

Involvement of mitogen-activated protein kinases and peroxisome proliferator-activated receptor γ in monosodium urate crystal-induced vascular cell adhesion molecule 1 expression in human rheumatoid arthritis synovial fibroblasts

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Abstract. To investigate whether monosodium urate (MSU) crystals could induce the production of VCAM-1 (vascular cell adhesion molecule 1) in human synovial cells and its possible signaling pathways, human synovial cells isolated from synovial tissue explants were stimulated with various doses of MSU crystals for different time intervals. Expression of VCAM-1 was evaluated with Western blotting. To explore the underlying mechanisms, VCAM-1 protein expression was also evaluated after activation of several signaling molecules including mitogen-activated protein kinases (MAPKs) and peroxisome proliferator-activated receptor γ (PPAR γ) were blocked. Exposure of synovial cells to MSU crystals induced VCAM-1 expression in culture medium in a dose- and time-dependent manner, reaching a plateau at 1000 μ M and 24 h. Inhibition of the activation of MAPKs and PPAR γ could block this increase. The present results demonstrated that MSU crystals could induce VCAM-1 expression. MAPKs and PPAR γ signaling pathways regulated the induced VCAM-1 expression.

Introduction

Acute gouty arthritis is the most common painful inflammatory arthritis and is clearly associated with hyperuricemia (1). Hyperuricemia is best defined as extracellular fluid urate supersaturation, reflects an enlarged body pool of uric acid and increases the risk for precipitation of monosodium urate (MSU) crystals in joints (2).

VCAM-1, a member of cell adhesion molecules, has been demonstrated to be expressed at a low level in vascular endo-

thelial cells and other cells. Up-regulation of VCAM-1 has been shown in the synovial lining of RA patients by immunohistochemical staining (3) and in cultured human RASFs by Western blotting (4). This up-regulation after inflammatory stimuli was believed to contribute to the development of rheumatoid arthritis (5). Whether VCAM-1 expression could change in acute gouty arthritis has not been studied. We cultured human synovial cells, established a gouty arthritis cell model by MSU stimulation, and examined VCAM-1 protein expression.

Induction of VCAM-1 by inflammatory cytokines such as TNF- α and interleukin-1 β has been well clarified in various cell types (6-8). Three MAPKs including ERK1/2 (9), p38 MAPK (10), and JNK (11) have been reported to be activated by inflammatory stimuli. The relationship between activation of these pathways and expression of VCAM-1, however, has been controversial. For example, TNF- α induced VCAM-1 expression in mouse Sertoli cells does not require the activation of p38 MAPK, whereas activation of JNK is essential for these responses (12). In endothelial cells, JNK and protein kinase C activation is required for TNF- α mediated ICAM-1 expression. In contrast, cardiac cells require p38 MAPK activation for VCAM-1 expression (13). Moreover, p38 MAPK and JNK oppositely regulate TNF- α induced VCAM-1 expression in chondrosarcoma cells.

In the present study, human synovial cells were isolated from synovial tissue explants, cultured and stimulated with MSU crystals. VCAM-1 protein expression and activation of related signaling molecules including ERK, p38 MAPK, JNK, and PPAR γ were evaluated with Western blotting. We observed that MSU crystals induced cells to increase VCAM-1. Activation of the MAPK signal pathway and PPAR γ may contribute to this increase. Our research provides a foundation for the prevention and drug development of anti-acute gouty arthritis.

Materials and methods

Human materials. Human synovial tissue from patients was obtained with the approval of the Institutional Review Board of Chongqing Medical University, which approved the study. An informed-consent form was signed by the patients.

