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Effects of ethanol on monosodium urate crystal-induced inflammation

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Abstract

To investigate whether ethanol is able to decrease monosodium urate ([illegible]SU) crystal-induced inflammation, differentiated THP1 cells from a human monocyte cell line were cultured in the presence or absenc[illegible] MSU crys[illegible]s with and without ethanol. In an *in vivo* experiment, MSU crystals were administered into subcutaneous air pouches c[illegible]ed in mice[illegible]llowing peritoneal injection of ethanol diluted with PBS. MSU crystals (0.75 mg/ml) stimulated the secretion of TNF-α, [illegible], and I[illegible]β from THP1 cells, while ethanol at a concentration of 0.8% reduced those increases by 1.79-, 1.63-, and [illegible]5-fold, resp[illegible]*n vitro*, MSU crystals (0.75 mg/ml) significantly increased the expression of phosphorylated JNK, ERK1/2, [illegible]teins in THP1 cells, while ethanol at a concentration of 0.8% reduced those increased expressions by 1.28-, 1.14-, and 1.68[illegible]ld, re[illegible]y. In addition, MSU crystals (0.75 mg/ml) significantly increased the expression of phosphorylated NF-κB protein in t[illegible]ar and cytosolic fractions and decreased the expression of IκBα in the cytosolic fraction. Ethanol at a concentrati[illegible]8% rec[illegible]d the MSU-increased expression of phosphorylated NF-κB in the nuclear and cytosolic fractions by 1.25- and [illegible]fold, spectiv[illegible] while it also reduced the MSU-decreased expression of IκBα in the cytosolic fraction by 1.12-fold. *In vivo*, [illegible]U crys[illegible]s increase[illegible] the number of leukocytes, as well as the concentrations of KC, MIP1α, and IL-6 in pouch fluids, while ethan[illegible]5 [illegible]ight) considerably inhibited the MSU crystal-induced inflammation. These results strongly suggest that eth[illegible] suppr[illegible] the secretion of inflammatory cytokines induced by MSU crystals via a pathway including MAPK (p38, JNK, and E[illegible]1/2, especia[illegible]38) and NF-κB.

Keywords: Monosodium urate[illegible]stal; MAPK; p[illegible]F-κB; Cytokine

1. Introduction

Hyper[illegible]cemia [illegible]defined a[illegible]a plasma urate level greater than 420[illegible]ol/[illegible]ates that total body urate is increased. W[illegible]hyperuricemia exists, plasma and extracellular fluids are [illegible]rsaturated with respect to urate, leading to crystal formati[illegible] and deposition. Therefore, gout develops on the background of hyperuricemia. Gout is a disease that is manifested by recurrent attacks of acute arthritis, tophaceous deposits of monosodium urate monohydrate (MSU) in and around the joints of the extremities, renal disease involving the interstitial tissues and blood vessels, and uric acid nephrolithiasis. During attacks of acute arthritis, MSU crystals are found in leukocytes in synovial fluids, indicating that they are involved with induction of those attacks. In previous studies [1,2], MSU crystals were demonstrated to stimulate synovial cells, monocytes–macrophages, and neutrophils to produce a variety of different cytokines including TNF-α, IL-8, IL-1β, IL-6, and monocyte chemotactic factor, which induce acute inflammation.

It is well known that ingestion of a large amount of alcoholic beverages containing ethanol causes hyperuricemia and gouty arthritis. Ethanol enhances adenine nucleotide degradation that leads to an increased production of uric acid [3–5], while it also increases the blood concentration of lactic acid, which inhibits the urinary excretion of uric

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acid [6]. These actions of ethanol raise the plasma concentration of urate, leading to gouty attack. Accordingly, patients with gout are recommended to refrain from ingestion of large amounts of alcoholic beverages.

Our experience has shown that patients with gout rarely experience gouty arthritis at the time of drinking, whereas they frequently experience a gouty attack due to hyperuricemia-induced crystal formation and deposition in the joints early the next morning during sleep after ingesting a large amount of alcoholic beverages the previous evening. Several reasons can be considered for why patients with gout rarely experience gouty arthritis when consuming alcohol. First, the associated increase in plasma concentration of urate is not enough to cause gouty arthritis. Second, ingested ethanol affects the sensory area of the central nervous system, leading to a decrease in the sense of pain. Third, ethanol may suppress inflammation due to urate crystal deposition via cytokine regulation. In the present study, we focused on the third reason, because a previous study demonstrated that excessive ethanol consumption depresses the immune system, thus increasing the susceptibility to infection [7], and other studies have reported that ethanol suppressed the immune responses of several immune cell types, including monocytes, macrophage, T lymphocytes, and neutrophils [8–12]. Recently, it was demonstrated that ethanol inhibits LPS-induced acc tion of leukocytes [13], which suggests that it may als hibit arthritis induced by MSU crystals. Therefore, e examined the effects of ethanol on MSU l-indu inflammation *in vitro* as well as *in vivo* in er lucida the mechanisms involved.

