Original Research

Soluble uric acid increases intracellular calcium through an angiotensin II-dependent mechanism in immortalized human mesangial cells

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Abstract

Hyperuricemia is associated with increases in cardiovascular risk and renal disease. Mesangial cells regulate glomerular filtration rates through the release of hormones and vasoactive substances. This study evaluates the signaling pathway of uric acid (UA) in immortalized human mesangial cells (ihMCs). To evaluate cell proliferation, ihMCs were exposed to UA (6–10 mg/dL) for 24–144 h. In further experiments, ihMCs were treated with UA (6–10 mg/dL) for 12 and 24 h simultaneously with losartan (10^{-7} mmol/L). Angiotensin II (AII) and endothelin-1 (ET-1) were assessed using the enzyme-linked immunosorbent assay (ELISA) technique. Pre-pro-ET mRNA was evaluated by the real-time PCR technique. It was observed that soluble UA (8 and 10 mg/dL) stimulated cellular proliferation. UA (10 mg/dL) for 12 h significantly increased AII protein synthesis and ET-1 expression and protein production was increased after 24 h. Furthermore, UA increased $[Ca^{2+}]_i$, and this effect was significantly blocked when ihMCs were preincubated with losartan. Our results suggested that UA triggers reactions including AII and ET-1 production in mesangial cells. In addition, UA can potentially affect glomerular function due to UA-induced proliferation and contraction of mesangial cells. The latter mechanism could be related to the long-term effects of UA on renal function and chronic kidney disease.

Keywords: uric acid, angiotensin II, intracellular calcium, endothelin, mesangial cells

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Introduction

Epidemiological studies have identified a strong association between increases in serum uric acid (UA) concentration and cardiovascular risks, such as hypertension and coronary artery disease. It is estimated that approximately 25% of hypertensive patients have hyperuricemia,[1,2] and this incidence of hypertension is significantly higher than that in the normal population. Hyperuricemia can induce, aggravate and predict renal injury, as well as result in the development of hypertension.[3] It has been demonstrated that hyperuricemia induces renocardiovascular damage, resulting in hypertension and renal injury due to vascular endothelial dysfunction.[4] There are several proposed mechanisms for the association between hypertension and hyperuricemia, including the reduction of renal blood flow and subsequent increase of urate reabsorption.[5] In addition, UA can cause local tissue ischemia, which inhibits renal urate secretions mediated by the presence of lactate,[6] alcoholism,[7] diuretic consumption, lead intoxication[3] or insulin resistance.[6,8]

Clinical studies have confirmed that the renin–angiotensin system is strongly related to elevated serum UA levels in hypertensive patients.[9] Both UA and angiotensin II (AII) stimulate vascular muscle cell proliferation and hypertrophy,[7,10] which are key steps in the atherosclerotic process. *In vitro* data demonstrate that UA inhibits the release of nitric oxide induced by vascular endothelial growth factor in cultured endothelial cells. UA could also increase blood platelet adhesion and stimulate the proliferation of vascular smooth muscle cells.[7,11] Thus, these observations suggest that hyperuricemia can induce endothelial dysfunction.

Mesangial cells are perivascular pericytes located within the central portion of the glomerular tuft between capillary loops. The main function of mesangial cells is to regulate the ultra filtration coefficient (Kf) via mesangial cell contraction or the release of vasoactive hormones. Mesangial cells play

a major role in glomerular contraction, filtration surface area and Kf regulation through the release of hormones such as AII. These cells express all components of renin–angiotensin system.[12] Endothelins (ETs) are also powerful vasoconstrictor peptides, and they act as modulators of vasomotor tone, cell proliferation and hormone production. Mesangial cells are the main site of ET production in the kidney. Activation of ET receptor in renal cells leads to a complex signaling cascade resulting in stimulation of mesangial cell hypertrophy, proliferation, contraction and extracellular matrix accumulation.[13,14] These hemodynamic renal alterations are associated with the onset and progression of renal disease.

At this time, no studies have been performed to evaluate the impact of UA on mesangial cells. Thus, the present study was carried out to examine the effects of UA on cell activity and the hormonal impact mediated by UA on mesangial cells.

Materials and methods

Cell culture

Immortalized human mesangial cells (ihMCs), kindly provided by Bernhard Banas (Nephrology Center, Medical Policlinic, Ludwig-Maximilians, University Munich, Germany), were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemicals, St Louis, MO, USA) with the addition of 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 24 mmol/L of $NaHCO_3$, 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 10,000 U/L of penicillin/streptomycin. The culture was incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide.

At a semi-confluent stage, the cells were detached from plastic flasks by incubation with trypsin (0.5%; Cultilab, Campinas-SP, Brazil). Cells were subsequently centrifuged, resuspended in DMEM and subcultured in 22 cm^2 plastic culture flasks for further experimental procedures.

Preparation of UA

UA was sterilized in a steam autoclave and subsequently added into the culture medium without FBS. The UA crystals were suspended in DMEM with 1% of FBS, incubated at 37°C and sonicated for 15 min to increase UA solubility.

Exposure of ihMCs to UA

Prior to the experiments, cells were transferred to a plastic plate (1×10^6 cells/plate) and maintained under culture conditions for three days. At confluence, ihMCs were exposed for 12 or 24 h to either DMEM (without FBS, for the control) or DMEM containing UA (6–10 mg/dL).

Proliferation

The ihMCs (1×10^3 cells) were seeded into 24-well plates and treated, after 24 h, with DMEM and 10% FBS (control situation) or UA (6–10 mg/dL). Cell cultures were counted in a Neubauer chamber with Trypan blue over a 24–144 h period.

