Effects of uric acid on oxidative stress in vascular smooth muscle cells

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Abstract. Hyperuricemia during hypertension is associated with aberrant vascular functions and increased oxidative stress, which affects endothelial dysfunction. Nevertheless, the molecular mechanisms underlying the effects of uric acid on vascular smooth muscle cells (VSMCs) through oxidative stress remain unclear. The aim of the present study was to investigate the dose- and time-dependent effects of uric acid on oxidative stress and p53 protein expression in VSMCs. VSMCs were incubated with various concentrations of uric acid (0-50 mg/dl) for different time periods (1-24 h). Thiobarbituric acid reactive substances (TBARs), protein carbonylation and nitric oxide (NO) levels were determined using appropriate assay kits. Superoxide anion release was detected using the Görlach method. Western blotting was performed to determine the protein expression levels of p53. The findings demonstrated that the application of uric acid led to an increase in protein carbonylation and superoxide anion levels while causing a decrease in NO levels. Conversely, no significant effect was observed on TBARS levels. Additionally, it was observed that high concentrations of uric acid suppressed p53 expression at 6, 12 and 24 h. The present study provided evidence that the influence of uric acid on oxidative stress was more closely associated with time than dose; however, not all effects observed were strictly time-dependent.

Introduction

Uric acid is the end product of purine metabolism in humans and higher primates, which is mainly synthesized in the liver and is predominantly excreted by the kidneys. Under normal physiological conditions, serum uric acid concentrations range from 3.5 to 7.2 mg/dl in men, and from 2.6 to 6.0 mg/l in women; these levels are maintained through a tightly regulated balance between uric acid production and excretion (1). When this balance is disrupted, leading to elevated levels of uric acid in the blood, hyperuricemia occurs (2,3). Clinically, hyperuricemia is defined as serum uric acid concentrations ≥7 mg/dl (0.42 mmol/l) at physiological temperature and pH (4).

Chronic hyperuricemia is implicated in the development of various pathophysiological conditions, including hypertension, chronic kidney disease, cardiovascular disease, nonalcoholic fatty liver disease, metabolic syndrome and diabetes (5,6). The association between hyperuricemia and these conditions is well known, making it a significant risk factor for cardiovascular and renal diseases. Despite this, the precise serum uric acid concentration at which the risk begins to increase remains unclear. Emerging evidence has suggested that the threshold for increased risk may be lower than previously considered, potentially <6 mg/dl (5). Consequently, this issue continues to be a focal point for clinical research, as understanding the exact uric acid levels that pose a risk is crucial for developing effective treatment and prevention strategies (5-7). Uric acid can interact with reactive oxygen species (ROS), such as hydroxyl radicals and hypochlorous acid, transforming them into less harmful substances such as allantoin, allantoate, glyoxylate, urea and oxalate (8). This antioxidant capability has been demonstrated in various experimental studies, both *in vitro* and *in vivo*, including studies on isolated organs and the human lung (9). Moreover, uric acid serves as an oxidizable co-substrate for cyclooxygenase enzymes, thereby contributing to the reduction of oxidative stress and the maintenance of endothelial function, particularly in coronary circulation (10). Despite its beneficial antioxidant properties, elevated uric acid levels, or hyperuricemia, are linked to numerous adverse health conditions.

Epidemiological studies have consistently identified hyperuricemia as a key contributor to hypertension (11-14). Experimental research in a rat model has shown that dietary supplementation with oxonic acid can lead to increased plasma uric acid levels and subsequent increased blood pressure (15-17). Additionally, hyperuricemia has been associated with endothelial dysfunction, impaired vasodilation and vascular injury, which are precursors to cardiovascular diseases, including hypertension and atherosclerosis (18-21).

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These findings highlight the complex role of uric acid in vascular biology, where its antioxidant functions are unclear due to its potential to contribute to vascular damage under hyperuricemic conditions. The exacerbation of endothelial dysfunction and vascular injury in the presence of elevated uric acid levels suggests a critical need to understand the underlying molecular mechanisms. Further research is needed to identify targeted therapeutic approaches that potentially reduce the deleterious effects of hyperuricemia.

Vascular smooth muscle cells (VSMCs) and endothelial cells (ECs), are integral components of blood vessels, serving crucial roles in vasoconstriction, vascular tone regulation and the development of vasculature (22). Nitric oxide (NO), a key cellular signaling molecule involved in both physiological and pathological processes, mediates vasodilation. Under normal conditions, NO is synthesized by endothelial NO synthase in ECs, diffuses to adjacent VSMCs and induces vasodilation (22-24). However, under oxidative stress, NO reacts with superoxide anions to form peroxynitrite (25,26). Additionally, uric acid leads to a reduction in NO levels because of the formation of peroxynitrite in vascular cells (27,28). Peroxynitrite is a potent oxidant known to cause oxidative damage and nitrosative stress to macromolecules, such as proteins, lipids and DNA (25,29,30). Previous studies have demonstrated that elevated uric acid levels decrease NO production in human umbilical vein ECs (HUVECs) (31-34). Furthermore, it has previously been indicated that uric acid increases oxidative stress, which triggers a phenotypic transition in vascular ECs (21). While the deleterious effects of uric acid-induced NO depletion and increased oxidative stress on EC function have been demonstrated (20,35-37), emerging evidence has suggested that high uric acid conditions may also affect VSMCs (38,39). However, the specific impact of uric acid on NO levels in VSMCs remains elusive. Further studies are required to elucidate the mechanisms through which uric acid influences NO bioavailability in VSMCs, and to understand the implications for vascular health and disease.