2. Materials and methods

2.1. Reagents

Uric acid was pu ased from Wa Pure Chemicals Ltd. (Osaka, Japa nd M U crystals were produced by a method previou es ed [14]. In brief, after treating uric acid for 2 h at C, a acid solution (0.03 M, pH 7.5) w p red b is ution of equimolar quantities of e acid nd sodi hydroxide. Sodium chloride (0.1 M l co n) was added to accelerate the formation o mic MSU crystallization. Endotoxin was not detected in g MSU crystals/ml in PBS (<0.8 endotoxin pg/ml by a lim s amoebocyte assay.

2.2. Cell culture conditions

THP1 cells from a human monocyte cell line, were grown in RPMI 1640 containing penicillin–streptomycin and 20% fetal cow serum (FBS) at 37 °C under 5% CO_2/95% air. Prior to the experiments, cells (0.5×10^6 cells) were plated in flat-bottomed 24-well plates (Iwaki Glass, Tokyo, Japan) in 1 ml of RPMI 1640 containing penicillin–streptomycin and 20% fetal cow serum, then pretreated for differentiation with 10 nM phorbol 12-myrisate 13-acetate (PMA) for 24 h. Thereafter, the culture medium was aspirated and 0.5 ml of fresh medium containing penicillin–streptomycin and 1% fetal cow serum was added, with or without MSU crystals (0.25, 0.5, or 0.75 mg/ml) in the presence or absence of ethanol (0%, 0.4%, or 0.8% concentration). Differentiated cells cultured with medium containing 20% fetal cow serum secreted considerable amounts of cytokines, while the increase in MSU crystals-induced cytokine secretion was slight, as compared with the baseline values. On the other hand, thos ltured with 1% fetal cow serum secreted negli e amo of cytokines, whereas the increase in M crystal-in ed cytokine secretion was significant There 1% fe cow serum was used instead of 2 fetal co ru n the present study. Twelve hours er the dition esh culture medium, the medium w pi ed to measure the secretion of cytokines. In dition inves ate the expression of phosphoryl p38, JNK and RK1/2 proteins, 1 h after the addi esh medi the medium was removed and THP1 cells w washed with PBS. The cells were then dis with RIP ffer consisting of PBS, 1% Nonidet 0, 0.5% sodium deoxycholate, and 0.1% SDS containing 0 μg of P SF/ml RIPA, 30 μl of aprotinin (Sigma, St. is, MO, A)/ml RIPA, and 1 μmol sodium orthovan- a /ml o IPA, after which the expression of the proteins etermined.

2.3. Cytokines

Human and mouse IL-8 levels were measured using Human IL-8 ELISA kit (BMI Medical & Biological Laboratories Co., Ltd., Nagoya, Japan). Human IL-1β, TNF-α, and IL-6 levels and those levels of mouse IL-1β, IL-6, KC, and MIP1α were determined with commercially available ELISA kits (human: BioSource International, Inc., CA, USA, mouse: R&D Systems Minneapolis, MN, USA).

2.4. Immunoblotting of phosphorylated p38, JNK, and ERK proteins

RIPA containing 5 μg of protein from the dissolved cells was applied to gels and electrophoresis was performed, after which immunoblotting was performed as described previously [15]. In brief, phosphorylated p38, JNK, and ERK1/2 proteins together with GAPDH protein as a control were each transferred electrophoretically from a 10% SDS–polyacrylamide gel to a PVDF membrane at 1 mA/cm^2 for 90 min, using Trans Blot SD (Bio-Rad Laboratories, Hercules, CA, USA). After incubation with Block Ace (Dainippon Pharmaceuticals, Osaka) overnight at 4 °C, the PVDF membrane was washed with 50 mM Tris-buffered saline (pH 7.4) (TBS) and incubated overnight at room temperature in a dilution of anti-phospho-SPARK/JNK (Thr183/Tyr185) (Cell Signaling Technology Inc., MA, USA), phospho-p44/42 map kinase (Thr202/Tyr204) (Cell Signaling Technology Inc.), p38 mitogen-activated protein kinase (MAPK) (pT180/pY182)

