JPET Fast Forward. Published on February 20, 2004 as DOI: 10.1124/jpet.103.062620

Dual Regulation of TNF-α-induced CCL2/Monocyte Chemoattractant Protein-1 Expression in Vascular Smooth Muscle Cells by NF-κB and AP-1: Modulation by Type III Phosphodiesterase Inhibition

Yung-Ming Chen, Wen-Chih Chiang, Shuei-Liong Lin, Kwan-Dun Wu, Tun-Jun Tsai, and Bor-Shen Hsieh

Department of Medicine, National Taiwan University Hospital and College of Medicine National Taiwan University, Taipei, Taiwan

This work was supported by grants from the National Taiwan University Hospital 93S027 (to Y-M Chen), the Ta-Tung Kidney Foundation, and the Mrs. Hsiu-Chin Lee Kidney Research Fund, Taipei, Taiwan.

Running title: Cilostamide and pentoxifylline inhibit CCL2/MCP-1

Address Correspondence to Dr. Kwan-Dun Wu, Department of Medicine, National Taiwan University Hospital, No. 7, Chung-Shan South Road, Taipei, 10016, Taiwan

E-mail: kdw@ha.mc.ntu.edu.tw
TEL: (02) 2312-3456 ext 5014; FAX: (02) 2322-2955

Number of text pages: 28
Number of tables: 0
Number of figures: 7
Number of references: 44
Number of words in the *Abstract*: 246
Number of words in the *Introduction*: 720
Number of words in the *Discussion*: 1090

Abbreviations: AP-1, activator protein-1; CCL2/MCP-1, CC chemokine ligand 2/monocyte chemoattractant protein-1; CCR2, chemokine receptor 2; DMEM, Dulbecco's modified Eagle's media; FCS, fetal calf serum; FCSA, fetal calf serum albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; I-κB, inhibitory protein of NF-κB; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; MG132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; NF-κB, nuclear factor-kappaB; PBS, phosphate-buffered saline; PD98059, 2-(2-amino-3-methoxyphenyl)-*4H*-1-benzopyran-4-one; PDE, phosphodiesterase; PTX, pentoxifylline; RT-PCR, reverse transcription-polymerase chain reaction; SB203580, 4-[5-(4-fluorophenyl)-2-[4-(methylsulfonyl)phenyl]-*1H*-imidazol-4-yl]pyridine; SP600125, anthra[1-9-cd]pyrazol-6(*2H*)-one; TNF, tumor necrosis factor; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene; VSMC, vascular smooth muscle cell.

Recommended section assignment: Cardiovascular

JPET Fast Forward. Published on February 20, 2004 as DOI: 10.1124/jpet.103.062620
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Monocytes/macrophages infiltration to the subendothelial space of arterial wall is a critical initial step in atherogenesis, in which CCL2/monocyte chemoattractant protein-1 (MCP-1) is thought to play a key role. This study investigated the effectiveness of phosphodiesterase inhibitors, including the nonselective pentoxifylline (PTX), and the selective type III (cilostamide) and type IV (denbufylline) inhibitors, on cytokine-induced CCL2/MCP-1 production in cultured rat vascular smooth muscle cells (VSMCs), and the signal transduction mechanisms whereby they act. Our results showed that tumor necrosis factor (TNF)-α induced a marked increase in CCL2/MCP-1 production in dose- and time-dependent manners. PD98059, U0126 (both inhibitors of p42/44 mitogen-activated protein kinase (MAPK) kinase) and SP600125 (an inhibitor of c-Jun NH_2-terminal kinases (JNK)) attenuated TNF-α-induced CCL2/MCP-1 production, without affecting I-κBα degradation or p65/NFκB nuclear trnaslocation. PD98059 abolished TNF-α-activated p42/44 MAPK phosphorylation and c-Fos upregulation, whereas SP600125 inhibited TNF-α-activated JNK and c-Jun phosphorylation. The NF-κB inhibitor, MG132, attenuated TNF-α-induced CCL2/MCP-1 production in the presence of increased phospho-JNK and phospho-c-Jun levels. When SP600125 was added simultaneously, MG132 completely inhibited TNF-α-induced CCL2/MCP-1 production. Finally, the pretreatment of VSMCs with PTX or cilostamide, but not denbufylline, reduced TNF-α-induced CCL2/MCP-1 production, which was preceded by attenuation of p65/NF-κB nuclear trnaslocation, p42/44 MAPK and JNK-c-Jun phosphorylation, and c-Fos upregulation. These data indicate that TNF-α-stimulated CCL2/MCP-1 production in rat VSMC is dually regulated by AP-1 and NF-κB pathways, and inhibition of type III PDE contributes substantially to the suppressive effect of PTX on CCL2/MCP-1 production via down-regulation of AP-1 and NF-κB signals.

Monocyte infiltration and accumulation in the subendothelial space of the arterial wall is a prominent pathobiologic feature in early atherogenesis (Ross, 1999), in which chemokines are thought to play a key role (Reape et al., 1999; Gerszten et al., 2000). Among these, the cytokine-induced CCL2/monocyte chemoattractant protein-1 (MCP-1) is noteworthy for its ability to promote migration of monocytes harboring its cognate receptor, chemokine receptor 2 (CCR2) (Reape et al., 1999). A growing body of evidence indicates that local overexpression of CCL2/MCP-1 by infiltrating monocytes or vascular cells induce accumulation of monocytes/macrophages and formation of atherosclerotic lesion, which appears to synergize with hypercholesterolemia (Namiki et al., 2002; Ikeda et al., 2002). Parallel to these findings, expression of CCR2 has also been documented in experimental atherosclerotic lesions in cholesterol-fed rabbits (Ohtsuki et al., 2001). Further *in vivo* studies have shown that genetically altered mice deficient for CCR2 and CCL2/MCP-1 are protected from development of atherosclerosis (Peters and Charo, 2001), and that anti-CCL2/MCP-1 gene therapy attenuates atherosclerosis in apolipoprotein E-knockout mice (Ni et al., 2001). In humans, CCL2/MCP-1 polymorphism (2518 G/G high MCP-1 producer genotype) has been implicated with elevated lipoprotein(a) levels and an increased susceptibility to coronary artery disease (Szalai et al., 2001). Taken together, these data support a critical role of the MCP-1/CCR2 system for atherogenesis.

CCL2/MCP-1 was originally discovered from mononuclear leukocytes (Robinson et al., 1989; Yoshimura et al., 1991), but is now known to be produced by many non-immune cells, including vascular smooth muscle cells (VSMCs) (Ortego et al., 1999; Iseki et al., 2000; de Keulenaer et al., 2000). Its expression is regulated by a number of stimuli, most notably the proinflammatory cytokine tumor necrosis factor (TNF)-α (Libby & Galis, 1995). Many of the cellular responses induced by TNF-α require cytoplasmic signals transduced to two major transcription factors, nuclear factor-kappaB (NF-κB) and activator protein (AP)-1 (Karin, 1995; Kyriakis, 1999; Leong & Karsan, 2000; Baud & Karin, 2001). NF-κB is consist of a homo- or

heterodimer formed from five possible subunits of the Rel family, with the p65/p50 heterdimer being the most common form. Its activation by TNF-α is associated with phosphorylation of inhibitory protein of NF-κB (I-κB) by I-κB kinase complexes, degradation of the phosphorylated I-κB, and translocation of the freed NF-κB to the nucleus, which triggers proinflammatory and anti-apoptotic gene expression (Leong & Karsan, 2000; Baud & Karin, 2001). By contrast, activation of AP-1 upon TNF-α treatment is regulated by three of the five known mammalian mitogen-activated protein kinases (MAPKs), the p42/44 MAPK, p38 MAPK and c-Jun NH_2-terminal kinase (JNK). Upon stimulation, these protein kinases enter the nucleus to induce or phosphorylate subunits of AP-1, including Jun and Fos proteins. The resultant enhanced AP-1 activity can then participate in the regulation of genes involved in inflammation and cell survival (Karin et al., 1995; Kyriakis, 1999). In cultured rat VSMCs, TNF-α has been shown to induce CCL2/MCP-1 expression via activation of the NF-κB signals (Ortego et al., 1999; Iseki et al., 2000), and the p42/44 MAPK pathways (de Keulenaer et al., 2000). However, information is limited regarding the role of AP-1 and other MAPK members in TNF-α-dependent CCL2/MCP-1 expression in VSMCs. We hypothesize that TNF-α may act on multiple kinase cascades which converge to AP-1 or NF-κB signals and activate CCL2/MCP-1 expression. Thus, measures capable of modulating these signaling pathways and the resultant CCL2/MCP-1 production may have therapeutic value in the prevention and treatment of atherosclerotic vascular diseases.

Recently, we have shown that pentoxifylline (PTX), a nonselective phosphodiesterase (PDE) inhibitor, is capable of suppressing the production of CX3CL1/fractalkine in TNF-α-activated VSMCs via down-regulation of the p42/44 MAPK and NF-κB signals (Chen et al., 2003). In VSMCs, there exist at least four PDE isozymes (types I, III, IV and V) (Rabe et al., 1994; Pauvert et al., 2000). Among these, the types III and IV PDE isozymes hydrolyse only cAMP, the type V PDE hydrolyzes only cGMP, and the type I PDE accepts both nucleotides as a substrate (Dousa, 1999). Based on our previous work that the incubation of VSMCs with PTX

leads to an increase in intracellular cAMP but not cGMP levels (Chen et al., 1999), it is speculated that PTX acts predominantly by inhibiting type III or IV PDE in VSMCs. In this study, we investigated (1) the role of the MAPK, NF-κB, and AP-1 pathways in TNF-α-dependent CCL2/MCP-1 production; (2) the effectiveness of PTX, cilostamide (a selective type III PDE inhibitor), and denbufylline (a selective type IV PDE inhibitor) on TNF-α-induced CCL2/MCP-1 production; and (3) the underlying signal transduction pathways whereby these PDE inhibitors act.

