to offer potential in the treatment of diabetes. If adopted for this use, it might be helpful in protecting against the development of atherosclerosis and microvascular complications via its improvement of insulin resistance. However, it has not yet been clarified whether troglitazone acts directly on the vascular cells and inhibits the progression of atherosclerosis, including glomerulosclerosis. Meanwhile, monocyte chemoattractant protein-1 (MCP-1) is known to play an important role in the pathogenesis of atherosclerosis and glomerulosclerosis through the induction of monocyte migration. Therefore, we investigated the effect of troglitazone on the expression of MCP-1 in human mesangial cells (HMCs). HMCs were treated with or without troglitazone (1 or 10 μmol/L) in the presence or absence of tumor necrosis factor alpha (TNF-α) at various concentrations (50 or 500 ng/mL), and then MCP-1 secretion from the HMCs was measured. We found that TNF-α increased the secretion of MCP-1 by 55-fold versus the control and troglitazone significantly inhibited this TNF-α–induced increase in MCP-1 secretion (49.3%). Moreover, Northern blot analysis showed that troglitazone decreased the MCP-1 mRNA level in HMCs. We demonstrated that α-tocopherol also inhibited TNF-α–induced MCP-1 production in HMCs, although its effects were not as strong as troglitazone. The present study indicates that troglitazone may prevent the progression of atherosclerosis by inhibiting MCP-1 expression in mesangial cells.

IN ADDITION TO its known role in the pathogenesis of type 2 diabetes mellitus, insulin resistance has been considered one of the risk factors for the development of atherosclerosis, including glomerulosclerosis.[1-3] Recently, troglitazone, a new thiazolidinedione derivative used as an oral insulin-sensitizing agent, has been reported to protect against atherosclerosis by improving insulin resistance and dyslipidemia.[4] In addition, like α-tocopherol, troglitazone has been shown to have antioxidative effects, which inhibit oxidative modification of human plasma low-density lipoprotein in vitro.[5,6] This antioxidative effect of troglitazone may also retard the progression of atherosclerosis. Several recent reports have shown that troglitazone inhibits mouse aortic endothelial cell proliferation[7] and endothelin-1 secretion from bovine vascular endothelial cells.[8] However, the direct effects of troglitazone on human mesangial cells (HMCs) have never been studied.

Since the first description of the in vitro production of monocyte chemoattractant protein-1 (MCP-1) in 1987, the monocyte-specific chemotaxis and activating factor has been detected in vascular endothelial cells,[9] smooth muscle cells,[10] and mesangial cells.[11] Induction of MCP-1 has been shown to be enhanced by proinflammatory factors such as tumor necrosis factor alpha (TNF-α) and interleukin-1β in HMCs.[11] While monocyte infiltration is a known characteristic of diabetic nephropathy and has been observed from the early stage,[12] the expression of MCP-1 has been demonstrated in macrophage-rich areas of human and rabbit atherosclerotic lesions,[13] human atherosclerotic plaques,[14] and vascular smooth muscle cells in hypercholesterolemic primates.[10] Therefore, MCP-1–induced monocyte infiltration is thought to play an important role in the progression of atherosclerosis and glomerulosclerosis. In this study, we investigated the effects of troglitazone on TNF-α–induced MCP-1 secretion and MCP-1 mRNA expression in HMCs.

MATERIALS AND METHODS

Materials

The materials used in this study were obtained from the following suppliers: HMCs and mesangial cell basal medium (MsBM) from Clonetics, San Diego, CA; TNF-α from Endogen, Cambridge, MA; troglitazone from Sankyo, Tokyo, Japan; α-tocopherol from Wako, Tokyo, Japan; and [γ-^{32}P]adenosine triphosphate (ATP) from Amersham Japan, Tokyo, Japan.

Cell Culture and Cytokine Activation

HMCs were grown in MsBM supplemented with 5% fetal bovine serum (FBS), 50 mg/mL gentamicin sulfate, and 50 μg/mL amphotericin-B under a humidified atmosphere containing 5% CO_2. Fresh MsBM was added every 3 to 4 days. Cells were used between the fifth and eighth passage. For the experiment, HMCs were plated on 12-well tissue-culture plates at a concentration of 20,000/well. The next day, the medium was exchanged with medium containing various concentrations of TNF-α (50 or 500 ng/mL) in the presence or absence of troglitazone (1 or 10 μmol/L), and then the cells were incubated for 24 hours at 37°C. After incubation, the cell culture supernatant of each well was stored at −20°C until assayed.

