SPANDIDOS PUBLICATIONS

MOLECULAR MEDICINE REPORTS 12: 6221-6226, 2015

Peroxisome proliferator-activated receptor γ prevents the production of NOD-like receptor family, pyrin domain containing 3 inflammasome and interleukin 1β in HK-2 renal tubular epithelial cells stimulated by monosodium urate crystals

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Received October 29, 2014; Accepted July 3, 2015

DOI: 10.3892/mmr.2015.4145

Abstract. Recent evidence showed that peroxisome proliferator-activated receptor γ (PPARγ) ameliorates a variety of inflammatory conditions. The present study aimed to investigate the role of PPARγ in regulating NOD-like receptor family, pyrin domain containing 3 (NALP3) inflammasome and interleukin (IL)-1β levels during monosodium urate (MSU) crystal-induced inflammation. HK-2 cells were incubated with or without 200 μg/ml MSU crystals, and mRNA and protein levels of PPARγ were determined using reverse transcription quantitative polymerase chain reaction and western blot analysis, respectively. To verify the role of PPARγ, HK-2 cells were pre-treated with PPARγ agonist pioglitazone, and the levels of NALP3 inflammasome and IL-1β were detected by western blot analysis and ELISA. The results showed that MSU crystals increased PPARγ expression in HK-2 cells at 24 h, while the expression decreased to normal levels at 48 h. It was also demonstrated that although the PPARγ agonist pioglitazone did not alter the mRNA and protein levels of PPARγ, it significantly reduced the MSU crystal-induced production of NALP3 inflammasome and IL-1β in HK-2 cells, possibly by increasing the level of PPARγ activity. In conclusion, the results of the present study indicated that PPARγ prevented NALP3 inflammasome formation and IL-1β production in HK-2 cells stimulated by MSU crystals, which indicated that PPARγ may represent a novel target for the treatment of hyperuricemic nephropathy.

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Key words: peroxisome proliferator-activated receptor γ, monosodium urate crystals, renal tubular epithelial cells, interleukin 1β, NALP3 inflammasome, pioglitazone

Introduction

The incidence of hyperuricemia is steadily increasing in the world population and has therefore become a focus of recent studies (1,2). Hyperuricemia is not only a marker of chronic kidney disease but is also an independent risk factor for numerous types of kidney disease (3,4). Experimental studies have demonstrated a variety of mechanisms by which hyperuricemia causes the development of renal disease. One of the key mechanisms of hyperuricemia-induced kidney injury is the inflammation provoked by monosodium urate (MSU) crystals (5-7). MSU crystals were first identified as the etiological agent of gout in the eighteenth century and more recently as a danger signal released from dying cells (8).

NOD-like receptor (NLR) family, pyrin domain containing 3 (NALP3) inflammasome and interleukin (IL)-1β were reported to be crucial molecules in MSU crystal-mediated inflammation. NALP3 inflammasome is an innate immune complex that contains NALP3, caspase-recruitment domain (CARD)-8, and apoptosis-associated speck-like protein containing a CARD (ASC). NALP3 is a member of the NLR family, which not only detects microbial structure but also senses endogenous danger signals such as uric acid released from injured cells (9). ASC is an essential component of the NALP3 inflammasome, as it can recruit caspase-1 to the inflammasome (10), while the protein CARD-8 normally inhibits activation of caspase-1 (11). NALP3 inflammasome controls IL-1β production by recruiting caspase-1, which directly cleaves cytokine IL-1β precursors into active forms (12). Martinon *et al* (9) reported that MSU crystals induced inflammation, activation of NALP3 inflammasome, and production of active IL-1β and IL-18. Furthermore, an impaired neutrophil influx was found in inflammasome-deficient mice. Miao *et al* (13) suggested that gene mutations in NALP3 and CARD-8 may contribute to susceptibility to gout.

IL-1β belongs to the IL-1 family of cytokines and is pivotal to the regulation of innate and adaptive immunity (14). Chen *et al* (15) showed that Il-1β is important in MSU crystal-induced inflammation based on findings of a markedly decreased inflammatory response to MSU crystals in IL-1R-deficient mice. The finding that IL-1β inhibition was

efficacious in the treatment of MSU crystal-induced inflammation suggested a crucial role for IL-1β in this type of inflammation (16).

