

# Angiotensin II enhances the increase in monocyte chemoattractant protein-1 production induced by tumor necrosis factor- $\alpha$ from 3T3-L1 preadipocytes

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## Abstract

Monocyte chemoattractant protein-1 (MCP-1) and angiotensin II (Ang II) in adipose tissue are thought to induce systemic insulin resistance in rodents; but the precise mechanism is not fully clarified. We examined the mechanism of Ang II-induced and/or tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )-induced MCP-1 production from 3T3-L1 preadipocytes. The MCP-1 protein and MCP-1 mRNA expression in 3T3-L1 preadipocytes were increased significantly by stimulation with TNF- $\alpha$ . We found no significant increase in MCP-1 concentrations by Ang II alone; but it enhanced the TNF- $\alpha$ -induced MCP-1 mRNA expression in a dose-dependent manner. Then, we examined the effect of Ang II and/or TNF- $\alpha$  on phosphorylation of extracellular

signal-regulated kinase (ERK), p38MAPK, and I $\kappa$ B- $\alpha$ . Ang II and TNF- $\alpha$  clearly enhanced ERK and p38MAPK phosphorylation. I $\kappa$ B- $\alpha$  phosphorylation was enhanced by TNF- $\alpha$ , but not by Ang II. The MCP-1 mRNA expression induced by TNF- $\alpha$  and co-stimulation with Ang II was inhibited by either ERK inhibitor, p38MAPK inhibitor or NF- $\kappa$ B inhibitor. Moreover, Ang II enhanced the activation of AP-1 (c-fos) induced by TNF- $\alpha$ . Our results suggest that Ang II may serve as an additional stimulus on the TNF- $\alpha$ -induced MCP-1 production through the ERK- and p38MAPK-dependent pathways probably due to AP-1 activation.

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## Introduction

Obesity and insulin resistance, the cardinal features of metabolic syndrome, are closely associated with a state of low-grade inflammation in adipose tissues (Hotamisligil 2006). These inflammatory changes are caused in part by the infiltrated macrophages in visceral adipose tissue through various inflammatory molecules including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ; Hotamisligil 2006), interleukin-6 (IL-6; Fernandez-Real & Ricart 2003), and monocyte chemoattractant protein-1 (MCP-1; Takahashi *et al.* 2003, Christiansen *et al.* 2005). Most of these inflammatory molecules are produced by non-adipose cells such as macrophages and preadipocytes (Fain *et al.* 2004). They might have local effects on white adipose tissue physiology as well as potential systemic effects on other organs, which culminate in insulin resistance (Kershaw & Flier 2004).

Although the signals that initiate the infiltration of macrophages are not well understood, it has been considered that MCP-1, a member of the CC chemokine family, is a key molecule (Kamei *et al.* 2006, Kanda *et al.* 2006, Weisberg *et al.* 2006). MCP-1 is reported to be produced and secreted by

macrophages, endothelial cells, preadipocytes, and mature adipocytes in adipose tissues (Christiansen *et al.* 2005) to promote migration of inflammatory cells (Ashida *et al.* 2001). The MCP-1 expression in adipose tissue and plasma MCP-1 levels are positively correlated with the degree of obesity (Sartipy & Loskutoff 2003, Weisberg *et al.* 2003, Xu *et al.* 2003, Christiansen *et al.* 2005). In addition, increased expression of this chemokine in adipose tissue precedes the expression of other macrophage markers during the development of obesity (Xu *et al.* 2003). Recent reports have suggested that the MCP-1/CCR2 pathway influences the development of insulin resistance via adipose macrophage accumulation and inflammation (Kamei *et al.* 2006, Kanda *et al.* 2006, Weisberg *et al.* 2006).

