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Uric acid activates extracellular signal-regulated kinases and thereafter endothelin-1 expression in rat cardiac fibroblasts☆

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Abstract

Background: The association between hyperuricemia and cardiovascular diseases has long been recognized. Elevated levels of uric acid may have a causal role in hypertension and cardiovascular diseases. However, the direct effect of uric acid on cardiac cells remains unclear. Therefore, this study was aimed to examine the effect of uric acid in rat cardiac fibroblasts and to identify the putative underlying signaling pathways.
Methods: Cultured rat cardiac fibroblasts were stimulated with uric acid; cell proliferation and endothelin-1 (ET-1) gene expression were examined. The effect of uric acid on NADPH oxidase activity, reactive oxygen species (ROS) formation, and extracellular signal-regulated kinases (ERK) phosphorylation were tested to elucidate the intracellular mechanism of uric acid in ET-1 gene expression.
Results: Uric acid-increased cell proliferation and ET-1 gene expression. Uric acid also increased NADPH oxidase activity, ROS formation, ERK phosphorylation, and activator protein-1 (AP-1)-mediated reporter activity. Antioxidants suppressed uric acid-induced ET-1 gene expression, and ERK phosphorylation, and AP-1 reporter activities. Mutational analysis of the ET-1 gene promoter showed that AP-1 binding site was an important *cis*-element in uric acid-induced ET-1 gene expression.
Conclusions: These results suggest that uric acid-induced ET-1 gene expression, partially by the activation of ERK pathway *via* ROS generation in cardiac fibroblasts.

Keywords: Uric acid; Endothelin-1; Cardiac fibroblasts; Reactive oxygen species; Extracellular signal-regulated kinases

1. Introduction

Uric acid is an intermedia product of the purine degradation pathway in the cell. Epidemiological evidence suggests an association between increasing uric acid levels and increased cardiovascular morbidity and mortality [1]. A recent clinical study reports that high plasma uric acid level, partly secreted from the failing heart, is a prognostic predictor in patients with congestive heart failure [2]. *In vitro* studies reveal that uric acid induces gene expression of chemokines and growt h factors, such as monocyte chemoattractant protein-1 (MCP-1) and platelet-derived growth factor [3,4], and stimulates proliferation of vascular smooth muscle cells [4,5]. Furthermore, uric acid-induced MCP-1 expression in vascular smooth muscle cells was attenuated by antioxidants, suggesting an involvement of redox-

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dependent mechanism [3]. However, the direct effect of uric acid on cardiac cells remains unclear.

The cardiac interstitium, which is mainly composed of fibroblasts, is a dynamic supportive structure for the heart. Unlike cardiac myocytes, cardiac fibroblasts can proliferate and increase the deposition of extracellular matrix proteins such as fibronectin and collagen, which leads to interstitial fibrosis [6]. There are growing recognitions and experimental evidences that oxidative stress mediated by reactive oxygen species (ROS) plays a role in the pathogenesis of myocardial fibrosis in various cardiac diseases [7]. Pro-fibrotic factors such as endothelin-1 (ET-1) stimulate fibrosis by modulating collagen synthesis and matrix metalloproteinases (MMPs)/tissue inhibitor of MMP activity [8]. We previously reported that ROS *via* the activation of extracellular signal-regulated kinases (ERK) and the nuclear transcription factor activator protein-1 (AP-1) are essential for cell proliferation and ET-1 gene expression in cardiac fibroblasts [9]. However, the effect of uric acid on cell proliferation and ET-1 gene expression remains to be elucidated.

In this study, we aimed to examine the hypothesis that uric acid has direct proliferative effects on cardiac fibroblasts. We report that uric acid can increase cell proliferation and ET-1 gene expression in rat cardiac fibroblasts *in vitro*. The mechanism involves the activation of ERK and AP-1. Redox pathways in cardiac fibroblasts are involved in this process. These findings may provide a mechanism through which uric acid could mediate cardiac diseases.

2. Methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and tissue culture reagents were from Life Technologies, Inc. A rat ET-1 cDNA probe (accession no. M64711) was obtained as previously described [9]. Wild type (204 bp) or AP-1 mutant of ET-1 promoter was fused to the chloramphenicol acetyltransferase (CAT) reporter gene (ET-1 promoter-CAT plasmid); PBLCAT2 (containing CAT reporter gene with its promoter) and PBLCAT3 (containing the CAT gene only) were constructed as previously described [10] 2′,7′-Dichlorofluorescin diacetate (DCF-DA) was obtained from Molecular Probes (Eugene, OR, U.S.A.). H_2O_2 was purchased from Acros Organics (Pittsburgh, PA, U.S.A.). Uric acid, *N*-acetyl-cysteine (NAC) and all other reagent-grade chemicals were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.). Uric acid solution for cell treatments was prepared in the prewarmed cell culture medium (Ultrapure, a microcrystal-free endotoxin-free solution; Sigma, St. Louis, MO, U.S.A.) and passed through a 20-μm sterile filter. The plasmid AP-1-Luc containing the firefly luciferase reporter gene driven by a basic promoter element (TATA box) joined to tandem repeats of AP-1 binding element were obtained from Stratagene (La Jolla, CA, U.S.A.).

2.2. Culture of cardiac fibroblasts

The investigation was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by the Institutional Animal Care and Use Committee of Taipei Medical University. Primary cultures of neonatal rat cardiac fibroblasts were prepared as previously described [9]. Cardiac fibroblasts grown in either 60- or 100-mm culture dishes from the second to fourth passage were used for the experiments. Cardiac fibroblasts were grown in DMEM containing antibiotics and 10% fetal calf serum until 24 h prior to experimentation, at which time point cells were placed in a defined serum-free medium containing insulin (0.5 μM) and transferrin (5 mg/ml) for all experiments. Cells were then treated as indicated, followed by harvesting. Cellular viability under all treatment conditions was determined by cell count, morphology, and trypan blue exclusion.

2.3. Cell proliferation

Proliferation was assessed by counting and [^{3}H]thymidine incorporation. The rate of cellular proliferation was determined by cell counting. Cells were removed from the culture dish by addition of trypsin, and pelleted by centrifugation. The pellet was resuspended in 1 ml DMEM and cells were counted in an automatic cell counter (S.ST.II/ZM, Coulter Electronics Ltd., Miami, FL, U.S.A.). To measure synthesis of new DNA, cells (1×10^5/well) were plated in six-well (35-mm) dishes 24 h before experiments as previously described [9]. Cells were incubated with [^{3}H]thymidine (5 μCi/ml). Following the indicated treatment, cells were harvested by incubation at 4 °C with trichloroacetic acid (5%) followed by solubilization in 0.1 N NaOH. Radioactivity was determined by scintillation counting. Data are presented as the mean ± SEM of 9–12 determinations for three to four different cell preparations and normalized to the untreated sample × 100 (*i.e.* percentage of control).

2.4. NADPH oxidase activity assay and detection of intracellular ROS

NADPH oxidase was measured as described previously [11]. In brief, cells were scraped into ice-cold phosphate-buffered saline buffer containing 1 mM EGTA and centrifuged for 10 min with 750 ×*g* at 4 °C. The pellet was resuspended in lysis buffer (20 mM potassium phosphate, 1 mM EGTA, 10 mM aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM leupeptin, pH 7.0) and sonicated. The protein concentration was adjusted to 2 mg/ml. Total cell suspension with a volume of 250 μl was mixed with 250 μl of Hanks buffered salt solution (HBSS: 136.6 mM NaCl, 5.4 mM KCl, 4.2 mM $NaHCO_3$, 2.7 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.44 mM KH_2PO_4, 0.41 mM $MgSO_4$,