The p53 gene is widely recognized as a tumor suppressor gene, playing a pivotal role in regulating key cellular processes, including cell cycle control, DNA repair, cell proliferation, apoptosis, aging and oxidative stress response, which are associated with diseases such as diabetes, cancer and hypertension (40,41). In VSMCs, p53 has been shown to promote senescence and apoptosis, and is actively involved in the pathogenesis of atherosclerotic plaques (42). However, there are conflicting reports in the literature regarding the role of p53 in VSMCs. Recent data have revealed that p53 deficiency reduces VSMCs senescence and calcification, and knockdown of p53 can decrease mitochondrial ROS (43), suggesting that it protects VSMCs against oxidative stress. Moreover, p53 has been shown to protect VSMCs from NO-mediated oxidative stress (44). Previous findings have also revealed that p53 can be directly targeted by uric acid (45). To the best of our knowledge, although numerous aspects of uric acid-induced cell proliferation (38,39,46), inflammation (47,48) and oxidative stress (38,48) have been studied in VSMCs, the effect of uric acid on p53 has not yet been demonstrated. The interplay between uric acid and p53 in VSMCs is of particular interest, given the potential implications for understanding how uric acid influences cellular senescence, oxidative stress and vascular pathophysiology.

In the present study, it was hypothesized that uric acid could elevate oxidative stress in rat VSMCs in a dose- and time-dependent manner. To investigate this hypothesis, the effects of various concentrations of uric acid on oxidative stress markers, including protein carbonylation, thiobarbituric acid reactive substances (TBARs) and superoxide anion levels, were examined. Additionally, the protein expression levels of p53 and NO levels were assessed in rat VSMCs. By comparing these parameters, the present study aimed to understand the association between uric acid exposure and oxidative stress, as well as its impact on p53 expression and NO levels over time and across different doses. The present findings provide preliminary novel insights into oxidative stress responses induced by uric acid and highlight the potential role of uric acid in mediating vascular cell function.

Materials and methods

Reagents and antibodies. Uric acid, fetal bovine serum (FBS), penicillin-streptomycin, HEPES, elastase, collagenase, cytochrome *c*, superoxide dismutase (SOD) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Merck KGaA). DMEM (cat. no. E0500-100) was obtained from Cegrogen Biotech GmbH and Hank's balanced salt solution (HBSS) was obtained from Biochrom, Ltd. Nitrate/Nitrite (NO detection) Colorimetric Assay Kit (cat. no. 780001), TBARS Assay Kit (cat. no. 10009055) and Protein Carbonyl Assay Kit (cat. no. 10005020) were purchased from Cayman Chemical Company. Primary antibodies against p53 (1C12) (cat. no. 2524) and β-actin (cat. no. 4967), as well as secondary antibodies [horseradish peroxidase (HRP)-linked goat anti-rabbit immunoglobulin G (IgG) (cat. no. 7074) and anti-mouse IgG (cat. no. 7076)] were purchased from Cell Signaling Technology, Inc.

Isolation and culture of primary rat vascular smooth muscle cells. All animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals (49) following experimental protocols approved by the Local Committee on Animal Research Ethics at Akdeniz University (approval no. 727/2018.01.024; Antalya, Turkey). For the present study, a total of 4 male Wistar rats (age, 8-10 weeks; weight, 200-300 g) were used. The male Wistar rats were obtained from the Local Committee on Animal Research Ethics at Akdeniz University. The rats were housed in a controlled environment with a temperature of 22±2˚C, a relative humidity of 50±10% and a 12-h light/dark cycle. The rats had *ad libitum* access to standard chow and water. According to the latest guidelines from the American Veterinary Medical Association (50) and guidelines adopted by institutions such as Boston University (51) and the University of Maryland (52), the rats were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg), in compliance with approved ethical standards (53,54). Upon confirmation of deep anesthesia, which was verified by the lack of response to painful stimuli and absence of the corneal reflex, the chest cavity was exposed to allow access to the aorta. The aorta was then carefully dissected, the adventitial layer was meticulously removed using forceps, and the tissue was subsequently transferred to cell culture dishes under sterile conditions. VSMCs

were isolated from the dissected aorta using enzymatic dissociation solution [HEPES dissolved in HBSS (15 mM, pH: 7.2-7.3), BSA (2 mg/ml), $CaCl_2$ (0.2 mM), Soybean Trypsin Inhibitor (0.25 mg/ml), elastase (0.0625 mg/ml) and collagenase (0.5 mg/ml)] (39). After the isolation of VSMCs, while the rats were still under deep anesthesia, they were sacrificed by cervical dislocation to ensure a humane and painless death. The isolated cells were subsequently transferred to cell culture dishes and cultured as previously described (39,47,55). VSMCs were maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin, and were cultured at 37˚C and 5% CO_2. VSMCs in passages 3 to 5 were used in all experiments and the cells were incubated up to 70-80% density. After the control and experimental groups were formed, all cells were incubated with FBS-free DMEM overnight for serum starvation. Uric acid was prepared by filtration through 0.2-mm sterile filters and VSMCs were stimulated at 37˚C with different uric acid doses (0-50 mg/dl) for various durations (1-24 h), excluding the control groups.