Exactly how MCP-1 expression is induced in adipose tissues of obese humans and animals is now under intensive investigation (Bruun *et al.* 2005, Fain & Madan 2005, Kamei *et al.* 2006). Such inflammatory cytokines as TNF- $\alpha$  and angiotensin II (Ang II) are molecules reported to induce MCP-1 gene expression (Funakoshi *et al.* 2001, Fain *et al.* 2004, Omura *et al.* 2004). The renin-angiotensin system (RAS) has been shown to be another important pathway in

the pathogenesis of metabolic syndrome, especially in hypertension. Circulating angiotensinogen levels are closely correlated with body weight/insulin sensitivity. Therefore, examination of those levels might provide a major link between visceral obesity and hypertension (Cooper *et al.* 1998, Massiera *et al.* 2001). Adipocytes and stromal cells including preadipocytes in adipose tissue are a major source of products of the RAS (Jones *et al.* 1997, Karlsson *et al.* 1998). In those cells, the expression of Ang II precursor, angiotensinogen, and enzymes required for its conversion to Ang II such as renin and cathepsins is reported, suggesting that Ang II, the active component of RAS, is produced in adipose tissues (Karlsson *et al.* 1998, Engeli *et al.* 1999, Schling *et al.* 1999). In rats, overfeeding engenders increased local formation of angiotensinogen and Ang II from adipocytes (Frederich *et al.* 1992). In human studies, local Ang II formation in adipose tissue is increased in obese hypertensive subjects (Giacchetti *et al.* 2002, Gorzelnik *et al.* 2002). These findings suggest that the adipose RAS may play important roles in the pathogenesis of obesity, obesity-associated hypertension, and insulin resistance. Several reports have described that MCP-1 production induced by Ang II is mediated by extracellular signaling-regulated kinase 1/2 (ERK1/2), p38MAPK or NF- $\kappa$ B pathway in cardiovascular cells such as smooth muscle cells (Funakoshi *et al.* 2001) and cardiac fibroblasts (Omura *et al.* 2004). However, the cellular signaling mechanism of Ang II-induced MCP-1 production has not been fully examined.

A proinflammatory cytokine, TNF- $\alpha$ , is an adipokine reported earlier to be related to obesity and metabolic syndrome (Hotamisligil *et al.* 1995). Recent studies have shown that TNF- $\alpha$  exerts local autocrine and/or paracrine effects to stimulate the production of other inflammatory cytokines, especially MCP-1 in adipose tissue (Bruun *et al.* 2005, Fain & Madan 2005). Although both Ang II and TNF- $\alpha$  are strong mediators to produce MCP-1 in adipose tissue causing insulin resistance, there is no report to examine the effect of Ang II co-stimulation with TNF- $\alpha$ .

Consequently, the present study was designed to investigate the cellular signaling mechanism of Ang II-induced MCP-1 production and examine the effect of Ang II co-stimulation with TNF- $\alpha$  on MCP-1 production in 3T3-L1 preadipocytes because preadipocytes are a major source of MCP-1 production in adipose tissue. We demonstrate herein, for the first time that Ang II enhances TNF- $\alpha$ -induced MCP-1 mRNA and protein expression in 3T3-L1 preadipocytes. We also demonstrate that Ang II-induced enhancement of TNF- $\alpha$ -stimulated MCP-1 expression may be mediated through ERK1/2- and p38MAPK-dependent pathways.

## Materials and Methods

### Cell culture

For this study, 3T3-L1 preadipocyte cells were purchased from the American Type Culture Collection (Rockville, MD, USA). Preadipocytes were cultured for 2 days in DMEM

(Invitrogen Corporation) containing 25 mM glucose (DMEM-H), 10% bovine serum (DBS; Sigma-Aldrich Corp.), and antibiotics. Passages 3–5 of the cells were used for the experiments. Passages 7 or 8 of cells were used for RT-PCR experiments.