phospho-specific (BD Transduction Laboratories, CA, USA) or GAPDH(V-18):sc-20357 (Santa Cruz Biotechnology Inc., CA, USA) antibodies in TBS containing 10% (V/V) Block Ace. The PVDF membrane was then washed three times for 10 min each, with TBS and incubated with a 1:500 dilution of a secondary antibody (biotinylated anti-rabbit IgG, anti-mouse IgG; or anti-goat antibody) for 3 h at room temperature. Next, the PVDF membrane was washed 4 times with TBS and incubated with avidin–biotin complex (Vectastain ABC Elite kit; Vector Laboratories, CA, USA). Finally, the phosphorylated p38, JNK, and ERK1/2 proteins along with the GAPDH protein were visualized by incubation with 0.01% H_2O_2 in TBS containing 0.05% diaminobenzidine.

2.5. Preparation of nuclear and cytoplasmic extracts and immunoblotting of phosphorylated NF-κB and nonphosphorylated IκBα

Differentiated THP1 cells were incubated with fresh medium containing penicillin–streptomycin and 1% fetal cow serum, with or without MSU crystals (0.75 mg/ml) in the presence or absence of 0.8% ethanol at 37 °C for 1 h, then nuclear and cytoplasmic extracts were prepared using an NE-PER kit (Pierce, Rockford, IL, USA). Thereafter, using 5 μg protein of each extract, western blot analyses of phosphorylated NF-κB and nonphosphorylated IκBα expressions were performed as described above, using phospho-NF-κB p65 (Ser 536) antibody (Cell Signaling Technology Inc.) and IκBα/MAD-3 (BD Transduction Laboratories, CA, USA). As controls for nuclear and cytoplasmic proteins, p44/p42 and β-actin, respectively, were used.

2.6. Mice

Specific pathogen-free female C57BL/6 mice (6–8 week old) were purchased from SLC (Shizuoka, Japan).

2.7. Treatment of mice

It is considered better to inject MSU crystals into mouse joints to investigate MSU crystal-induced arthritis. However, it is very difficult to inject MSU crystals into mouse joints and aspirate joint fluids after such an injection. Therefore, we performed the present *in vivo* experiment using a subcutaneous air pouch, which is similar to a joint in terms of MSU crystal-induced inflammation [16,17].

Subcutaneous air pouches were created in the backs of the mice according to a method previously described [16]. Three milliliters of air was injected into the subcutaneous tissue, followed by a second injection of 5 ml of air after 3 days. Seven days after the first injection, a preparation of MSU crystals (3 mg in a volume of sterile PBS) was injected into the air pouches 15 min after intraperitoneal injection of sterile PBS containing ethanol (5 ml/kg body weight) or sterile PBS alone. As a control, the same volume of sterile PBS was injected into the air pouches 15 min after intraperitoneal injection of sterile PBS. Eight hours later, the pouch fluid was harvested by injecting 3 ml of PBS. The infiltrating cells were then counted using a hemocytometer and stained with Wright–Giemsa solution to perform a differential count of leukocytes. In addition, the pouch fluid sample was centrifuged at 1500*g* for 10 min and the supernatant was obtained.

2.8. Statistical analysis

Values are expressed as median (range) or mean ± SD, and significant differences between the variables were analyzed using a nonparametric method or ANOVA. Significance was defined as $P < 0.05$.

3. Results

3.1. In vitro data

MSU crystals stimulated the secretion of TNF-α from THP1 cells, which were enhanced by increases in MSU crystal concentration in medium in a range of 0.25–0.75 mg/ml (Table 1). On the other hand, ethanol at concentrations of 0.4% and 0.8% reduced the secretion of TNF-α induced by 0.75 mg/ml MSU crystals by 1.51-fold ($P < 0.01$) and 1.79-fold ($P < 0.01$), respectively (Table 1).