JPET Fast Forward. Published on February 20, 2004 as DOI: 10.1124/jpet.103.062620
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods

Reagents

Dulbecco's modified Eagle's media (DMEM), penicillin/streptomycin, fetal calf serum (FCS), and other tissue culture reagents were purchased from Gibco BRL (Rockville, MD, USA). The FCS was heat-inactivated before use. Culture flasks and plates were purchased from Costa Corning (Cambridge, MA, USA). Cilostamide and PTX were purchased from Sigma (St. Louis, MO, USA). Denbufylline, PD98059, U0126, SB203580, and MG132 were obtained from Calbiochem (La Jolla, CA, USA). SP600125 was purchased from Tocris Cookson Ltd (Avonmouth, UK). Recombinant rat TNF-α was obtained from R & D Systems (Minneapolis, MN, USA). Rabbit anti-rat MCP-1 was purchased from PetroTech EC LTD (London, UK). Rabbit anti-p44/42 MAPK**,** and mouse anti-phosphorylated p44/42 MAPK were obtained from New England BioLab (Beverly, MA, USA). Mouse anti-phosphorylated JNK and phosphorylated c-Jun, and rabbit anti-JNK and anti-c-Jun, anti-c-Fos, anti-p65/NF-κB and anti-I-κBα were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Mouse anti-β-actin was obtained from Sigma. All chemicals used for total RNA isolation, reverse transcription-polymerase chain reaction, Northern blot analysis, whole cell lysate extraction, and Western blot analysis were of molecular grade and were obtained from Sigma or Roche Molecular Biochemicals (Mannheim, Germany) unless otherwise specified.

Cell culture and experimental conditions

Primary culturing of rat aortic VSMCs was performed and characterized as described previously (Chen et al., 1999). Briefly, the thoracic aortas were removed from male Sprague Dawley rats immediately after the rats were decapitated. The endothelium and adventitia were removed gently by scratching with forceps. Specimens were cut into 5 mm rings and digested in Hank's balanced salt solution containing 15 mM HEPES, 0.2 mM $CaCl_2$, 1 mg/ml collagenase, 0.125 mg/ml elastase, 0.375 mg/ml soybean trypsin inhibitor and 2 mg/ml bovine serum albumin

JPET Fast Forward. Published on February 20, 2004 as DOI: 10.1124/jpet.103.062620
This article has not been copyedited and formatted. The final version may differ from this version.

at 37°C for 90 minutes. The digested tissue was strained through a 180 μm steel mesh. After centrifugation at 200 x g for 5minutes, the cells were resuspended in DMEM containing 20% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were then seeded on flasks at a density of 1 x 10^4 cells/cm^2 and incubated at 37°C in a humidified 5% CO_2-95% O_2 air atmosphere. Cells were confirmed to be VSMC by morphological criteria, by the presence of smooth muscle α-actin staining, and by the absence of staining for cytokeratin and factor VIII-related antigen with the avidin-biotin-peroxidase method. Cells between 10-20 passages were used and grown in DMEM containing 10% FCS. To determine the regulatory role of various MAPKs on TNF-α-stimulated CCL2/MCP-1 expression by VSMCs, cells were first grown in DMEM containing 10% FCS until reaching 90% confluency. The medium was then replaced by DMEM containing 0.5% FCS for 24 hours before treatment with a p44/42 MAPK kinase inhibitor PD98059 or U0126 (10-40 μM and 5-20 μM, respectively, for 30 minutes), a p38 MAPK inhibitor SB203580 (5-20 μM for 30 minutes), or a broad-spectrum JNK inhibitor SP600125 (5-20 μM for 30 minutes). After preincubation, cells were stimulated with TNF-α (5 ng/ml) for 4 or 24 hours. Further experiments were conducted to examine the role of NF-κB and AP-1 on TNF-α-stimulated CCL2/MCP-1 expression by VSMCs. The NF-κB inhibitor, MG132 (5-20 μM), and the c-Jun/AP-1 inhibitor, SP600125 (5-20 μM) were incubated with VSMCs for 1.5 hours and 30 minutes, respectively. Then, cells were stimulated with TNF-α (5 ng/ml) for 7.5 to 120 minutes for Western blot analysis (signal transduction pathways) and immunocytochemistry (nuclear p65/NF-kB), for 4 hours for Northern blot analysis (CCL2/MCP-1 mRNA), or for 24 hours for Western blot analysis (CCL2/MCP-1 protein).

Additional studies were designed to examine the role of PDE inhibitors in VSMC CCL2/MCP-1 expression. Cells were preincubated with PTX (0.5-2 mM) for 45 minutes, or cilostamide (10-40 μM) or denbufylline (10-40 μM) for 30 minutes, followed by TNF-α (5 ng/ml) for 7.5 to 120 minutes, 4 hours, or 24 hours. After incubation for the given periods, cell

JPET Fast Forward. Published on February 20, 2004 as DOI: 10.1124/jpet.103.062620
This article has not been copyedited and formatted. The final version may differ from this version.

monolayers were washed and used for Northern blot analysis (4-hour stimulation) or Western blot analysis (7.5 to 120-minute or 24-hour stimulation).

In all experiments mentioned above, corresponding controls were performed by incubating VSMCs with vehicles (either dimethyl sulfoxide or phosphate-buffered saline, PBS) wherever appropriate.

Northern blot analysis

Total RNA was extracted by the acid guanidinium thiocyanate phenol chloroform method as described previously (Chen et al., 1999). Ten μg of total RNA were electrophoresed on formaldehyde-denatured 1% agarose gels and subsequently transferred to nylon membranes. To synthesize riboprobes for Northern blot hybridization, cDNA fragments of rat CCL2/MCP-1, c-*jun*, c-*fos*, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were first amplified by reverse transcription-polymerase chain reaction (RT-PCR) from glomerular RNA of Wistar rats using the following specific primer pairs: rat CCL2/MCP-1, upstream 5'-TCAGCCAGATGCAGTTAATG-3', downstream 5'-TTCTCTGTCATACTGGTCAC-3' (GeneBank accession number, M57441), rat c-*jun*, upstream 5'-TTCTGAAGCAGAGCATGACC-3', downstream, 5'-TTGAAGTTGCTGAGGTTGGC-3' (GeneBank accession number, X17163), rat c-*fos*, upstream, 5'-GCCTTTCCTACTACCATTCC-3', downstream, AGTTGATCTGTCTCCGCTTG-3' (GeneBank accession number, X06769), and rat GAPDH, upstream 5'-tcattgacctcaactacatg-3', downstream 5'-caaagttgtcatggatgacc-3' (GeneBank accession number, NM_017008). RT-PCR was performed as described previously (Chen et al., 2003). The amplified products were eluted from polyacrylamide gels, and subcloned into pGEM-T vectors (Promega). The accuracy of the inserts were confirmed by sequence analysis, and the cloned cDNAs were linearized and used as templates for *in vitro* transcription of antisense digoxigenin-conjugated riboprobes, following the manufacturer's instructions (Roche Molecular Biochemicals). After hybridization, the blots were developed using CSPD® (Roche Molecular Biochemicals) as the substrate for alkaline

phosphatase. The intensity of the signal was then quantified with computerized densitometry, and normalized against the signal of GAPDH messages.

Western blot analysis

VSMCs were washed with ice-cold 1x PBS and lysed at 4°C for 15 minutes in lysis buffer (pH 7.4) containing 50 mM Tris HCl, 150 mM NaCl, 1% Igepal CA-630 (Sigma), 0.25% sodium deoxycholate (Sigma), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 1 μg/mL each for aprotinin, leupeptin and pepstatin. Thirty μg of cell lysates were heated at 100°C for 10 minutes, applied to 9% SDS-polyacrylamide gels, and electrophoresed for detection of p44/42 MAPK, JNK, c-Jun, c-Fos, IκBα and β-actin. To detect CCL2/MCP-1, the conditioned media of TNF-α-activated VSMCs were concentrated with Centricon-10® (Millipore, Bedford, MA, USA), and 20 μg of protein were electrophoresed on 12% SDS-polyacrylamide gels. A prestained marker was also electrophoresed as a molecular weight marker. After electrophoresis, proteins were transferred onto a PVDF membrane (Millipore) using a transblot chamber with Tris buffer. Western blots were incubated at 4°C overnight with primary antibodies. The next morning, membranes were washed with 1x PBS/5% Tween-20 at room temperature for 40 minutes, and incubated with peroxidase-conjugated second antibodies at room temperature for 1 hour. After washing, the membranes were incubated with Renaissance® (NEN™ Life Science, MA, USA) according to the manufacturer's instructions. The intensity of the signal was then quantified with computerized densitometry, and normalized against the signal of β-actin.

Immunocytochemistry

To demonstrate p65/ NF-κB nuclear translocation, VSMCs, with or without pretreatment with the given inhibitors, were incubated with TNF-α (5 ng/mL) or vehicles for 7.5, 15, or 30 minutes before fixation with 4% paraformaldehyde for 1 hour at 4°C. The cells were then washed by 1x PBS/0.2% TritonX-100 for 15 minutes and incubated with a rabbit anti-p65/NF-κB

antibody at 4°C overnight. The next day, after washing for 15 minutes, the cells were incubated with biotin-conjugated anti-rabbit IgG at room temperature for 1 hour. Then, the cells were washed and incubated with the avidin-biotin-peroxidase reagent (Dakopatts, Glostrup, Denmark) at room temperature for 1 hour. After washing, immunodetection for p65/NF-κB was performed by adding 3-amino-9-ethylcarbazole chromogen as substrate according to the manufacturer's instructions.

Statistics

Data are expressed as mean ± sem. All comparisons were done by analysis of variance using the StatView® package for the Macintosh computer (Abacus Concepts, CA, USA). A probability value of less than 0.05 was considered statistically significant.