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Drugs and reagents. α -MEM culture medium was provided by Gibco, fetal bovine serum and horse serum were purchased from Hangzhou Sijiqing Co. Ltd., the ECL kit was from Pierce, and type I collagen was from Invitrogen. NP-40, leupeptin, PMSF were from Sigma, the protein quantification kit was from Pierce, polyvinylidene difluoride filters were from Bio-Rad, primary antibodies were from Cell Signaling, secondary antibodies were from Santa Cruz Biotechnology, Inc., inhibitors of MAPKs were from Sigma, and the optical scanner was from Bio-Imaging Systems, Rhenium, Israel.

Synthesis of MSU crystals. Uric acid (4 g) was dissolved in 800 ml deionized water, heated to 60°C, adjusted to pH 8.9 with 0.5 N NaOH, and allowed to crystallize overnight at room temperature. MSU crystals were recovered by centrifugation, washed with distilled water and dried at 40°C for 24 h. Crystal shape and birefringence were assessed by compensated polarized light microscopy. MSU crystals were milled and then sterilized by heating at 180°C for 2 h before each experiment. Less than 0.015 EU/ml endotoxin were measured in MSU crystal preparations by Limulus amoebocyte lysate assay (E-toxate kit, Sigma- Aldrich, SRL, Milano, Italy).

Western blotting. After drug treatment, cells were homogenized in buffer containing 50 mmol/l Tris (pH 7.5), 250 mmol/l NaCl, 10 mmol/l EDTA (pH 8.0), 0.5% NP-40, 10 μ g/ml leupeptin, 1 mmol/l PMSF, 4 mmol/l NaF. The supernatants were centrifuged at 12,000 g for 10 min at 4°C. The protein concentration was determined with a protein quantification kit. Protein samples (20 μ g/well) were separated through sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (10% gel) and then transferred to polyvinylidene difluoride filters. The blots were blocked with 5% milk for 60 min at room temperature, and incubated with a primary antibody for 2 h at room temperature. The blots were then incubated in horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies or HRP-conjugated anti-mouse secondary antibodies for 60 min at room temperature, and developed in ECLTM (enhanced chemiluminescence) solution. Quantification of protein levels was carried out by scanning the films with an optical scanner and then analyzing band intensities with the Quantity One software.

Isolation and culture of human synovial cells. Synovial tissues were obtained, washed with α -MEM and cut into 0.5 mm³ under sterility conditions. After centrifugation at 1,200 rpm for 5 min, the precipitate was digested with type I collagen at a dose of 4 mg/ml in a water bath at 37°C for 30 min, the cell suspension was filtered through 70 μ m cell mesh, centrifugation at 1,500 rpm for 6 min and the supernatant was digested with 0.2% type I collagen at 37°C until the tissue turned flocculent and the solution turned cloudy. The precipitate was washed with PBS after centrifugation at 1,200 rpm for 5 min, then the single cell suspension was obtained vigorously by transferring the precipitate in α -MEM culture media (supplemented with 15% FBS) and passing through a metal screen, followed by centrifugation at 1,500 rpm for 5 min. After washing with PBS twice, the precipitate was added with 5 ml α -MEM supplemented with 15% FBS. The cells were plated in 25 cm² culture bottles with 5x10⁵ cells in each bottle and were

