

Full-length article

Uric acid stimulates endothelin-1 gene expression associated with NADPH oxidase in human aortic smooth muscle cellsHung-hsing CHAO^{1,2,4}, Ju-chi LIU^{2,4}, Jia-wei LIN², Cheng-hsien CHEN², Chieh-hsi WU³, Tzu-hung CHENG^{3,5}¹Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan, China; ²Department of Medicine, Taipei Medical University, Taipei, Taiwan, China; ³Department of Biological Science and Technology, College of Life Sciences, China Medical University, Taichung, Taiwan 40402, China**Key words**

uric acid; endothelin-1; human aortic smooth muscle cells; reactive oxygen species; extracellular signal-regulated kinases; NADPH oxidase

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Received 2008-04-20

Accepted 2008-08-05

doi: 10.1111/j.1745-7254.2008.00877.x

Abstract

Aim: Recent experimental and human studies have shown that hyperuricemia is associated with hypertension and cardiovascular diseases. Elevated levels of endothelin-1 (ET-1) has been regarded as one of the most powerful independent predictors of cardiovascular diseases. For investigating whether uric acid-induced vascular diseases are related to ET-1, the uric acid-induced ET-1 expression in human aortic smooth muscle cells (HASMC) was examined. **Methods:** Cultured HASMC treated with uric acid, cell proliferation and ET-1 expression were examined. Antioxidant pretreatments on uric acid-induced extracellular signal-regulated kinases (ERK) phosphorylation were carried out to elucidate the redox-sensitive pathway in proliferation and ET-1 gene expression. **Results:** Uric acid was found to increase HASMC proliferation, ET-1 expression and reactive oxygen species production. The ability of both N-acetylcysteine and apocynin (1-[4-hydroxy-3-methoxyphenyl]ethanone, a NADPH oxidase inhibitor) to inhibit uric acid-induced ET-1 secretion and cell proliferation suggested the involvement of intracellular redox pathways. Furthermore, apocynin, and p47^{phox} small interfering RNA knockdown inhibited ET-1 secretion and cell proliferation induced by uric acid. Inhibition of ERK by U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene) significantly suppressed uric acid-induced ET-1 expression, implicating this pathway in the response to uric acid. In addition, uric acid increased the transcription factor activator protein-1 (AP-1) mediated reporter activity, as well as the ERK phosphorylation. Mutational analysis of the ET-1 gene promoter showed that the AP-1 binding site was an important *cis*-element in uric acid-induced ET-1 gene expression. **Conclusion:** This is the first observation of ET-1 regulation by uric acid in HASMC, which implicates the important role of uric acid in the vascular changes associated with hypertension and vascular diseases.

Introduction

Uric acid is an intermediate product of the purine degradation pathway in the cell. An elevated serum uric acid in humans is associated with hypertension^[1] and cardiovascular diseases^[2]. All of these conditions are thought to be mediated by oxidative stress^[3]. Experimental studies have shown that hyperuricemic rats (induced by blocking uricase) also develop hypertension, and vascular diseases^[4].

Physiological concentrations of soluble microcrystal-free uric acid induce gene expression of chemokines and growth factors, such as monocyte chemoattractant protein (MCP)-1 and platelet-derived growth factor^[5,6], and stimulate proliferation of vascular smooth muscle cells with the activation of mitogen-activated protein kinases (MAPK)^[6,7]. The effects of uric acid may involve complex but poorly understood redox-dependent pathways. Uric acid-induced MCP-1 expression in vascular smooth muscle cells was

attenuated by antioxidants, suggesting the involvement of a redox-dependent mechanism^[5]. More recently, it has been shown that soluble uric acid stimulated an increase in NADPH oxidase activity and reactive oxygen species (ROS) production in mature adipocytes^[8]. What remains unclear is whether ROS also play a role in the direct effects of uric acid on human aortic smooth muscle cells (HASMC).

Endothelin-1 (ET-1) is a potent vasoconstrictor derived from the endothelium^[9]. Patients with moderate to severe hypertension reveal increased vascular levels of prepro-ET-1 mRNA^[9]. Elevated levels of ET-1 have emerged as one of the most important predictors of myocardial infarction, stroke, and vascular death^[10]. It has been shown that the plasma levels of ET-1 were significantly associated with uric acid by univariate analysis in an epidemiological study^[11]. We previously reported that ROS *via* the activation of extracellular signal-regulated kinase (ERK) and the nuclear transcription factor activator protein-1 (AP-1) are essential for cell proliferation and ET-1 gene expression in rat aortic smooth muscle cells^[12]. However, the effect of uric acid on cell proliferation and ET-1 gene expression in HASMC remains to be elucidated.

Despite the striking common features of uric acid and ET-1 in the development of hypertension and cardiovascular diseases, there are no data on whether uric acid-induced vascular changes in blood vessels are also related to ET-1 expression. In this study we attempted to clarify the relationship of uric acid with ET-1 expression, and investigated the effect of uric acid on ET-1 synthesis in human vascular cells.

Materials and methods

Materials Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and tissue culture reagents were from Life Technologies, Inc (Gaithersburg, MD, USA). An ET-1 cDNA probe was obtained as previously described^[13]. Wild type (204 bp) or AP-1 mutant of ET-1 promoter was fused with the chloramphenicol acetyltransferase (CAT) reporter gene (ET-1 promoter-CAT plasmid); PBLCAT2 (containing CAT reporter gene with its promoter) and PBLCAT3 (containing CAT gene only) were constructed as previously described^[13]. 2',7'-Dichlorofluorescein diacetate (DCF-DA) was obtained from Molecular Probes (Eugene, OR, USA). H₂O₂ was purchased from Acros Organics (Pittsburgh, PA, USA). The ECL detection system was from Amersham Pharmacia Biotech (Uppsala, Sweden). Bosentan (*N*-[6-(2-hydroxyethoxy)-5-(2-methoxyphenoxy)-2-pyrimidin-2-yl-pyrimidin-4-yl]-

4-*tert*-butyl-benzenesulfonamide), a nonselective ET_A/ET_B receptor antagonist, was obtained from Actelion Pharmaceuticals (Allschwil, Switzerland). U0126 (1,4-diamino-2,3-dicyano-1,4-*bis*[2-aminophenylthio]butadiene) was obtained from Tocris Cookson Ltd (Bristol, UK). 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, USA). Uric acid, *N*-acetylcysteine (NAC), apocynin, probenecid (4-[dipropylsulfamoyl]benzoic acid), benzbromarone ([3,5-dibromo-4-hydroxyphenyl]-[2-ethyl-3-benzofuranyl]methanone) and all other reagent-grade chemicals were purchased from the Sigma-Aldrich Chemical Co (St Louis, MO, USA).