MSU crystals also stimulated the secretion of IL-8 from THP1 cells, which was enhanced by increases in MSU crystal concentration in medium in a range of 0.25 to 0.75 mg/ml (Table 2). On the other hand, ethanol at concentrations of 0.4% and 0.8% reduced the secretion of IL-8 induced by 0.75 mg/ml of MSU crystals by 1.52-fold ($P < 0.01$) and 1.63-fold ($P < 0.01$), respectively, that induced by 0.5 mg/ml of MSU crystals by 1.47-fold ($P < 0.01$) and 1.92-fold ($P < 0.01$), respectively, and that induced by 0.25 mg/ml of MSU crystals by 1.21-fold ($P < 0.01$) and 1.89-fold ($P < 0.01$), respectively (Table 2).

We also noted that MSU crystals stimulated the secretion of IL-1β from THP1 cells, which was enhanced by increases in MSU crystal concentration in medium in a range of 0.25–0.75 mg/ml (Table 3). On the other hand, ethanol at concentrations of 0.4% and 0.8% reduced the secretion of IL-1β induced by 0.75 mg/ml of MSU crystals by 1.49-fold ($P < 0.01$) and 1.75-fold ($P < 0.01$), respec-

Table 1
Effects of ethanol on MSU-increased secretion of TNF-α (pg/ml) from THP1 cells

	0% Ethanol	0.4% Ethanol	0.8% Ethanol
0.75 mg/ml MSU	1732 ± 78	1148 ± 68**	965 ± 51**
0.5 mg/ml MSU	1213 ± 78	787 ± 58**	577 ± 32**
0.25 mg/ml MSU	633 ± 47	520 ± 82**	306 ± 37**
0 mg/ml MSU	ND	ND	ND

Values are expressed as the mean ± SD (number of samples = 5). ND denotes below the limits of detection of TNF-α (<0.9 pg/ml).
** $P < 0.01$, as compared with the respective value with 0% ethanol.

Table 2
Effects of ethanol on MSU-increased secretion of IL-8 (pg/ml) from THP1 cells

	0% Ethanol	0.4% Ethanol	0.8% Ethanol
0.75 mg/ml MSU	81,605 ± 3503	53,768 ± 4045**	49,985 ± 5031**
0.5 mg/ml MSU	62,628 ± 9150	42,714 ± 4093**	31,830 ± 1468**
0.25 mg/ml MSU	54,873 ± 5631	45,333 ± 6529**	28,978 ± 2726**
0 mg/ml MSU	ND	ND	ND

Values are expressed as the mean ± SD (number of samples = 5). ND denotes below the limits of detection of IL-8 (<32 pg/ml).
** $P < 0.01$, as compared with the respective values in 0% ethanol.

Table 3
Effects of ethanol on MSU-increased secretion of IL-1β (pg/ml) from THP1 cells

	0% Ethanol	0.4% Ethanol	0.8% Ethanol
0.75 mg/ml MSU	10,579 ± 852	7075 ± 414**	6046 ± 397**
0.5 mg/ml MSU	8032 ± 1079	6039 ± 576**	5000 ± 352**
0.25 mg/ml MSU	5502 ± 136	4844 ± 299**	3913 ± 133**
0 mg/ml MSU	ND	ND	ND

Values are expressed as the mean ± SD (number of samples = 5). ND denotes below the limits of detection of IL-1β (<4 pg/ml).
** $P < 0.01$, as compared with the respective value with 0% ethanol.

tively, that induced by 0.5 mg/ml of MSU crystals by 1.33-fold ($P < 0.01$) and 1.61-fold ($P < 0.01$), respectively, that induced by 0.25 mg/ml of MSU crystals by 1.14-fold ($P < 0.01$) and 1.41-fold ($P < 0.01$), respectively (Table

3.2. Effects of ethanol on MSU crystal-increased expression of phosphorylated JNK, ERK1/2, and p38 protein

MSU crystals at 0.75 mg/ml increased the expression of phosphorylated JNK, ERK1/2, and p38 proteins by 27.21 ± 2.76-fold (number of samples = 3) ($P < 0.01$), 2.16 ± 0.23-fold (number of samples = 3) ($P < 0.01$), and 1.82 ± 0.08-fold (number of samples = 3) ($P < 0.01$), respectively (Fig. 1). Further, ethanol at a concentration of 0.4% reduced the MSU crystal-increased expression of those by 1.13 ± 0.07-fold (number of samples = 3) ($P < 0.05$), 1.18 ± 0.04-fold (number of samples = 3) ($P < 0.01$), and 1.21 ± 0.04-fold (number of samples = 3) ($P < 0.01$), respectively, and ethanol at 0.8% reduced them by 1.28 ± 0.04-fold (number of samples = 3) ($P < 0.01$), 1.14 ± 0.03-fold (number of samples = 3) ($P < 0.01$), and 1.68 ± 0.06 (number of samples = 3) ($P < 0.01$), respectively (Figs. 1 and 2).