RNA isolation, reverse transcription and quantitative real-time PCR

Total RNA was purified from ihMCs with a phenol and guanidine isothiocyanate–cesium chloride method, using the appropriate kit (Trizol; Life Technologies, Rockville, MD, USA). Two micrograms of total RNA were treated with DNase (RQ1 RNase-free DNase; Promega, Madison, WI, USA) to prevent genomic DNA contamination. The RNA pellet was resuspended in RNase-free water. RNA was reverse transcribed into cDNA by the addition of a mixture containing 0.5 mg/mL oligo-d(T), 10 mmol/L dithiothreitol, 0.5 mmol/L dNTPs (Pharmacia Biotech, Uppsala, Sweden) and 200 U of reverse transcriptase enzyme (SuperScript RT, Gibco-BRL, Gaithersburg, MD, USA).

Real-time amplification was performed using a 7500 Real-Time Sequence Detection System (SDS; ABI Prism 7500; Applied Biosystems, Foster City, CA, USA). Real-time PCR product accumulation was monitored using an intercalating dye (SYBR Green I; Molecular Probes Inc, Eugene, OR, USA), which exhibits increased fluorescence upon binding to double-stranded DNA.

Relative gene expression was calculated using conditions at the early stages of PCR. At this point, amplification is logarithmic and can thus be correlated to the initial copy number of gene transcripts. Reactions were cycled 40 times under the conditions previously determined by conventional PCR. At the end of the PCR reaction, the temperature was increased from 60°C to 95°C at a rate of 2°C/min. During this time fluorescence was measured every 15 s to construct a melting curve. A non-templated control was run with each assay.

PCR was performed with primers selective for ET, ET-converting enzyme (ECE) and β-actin. The primer sequences were pre-pro-ET sense (5′-GAGAAACCCACTC CCAGTCC-3′) and antisense (5′-GATGTCCAGGTGGCA GAAGT-3′); β-actin sense (5′-CCTCTATGCCAACACAGT GC-3′) and antisense (5′-ACATCTGCTGGAAGGTGGAC-3′); and ECE sense (5′-AATGGAGACCACACTCATGGCTCA-3′) and antisense (5′-TTTCCTTAAAGCTCTGGAGCGGGT-3′). The results from these experiments, per group, are reported as the relative expression normalized with the β-actin housekeeping gene. This gene was used as an endogenous control and is expressed here as a percentage of the treatment control group (CN).[15]

Real-time data analysis

The cycle threshold (C_t) values were subtracted from the C_t value for each gene to yield ΔC_t values. These values were used to carry out statistical comparisons. For graphical representations, the fold variation was determined using the $2^{-(\Delta\Delta C_t)}$ method according to published protocols and manufacturer recommendations. Fold variations were calculated by determining the difference in ΔC_t values between chosen reference and test samples ($\Delta\Delta C_t$ value), with subsequent application of the $2^{-(\Delta\Delta C_t)}$ formula.[16]

Intracellular calcium measurements

Confluent ihMCs (at approximately 10^6 cells/mL) were resuspended in 2.5 mL of Tyrode buffer (137 mmol/L

NaCl, 2.68 mmol/L KCl, 1.36 mmol/L $CaCl_2$ $2H_2O$, 0.49 mmol/L $MgCl_2$ $6H_2O$, 12 mmol/L $NaHCO_3$, 0.36 NaH_2PO_4 and 5.5 mmol/L D-glucose), containing 0.2% bovine serum albumin, and left to stand in a CO_2 incubator at 37°C for 20 min. The suspension was then centrifuged at 1500 rpm for four minutes. Subsequently, the supernatant was aspirated and the pellet resuspended in 2.5 mL of albumin-free Tyrode. This solution was transferred into the quartz cuvette of a SPEX fluorimeter (AR CM System, Vernon, NJ, USA) for autofluorescence determination. Measurements were made at 340- and 380-nm excitation wavelengths, with emission at 505 nm. The autofluorescence ratio was less than 10%. The ihMCs were then incubated with 0.01% Pluronic 127 detergent and 2 μmol/L fura-2/AM. Afterwards, the cuvette was transferred onto a Perkin-Elmer spectrofluorimeter (LS 5B, Buckinghamshire, UK) to record the fluorescence spectrum of the indicator (in the excitation range from 300 to 400 nm, with emission at 520 nm). In the esterified form, the maximum fluorescence of fura-2/AM was observed at 390 nm. As the indicator was transformed into the acid form (fura-2), the fluorescence peak shifted to 350 nm in wavelength within a mean period of three hours, thus indicating the maximum amount of indicator incorporated into the cell suspension. Cells were washed with 15 mL of Tyrode and centrifuged at 1500 rpm for four minutes. The supernatant was discarded and the pellet was resuspended in 2.5 mL of Tyrode. This solution was transferred to a SPEX fluorimeter programmed for excitation at two wavelengths (340 and 380 nm), with emission at 505 nm, under constant stirring at 37°C. The first reading of this phase corresponded to basal intracellular calcium. The cells were then stimulated with UA (8–10 mg/dL) and AII (10^{-7} mmol/L) in the presence or absence of losartan (10^{-7} mmol/L). At the end of each experiment, 50 μmol/L digitonin, 1 mmol/L manganese and 2 mmol/L ethylene glycol tetraacetic acid were added to the cells. The results are reported as the relative ratio of excitation at wavelengths of 340 and 380 nm (considering the reading for digitonin to be 100%). The calcium concentration was estimated using the formula of Grynkiewicz *et al*.[17]

Enzyme-linked immunosorbent assay

The concentrations of AII and ET-1 were assessed in a cellular culture treated with UA using commercially available, competitive enzyme-linked immunosorbent assays (ELISAs) (Cayman Chemical, Ann Arbor, MI, USA). All assays were performed according to the manufacturer's protocols. The optical density of each sample was determined using an Ultra Microplate Reader (EL808; Bio-Tek Instruments, Winooski, VT, USA) and expressed as picograms per milliliter.

Statistical analysis

Data were expressed as the mean ± SEM (standard error of the mean). Experimental and control groups were compared via the Student's *t*-test. The significance level for a null hypothesis was set at 5% ($P < 0.05$).