Culture of human aortic smooth muscle cells and treatments Primary cultures of HASMC were obtained from PromoCell GmbH (Heidelberg, Germany) and cultured as per the manufacturer instructions. Cells were passaged every 3–4 d at a split ratio of 1:4 in growth medium (SMCGM2; Promocell). SMCGM2 is a commercially produced medium supplemented with 5% fetal calf serum (FCS), epidermal growth factor (EGF) (0.5 ng/mL), basic fibroblast growth factor (bFGF) (2 ng/mL), insulin (5 µg/mL), amphotericin B (50 ng/mL) and gentamicin (50 µg/mL). Cells were then treated with basal medium containing no serum or growth factors in the following experiments. All experiments were carried out between passages 5 and 16. For analysis of the effects of uric acid, cells were seeded in growth medium at a pre-optimized density of 5×10³ cells/cm² in 96-well, 12-well or 6-well plates or 60-mm culture dishes, as appropriate. After 3 d (about 70%–80% confluency) existing medium was replaced with basal medium for 24 h. Monolayers were then treated in fresh basal medium, alone (control) or with reagents as indicated. Inhibitor concentrations used were bosentan 3 or 30 µmol/L, U0126 10 µmol/L, probenecid 1 mmol/L and benzbromarone 50 µmol/L (30 min preincubation). Antioxidant concentrations were NAC 10 mmol/L and apocynin 10 µmol/L (30 min preincubation).

Cell proliferation Proliferation was assessed by counting and [³H]thymidine incorporation of cells incubated with or without uric acid, in the presence or absence of reagents as indicated. 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, USA). To measure synthesis of new DNA, cells were plated on 6-well (35 mm)

dishes 24 h before experiments as previously described^[12]. Cells were incubated with [³H]thymidine (5 μ Ci/mL). After addition of agent indicated, cells were harvested by incubation at 4 °C with trichloroacetic acid (5%) followed by solubilization in NaOH (0.1 mol/L), and radioactivity was determined by scintillation counting. Data are presented as the mean \pm SEM of 9–12 determinations in 3–4 different cell preparations and normalized to the untreated sample \times 100 (i.e. percentage of control).

Assay of ET-1 peptide secretion Endothelin-1 levels were measured in culture medium using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Amersham-Pharmacia, Amersham, UK) as previously described^[12]. Results were normalized to cellular protein content in all experiments and expressed as a percentage relative to the cells incubated with the vehicle.

Detection of intracellular ROS and NADPH oxidase activity assay Reactive oxygen species were measured using a previously described method^[12]. Prior to the chemical treatment, cells were incubated in culture medium containing a fluorescent dye, DCF-DA (30 μ mol/L) for 1 h to establish a stable intracellular level of the probe. The same concentration of DCF-DA was maintained during the chemical treatment. Subsequently, the cells were washed with phosphate-buffered saline (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, USA) and used to normalize the fluorescence intensity of DCF. Chemiluminescence assay of superoxide production was measured as described previously^[14]. NADPH oxidase activity was measured as described previously^[15]. NADPH oxidase activity was measured using the lucigenin-enhanced chemiluminescence method in microsomal membrane fractions. To prepare cell homogenates, the cell monolayer was washed three times with ice-cold PBS and scraped on ice in lysis buffer containing 20 mmol/L K-phosphate buffer (pH 7.0), 1 mmol/L glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 mmol/L phenylmethanesulfonyl fluoride (PMSF), 10 μ g/mL aprotinin, and 5 μ g/mL leupeptin, followed by homogenization with 100 strokes in a Dounce homogenizer on ice. For the isolation of microsomal membranes, cell homogenates were prepared in 250 mmol/L sucrose, 5

mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4), 1 mmol/L PMSF, 10 μ g/mL aprotinin, and 5 μ g/mL leupeptin, followed by centrifugation at 1000 \times g (10 min, 4 °C). The pellet was discarded, and the supernatant was spun at 8000 \times g (10 min, 4 °C). The microsomal fraction was separated from cytosol by centrifugation of the supernatant at 105 000 \times g (45 min, 4 °C). The pellet was resuspended in the homogenization buffer by using a Hamilton glass syringe. The cell homogenate and microsomal fraction were used immediately. The assay was started in an Orion microplate luminometer (Berthold Detection Systems, GmbH of Pforzheim, Germany) by automatic injection of the 150 μ L reaction buffer (50 mmol/L K-phosphate buffer [pH 7.0] containing 1 mmol/L EGTA, 150 mmol/L sucrose, 5 μ mol/L lucigenin, and 100 μ mol/L NADPH) into 10 μ L of the homogenate or membrane suspension (5–20 μ g protein). Photon emission in response to superoxide generation was measured every 60 s with a 5 s signal integration time for 20 min. The activity is expressed in relative light units per milligram of protein. The protein concentration was measured using the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA).

RNA isolation and Northern blot analysis Total RNA was isolated from cells by the guanidine isothiocyanate/phenol chloroform method as previously described^[12]. Blots were stripped and reprobed for 18S cDNA probe (obtained from American Type Culture Collection [Rockville, MD, USA]) to control for loading. Expression of ET-1 mRNA was quantitated and was normalized to the 18S signal.

siRNA-mediated gene knockdown p47^{phox} siRNA (sc-36157), and control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and used for p47^{phox} knockdown, and mock control, respectively^[16]. Transfection of siRNA in HASMC was carried out using nucleofection according to the manufacturer's instruction (Amaxa, Gaithersburg, MD, USA). Transfected cells were applied to advanced assays and Western blot analysis.

Western blot analysis Rabbit polyclonal anti-phospho-specific ERK antibodies were purchased from New England Biolabs (Beverly, MA, USA). Anti-ERK antibodies, anti-p47^{phox} antibodies and anti- α tubulin antibodies were purchased from Santa Cruz Biotechnology. Western blot analysis was carried out as previously described^[12]. Cells were rinsed twice with ice-cold PBS following treatment and scraped into ice-cold buffer containing 50 mmol/L Tris·HCl (pH 7.6), 120 mmol/L NaCl, 1% Nonidet P-40, 10% glycerol, 1 mmol/L PMSF, 2 mmol/L sodium

orthovanadate, 10 mmol/L sodium pyrophosphate, 40 $\mu\text{g/mL}$ leupeptin, 5 $\mu\text{g/mL}$ aprotinin, 1 $\mu\text{g/mL}$ pepstatin, 100 mmol/L NaF, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), and 1 mmol/L EGTA. After lysis on ice for 60 min, extracts were centrifuged for 10 min at $14\,000\times g$ at 4 °C. The protein concentration of the supernatants was measured, and 20 μg protein samples of cell lysate were mixed (1:1) with Laemmli sample buffer and incubated at 95 °C for 5 min. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electroblotting onto polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in 10 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, and 0.1% Tween 20 containing 5% non-fat dry milk, followed by incubation with primary antibody. Membranes were washed 3 times and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. The immunocomplexes were visualized by chemiluminescence with the Phototope Western blot detection system (Cell Signaling Technology, Beverly, Massachusetts, USA). The images were digitalized using the AlphaEase FluorChem digital imaging system (Alpha Innotech, San Leandro, CA, USA). Band densitometry was carried out using NIH Image software. Ratios of phosphorylated kinases to total kinases or to α tubulin were calculated.