3.3. Effects of ethanol on MSU crystal-increased expression of phosphorylated NF-κB protein in nuclear and cytosolic fractions, and on MSU crystal (0.75 mg/dl)-decreased expression of IκBα proteins in cytosolic fraction

MSU crystals at 0.75 mg/ml increased the expression of phosphorylated NF-κB protein in nuclear and cytosolic fractions by 1.25 ± 0.03-fold (number of samples = 3) ($P < 0.01$) and 1.27 ± 0.04-fold (number of samples = 3)

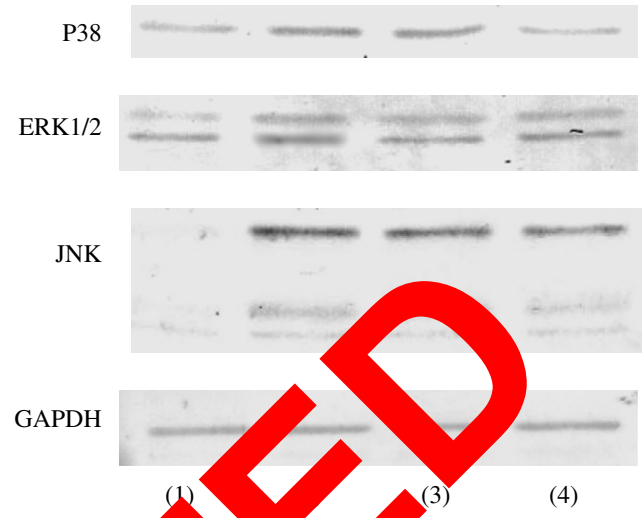

Fig. 1. Immunoblots of phosphorylated p38, ERK1/2, and JNK (1), control; (2), with MSU crystal (0.75 mg) (3), with MSU crystals (0.75 mg) and 0.4% ethanol; and with MSU crystals (0.75 mg) and 0.8% ethanol.

($P < 0.01$), respectively, and decreased the expression of IκBα protein in the cytosolic fraction by 1.63 ± 0.16-fold (number of samples = 3) ($P < 0.01$). Further, ethanol at 0.8% reduced the MSU-increased expression of phosphorylated NF-κB protein in the nuclear and cytosolic fractions by 1.25 ± 0.03-fold (number of samples = 3) ($P < 0.05$)

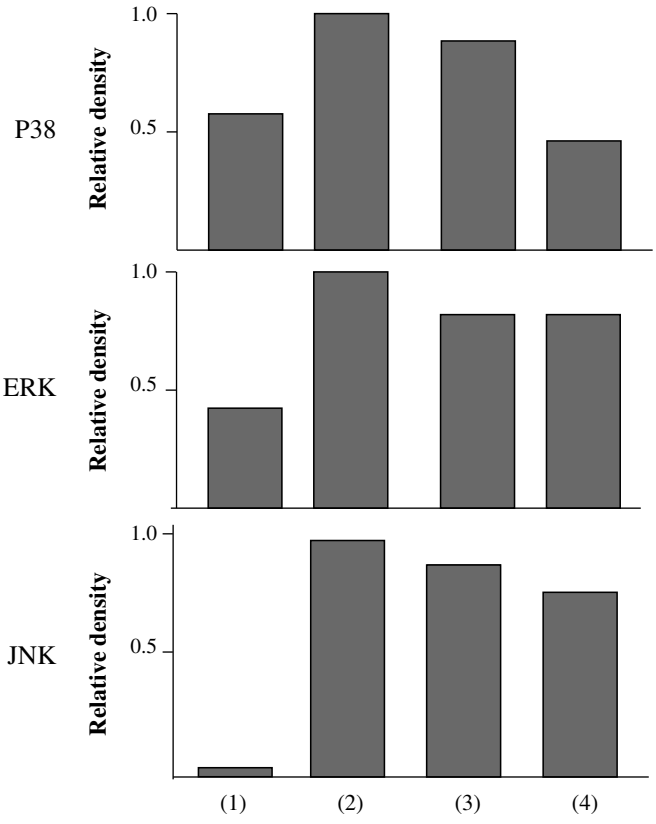

Fig. 2. Effects of ethanol on MSU-increased expression of phosphorylated p38, ERK1/2, and JNK. Bars labeled (1), (2), (3), and (4) are the same as in Fig. 1.