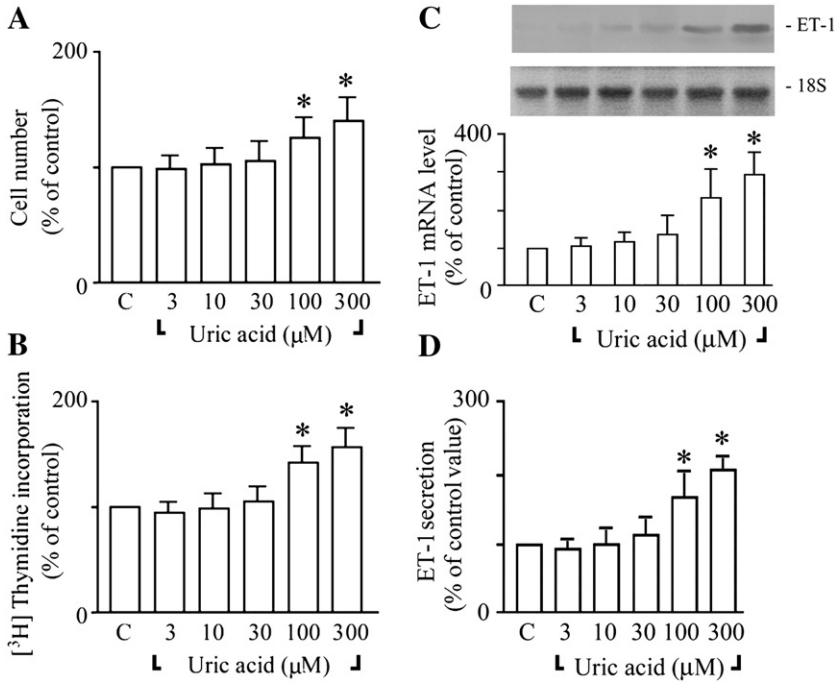

Fig. 1. Effects of uric acid on cell proliferation and ET-1 gene expression in neonatal rat cardiac fibroblasts. $*P<0.05$ vs. control (Student's *t* test). (A) Effect of uric acid on cell proliferation. Cells were cultured in the absence (C) or presence of uric acid (24 h) as indicated. Cell number was expressed as percentage of control. The results are shown as the mean±S.E.M. ($n=5$). (B) Effect of uric acid on the DNA synthesis. Cells were incubated with the indicated concentrations of uric acid for 24 h and then assayed [^{3}H]thymidine incorporation. [^{3}H]thymidine incorporation is expressed as percentage increase relative to the [^{3}H] content (100%) in their respective control (C). All data are shown as the means±SEM of 9–12 determinations in six different cell preparations. (C) Effect of uric acid on ET-1 mRNA expression. Cells were incubated with indicated concentrations of uric acid for 6 h. Data are represented as difference relative to control groups. The results are shown as mean±SEM ($n=6$). (D) Induction of ET-1 peptide secretion by different concentrations of uric acid. Cells were incubated with various concentrations of uric acid for 24 h. Data are represented as the difference relative to the data in the control groups. The results are shown as the mean±SEM ($n=5$).

pH 7.8) containing 500 μM lucigenin and kept at 37 °C for 10 min. NADPH oxidase activity assay was initiated by adding 10 μl of NADPH (100 μM) as substrate. The photon emission was measured using a microplate scintillation counter (Topcount, Packard Instrument Co., Meriden, CT, U.S.A.), and the respective background counts were subtracted. Neither the cellular fraction alone nor NADPH alone evoked any lucigenin chemiluminescence signal. ROS were measured using a previously described method [10]. Prior to the chemical or uric acid treatment, cells were incubated in culture medium containing a fluorescent dye, 2′,7′-dichlorofluorescin diacetate (DCF-DA) (Molecular Probes, Eugene, OR, U. S. A.) of 30 μM for 1 h to establish a stable intracellular level of the probe. The same concentration of DCF-DA was maintained during the chemical or uric acid treatment. Subsequently, the cells were washed with PBS, removed from Petri dishes by brief trypsinization, and measured for 2′,7′-dichlorofluorescein (DCF) fluorescence intensity. The DCF fluorescence intensity of the cells is an index of intracellular levels of ROS; and it can be determined by fluorescence spectrophotometry with excitation and emission wavelengths at 475 and 525 nm, respectively. The cell number in each sample was counted in an automatic cell counter (S.ST.II/ZM, Coulter Electronics Ltd., Miami, FL, U.S.A.) and utilized to normalize the fluorescence intensity of DCF.

2.5. RNA isolation and Northern blot analysis

Preparation of total RNA and Northern blot analyses of ET-1 and 18S RNA were performed as described previously [9]. Total RNA was isolated from cardiac fibroblasts by the guanidine isothiocyanate/phenol chloroform method. The RNA (10 μg/lane) was separated by electrophoresis on a 1% agarose formaldehyde gel and transferred onto a nylon membrane (Nytran, Schleicher & Schuell, Inc) by a vacuum blotting system (VacuGene XL, Pharmacia). After hybridization with the ^{32}P-labeled ET-1 cDNA probes, the membrane was washed with 1× SSC containing 1% SDS at room temperature for 30 min and then exposed to X-ray film at −70 ° C. Blots of specific mRNA bands were detected by autoradiography and analyzed with a densitometer (Computing Densitometer 300S, Molecular Dynamics). Blots were stripped and reprobed for 18S cDNA probe (obtained from American Type Culture Collection) to control for loading. Expression of ET-1 mRNA was quantitated and was normalized to the 18S signal.