### Measurement of MCP-1 in the media

The 3T3-L1 preadipocytes were cultured in 10% DBS media in 24-well culture plates for 2 days and then in the 0.5% DBS media overnight. After that, Ang II (Merck and Co., Ltd) and/or TNF- $\alpha$  (MorphoSys AbD) was added to the medium and incubated for 8 h. The media were collected after stimulation with Ang II and/or TNF- $\alpha$  and the concentrations of MCP-1 in the media were measured using a mouse MCP-1 ELISA kit (Biosource International Inc).

### Quantitative real-time RT-PCR

The MCP-1 mRNA expression was determined using quantitative real-time RT-PCR with an Mx3005P real-time PCR system. The 3T3-L1 preadipocytes were cultured in 10% DBS media in six-well culture plates for 2 days and then in 0.5% DBS media overnight. After that, Ang II and/or TNF- $\alpha$  were added to the medium and incubated for 3 h. A specific inhibitor of mitogen-activated protein kinase (MAPK) ERK kinase, PD98059 (Cell Signaling Technology) and a specific inhibitor of p38MAPK, SB203580 (Cell Signaling Technology), and NF- $\kappa$ B inhibitor *N*-tosyl-L-phenylalanine-chloromethyl ketone (TPCK, Sigma-Aldrich Corp.) were added to the medium 1 h before stimulation with Ang II and/or TNF- $\alpha$ . Total RNA was isolated from 3T3-L1 preadipocytes with Isogen (Nippon Gene Co. Ltd) and 0.1  $\mu$ g RNA was reverse transcribed using Taqman Reverse Transcription Reagents (Applied Biosystems). Subsequently, 4.5  $\mu$ l of each reverse-transcribed reaction were amplified in a total volume of 10  $\mu$ l per sample using the Taqman Universal PCR Master Mix Reagents (Applied Biosystems) according to the manufacturer's instructions. Samples were incubated for initial denaturation at 95 °C for 10 min. Then, 40 PCR cycles were performed, each cycle consisting of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 30 s. Data were corrected to glyceraldehyde-3-phosphate dehydrogenase and expressed as a fold increase over control.

### Western blot analysis

Phosphorylation of MAPK and I $\kappa$ B analysis were performed as described in our previous report (Kobashi *et al.* 2005) using a non-radioactive method with a commercial kit (New England Biolabs Inc., Cell Signaling Technologies). The 3T3-L1 preadipocytes were cultured in 10% DBS media in six-well culture plates for 2 days and then in 0.5% DBS media overnight. After that, Ang II and/or TNF- $\alpha$  was added to

the medium and incubated for 10 min. Then cell lysates were prepared using lysis buffer. Protein (40 µg/lane) was subjected to 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Millipore Corp). The membrane was blocked with 5% non-fat dry milk in Tris buffered saline (TBS-T; 50 mM Tris-HCl, pH 7.6, NaCl, 0.05% Tween-20). Then the membrane was incubated with the following antibodies from Cell Signaling Technology: phospho-specific p44/42 MAP kinase (Thr202 and Tyr204) antibody, phospho-p38MAP kinase (Thr180 and Tyr182) antibody, phospho-specific IκB-α (Ser32) antibody, p44/42MAPK antibody, p38MAPK antibody, and IκB-α antibody overnight. The membrane was incubated with anti-rabbit IgG HRP-linked secondary antibody (Cell Signaling Technology) for an hour after washing with TBS-T. The membrane was then visualized using ECL detection system (Amersham). For re-blotting, the membrane was stripped with a strip solution at room temperature for 20 min and then re-blotting with primary antibodies.

#### Immunofluorescence cell staining

The 3T3-L1 preadipocytes were seeded on Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL, USA), and then treated with Ang II (Merck and Co. Inc.) and/or TNF-α (MorphoSys AbD) for 30 min. After that, cells were fixed with 2% paraformaldehyde in PBS for 10 min. Cells were washed three times with 10% calf serum in PBS for 5 min each, and then primary antibody in 10% calf serum containing 0.2% saponin in PBS was applied and incubated at room temperature for 1 h with gentle rocking. Cells were washed three times with 10% calf serum in PBS and incubated at room temperature for 1 h after adding fluorescence-labeled secondary antibody (FITC-conjugated anti-rabbit IgG from Sigma Chemical Co., Alexa Fluor 488 anti-mouse IgG from Molecular Probes Inc). Cells were washed with 10% calf serum in PBS and covered with mounting solution and a cover glass until examination by fluorescent microscopy.