JPET Fast Forward. Published on February 20, 2004 as DOI: 10.1124/jpet.103.062620
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Induction of CCL2/MCP-1 mRNA and protein expression by TNF-α. The incubation of VSMCs with different concentrations of TNF-α (0.5 to 50 ng/ml) for varying time periods (2 to 24 hours) resulted in marked upregulation of a single 0.9 kb CCL2/MCP-1 mRNA species and approximately 16 to 30 kDa CCL2/MCP-1 proteins in time- and dose-dependent manners (Fig. 1). The presence of several molecular masses of CCL2/MCP-1 protein probably represent different degrees of glycosylation as reported previously (Satriano et al., 1993; Rovin et al., 1994).

Roles of MAPKs in TNF-α-induced CCL2/MCP-1 mRNA and protein expression. To examine the roles of p42/44 MAPK, p38 MAPK, and JNK in TNF-α-dependent CCL2/MCP-1 production, VSMCs were incubated with PD98059, an inhibitor of p42/44 MAPK kinase (Means et al, 2000), SB203580, an inhibitor of p38 MAPK (Davies et al, 2000), or SP600125, an inhibitor of JNK (Bae & Song, 2003). The results showed that PD98059 (10-40 μM) and SP600125 (5-20 μM) dose-dependently attenuated TNF-α-stimulated CCL2/MCP-1 mRNA induction (Figs. 2A). The incubation of VSMCs with another potent inhibitor of p42/44 MAPK kinase, U0126, also resulted in dose-dependnet (5-20 μM) attenuation of MCP-1 gene and protein expression (data not shown). When VSMCs were pretreated with both PD98059 and SP600125 in combination, TNF-α-induced CCL2/MCP-1 mRNA expression was additively inhibited (Fig. 2B). These effects of PD98059 and SP600125 were also confirmed at the protein level (Fig. 2B). By contrast, SB203580 (5-20 μM) did not have discernible effects on TNF-α-induced CCL2/MCP-1 mRNA expression (Fig. 2A).

Additional experiments were performed to examine the differential effects of PD98059 and SP600125 on TNF-α-activated signaling cascades. The pretreatment of PD98059 (20 μM) abolished TNF-α-activated p42/44 MAPK phosphorylation and c-Fos upregulation without affecting JNK-c-Jun phosphorylation, I-κBα degradation, or p65/NFκB nuclear trnaslocation (Figs. 3A and C). The preincubation of VSMCs with U0126 (10 μM) resulted in a similar pattern

JPET Fast Forward. Published on February 20, 2004 as DOI: 10.1124/jpet.103.062620
This article has not been copyedited and formatted. The final version may differ from this version.

of inhibition in TNF-α-activated signaling pathways as did PD98059 (data not shown). By contrast, the pretreatment of VSMC with SP600125 (10 μM) completely inhibited TNF-α-activated phosphorylation of JNK2/3 and c-Jun, without affecting p42/44 MAPK phosphorylation, c-Fos upregulation, I-κBα degradation, or p65/NFκB nuclear trnaslocation (Figs. 3B and C).

Roles of NF-κB and c-Jun/AP-1 in TNF-α-Induced CCL2/MCP-1 mRNA and protein expression. The ubiquitin/proteosome inhibitor, MG132 (Nakayama *et al.*, 2001), was used to examine the role of NF-κB in TNF-α-dependent CCL2/MCP-1 production. Fig. 4A shows that preincubation of VSMCs with MG132 (5-20 μM) dose-dependently attenuated TNF-α-stimulated CCL2/MCP-1 mRNA expression (Fig. 4A). When VSMCs were preincubated with SP600125 (10 μM) and MG132 (20 μM) in combination, TNF-α-induced CCL2/MCP-1 production was completely inhibited (Figs. 4B and C). At the concentrations used in this study, our previous work has shown that MG132 abolishes TNF-α-induced p65/NF-κB nuclear translocation in the presence of an augmented phospho-c-Jun level (Chen et al., 2003). This study further demonstrates that MG132 augmented TNF-α-induced JNK2/3 phosphorylation without affecting c-Fos upregulation (Fig.5). These results are consistent with our current knowledge that MG132, besides antagonizing NF-κB activity, also activates the JNK-c-Jun/AP-1 cascade (Nakayama *et al.*, 2001). The MG132-augmented JNK and c-Jun phosphorylation was abolished by the addition of the JNK-c-Jun inhibitor, SP600125 (10 μM) (Fig. 5).

Effects of PDE Inhibitors on TNF-α-Induced CCL2/MCP-1 mRNA and protein expression. Fig. 6A shows that pretreatment of VSMCs with PTX (0.5-2 mM) and cilostamide (10-40 μM) but not denbufylline (10-40 μM), dose-dependently attenuated TNF-α-stimulated CCL2/MCP-1 mRNA expression. Consistent with that finding, cilostamide (40 μM) and PTX (2 mM), but not denbufylline (40 μM), attenuated TNF-α-stimulated CCL2/MCP-1 protein

JPET Fast Forward. Published on February 20, 2004 as DOI: 10.1124/jpet.103.062620
This article has not been copyedited and formatted. The final version may differ from this version.

production (Fig. 6B).

To explore the underlying mechanisms whereby PTX and cilostamide act, VSMCs were pretreated with PTX (2 mM) or cilostamide (40 μM), followed by TNF-α (5 ng/mL) stimulation for various time points. The results showed that both PTX and cilostamide partially prevented TNF-α-induced degradation of I-κBα and nuclear translocation of p65/NF-κB (Figs. 7A, B and G). Additionally, PTX and cilostamide inhibited TNF-α-stimulated c-*fos* and c-*jun* mRNA expression (Figs. 7D and E), attenuated TNF-α-activated p42/44 MAPK phosphorylation and c-Fos upregulation, and suppressed TNF-α-induced JNK2/3 and c-Jun phosphorylation (Figs. 7A and B). By contrast, the pretreatment of VSMCs with denbyfylline, while attenuating TNF-α-activated p42/44 MAPK phosphorylation, did not affect the other TNF-α-dependent signaling pathways or TNF-α-induced c-*fos* and c-*jun* mRNA expression (Figs. 7C, F and G).

JPET Fast Forward. Published on February 20, 2004 as DOI: 10.1124/jpet.103.062620
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

The present study demonstrates that cultured rat VSMCs at steady-state expressed a very low level of CCL2/MCP-1 mRNA and protein, which could be up-regulated substantially by TNF-α in both time- and dose-dependent manners. This finding is consistent with previous reports which show CCL2/MCP-1 induction by proinflammatory cytokines in a variety of non-immune tissue cells, including VSMCs (Hurwitz et al., 1995; Parry et al., 1998; Ortego et al., 1999; Iseki et al., 2000; de Keulenaer et al., 2000; Momoi et al., 2001; Chen et al., 2001; Loghmani et al., 2002).

The intracellular signaling pathways leading to TNF-α-dependent CCL2/MCP-1 expression may include those that activate protein kinases such as p42/44 MAPK, p38 MAPK, and JNK (Leong & Karsan, 2000). This study shows that TNF-α-stimulated CCL2/MCP-1 mRNA and protein expression was attenuated by pharmacologic inhibitors of p42/44 MAPK kinase (PD98059 or U0126), but not p38 MAPK (SB203580), suggesting that activation of the p42/44 MAPK pathway is involved in TNF-α-stimulated CCL2/MCP-1 expression in rat VSMCs in culture. This finding is consistent with that reported by de Keulenaer et al. (2000). However, in other rat cell types or in human cell systems, p38 MAPK instead of p42/44 MAPK was reported to mediate the induction of CCL2/MCP-1 elicited by TNF-α (Rovin et al., 1999; Goebeler et al., 1999; Blinman et al., 2000). Thus, the MAPK signals mediating TNF-α-dependent CCL2/MCP-1 expression may vary by cell types and species. Besides p42/44 and p38 MAPKs, the present study shows that SP600125, a potent JNK inhibitor, attenuated TNF-α-induced CCL2/MCP-1 expression. This suggests that the JNK pathway may also participate in TNF-α-dependent CCL2/MCP-1 expression in VSMC. Because PD98059 and SP600125 inhibited distinct TNF-α-dependent MAPK pathways, we surmise that both the p42/44 MAPK and the JNK pathways coordinately modulate TNF-α-induced CCL2/MCP-1 expression in rat VSMCs. In support for this notion, the pretreatment of VSMCs with PD98059 and SP600125 in combination resulted in additive inhibition of TNF-α-induced CCL2/MCP-1 expression than either agent

alone.

Many of the cellular responses induced by TNF-α require intracellular signals transduced to two major transcription factors, NF-κB and AP-1 (Karin, 1995; Kyriakis, 1999; Leong & Karsan, 2000; Baud & Karin, 2001). Upon stimulation with TNF-α, activated p42/44 MAPK and JNK can enter the nucleus to induce expression of c-Fos and c-Jun proteins, respectively. These proteins then combine to form c-Jun-based AP-1 complexes. In addition, the JNKs, especially JNK2 isoform, can efficiently phosphorylate the N-terminal sites of c-Jun and enhance AP-1 transcriptional activity (Kallunki et al., 1994). In this study, we show that pretreatment with SP600125 completely inhibited TNF-α-activated phosphorylation of JNK2/3 and c-Jun. This is consistent with our current understanding that SP600125 is a potent inhibitor for the JNK-c-Jun pathway (Bennett et al., 2001). By contrast, PD98059 was found to abolish TNF-α-induced p42/44 MAPK phosphorylation and the resultant c-Fos upregulation. Because neither SP600125 nor PD98059 affected TNF-α-induced p65/NF-κB nuclear translocation, their anti-CCL2/MCP-1 action might be mediated predominantly through down-regulation of AP-1 activity. However, AP-1 proteins are known to interact with NF-κB and modulate κB-dependent gene transcription (Kyriakis, 1999), the possibility that SP600125 and PD98059 may act in part through attenuation of nuclear NF-κB activity cannot be ruled out completely.