Peroxisome proliferator-activated receptor γ (PPARγ) belongs to the nuclear hormone receptor superfamily and acts as a transcriptional regulator of numerous target genes by forming heterodimers with the retinoid X receptor (17). PPARγ is expressed not only in white adipose tissue but also in proximal tubular cells (18). It has been evidenced that activation of PPARγ attenuated the expression of pro-inflammatory mediators (19,20). In addition, an increasing number of studies suggested that PPARγ agonists, including pioglitazone and troglitazone, have a protective effect on renal function in various models of acute and chronic renal injury (21,22). However, the effects of PPARγ ligand on NALP3 inflammasome and IL-1β production in MSU crystal-stimulated HK-2 cells have remained elusive. The present study was therefore performed to investigate the expression of PPARγ in MSU crystal-stimulated HK-2 cells. The PPARγ agonist pioglitazone was used to assess the regulatory effects of PPARγ on NALP3 inflammasome and IL-1β expression levels.

Materials and methods

Cell line and culture. The primary human proximal tubular cell line HK-2 was obtained from the American Type Culture Collection (Manassas, VA, USA). HK-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Gibco-Brl, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FBS; Gibco-BRL) at 37˚C in a humidified 5%-CO_2 incubator.

Preparation of MSU crystals. MSU crystals were prepared according to the following process: First, 0.8 g uric acid (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 200 ml 0.1 M borate buffer (pH 8.5; Thermo Fisher Scientific, Waltham, MA, USA). Through the addition of HCl, the pH of the solution was adjusted to 8.0. The solution was then passed through a 0.22-μm filter (Millipore, Billerica, MA, USA), and the supersaturated uric acid solution was left at room temperature for seven days to allow the formation of fine crystals. After two washes with absolute ethanol and one wash with acetone, the crystals were allowed to air-dry and were suspended in phosphate-buffered saline (PBS) at a concentration of 8 mg/ml. All MSU crystals were verified to be endotoxin-free by the Limulus amebocyte cell lysate assay (Xiamen Limulus Reagent Company, Xiamen, China).

Cell treatments. Upon 80%-confluency, cells were divided into four groups, which were incubated in serum-free medium for 24 h as follows: (A) FBS-free medium only; (B) MSU crystals (200 μg/ml); (C) lipopolysaccharide (LPS) (100 μg/ml; Sigma-Aldrich); (D) pre-treatment with pioglitazone for 12 h (5 μmol/l; Sigma-Aldrich) followed by incubation with MSU crystals (200 μg/ml).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from HK-2 cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized using the Reverse Transcription system (PrimeScript™ RT master mix; Takara Bio Inc., Shiga, Japan) consisting of 10 μl reaction mixture (5 μl 5X PrimeScript buffer, 1 μl PrimeScript® RT enzyme mix I, 1 μl oligo dT Primer, 1.5 μl Random 6 mers and 1.5 μl Total RNA) incubated at 37˚C for 15 min followed by 85˚C for 5 sec. PCR amplification of cDNA was performed according to the instructions in the Takara Taq™ HS PCR kit (Takara Bio Inc.) with 1 μl cDNA in a final volume of 25 μl. The sequences of primers for PCR were as follows: PPARγ (313 bp) forward, 5'-AGCCAACACTAAACCACA-3' and reverse, 5'-AGAAACCCTTGCATCCT-3'; GAPDH (638 bp) forward, 5'-AGTCCACTGGCGTCTTCAC-3' and reverse, 5'-GCTTGACAAAGTGGTCGTTGAG-3' (Sangon Biotech, Shanghai, China). PCR was carried out in the GeneAmp®PCR System 9700 (Applied Biosystems, Foster City, CA, USA) and PCR products were electrophoresed on 1% agarose gel (Biowest, Barcelona, Spain) using HE-120 Electrophoresis Cell (Shanghai Tanon Science and Technology Ltd., Shanghai, China), and detected by ultraviolet transillumination (Shanghai Furi Science & Technology Co. Ltd, Shanghai, China). Gel-Pro Analyzer 4.0 (Media Cybernetics, Bethesda, MD, USA) was used for quantification of the bands.