#### Microplate assay for transcription factor NF-κB (p65) and AP-1 (c-fos)

The measurement of activated transcription factor NF-κB and AP-1 from nuclear extracts was performed using Transcription Factor Microplate Assay (Marligen Biosciences, Inc). Briefly, 3T3-L1 preadipocytes were cultured in 10% DBS media in 10 cm culture dish for 2 days and then in 0.5% DBS media overnight. Then Ang II (Merck and Co. Ltd) and/or TNF-α (MorphoSys AbD) were added to the medium and incubated for 30 min. After that, nuclear extracts were prepared, and the binding of transcription factor (p65 or c-fos) to their cognate DNA-binding sequences was analyzed according to the manufacturer's recommended method.

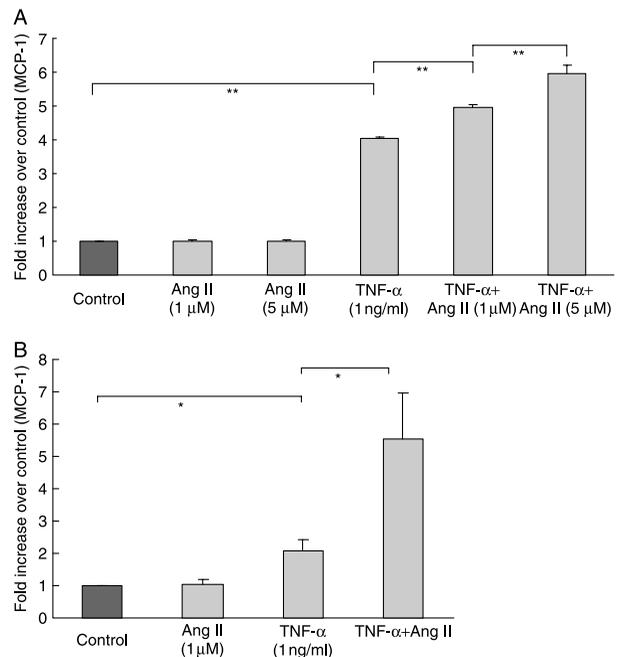
#### Data analyses

Data are presented as the mean ± s.d. Statistical analyses were performed by ANOVA with subsequent Scheffe's *t*-test. A value of  $P < 0.05$  was taken as significant.