Results

Incubation of ihMCs with UA increased cellular proliferation at concentrations of 6, 8 and 10 mg/dL (Table 1 and Figures 1a–c). UA at 6 mg/dL increased cellular proliferation in 5% ($7.13 \times 10^5 \pm 9$ cells versus $6.73 \times 10^5 \pm 12$ cells in the control situation) after 144 h. The incubation of UA at 8 and 10 mg/dL resulted in an increase of cellular proliferation in 17% ($7.90 \times 10^5 \pm 6$ cells versus $6.73 \times 10^5 \pm 12$ cells in the control) and 121% ($8.17 \times 10^5 \pm 9$ cells versus $6.73 \times 10^5 \pm 12$ cells in the control), respectively, after 144 h. The effect of UA on cellular proliferation was directly proportional to its concentration. The greatest increase in cellular proliferation was observed with the highest concentration of UA.

AII synthesis increased when the incubation of ihMCs was performed with 6, 8 and 10 mg/dL of UA (Table 2 and Figure 2). At 6 mg/dL, UA increased AII synthesis in 212% (0.34 ± 0.01 versus 0.16 ± 0.01 ng/mL in the control), while incubation at 8 mg/dL UA resulted in an increase of 244% (0.39 ± 0.03 versus 0.16 ± 0.01 ng/mL in the control). The incubation of UA at 10 mg/dL resulted in an increase in AII synthesis of 181% (0.29 ± 0.007 versus 0.16 ± 0.01 ng/mL in the control) in 12 h. In 24 h there was no significant change in AII synthesis compared with the control situation (data not shown).

UA increased pre-pro-ET mRNA in ihMCs (Table 3 and Figure 3). UA incubation (10 mg/dL) increased pre-pro-ET expression in 344% (4.34 ± 0.78 versus 1.26 ± 0.18 arbitrary units in the control) in 24 h. There was no significant difference in pre–pro-ET expression when ihMCs were exposed to UA for 12 h (data not shown).

Figure 4 and Table 4 show ET-1 protein production in ihMCs exposed to UA. Interestingly, UA (10 mg/dL) or losartan alone did not significantly change the ET-1 protein production after 24 h of exposure. Nevertheless,

Table 1 Effects of UA on mesangial cell proliferation

1×10^3 cells	24 h	48 h	72 h	96 h	120 h	144 h
CN	116 ± 07	202 ± 18	308 ± 08	459 ± 22	553 ± 08	673 ± 12
6 mg/dL	139 ± 5	227 ± 7	372 ± 31	496 ± 21	630 ± 23*	713 ± 9
8 mg/dL	132 ± 4	224 ± 6	342 ± 13	558 ± 28	737 ± 13*	790 ± 6*
10 mg/dL	103 ± 22	208 ± 19	397 ± 13	619 ± 22*	772 ± 14*	817 ± 9*

UA, uric acid; CN, control

*$P < 0.05$ versus CN

Data were expressed as the mean ± standard error of the mean. Experimental and control groups were compared via the Student's *t*-test. The significance level for a null hypothesis was set at 5% ($P < 0.05$)

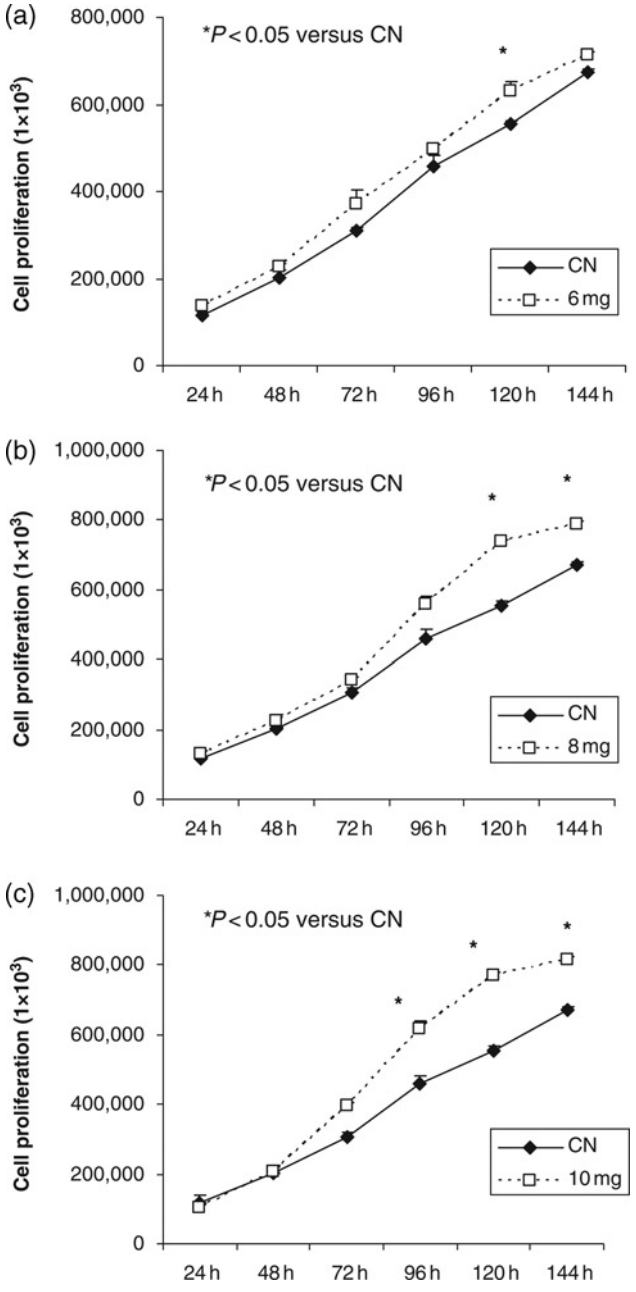

Figure 1 Effect of UA treatment upon the proliferation of ihMCs. Cells were seeded into 24-well plates and, after 24 h, were exposed to DMEM with 10% FBS containing (a–c) UA (soluble; 6, 8 or 10 mg/dL). Cells were detached with trypsin, centrifuged and incubated with trypan blue. White cells were considered viable and counted. Blue cells were considered non-viable. Data are expressed in cell numbers. UA, uric acid; ihMC, immortalized human mesangial cells; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum

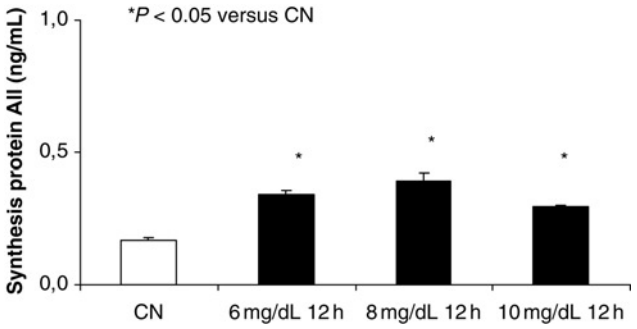

Figure 2 Effect of UA treatment on AII production in ihMCs. Briefly, semi-confluent ihMCs were treated with UA (soluble; 6, 8 or 10 mg/dL for 12 h). AII concentrations were assessed with commercially available competitive ELISAs and all assays were performed according to the manufacturer's protocols. The results are expressed as nanograms of AII per microliter (mean ± SEM). UA, uric acid; ihMC, immortalized human mesangial cells; AII, angiotensin II; ELISA, enzyme-linked immunosorbent assay; SEM, standard error of the mean

incubation with UA and losartan together significantly decreased the amount of ET-1 protein in ihMCs.

Despite the significant increase in ET mRNA, there was no difference in ET-1 protein concentrations when mesangial cell were exposed to UA. This could be attributed to a reduction in the activity or expression of ECE. Figure 5 shows the effect of UA (6–10 mg/dL) on the expression of ECE. There was no difference in ECE expression induced with 24 h exposure to UA (3.76 ± 1.7 arbitrary units) compared with that of the control (3.35 ± 0.67 arbitrary units) in mesangial cells.

In ihMCs, treatment of ihMCs with UA (6–10 mg/dL) elicited a rapid increase followed by a decrease in $[Ca^{2+}]_i$. The same pattern of $[Ca^{2+}]_i$ fluctuation was observed with AII treatment, as already demonstrated by others,[18,19] but the intracellular calcium concentration was higher when ihMCs were stimulated with AII than it was when cells were stimulated with UA (Figure 6). The effect of UA was concentration dependent, reaching a plateau at 8 mg/dL and decreasing at 10 mg/dL. The increase in $[Ca^{2+}]_i$ was almost completely blocked when ihMCs were exposed to losartan (an antagonist of the AII receptor AT1). As expected, the AT1 antagonist completely blocked the increase in $[Ca^{2+}]_i$ induced by AII (Figure 6).

Figure 7 shows a quantitative analysis of the effect of UA (6–10 mg/dL) and AII (100 nmol/L) on the intracellular calcium concentration in ihMCs. UA (6–10 mg/dL) induced an increase in $[Ca^{2+}]_i$, and the maximum increase in $[Ca^{2+}]_i$ induced by UA was observed with 8 mg/dL ($[Ca^{2+}]_i$ was increased by 174.1 nmol/L or 14.4%).

Table 2 Angiotensin II induced by UA on mesangial cells

	Protein (ng/mL)	CN	6 mg/dL	8 mg/dL	10 mg/dL
UA	AII 12 h	0.16 ± 0.01	0.34 ± 0.01*	0.39 ± 0.03*	0.29 ± 0.007*

UA, uric acid; CN, control
*$P < 0.05$ versus CN
Data were expressed as the mean ± SEM (standard error of the mean). Experimental and control groups were compared via the Student's *t*-test. The significance level for a null hypothesis was set at 5% ($P < 0.05$)

Table 3 Effects of UA on pre-pro-ET mRNA in mesangial cells

	mRNA	CN	6 mg/dL	8 mg/dL	10 mg/dL
UA	Pre-pro-ET 24 h	1.26 ± 0.18	2.05 ± 0.42	1.33 ± 0.36	4.34 ± 0.78*

UA, uric acid; CN, control; ET, endothelin
*$P < 0.05$ versus CN
Data were expressed as the mean ± SEM (standard error of the mean). Experimental and control groups were compared via the Student's *t*-test. The significance level for a null hypothesis was set at 5% ($P < 0.05$)

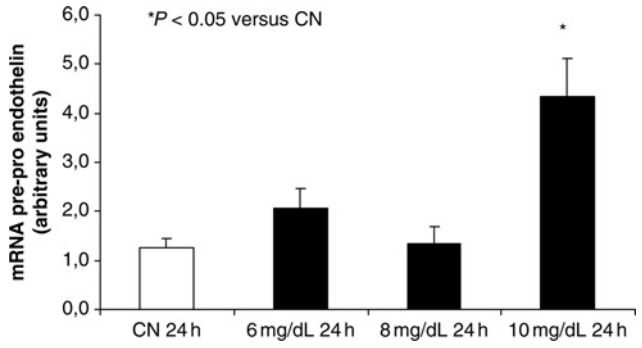

Figure 3 Effect of UA treatment on pre-pro-ET mRNA expression in ihMCs. Briefly, semi-confluent ihMCs were treated with UA (soluble; 6, 8 or 10 mg/dL for 24 h). Pre-pro-ET-1 concentrations were assessed with RT-PCR and all assays were performed as described in Materials and methods. The results are expressed as the mean ± SEM (arbitrary units). UA, uric acid; ihMC, immortalized human mesangial cells; ET-1, endothelin I; RT-PCR, real-time PCR