2.6. ET-1 enzyme-linked immunosorbent assay (ELISA)

Cardiac fibroblasts were seeded in twelve-well plates and grown to confluence in 2–3 days. After overnight serum-

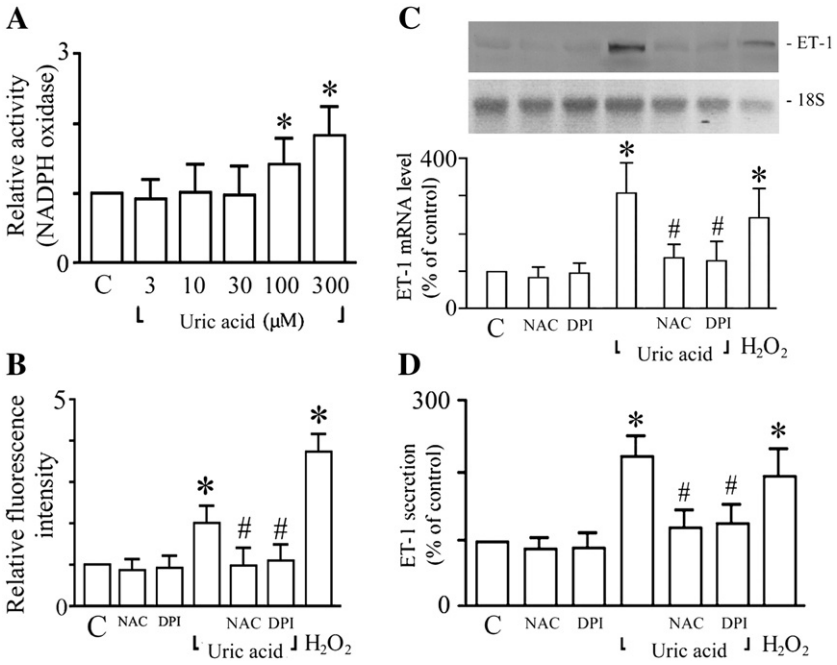

Fig. 2. Involvement of ROS in uric acid-induced ET-1 gene expression in cardiac fibroblasts. $*P<0.05$ vs. control (Student's t test); $\#P<0.05$ vs. Uric acid alone (ANOVA). (A) Uric acid-increased NADPH oxidase activity in cardiac fibroblasts. NADPH oxidase activity was measured after uric acid (1–300 μM) treatment for 30 min. The results are shown as the mean±S.E.M. ($n=6$). (B) Uric acid-increased intracellular ROS in cardiac fibroblasts. Cardiac fibroblasts were loaded with DCF-DA for 30 min and stimulated with uric acid (300 μM) for 30 min. Uric acid (300 μM) increased ROS levels in cardiac fibroblasts, and these increases were inhibited by NAC (10 mM), or the NAD(P)H oxidase inhibitor DPI (10 μM). Cells treated with H2O2 (25 μM) for 30 min were used as a positive control. The results are shown as the mean±SEM ($n=6$). (C) Effect of antioxidants on uric acid-induced ET-1 mRNA in cardiac fibroblasts. Cells were preincubated with either the NAC (10 mM) or DPI (10 μM) for 30 min followed by incubation with 300 μM uric acid for 6 h. The results are shown as the mean±SEM ($n=5$). (D) Effect of antioxidants on uric acid-increased ET-1 peptide secretion in cardiac fibroblasts. Cells were preincubated with either the NAC (10 mM) or DPI (10 μM) for 30 min followed by incubation with 300 μM uric acid for 24 h. The results are shown as mean±SEM ($n=5$).

starvation, medium in each well was changed to include uric acid and/or the indicated treatment. In each set of experiments, there was also one well with no cells and medium with no uric acid (blank). Medium in each well was collected after the indicated treatment and was briefly centrifuged to remove floating cellular debris that was present. Supernatants were transferred to fresh Eppendorf tubes and stored frozen at −70 °C. ET-1 levels were measured in culture medium using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Amersham-Pharmacia, Amersham, U.K.) following the manufacturer's instructions as previously described [12]. For normalization purposes, cells in each well were lysed in 0.2 N NaOH and quantitated for protein content with Pierce BCA (bicinchoninic acid) protein assays (Thermo Fisher Scientific Inc., Rockford, IL, U.S.A.). Results were normalized to cellular protein content in all experiments and expressed as a percentage relative to the cells incubated with the vehicle.