Western blot analysis. Protein concentrations were determined using the Pierce BCA protein assay reagent kit (Thermo Fisher Scientific) following the manufacturer's instructions. Proteins (20 μg per lane) were separated by 12% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore). The membranes were blocked in 5% bovine serum albumin (Sigma-Aldrich), and incubated with mouse monoclonal anti-human NALP3 antibody (1:1,000; cat. no. ab17267; Abcam, Cambridge, UK), mouse monoclonal anti-human PPARγ antibody (1:1,000; cat. no. ab70405; Abcam) and GAPDH antibody (1:5,000, cat. no. ab8245; Abcam) overnight at 4˚C, followed by incubation with a horseradish peroxidase-labeled anti-mouse antibody (1:5,000; cat. no. A0216; Beyotime Institute of Biotechnology, Haimen, China) for 1 h at room temperature. Protein was detected using an ECL western blotting kit (Thermo Fisher Scientific) and X-OMAT BT film (Carestream, Xiamen, China) in an X-ray film cassette (Shanghai Kunlei Medical Instrument Co., Ltd., Shanghai, China). The bands were quantified using Gel-Pro Analyzer 4.0. The results of protein expression were normalized to GAPDH in all figures.

ELISA for detection of IL-1β. HK-2 cells were treated for 48 h as described above. Supernatants were then collected, and the levels of IL-1β protein were measured using an IL-1β (human) ELISA kit according to the manufacturer's instructions (BioVision, San Francisco, CA, USA). OD was determined by a microplate reader (Multiskan MK3; Thermo Fisher Scientific) at 450 nm.

Statistical analysis. Statistical analysis was performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Values are expressed as the mean ± standard deviation. The standard error of the mean was shown for all experiments. Comparisons between groups were evaluated by analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference.

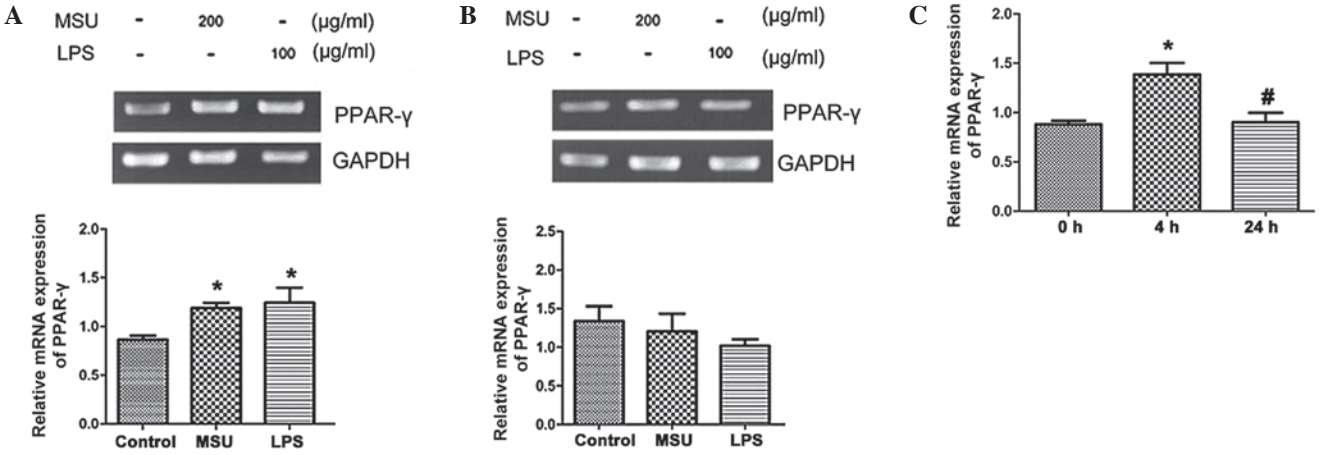

Figure 1. Levels of PPARγ mRNA in MSU crystal-treated HK-2 cells were unstable. (A) HK-2 cells were treated with 200 μg/ml MSU crystals or 100 μg/ml LPS as a positive control for 12 h. Levels of PPARγ mRNA were detected using reverse transcription quantitative polymerase chain reaction. *P<0.05 vs. control. (B) HK-2 cells were treated with 200 μg/ml MSU crystals or 100 μg/ml LPS as a positive control for 24 h. (C) HK-2 cells were treated with 200 μg/ml MSU crystals for 0, 4 or 24 h and mRNA levels of PPARγ were detected. *P<0.05 vs. 0 h and #P<0.05 vs. 4 h. Values are stated as the PPARγ/GAPDH ratio and are expressed as the mean ± standard deviation of results from three experiments. LPS, lipopolysaccharide; PPARγ, peroxisome proliferator-activated receptor γ; MSU, monosodium urate.