NO determination. Once cells reached 80% confluence, NO levels were detected. NO (total nitrate + nitrite) levels were determined using a colorimetric assay kit that included Griess reagents (Griess 1 and 2), according to the manufacturer's instructions. NO content was determined as the total value measured in the presence of cells minus the value determined from the media alone in the absence of cell growth, according to the manufacturer's protocol. Each sample absorbance was measured by spectrophotometry (540 nm) and NO concentrations were calculated using a standard curve. The levels of NO are shown in μM. Experiments were repeated four times and the results are presented as the mean ± standard error of the mean (SEM).

Superoxide anion accumulation levels. For the determination of superoxide anion production, after reaching 80% cell confluence, the Görlach method of spectrophotometric SOD-inhibitable reduction of cytochrome *c* was performed for each sample, with a blank for each one (negative control), as previously described (47,56). VSMCs were cultured in 12-well plates for 48 h. Superoxide anion related to cytochrome *c* reduction was calculated for each sample by measuring between cells incubated with SOD and without SOD. Superoxide anion accumulation levels are shown in nmol/μg. Experiments were repeated five times and the results are presented as the mean ± SEM.

Quantification of TBARS levels. Lipid peroxidation was detected by measuring the amount of malondialdehyde (MDA)-TBA adduct in the cell homogenates. The cells were collected ($2x10^7$) with 1 ml PBS buffer according to the whole cell lysis procedure indicated in the TBARS assay kit. The cells were homogenized on ice with an ultrasonic homogenizer (UW2070; BANDELIN electronic GmbH & Co. KG), SDS was then added and the cells were mixed with the color reagent (TBA in acetic acid and sodium hydroxide) in a boiling water bath for 1 h, cooled in an ice-water bath, and then incubated on ice. After cooling, the sample was centrifuged at 1,600 x g for 10 min at 4˚C and was maintained at room temperature for 30 min. TBARS was measured by spectrophotometry (530 nm) and each sample concentration was determined using the MDA colorimetric standard curve. Results are expressed as μM. Experiments were repeated three times and the results are presented as the mean ± SEM.

Quantification of protein carbonyl content. The concentration of protein carbonyl was determined spectrophotometrically using a Protein Carbonyl Assay Kit according to the manufacturer's instructions. Briefly, cells at 80% confluence were collected and then homogenized in phosphate buffer (pH 6.7; containing 1 mM EDTA), after which, the sample was centrifuged at 10,000 x g for 15 min at 4˚C and the supernatant was removed. The lysates were incubated with dinitrophenylhydrazine for 1 h at room temperature in the dark. The protein was precipitated twice with trichloroacetic acid (first 20%, second 10%) and was then washed in an ethanol/ethyl acetate mixture. After being washed, the sample was resuspended in guanidine hydrochloride and centrifuged at 10,000 x g for 10 min at 4˚C. The protein carbonyl content was measured at 360 nm using spectrophotometry and was calculated according to the manufacturer's instructions. Results are expressed as nmol/mg protein. The protein content was determined using the BCA Protein Assay Kit (Takara Bio, Inc.), with BSA solution as the standard. This assay had a detection limit of 1-10 mg protein. Experiments were repeated three times and the results are presented the mean ± SEM.

Western blot analysis. Primary rat VSMCs were seeded in 6-well plates. After the cells reached 80% confluence, sample preparation for western blotting was performed using lysis buffer [50 mmol/l HEPES, 50 mmol/l NaCl, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1.5 mmol/l MgCl2, 1 mmol/l EDTA, 10 mmol/l sodium pyrophosphate, 1 mmol/l Na_3VO_4, 100 mmol/l NaF, 30 mmol/l 2-(p-nitrophenyl) phosphate, 1 mmol/l phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin and 10 mg/ml aprotinin (pH 7.4)] as previously described (39). After the sample protein concentrations were calculated using the BCA Protein Assay Kit, total proteins (25 μg) were separated by SDS-PAGE on 10% gels and were then transferred onto 0.2-μm nitrocellulose membranes (Whatman plc; Cytiva). The membranes were blocked for 1 h at room temperature with 5% w/v nonfat dry milk solution, followed by incubation with primary antibodies against p53 (1:1,000) and β-actin (1:5,000) at 4˚C overnight. The following day, membranes were washed three times for 5 min in Tris-buffered saline with 0.01% Tween-20 (TBST) and then incubated with anti-mouse and anti-rabbit secondary antibodies for 2 h at room temperature. Subsequently, the membranes were washed three times for 5 min in TBST. The protein bands were detected using an enhanced chemiluminescence reagent-based Super Signal West Pico HRP Substrate System (Thermo Fisher Scientific, Inc.). Semi-quantification of the protein bands was performed using Alpha Digi Doc 1,000 Gel Documentation Unit (AlphaEaseFC™; Alpha Innotech Corporation). After stripping, the membranes were probed with anti-β-actin antibody for the same duration and at the same temperature as with the anti-p53 antibody to confirm equal protein loading. All experiments were performed in triplicate.