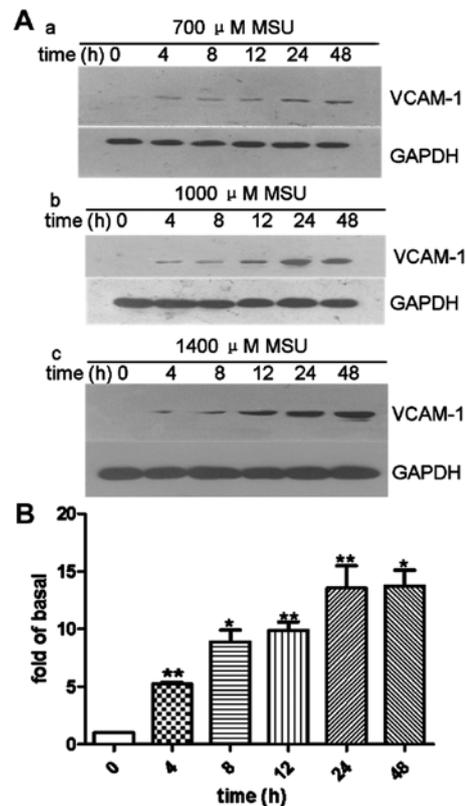


Figure 1. MSU-induces VCAM-1 protein expression in a time-dependent manner in human synovial cells. Human synovial cells were exposed to (a) 700 μ M; (b) 1,000 μ M; (c) 1,400 μ M, of MSU for 0, 4, 8, 12, 24 and 48 h, and VCAM-1 expression was determined. (A) Representative immunoblots for VCAM-1 (upper panel) and GAPDH (lower panel), respectively. The positions of VCAM-1 and GAPDH are indicated on the right. Time and dose are indicated above the panels. (B) Results were quantified by densitometric analysis. Data were normalized to GAPDH and expressed as the fold of VCAM-1 expression over basal in 0 h-treated cells. Values represent means \pm SEM of 3 independent experiments. * P <0.05, ** P <0.01 compared with 0 h-treated cells, n =3.

grown in a humidified atmosphere with 5% CO₂ at 37°C. The culture medium was changed 48-72 h later. Synovial cells were used between the 4th and 22nd passages.

Kinase activation analysis. Following drug treatment, cells were lysed with ice cold lysis buffer containing 50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1.5 mM MgCl₂, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 μ g/ml leupeptin and 100 μ M sodium orthovanadate. Solubilized cell extracts were then centrifuged at 12,000 g for 10 min, and protein concentrations were determined with the Bradford Reagent. A volume of 33 μ l of the supernatants, that contained about 20 μ g protein, were added to 10 μ l of sample buffer (200 mM Tris, pH 6.8, 40% glycerol, 20% mercaptoethanol, 8% SDS and bromophenol blue) and subjected to Western blot analysis. Following separation on 8% SDS-polyacrylamide gels, the proteins were electrophoretically transferred to nitrocellulose membranes for immunoblotting. Phosphorylated ERK1/2, p38 MAPK, JNK were detected by immunoblotting with mouse monoclonal anti-phospho- ERK1/2, p38 MAPK, or JNK (Santa Cruz Biotechnology, Inc., 1:1,000). Subsequently, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgGs. Antibody binding was visualized by an ECL