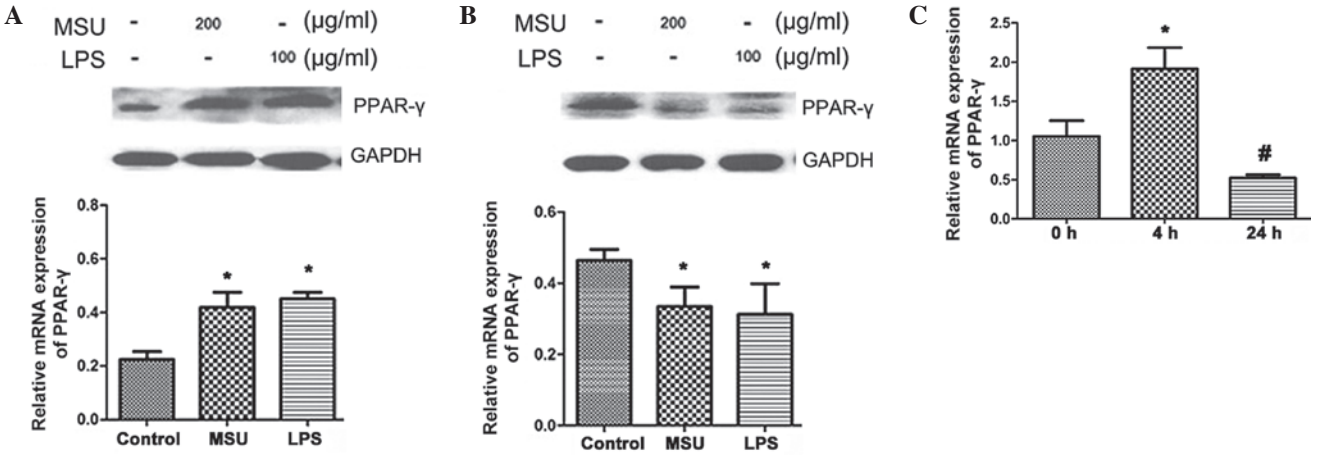

Figure 2. Protein levels of PPARγ in MSU crystal-treated HK-2 cells were unstable. (A) HK-2 cells were treated with 200 μg/ml MSU crystals or 100 μg/ml LPS as a positive control for 24 h. Protein levels of PPARγ were detected using western blot analysis. *P<0.05 vs. control. (B) HK-2 cells were treated with 200 μg/ml MSU crystals or 100 μg/ml LPS as a positive control for 48 h. *P<0.05 vs. control. (C) HK-2 cells were treated with 200 μg/ml MSU crystals for 0, 4 or 24 h, and protein levels of PPARγ were detected. *P<0.05 vs. 0 h and #P<0.05 vs. 24 h. Values are stated as the PPARγ/GAPDH ratio and are expressed as the mean ± standard deviation from three experiments. LPS, lipopolysaccharide; PPARγ, peroxisome proliferator-activated receptor γ; MSU, monosodium urate.

Results

Effects of MSU crystals on PPARγ mRNA expression in HK-2 cells. After 12-h stimulation, MSU crystals (200 μg/ml) and LPS (100 μg/ml) increased the levels of PPARγ mRNA expression in HK-2 cells compared to that in the untreated control group (P<0.05) (Fig. 1A). However, when HK-2 cells were stimulated for 24 h, PPARγ expression levels in the MSU crystal- and LPS-treated groups were slightly decreased compared with those in the control group; however, the differences were not significant (Fig. 1B). Since MSU crystals regulated the expression of PPARγ in a time-dependent manner, the present study further investigated the effects of MSU crystals on the mRNA expression of PPARγ at 0, 4 and 24 h. As shown in Fig. 1C, MSU crystals (200 μg/ml) significantly induced PPARγ mRNA expression at 4 h after stimulation, while gene expression declined to basal levels at 24 h. Therefore, MSU crystals increased PPARγ mRNA expression at early stages, while at later stages, PPARγ expression returned to basal levels and declined eventually.

