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ORIGINAL PAPER

Pro-inflammatory and oxidative effects of noncrystalline uric acid in human mesangial cells: contribution to hyperuricemic glomerular damage

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Abstract Hyperuricemia is associated with cardiovascular and renal diseases, as glomerulosclerosis. Noncrystalline uric acid induces deleterious effects on endothelial and vascular smooth muscle cells. In the present study, we analyzed the damage induced by UA on human mesangial cells (HMC), the potential mechanism involved in this injury, and its consequences during infection. HMC were exposed to noncrystalline UA (8 mg/dl) and/or lipopolysaccharide (LPS, 100 μg/ml) for 24 h. In the experiments of cellular viability, HMC were exposed to 8–50 mg/dl of UA. Necrosis was assessed by acridine orange and ethidium bromide. Reactive oxygen species (ROS) were analyzed by 2′,7′-dichlorofluorescein. Prostaglandin E2 (PGE2) was evaluated by ELISA. Cyclooxygenase 2 (COX-2) expression was assessed by real-time PCR. UA induced necrosis only at supraphysiological concentrations. Nevertheless, it significantly increased ROS production at 8 mg/dl. LPS increased necrosis and ROS production. Interestingly, the association between UA and LPS decreased ROS and necrosis. UA associated or not with LPS induced COX-2 expression and PGE2 increases in HMC. Results suggest that UA has pro- and anti-oxidant effects in HMC. During infections, it acts like scavenger increasing cellular viability, but alone it can induce ROS production and cellular death in higher concentrations. Additionally, UA has direct pro-inflammatory effects inducing COX-2 expression and PGE2 synthesis. It is concluded that elevated concentrations of uric acid potentially contributes to glomerular damage.

Keywords Reactive oxygen species · Noncrystalline uric acid · Cyclooxygenase 2 · Mesangial cells · Lipopolysaccharide

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Introduction

Uric acid is the end product of purine metabolism in mammals. In most mammals excluding man and related primates, UA is broken down to allantoin, a more soluble metabolic product by the enzyme uricase. The consequence is that humans have relative higher levels of serum uric acid, compared to others mammals.

Soluble UA was considered biologically inert or possibly anti-inflammatory, since it may act as an antioxidant. It is the main serum scavenger against oxygen radicals. At physiologic concentrations, UA protects erythrocyte against peroxidative damage that leads to lysis [1].

Controversially, elevated UA has been associated with increased risk of hypertension [2] and renal disease [3]. Johnson et al. have demonstrated that hyperuricemia predicts cardiovascular events in the general population, the hypertensive population, and patients with pre-existing cardiovascular disease. Furthermore, hyperuricemia predicts the development of future hypertension [4].

Serum UA could be considered an injurious stimulus to the arterial vessel walls and capillaries, which may contribute to the dysfunction of endothelial, arterial, and capillary vessel walls by remodeling through oxidative stress [5, 6]. Noncrystalline UA stimulates rat vascular smooth muscle cell proliferation with the activation of mitogen-activated protein kinases (MAPK), growth factors

(PDGF), chemokines (monocyte chemoattractant protein-1), and inflammatory enzymes cyclooxygenase 2 (COX-2) [7–9]. Soluble UA also activates primary human vascular endothelial cells, blocks NO release, inhibits endothelial proliferation, and stimulates C-reactive protein production [7, 10].

Uric acid is freely filtered by the glomerulus, and is reabsorbed in the early renal proximal convoluted tubule via a UA transporter. It is followed by secretion and postsecretory reabsorption. UA could be retained in renal proximal tubule epithelial cells, causing renal tubular dysfunction through DNA synthesis inhibition. At least two mechanisms are described in the inhibition of proliferation induced by UA in proximal tubular cells: NF-kB and protein kinase C, MAPK, and cPLA2 [11]. Additionally, serum UA contributes to the metabolic syndrome, term that defines people who are at greatest risk of cardiovascular events due to the common pathophysiologic basis (visceral obesity, atherogenic dyslipidemia, hypertension, and insulin resistance).

Serum UA is higher in subjects with abnormal triglyceride, low-density lipoprotein cholesterol, and blood pressure. Treatment of hyperuricemia could minimize the development of metabolic syndrome [12].