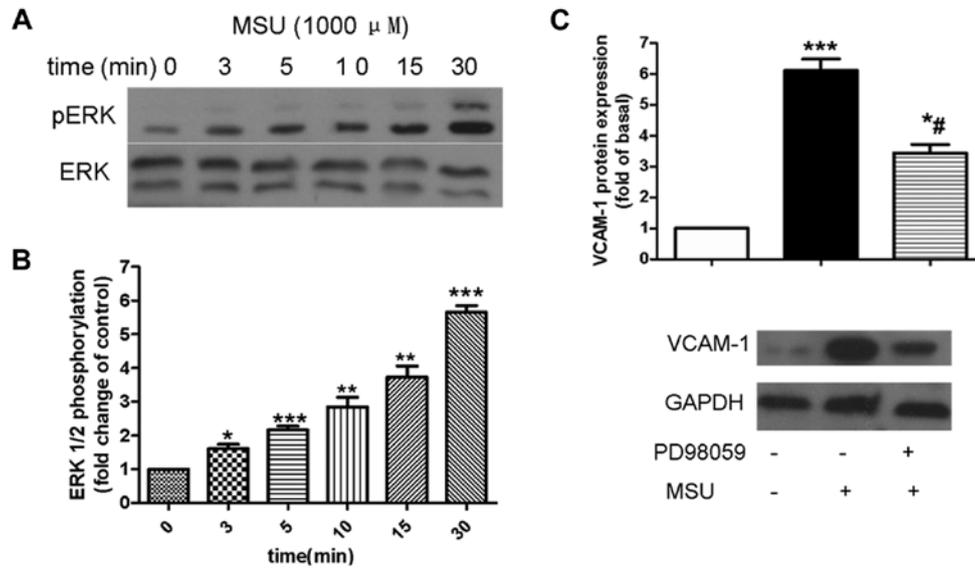


Figure 2. Involvement of ERK1/2 in MSU-induced VCAM-1 expression in human synovial cells. (A) Cells were treated with 1,000 μ M MSU for different time intervals from 0 to 30 min and subjected to Western blotting to determine ERK1/2 activation. Representative immunoblots for phospho-ERK1/2 (upper panel) and total ERK1/2 (lower panel) are presented. The positions of pERK1/2 and total ERK1/2 are indicated on the left. Results were quantified by densitometric analysis. (B) Data were normalized to total ERK1/2 and expressed as the fold of ERK1/2 phosphorylation over basal levels in untreated cells. Values represent means \pm SEM of 3 independent experiments. * P <0.05, ** P <0.01, *** P <0.001 compared with 0 h-treated cells, n =3. (C) Human synovial cells were pretreated with vehicle or PD98059 for 1 h and then exposed to 1,000 μ M MSU for 24 h, VCAM-1 expression was determined. * P <0.05, *** P <0.001 compared with 0 h-treated cells, # P <0.05 compared with cells pretreated with vehicle, n =3.

substrate for detecting HRP. Total ERK1/2, p38 MAPK, JNK levels were determined after stripping by 10 min incubation in 0.1 M NaOH containing 0.2% SDS and then reprobing with a rabbit polyclonal antibody raised against total ERK1/2, p38 MAPK, JNK (Santa Cruz Biotechnology Inc., 1:1,000). Quantification of phosphorylated kinase levels was carried out by scanning the films with an optical scanner and then analyzing band intensities with TINA 2.07 software. The results were statistically analyzed by paired t-test and the P-value refers to the comparison between the effect of an agonist (opioid or cannabinoid) and its matched control that was run within the same experiment.

Statistical analysis. All data are presented as mean \pm standard deviation. Data were statistically analyzed by Student's t-test with the use of the Prism program. P <0.05 was considered statistically significant.

Results

VCAM-1 protein expression increases after urate treatment in human synovial cells. To assess whether VCAM-1 protein expression could be affected by MSU, purified human synovial cells were treated with different doses of urate for different time intervals, then the cell protein was extracted and submitted to Western blotting, and VCAM-1 expression was evaluated. Exposure to 700, 1,000, or 1,400 μ M MSU for 0, 4, 8, 12, 24 and 48 h induced a dose- and time-dependent increase of VCAM-1 expression in synovial cells, reaching a plateau at 1,000 μ M for 24 h (Fig. 1).

Involvement of ERK1/2 in MSU-induced VCAM-1 expression in human synovial cells. Previous studies have demonstrated

the involvement of the ERK1/2-Ets-1 pathway in inducing the outgrowth of synovial cells. We next investigated whether ERK1/2 is involved in MSU-induced VCAM-1 expression using Western blot analysis. As shown in Fig. 2A, treatment with MSU for different time intervals from 3 to 30 min increased ERK1/2 phosphorylation in a time-dependent manner, with the maximum response at 30 min. We pretreated cells with the ERK1/2 inhibitor PD98059 for 1 h to block ERK1/2 activation and examined VCAM-1 expression. As can be seen from Fig. 2C, after pretreatment with PD98059, VCAM-1 expression was significantly reduced, suggesting that ERK1/2 is essential for the increased VCAM-1 expression induced by MSU.