2.7. Western blot analysis

Rabbit polyclonal anti-phospho-specific ERK antibodies were purchased from New England Biolabs (Beverly, MA, U.S.A.). Anti-ERK antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). Western blot analysis was performed as previously described [9].

2.8. Luciferase assay

Cardiac fibroblasts plated on six-well (35-mm) dishes were transfected with the luciferase reporter construct possessing consensus AP-1 binding sites (AP-1-Luc) (Stratagene, La Jolla, CA, U.S.A.). Following incubation for 24 h in serum-free DMEM, cardiac fibroblasts were cultured under various conditions as indicated for a period of 48 h, and then assayed for luciferase activities with a luciferase reporter assay kit (Stratagene). As was the case for AP-1 transcriptional activity, the specific firefly luciferase activity was normalized for transfection efficiency to its respective β-galactosidase activity and expressed relatively to the control.

2.9. Transfection and chloramphenicol acetyltransferase assays

For the transient transfections, cells were transfected with different expression vectors by the calcium phosphate method [12]. DNA concentration for all samples was adjusted to equal amount with empty vector pSRα in each experiment. Briefly, cardiac fibroblasts were maintained in culture for 24 h prior to transfection. The indicated expression vectors were mixed with calcium phosphate and immediately added to the cardiac fibroblast cell culture. After incubation for 5 h, cells were then washed three times with PBS and incubated with 10% serum DMEM. After 12 h, cells were washed with serum-free