Effects of MSU crystals on PPARγ protein expression in HK-2 cells. Next, the protein expression of PPARγ in HK-2 cells was assessed using western blot analysis. As shown in Fig. 2A, after 24 h of treatment, PPARγ protein levels were increased by MSU crystals (200 μg/ml) or LPS (100 μg/ml) (P<0.05). However, after 48 h incubation, the PPARγ protein expression levels in the MSU crystal- and LPS-treated groups were decreased compared with those in the control group (P<0.05) (Fig. 2B). Similarly to the effects of MSU crystals on mRNA levels, PPARγ protein expression was affected in a time-dependent manner: Increased PPARγ protein expression occurred at 4 h (P<0.05), but at 24 h, expression was decreased compared with that at 0 h (P<0.05) (Fig. 2C).

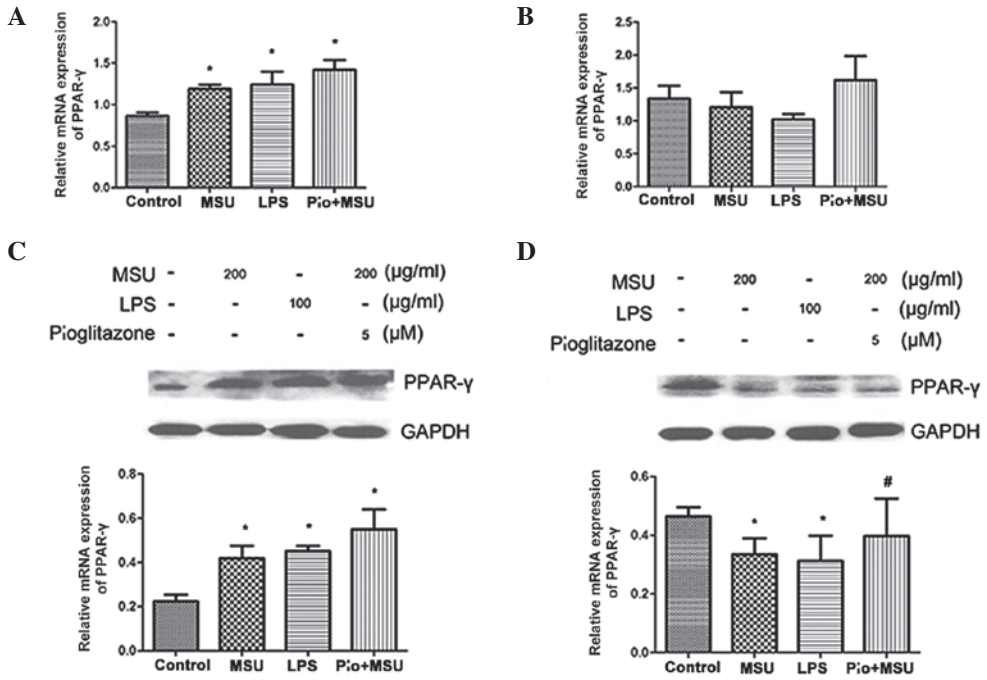

Figure 3. PPARγ ligand Pio improved MSU crystal-induced PPARγ up-regulation in the early stage but had no effect in the late stage. (A) HK-2 cells were treated with 200 μg/ml MSU crystals or 100 μg/ml LPS for 12 h or pre-treated with 5 μmol/l Pio for 12 h and then treated with 200 μg/ml MSU crystals for 12 h. Levels of PPARγ mRNA were detected using reverse transcription quantitative polymerase chain reaction. (B) HK-2 cells were treated with 200 μg/ml MSU crystals or 100 μg/ml LPS for 24 h or pre-treated with 5 μmol/l Pio for 12 h and then treated with 200 μg/ml MSU crystals for 24 h. Protein levels of PPARγ in HK-2 cells treated with 200 μg/ml MSU crystals or 100 μg/ml LPS for (C) 24 h or (D) 48 h, or pre-treated with 5 μmol/l Pio for 12 h and then incubated with 200 μg/ml MSU crystals for (C) 24 h or (D) 48 h. Protein levels of PPARγ were detected using western blot analysis. Values are stated as the PPARγ/GAPDH ratio and are expressed as the mean ± standard deviation from three experiments. *P<0.05 vs. control; #P<0.05 vs. MSU. LPS, lipopolysaccharide; PPARγ, peroxisome proliferator-activated receptor γ; MSU, monosodium urate; Pio, pioglitazone.