Involvement of p38 MAPK in MSU-induced VCAM-1 expression in human synovial cells. p38, another member of the MAPK family, was widely recognized as a central mediator of many inflammatory responses. We investigated whether p38 MAPK could be activated by MSU using Western blot analysis. As shown in Fig. 3A, treatment with MSU for different time intervals from 3 to 30 min increased p38 MAPK phosphorylation in a time-dependent manner, with the maximum response at 30 min. We used the p38 MAPK inhibitor SB203580 to block p38 MAPK activation for 1 h before MSU incubation. As can be seen from Fig. 3C, pretreatment with SB203580 almost completely blocked the increase of VCAM-1 protein induced by MSU, suggesting that p38 MAPK is essential for the increased VCAM-1 expression.

Involvement of JNK in MSU-induced VCAM-1 expression in human synovial cells. JNK, another member of MAPK family, is widely recognized as a central mediator of many inflammatory responses. We next investigated whether JNK could be

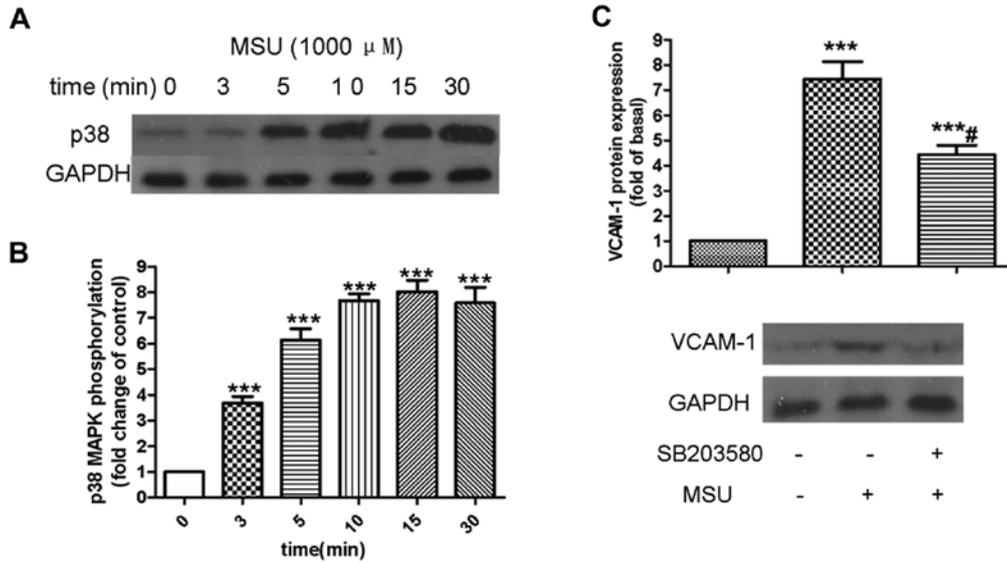


Figure 3. Involvement of p38 in MSU-induced VCAM-1 expression in human synovial cells. (A) Cells were treated with 1,000 μ M MSU for different time intervals from 0 to 30 min. Representative immunoblots for phospho-p38 (upper panel) and GAPDH (lower panel) are presented. The positions of phospho-p38 and GAPDH are indicated on the left. Results were quantified by densitometric analysis. (B) Data were normalized to GAPDH and expressed as the fold of p38 phosphorylation over basal levels in untreated cells. Values represent means \pm SEM of 3 independent experiments. *** P <0.001 compared with 0 h-treated cells, $n=3$. (C) Human synovial cells were pretreated with vehicle or SB203580 for 1 h and then exposed to 1,000 μ M MSU for 24 h, VCAM-1 expression was determined. *** P <0.001 compared with 0 h-treated cells, # P <0.05 compared with cells pretreated with vehicle, $n=3$.