The UA is also a frequent component of urinary stones. The lesion observed in gouty nephropathy consists of glomerulosclerosis, interstitial fibrosis, and renal arteriolosclerosis. This often occurs with interstitial urate crystal deposition [13, 14]. It can be a contributing factor in the formation of calcium oxalate stones themselves.

Additionally, there is an association between nephrolithiasis and infections. It seems that urinary infections are risk factors to stones development [15].

The goal of the present study was to analyze the effect of noncrystalline UA in human mesangial cells, the potential mechanism involved in this effect, and the consequences of its accumulation during infection.

We found that UA has pro- and anti-oxidant effects in HMC. During infection, it acts like scavenger increasing cellular viability, but alone it can induce ROS production and cellular death in higher concentrations. Additionally, UA has direct pro-inflammatory effects inducing cyclooxygenase expression and PGE2 synthesis that potentially contribute to glomerular sclerosis observed during hyperuricemia.

Materials and methods

Cell culture

All experiments were performed with immortalized HMC, kindly donated by Dr. Bernhard Banas (Nephrological Center, Medical Policlinic, Ludwig-Maximilians University, Munich, Germany) [16]. Cells were grown in Dulbeco's Modified Eagle Medium (DMEM, Sigma Chemicals) with the addition of 5% of fetal bovine serum (FBS, Gibco), 24 mM of NaHCO3, 10 mM of HEPES, and 10,000 U/L of penicillin/streptomycin. The culture was incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide.

At semi-confluence stage, cells were detached from plastic flasks utilizing trypsin (Cultilab) (0.5%), centrifuged, resuspended in DMEM, and subcultured in 25 cm^2 culture plastic flasks for the experimental procedures.

Preparation of uric acid

The UA (Merck EGA) was weighed, ethanol solution (100 mg/dl) was added, and the solution was continuously stirred for 24 h. UA crystals were dried, sterilized in ethylene oxide or steam autoclave, and culture medium with 1% of FBS was added (100 ml). The suspension was sonicated for 15 min, at 37°C, to solubilize the crystals and filtered (22 μm pore size). After filtration, the concentration of uric acid was quantified and, when necessary, was increased to correct it and filtered again.

Exposure of human mesangial cells to UA

Prior to the experiments, cells were transferred to a 6-well plate (1×10^6 cells/well) or culture flasks and maintained under the culture conditions for 3 days. At confluence, HMC were exposed for 24 h to either DMEM (1% FBS, control), DMEM containing noncrystalline UA (0.8–50 mg/dl), LPS (100 μg/ml) or the association between LPS and UA.

Cellular viability experiments

Necrotic and apoptotic cells were evaluated, respectively, by acrydine orange/ethidium bromide dyes. HMC suspensions were centrifuged, resuspended in PBS, and incubated with acrydine orange/ethidium bromide solution for 5–15 min. Cells were than observed under light microscope. Ethidium bromide stains all cells in green, while acrydine orange is excluded from viable cells. At least 100 cells per culture were counted, and the results were expressed as percentage of necrotic cells.

Hydrogen peroxide determination

Hydrogen peroxide production was determined by 2′,7′-dichlorofluorescin (DCFH) (Sigma Chemicals) that undergoes oxidation and becomes fluorescent in hydrogen peroxide presence [17].

Cellular cultures treated with noncrystalline UA (8 md/dl), associated or not with lipopolysaccharide (100 μg/ml) during 24 h, were detached from culture flasks by trypsinization, counted (1×10^6 cells) and resuspended in 800 μL of PBS. A solution of DCFH (200 μL) at final concentration 20 μmol/mL were added to the samples, and incubated with agitation at 37°C for 30 min. The cells were centrifuged, resuspended in EDTA, and carried out for FACS analysis. The results were expressed by geometric media of luminous intensity (MIL).

Lipid peroxidation

Lipid peroxidation was determined by the malondialdehyde (MDA) quantification. The MDA combines with thiobarbituric acid (TBA) forming a red compound, whose concentration was assessed by spectrophotometry with reading at 535 nm [18].