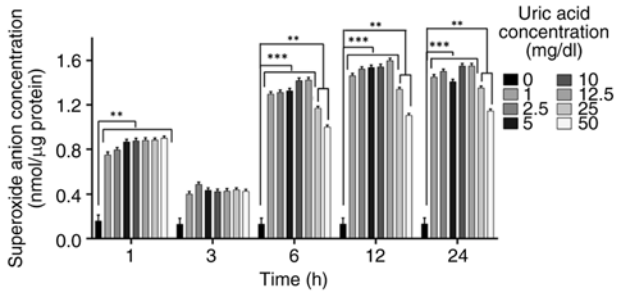


Figure 1. Uric acid exposure promotes superoxide anion accumulation in VSMCs. VSMCs were cultured in 12-well plates for 48 h and were then incubated with uric acid (1, 2.5, 5, 10, 12.5, 25 and 50 mg/dl) for different durations (1, 3, 6, 12 and 24 h). The control group was not treated with uric acid. The differences among quantitative variables were evaluated by one-way ANOVA followed by Dunnett's test. The data are presented as the mean ± standard error of the mean from five independent experiments. **P<0.01 and ***P<0.001 vs. control. VSMCs, vascular smooth muscle cells.

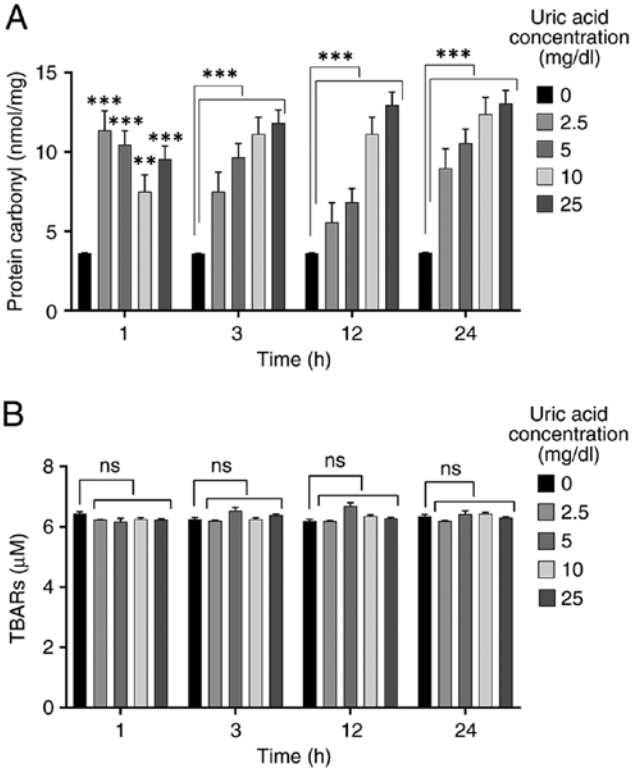


Figure 2. Effects of uric acid stimulation on protein carbonylation and lipid peroxidation in VSMCs. To identify the effects of uric acid on protein carbonylation and lipid peroxidation, VSMCs were stimulated with uric acid (2.5, 5, 10 and 25 mg/dl) for 1, 3, 12 and 24 h. The control group was not treated with uric acid. (A) Protein carbonylation was detected using the commercial Protein Carbonyl Assay Kit. (B) Lipid peroxidation was measured using the commercial TBARS Assay Kit. The differences among quantitative variables were evaluated by one-way ANOVA followed by Dunnett's test. The data are presented as the mean ± standard error of the mean from three independent experiments. **P<0.01 and ***P<0.001 vs. control. VSMCs, vascular smooth muscle cells; TBARS, thiobarbituric acid reactive substances; ns, not significant.

Statistical analysis. Statistical analysis was performed with GraphPad Prism (version 8.01; Dotmatics). The results are presented as the mean ± SEM. The differences among the control and experimental groups were evaluated by one-way ANOVA followed by Dunnett's multiple comparisons test. P<0.05 was considered to indicate a statistically significant difference.

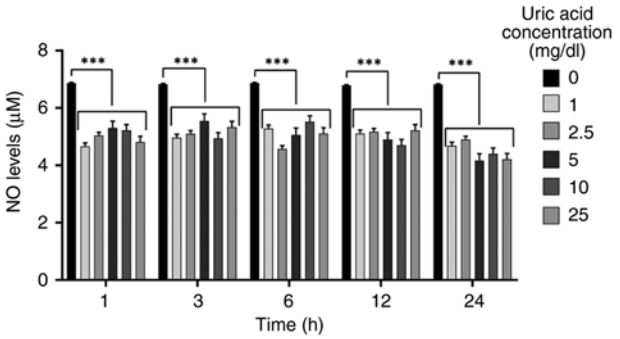


Figure 3. Stimulation of VSMCs with uric acid reduces NO levels in a dose- and time-independent manner. VSMCs were treated with uric acid at 1, 2.5, 5, 10 and 25 mg/dl for 1, 3, 6, 12 and 24 h. The control group was not treated with uric acid. The NO Assay kit was used to detect NO production. The differences among quantitative variables were evaluated by one-way ANOVA followed by Dunnett's test. The data are presented as the mean ± standard error of the mean from four independent experiments. ***P<0.001 vs. control. VSMCs, vascular smooth muscle cells; NO, nitric oxide.