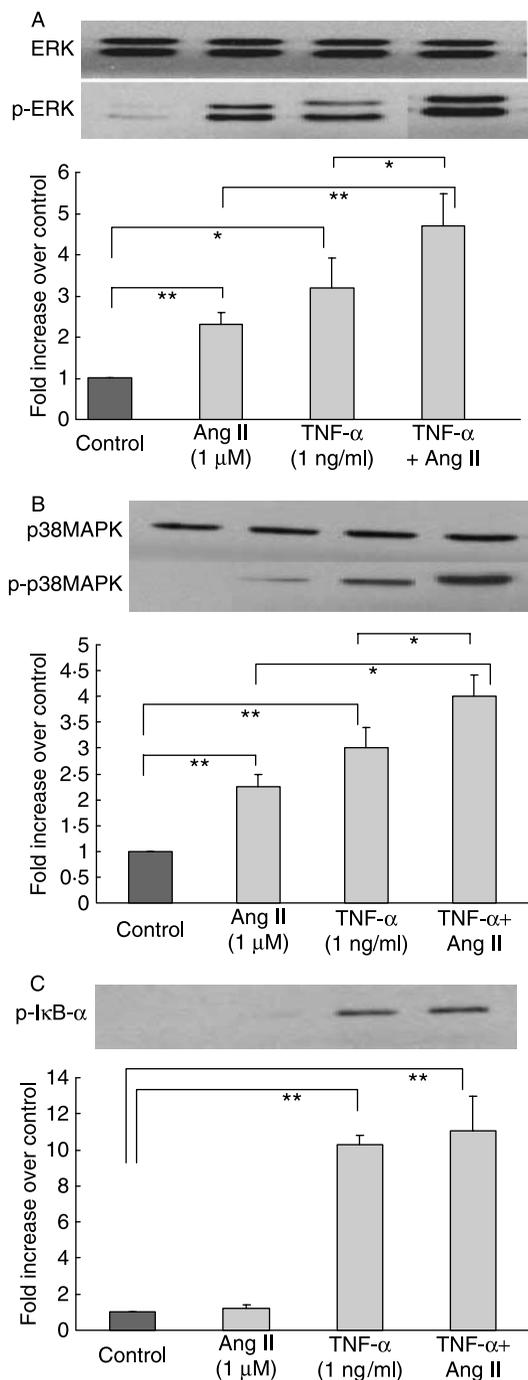
## Results

### TNF-α stimulated the MCP-1 production from 3T3-L1 preadipocytes and Ang II enhanced the stimulating effect of TNF-α

We first examined the effect of TNF-α on MCP-1 production from 3T3-L1 preadipocytes. As shown in Fig. 1A and B, TNF-α (1 ng/ml) increased the concentration of MCP-1 in the medium by 4.1-fold after 8 h incubation, and increased the expression of MCP-1 mRNA in 3T3-L1 preadipocytes by 2.1-fold after 3 h incubation. We then examined the effect of Ang II on MCP-1 production from 3T3-L1 preadipocytes. Ang II alone did not affect MCP-1 production from 3T3-L1 preadipocytes, but Ang II enhanced the effect of TNF-α on MCP-1 production and MCP-1 mRNA expression (Fig. 1).



**Figure 1** Effect of Ang II and/or TNF-α on monocyte chemoattractant protein-1 (MCP-1) production in 3T3-L1 preadipocytes. (A) 3T3-L1 preadipocytes were incubated with Ang II and/or TNF-α of the indicated concentrations for 8 h for measurement of protein levels in conditioned medium using ELISA. Data were obtained from three independent experiments. Protein levels are expressed as a fold increase over control. (B) 3T3-L1 preadipocytes were incubated with Ang II (1 µM) and/or TNF-α (1 ng/ml) for 3 h. MCP-1 mRNA levels were measured using real-time RT-PCR. Data were obtained from three independent experiments and expressed as a fold increase over control. \* $P < 0.05$ , \*\* $P < 0.005$ .



**Figure 2** Effect of Ang II and/or TNF- $\alpha$  on phosphorylation of ERK1/2, p38MAPK and I $\kappa$ B- $\alpha$  in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were incubated with Ang II (1  $\mu$ M) and/or TNF- $\alpha$  (1 ng/ml) for 10 min. Phosphorylation of ERK1/2 (A), p38MAPK (B), and I $\kappa$ B- $\alpha$  (C) was evaluated using western blot. Data were obtained from four independent experiments and expressed as a fold increase over control. \* $P$ <0.05, \*\* $P$ <0.005.