Enzyme-Linked Immunosorbent Assay for MCP-1

MCP-1 protein concentrations in HMCs culture supernatants were estimated by enzyme-linked immunosorbent assay (ELISA) using a soluble MCP-1 kit (R&D Systems, Minneapolis, MN). This assay uses the quantitative sandwich-enzyme immunoassay technique. Two hundred microliters of diluted culture medium from each sample was dispensed into a 96-well polystyrene microtiter plate (12 strips of 8 wells) coated with a murine monoclonal antibody against MCP-1. After incubation for 2 hours at room temperature, the media were removed and the wells were washed and aspirated 3 times consecutively. Polyclonal antibody against MCP-1 conjugated to horseradish peroxidase (200 μL per well) was then added to the plate, and the plate was incubated for 1 hour at room temperature. After repeating the same washing process to remove any unbound antibody-enzyme reagent, a

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substrate solution (the resultant mixture of stabilized hydrogen peroxide and chromogen) was added to the wells and the color was developed in proportion to the amount of MCP-1 bound in the initial step. The reaction was stopped by the addition of 50 μL 2N sulfuric acid 20 minutes later. The plates were read at 450 nm by a microplate reader (Corona Electric, Ibaragi, Japan). The standard concentration curve for MCP-1 measured by this ELISA was linear from 5.0 to 5,000 pg/mL. Data represent the mean ± SEM of triplicate samples. The validity of the ELISA was confirmed by the intra-assay and interassay coefficient of variation of 4.2% to 5.9% and 4.5% to 5.9%, respectively. Cellular protein content was measured by a bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL). All MCP-1 protein concentrations are expressed as nanograms per milligram of cellular protein.

Determination of Viable Cell Number

Cells cultured for 24 hours in the presence or absence of troglitazone (1 or 10 μmol/L) were dislodged with trypsin, and cell viability was determined by the trypan blue exclusion method.

Preparation of Total RNA and Northern Blot Analysis

Subconfluent HMCs in culture were incubated with culture medium containing FBS (5%) and TNF-α 500 ng/mL in the presence or absence of troglitazone (10 μmol/L) for 6 hours. Total RNA was prepared by a guanidinium isothiocyanate–cesium chloride method.[15] Total RNA (10 μg per lane) was size-fractionated electrophoretically to Hybond-N+ membrane (Amersham Japan) and fixed to the membrane. The probe used in this study was a synthetic oligonucleotide (5′-TTG GGT TTG CTT GTC CAG GTG GTC CAT GGA-3′) complementary to nucleotides 256 to 285 in the coding region of the human MCP-1 mRNA sequence.[16,17] The probe was labeled with [γ-^{32}P]ATP by a random oligonucleotide priming method to a specific activity of approximately 1 to 2 × 10^8 cpm/μg DNA. The membrane was hybridized with a ^{32}P-labeled human MCP-1 cDNA probe for 16 hours at 65°C. The mRNA signals were quantified by densitometric scanning of the autoradiographs.

Statistical Analysis

All data are expressed as the mean ± SEM. Groups were compared using one-way ANOVA followed by Scheffe's F test for multiple comparisons. A P level less than .05 was considered significant.

RESULTS

Effect of Troglitazone on TNF-α–Induced MCP-1 Secretion in HMCs

To determine whether troglitazone inhibits TNF-α–induced secretion of MCP-1 protein (50 or 500 ng/mL) in HMCs, the amount of MCP-1 in the media was measured using ELISA.

After incubation with 500 ng/mL TNF-α alone, significant elevations of MCP-1 were observed in a time-dependent manner: 21.6 ± 2.46 ng/mg protein at 6 hours, 62.4 ± 16.2 ng/mg protein at 12 hours, and 275.3 ± 19.8 ng/mg protein at 24 hours (Fig 1). Troglitazone (10 μmol/L) significantly suppressed the TNF-α–induced production of MCP-1 in HMCs at all time points examined for 24 hours. Pretreatment of HMCs with TNF-α for 24 hours resulted in a marked significant dose-dependent increase in the MCP-1 protein concentration (36- to 55-fold, $P < .001$) compared with the increases induced by the media alone (control). Troglitazone 10 μmol/L significantly inhibited TNF-α–induced MCP-1 secretion in HMCs as compared with MCP-1 secretion in the absence of troglitazone,

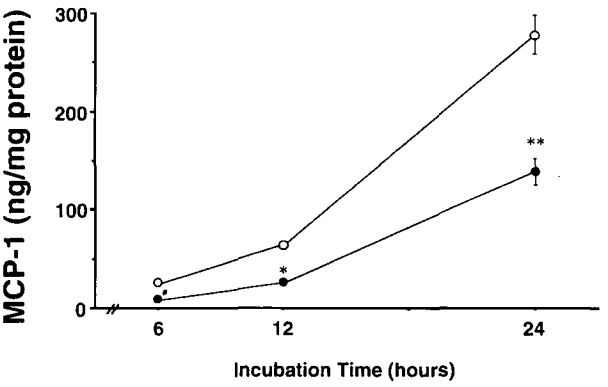

Fig 1. Time course of TNF-α (500 ng/mL)–induced soluble MCP-1 secretion from HMCs. Results are the mean ± SEM of triplicate values from 3 separate experiments. #$P < .05$, *$P < .005$, **$P < .001$ *v* TNF-α alone.