Transfection and chloramphenicol acetyltransferase assays For the transient transfections, cells transfected with different expression vectors were obtained with the Amaxa Nucleofector system (Amaxa, Gaithersburg, MD, USA), which allows non-viral gene to be directly transferred into the nucleus using proprietary Nucleofector solutions. To correct for variability in transfection efficiency, 5 μg of pSV- β -galactosidase plasmid DNA was cotransfected in all of the experiments. The CAT and β -galactosidase assays were carried out as described previously^[17]. 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 for positive and negative controls, pBLCAT2 (with thymidine kinase promoter) and pBLCAT3 (without promoter) were included in each assay.

Luciferase assay Human aortic smooth muscle cells plated on 6 well (35 mm) dishes were transfected with the luciferase reporter construct possessing consensus AP-1 binding sites (AP-1-Luc) (Stratagene). HASMC were assayed for luciferase activity with a luciferase reporter assay kit (Stratagene) as previously described^[12]. The firefly

luciferase activities as AP-1 transcriptional activity were normalized for transfection efficiency to its respective β -galactosidase activity and expressed as relative activity to control.

Statistical analysis Results are expressed as mean \pm SEM. Statistical analysis was carried out using Student's *t* test and ANOVA followed by a Dunnett multiple comparison test using Prism version 3.00 for Windows (GraphPad Software, San Diego, CA, USA). A value of $P < 0.05$ was considered to be statistically significant.

Results

Effect of uric acid on cell proliferation and ET-1 expression in human aortic smooth muscle cells The effect of uric acid on cell proliferation was evaluated by means of cell count and [³H]thymidine incorporation studies. Uric acid (300 and 600 $\mu\text{mol/L}$, 24 h) significantly increased cell number and DNA synthesis in HASMC (Figure 1A, 1B). Uric acid (300 $\mu\text{mol/L}$) also significantly increased the proliferative activity of HASMC in a time-dependent manner (Figure 1C, 1D). Uric acid (300 $\mu\text{mol/L}$, 24 h)-increased cell number and DNA synthesis in HASMC was inhibited by pan-ET-receptor antagonist bosentan (30 $\mu\text{mol/L}$) treatment (Figure 1E, 1F). These data suggest the possible role of endogenous ET-1 as an autocrine growth factor for the proliferation of HASMC under uric acid stimulation.

To determine whether uric acid would increase ET-1 mRNA levels in HASMC or not, we carried out Northern blot analysis (Figure 2A, B). In HASMC, uric acid induced ET-1 mRNA expression in a time-dependent manner (uric acid 300 $\mu\text{mol/L}$) (Figure 2A). When cells were treated with uric acid for 6 h, uric acid (100–600 $\mu\text{mol/L}$) significantly increased ET-1 mRNA expression (Figure 2B). To examine whether uric acid would increase ET-1 expression in HASMC, concentration-dependency of ET-1 secretion by uric acid had been carried out by ELISA (Figure 2C). HASMC exposed to 24 h of uric acid (300 and 600 $\mu\text{mol/L}$) significantly increased ET-1 peptide secretion (Figure 2C). To determine whether transport of uric acid into HASMC required the stimulation of ET-1 peptide secretion, we pretreated cells with probenecid and benzbromarone, two structurally unrelated organic anion transporter inhibitors of the transmembrane transport of urate^[18]. As shown in Figure 2D, both inhibitors prevented stimulation of ET-1 peptide secretion in HASMC in response to uric acid, suggesting that uric acid must have entered the cell to induce ET-1 peptide secretion. These data show that uric acid induces cell proliferation and ET-1 gene expression in