### ERK1/2, p38MAPK, and I $\kappa$ B- $\alpha$ pathways are involved in the TNF- $\alpha$ -induced MCP-1 mRNA expression in 3T3-L1 preadipocytes

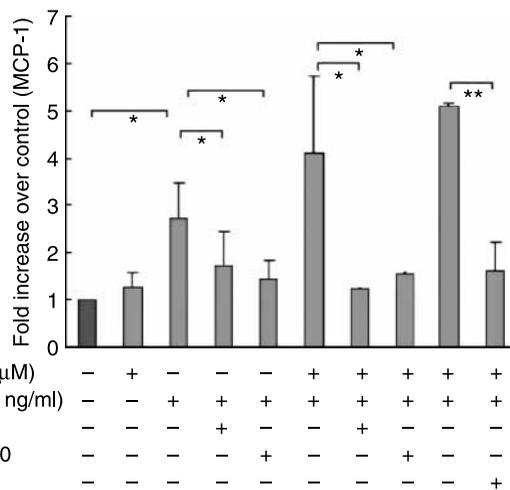
Then, we examined whether ERK1/2, p38MAPK- or NF- $\kappa$ B-dependent signaling pathway is involved in the TNF- $\alpha$ -induced MCP-1 mRNA using a specific chemical inhibitor of each pathway. The TNF- $\alpha$  enhanced the phosphorylation of ERK1/2, p38MAPK, and I $\kappa$ B- $\alpha$  (Fig. 2). As shown in Fig. 3, PD98059, SB203580, and TPCK respectively inhibited the MCP-1 mRNA expression induced by TNF- $\alpha$ .

### Ang II activated ERK1/2 and p38MAPK, but not I $\kappa$ B- $\alpha$

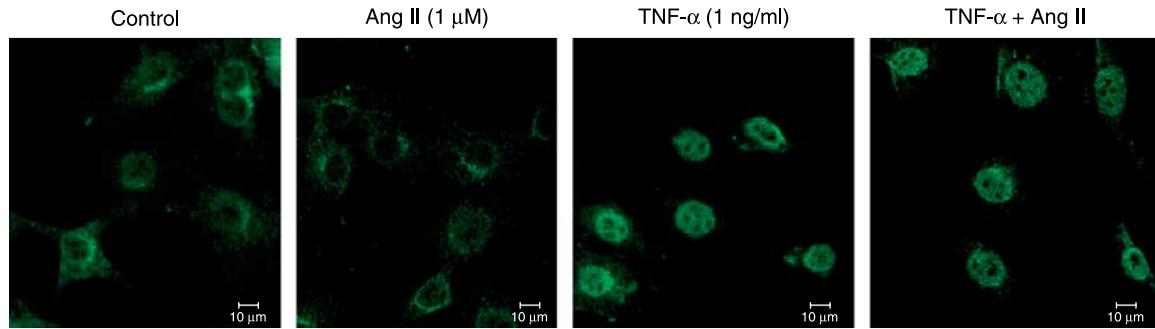
On the other hand, as shown in Fig. 2, Ang II enhanced phosphorylation of ERK1/2 and p38MAPK, but not phosphorylation of I $\kappa$ B- $\alpha$ . Moreover, Ang II co-stimulation with TNF- $\alpha$  further enhanced phosphorylation of ERK1/2 and p38MAPK, but phosphorylation of I $\kappa$ B- $\alpha$  was not enhanced even by Ang II co-stimulation with TNF- $\alpha$ .

### Ang II activated transcription factor AP-1, not NF- $\kappa$ B

Finally, we examined the effect of TNF- $\alpha$  and/or Ang II on the activation of transcription factor NF- $\kappa$ B (p65) and AP-1 (c-fos). As shown in Fig. 4, TNF- $\alpha$  enhanced the translocation of p65 from cytosol to the nucleus, but Ang II did not. As shown in Fig. 5, activation of c-fos was enhanced by stimulation with TNF- $\alpha$  or Ang II. It is particularly



**Figure 3** Effect of ERK1/2 inhibitor (PD98059), p38MAPK inhibitor (SB203580), or NF- $\kappa$ B inhibitor (TPCK) on Ang II and/or TNF- $\alpha$ -induced MCP-1 mRNA expression in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes pretreated with PD98059 (20  $\mu$ M), SB203580 (20  $\mu$ M), or TPCK (20  $\mu$ M) for 1 h were stimulated with Ang II (1  $\mu$ M) and/or TNF- $\alpha$  (1 ng/ml) for 2 h for measurement of MCP-1 mRNA levels using real-time RT-PCR. Data were obtained from three independent experiments and expressed as a fold increase over control. \* $P$ <0.05, \*\* $P$ <0.005.