Table 4
Effects of ethanol on MSU-induced inflammation in mice

	MSU crystals	MSU crystals with ethanol	Vehicle (PBS)
KC (pg/ml)	371 (352–1836)	127 (48–139)**	20 (10–30)**
MIP1α (pg/ml)	19.9 (11.0–57.7)	4.1 (3.3–5.5)**	2.3 (1.3–2.4)**
IL-1β (pg/ml)	109.2 ± 36.9	6.1 ± 5.2*	ND*
IL-6 (pg/ml)	502 ± 284	27 ± 15*	ND*
TNF-α (pg/ml)	ND	ND	ND
Infiltrating cells	$11.6 \pm 5.3 \times 10^5$	$2.7 \pm 1.0 \times 10^{5*}$	$1.1 \pm 0.7 \times 10^{5*}$
Neutrophils	$11.0 \pm 5.4 \times 10^5$	$2.0 \pm 1.0 \times 10^{5*}$	$0.7 \pm 0.3 \times 10^{5*}$

Values are expressed as the mean ± SD or median (range) (number of samples = 5).
*,**$P < 0.05$ and $P < 0.01$, respectively, as compared with the respective values in the presence of MSU crystals. ND denotes below the limit of detection of IL-6, IL-1β, and TNF-α (each <16 pg/ml).

and 1.27 ± 0.04-fold (number of samples = 3) ($P < 0.05$) respectively, and also reduced the MSU-decreased expression of IκBα in cytosolic fraction by 1.12 ± 0.02-fold (number of samples = 3) ($P < 0.01$).

3.4. Effects of ethanol on MSU crystal-induced inflammation in vivo

In the subcutaneous pouches of mice, MSU crystals-induced acute inflammation, as compared with the control group. Our results showed that KC, MIP1α, IL-1β, and IL-6 were increased, together with increases in the numbers of leukocytes and neutrophils (Table 4). On the other h ethanol considerably reduced the MSU crystal-indu inflammation by reducing the secretion of KC, MIP IL-1β, and IL-6, as well as numbers of leukocy nd neu trophils by 2.92-fold ($P < 0.01$), 4.85-fold (0. 17.9- fold ($P < 0.05$), 18.6-fold ($P < 0.05$), 4. old (0.05), and 5.5-fold ($P < 0.05$), respectively (Tab).

4. Discussion

A number of studies h e reporte t ethanol inhibited LPS-induced incre in inflammat cytokine production in macroph –mon yte lineage cells [18–21], which was shown t cu a decreases in mitogen-activated protein kinase PK) [–23]. In addition, it was reporte anol fect on TLR4, probably by chang the c membra , and inhibits LPS-induced p38 ph ory ding to a reduction in LPS-induced sec n of IL-8 and TNF-α [23]. These findings suggest that e ol reduces LPS-induced inflammation.

Recently, it w reported that MSU crystals activated caspase-1 [16,24] and also processed the inactive form of IL-1β to the active form via the NACHT-LRR-PYD-containing protein-3 inflammasome [24]. In addition, it was demonstrated that MSU crystals stimulated the secretion of cytokines containing IL-1β and TNF-α from monocytes via TLR2 and TLR4 [25], while another study found that MSU stimulated the secretion of IL-1β from resident cells and the secreted IL-1β stimulated an MyD88-dependent amplification of proinflammatory responses via the IL-1β receptor (IL-1 R) on non-bone marrow-derived cells, suggesting that the secretion of IL-1β from resident cells induced by MSU crystals ays an im tan le in gouty attack [26]. These findi s indic e that p produced by MSU crystal-induced tiv n of caspase-1 may cause proinflammatory spon a a M 8-dependent signal pathway inclu g MAPK κB. They also suggest that ethano MSU-ind d inflammation. Accordingly, we conducte present study to determine whether ethan hibits the SU crystal-induced secretion of inf matory cytokines *in vitro* and *in vivo*.