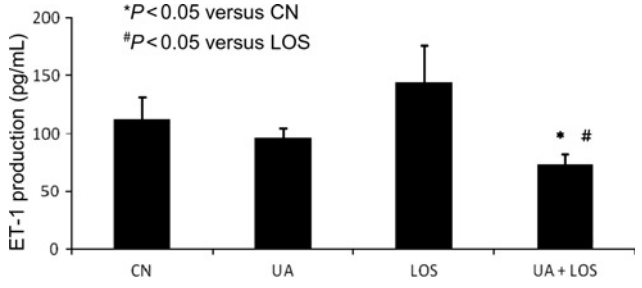

Figure 4 Effect of UA treatment on ET production by ihMCs. Briefly, semi-confluent ihMCs were treated with UA (soluble; 10 mg/dL), losartan (LOS, 10^{-7} mmol/L) or UA and LOS for 24 h. ET-1 concentrations were assessed with commercially available sandwich ELISAs and all assays were performed according to the manufacturer's protocols. The results are expressed as the mean ± SEM (in picograms of ET-1 per microgram of total protein). The data are marked as significantly different from the control (*) or LOS (#). UA, uric acid; ihMC, immortalized human mesangial cells; ET-1, endothelin I; RT-PCR, real-time PCR; SEM, standard error of the mean

Nevertheless, AII increased $[Ca^{2+}]_i$ more than UA ($[Ca^{2+}]_i$ was increased by 312.4 nmol/L or 33.0% with AII versus 14.4% with UA).

Discussion

Hyperuricemia induces renocardiovascular damage resulting in hypertension and glomerular damage, which decreases the ability of the kidneys to eliminate UA, and progressively increases serum UA levels. A vicious cycle of hyperuricemia, hypertension and renal damage is therefore observed. In this complex pathophysiological pathway, endothelial dysfunction plays an important role in the development of hypertension and renal disease.[20] The present study hypothesizes that mesangial cells also participate in the hypertension and renal damage observed in hyperuricemia.

Table 4 Endothelin levels in mesangial cells exposed to UA and/or LOS

Protein (pg/mL)	CN	UA (10 mg/dL)	LOS 10^{-3}(mmol/L)	UA + LOS
	112.3 ± 18.6	96.6 ± 10.6	144.4 ± 31.7	78.8 ± 11.0*†

UA, uric acid; CN, control; LOS, losartan
*$P < 0.05$ versus CN
†$P < 0.05$ versus LOS
Data were expressed as the mean ± SEM (standard error of the mean). Experimental and control groups were compared via the Student's *t*-test. The significance level for a null hypothesis was set at 5% ($P < 0.05$)

Evidence for the pathogenic role of UA has been building. Recent studies have identified serum UA as a predictor of hypertension,[20,21] including observations from the Sundstrom *et al.*[22] and Bogalusa *et al.*[23] Heart Studies. UA has also been found to predict insulin resistance[15,24,25] and obesity.[26] In addition, UA is elevated in a high percentage of individuals having new onset hypertension, particularly in adolescents.[27] Preliminary studies have found that lowering UA can reduce blood pressure.[28] In addition, recent experimental studies suggest that UA may have a causal role in hypertension,[29] salt-sensitivity,[30] arteriosclerosis[31] and renal disease.[32,33]

Studies have focused on the mechanisms of how UA causes these conditions. UA is uptaken by an organic anion transport system and can activate vascular smooth muscle cells.[32] This process includes the activation of specific mitogen-activated protein kinase (MAP) kinases[30,33] and nuclear transcription factors,[30] with the stimulation of cyclooxygenase-2 (COX-2),[33] platelet-derived growth factor (PDGF) A and C chains,[7,30] PDGF α receptors[30] and various inflammatory mediators, including C-reactive protein and monocyte chemoattractant protein-1 (MCP-1).[18] Soluble UA also activates primary human endothelial cells, where it blocks nitric oxide release and inhibits endothelial proliferation.[11] The combination of a proliferative effect on vascular smooth muscle cells (VSMCs) and an inhibitory effect on endothelial cells could explain why UA is particularly effective for inducing small vessel arteriolar disease in experimental models. The classical explanation for UA stimulation of VSMC proliferation after uptake is that it increases PDGF A and C chain expression, local thromboxane production and COX-2 stimulation.[34] These are all factors that induce specific MAP kinases.[31] Nakagawa *et al.*[32] showed in renal biopsies that hyperuricemic rats had a 30% increase in glomerular tuft area. Our experimental model also demonstrates that UA induces the proliferation of mesangial cells and that this effect is associated with mesangial contraction induced by UA, which could contribute to a decrease in Kf and glomerular filtration.

Corry *et al.*[35] showed that the proliferative effects of UA on VSMCs indicate its contribution to vascular growth. A recent clinical study has demonstrated indirectly that UA stimulates RAS in the renal circulatory system.[36] These observations from Corry *et al.*[35] were significantly reduced by agents that block the RAS cascade, including angiotensin-converting enzyme inhibitors and AT1 receptor blockers. The study observed that PD 98059, an AT2 receptor blocker, inhibits the increased expression of angiotensinogen mRNA in UA-exposed VSMC. This indicates that MAP kinase also mediates the stimulation of vascular RAS, as well as MCP-1.[37] We demonstrated that UA stimulates AII production and increases $[Ca^{2+}]_i$ in mesangial cells.