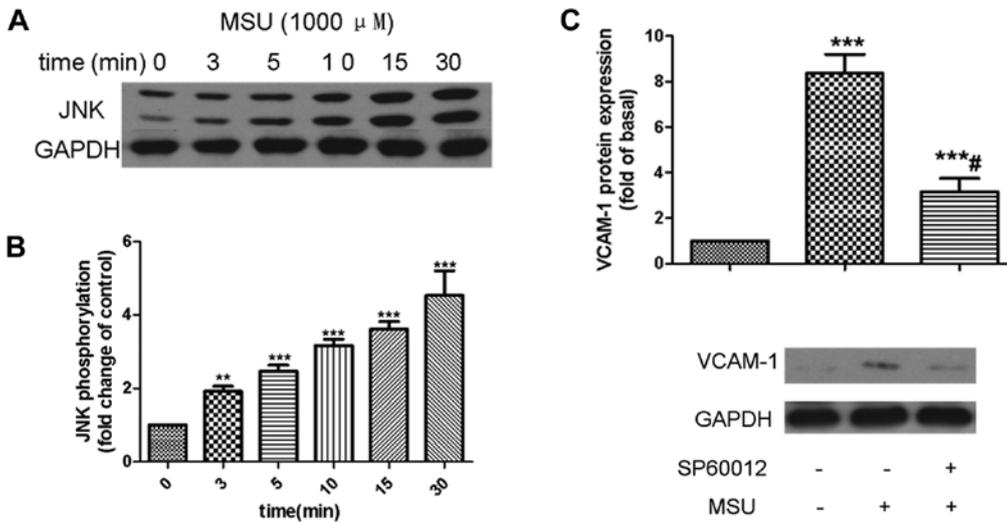


Figure 4. Involvement of JNK in MSU-induced VCAM-1 expression in human synovial cells. (A) Cells were treated with 1,000 μ M MSU for different time intervals from 0 to 30 min and then subjected to Western blotting. Representative immunoblots for phospho-JNK (upper panel) and GAPDH (lower panel) are presented. The positions of phospho-JNK and GAPDH are indicated on the left. Results were quantified by densitometric analysis. (B) Data were normalized to GAPDH and expressed as the fold of JNK phosphorylation over basal levels in untreated cells. Values represent means \pm SEM of 3 independent experiments. ** P <0.01, *** P <0.001 compared with 0 h-treated cells, $n=3$. (C) Human synovial cells were pretreated with vehicle or SP60012 for 1 h and then exposed to 1,000 μ M MSU for 24 h, VCAM-1 expression was determined. *** P <0.001 compared with 0 h-treated cells, # P <0.05 compared with cells pretreated with vehicle, $n=3$.

activated by MSU using Western blot analysis. As shown in Fig. 4A and B, consistent with the results of ERK1/2 and p38 MAPK, treatment with MSU for different time intervals from 3 to 30 min increased JNK phosphorylation in a time-dependent manner, with the maximum response for 30 min, demonstrating that MSU could activate JNK, which might be the underlying signaling molecule mediating the increased VCAM-1 protein expression. We used the JNK inhibitor SP60012 to block JNK activation and examined VCAM-1 expression. As can be seen from Fig. 4C and D, pretreatment with SP600125 almost

completely blocked JNK activation as well as the increase of VCAM-1 protein induced by MSU, suggesting that JNK is essential for the increased VCAM-1 expression.

PPAR γ expression decreased after MSU treatment in human synovial cells. PPAR γ is a member of the nuclear hormone receptor superfamily and functions as a key regulator in inflammatory responses (14). PPAR γ expression could be induced by MSU in human monocytes (15). We next investigated whether PPAR γ expression could be activated by MSU

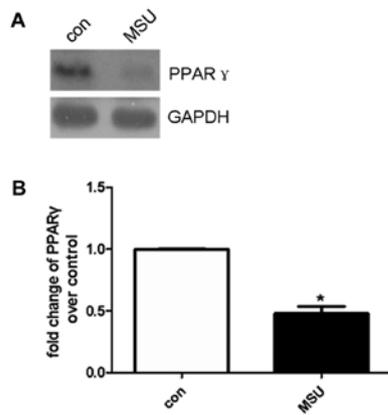


Figure 5. PPAR γ expression decreased after MSU treatment in human synovial cells. Human synovial cells were exposed to 1,000 μ M MSU for 24 h, and PPAR γ activation was determined. (A) Representative immunoblots for PPAR γ (upper panel) and GAPDH (lower panel), respectively. The positions of PPAR γ and GAPDH are indicated on the right. (B) Results were quantified by densitometric analysis. Data were normalized to GAPDH and expressed as the fold PPAR γ over GAPDH in untreated cells. Values represent means \pm SEM of 3 independent experiments. * P <0.05 compared with 0 h-treated cells, n=3.