the pres *in vitro* study, MSU crystals stimulated th cretion o L-8, TNF-α, and IL-1β from THP1 cells, whi hanol ibited the MSU-induced secretion of these cytoki bles 1–3). A previous study demonstrated MSU crystals-induced the activation of JNK, , and p38 in THP1 cells, and also increased their secretion of IL-8 [27]. Further, a recent more study showed that MSU crystals promoted MSK1/2, ERK1/2, and IκBα phosphorylation as well as NF-κB and AP-1 translocation in the murine macrophage cell line B10R [28]. Our findings also indicate that MSU crystals stimulate IL-8 secretion from THP1 cells together with increased phosphorylation of ERK1/2, JNK, and p38 (Figs. 1 and 2). In addition, they increased the levels of phosphorylated NF-κB in the cytosolic and nuclear fractions and decreased IκBα in the cytosolic fraction (Figs. 3 and 4). Therefore, the present results

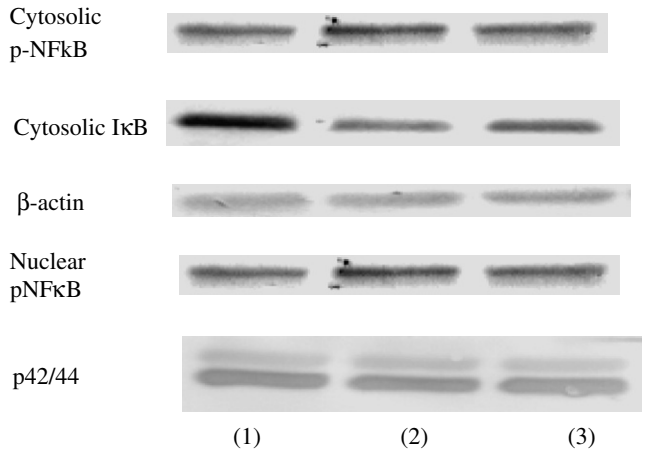

Fig. 3. Effects of ethanol on MSU-increased expression of phosphorylated NF-κB in nuclear and cytosolic fractions, and on MSU-decreased expression of IκBα in cytosolic fraction. (1) Control; (2) with MSU crystal (0.75 mg); (3) with MSU crystals (0.75 mg) and 0.8% ethanol.

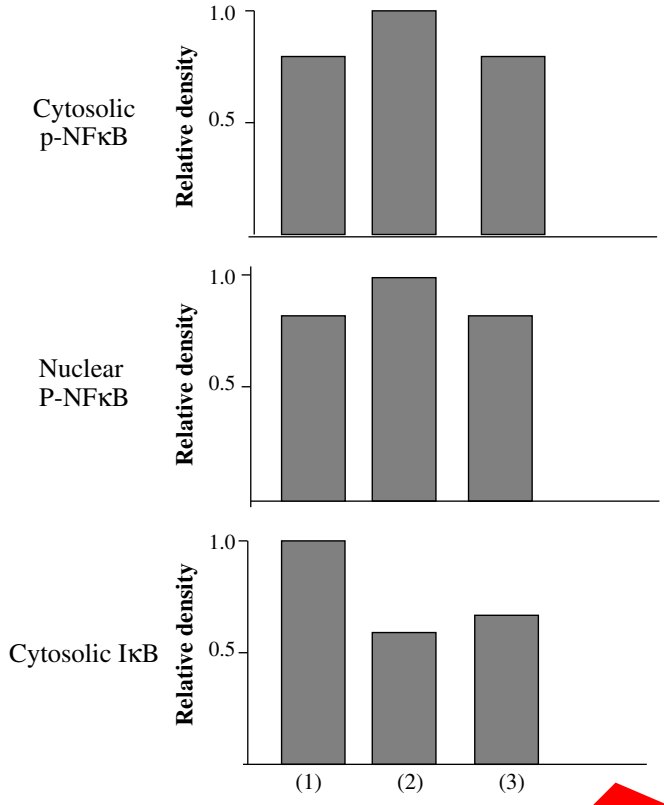

Fig. 4. Effects of ethanol on MSU-increased expression of phospho[rylated] NF-κB in nuclear and cytosolic fractions and on MSU-de[creased] expression of IκBα in cytosolic fraction. Bars labeled (1), (2), an[d (3)] are the same as in Fig. 3.

showed that MSU crystals produce pro[-in]kines via a pathway including M[AP]K and [NF]-κB in THP1 cells, as described above [27]. Further, [in] the present experiments, ethanol slight[ly] inhibited the MSU crystal-induced increases in phosphorylated ERK1/2 and JNK, while it considerably inhibited the increase in phosphorylated p38. In addition, ethanol inhibited the MSU crystal-induced increases in phosphorylated NF-κB in the cytosolic and nuclear fractions as well as MSU crystal-induced d[egradation] of IκBα in the cytosolic fraction. Ethanol also inhibited the secretion of IL-8, TNF-α, and IL-1β in THP1 cells induced by MSU crystals. Thus, we concluded that ethanol decreases the MSU-induced secretion of IL-8, TNF-α, and IL-1β via pathways, including MAPK (p38, JNK, and ERK1/2, especially p38) and NF-κB.