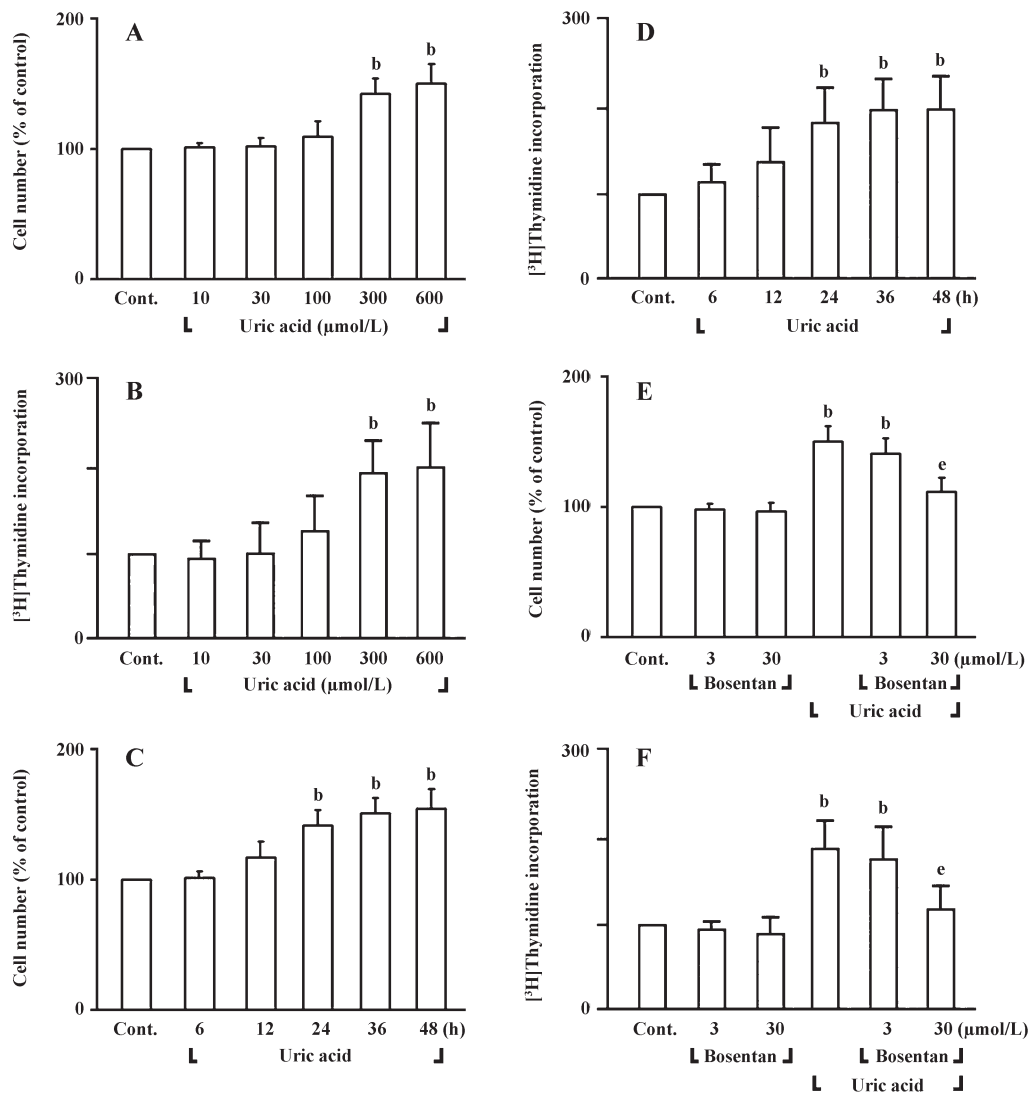


Figure 1. Effects of uric acid on cell proliferation in HASMC. The results shown are the mean±SEM (n=6). ^bP<0.05 vs control (Student *t* test); ^eP<0.05 vs uric acid alone (ANOVA). (A) Concentration-response effect of uric acid on cell proliferation. Cells were cultured in control conditions (cont) or incubated with various concentrations of uric acid for 24 h then counted for cell number. Cell number was expressed as percentage of control (100%). (B) Effect of uric acid on the DNA synthesis. Cells were incubated with the indicated concentrations of uric acid for 24 h and then for assay of [³H]thymidine incorporation. Experimental details are given in Materials and methods. [³H]thymidine incorporation is expressed as percentage increase relative to the [³H] content (100%) in their respective control (cont). All data shown are the mean±SEM of 9–12 determinations in 6 different cell preparations. (C) Time course of uric acid on cell proliferation. Cells were incubated with uric acid (300 μmol/L) for the indicated time slots. (D) Time course of uric acid on DNA synthesis. Cells were incubated with uric acid (300 μmol/L) for the indicated time slots. (E) Effect of bosentan on cell proliferation. Cells were preincubated with bosentan (3 or 30 μmol/L) for 1 h before their incubation with uric acid (300 μmol/L) for 24 h. (F) Effect of bosentan on [³H]thymidine incorporation. Cells were preincubated with bosentan (3 or 30 μmol/L) for 1 h before their incubation with uric acid (300 μmol/L) for 24 h.

HASMC.

Uric acid-induced cell proliferation and ET-1 gene expression is redox-sensitive To determine whether uric acid would induce intracellular ROS in HASMC or not, we measured intracellular ROS levels by analyzing the fluorescent product DCF, a peroxidative product of DCF-DA. HASMC treated with uric acid (100–600 μmol/L)

for 30 min had ROS levels significantly higher than those cells treated with vehicle only (Figure 3A). Similar results were obtained with the use of lucigenin-enhanced chemiluminescent superoxide detection (Figure 3B). Next, we examined the effect of uric acid on the enzymatic activity of NADPH oxidase. Since active NADPH oxidase is a membrane-associated enzyme^[19], we tested the effect of

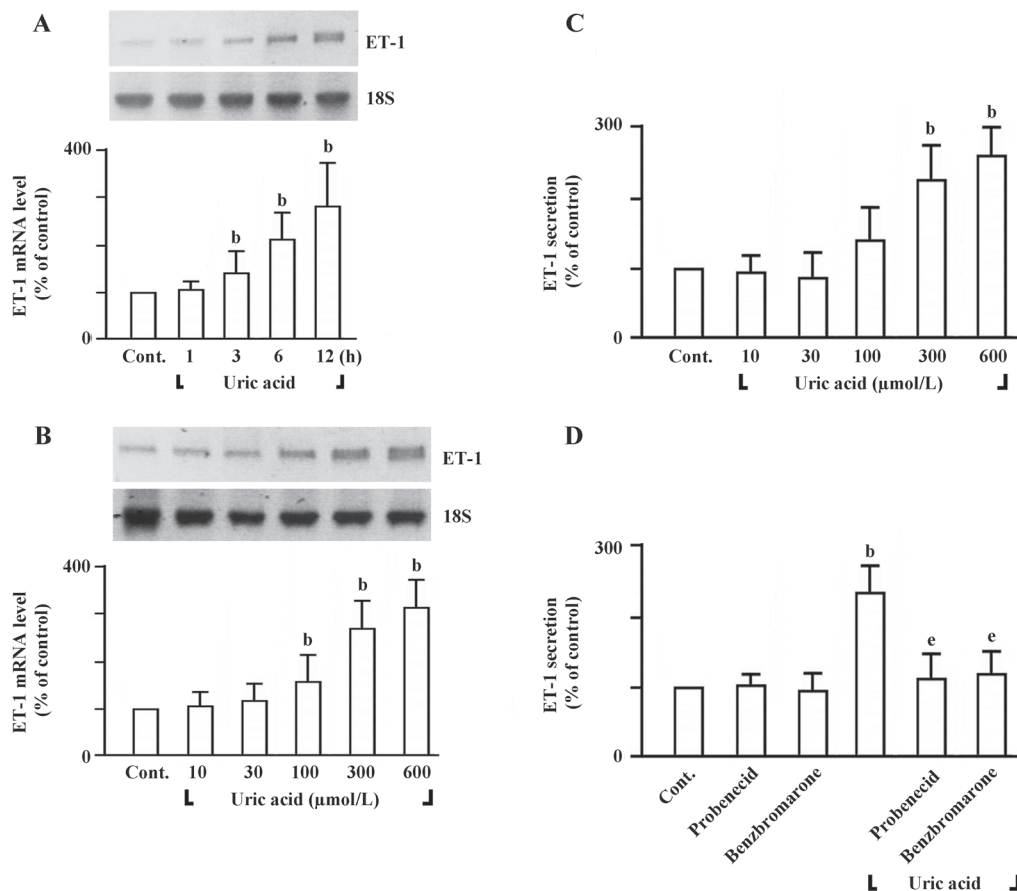


Figure 2. Effects of uric acid on ET-1 gene expression in HASMC. The results shown are the mean±SEM ($n=6$). ^b $P<0.05$ vs control (Student *t* test); ^e $P<0.05$ vs uric acid alone (ANOVA). (A) Time- and (B) Concentration-dependent effect of uric acid on ET-1 mRNA expression in HASMC. Uric acid increased ET-1 mRNA expression at 3 h and at concentrations of 100 μmol/L or higher compared with control (Cont). Data are represented as difference relative to control groups. (C) 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. (D) Effect of organic anion transporter inhibitors on ET-1 peptide secretion. Cells were preincubated with probenecid (1 mmol/L) and benzbromarone (50 μmol/L) for 30 min before their incubation with uric acid (300 μmol/L) for 24 h.