Results

Superoxide anion accumulation is affected by uric acid in VSMCs independent of dose. Uric acid stimulation can promote the occurrence of oxidative stress in VSMCs and superoxide anion release is an upstream target mediator of oxidative stress (57-59). It was thus hypothesized that uric acid may promote the occurrence of superoxide anion release by upregulating oxidative stress. To understand whether superoxide anion release is affected by uric acid in VSMCs in a dose- and time-dependent manner, the accumulation of superoxide anion was determined using the Görlach method (56). Uric acid increased superoxide anion release at 1, 6, 12 and 24 h in a dose-independent manner compared with that in the control groups, although there was no significant difference observed at 3 h (Fig. 1). All uric acid doses decreased superoxide anion levels at 1, 3, 6, 12, and 24 h compared with the control; however, the increase observed at 3 h was less pronounced than at the other time points. In addition, in response to 25 and 50 mg/dl uric acid, superoxide anion accumulation was decreased compared with in response to the other uric acid concentrations (1, 2.5, 5, 10 and 12.5 mg/dl uric acid) at 6, 12 and 24 h.

Uric acid promotes protein carbonylation but does not affect lipid peroxidation. High levels of uric acid can be a key regulator for oxidative stress (21,35). In order to evaluate the effects of uric acid on oxidative stress in VSMCs, protein carbonylation and lipid peroxidation assays were performed. VSMCs were incubated with different uric acid concentrations (2.5, 5, 10 and 25 mg/dl) for various durations (1, 3, 12 and 24 h). The control group (0 mg/dl uric acid concentration) was not treated with uric acid. Treatment with all uric acid concentrations caused a significant increase in protein carbonyl levels at 1 h compared with those in the control group, but 10 mg/dl uric acid dose resulted in a reduction compared with the other doses