by 49.3% with 50 ng/mL TNF-α ($P < .005$) and by 52.5% with 500 ng/mL TNF-α ($P < .001$) (Fig 2). One micromole of troglitazone was also inhibitory, although not significantly. While troglitazone (10 μmol/L) inhibited TNF-α (50 ng/mL)–induced MCP-1 protein synthesis in HMCs to 49.3%, α-tocopherol also (10 μmol/L) inhibited TNF-α (50 ng/mL)–induced MCP-1 secretion to 86.3% (Fig 3). α-Tocopherol also inhibited TNF-α–induced MCP-1 production, although its effects were not as strong as troglitazone. Cell viability, assessed by the trypan blue exclusion method, did not differ between control and troglitazone-treated cells (>95%), indicating that the doses of troglitazone used in this study were not cytotoxic to HMCs. We also confirmed that troglitazone and TNF-α did not influence the MCP-1 assay system.

Effect of Troglitazone on TNF-α–Induced MCP-1 mRNA Expression in HMCs

To evaluate whether troglitazone influences TNF-α–induced MCP-1 mRNA expression, total cellular RNA was isolated from control or TNF-α–treated HMCs and probed for MCP-1

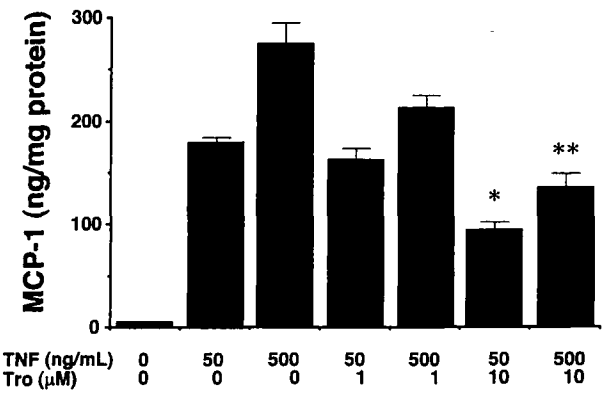

Fig 2. Effect of troglitazone on TNF-α–induced MCP-1 secretion from HMCs. Troglitazone (Tro) 10 μmol/L significantly reduced TNF-α (50 or 500 ng/mL)–stimulated MCP-1 secretion from HMCs for a period of 24 hours. Results are the mean ± SEM of triplicate values from 3 separate experiments. *$P < .005$, **$P < .001$ *v* TNF-α alone.

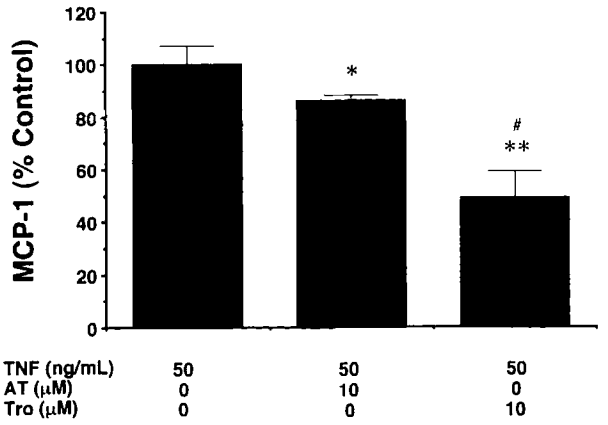

Fig 3. Effect of troglitazone and α-tocopherol on TNF-α–induced MCP-1 secretion from HMCs. Troglitazone (Tro) 10 μmol/L significantly reduced TNF-α (50 ng/mL)-stimulated MCP-1 secretion from HMCs for a period of 24 hours *v* 10 μmol/L α-tocopherol (AT). MCP-1 secretion is expressed as a percentage of the amount secreted by TNF-α (50 ng/mL) alone. Results are the mean ± SEM of triplicate values from 3 separate experiments. *$P < .05$, **$P < .001$ *v* TNF-α alone, #$P < .001$ *v* TNF-α + α-tocopherol.

message (Fig 4). The treatment with 500 ng/mL TNF-α for 6 hours induced a 2.0-fold increase of MCP-1 mRNA relative to the control condition. Troglitazone (10 μmol/L) partially suppressed TNF-α–induced MCP-1 mRNA expression (73.0%).