Culture medium and cell homogenate samples of cell cultures were treated with noncrystalline UA (8 mg/dl), lipopolysaccharide (100 μg/ml). Cellular homogenates were obtained by scrapping cells from the flasks with PBS. Samples were added to a solution of 0.375% TBA, 15% trichloroacetic acid, and 0.25 N HCl (Sigma Chemicals), kept in continual agitation, heated in 95°C for 20 min and subsequently cooled to room temperature. The assessment of protein level in the solution was carried out by the Lowry method [19]. Positive control was obtained by incubating the cell samples with H_2O_2 at 1% for 1 h before the assay.

RNA isolation, reverse transcription and quantitative real-time PCR

Total RNA was purified from HMC cells by phenol and guanidine isothiocyanate-cesium chloride method using an appropriate kit (Trizol, Life Technologies, USA). Two micrograms of total RNA were treated with DNase (RQ1 Rnase–Free Dnase, Promega) to prevent genomic DNA contamination. The RNA pellet was resuspended in RNase-free water. Reverse transcribed into cDNA by the addition of a mix containing 0.5 mg/ml oligo d(T), 10 mM DTT, 0.5 mM dNTPs (Pharmacia Biotech), and 200 U of reverse transcriptase enzyme (SuperScript RT, Gibco-BRL).

Real-time amplification was obtained using a GeneAmp 5700 Sequence Detection System SDS (ABI Prism 7700, Applied Biosystems, CA, USA). Real-time PCR product accumulation was monitored using the intercalating dye, SYBR Green I (Molecular Probes Inc., USA), which exhibits a higher fluorescence upon binding of double-stranded DNA.

Relative gene expression was calculated using conditions at the early stages of PCR, when amplification was logarithmic, and thus, could be correlated with the initial copy number of gene transcripts. The reactions were cycled 40 times under the conditions previously determined by conventional PCR. Fluorescence for each cycle was quantitatively analyzed by ABI Prism 7700 SDS (Applied Biosystems, CA, USA). At the end of the PCR, the temperature was increased from 60 to 95°C at a rate of 2°C/min, and fluorescence was measured every 15 s to construct the melting curve. A nontemplate control (NTC) was run with each assay.

PCR was performed with primers selective for cyclooxygenase 2 (COX-2) (Table 1). Results of four experiments per group were reported as relative expression normalized with the β-actin housekeeping gene, used as an endogenous control and expressed as percentage to the control situation.

Real-time data analysis

The cycle threshold (Ct) values were subtracted from the Ct value for each gene (to give ΔCt values). These values were used to carry out statistical comparisons. For graphical representation, the fold variation was then determined using the $2^{-(\Delta\Delta Ct)}$ method according to published protocols [20] and manufacturer's recommendations. Fold variation was calculated by determining the difference in ΔCt values between a chosen reference and test sample (ΔΔCt value), and applying the $2^{-(\Delta\Delta Ct)}$ formula.

PGE2 production

The concentrations of PGE2 were assessed in cellular culture treated with UA alone or associated with LPS through commercially available competitive ELISAs (Cayman Chemical, Michigan, USA). All assays were performed according to the manufacturer's protocols. The

Table 1 Primers sequence and annealing temperature for β-actin and COX-2 genes in human

Gene	Primer	Sequence (5′–3′)	Product (bp)	Annealing temperature (°C)
COX-2 human	5′	AAG TCC CTG AGC ATC TAC GGT TT GTT GTG TTC CCT CAG CCA GAT T	98	55
β-actin	5′	CCT CTA TGC CAA CAC AGT GC ACA TCT GCT GGA AGG TGG AC	191	56.9

optical density of each sample was read by the Ultra Microplate Reader EL808 (Bio-Tek Instruments, Winooski, VT, USA) and expressed as picograms per milliliter.

Statistical analysis

Data were expressed as media ± SEM (standard error mean). Experimental and control groups were compared by Student *t* test. Significance level for nullity hypothesis was set at 5% ($p < 0.05$).

Results

Figure 1a shows the effect of UA (8 mg/dl) associated or not with LPS (100 μg/ml) in cellular viability. In this concentration, UA did not induce cellular death in mesangial cells. There was significant increase in necrosis rate only at supraphysiological concentrations (above 20 mg/dl) (Fig. 1b). LPS alone induced more cell death in mesangial cells (16.5 ± 1.5%) compared to control (5.8 ± 1.6%), but the association between LPS and UA significantly inhibited cellular death induced by UA (6.9 ± 1.4%).