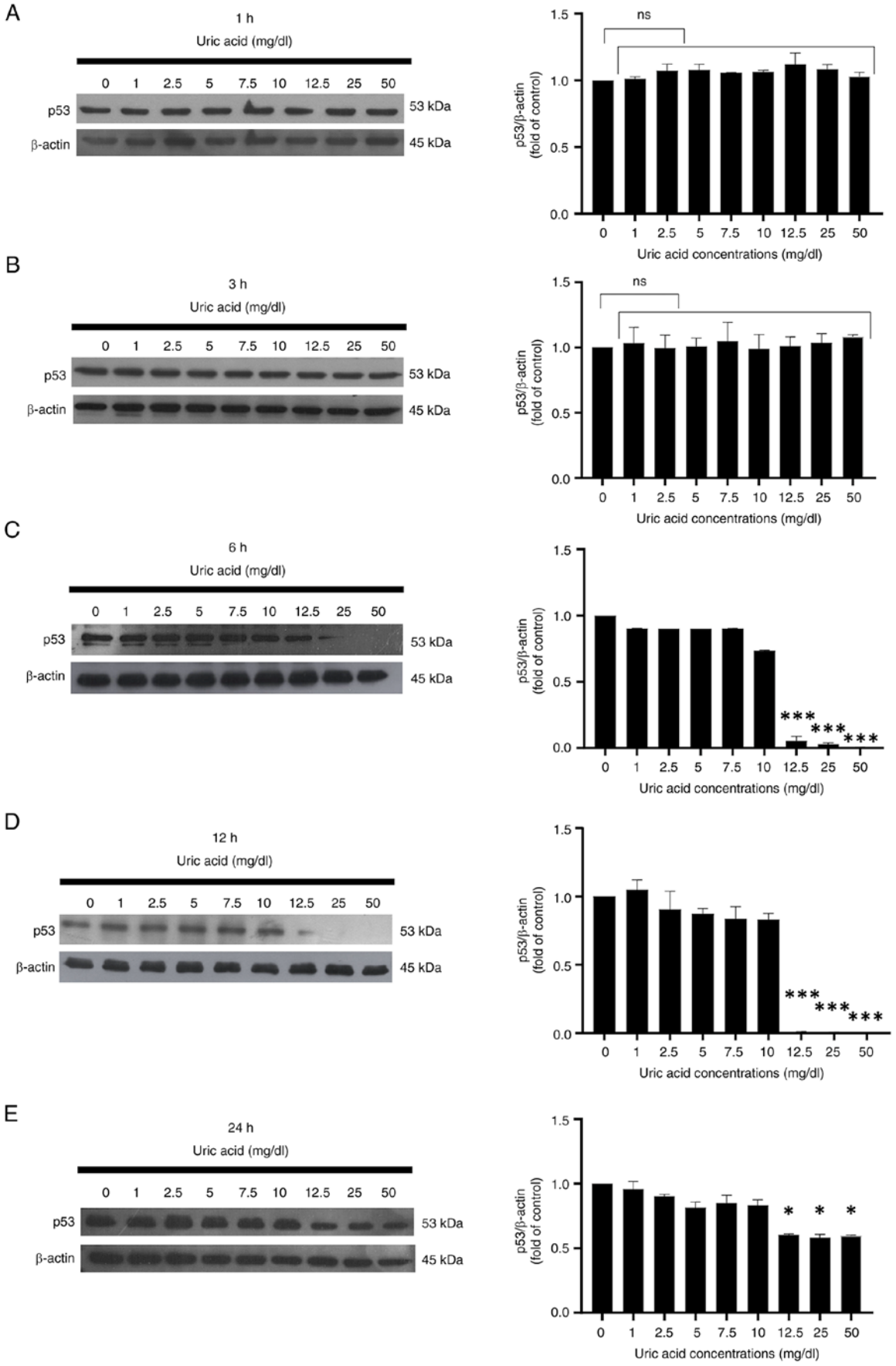


Figure 4. p53 expression is suppressed in VSMCs in response to long-term treatment with high concentrations of uric acid. VSMCs were treated with uric acid (1-50 mg/dl) for (A) 1, (B) 3, (C) 6, (D) 12 and (E) 24 h, and total protein was collected. The control group not treated with uric acid. The p53 protein expression levels were detected by western blotting and β-actin was used as the internal control. The differences among quantitative variables were evaluated by one-way ANOVA followed by Dunnett's test. The data are presented as the mean ± standard error of the mean from four independent experiments. *P<0.05 and ***P<0.001 vs. control. VSMCs, vascular smooth muscle cells; ns, not significant.

(2.5, 5 and 25 mg/dl uric acid) (Fig. 2A). Protein carbonylation in VSMCs was significantly increased by all concentrations of uric acid at 3, 12 and 24 h compared with that in the control groups in a dose-dependent manner. As shown in Fig. 2B, uric acid stimulation had no effect on TBARS levels in VSMCs compared with those in the control group, thus indicating that no dose of uric acid promoted lipid peroxidation in VSMCs.

Uric acid decreases NO levels independent of dose and time. NO is a vasodilator that modulates important processes, such as vascular tone, inflammation and oxidation-sensitive mechanisms (60,61). To determine if higher uric acid concentrations modify NO levels in VSMCs, NO levels were analyzed in response to different uric acid doses for various durations. As shown in Fig. 3, all uric acid doses significantly diminished NO levels in a dose- and time-independent manner.

Long-term exposure to high uric acid levels suppresses p53 expression in VSMCs. Uric acid affected the protein expression levels of p53 in rat primary VSMCs in a time-dependent manner. As determined by western blotting, it was determined that all uric acid concentrations did not affect the expression levels of p53 in VSMCs at 1 and 3 h compared with those in the control group (0 mg/dl uric acid) (Fig. 4A and B). As shown in Fig. 4C, p53 expression was suppressed in VSMCs stimulated with high uric acid doses (12.5, 25 and 50 mg/dl uric acid concentrations) at 6 h. Long-term uric acid stimulation of VSMCs indicated that high concentrations of uric acid (12.5, 25 and 50 mg/dl uric acid) abolished p53 expression at 12 h (Fig. 4D), but the same doses of uric acid only slightly reduced the protein expression levels of p53 at 24 h (Fig. 4E).

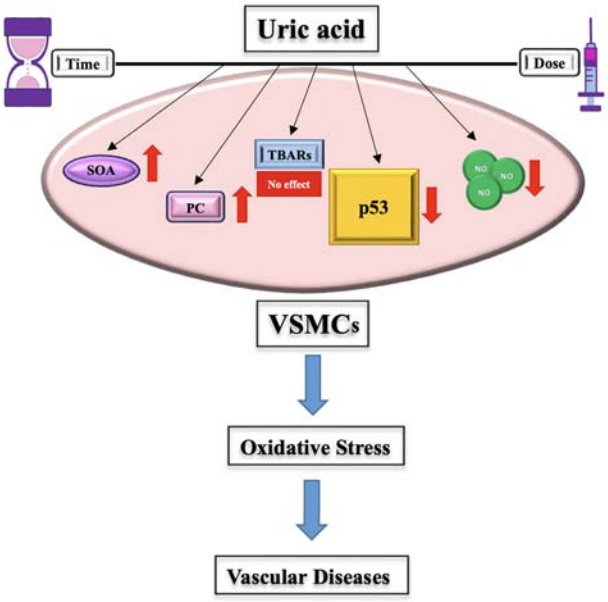


Figure 5. Effects of uric acid on oxidative stress in VSMCs. Uric acid significantly promoted an increase in SOA levels and PC, which are key indicators of oxidative stress. It also resulted in a marked reduction in NO concentrations, a molecule essential for vascular homeostasis. Additionally, uric acid did not significantly alter TBARs levels. Uric acid exacerbated oxidative stress, with its inhibitory effects on p53 potentially contributing to this process. Given these observations, uric acid may serve as a reliable biomarker for oxidative stress in VSMCs. VSMCs, vascular smooth muscle cells; TBARs, thiobarbituric acid reactive substances; SOA, superoxide anion; PC, protein carbonylation; NO, nitric oxide.