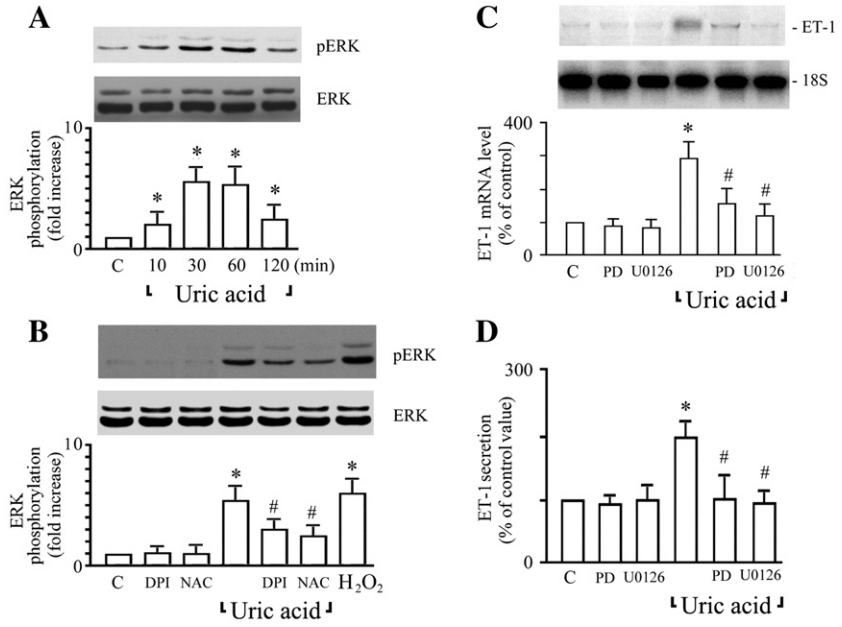

Fig. 3. Uric acid-increased ET-1 gene expression *via* ERK in a redox-sensitive manner. $*P<0.05$ vs. control (Student's *t* test); $\#P<0.05$ vs. Uric acid alone (ANOVA). (A) Time course of uric acid on ERK phosphorylation. Cells were exposed to uric acid (300 μM) for times indicated. Data are presented as differences relative to control groups. The results are shown as mean±SEM ($n=5$). (B) Effect of antioxidants on uric acid-increased ERK phosphorylation in cardiac fibroblasts. Cells were preincubated with either the NAC (10 mM) or DPI (10 μM) for 30 min and stimulated with uric acid (300 μM) for 30 min. Cells treated with H_2O_2 (25 μM) for 30 min were used as a positive control. The results are shown as mean±SEM ($n=4$). (C) Uric acid-induced ET-1 mRNA was attenuated by PD98059 (20 μM) or U0126 (10 μM) in cardiac fibroblasts. Cardiac fibroblasts were stimulated with uric acid (300 μM) in the presence of PD98059 (PD; 20 μM) or U0126 (10 μM) for 6 h. The results are shown as mean±SEM ($n=4$). (D) Uric acid-induced ET-1 peptide secretion was attenuated by PD98059 (20 μM) or U0126 (10 μM) in cardiac fibroblasts. Cardiac fibroblasts were stimulated with uric acid (300 μM) in the presence of PD98059 (PD; 20 μM) or U0126 (10 μM) for 24 h. The results are shown as mean±SEM ($n=5$).

media and further incubated for 48 h in serum-free medium. Cells were then treated with different agents. To correct for variability in transfection efficiency, 5 μg of pSV-β-galactosidase plasmid DNA was cotransfected in all the experiments. The CAT and β-galactosidase assays were performed as described previously [12]. The relative CAT activity was corrected by normalizing the respective CAT value to that of β-galactosidase activity. Cotransfected β-galactosidase activity varied by <10% within a given experiment and was not affected by any of the experimental manipulations described. As positive and negative controls, pBLCAT2 (with thymidine kinase promoter) and pBLCAT3 (without promoter) were included in each assay.

2.10. Statistical analysis

Results are expressed as mean±S.E.M. Statistical analysis was performed using Student's *t* test and analysis of variance (ANOVA) followed by a Dunnett multiple comparison test using GraphPad Prism (GraphPad Software, San Diego, CA, USA). A value of $p<0.05$ was considered to be statistically significant.

3. Results

3.1. Uric acid-induced proliferation and ET-1 expression in cardiac fibroblasts

Uric acid-stimulated cardiac fibroblast proliferation was assessed by cell counting and analyzing DNA synthesis with [^{3}H]thymidine incorporation. Uric acid (100–300 μM) significantly increased cell number and DNA synthesis in neonatal rat cardiac fibroblasts (Fig. 1A, B). To determine whether uric acid increases ET-1 mRNA levels in cardiac fibroblasts, we performed Northern blot analysis (Fig. 1C). When cardiac fibroblasts were treated with uric acid for 6 h, uric acid (100–300 μM) significantly increased ET-1 mRNA expression (Fig. 1C). ET-1 peptide secretion also significantly increased in cardiac fibroblasts which have been exposed to uric acid (30–100 μM) for 24 h (Fig. 1D). These data have shown that uric acid induces cell proliferation and ET-1 gene expression in cardiac fibroblasts.

3.2. Uric acid-induced ET-1 gene expression is redox-sensitive

As shown in Fig. 2A, uric acid-increased NADPH oxidase activity in cardiac fibroblasts in a concentration-dependent manner. We next determine whether uric acid induces intracellular ROS in cardiac fibroblasts. We measured the intracellular ROS level by analyzing the fluorescent product DCF, a peroxidative product of DCF-DA. Cardiac fibroblasts treated with uric acid (300 μM) for 30 min had significantly higher ROS levels than those cells treated with vehicle only (Fig. 2B). Cells pretreated with antioxidants such as *N*-acetylcysteine (NAC; 10 mM) or a flavoprotein containing the NADH/NADPH oxidase inhibitor, diphenyleneiodonium (DPI; 10μM) for 30 min showed