**Figure 4** Translocation of NF- $\kappa$ B p65 from cytosol to nucleus induced by Ang II and/or TNF- $\alpha$ . 3T3-L1 preadipocytes were seeded on Lab-Tek chamber slides, and then treated with Ang II and/or TNF- $\alpha$ . After that, cells were fixed, and analyzed for the localization of NF- $\kappa$ B p65 (green) by immunofluorescence staining. Representative photographs from one experiment are presented. Similar results were observed in three independent experiments. Scale bar, 10  $\mu$ m.

interesting that Ang II co-stimulation with TNF- $\alpha$  further activated c-fos compared with that by either TNF- $\alpha$  or Ang II alone.

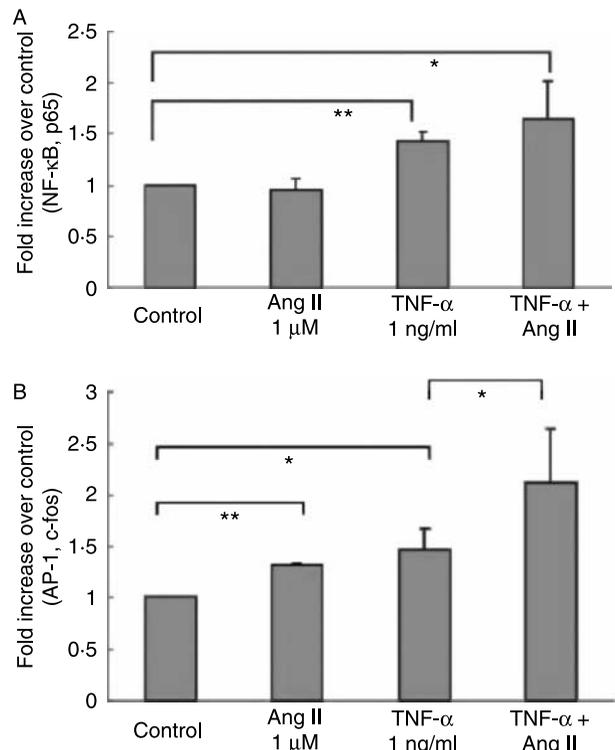
## Discussion

Cytokine production by adipose tissue has become a focus of present research because preadipocytes and adipocytes, as well as macrophages infiltrating into adipose tissue, are thought to produce various proinflammatory cytokines including TNF- $\alpha$  and MCP-1 (Bruun *et al.* 2005, Fain & Madan 2005). It is reported that MCP-1 is overexpressed in the adipose tissues of obese mice and humans, promoting macrophage infiltration in adipose tissue, thereby causing insulin resistance (Kamei *et al.* 2006, Kanda *et al.* 2006, Weisberg *et al.* 2006). Macrophages within adipose tissue secrete TNF- $\alpha$ , a proinflammatory cytokine that activates MCP-1 production from adipocytes and causes insulin resistance (Kanda *et al.* 2006). Preadipocytes are another major source of MCP-1 production in adipose tissue (Christiansen *et al.* 2005). Therefore, we examined the MCP-1 production from 3T3-L1 preadipocytes. It has been shown that CCR2, the main receptor of MCP-1, is also expressed in adipocytes (Tsuchiya *et al.* 2006). Taken together, it is postulated that MCP-1 is a novel proinflammatory cytokine that alters metabolism and insulin sensitivity of fat cells in a paracrine manner, thereby influencing whole-body glucose homeostasis.