Activation of NF-κB by TNF-α involves phosphorylation-dependent ubiquitination and degradation of I-κB proteins, which normally trap NF-κB within the cytoplasm of quiescent cells (Chen & Goeddel, 2002). Freed NF-κB can enter the nucleus which in turn regulates the transactivation of κB-dependent genes involved in a variety of inflammatory disorders, including atherosclerosis (Barnes & Karin, 1997; Bourcier *et al.*, 1997). This study shows that the ubiquitin/proteosome inhibitor, MG132, attenuated but did not abolish TNF-α-stimulated CCL2/MCP-1 production. The incomplete inhibition might be explained in part by the realization

that MG132 also activates the c-Jun/AP-1 pathway, which by itself could lead to transcriptional induction of CCL2/MCP-1 (Nakayama *et al*., 2001). When the activated c-Jun/AP-1 signal was blocked by simultaneous pretreatment of SP600125, MG132 completely inhibited TNF-α-stimulated CCL2/MCP-1 induction. Taken together, these findings indicate that TNF-α-induced CCL2/MCP-1 production in rat VSMCs is dually regulated by both AP-1 and NF-κB pathways.

This study shows that PTX, a nonselective PDE inhibitor, suppressed TNF-α-induced CCL2/MCP-1 production that was preceded by attenuation of TNF-α-activated phosphorylation of p42/44 MAPK, JNK2/3 and c-Jun, inhibition of TNF-α-upregulated c-Fos expression, as well as attenuation of TNF-α-induced degradation of I-κBα and nuclear translocation of p65/NF-κB. Thus, the anti-CCL2/MCP-1 activity of PTX appears to be mediated by its ability to counteract both AP-1 and NF-κB activation triggered by TNF-α. This study further demonstrates that the selective type III PDE inhibitor, cilostamide, but not the type IV inhibitor, denbufylline, attenuated TNF-α-stimulated CCL2/MCP-1 induction and down-regulated TNF-α-dependent signaling pathways, similarly to that achieved by PTX treatment. This suggests that type III PDE may be a major, if not the only, target whereby PTX acts to suppress TNF-α-stimulated CCL2/MCP-1 induction. Our result was consistent in part with that observed by Aizawa et al (2003) who showed that inhibition of PDE3 by cilostamide activated PKA and dose-dependently decreased TNF-α-induced NF-κB-dependent reporter gene expression in VSMC. In view of the critical role of CCL2/MCP-1 in atherogenesis, the ability of PTX and cilostamide to antagonize cytokine-induced CCL2/MCP-1 expression may have implications in the prevention and treatment of atherosclerotic vascular disorders.

In summary, TNF-α-stimulated CCL2/MCP-1 induction in rat VSMC is dually regulated by AP-1 and NF-κB pathways. Inhibition of type III PDE may contribute substantially to the

suppressive effect of PTX on TNF-α-dependent CCL2/MCP-1 production via down-regulation of AP-1 and NF-κB signals.

Acknowledgments

We thank Ms. Yi-Hsuan Lee for excellent technical assistance.

References

Aizawa T, Wei H, Miano JM, Abe J, Berk BC, and Yan C (2003) Role of phosphodiesterase 3 in NO/cGMP-mediated antiinflammatory effects in vascular smooth muscle cells. *Circ Res* **93**:406-413.

Bae MA and Song BJ (2003) Critical role of c-Jun N-terminal protein kinase activation in troglitazone-induced apoptosis of human HepG2 hepatoma cells. *Mol Pharmacol* **63**:401-408.

Barnes PJ and Karin S (1997) Nuclear factor-κB: A pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* **336**:1066-1071.

Baud V and Karin M (2001) Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol* **11**:372-377.

Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, and Anderson DW (2001) SP600125, an anthrapyrazolone inhibitor of Jun N-ternimal kinase. *Proc Natl Acad Sci USA* **98**:13681-13686.

Blinman TA, Gukovsky I, Mouria M, Zaninovic V, Livingston E, Pandol SJ, and Gukovskaya AS (2000) Activation of pancreatic acinar cells on isolation from tissue: cytokine upregulation via p38 MAP kinase. *Am J Physiol Cell Physiol* **279**:C1993-2003.

Bourcier T, Sukhova G, and Libby P (1997) The nuclear factor kappa-B signaling pathway participates in dysregulation of vascular smooth muscle cells in vitro and in human atherosclerosis. *J Biol Chem* **272**:15817-15824.

Chen G and Goeddel DV (2002) TNF-R1 signaling: a beautiful pathway. *Science* **296**:1634-1635.

Chen MC, Proost P, Gysemans C, Mathieu C, and Eizirik DL (2001) Monocyte chemoattractant protein-1 is expressed in pancreatic islets from prediabetic NOD mice and in interleukin-1 beta-exposed human and rat islet cells. *Diabetologia* **44**:325-332.

Chen Y-M, Wu K-D, Tsai T-J, Hsieh B-S (1999) Pentoxifylline inhibits PDGF-induced proliferation of and TGF-β-stimulated collagen synthesis by vascular smooth muscle cells. *J Mol*

Cell Cardiol **31**:773-783.

Chen Y-M, Tu C-J, Hung K-Y, Wu K-D, Tsai T-J, and Hsieh B-S (2003) Inhibition by pentoxifylline of TNF-α-stimulated fractalkine production in vascular smooth muscle cells: evidence for mediation by NF-κB down-regulation. *Br J Pharmacol* **138**:950-958.

Davies SP, Reddy H, Caivano M, and Cohen P (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* **351**:95-105.

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

Dousa TP (1999) Cyclic-3',5'-nucleotide phosphodiesterase isozymes in cell biology and pathophysiology of the kidney. *Kidney Int* **55**:29-62.

Gerszten RE, Mach F, Sauty A, Rosenzweig A, and Luster AD (2000) Chemokines, leukocytes, and atherosclerosis. *J Lab Clin Med* **136**:87-92.

Goebeler M, Kilian K, Gillitzer R, Kunz M, Yoshimura T, Brocker EB, Rapp UR, and Ludwig S (1999) The MKK6/p38 stress kinase cascade is critical for tumor necrosis factor-α-induced expression of monocyte-chemoattractant protein-1 in endothelial cells. *Blood* **93**:857-865.

Hurwitz AA, Lyman WD, and Berman JW (1995) Tumor necrosis factor α and transforming growth factor β upregulate astrocyte expression of monocyte chemoattractant protein-1. *J Neuroimmunol* **57**:193-198.

Ikeda U, Matsui K, Murakami Y, and Shimada K (2002) Monocyte chemoattractant protein-1 and coronary artery disease. *Clin Cardiol* **25**: 143-147.

Iseki A, Kambe F, Okumura K, Niwata S, Yamamoto R, Hayakawa T, and Seo H (2000) Pyrrolidine dithiocarbamate inhibits TNF-α-dependent activation of NF-κB by increasing intracellular copper level in human aortic smooth muscle cells. *Biochem Biophys Res Commun*

276:88-92.

Kallunki T, Su B, Tsigelny I, Sluss HK, Derijard B, Moore G, Davis R, and Karin M (1994) JNK2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation. *Genes Dev* **8**:2996-3007.

Karin M (1995) The regulation of AP-1 activity by mitogen-activated protein kinases. *J Biol Chem* **270**:16483-16486.

Kyriakis JM (1999) Activation of the AP-1 transcription factor by inflammatory cytokines of the TNF family. *Gene Express* **7**:217-231.

Leong KG and Karsan A (2000) Signalling pathways mediated by tumor necrosis factor α. *Histol Histopathol* **15**:1303-1325.

Libby P and Galis ZS (1995) Cytokines regulate genes involved in atherosclerosis. *Ann N Y Acad Sci* **748**:158-170.

Loghmani F, Mohammed KA, Nasreen N, Van Horn RD, Hardwick JA, Sanders KL, and Antony VB (2002) Inflammatory cytokines mediate C-C (monocyte chemotactic protein 1) and C-X-C (interleukin 8) chemokine expression in human pleural fibroblasts. *Inflammation* **26**:73-82.

Means TK, Pavlovich RP, Roca D, Vermeulen MW, and Fenton MJ (2000) Activation of TNF-alpha transcription utilizes distinct MAP kinase pathways in different macrophage populations. *J Leukoc Biol* **67**:885-893.

Momoi A, Murao K, Imachi H, Ishida T, Cao WM, Sato M, and Takahara J (2001) Inhibition of monocyte chemoattractant protein-1 expression in cytokine-treated human lung epithelial cells by thiazolidinedione. *Chest* **120**:1293-1300.

Nakayama K, Furusu A, Xu Q, Konta T, and Kitamura M (2001) Unexpected transcriptional induction of monocyte chemoattractant protein 1 by proteosome inhibition: Involvement of the c-Jun N-terminal kinase-activator protein 1 pathway. *J Immunol* **167**:1145-1150.

Namiki M, Kawashima S, Yamashita T, Ozaki M, Hirase T, Ishida T, Inoue N, Hirata K,

Matsukawa A, Morishita R, Kaneda Y, and Yokoyama M (2002) Local overexpression of monocyte chemoattractant protein-1 at vessel wall induces infiltration of macrophages and formation of atherosclerotic lesion: synergism with hypercholesterolemia. *Arterioscler Thromb Vasc Biol* **22**: 115-120.

Ni W, Egashira K, Kitamoto S, Kataoka C, Koyanagi M, Inoue S, Imaizumi K, Akiyama C, Nishida KI, and Takeshita A (2001) New anti-monocyte chemoattractant protein-1 gene therapy attenuates atherosclerosis in apolipoprotein E-knockout mice. *Circulation* **103**: 2096-2101.

Ohtsuki K, Hayase M, Akashi K, Kopiwoda S, and Strauss HW (2001) Detection of monocyte chemoattractant protein-1 receptor expression in experimental atherosclerotic lesions: an autoradiographic study. *Circulation* **104**: 203-208.

Ortego M, Bustos C, Hernandez-Presa MA, Tunon J, Diaz C, Hernandez G, and Egiso J (1999) Atorvastatin reduces NF-kappaB activation and chemokine expression in vascular smooth muscle cells and mononuclear cells. *Arteriosclerosis* **147**:253-261.

