Uric Acid Stimulates Monocyte Chemoattractant Protein-1 Production in Vascular Smooth Muscle Cells Via Mitogen-Activated Protein Kinase and Cyclooxygenase-2

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Abstract—Previous studies have reported that uric acid stimulates vascular smooth muscle cell (VSMC) proliferation in vitro. We hypothesized that uric acid may also have direct proinflammatory effects on VSMCs. Crystal- and endotoxin-free uric acid was found to increase VSMC monocyte chemoattractant protein-1 (MCP-1) expression in a time- and dose-dependent manner, peaking at 24 hours. Increased mRNA and protein expression occurred as early as 3 hours after uric acid incubation and was partially dependent on posttranscriptional modification of MCP-1 mRNA. In addition, uric acid activated the transcription factors nuclear factor-κB and activator protein-1, as well as the MAPK signaling molecules ERK p44/42 and p38, and increased cyclooxygenase-2 (COX-2) mRNA expression. Inhibition of p38 (with SB 203580), ERK 44/42 (with UO126 or PD 98059), or COX-2 (with NS398) each significantly suppressed uric acid–induced MCP-1 expression at 24 hours, implicating these pathways in the response to uric acid. The ability of both N-acetyl-cysteine and diphenyleneionium (antioxidants) to inhibit uric acid–induced MCP-1 production suggested involvement of intracellular redox pathways. Uric acid regulates critical proinflammatory pathways in VSMCs, suggesting it may have a role in the vascular changes associated with hypertension and vascular disease. (Hypertension. 2003;41:1287-1293.)

Key Words: atherosclerosis ■ chemokines ■ hyperuricemia ■ arteriolosclerosis
Methods

Cell Culture
Primary rat aortic VSMCs were obtained from Dr Andrew Kahn (University of Texas, Houston), maintained as described1,10, and strictly used between passages 5 to 12. Before incubation with uric acid or other compounds, VSMCs were serum-deprived in 0.4% FBS for 48 to 72 hours.

Tests for Uric Acid Crystals, Endotoxin, Mycoplasma, and Cell Toxicity
In experiments requiring uric acid-incubation, media was prewarmed (37°C), and uric acid (Ultrapure, Sigma; 2.5 to 10 mg/dL) was added. The mixture was again warmed (37°C, 30 minutes) and passed through sterile 0.20-μm filters. Control media was treated the same way. Crystals were not detectable under these conditions (polarizing microscopy), nor did they develop during cell incubation. Assessment of conditions required to generate crystals revealed that refrigeration, time, and urate concentration were important factors. Media containing 20 to 30 mg/dL uric acid showed the sudden appearance of multiple, negatively birefringent crystals after 3-day refrigeration. Warming (37°C) resulted in their complete disappearance, usually within 15 to 30 minutes.

Endotoxin was not detectable in all batches of uric acid (limulus amebocyte assays; BioWhittaker Inc) on 10 mg/dL uric acid media (indicating <0.015 endotoxin U/mL). Mycoplasma contamination was also excluded (Inmu-Mark Myco-Test; ICN Biomedicals). Uric acid, dimethyl sulfoxide, inhibitor, and antioxidant doses reported were not associated with increased toxicity (TOX-7 lactate dehydrogenase assay; Sigma) compared with media without these compounds.

RNase Protection Assay
RNase protection assays (RPAs) were performed on 2- to 4-μg RNA by using the RPA I kit (Torrey Pines Biolabs). The rat riboprobes used have all been described previously.15,16

Real-Time Reverse Transcriptase–Polymerase Chain Reaction
Real-time, 1-step reverse transcriptase–polymerase chain reaction (RT-PCR) was performed with SYBR green PCR reagents (Sigma), the Thermoscript RT-PCR system (Invitrogen), and the Opticon (RT-PCR) was performed with SYBR green PCR reagents (Sigma), and the Opticon polymerase chain reaction–Real-Time Reverse Transcriptase–Polymerase Chain Reaction method was chosen instead of RPA but were easily demonstrated in rat macrophages further delineate the early time-course of the MCP-1 mRNA RPA sensitivity did not allow this to be demonstrated. To than controls at 3 hours (data not shown, both MCP-1 mRNA expression compared with control media, after 24 hours of incubation (Figure 1A). MCP-1 mRNA was not usually detectable in VSMCs at baseline or after incubation with control media. In multiple time-course experiments with uric acid incubations ranging between 1 and 48 hours (5 separate experiments), MCP-1 mRNA expression was generally first detectable at 6 hours and peaked at 24 hours (Figure 1A). On occasion, MCP-1 mRNA was detectable 3 hours after uric acid incubation (data not shown), but usually, the RPA sensitivity did not allow this to be demonstrated. To further delineate the early-time-course of the MCP-1 mRNA upregulation, real-time RT-PCR was used as an alternative more-sensitive technique. This showed uric acid–incubated cells to have 1.8-fold higher MCP-1 mRNA expression than that of controls at 3 hours (data not shown, P<0.05). Both IL-1β and TNF-α mRNA were not detectable in VSMCs by RPA but were easily demonstrated in rat macrophages (NR8383) after lipopolysaccharide stimulation (Figure 1A).