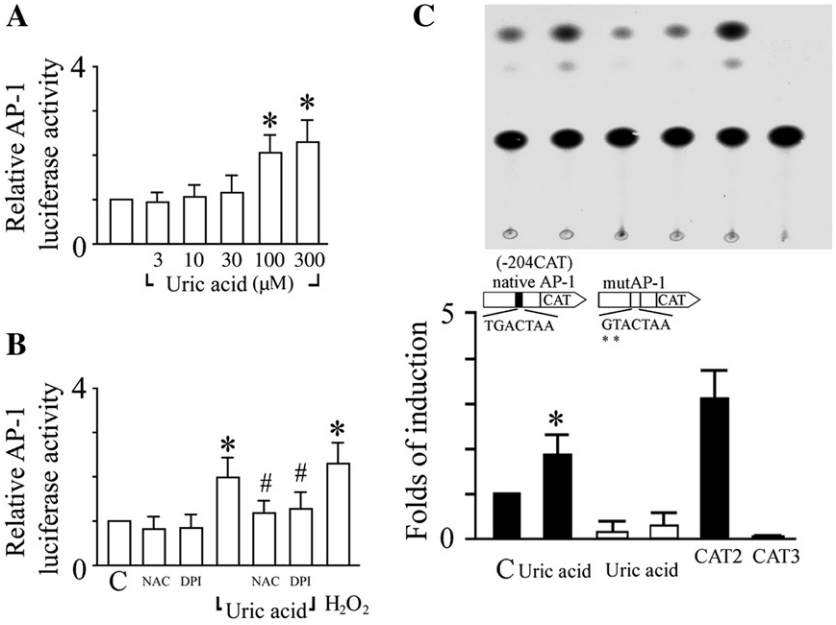

Fig. 4. Role of AP-1 motif in uric acid-increased ET-1 promoter activity. $*P<0.05$ vs. control (Student's t test); $\#P<0.05$ vs. Uric acid alone (ANOVA). (A) Effect of uric acid on AP-1-mediated reporter activity. Cells transfected with AP-1-Luc were stimulated with uric acid (3–300 μM) for 24 h, and luciferase activities were measured. Data are presented as differences relative to control groups. The results are shown as mean±SEM ($n=5$). (B) Effect of antioxidants on uric acid-increased AP-1-mediated reporter activity in cardiac fibroblasts. Cells were preincubated with either the NAC (10 mM) or DPI (10 μM) for 30 min and stimulated with uric acid (300 μM) for 24 h. Cells treated with H_2O_2 (25 μM) for 24 h were used as a positive control. The results are shown as mean±SEM ($n=6$). (C) Wild type (204 bp) or AP-1 mutant of ET-1 promoter-CAT plasmid was transfected into cardiac fibroblasts. Cells were stimulated with uric acid (300 μM) for 24 h. CAT 2 and CAT 3 were used as positive or negative controls for CAT assay respectively. Data are presented as differences relative to control groups. The results are shown as mean±SEM ($n=5$).

a significant reduction in ROS production (Fig. 2B). To elucidate the involvement of ROS in the uric acid-induced ET-1 expression, cardiac fibroblasts were pretreated with NAC or DPI for 30 min followed by uric acid treatment. Cardiac fibroblasts pretreated with NAC or DPI significantly suppressed uric acid-increased ET-1 mRNA level and ET-1 peptide secretion (Fig. 2C, D). These findings suggest that intracellular ROS generation plays a role in uric acid-induced ET-1 gene expression in rat cardiac fibroblasts.

3.3. Uric acid-induced ET-1 gene expression is mediated via ERK pathway

To study the intracellular pathways which were involved in uric acid-induced ET-1 gene expression in cardiac fibroblasts, we examined the effect of uric acid on ERK phosphorylation and determined the effect of ERK inhibitors on uric acid-induced ET-1 gene expression. We found that uric acid-increased ERK phosphorylation in cardiac fibroblasts (Fig. 3A). Both NAC and DPI significantly inhibited uric acid-induced ERK phosphorylation (Fig. 3B). Next, we determined the role of redox-sensitive activation of ERK in uric acid-induced ET-1 gene expression. PD98059 (20 μM) or U0126 (10 μM), both are inhibitors of MKK-1 (MEK), inhibited augmentation of ET-1 mRNA expression and ET-1 secretion stimulated by uric acid (Fig. 3C, D). These findings suggest that the activation of ERK is a necessary step for ET-1 gene expression induced by uric acid.

3.4. Identification of uric acid-responsive regulatory elements in the ET-1 promoter

The ET-1 promoter contains a number of AP-1 and GATA sites, which can be regulated by multiple activation pathways [13,14]. As shown in Fig. 4A, uric acid (100–300 μM) significantly increased AP-1-mediated reporter activity in neonatal rat cardiac fibroblasts. Moreover, pretreated cells with antioxidants, NAC or DPI attenuated the uric acid-stimulated AP-1-mediated reporter activity (Fig. 4B). We further examined whether AP-1 site is essential for the induction of ET-1 gene by uric acid. In cells transfected with reporter construct −204CAT with two-bp mutation in the AP-1 site, the uric acid-induced ET-1 promoter activity was completely abolished. In addition, the basal promoter activity also decreased as compared with control (Fig. 4C).