Discussion

Uric acid is known to induce oxidative stress, which can pathologically contribute to hypertension in VSMCs (62). Given its significant role in causing hypertension, the vascular damage and endothelial injury induced by high uric acid levels was further assessed in the present study. VSMCs have a crucial role in maintaining endothelial homeostasis and in the development of blood vessels (22). Although research on the effects of uric acid on VSMCs is insufficient, numerous *in vitro* and *in vivo* studies have demonstrated that ECs continuously interact with VSMCs. These studies also showed that large amounts of NO produced by ECs diffuse into VSMCs, where NO is essential for regulating vascular contraction and relaxation (63-65). Despite these findings, the precise molecular mechanisms by which NO operates in uric acid-stimulated VSMCs under oxidative stress conditions remain elusive. Understanding these mechanisms is crucial as NO serves a pivotal role in vascular function and integrity. The interactions between ECs and VSMCs, particularly the transfer and effects of NO, are central to vascular health, and disruptions can lead to pathological states. Further research is needed to elucidate the pathways involved in NO signaling within VSMCs exposed to high levels of uric acid, as this knowledge could contribute to the development of therapeutic strategies aimed at mitigating uric acid-induced vascular damage and hypertension.

Oxidative stress is a pathological condition characterized by the excessive production of ROS, such as superoxide anion, hydroxyl radicals and hydrogen peroxide, along with oxidative damage to macromolecules (66-69). This process has been identified as a significant clinical risk factor in contributing to vascular damage, endothelial injury and the progression of vascular diseases (70-73). Emerging evidence has consistently demonstrated that uric acid is a critical factor in the development of endothelial dysfunction by regulating the oxidative stress of HUVECs (18,21,35,36). Specifically, studies have shown that uric acid stimulation leads to increased oxidative stress. Research has also indicated that uric acid-stimulated VSMCs have a marked increase in ROS production, and uric acid has been shown to affect oxidative stress-related signaling pathways within VSMCs (48,74).

The present study focused on the effect of uric acid on various oxidative stress parameters, including protein carbonylation, lipid peroxidation and superoxide anion levels in VSMCs after 1-24 h of treatment. Uric acid increased protein carbonylation levels in a dose-dependent manner. Specifically, protein carbonyl levels showed a statistically significant increase even at the 1-h time point, and this increase was almost maintained at 3, 6, 12 and 24 h, especially in response to higher concentrations of uric acid. The results at the short-term time point (1 h) suggested that uric acid may induce oxidative stress, leading to protein carbonylation in VSMCs and a high reactivity of uric acid with cellular proteins indicating that it may rapidly cause oxidative modifications. Additionally, the results in response to prolonged exposure (3, 12 and 24 h) provide a more comprehensive understanding of the impact of uric acid on oxidative stress. These findings emphasize the necessity of examining both short-term and long-term

exposures to fully elucidate the biochemical pathways and molecular mechanisms. By contrast, the present analysis of lipid peroxidation, as measured by TBARs levels, revealed that uric acid did not significantly affect lipid peroxidation in VSMCs. This indicated that uric acid may preferentially induce oxidative stress in proteins rather than lipids. This selectivity may be attributed to differences in the susceptibility of proteins and lipids to oxidative damage, or it may be related to the specific localization of uric acid within cellular compartments. Moreover, VSMCs may possess robust antioxidant defense mechanisms, such as glutathione peroxidase and catalase, which effectively mitigate lipid peroxidation but are less effective against protein oxidation. This could explain the observed increase in protein carbonylation despite unchanged TBARs levels (75). To the best of our knowledge, the present study is the first to present data on the effects of uric acid stimulation on protein carbonylation and lipid peroxidation in rat primary VSMCs.

The present study indicated that all doses of uric acid increased superoxide anion release compared with that in the control groups at most time points (1-24 h, with the exception of at 3 h). Previous studies showed that a uric acid dose of 5 mg/dl significantly increased superoxide anion accumulation at 1 h in primary rat VSMCs (47). Consistent with previous findings (47), the current results demonstrated that all doses of uric acid caused a transient reduction of superoxide anion production in VSMCs at 3 h compared with at the other time points (1, 6, 12 and 24 h). Although there is no direct evidence to explain the transient decrease in superoxide anion levels at 3 h, it may be that the protective effect of the cells against oxidative damage is related to its ability to reduce oxidative stress in the early time intervals. These findings have the potential to provide evidence for the time-dependent effects of uric acid on superoxide anion production in VSMCs.