In order to investigate the ROS participation in cellular damage induced by UA in mesangial cells, we evaluated hydrogen peroxide production (Fig. 2). UA (1,712 ± 619 MIL) and LPS (2,486 ± 803 MIL) increased hydrogen peroxide production in this cell compared to control (919 ± 57 MIL). The association between UA and LPS decreased ROS production (914 ± 83 MIL). These findings were corroborated by lipidic peroxidation results (Fig. 3). UA (0.24 ± 0.04 μM/mg) and LPS (0.36 ± 0.06 μM/mg) significantly increased malondialdehyde compared to control (0.11 ± 0.02 μM/mg). But when UA was exposed to LPS there was no increase in malondialdehyde production, indicating the scavenger action of UA.

Oxygen species are important inductors of cyclooxygenase gene expression and contribute strictly to the amplification of prostanoids in inflammatory process. Figure 4 shows the expression of cyclooxygenase 2 in mesangial cells stimulated by UA alone or in the presence of LPS. UA alone induced cyclooxygenase 2 expression in mesangial cells (3.6 ± 1.6 arbitrary units) compared to control (1.47 ± 0.5 arbitrary units). Moreover, UA effects significantly potentiate the LPS effects (44.5 ± 13.7 arbitrary units).

Figure 5 shows UA alone or with LPS effects on PGE2 synthesis in mesangial cells. UA itself significantly increased PGE2 production compared to control in this cell line (323.8 ± 86.4%), the association between UA and LPS also enhanced PGE2 production (278.4 ± 59.8%). This result is in agreement with cyclooxygenase expression induced by UA alone or associated with LPS in this cell.

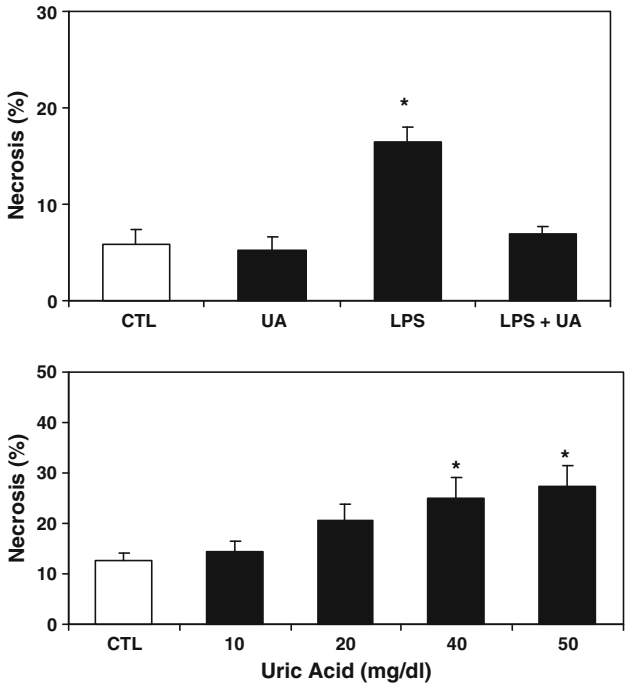

Fig. 1 Effect of UA treatment upon necrotic cell death on HMC. HMC were exposed to **a** uric acid (UA, 8 mg/dl) and/or LPS (100 μg/ml) or **b** increasing concentrations of UA (10–50 mg/dl). Suspensions were centrifuged and incubated with acrydine orange/ethidium bromide solutions. At least 100 cells were counted. Data were expressed as mean ± SEM of cellular percentage. The *bars* assigned are significantly different from the respective control condition

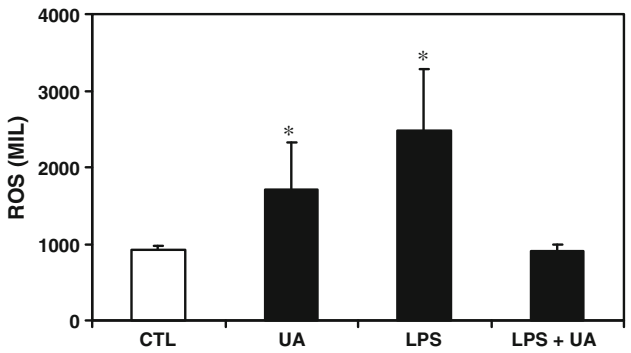

Fig. 2 Effect of UA treatment on hydrogen peroxide production by HMC. In brief, HMC cells were treated with uric acid (UA, 8 mg/dl) and/or LPS (100 μg/ml) during 24 h. Cells were treated with DCFH and analyzed by FACS. Data represent at least four independent assays. Data were expressed as mean ± SEM of geometric media of luminous intensity (MIL). The *bars* assigned are significantly different from the respective control condition