DISCUSSION

Overexpression of TNF-α in adipose tissue has been shown to induce insulin resistance by suppressing the phosphorylation of insulin receptor and the insulin receptor signaling pathway,[18] and it has also been found to reduce the transcriptional activity of the glucose transporter 4 (GLUT 4) gene.[19] Thiazolidinedione derivatives are potent and selective activators of peroxisome proliferator–activated receptor-γ (PPAR-γ), a member of a nuclear superfamily recently shown to function in adipocyte differentiation, and activated PPAR-γ is known to alleviate insulin resistance.[20,21] The improvement of insulin resistance by troglitazone has been proved to increase glucose utilization, reduce hepatic glucose production, and improve both fasting and postprandial glycemia in type 2 diabetic patients.[4,22] Troglitazone has also been reported to inhibit atherosclerosis.[23,24] However, these effects may be due to a troglitazone-induced reduction in insulin resistance rather than a direct action of troglitazone on the vascular cells.

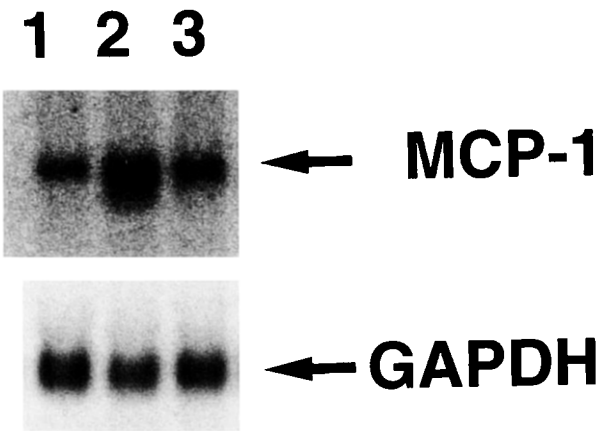

Fig 4. Northern blot analysis of MCP-1 mRNA in cytokine-treated HMCs. The presence of 10 μmol/L troglitazone reduced TNF-α–induced MCP-1 mRNA expression. MCP-1 mRNA expression of the samples: lane 1, control; lane 2, 500 ng/mL TNF-α; lane 3, 500 ng/mL TNF-α + 10 μmol/L troglitazone.

On the other hand, several reports have recently shown that troglitazone upregulates vascular smooth muscle cell nitric oxide synthesis[25] and inhibits vascular smooth muscle cell growth and intimal hyperplasia,[26] mouse aortic endothelial cell mitogenesis,[7] and bovine vascular endothelial cell endothelin-1 secretion.[8] Moreover, troglitazone reportedly has potential in the treatment of diabetic nephropathy to ameliorate albuminuria in vivo without an improvement of insulin resistance and hyperglycemia.[27] Thus, this agent also may have a direct effect on vascular cells.

In contrast, atherosclerosis is characterized by the presence of monocyte-macrophage in the lesions. MCP-1 is believed to be an important mediator in monocyte migration and accumulation in the atherogenic tissue. Thus, in the present study, we investigated whether troglitazone can inhibit TNF-α–induced MCP-1 expression in HMCs. Our results demonstrate for the first time that the TNF-α–induced increase of MCP-1 mRNA expression and protein synthesis in mesangial cells is indeed inhibited by troglitazone.

While the nuclear factor-kappa B (NF-κB) has been reported to mediate MCP-1 gene transcription in HMCs,[28] troglitazone and other PPAR-γ agonists have been demonstrated to inhibit the activation of NF-κB and the production of monocyte inflammatory cytokines.[29] Thus, it is likely that troglitazone inhibits MCP-1 gene expression by inhibiting the activation of NF-κB as a PPAR-γ agonist. On the other hand, it has been reported that the antioxidant agent α-tocopherol inhibits radical-induced MCP-1 mRNA expression in cultured human endothelial cells,[30] and troglitazone has already been shown to have an antioxidative effect similar to that of α-tocopherol.[5,6] Therefore, the inhibitory effects of troglitazone on MCP-1 mRNA expression may be derived not only from the inhibition of NF-κB activation but also from the antioxidative effects. In fact, we have demonstrated for the first time that α-tocopherol also inhibited TNF-α–induced MCP-1 production in HMCs. However, considering that the typical therapeutic levels of troglitazone in serum are 1 μmol/L or less,[31] these lower concentrations of troglitazone may not ediate the effects reported in response to 10 μmol/L.

In addition, since the MCP-1–induced migration of monocytes is found in the early stage of atherosclerosis and is thought to play an important role in its development, we also investigated the effects of troglitazone on the cytokine-induced MCP-1 protein synthesis in human endothelial cells. We confirmed that troglitazone inhibits the cytokine-induced increase of MCP-1 mRNA expression and protein synthesis (Ohta M.Y., unpublished data, May 1998).

The results presented herein clearly suggest that troglitazone may impart a protective effect against the progression of diabetic glomerulosclerosis via its direct action.

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