uric acid on the NADPH oxidase activity in microsomal membranes. Treatment of HASMC with uric acid (100–600 μmol/L) for 30 min stimulated NADPH oxidase activity in the microsomal fraction (Figure 3C). NADPH oxidase is composed of several regulatory subunits, including those involved in the formation of the classic phagocyte-type NADPH oxidase (gp91^{phox}, p67^{phox}, p47^{phox}, p40^{phox}, and p22^{phox})^[19]. We assessed the effect of uric acid on translocation of p47^{phox} to the membranes. Immunoblot analysis of subcellular fractions of HASMC treated with uric acid revealed that uric acid (100–600 μmol/L; for 30 min) induced a dramatic dose-dependent increase in the content of p47^{phox} in the microsomal fraction with a simultaneous decrease in the cytosol (Figure 3D), indicating translocation

of the subunit from the cytosol to membranes.

In addition, cells pretreated with antioxidants such as NAC (10 mmol/L) or a catechol-methyl derivative that blocks p47^{phox} interaction with gp91^{phox}/p22^{phox}, apocynin (10 μmol/L) for 30 min showed a significant reduction in ROS production (Figure 4A). To elucidate the involvement of ROS in the uric acid-induced cell proliferation and ET-1 expression, HASMC were pretreated with NAC or apocynin for 30 min followed by uric acid treatment. HASMC pretreated with NAC or apocynin significantly suppressed uric acid-increased cell proliferation, ET-1 mRNA level, and ET-1 peptide secretion (Figure 4B, 4C, 4D). These findings suggest that intracellular ROS generation plays a role in uric acid-induced cell proliferation and ET-1 gene

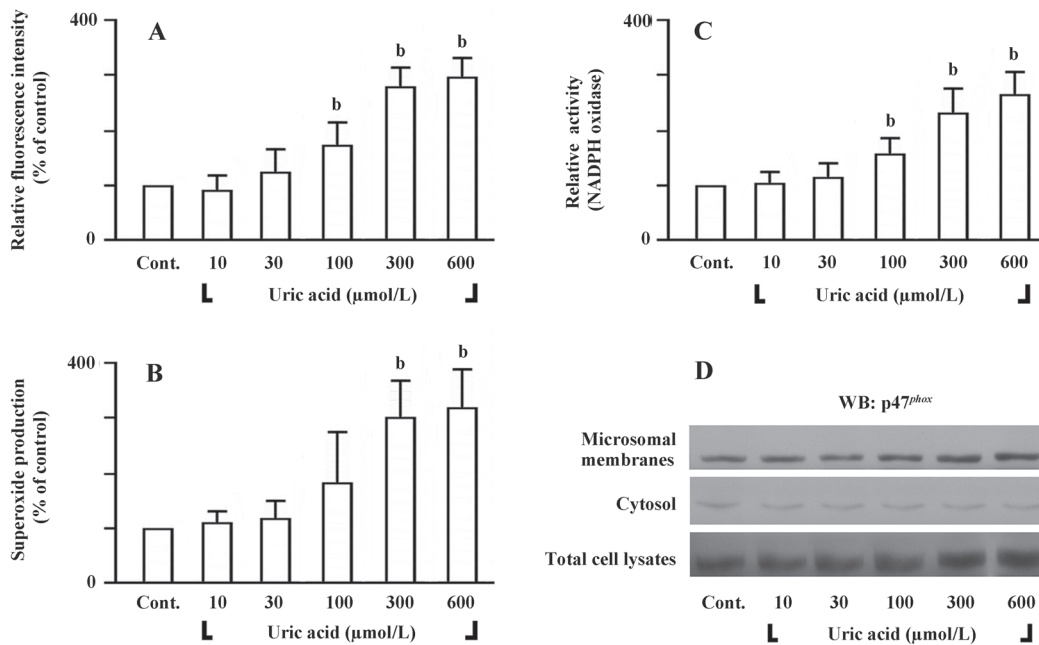


Figure 3. Effects of uric acid on ROS production in HASMC. The results shown are the mean±SEM (*n*=6). ^b*P*<0.05 vs control (Student *t* test). (A) Concentration-response effect of uric acid on intracellular ROS in HASMC. Cells were loaded with DCF-DA for 30 min and cultured in control conditions (Cont.) or incubated with various concentrations of uric acid for 30 min. (B) Concentration-response effect of uric acid on superoxide production in HASMC. Cells were in control conditions (Cont.) or incubated with various concentrations of uric acid for 30 min. (C) Concentration-response effect of uric acid on NADPH oxidase activity in HASMC. Cells were in control conditions (Cont.) or incubated with various concentrations of uric acid for 30 min. (D) Concentration-response effect of uric acid on translocation of p47^{phox} to the membranes in HASMC. Cells were in control conditions (Cont.) or incubated with various concentrations of uric acid for 30 min.

expression in HASMC.

Uric acid-induced cell proliferation and ET-1 gene expression is associated with NADPH oxidase To confirm the role of NADPH oxidase in uric acid-induced ET-1 expression and cell proliferation, NADPH oxidase subunit p47^{phox} siRNAs were used for NADPH oxidase knockdown cells. As shown in Figure 5A, the expression level of p47^{phox} was significantly reduced in p47^{phox} siRNA transfection cells compared with the mock controls. The uric acid-induced ET-1 peptide secretion and cell proliferation were also decreased apparently in siRNA transfection cells (Figure 5B, 5C). These results revealed that uric acid induces ET-1 expression and cell proliferation via NADPH oxidase in HASMC.

Involvement of ERK phosphorylation in uric acid-induced ET-1 gene expression To study the intracellular pathways that were involved in uric acid-induced ET-1 gene expression in HASMC, 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 HASMC (Figure 6A). Both NAC and apocynin

significantly inhibited uric acid-induced ERK phosphorylation (Figure 6A). We next determined the role of redox-sensitive activation of ERK in uric acid-induced ET-1 gene expression. NAC or U0126 (10 μmol/L), an inhibitor of MKK-1 (MEK), inhibited the augmentation of ET-1 mRNA expression stimulated by uric acid (Figure 6B). These findings implicate the redox-sensitive activation of ERK signaling pathways in uric acid-induced ET-1 mRNA expression.