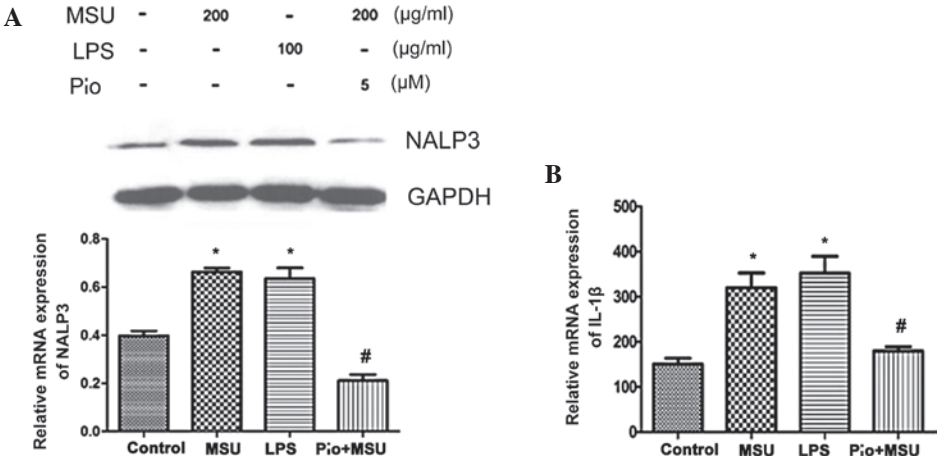

Figure 4. PPARγ regulates NALP3 and IL-1β production in MSU crystal-stimulated HK-2 cells. HK-2 cells were treated with 200 μg/ml MSU crystals or 100 μg/ml LPS for 48 h, or pre-treated with 5 μmol/l Pio for 12 h and then incubated with 200 μg/ml MSU crystals for 48 h. (A) Levels of NALP3 protein were determined using western blot analysis. Values are stated as theNALP3/GAPDH ratio and are expressed as the mean ± standard deviation from three experiments. (B) Levels of IL-1β were determined using ELISA. *P<0.05 vs. control group; #P<0.05 vs. MSU crystals group. LPS, lipopolysaccharide; PPARγ, peroxisome proliferator-activated receptor γ; MSU, monosodium urate; NALP3, NLR family, pyrin domain containing 3; IL, interleukin; Pio, pioglitazone.

Effect of PPARγ agonist pioglitazone on the expression of PPARγ in MSU crystal-stimulated HK-2 cells. To verify the role of PPARγ expression in HK-2 cells stimulated by MSU crystals, the present study further investigated the mRNA and protein levels of PPARγ in HK-2 cells which were pre-treated with PPARγ agonist pioglitazone for 12 h and then treated with 200 μg/ml MSU crystals. Pioglitazone pre-treatment induced a further increase in PPARγ mRNA expression at 12 h and partly restored basal PPARγ mRNA expression at 24 h; however, the results were not significantly different from those of the MSU crystals only-treated group (Fig. 3A and B). Similar results were obtained by western blot analyses of protein levels of PPARγ (Fig. 3C and D).

Effects of MSU crystals and PPARγ agonist pioglitazone on NALP3 inflammasome expression and IL-1β secretion.

Studies have shown that the biological activity of MSU crystals mainly depends on the activation of NALP3 inflammasome, while IL-1β mediates the release of cytokines in MSU crystal-induced inflammation (23). Therefore, the present study examined the effect of MSU crystals and pioglitazone on NALP3 and IL-1β production in MSU crystal-induced HK-2 cells. NALP3 protein levels were detected using western blot analysis and IL-1β levels were examined by ELISA. As shown in Fig. 4A, MSU crystals elevated NALP3 protein expression in HK-2 cells compared to that in the untreated control cells at 48 h ($P<0.05$), while pre-incubation with PPARγ agonist pioglitazone resulted in a significant decrease of NALP3 protein expression ($P<0.05$). IL-1β secretion increased following MSU-crystal treatment compared with that in the untreated control group. Pioglitazone almost fully inhibited MSU crystal-induced increases in IL-1β ($P<0.05$) (Fig. 4B).