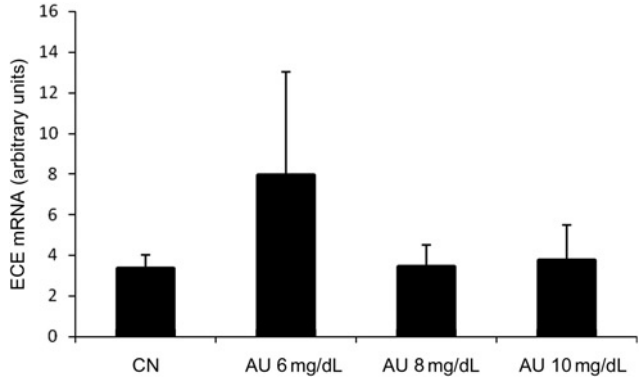

Figure 5 Effect of UA treatment on ECE expression in ihMCs. Briefly, semi-confluent ihMCs were treated with UA (soluble; 6, 8 or 10 mg/dL) for 24 h. ECE concentrations were assessed with RT-PCR and all assays were performed as described in Materials and methods. The results are expressed as the mean ± SEM (using arbitrary units). UA, uric acid; ECE, endothelin-converting enzyme; ihMC, immortalized human mesangial cells; RT-PCR, real-time PCR; SEM, standard error of the mean

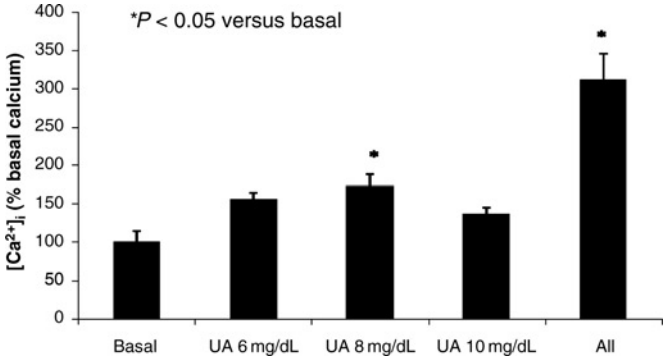

Figure 7 Intracellular calcium concentration in ihMCs treated with UA or AII. Briefly, semi-confluent ihMC cells were detached with trypsin and the level of intracellular calcium was determined by fluorescence in the presence of UA (6–8 mg/dL) and AII (10^{-7} mmol/L). The results were expressed as the mean ± SEM (relative to basal intracellular calcium normalized to 100%). ihMC, immortalized human mesangial cells; UA, uric acid; AII, angiotensin II; SEM, standard error of the mean

These increases were almost completely inhibited by the AT1 antagonist losartan.

An *et al.*[24] demonstrated for the first time that adventitial fibroblasts, after being stimulated by external factors, expressed and synthesized ET. The expression of pre-pro-ET mRNA increased within the first 30 min of incubation with AII (100 nmol/L) and continued to increase further, reaching a maximum at the 1.5 h mark. Thereafter, pre-pro-ET mRNA expression decreased progressively. After 12 h, expression of pre-pro-ET mRNA was similar to that observed in the control group. They found that the increase of AII concentration was responsible, in a dose-dependent manner, for the increase in pre-pro-ET mRNA. In addition, ET-1 protein synthesis was also observed. Thus, they have provided the evidence that AII stimulates ET synthesis in response to AII stimulation in adventitial fibroblasts.

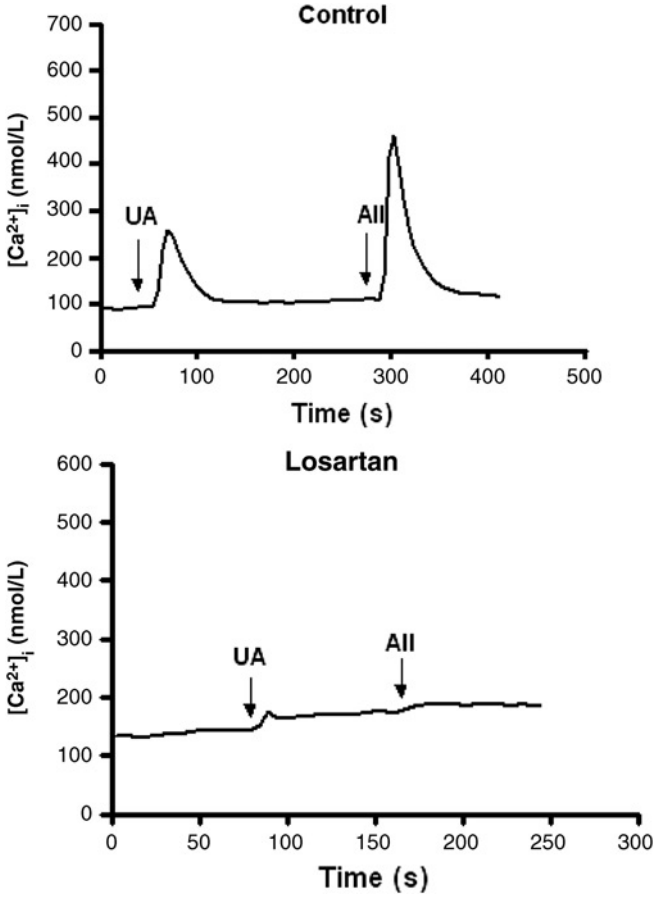

Figure 6 Intracellular calcium recordings of ihMCs. Representative $[Ca^{2+}]_i$ recordings obtained with ihMCs in response to successive applications of increasing concentrations of UA (8 mg/dL) and AII (10^{-7} mmol/L) in the absence (control) or in the presence of losartan (10^{-7} mmol/L) (losartan). Each application lasted three minutes. ihMC, immortalized human mesangial cells; UA, uric acid; AII, angiotensin II

ET-1 is a potent vasoconstrictor agent produced by endothelial cells that induces proliferation and the production of extracellular matrix in mesangial cells, and both of these events are important for the pathogenesis of glomerular sclerosis.[38,39] In our experimental model, hyperuricemia-induced ET dysfunction was observed with increased pre-pro-ET mRNA expression. Interestingly, in spite of this increase, there was no difference in ET-1 protein production, suggesting an impairment of the translation mechanism in the ihMCs, increase in ET-1 degradation or inhibition of ECE. This possibility was also observed by López-Ongil *et al.*[40] in human umbilical vein endothelial cells; however, there was no difference in ECE expression after treatment with UA in our experimental conditions.