Identification of uric acid-responsive regulatory elements in the ET-1 promoter The ET-1 promoter contains an AP-1 site, which can be regulated by multiple activation pathways^[20,21]. As shown in Figure 7A, uric acid (300 and 600 μmol/L) significantly increased AP-1-mediated reporter activity in HASMC. Moreover, pretreated cells with antioxidants, NAC or apocynin attenuated the uric acid-stimulated AP-1-mediated reporter activity (Figure 7B). We further examined whether the 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 the control (Figure

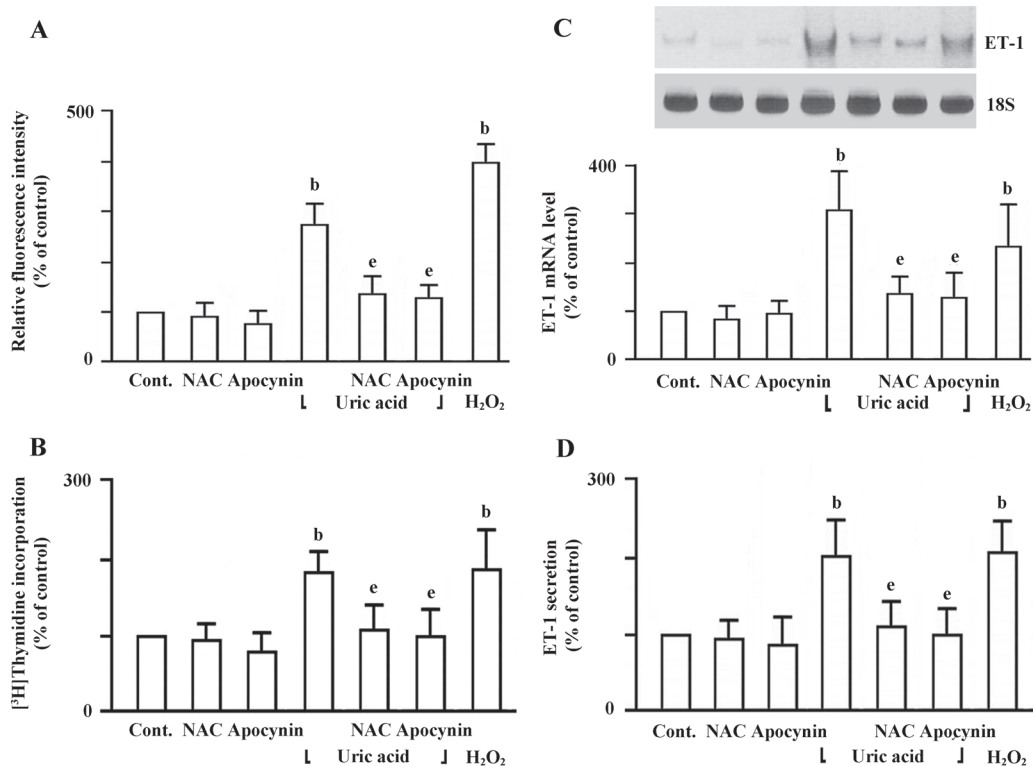


Figure 4. Involvement of ROS in uric acid-induced ET-1 gene expression in HASMC. ^b $P < 0.05$ vs control (Student *t* test); ^c $P < 0.05$ vs uric acid alone (ANOVA). (A) Uric acid increased intracellular ROS in HASMC. Cells were loaded with DCF-DA for 30 min and stimulated with uric acid (300 $\mu\text{mol/L}$) for 30 min. Uric acid increased ROS levels in HASMC, and this increment was inhibited by NAC (10 mmol/L), or the NAD(P)H oxidase inhibitor apocynin (10 $\mu\text{mol/L}$). Cells treated with H₂O₂ (25 $\mu\text{mol/L}$) for 30 min were used as positive control. The results shown are the mean \pm SEM ($n=6$). (B) Effect of antioxidants on uric acid-increased DNA synthesis in HASMC. Cells were preincubated with either the NAC (10 mmol/L) or apocynin (10 $\mu\text{mol/L}$) for 30 min followed by incubation with 300 $\mu\text{mol/L}$ uric acid for 24 h. The results shown are the mean \pm SEM ($n=6$). (C) Effect of antioxidants on uric acid-induced ET-1 mRNA in HASMC. Cells were preincubated with either the NAC (10 mmol/L) or apocynin (10 $\mu\text{mol/L}$) for 30 min followed by incubation with 300 $\mu\text{mol/L}$ uric acid for 6 h. The results shown are the mean \pm SEM ($n=5$). (D) Effect of antioxidants on uric acid-increased ET-1 peptide secretion in HASMC. Cells were preincubated with either the NAC (10 mmol/L) or apocynin (10 $\mu\text{mol/L}$) for 30 min followed by incubation with 300 $\mu\text{mol/L}$ uric acid for 24 h. The results shown are the mean \pm SEM ($n=5$).

7C). These findings suggest that the AP-1 binding element is essential for the induction of the 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 HASMC.

Discussion

The results of the present study demonstrate that uric acid exerts direct effects on HASMC. In this study, we report that soluble uric acid stimulates cell proliferation and increases ET-1 expression in HASMC. The increase in ET-1 expression with both mRNA and protein were up-regulated within a few hours of incubation of the HASMC with uric acid. The increase led us to examine the possible

posttranscriptional effects of uric acid, as well as various signaling pathways and transcription factors known to be important in ET-1 regulation.

We observed concentration-dependent effects of uric acid in HASMC within a concentration range of 100–600 $\mu\text{mol/L}$, with distinct effects observed at concentrations as low as 300 $\mu\text{mol/L}$. It is important to note that uric acid levels in most mammals differ from that seen in humans. In humans, hyperuricemia is defined as uric acid levels >415 $\mu\text{mol/L}$ in men and >360 $\mu\text{mol/L}$ in women (with a normal range of ~ 120 – 415 $\mu\text{mol/L}$)^[2], and has been identified as a risk factor in the development of hypertension^[22,23].