Parry GC, Martin T, Felts KA, and Cobb RR (1998) IL-1beta-induced monocyte chemoattractant protein-1 gene expression in endothelial cells is blocked by proteosome inhibitors. *Arterioscler Thromb Vasc Biol* **18**:934-940.

Pauvert O, Salvail D, Rousseau E, Lugnier C, Marthan R, and Savineau JP (2002) Characterisation of cyclic nucleotide phosphodiesterase isoforms in the media layer of the main pulmonary artery. *Biochem Pharmacol* **63**:1763-1772.

Peters W and Charo IF (2001) Involvement of chemokine receptor 2 and its ligand, monocyte chemoattractant protein-1, in the development of atherosclerosis: lessons from knockout mice. *Curr Opin Lipidol* **12**: 175-180.

Rabe KF, Tenor H, Dent G, Schudt C, Nakashima M, and Magnussen H (1994) Identification of PDE isozymes in human pulmonary artery and effect of selective PDE inhibitors. *Am J Physiol* **266**:L536-L543.

Reape TJ and Groot PHE (1999) Chemoknes and atherosclerosis. *Atherosclerosis* **147**:213-225.

Robinson EA, Yoshimura T, Leonard EJ, Tanaka S, Griffen PR, Shabanowitz J, Hunt DF, and Appella E (1989) Complete amino acid sequence of a human monocyte chemoattractant, a putative mediator of cellular immune reactions. *Proc Natl Acad Sci USA* **86**:1850-1854.

Rovin BH, Rumancik M, Tan L, Dickerson J (1994) Glomerular expression of monocyte chemoattractant protein-1 in experimental and human glomerulonephritis. *Lab Invest* **71**:536-542.

Rovin BH, Wilmer WA, Danne M, Dickerson JA, Dixon CL, and Lu L (1999) The mitogrn-activated protein kinase p38 is necessary for interleukin 1β-induced monocyte chemoattractant protein 1 expression by human mesangial cells. *Cytokine* **11**:118-126.

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

Satriano JA, Hora K, ShanZ, Stanley ER, Mori T, and Schlondorff D (1993) Regulation of monocyte chemoattractant protein-1 and macrophage colony-stimulating factor-1 by IFN-γ, tumor necrosis factor-α, IgG aggregates, and cAMP in mouse mesangial cells. *J Immunol* **150**:1971-1978.

Szalai C, Duba J, Prohaszka Z, Kalina A, Szabo T, Nagy B, Horvath L, and Csaszar A (2001) Involvement of polymorphisms in the chemokine system in the susceptibility for coronary artery disease (CAD). Coincidence of elevated Lp(a) and MCP-1 -2518 G/G genotype in CAD patients. *Atherosclerosis* **158**:233-239.

Yoshimura T, Takeya M, and Takahashi K (1991) Molecular cloning of rat monocyte chemoattractant protein-(MCP-1) and its expression in rat spleen cells and tumor cell lines. *Biochem Biophys Res Commun* **174**:504-509.

Footnotes

(a) This work was supported by grants from the National Taiwan University Hospital 93S027 (to Y-M Chen), the Ta-Tung Kidney Foundation, and the Mrs. Hsiu-Chin Lee Kidney Research Fund, Taipei, Taiwan.

(b) Address Correspondence to: Dr. Kwan-Dun Wu, Department of Medicine, National Taiwan University Hospital, No. 7, Chung-Shan South Road, Taipei, 10016, Taiwan. E-mail: kdw@ha.mc.ntu.edu.tw.

JPET Fast Forward. Published on February 20, 2004 as DOI: 10.1124/jpet.103.062620
This article has not been copyedited and formatted. The final version may differ from this version.

Figure legends

Figure. 1 Expression of CCL2/MCP-1 mRNA and protein by TNF-α.

VSMCs were incubated with TNF-α for the given periods. Ten μg of total RNA and twenty μg of concentrated condition media per lane were analyzed for expression of CCL2/MCP-1 mRNA and protein, respectively, as described in **Materials and Methods**. (A) Representative time-course Northern blots for CCL2/MCP-1 mRNA in response to TNF-α (5 ng/mL). (B) Representative dose-response Northern blots for CCL2/MCP-1 mRNA in response to different concentrations of TNF-α at 4 hours. (C) Representative time-course and dose-response Western blots for CCL2/MCP-1 protein induced by TNF-α. Bar graphs show corresponding quantitative results of MCP-1/GAPDH mRNA and MCP-1/FCSA protein ratios relative to that of control. Values are mean ± sem of four experiments. *: $P<0.05$ vs control at zero time. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FCSA, fetal calf serum albumin.

Figure. 2 Roles of MAPK pathways in TNF-α-induced CCL2/MCP-1 mRNA and protein expression

VSMCs were incubated with TNF-α (5 ng/mL) for 4 or 24 hours, with or without pretreatment with the given pharmacologic inhibitors. (A) Representative Northern blots. Bar graphs show corresponding quantitative results corrected for GAPDH, and relative to that of control. Values are mean ± sem of four experiments. +: $P<0.05$ versus control; *: $P<0.05$ versus TNF-α-treated cells. (B) Representative Northern and Western blots showing additive inhibition of CCL2/MCP-1 expression by combined pretreatment of PD98059 and SP600125. Bar graphs show quantitative results of MCP-1/GAPDH mRNA and MCP-1/FCSA protein ratios, respectively, relative to that of control. Values are mean ± sem of four experiments. +: $P<0.05$ versus control; *: $P<0.05$ versus TNF-α-treated cells.

Figure. 3 Effects of PD98059 and SP600125 on TNF-α-activated signaling cascades

(A and B) Representative Western blots detected with antibodies targeting phosphorylated and unphosphorylated p42/44 MAPK, phosphorylated and unphosphorylated JNK2/3, phosphorylated and unphosphorylated c-Jun, c-Fos, I-κBα and β-actin. VSMCs were pre-incubated with PD98059 (20 μM) or SP600125 (10 μM) for 30 minutes, followed by stimulation with TNF-α (5 ng/mL) for 7.5, 15 and 30 minutes (all but c-Fos), or 60, 90 and 120 minutes (c-Fos only). These experiments were performed three times and similar results were obtained. (C) Immunostaining showing no effects of PD98059 (40 μM) and SP600125 (20 μM) on TNF-α (5 ng/mL)-induced nuclear translocated p65/NF-κB at 15 minutes. (original magnification x 150).

Figure. 4 Roles of NF-κB and c-Jun/AP-1 pathways in TNF-α-induced CCL2/MCP-1 mRNA and protein expression

(A) Representative Northern blots showing VSMCs pretreated with MG132 (5-20 μM) for 1.5 hours, followed by stimulation with TNF-α (5 ng/mL) for 4 hours. Lower panel shows quantitative results of CCL2/MCP-1/GAPDH mRNA ratios relative to that of control. Values are mean ± sem of four experiments. +: $P<0.05$ versus control; *: $P<0.05$ versus TNF-α-treated cells. (B and C) Representative Northern and Western blots, respectively, showing complete inhibition of CCL2/MCP-1 production by combined pretreatment of MG132 (20 μM) and SP600125 (10 μM). Lower panels show corresponding quantitative results of MCP-1/GAPDH mRNA and MCP-1/FCSA protein ratios relative to that of control. Values are mean ± sem of four experiments. +: $P<0.05$ versus control; *: $P<0.05$ versus TNF-α-treated cells.

JPET Fast Forward. Published on February 20, 2004 as DOI: 10.1124/jpet.103.062620
This article has not been copyedited and formatted. The final version may differ from this version.

Figure. 5 Effects of MG132 alone or in combination with SP600125 on TNF-α-activated signaling cascades

VSMCs were incubated with TNF-α (5 ng/mL) with or without pretreatment with MG132 (20 μM) or SP600125 (10 μM). Representative Western blots were detected with antibodies targeting phosphorylated and unphosphorylated JNK2/3 (at 7.5 minutes), phosphorylated and unphosphorylated c-Jun (at 15 minutes), c-Fos (at 120 minutes), and I-κBα (at 15 minutes). These experiments were performed three times and similar results were obtained.

Figure. 6 Effects of cilostamide and pentoxifylline on TNF-α-induced CCL2/MCP-1 mRNA and protein expression

VSMCs were incubated with TNF-α (5 ng/mL) for 4 or 24 hours, with or without pretreatment with cilostamide or denbufylline (10-40 μM) for 30 minutes, or pentoxifylline (0.5-2 mM) for 45 minutes. (A) Representative Northern blots. Bar graphs show quantitative results of MCP-1/GAPDH mRNA ratios relative to that of control. Values are mean ± sem of four experiments. +: $P<0.05$ versus control; *: $P<0.05$ versus TNF-α-treated cells. (B) Representative Western blots. Bar graph shows quantitative results of MCP-1/FCSA protein ratios relative to that of control. Values are mean ± sem of four experiments. +: $P<0.05$ versus control; *: $P<0.05$ versus TNF-α-treated cells.

Figure. 7 Effects of cilostamide, denbufylline and pentoxifylline on TNF-α-activated signaling cascades.

VSMCs were incubated with TNF-α (5 ng/mL) for the given periods with or without pretreatment with pentoxifylline (2 mM), cilostamide (40 μM) or denbufylline (40 μM). (A, B and C) Representative Western blots detected with antibodies targeting

phosphorylated and unphosphorylated p42/44 MAPK, phosphorylated and unphosphorylated JNK2/3, phosphorylated and unphosphorylated c-Jun, c-Fos, I-κBα, and β-actin. VSMCs were incubated with TNF-α (5 ng/mL) for 7.5, 15 and 30 minutes (all but c-Fos), or 60, 90 and 120 minutes (c-Fos only). These experiments were performed three times and similar results were obtained. (D, E and F) Representative Northern blots detected with a c-*jun* or c-*fos* riboprobe. Ten μg of total RNA per lane were analyzed as described in **Materials and Methods**. These experiments were performed three times and similar results were obtained. (G) Immunostaining showing partial inhibitory effects of pentoxifylline and cilostamide, but not denbufylline, on TNF-α-induced nuclear translocated p65/NF-κB at 15 minutes. (original magnification x 120).