When the AII receptor AT1 was blocked, there was a significant decrease in ET-1 production induced by UA in ihMCs, although no difference was observed after exposure of ihMCs to UA or losartan alone. It has already been shown that AII stimulates ET-1 synthesis.[41] We have shown indirect evidence that (under our experimental conditions) UA stimulates ET-1 production by increasing AII production.

We also demonstrated that the increase in the concentration of intracellular calcium was mediated by AII. When ihMCs were exposed to the AT1 receptor antagonist, the increase in intracellular calcium was inhibited. The results from the present study are the first direct evidence that UA induces $[Ca^{2+}]_i$ increase through AII production

in ihMCs. These findings constitute novel evidence indicating that the proliferative effect of UA associated with an increase in intracellular calcium could be responsible, at least in part, for the glomerulosclerosis observed in hyperuricemia. Additionally, RAS blockers (such as ACE inhibitors and antagonists of AT1 receptors) could be potential mechanisms to attenuate the effects of hyeruricemia in the glomerulus.

In conclusion, this study has shown that UA is not just a marker of other coronary or metabolic disease risk factors, but that UA is a stimulator of the renin–angiotensin system and of ET-1 in mesangial cells. This leads to the production of AII and ET-1, causing increases in glomerular cell proliferation as well as the $[Ca^{2+}]_i$. These effects could be responsible, at least in part, for the glomerulosclerosis and renal damage observed in long-term hyperuricemia.

The Research Ethics Committee of University Federal of São Paulo/Sao Paulo Hospital examined and approved this research project (#0227/05).

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. GA, EM, EP, JAB, FB and NS conducted the experiments and GA and FB wrote the manuscript. All the authors declare that they have no competing interests.

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REFERENCES

1 Tykarski A. Evaluation of renal handling of uric acid in essential hypertension: hyperuricemia related to decreased urate secretion. *Nephron* 1991;**59**:364–8
2 Selby JV, Friedman GD, Quesenberry CPJ. Precursors of essential hypertension pulmonary function, heart rate, uric acid, serum cholesterol, and other serum chemistries. *Am J Epidemiol* 1990;**131**:1017–27
3 Sánchez-Lozada LG, Tapia E, Avila-Casado C, Soto V, Franco M, Santamaría J, Nakagawa T, Rodríguez-Iturbe B, Johnson RJ, Herrera-Acosta. Mild hyperuricemia induces glomerular hypertension in normal rats. *Am J Physiol Renal Physiol* 2002;**283**:1105–10
4 Kang DH, Nakagawa T, Feng L, Watanabe S, Han L, Mazzali M, Truong L, Harris R, Johnson RJ. A role for uric acid in the progression of renal disease. *J Am Soc Nephrol* 2002;**13**:2888–97
5 Messerli FH, Frohlich ED, Dreslinski GR, Suarez DH, Aristimuno GG. Serum uric acid in essential hypertension: an indicator of renal vascular involvement. *Arch Intern Med* 1980;**93**:817–21
6 Quiñones GA, Natali A, Baldi S, Frascerra S, Sanna G, Ciociaro D, Ferrannini E. Effect of insulin on uric acid excretion in humans. *Am J Physiol* 1995;**268**:1–5
7 Rao GN, Corson MA, Berk BC. Uric acid stimulates vascular smooth muscle cell proliferation by increasing platelet derived growth factor A-chain expression. *J Biol Chem* 1991;**266**:8604–8
8 Tsunoda S, Kamide K, Minami J, Kawano Y. Decreases in serum uric acid by amelioration of insulin resistance in overweight hypertensive patients: effect of a low-energy diet and an insulin-sensitizing agent. *Am J Hypertens* 2002;**15**:697–701
9 Saito I, Saruta T, Kondo K, Nakamura R, Oguro T, Yamagami K, Ozawa Y, Kato E. Serum uric acid and the renin–angiotensin system in hypertension. *J Am Geriatr Soc* 1978;**26**:241–7
10 Feig DI, Nakagawa T, Karumanchi SA, Oliver WJ, Kang DH, Finch J, Johnson RJ. Uric acid, nephron number, and the pathogenesis of essential hypertension. *Kidney Int* 2004;**66**:281–7
11 Khosla UM, Zharikov S, Finch JL, Nakagawa T, Roncal C, Mu W, Krotova K, Block ER, Prabhakar S, Johnson RJ. Hyperuricemia induces endothelial dysfunction. *Kidney Int* 2005;**67**:1739–42
12 Ardaillou R, Sraer J, Chansel D, Ardaillou N, Sraer JD. The effects of angiotensin II on isolated glomeruli and cultured glomerular cells. *Kidney Int* 1987;**31**:574–80
13 Kohno M, Horio T, Ikeda M, Yokokawa K, Fukui T, Yasunari K, Kurihara N, Takeda T. Angiotensin II stimulates endothelin I secretion in cultured rat mesangial cells. *Kidney Int* 1992;**42**:860–6
14 Cosenzi A, Bernobich E, Trevisan R, Milutinovic N, Borri A, Bellini G. Nephroprotective effect of bosentan in diabetic rats. *J Cardiovasc Pharmacol* 2003;**42**:752–6
15 Selby JV, Friedman GD, Quesenberry CPJ. Precursors of essential hypertension: pulmonary function, heart rate, uric acid, serum cholesterol, and other serum chemistries. *Am J Epidemiol* 1990;**131**:1017–27
16 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods* 2001; **25**:402
17 Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 1985;**260**:3440–50
18 Hadad SJ, Ferreira AT, Oshiro ME, Neri R, Schor N. Alteration of cytosolic calcium induced by angiotensin II and norepinephrine in mesangial cells from diabetic rats. *Kidney Int* 1997;**51**:87–93
19 Ferreira VM, Gomes TS, Reis LA, Ferreira AT, Razvickas CV, Schor N, Boim MA. Receptor-induced dilatation in the systemic and intrarenal adaptation to pregnancy in rats. *PLoS One* 2009;**3**:4845
20 Kanellis J, Watanabe S, Li JH, Kang DH, Li P, Nakagawa T, Wamsley A, Sheikh-Hamad D, Lan HY, Feng L, Johnson RJ. Uric acid stimulates monocyte chemoattractant protein-1 production in vascular smooth muscle cells via mitogen-activated protein kinase and cyclooxygenase-2. *Hypertension* 2003;**41**:1287–93
21 Johnson RJ, Feig DI, Herrera-Acosta J, Kang DH. Resurrection of uric acid as a causal risk factor for essential hypertension. *Hypertension* 2005;**45**:18–20
22 Sundstrom J, Sullivan L, D'Agostino RB, Levy D, Kannel WB, Vasan RS. Relations of serum uric acid to longitudinal blood pressure tracking and hypertension incidence. *Hypertension* 2005;**45**:28–33
23 Alper AB, Chen W, Yau L, Srinivasan SR, Berenson GS, Hamm LL. Childhood uric acid predicts adult blood pressure: The Bogalusa Study. *Hypertension* 2005;**45**:34–8
24 An SJ, Boyd R, Wang Y, Qiu X, Wang HD. Endothelin-1 expression in vascular adventitial fibroblasts. *Am J Physiol Heart Circ Physiol* 2006;**290**:700–8
25 Nakanishi N, Okamoto M, Yoshida H, Matsuo Y, Suzuki K, Tatara K. Serum uric acid and risk for development of hypertension and impaired fasting glucose or type II diabetes in Japanese male Office workers. *Eur J Epidemiol* 2003;**18**:523–30
26 Masuo K, Kawaguchi H, Mikami H, Ogihara T, Tuck ML. Serum uric acid and plasma norepinephine concentrations predict subsequent weight gain and blood pressure elevation. *Hypertension* 2003;**42**:474–80
27 Feig DI, Johnson RJ. Hyperuricemia in childhood essential hypertension. *Hypertension* 2003;**42**:247–52
28 Feig DI, Nakagawa T, Karumanchi SA, Oliver WJ, Kang DH, Finch J, Johnson RJ. Uric acid, nephron number, and the pathogenesis of essential hypertension. *Kidney Int* 2004;**66**:281–7
29 Mazzali M, Hughes J, Kim YG, Jefferson JA, Kang DH, Gordon KL, Lan HY, Kivlighns S, Johnson RJ. Elevated uric acid increases blood pressure in the rat by a novel crystal-independent mechanism. *Hypertension* 2001;**38**:1101–6
30 Watanabe S, Kang DH, Feng L, Nakagawa T, Kanellis J, Lan H, Mazzali M, Johnson RJ. Uric acid, hominoid evolution and the pathogenesis salt-sensitivity. *Hypertension* 2002;**40**:355–60