Arbabi et al. reported that ethanol inhibited LPS-induced p38 phosphorylation in human mononuclear cells [8], whereas Oak et al. showed that ethanol did not have an effect on LPS-induced p38 phosphorylation [29]. Those contrasting results may be related to the timing differences between the administration of ethanol and TLR ligand utilized in their experiments, and/or differences in ethanol concentration in the media. In the former study, ethanol ranging from 46 to 174 mM was added to culture media that included monocytes at 1 h before the addition of LPS, while in the latter, ethanol at 25 mM and LPS were administered at the same time. In the present study, we added ethanol at 46–174 mM to culture media that included THP1 cells at 1 h before the addition of MSU crystals, since patients with gout typically have a gouty attack following the ingestion of alcoholic beverages. Our results are similar to those of Arbabi et al. [8], though we used THP1 cells instead of monocytes.

Oak et al. also demonstrated that ethanol enhanced increases in phosphorylated ERK1/2 and JNK proteins, whereas it did not have an effect on phosphorylated p38 protein in monocytes in the presence of TLR2 and TLR4 ligands (PGN and LPS) [29]. Further, they demonstrated that ethanol inhibited the increase in phosphorylated ERK1/2 proteins, but did not have an effect on phosphorylated JNK protein in monocytes in the presence of a TLR4 ligand (LPS) [29]. In contrast, in the present study, ethanol slightly inhibited MSU crystal-induced increases in phosphorylated ERK1/2 and JNK proteins, while it considerably inhibited the increase in phosphorylated p38 protein in THP1 cells. The differences between the results may be attributable to the different ligands used. In our *in vivo* experiment, MSU crystals caused neutrophil accumulation and the secretion of KC, IL-6, IL-1β, and MIP1α when injected into subcutaneous air pouches in mice. However, TNF-α secretion from THP1 cells induced by administration of the MSU crystals was not detected in the pouches 8 h after injection. In a previous rabbit study, the secretion of TNF-α in joints increased and peaked at 2 h after intra-articular administration of MSU crystals, and then rapidly decreased until only small amounts of TNF-α were detected after 4 h [30]. Together, these results suggest that TNF-α is abruptly secreted immediately after the administration of MSU crystals for a period of a few hours. However, since intra-articular administration of MSU crystals together with the anti-TNF-α antibody reduced the number of inflammatory cells in the rabbit joints, as compared with administration of MSU crystals alone, it was suggested that TNF-α plays a role in MSU crystal-induced arthritis. In addition, intra-articular administration of MSU crystals together with the anti-IL-8 antibody or IL-1β receptor antagonist also reduced the number of inflammatory cells, as compared with administration of MSU crystals alone. Those results also suggest that IL-8 and IL-1β play roles in MSU crystal-induced arthritis. Therefore, even though TNF-α was not detected in our *in vivo* experiment, it may enhance MSU crystal-induced inflammation together with other cytokines (KC, IL-6, IL-1β, and MIP1α).

A recent study of IL-1β receptor-defective type mice found that inflammatory reactions, including neutrophil accumulation, due to MSU were markedly decreased, as compared with wild type mice, indicating that IL-1β is an important factor in gouty arthritis and that MyD88-dependent IL-1β receptor signaling is essential for that condition [26]. Since KC and MIP1α are also neutrophil chemoattractants, in addition to IL-1β, the present results suggest that ethanol inhibited the MSU crystal-induced secretion

of KC, MIP1α, and IL-1β from resident cells such as macrophages via a pathway, including MAPK and NF-κB and suppressed neutrophil accumulation in the subcutaneous air pouches created in mice. They also show that though the effect is short-lived, the anti-inflammatory action of ethanol may retard the onset of ethanol-induced gouty arthritis despite an ethanol-induced increase in the serum concentration of urate.

Nevertheless, additional examinations are needed to elucidate the acute effects of ethanol on gouty inflammation.

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