To assess whether uric acid was responsible for altering MCP-1 mRNA stability, cells were incubated with actinomycin D (5 μg/mL) in the presence or absence of uric acid (5 mg/dL), and MCP-1 mRNA decay was assessed by real-time RT-PCR (Figure 1B). This method was chosen instead of RPA because of its greater sensitivity and its ability to assess MCP-1 mRNA decay without prior upregulation of MCP-1 mRNA. MCP-1 mRNA half-life approximately doubled from 2 hours in controls to 4 hours in uric acid–incubated cells. MCP-1/GAPDH mRNA ratios were significantly different between the 2 groups of samples at 2 and 4 hours, with control-incubated cells showing more rapid MCP-1 mRNA

Statistical Analysis
All values are expressed as mean±SD. ANOVA followed by Bonferroni correction was used in all instances, except in the uric acid dose-response ELISA, which was assessed with the Pearson correlate. Significance was defined as P<0.05.
decay than did uric acid–incubated cells (Figure 1B, \( P < 0.05, n = 4 \)).

### Uric Acid Increases Production of MCP-1 Protein by Rat VSMC

The associated uric acid–induced increase in MCP-1 expression was assessed by ELISA on media supernatants collected at various time points (Figure 2A). Uric acid (5 mg/dL) significantly increased MCP-1 production at 3, 6, and 24 hours when compared with that of control media (Figure 2A). The effect of uric acid was also found to be dose-dependent (Figure 2B), with a progressive increase in supernatant MCP-1 being observed at 24 hours, as uric acid concentration in the media was increased (Figure 2B; Pearson correlate, \( r = 0.88, P < 0.0001 \); \( n = 20 \)). For all experiments, total supernatant MCP-1 was corrected for cell protein concentration and expressed as mean±SD.

### NF\( \kappa \)B and AP-1 Are Activated in Rat VSMCs by Uric Acid

The transcription factors NF-\( \kappa \)B and AP-1 have been reported to be involved in the regulation of MCP-1 expression. The effect of uric acid on these molecules was therefore examined in VSMCs by electrophoretic mobility shift assay. Nuclear lysates from uric acid–incubated cells (5 mg/dL) showed greater activation of both NF-\( \kappa \)B and AP-1 than did control lysates (Figures 3A and 3C). In both instances, increased activation was early, evident after 15 minutes of incubation with uric acid and peaked at 30 minutes. By using supershift
Uric Acid–Induced MCP-1 Upregulation in Rat VSMCs Is Dependent on p38 and ERK44/42 MAPK

To identify potential signaling pathways involved in the uric acid–induced MCP-1 upregulation, p38 and ERK44/42 MAPK activation was examined. We have recently reported that uric acid (3 mg/dL) can activate ERK44/42 MAPK in rat aortic VSMCs at 30 and 120 minutes, and these findings were confirmed in this group of studies with activation seen as early as 15 minutes with 5 mg/dL uric acid (data not shown). Activation of p38 MAPK was also seen after incubation of VSMCs with uric acid (2.5 and 5 mg/dL) for 15 and 30 minutes (Figure 4A).

To assess the role of these 2 pathways, the effect of p38 and ERK44/42 MAPK inhibitors on uric acid–induced upregulation of MCP-1 was examined at the time of peak MCP-1 expression (24h). Supernatant MCP-1 was measured by ELISA (Figure 4B). SB 203580 (p38 pathway inhibitor), U0126, and PD 98059 (ERK pathway inhibitors) all had a similar effect, decreasing the total MCP-1 secreted protein by ~50% (B; solid bars, **P<0.01, n=3). MCP-1 measurements from control samples (no uric acid) with or without inhibitors are also shown (B; open bars). The fold-increase in supernatant MCP-1 relative to controls is indicated at the top of each solid bar.
lates increased production of the chemokine MCP-1, by rat

In the present study, we report that soluble uric acid stimu-

lates increased production of the chemokine MCP-1, by rat

VSMCs in vitro. The increase in MCP-1 production occurred

in a time- and dose-dependent manner, with both mRNA and

protein being upregulated within a few hours of incubation

of the VSMCs with uric acid. The rapidity of the increase led

us to examine possible posttranscriptional effects of uric acid, as

well as various signaling pathways and transcription factors

known to be important in MCP-1 regulation.

Our initial findings were that the uric acid–induced

increase in MCP-1 synthesis was partially dependent on in-

creased MCP-1 mRNA half-life. We subsequently found that

transcription factors known to be involved in MCP-1 regula-

tion (NF-κB and AP-1) were also activated very early (at 15

minutes), suggesting that both transcriptional and posttran-

scriptional factors were involved in the increased MCP-1

synthesis. The possibility that specific MAPK was involved

in this process was also examined. We have previously shown

that uric acid activates ERK44/42 MAPK in rat VSMCs in

vitro, and that this mediates uric acid–induced VSMC prolif-

eration.12 In these studies, we confirmed the activation of

ERK44/42 MAPK and showed that this partially mediated the

uric acid–induced increase in MCP-1 synthesis. We also

examined p38 MAPK, which is strongly linked to inflamma-

tion and has been shown to regulate MCP-1 expression in

several cell types in response to various mediators.19 P38

MAPK was also rapidly activated in rat VSMCs by uric acid,

and its inhibition partially blocked the increase in MCP-1

production. We additionally found that COX-2 partially

mediated the uric acid–induced increase in MCP-1 synthesis.