Direct effects of soluble uric acid were characterized in various cell types, including vascular smooth muscle cells, endothelial cells, and adipocytes. The observation that ET-1 peptide secretion in response to uric acid is sensitive

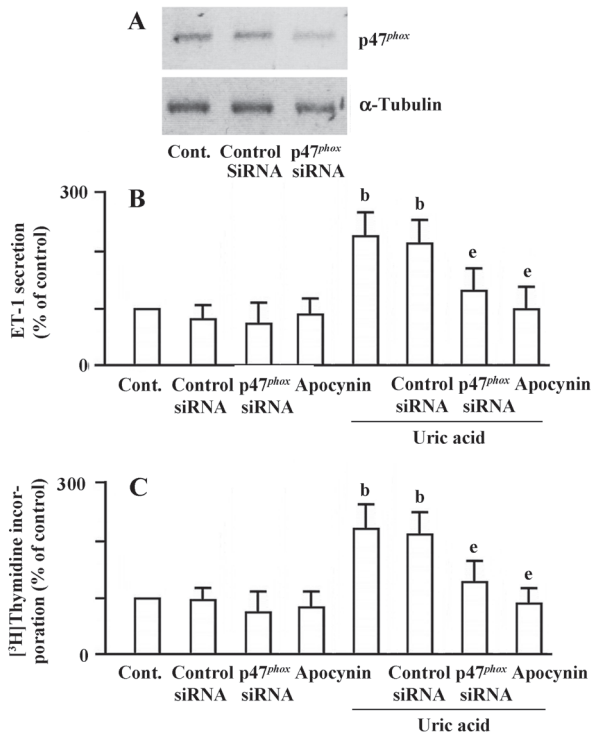


Figure 5. Effect of p47^{phox} knockdown in uric acid-induced ET-1 gene expression and cell proliferation in HASMC. ^b*P*<0.05 vs control (Student *t* test); ^e*P*<0.05 vs uric acid alone (ANOVA). (A) Western blots of p47^{phox} in p47^{phox} knockdown HASMC. Control siRNA and p47^{phox} siRNA were used for mock control and p47^{phox} siRNA knockdown, respectively. Proteins from HASMC without treatment (Cont.) or with control siRNA or p47^{phox} siRNA transfection were detected by using polyclonal goat anti-p47^{phox} antibody. (B) Effect of uric acid on ET-1 peptide secretion in p47^{phox} knockdown HASMC. HASMC with control siRNA or p47^{phox} siRNA transfection or preincubated with apocynin (10 μmol/L; 30 min) followed by incubation with 300 μmol/L uric acid for 24 h. The results shown are the mean±SEM (*n*=5). (C) Effect of uric acid on DNA synthesis in p47^{phox} knockdown HASMC. HASMC with control siRNA or p47^{phox} siRNA transfection or preincubated with apocynin (10 μmol/L; 30 min) followed by incubation with 300 μmol/L uric acid for 24 h. The results shown are the mean±SEM (*n*=6).

to blockade of urate transport with probenecid and benzbromarone suggests that entry of uric acid into the cell is required for these effects. In vascular smooth muscle cells, uric acid activates critical proinflammatory pathways^[5] and stimulates cell proliferation^[7]. 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 protein^[7]. In adipocytes, the redox-dependent effects of uric acid are mediated by the activation of intracellular oxidant production via NADPH oxidase^[8]. Activation of ERK in response to uric acid has been shown in vascular smooth muscle cells

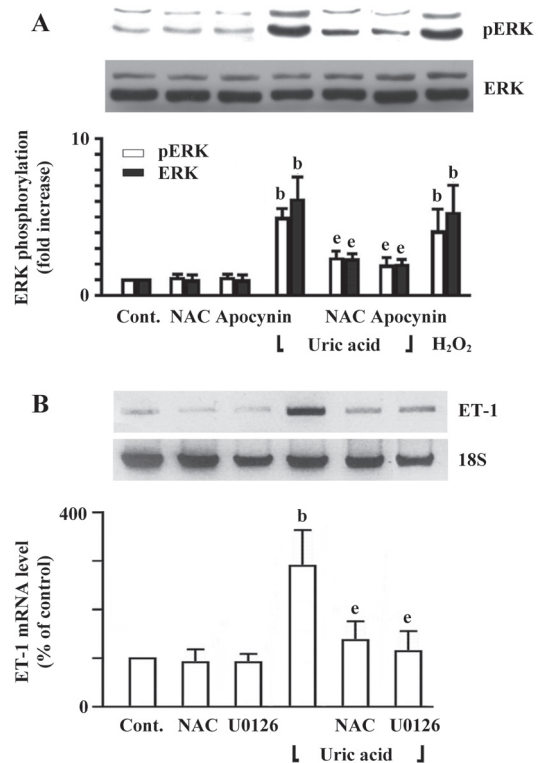


Figure 6. Uric acid increased ET-1 gene expression via ERK in a redox-sensitive manner. ^b*P*<0.05 vs control (Student *t* test); ^e*P*<0.05 vs uric acid alone (ANOVA). (A) Effect of antioxidants on uric acid-increased ERK phosphorylation in HASMC. Cells were preincubated with either the NAC (10 mmol/L) or apocynin (10 μmol/L) for 30 min and stimulated with uric acid (300 μmol/L) for 30 min. Cells treated with H₂O₂ (25 μmol/L) for 30 min are used as positive control. White or black columns denote the degree of ERK or ERK phosphorylation, respectively. Data are represented as fold increase relative to control groups. The results shown are the mean±SEM (*n*=6). (B) Uric acid-induced ET-1 mRNA was attenuated by NAC (10 mmol/L) or U0126 (10 μmol/L) in HASMC. HASMC were stimulated with uric acid (300 μmol/L) in the absence or presence of NAC or U0126, and total RNA was isolated at 6 h. The results shown are the mean±SEM (*n*=4).

and adipocytes^[5,8]. In assessing the mechanism through which uric acid mediated its effects in HASMC, we also found that redox pathways were implicated. The vascular NAD(P)H oxidases have been found to be essential in the physiological and pathological responses of vascular cells, including growth, migration, and modification of the extracellular matrix^[24-26].

Uric acid induced activation of NADPH oxidase in crude homogenates and isolated microsomal membranes of HASMC. Mechanisms of activation of NADPH oxidase (NOX) enzymes vary greatly depending on the spectrum of expressed isoforms and cytoplasmic regulators.