p53 is a central mediator of oxidative stress and apoptosis signaling in vascular functions (76), but its role in the pathogenesis of vascular damage remains insufficiently understood. Previous studies have linked p53 to apoptosis in VSMCs (77,78), although these investigations have been largely confined to the process of atherosclerotic plaque formation. Notably, p53 exhibits bidirectional functions in various biological processes, and its paradoxical role in metabolic pathways is attributed to the context-dependent nature of its activity (79,80). Despite this complexity, p53 signaling is recognized as a crucial regulator of oxidative stress, proliferation and inflammation. Furthermore, p53 activity has been implicated in uric acid-induced oxidative stress (45,81,82). Previous studies have shown that in response to mild ROS concentrations, p53 promotes cell survival by exerting an anti-oxidative effect to protect cells from damage. However, when cells are exposed to excessive and/or prolonged ROS levels, which can cause uncontrollable damage, p53 activity is inhibited by ROS, leading to cell death pathways to protect adjacent undamaged cells (83,84). In light of these findings, the present study aimed to evaluate the changes in p53 protein signaling in VSMCs stimulated with uric acid in a dose- and time-dependent manner. The results demonstrated that p53 protein expression was significantly suppressed by high doses of uric acid (12.5, 25 and 50 mg/dl) during prolonged stimulations (6-24 h). These results provided preliminary findings indicating a dose- and time-dependent relationship between uric acid exposure and p53 activity in VSMCs.; with p53 expression remaining stable at early time points (1 and 3 h) but progressively decreasing at 6 and 12 h, especially in response to higher uric acid concentrations. Furthermore, when comparing the results of p53 protein expression with oxidative stress parameters, it was observed that the suppression of p53 protein expression was associated with increased superoxide anion accumulation during long-term stimulation of high uric acid doses. These findings suggested that the accumulation of superoxide anions induced by uric acid may be associated with the suppression of p53 activation in VSMCs. Although the data provide valuable information about the molecular mechanisms by which uric acid affects vascular cell function, further studies are needed to explore p53 and oxidative stress pathways in vascular diseases.

It has been determined that NO-mediated apoptosis is increased by p53 deficiency in VSMCs (85,86). Furthermore, it has been shown that p53 deficiency can be increased by NO-mediated oxidative stress (44). These findings highlight the protective role of p53 in VSMCs; however, the precise relationship between p53 and NO in these cells remains unclear. It was hypothesized that p53 might also modulate the levels of oxidative stress in VSMCs, thereby controlling NO levels, given that one of the main functions of p53 is the regulation of oxidative stress. To explore this hypothesis, VSMCs were stimulated with various doses of uric acid over different time periods and superoxide anion, TBARS and protein carbonylation levels were measured. The results of the present study showed that uric acid concentrations decreased NO levels in VSMCs in a dose-independent manner at all time points. Based on these findings, it was concluded that the protective effect of p53 against uric acid-induced oxidative stress in VSMCs may not be mediated through the regulation of NO levels. This suggests that p53 may exert its protective effects through alternative pathways or mechanisms that do not directly involve NO. Additionally, the study observed increases in protein carbonyl levels and superoxide anion in response to uric acid exposure, which are all indicators of oxidative stress. Furthermore, TBARS was not directly related to the concentration of uric acid, indicating that TBARS levels were not sensitive to changes in uric acid concentration. This conclusion is visually summarized in Fig. 5, which illustrates the complex interactions between uric acid, oxidative stress markers and p53 activity.

To the best of our knowledge, the present study is the first to show that uric acid affected NO levels in VSMCs in a dose- and time-independent manner, while also providing comprehensive evidence that uric acid increased superoxide anion levels at 1, 3, 6, 12 and 24 h, with a less pronounced increase at 3 h compared with the other time points, and its dose-dependent effects on protein carbonyl levels. The results demonstrated that the doses of uric acid that induced accumulation of superoxide anion may also inhibit p53 protein activity in the long term, independent of NO levels. Additionally, a significant finding of the present study was that uric acid reduced NO levels in VSMCs regardless of the exposure time and dose. The effects of uric acid on protein carbonylation and lipid peroxidation in VSMCs are

little-known oxidative damage parameters that need further investigation.

The results of the present study indicated that uric acid may significantly increase oxidative stress in VSMCs, suggesting that controlling uric acid levels could represent a potential therapeutic target for preventing hypertension and related diseases. Furthermore, it is clear that additional research is needed to understand the role of the p53 signaling pathway in uric acid-induced oxidative stress-mediated vascular damage and to overcome cardiovascular diseases.

The primary aim of the present study was to provide a detailed analysis of the cellular mechanisms involved. Therefore, *in vivo* experiments and clinical studies are essential to translate these findings into real-world applications. In future studies, further analyses, including assessing the activities of antioxidant enzymes such as SOD, catalase and glutathione peroxidase, may provide more comprehensive data on the effects of uric acid, and these additional markers will help to elucidate cellular antioxidant defense mechanisms and their response to uric acid-induced oxidative stress. The integration of such data with the current study could provide a more comprehensive understanding of the effects of uric acid on vascular health.

In conclusion, while the current study lays the groundwork by elucidating the cellular effects of uric acid in VSMCs, further *in vivo* studies and clinical research are required for a complete understanding of these mechanisms. The data presented in the current study may serve as a foundation for future investigations, and the findings could have broader implications when integrated with additional studies. Therefore, future studies will aim to include investigations on ECs and immune cells to provide a comprehensive understanding of the effects of uric acid on vascular health. These future studies are crucial for developing effective therapeutic strategies to mitigate the adverse effects of uric acid on vascular health.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

SD was involved in data curation, formal analysis, investigation, methodology and the use of software. SD also contributed to the writing of the original draft, and participated in the review and editing process. EY contributed to data curation, formal analysis and methodology, as well as writing the original draft. AY was responsible for conceptualization, funding acquisition, investigation, project administration and providing resources. Additionally, AY provided supervision and validation, and was involved in the review and editing of the manuscript. SD and AY confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals following experimental protocols approved by the Local Committee on Animal Research Ethics at Akdeniz University (approval no. 727/2018.01.024).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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