In the present study, we observed that TNF- $\alpha$  enhanced both the level of protein and mRNA of MCP-1 from 3T3-L1 preadipocytes, which is consistent with previous reports in human adipocytes (Gerhardt *et al.* 2001, Wang *et al.* 2005). We also demonstrated that TNF- $\alpha$  significantly stimulates MCP-1 mRNA expression in 3T3-L1 preadipocytes via three different signal pathways: ERK1/2 (p44/42), p38MAPK, and NF- $\kappa$ B pathways. As shown in Fig. 3, respective pharmacological inhibition of each signal pathway by PD98059, SB203580, and TPCK suppressed the MCP-1 mRNA expression induced by TNF- $\alpha$ . These results

suggest that the ERK, p38MAPK, and NF- $\kappa$ B pathways are all required for TNF- $\alpha$ -induced MCP-1 mRNA expression in 3T3-L1 preadipocytes.

It has been reported that Ang II stimulates MCP-1 production from various cells including mononuclear cells, cardiovascular cells, preadipocytes, and adipocytes (Funakoshi *et al.* 2001, Omura *et al.* 2004, Tsuchiya *et al.* 2006). In our



**Figure 5** Effect of Ang II and/or TNF- $\alpha$  on activation of transcription factor (NF- $\kappa$ B and AP-1) in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were incubated with Ang II (1  $\mu$ M) and/or TNF- $\alpha$  (1 ng/ml) for 10 min. Activation of transcription factor (p65 and c-fos) was measured using microplate assay. Data were obtained from four independent experiments and expressed as a fold increase over control. \* $P$ <0.05, \*\* $P$ <0.005.

experiment, however, Ang II alone did not stimulate MCP-1 production in 3T3-L1 preadipocytes, which is inconsistent with the report in rat primary preadipocytes (Tsuchiya *et al.* 2006). The reason why Ang II alone did not stimulate the MCP-1 production in our study remains unclear, but one possibility is that the responses to Ang II of 3T3-L1 preadipocytes, a cell line from rat fibroblasts, and rat primary preadipocytes might differ.

It is particularly interesting that Ang II enhanced the TNF- $\alpha$ -induced MCP-1 mRNA expression in a dose-dependent manner (Fig. 1). Examination using chemical inhibitors PD98059 and SB203580 revealed that the stimulatory effect of Ang II on TNF- $\alpha$ -induced MCP-1 mRNA expression is mediated through ERK1/2 and p38MAPK pathways (Fig. 3). MCP-1 promoter has both NF- $\kappa$ B sites and AP-1 sites. AP-1 is another important transcription factor for MCP-1 mRNA expression, which is known to be activated by ERK1/2 and p38MAPK. Martin *et al.* (1997) reported that both NF- $\kappa$ B and AP-1 are required for maximal induction of the human MCP-1 promoter by IL-1 $\beta$  in human endothelial cells. Therefore, we speculate that the activation of AP-1 through ERK- and p38MAPK-dependent pathways induced by Ang II has an additive effect of the TNF- $\alpha$ -induced MCP-1 mRNA expression (Fig. 5B), and that in 3T3-L1 preadipocytes the activation of NF- $\kappa$ B might be critical for expression of MCP-1 mRNA.

Although our study does not clarify whether Ang II regulates MCP-1 *in vivo*, we were able to demonstrate that Ang II may serve as an additional stimulus of MCP-1 production in adipose tissue in the presence of obesity-related chronic inflammation, thereby maintaining the process.

## Conclusion

We have demonstrated for the first time that Ang II enhances TNF- $\alpha$ -induced MCP-1 production through ERK1/2- and p38MAPK-dependent mechanisms but not through NF- $\kappa$ B-dependent mechanisms in 3T3-L1 preadipocytes. Our results suggest that Ang II may serve as an additional stimulus on the TNF- $\alpha$ -induced MCP-1 production through the ERK- and p38MAPK-dependent pathways, probably due to AP-1 activation.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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