Figure 1

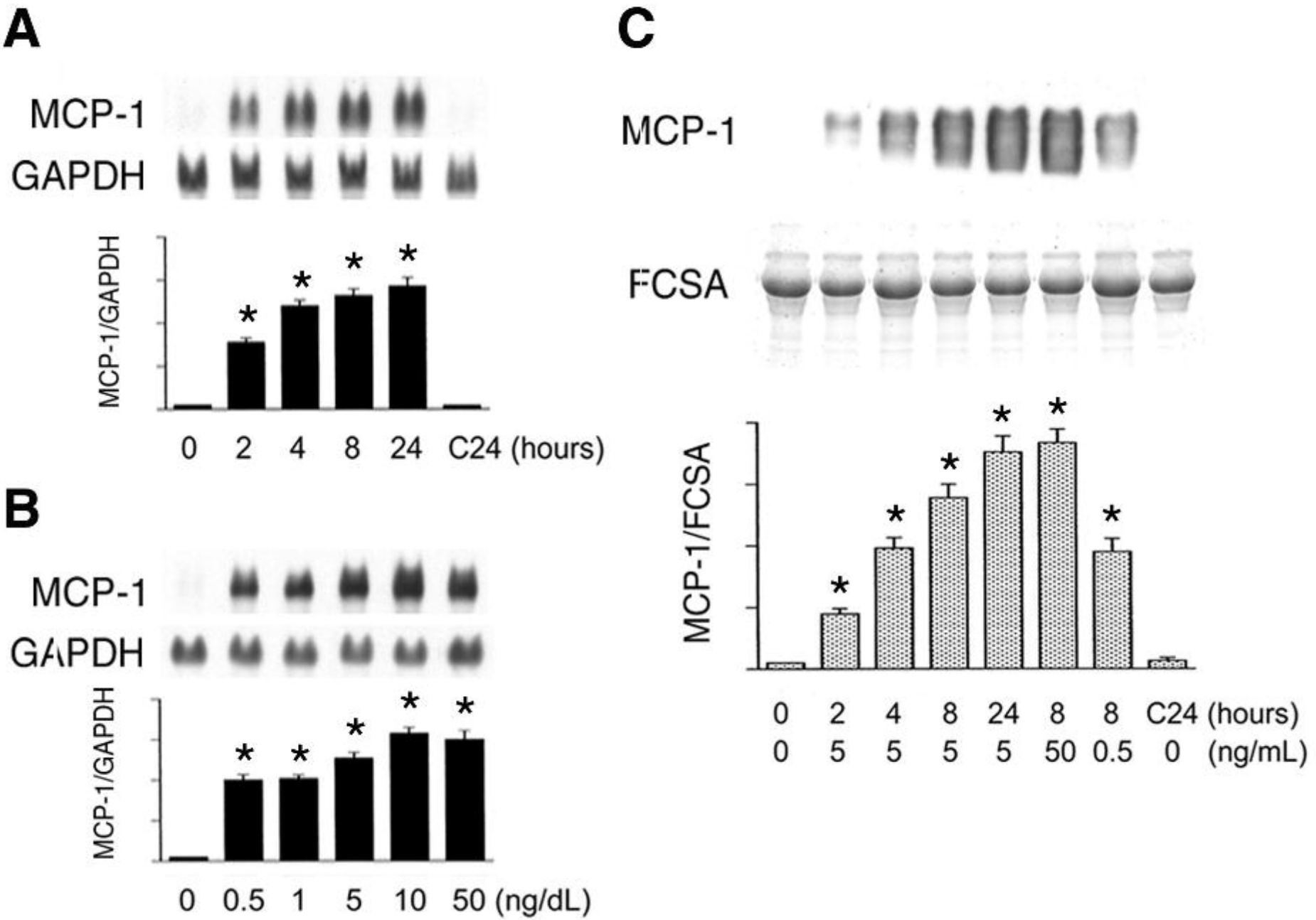

A
MCP-1
GAPDH
MCP-1/GAPDH
0 2 4 8 24 C24 (hours)
B
MCP-1
GAPDH
MCP-1/GAPDH
0 0.5 1 5 10 50 (ng/dL)
C
MCP-1
FCSA
MCP-1/FCSA
0 2 4 8 24 8 8 C24 (hours)
0 5 5 5 5 50 0.5 0 (ng/mL)

Figure 2

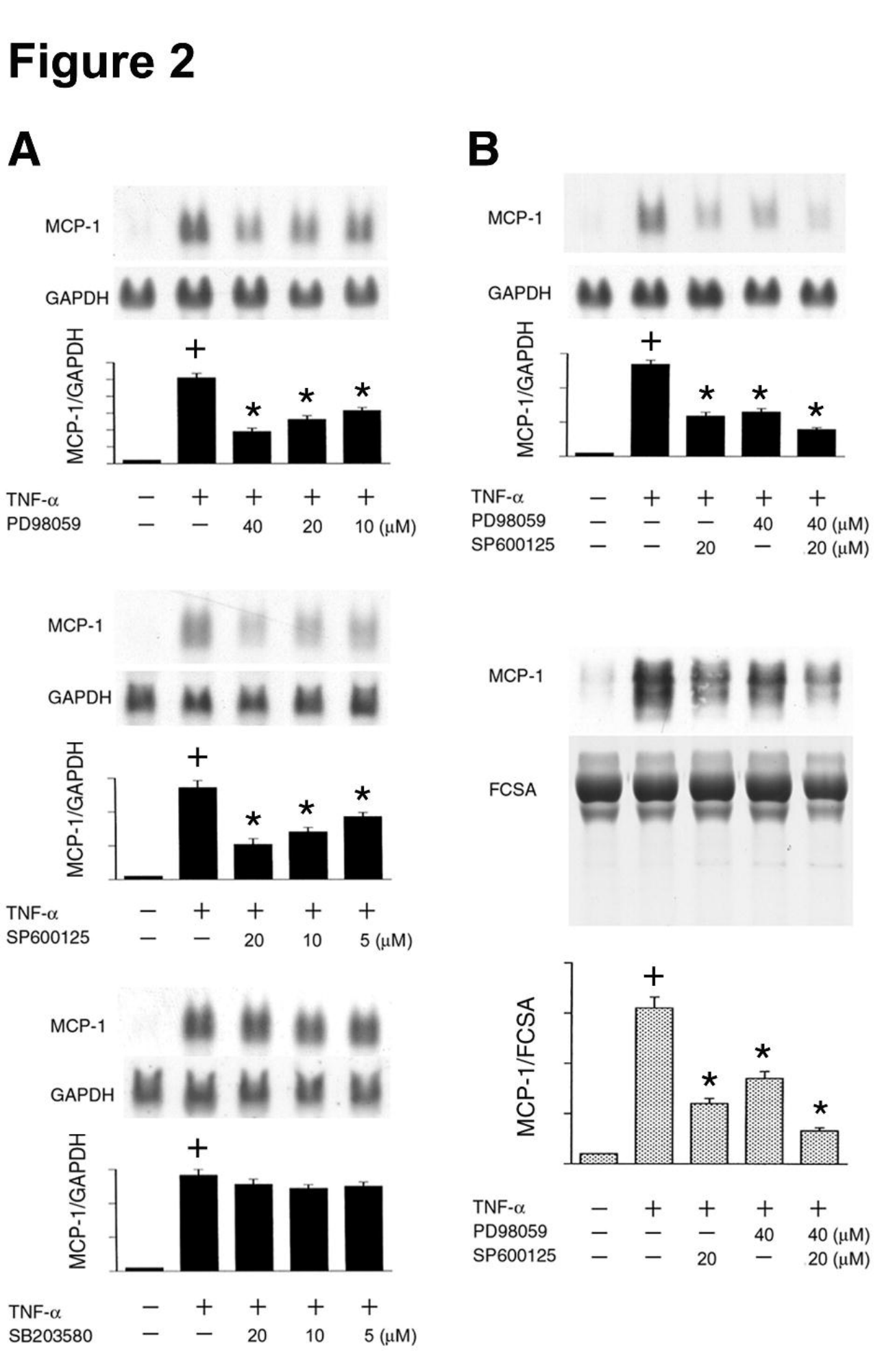
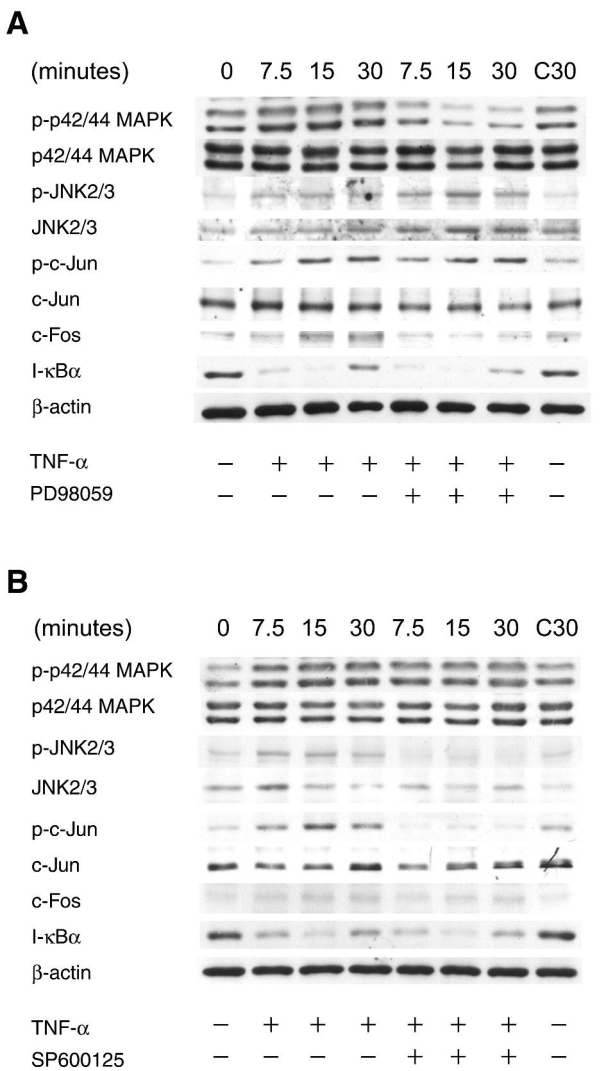