This is of interest as we have previously shown that COX-2

mediates uric acid–induced VSMC proliferation, in part,

through thromboxane generation.11

It is important to note that uric acid levels in most

mammals differ from that seen in humans. This difference

arises because in most mammals, the uricase (urate oxidase)

enzyme metabolizes uric acid to allantoin. By comparison, in

higher hominoids (great apes and humans) this enzyme has

been mutated,20 resulting in higher serum uric acid levels. In

humans, hyperuricemia is defined as a uric acid level >7

mg/dL (with a normal range of ~2 to 7 mg/dL). In rats, the

normal uric acid level is much lower (~0.8 to 1.5 mg/dL).

Redox-Sensitive Pathways Are Implicated in Uric

Acid–Induced MCP-1 Upregulation

As p38 MAPK, AP-1, NF-κB, and MCP-1 are all known to be

regulated through redox-sensitive pathways, the effect of

antioxidants on the uric acid–induced MCP-1 upregulation

was examined. NAC and DPI were both found to inhibit uric

acid–induced MCP-1 production by ~50% at 24 hours

(Figure 6, P<0.01, n=4). This occurred without evidence of

cell toxicity by lactate dehydrogenase assay.

Discussion

In the present study, we report that soluble uric acid stimu-
lates increased production of the chemokine MCP-1, by rat
Uric acid concentrations used in the in vitro studies presented here are 2- to 3-fold that observed in the normal rat and, therefore, are in the range of what may be observed in disease in these animals. The possibility that this may be clinically relevant is supported by a recent study in our laboratory in which the effect of hyperuricemia was examined in a model of progressive renal disease (the remnant kidney or RK model).11 In these studies, RK rats that received the uricase inhibitor oxonic acid developed modest hyperuricemia (4.0 ± 0.6 mg/dL) compared with that of control RK rats (2.7 ± 0.6 mg/dL; P < 0.05 at 2 weeks). Interestingly, these rats showed much more severe vascular disease and intrarenal macrophage accumulation.11 We have retrospectively tested sera and found that hyperuricemic RK rats also had higher serum MCP-1 levels at 2 weeks (136.8 ± 39.7 ng/mL) compared with that of control RK rats (82.8 ± 8.3 ng/mL; P < 0.05). This was associated with substantial macrophage accumulation in the intrarenal blood vessels (data not shown). This emphasizes the need to address the role of uric acid in the systemic inflammatory response in patients with cardiovascular and renal disease.

Further assessing the mechanism through which uric acid mediated its effects, we found that redox pathways were implicated. The ability of both NAC and DPI to inhibit the uric acid–induced increase in MCP-1 suggested that uric acid was acting in a prooxidative manner. This is of interest because uric acid is often thought of as an antioxidant.21 Despite this, several studies have demonstrated that uric acid can be prooxidative and may generate free radicals.22–25 The ability to activate p38 MAPK, NF-kB, and AP-1, as well as to increase MCP-1 expression, is consistent with an oxidant-driven pathway.26,27

In conclusion, we have found that uric acid can induce inflammatory pathways in rat VSMCs in vitro, with activation of p38 MAPK, NF-kB, and AP-1 and increased expression of COX-2 and MCP-1. Although the inflammatory potential of crystalline uric acid has been well known, this study provides the first evidence that soluble uric acid can also engage these pathways. These findings add further intrigue to the controversy surrounding the possible pathogenetic role of uric acid in hypertension, vascular disease, and atherosclerosis in man.28–31

Perspectives

The association between hyperuricemia, hypertension, and vascular disease has been appreciated for many decades.32 More than a century ago, Mahomed33 alluded to this association by noting that hypertensive subjects often had a family history of gout. Since then, several epidemiological studies have shown elevated serum uric acid levels to be independently associated with the incidence of hypertension, coronary artery disease, cardiovascular disease, and death.28–30 Recent studies also show elevated serum uric acid levels to be associated with greater renal disease progression in patients with immunoglobulin A nephropathy.34,35 Despite these associations, the possible pathogenetic role of uric acid in these disorders has long been debated. Much of the controversy has centered around the fact that hyperuricemia is seen to “cluster” with various known and accepted cardiovascular risk factors. In addition, several other epidemiological studies have not clearly shown the “independence” of hyperuricemia as a risk factor in multivariate analyses, particularly in the male population.36,37 Although speculative, this data suggests that the induction of inflammatory pathways in VSMCs by uric acid may have a role in the initiation of vascular disease and hypertension. Future studies will be necessary to determine the relevance of these findings to cardiovascular disease in man.

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References

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