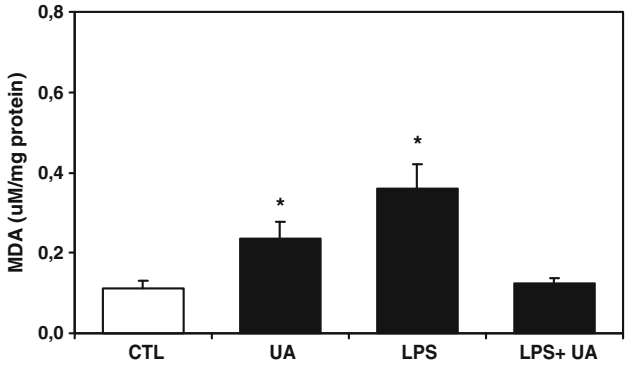

Fig. 3 Effect of UA treatment on lipid peroxidation by HMC. In brief, HMC were treated, respectively, with DMEM without FBS, uric acid (UA, 8 mg/dl) and/or LPS (100 μg/ml) for 24 h. Samples were added to a solution of 0.375% thiobarbituric acid, 15% trichloroacetic acid, and 0.25 N HCl. Levels of MDA were calculated by measuring sample absorbance at 535 nm. Data are expressed as mean ± SEM. The *bars* assigned are significantly different from the respective control condition

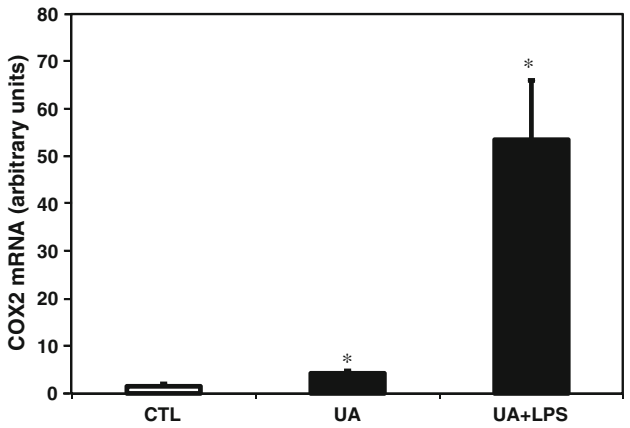

Fig. 4 Effect of UA on RNA messenger expression of cyclooxygenase 2 in HMC cells. Briefly, semi-confluent HMC were treated with uric acid (UA, 8 mg/dl) and or LPS (100 μg/ml) for 24 h. RT-PCR was performed with primers selective for cyclooxygenase 2 (COX-2). Results of four experiments per group are reported as relative expression normalized with the β-actin housekeeping gene. Data were expressed as mean ± SEM of arbitrary units. The *bars* assigned are significantly different from the respective control condition

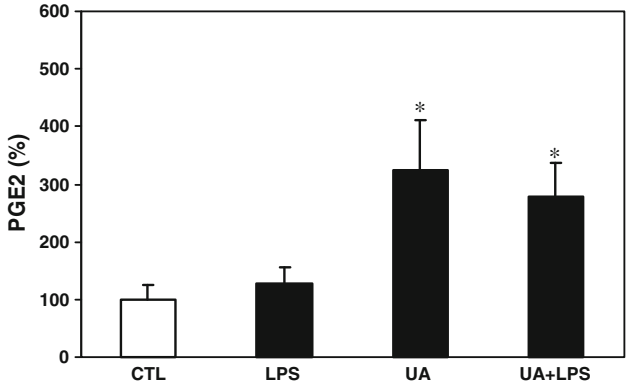

Fig. 5 Effect of UA treatment on PGE2 production in HMC cells. Briefly, semi-confluent HMC were treated with uric acid (UA, 8 mg/dl) and or LPS (100 μg/ml) for 24 h. The concentrations of PGE2 were assessed by commercially competitive ELISA. The results were expressed as mean ± SEM of picograms of PGE2 per milliliter. The *bars* assigned are significantly different from the respective control condition

Discussion

The present work suggests that noncrystalline UA can damage mesangial cells and may have several potential effects in the progression of renal disease observed during hyperuricemia [1, 13]. Low concentrations of uric acid were tested, and no toxic or oxidant effect was observed at concentrations of 1.0 mg/dl (data not shown), suggesting that these effects occur in concentrations observed in clinical practice.