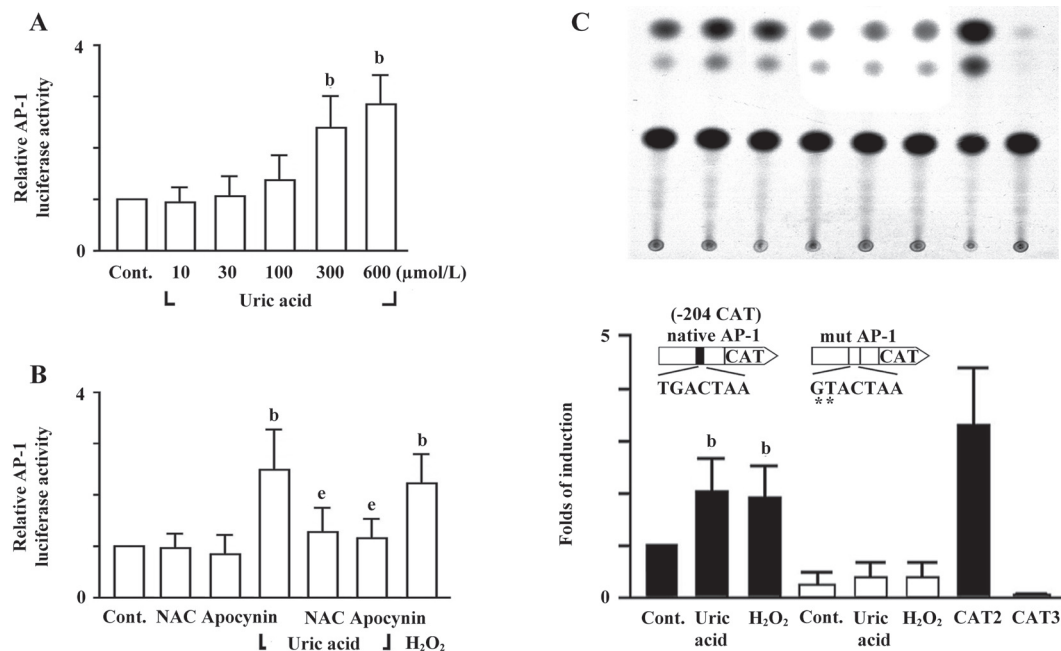


Figure 7. Role of AP-1 motif in uric acid-increased ET-1 promoter activity. ^b $P < 0.05$ vs control (Student *t* test); ^{*} $P < 0.05$ vs uric acid alone (ANOVA). (A) Effect of uric acid on AP-1 mediated reporter activity. Cells transfected with AP-1-Luc were stimulated with uric acid (10–600 μmol/L) for 24 h, and luciferase activities were measured. Data are represented as difference relative to control groups. The results shown are the mean±SEM ($n=6$). (B) Effect of antioxidants on uric acid-increased AP-1-mediated reporter activity in HASMC. Cells were preincubated with either the NAC (10 mmol/L) or apocynin (10 μmol/L) for 30 min and stimulated with uric acid (300 μmol/L) for 24 h. Cells treated with H₂O₂ (25 μmol/L) for 24 h are used as positive control. The results shown are the mean±SEM ($n=6$). (C) Wild type (204 bp) or AP-1 mutant of ET-1 promoter-CAT plasmid was transfected into HASMC. In the upper panels, HASMC (transfected with wild type [204 bp] of ET-1 promoter-CAT plasmid, middle left inset) were either vehicle controls (C, lane 1), stimulated with uric acid (300 μmol/L) (lane 2) or with H₂O₂ (25 μmol/L) (lane 3) for 24 h; and HASMC (transfected with AP-1 mutant of ET-1 promoter-CAT plasmid, middle right inset) treated with vehicle (lane 4), or stimulated with uric acid (300 μmol/L) (lane 5), or with H₂O₂ (25 μmol/L) (lane 6) for 24 h. CAT 2 (lane 6) and CAT 3 (lane 7) were used as positive or negative controls for CAT assay, respectively. The mutation of AP-1 strongly abolished the responsiveness to uric acid stimulation. Data are represented as difference relative to control groups. The results shown are the mean±SEM ($n=5$).

NOX1–NOX3 require the presence of p22^{phox} on the membrane and are more or less dependent on cytoplasmic subunits p47^{phox}, p67^{phox}, and p40^{phox}, which translocate to the membrane and form with NOX and p22^{phox} an active holoenzyme complex in response to cell stimulation^[19]. These NOX isoforms can provide constitutive and stimulated ROS production in HASMC, and different mechanisms may be involved in the activation of NADPH oxidase in response to stimulation. Our data on translocation of p47^{phox} in response to uric acid indicate the possibility of assembly of the classic phagocyte-type NADPH oxidase based on gp91^{phox} or another NOX protein. The upstream signaling mechanism linking urate transport into the cell and formation of the active NOX complex in HASMC remains largely unknown. The p47^{phox} plays a major role in functionally active NAD(P)H oxidase activity in cardiovascular cells, as evidenced by studies using p47^{phox}–/– mice^[27,28]. Furthermore, in vascular smooth muscle cells from human

resistance arteries that c-Src-mediated phosphorylation of p47^{phox} is a prerequisite for assembly and translocation of the p40-p47-p67^{phox} complex^[29]. The ability of NAC, and apocynin, which blocks association of p47^{phox} with membrane-associated subunits^[29,30], and NADPH oxidase subunit p47^{phox} knockdown to inhibit the uric acid-induced increase in cell proliferation and ET-1 expression suggested that uric acid was acting in a pro-oxidative manner associated with NADPH oxidase. Uric acid is often thought of as a chemical antioxidant, and is considered to be a major antioxidant in the human plasma^[11]; however, available data suggest that uric acid is not necessarily an antioxidant and, depending on the chemical milieu, may become a pro-oxidant^[8]. Several studies consistent with our present findings have demonstrated that uric acid can be pro-oxidative and may even generate free radicals^[5,8]. 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.

Our results support previous epidemiological studies of hyperuricemia, which suggest an involvement of uric acid in the pathogenesis of the cardiovascular diseases, and provide a possible molecular mechanism for this role based on the finding that soluble uric acid affects HASMC directly by inducing ROS production. We suggest that hyperuricemia can be one of the causal factors inducing intracellular oxidative stress followed by a mitogenic process in the vascular tissue, thereby contributing to the pathogenesis of the cardiovascular diseases. However, further experiments will be necessary to identify the precise mechanisms by which uric acid is associated with vascular diseases. Thus, this study delivers important new insight into the molecular pathways that may contribute to the deleterious effects of uric acid in regard to hypertension and cardiovascular diseases.

Acknowledgements

This study was supported by grants from the National Science Council (NSC 93-2320-B-038-020-; NSC 94-2314-B-038-062-) and Shin Kong Wu Ho-Su Memorial Hospital (SKH-TMU-94-16), Taipei, Taiwan, China. The authors wish to thank Miss Sherry LO for assistance in manuscript editing and revision.

Author contribution

Tzu-hung CHENG designed research; Hung-hsing CHAO and Ju-chi LIU performed research; Jia-wei LIN contributed new analytical tools and reagents; Cheng-hsien CHEN analyzed data; Chieh-hsi WU and Tzu-hung CHENG wrote the paper.

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