in human synovial cells using Western blot analysis. As shown in Fig. 5, treatment with MSU for 24 h significantly increased PPAR γ expression.

Discussion

MSU crystal is an inflammatory agent which has strong potential to stimulate various types of cells to produce a number of proinflammatory cytokines and chemokines (16), these proinflammatory cytokines and chemokines play an important role in the infiltration and activation of inflammatory cells in patients with acute gout (17). An essential part of the pathogenesis of acute and chronic gouty arthritis (17) is deposition of MSU crystals in the articular and periarticular tissues. We established a cell arthritis model by incubating cultured human synovial cells with MSU crystals, and determined VCAM-1 expression. VCAM-1 is a member of cell adhesion molecules and has been demonstrated to play an important role in cellular infiltration, which is a characteristic feature of rheumatoid arthritis (3,18,19). VCAM-1 was not expressed on resting vascular endothelium, but was rapidly induced in response to a number of inflammatory stimuli, consistent with our observation that VCAM-1 expression was maintained at a low level and 700, 1,000, 1,400 μ M MSU induced a dose- and time-dependent increase of VCAM-1 expression.

Expression of VCAM-1 has been reported to be highly regulated by MAPKs and NF- κ B (20-22). To find out signaling pathways that mediate increased VCAM-1 expression in human synovial cells, we performed Western blotting to examine the activation of several signaling molecules including 3 MAPKs (ERK1/2, p38 MAPK, and JNK), and PPAR γ . Our results demonstrated that these 3 MAPKs are activated after MSU treatment in human synovial cells, consistent with a previous study on tumor necrosis factor- α (TNF- α)-induced expression of VCAM-1 in human rheumatoid arthritis synovial fibroblasts (23).

Since ERK1/2, p38 MAPK, and JNK were involved in MSU-induced VCAM-1 expression in human synovial cells,

it was important to determine whether these MAPKs were associated with NF- κ B activation. It has been well established that inflammatory responses following exposure to cytokines are highly dependent on activation of NF- κ B, which plays an important role in expression of several inflammatory genes (4,24,25). Previous studies also demonstrated that activation of NF- κ B is essential for expression of VCAM-1 induced by TNF- α in human tracheal smooth muscle cells (20). Besides, VCAM-1 promoters contain NF- κ B binding sites, which are regulated through MAPKs in several cell types (26). We thus hypothesized that increased VCAM-1 expression may result from NF- κ B activation through MAPKs.

PPAR γ is a member of the nuclear hormone receptor superfamily and acts as a ligand-dependent transcription factor (27). PPAR γ is primarily found in adipose tissue and is well-characterized as a regulator of various genes related to lipid and glucose metabolism. It has recently been demonstrated that this receptor is also expressed in a wide variety of cells including monocytes and macrophages. Increased expression of PPAR has also been documented at sites of inflammation in arthritis and colitis and in foam cells from atherosclerotic plaques (28-31). PPAR γ has recently been suggested to function as a negative regulator of inflammatory responses, consistent with our present observation that PPAR γ expression could be decreased by MSU treatment.

In conclusion, we first demonstrated that exposure to MSU crystals induced VCAM-1 expression in human synovial cells culture medium in a dose- and time-dependent manner. We further revealed involvement of signaling molecules including ERK, p38 MAPK, JNK, and PPAR γ in the increased VCAM-1 expression.

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