The UA alone stimulated reactive oxygen species, mainly hydrogen peroxide, lipidic peroxidation, and at supraphysiologic concentrations significantly injures mesangial cells. LPS alone increased cellular death, reactive oxygen species production, and lipidic peroxidation [21]. Nevertheless, noncrystalline UA and LPS together significantly decreased hydrogen peroxide synthesis, lipidic peroxidation, and cellular death, suggesting the predominant antioxidant effects of UA. These effects disclose a unique amphipathic action by UA.

The dual effect of UA has already been demonstrated [22, 23]. Sautin and Johnson suggests that UA may function as an antioxidant primarily in plasma or pro-oxidant primarily within the cells. Gersch et al. [23] demonstrated that under oxidant conditions, UA can form reactive intermediates, including potential alkylating species, by reacting with peroxynitrite, and suggested that these reactive intermediates could explain how uric acid contributes to the pathogenesis of diseases such as the metabolic syndrome and of hypertension.

To our knowledge, this is the first study demonstrating that UA has pro- and antioxidant effects on mesangial cells. The antioxidant effect of UA is well known, and it is considered the main serum scavenger [1]. But the oxidant effect of UA was less demonstrated and could contribute to the renal damage like glomerulosclerosis observed in hyperuricemic patients [24].

The present study analyzed the effect of UA in the production of hydrogen peroxide induced by LPS, but the molecular mechanism of this interaction remains to be elucidated.

The results here presented suggested that UA increased cyclooxygenase 2 expression and prostanoid synthesis. Other studies have shown that hyperuricemic rats showed increased COX-2 expression in preglomerular arterial

vessels [25]. This finding indicates that UA has direct pro-inflammatory effects, even at low concentrations. Despite this, PGE2 production may serve to maintain glomerular filtration rate and renal plasma flow during inflammation.

Additionally, cyclooxygenase expression and PGE2 synthesis induced by UA did not depend on hydrogen peroxide production since the association between UA and LPS significantly decreased hydrogen peroxide production but stimulated COX-2 expression indicating another metabolic path of stimulation.

In clinical practice, antioxidant effect of UA has already been studied. Mackinnon et al. [26] observed an increase in serum antioxidant capacity and UA concentration in critical illness patients in intensive care unit, but it was attributed to the renal dysfunction.

Chuang et al. [27] demonstrated that serum total antioxidant capacity enhances with the increased UA concentration in several septic patients with preserved renal function. But the increase in serum antioxidant capacity and UA levels were correlated with the severity of illness and poor outcome of patients with severe sepsis and septic shock. Moreover, Chuang and co-workers suggested that anti-inflammatory processes were activated to counterbalance excessive levels of proinflammatory cytokines, oxidative stress or altered antioxidant defense (i.e. a significant change in serum antioxidant capacity) eventually leading to immune dysfunction and a poor outcome.

Despite the protective antioxidant effect of UA, its direct pro-inflammatory action may be more prominent and contributes to the progression of renal disease observed in hyperuricemia. It is already known that UA has direct pro-inflammatory action in vascular smooth muscle [8], proximal tubular [11], and endothelial cells [10] as stimulation of cytokines (IL-6) and transcription factor (NF-kB). Additional experiments using in vivo experimental model of hyperuricemia and sepsis will be necessary to clarify this issue.

Taken together, the results of the present study indicate that noncrystalline UA has antioxidant effects and during infection decreases ROS production and cellular death induced by LPS. Nevertheless, UA also has direct pro-inflammatory effects like COX-2 expression, PGE2 production, as well as pro-oxidant effects like the production of reactive oxygen species and lipidic peroxidation. Our results suggest that noncrystalline UA could be implicated in renal disease progression even at mild levels of hyperuricemia frequently observed in many pathological situations.

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