Discussion

The present study demonstrated for the first time, to the best of our knowledge, that MSU crystals affected PPARγ expression in HK-2 cells. MSU crystals exerted a biphasic effect on PPARγ expression, causing an increase during the first hours of exposure, while later inhibiting PPARγ expression. In addition, it was observed that the PPARγ agonist pioglitazone mildly, but not significantly increased the MSU crystal-induced expression of PPARγ in HK-2 cells at the mRNA and protein level. However, pioglitazone significantly decreased the amount of NALP3 and IL-1β protein in MSU crystal-stimulated HK-2 cells. Zou *et al* (24) reported that PPARγ ligand troglitazone enhanced the activity of PPARγ in mesangial cells. Therefore, it was hypothesized that pioglitazone inhibits NALP3 inflammasomes and IL-1β not by increasing the expression of the transcriptional regulator PPARγ but, similarly to the effect of troglitazone, by enhancing its activity.

The findings of the present study were consistent with a number of studies focusing on the expression of PPARγ. Akahoshi *et al* (25) have shown that MSU crystals can induce PPARγ gene expression by mononuclear cells in a time-dependent manner: mRNA expression was rapidly increased and subsequently declined. Wang *et al* (26) detected changes in PPARγ activity using an electrophoretic mobility shift assay (EMSA), which indicated that LPS upregulated PPARγ activity in HK-2 cells at 6 h, which then decreased at 48 h. Similar findings were reported by Bhatt *et al* (27), who examined the effect of peptidoglycan (PGN) on PPARγ production. PGN induced a biphasic effect on PPARγ expression in macrophages, leading to increases in the early stage followed by suppression of PPARγ expression. Further investigation of the mechanism of the late-phase inhibition of PPARγ expression showed that the early increase is mediated by extracellular signal-regulated kinase, while the late repression occurs via c-Jun N-terminal kinase activation.

Since the results of the present study showed that MSU crystals inhibited PPARγ expression at a later stage, MSU crystal-stimulated HK-2 cells were pre-treated with PPARγ agonist pioglitazone to investigate its effects on the expression of PPARγ, NALP3 and IL-6. The results were consistent with previous studies which explored the effect of PPARγ agonists. Jiang *et al* (28) reported that troglitazone inhibited LPS-induced IL-6, IL-8 and TNF-α secretion in macrophages. Wang *et al* (26) demonstrated that rosiglitazone inhibited LPS-induced IL-6 and IL-8 expression in HK-2 cells. In cultured human proximal tubular epithelial cells (HPTECs), rosiglitazone was reported to attenuate high-glucose-induced IL-6, CCL-2 and transforming growth factor (TGF)-β expression (29). The same conclusions were inferred from animal studies. Yang *et al* (30) showed that pioglitazone attenuated podocyte injury-associated glomerulosclerosis by reducing macrophage infiltration and inhibiting TGF-β and plasminogen activator inhibitor-1 expression. Pioglitazone was also reported to significantly decrease matrix metalloproteinase expression and oxidative stress, and to reduce renal ischemia/re-perfusion injury and acute inflammation in rats (31). In hyperoxaluric rats, Taguchi *et al* (32) demonstrated that pioglitazone suppressed kidney crystal formation through renal tubular cell protection as well as anti-oxidative and anti-inflammatory effects. All of these observations suggested that PPARγ agonists have a protective effect on renal function through the inhibition of inflammation.

However, in a number of studies, certain biochemical stimuli significantly reduced PPARγ expression, which was contrary to the results of the present study. Li *et al* (33) reported that the amount of PPARγ in hypoxia-induced HPTECs was significantly decreased. PPARγ expression in cyclosporine-treated rat kidneys was significantly lower than that in the control groups (34). Matsuyama *et al* (35) also showed that PPARγ expression was reduced in rats following ischemia/re-perfusion. The discrepancies between these previous studies and the results of the present study may be due to differences in treatment or stimulus intensity, or due to differences between experimental *in vitro* and *in vivo* models.

Based on the results of the present study, it is hypothesized that the rapid induction of PPARγ may contribute to the self-limiting nature of hyperuricemia-induced acute inflammation in gouty patients, while the later suppression of PPARγ may result in chronic renal injury in hyperuricemia patients. The results of the present and other studies have shown that PPARγ agonists downregulate MSU crystal-induced pro-inflammatory cytokines. Since MSU crystal-induced inflammation in kidneys has an important role in hyperuricemic nephropathy, PPARγ agonists have a potential therapeutic value in preventing tubular injury associated with hyperuricemia-associated renal disease.

Acknowledgements

This study was supported by the Shanghai Medical Guide Science and Technology Project (no. 114119a6200).

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