31 Mazzali M, Kanellis J, Han L, Feng L, Xia YY, Chen O, Kang DH, Gordon KL, Watanabe S, Nakagawa T, Lan HY, Johnson RJ. Hyperuricemia induces a primary arteriolopathy in rats by a blood pressure-independent mechanism. *Am J Physiol Renal Physiol* 2002;**282**:991–7
32 Nakagawa T, Mazzali M, Kang DH, Kanellis J, Watanabe S, Sanchez-Lozada LG, Rodriguez-Iturbe B, Herrera-Acosta J, Johnson RJ. Hyperuricemia causes glomerular hypertrophy in rat. *Am J Nephrol* 2003;**23**:2–7
33 Kang DH, Nakagawa T, Feng L, Watanabe S, Han L, Mazzali M, Truong L, Harris R, Johnson RJ. A role for uric acid in the progression of renal disease. *J Am Soc Nephrol* 2002;**13**:2888–97
34 Kang DH, Han L, Ouyang X, Kahn AM, Kanellis J, Li P, Feng L, Nakagawa T, Watanabe S, Hosoyamada M, Endou M, Lipkowitz M, Abramson R, Mu W, Johnson RJ. Uric acid causes vascular smooth muscle cell proliferation by entering cells via a functional urate transporter. *Am J Nephrol* 2005;**25**:425–33
35 Corry DB, Eslami P, Yamamoto K, Nyby MD, Makino H, Tuck ML. Uric acid stimulates vascular smooth muscle cell proliferation and oxidative stress via the vascular rennin–angiotensin system. *J Hypertens* 2008;**26**:269–75
36 Perlstein TS, Gumieniak O, Hopkins PN, Murphey LJ, Brown NJ, Williams GH, Hollenberg NK, Fisher ND. Uric acid and the state of the intrarenal renin–angiotensin system in humans. *Kidney Int* 2004;**66**: 1465–70
37 Long CL, Qin XC, Pan ZY, Chen K, Zhang YF, Cui WY, Liu GS, Wang H. Activation of ATP-sensitive potassium channels protects vascular endothelial cells from hypertension and renal injury induced by hyperuricemia. *J Hypertens* 2008;**26**:2326–38
38 Tuck ML, Bounoua F, Eslami P, Nyby MD, Eggena P, Corry DB. Insulin stimulates endogenous angiotensin II production via a mitogen-activated protein kinase pathway in vascular smooth muscle cells. *J Hypertens* 2004;**22**:1779–85
39 Marsen TA, Schramek H, Dunn MJ. Renal action of endothelin: linking cellular signaling pathways to kidney disease. *Kidney Int* 1994;**45**:336–44
40 López-Ongil S, Díez-Marqués ML, Griera M, Rodríguez-Puyol M, Rodríguez-Puyol D. Crosstalk between mesangial and endothelial cells: angiotensin II down-regulates endothelin-converting enzyme 1. *Cell Physiol Biochem* 2005;**1–4**:135–44
41 Lee JJ, Shin SJ, Chiu YW, Chen HC. Endothelin 1 antisense oligonucleotide suppresses the proliferation of glomerular mesangial cells stimulated with angiotensin II. *Kaohsiung J Med* 2007;**23**:170–5

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