These findings suggest that the AP-1 binding element is essential for the induction of ET-1 gene by uric acid. These results clearly indicate that ROS mediate the transcriptional activity of AP-1 induced by uric acid and the AP-1 binding element is responsible for the induction of ET-1 gene expression by uric acid in cardiac fibroblasts.

4. Discussion

4.1. Mechanism

The presented study is the first demonstration that uric acid exerts direct effects in cardiac fibroblasts. The heart is

composed of not only cardiac myocytes but also nonmyocytes, particularly fibroblasts [15]. Unlike cardiac myocytes, cardiac fibroblasts can proliferate and increase the deposition of extracellular matrix (ECM) proteins such as fibronectin and collagen, which leads to interstitial fibrosis [6]. Therefore, fibroblasts play a critical role in the development of cardiac fibrosis which enhances intrinsic myocardial stiffness and results in diastolic dysfunction, accounting for 30 to 50% of congestive heart failure in clinical practice [16,17]. In this study, we report that soluble uric acid stimulates cell proliferation and increases ET-1 expression, by rat cardiac fibroblasts *in vitro*. The increase in ET-1 expression with both mRNA and proteins are upregulated within a few hours of incubation of the cardiac fibroblasts with uric acid. 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 ET-1 regulation.

Direct effects of soluble uric acid were characterized in vascular smooth muscle cells, endothelial cells, and adipocytes. In vascular smooth muscle cells, uric acid activates critical proinflammatory [3] pathways and stimulates cell [5] proliferation. In endothelial cells, uric acid decreases nitric oxide bioavailability and inhibits cell migration and proliferation, which are mediated in part by the expression of C-reactive [5] protein. In adipocytes, the redox-dependent effects of uric acid are mediated by the activation of intracellular oxidant production *via* NADPH oxidase. Activation of ERK in response to uric acid has been shown in vascular smooth muscle cells and [3,18] adipocytes. Increased NADPH oxidase-derived ROS have recently been reported to be potentially linked with a defect in cardiac contractile function in a pathological [19] setting. ROS may act as second messengers that regulate various intracellular signal transduction cascades and the activity of various transcription factors. In assessing the mechanism through which uric acid mediated its effects in cardiac fibroblasts, we also found that redox pathways were implicated. The ability of both NAC and DPI to inhibit the uric acid-induced increase in ERK phosphorylation and ET-1 expression suggested that uric acid was acting in a prooxidative manner. Although uric acid is often thought of as an antioxidant, however, several studies have demonstrated that uric acid can be prooxidative and may generate free [3,18] radicals.

The ET-1 promoter contains AP-1 element that could be activated by [13] ROS. Several evidences suggest that ROS serve as messengers in AP-1 [20] activation. The *cis*-acting AP-1 element binds the protooncogene products jun and [21] fos and it is well known that the genes for jun and fos are activated by [22] ROS. Mutational analysis of the ET-1 gene promoter showed that AP-1 binding sequence is an important *cis*-element for uric acid-induced ET-1 gene expression. The ability of uric acid to activate ERK, and AP-1, as well as to increase ET-1 expression, is consistent with an oxidant-driven pathway. However, the mechanism by which uric acid activated various signaling pathways in cardiac fibroblasts remains further determined.

4.2. Limitations

We observed concentration-dependent effects of uric acid in rat cardiac fibroblasts within a concentration range of 3–300 μM, with distinct effects observed at a concentration as low as 100 μM (1.68 mg/dL). It is important to note that the uric acid levels in most mammals differ from that seen in humans. 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). Therefore, it is possible that the concentration dependence of the effects of uric acid in human cardiac fibroblasts will be different.

5. Conclusions

Our results support previous epidemiological studies of hyperuricemia, which suggest an involvement of uric acid in the pathogenesis of the cardiac diseases, and provide a possible molecular mechanism for this role based on the finding that soluble uric acid affects cardiac fibroblasts directly by inducing ROS production. We suggest that hyperuricemia can be one of the causal factors inducing oxidative stress followed by a pro-fibrotic process in the cardiac tissue, thereby contributing to the pathogenesis of the cardiac diseases.

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