A
MCP-1
GAPDH
MCP-1/GAPDH
TNF-α
PD98059
10 (μM)
MCP-1
GAPDH
MCP-1/GAPDH
TNF-α
SP600125
5 (μM)
MCP-1
GAPDH
MCP-1/GAPDH
TNF-α
SB203580
5 (μM)
B
MCP-1
GAPDH
MCP-1/GAPDH
TNF-α
PD98059
SP600125
40 (μM)
20 (μM)
MCP-1
FCSA
MCP-1/FCSA
TNF-α
PD98059
SP600125
40 (μM)
20 (μM)

Figure 3


A
(minutes)
0
7.5
15
30
7.5
15
30
C30
p-p42/44 MAPK
p42/44 MAPK
p-JNK2/3
JNK2/3
p-c-Jun
c-Jun
c-Fos
I-κBα
β-actin
TNF-α
PD98059
B
(minutes)
0
7.5
15
30
7.5
15
30
C30
p-p42/44 MAPK
p42/44 MAPK
p-JNK2/3
JNK2/3
p-c-Jun
c-Jun
c-Fos
I-κBα
β-actin
TNF-α
SP600125

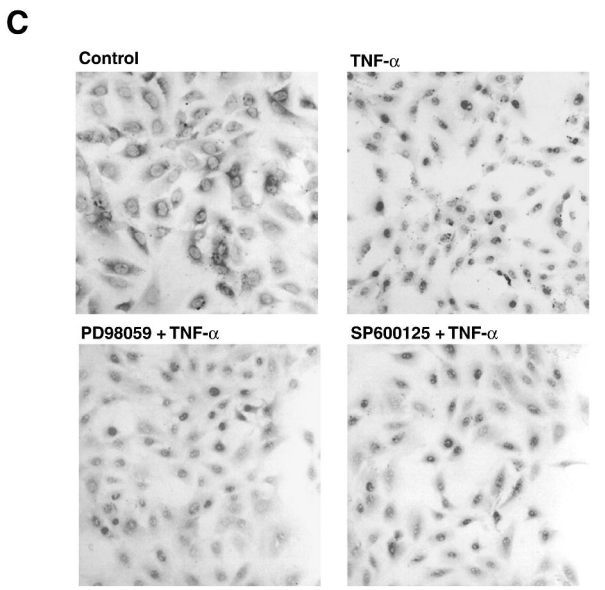

C
Control
TNF-α
PD98059 + TNF-α
SP600125 + TNF-α

Figure 4

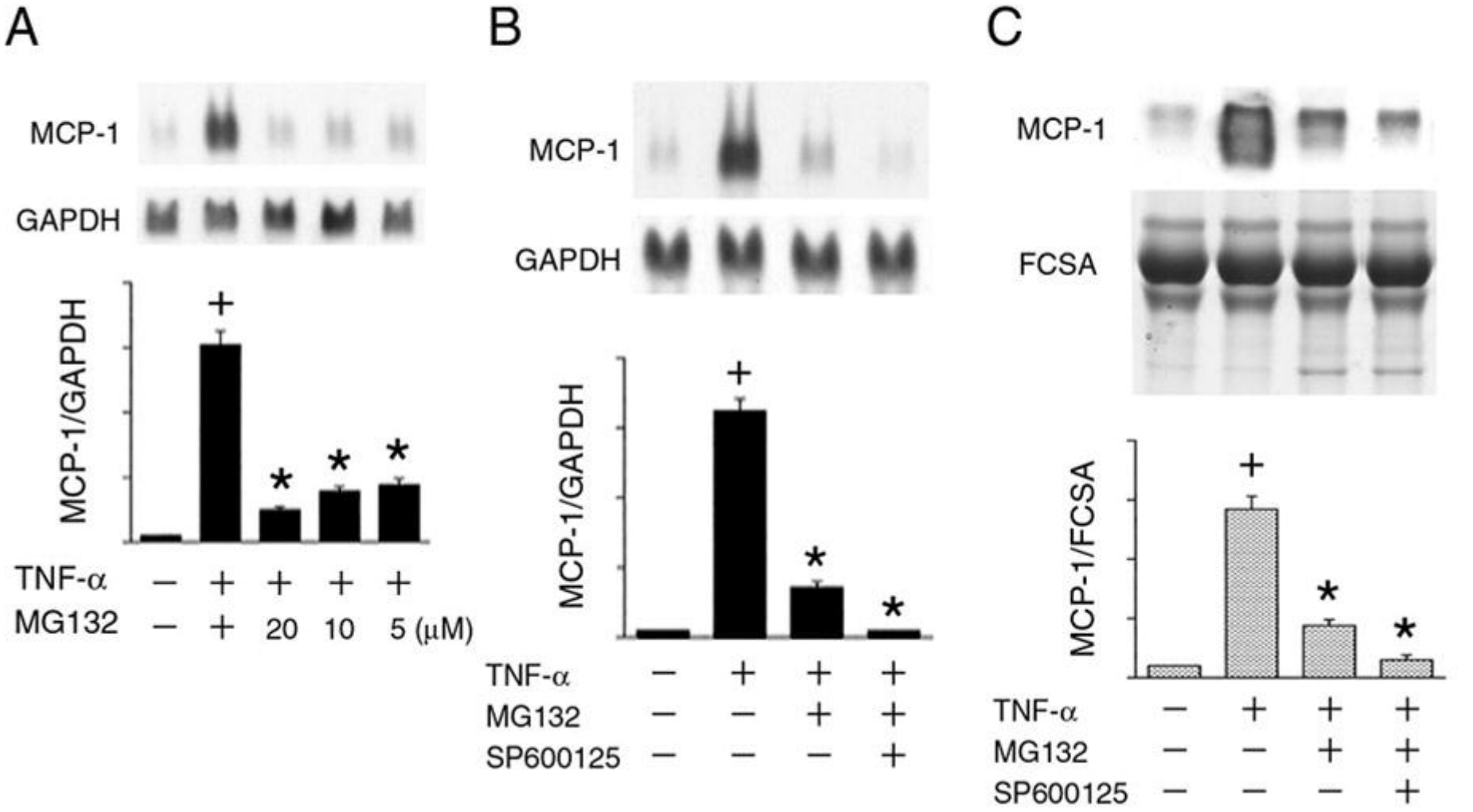

A
MCP-1
GAPDH
MCP-1/GAPDH
TNF-α – + + + +
MG132 – + 20 10 5 (μM)
B
MCP-1
GAPDH
MCP-1/GAPDH
TNF-α – + + +
MG132 – – + +
SP600125 – – – +
C
MCP-1
FCSA
MCP-1/FCSA
TNF-α – + + +
MG132 – – + +
SP600125 – – – +

Figure 5

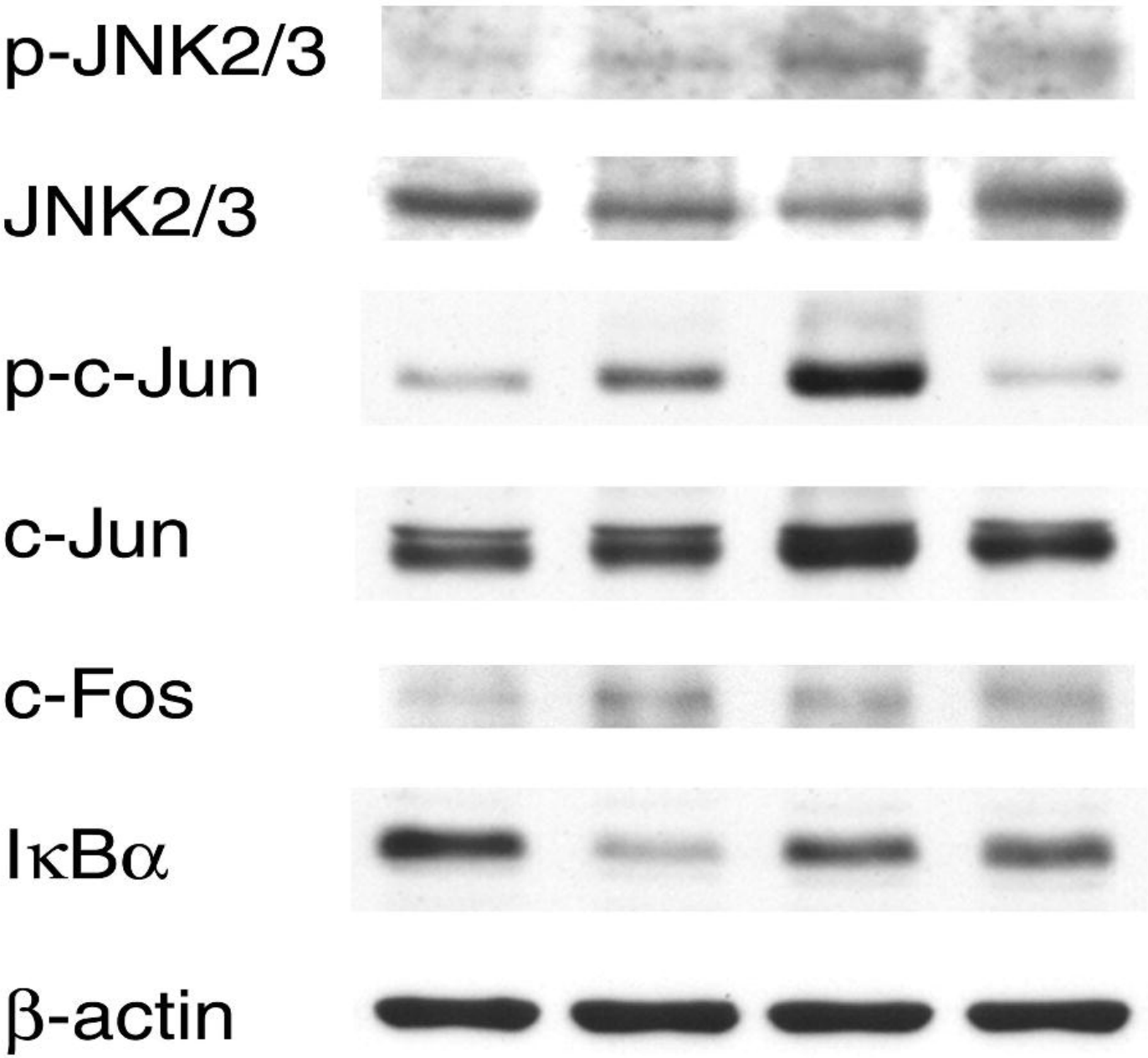

p-JNK2/3
JNK2/3
p-c-Jun
c-Jun
c-Fos
IκBα
β-actin
TNF-α − + + +
MG132 − − + +
SP600125 − − − +

Figure 6

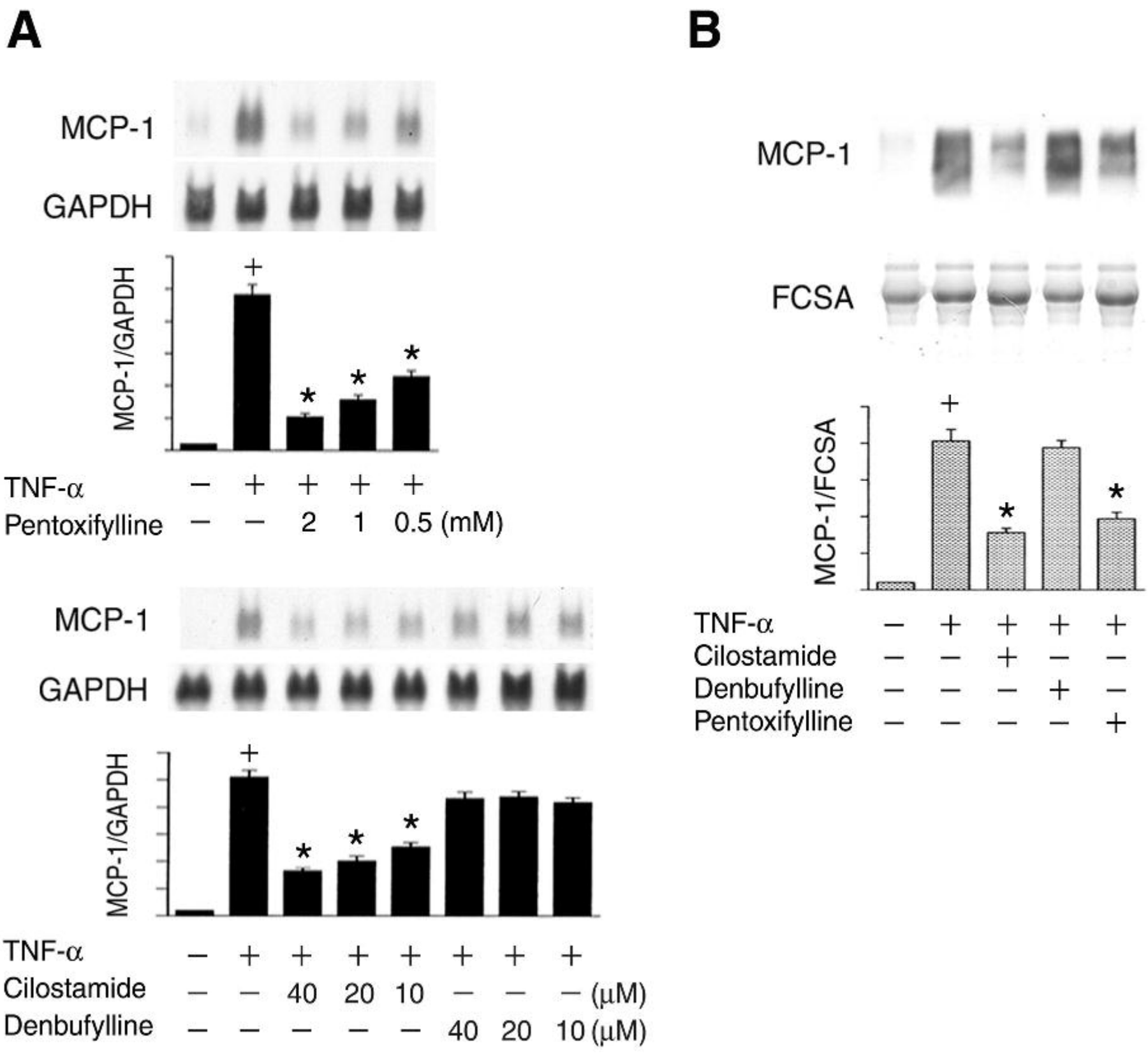
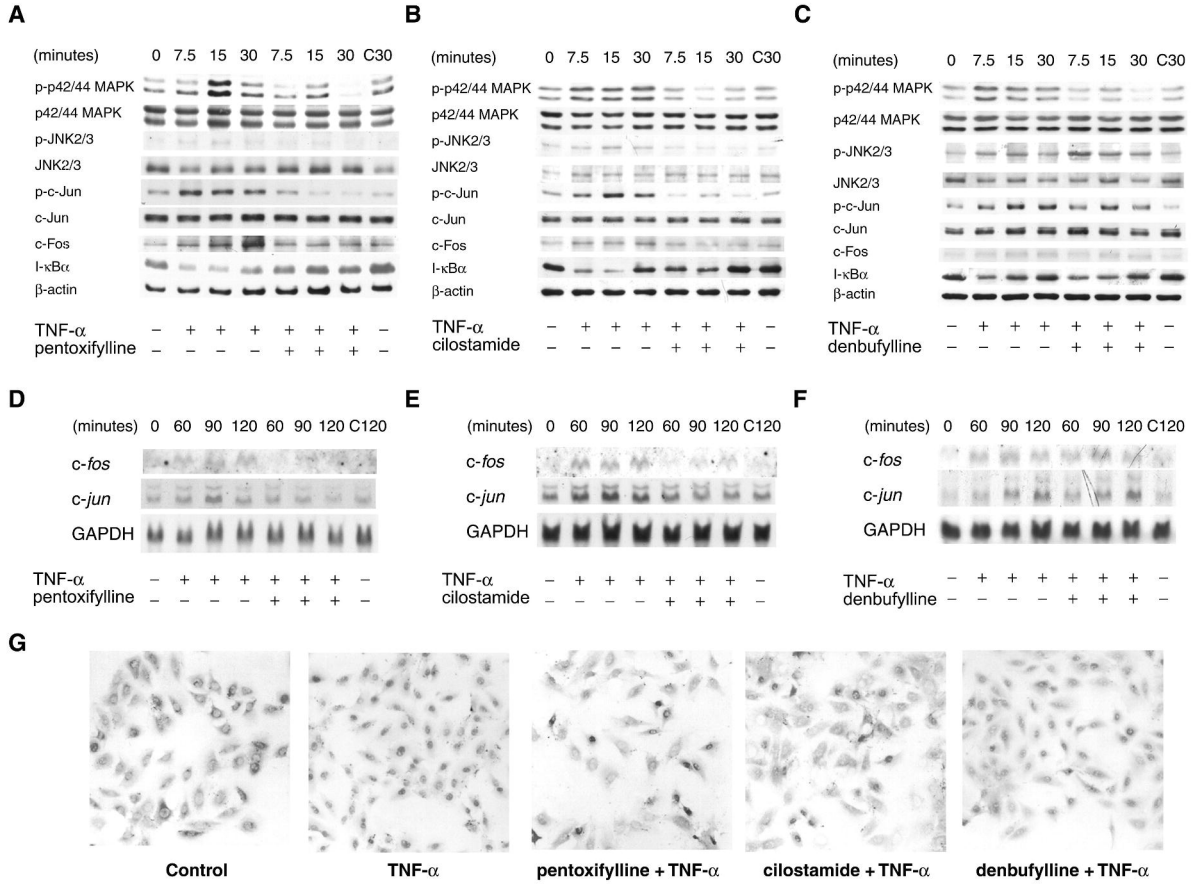

A
MCP-1
GAPDH
MCP-1/GAPDH
TNF-α
Pentoxifylline
2
1
0.5 (mM)
MCP-1
GAPDH
MCP-1/GAPDH
TNF-α
Cilostamide
Denbufylline
40
20
10 (μM)
B
MCP-1
FCSA
MCP-1/FCSA
TNF-α
Cilostamide
Denbufylline
Pentoxifylline

Figure 7


A
(minutes) 0 7.5 15 30 7.5 15 30 C30
p-p42/44 MAPK
p42/44 MAPK
p-JNK2/3
JNK2/3
p-c-Jun
c-Jun
c-Fos
I-κBα
β-actin
TNF-α
pentoxifylline
B
(minutes) 0 7.5 15 30 7.5 15 30 C30
p-p42/44 MAPK
p42/44 MAPK
p-JNK2/3
JNK2/3
p-c-Jun
c-Jun
c-Fos
I-κBα
β-actin
TNF-α
cilostamide
C
(minutes) 0 7.5 15 30 7.5 15 30 C30
p-p42/44 MAPK
p42/44 MAPK
p-JNK2/3
JNK2/3
p-c-Jun
c-Jun
c-Fos
I-κBα
β-actin
TNF-α
denbufylline
D
(minutes) 0 60 90 120 60 90 120 C120
c-fos
c-jun
GAPDH
TNF-α
pentoxifylline
E
(minutes) 0 60 90 120 60 90 120 C120
c-fos
c-jun
GAPDH
TNF-α
cilostamide
F
(minutes) 0 60 90 120 60 90 120 C120
c-fos
c-jun
GAPDH
TNF-α
denbufylline
G
Control
TNF-α
pentoxifylline + TNF-α
cilostamide + TNF-α
denbufylline + TNF-α