

Uric acid decreases NO production and increases arginase activity in cultured pulmonary artery endothelial cells

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Zharikov S, Krotova K, Hu H, Baylis C, Johnson RJ, Block ER, Patel J. Uric acid decreases NO production and increases arginase activity in cultured pulmonary artery endothelial cells. *Am J Physiol Cell Physiol* 295: C1183–C1190, 2008. First published September 10, 2008; doi:10.1152/ajpcell.00075.2008.—Elevated levels of serum uric acid (UA) are commonly associated with primary pulmonary hypertension but have generally not been thought to have any causal role. Recent experimental studies, however, have suggested that UA may affect various vasoactive mediators. We therefore tested the hypothesis that UA might alter nitric oxide (NO) levels in pulmonary arterial endothelial cells (PAEC). In isolated porcine pulmonary artery segments (PAS), UA (7.5 mg/dl) inhibits acetylcholine-induced vasodilation. The incubation of PAEC with UA caused a dose-dependent decrease in NO and cGMP production stimulated by bradykinin or Ca²⁺-ionophore A23187. We explored cellular mechanisms by which UA might cause reduced NO production focusing on the effects of UA on the L-arginine-endothelial NO synthase (eNOS) and L-arginine-arginase pathways. Incubation of PAEC with different concentrations of UA (2.5–15 mg/dl) for 24 h did not affect L-[³H]arginine uptake or activity/expression of eNOS. However, PAEC incubated with UA (7.5 mg/dl; 24 h) released more urea in culture media than control PAEC, suggesting that arginase activation might be involved in the UA effect. Kinetic analysis of arginase activity in PAEC lysates and rat liver and kidney homogenates demonstrated that UA activated arginase by increasing its affinity for L-arginine. An inhibitor of arginase (S)-(2-boronoethyl)-L-cysteine prevented UA-induced reduction of A23187-stimulated cGMP production by PAEC and abolished UA-induced inhibition of acetylcholine-stimulated vasodilation in PAS. We conclude that UA-induced arginase activation is a potential mechanism for reduction of NO production in PAEC.

nitric oxide; endothelial dysfunction; pulmonary hypertension

URIC ACID (UA) is the final enzymatic end product of purine degradation in the human body. In most animals, UA is degraded by the hepatic enzyme urate oxidase (uricase) to generate allantoin, which is freely excreted in the urine. In humans, the uricase gene was mutated during primate development (34) with the consequence that humans have much higher serum concentrations of UA (3.4 mg/dl or higher) than other mammals (<2 mg/dl).

Up to 79% of patients with primary pulmonary hypertension have hyperuricemia (UA concentrations in the blood more than 5.5 mg/dl) (2, 6, 10). Although some investigators consider UA in the blood as a bystander in the pathophysiology of pulmo-

nary hypertension (9) or even as a beneficial component (29), clinical studies have demonstrated that the serum concentration of UA defines the severity of pulmonary hypertension and may predict mortality of patients with this disease (3, 23, 31). The mechanisms responsible for the UA contribution to the severity of pulmonary hypertension are unknown.

Experimental studies have suggested that UA may affect various vasoactive mediators. Recently, we have shown that UA affects acetylcholine (ACh)-induced vasodilation in rat aortic segments in a concentration-dependent manner (24) and inhibits nitric oxide (NO) production in cultured aortic endothelial cells (14). We hypothesize that UA may impair NO production in pulmonary endothelial cells and in this way contributes to the severity of pulmonary hypertension. The goal of this study was to test whether UA alters NO production by pulmonary arterial endothelial cells (PAEC) and to clarify possible mechanisms of the UA action.

Endothelial cells generate NO from the amino acid L-arginine via the catalytic action of endothelial NO synthase (eNOS) (1). L-Arginine is the only substrate for NO synthesis by eNOS. On the other hand, L-arginine is metabolized by several pathways including intracellular arginase, the final enzyme in the L-arginine-urea cycle (35). By competing for a common substrate, arginase may affect NO production by endothelial cells (4, 7). In this paper, we studied effects of UA on vasodilation of isolated porcine pulmonary artery segments (PAS), on NO production, on the L-arginine-eNOS pathway, and on arginase activity in cultured PAEC.

EXPERIMENTAL PROCEDURES

Cell culture. PAEC were isolated from the main pulmonary artery of 6- to 7-mo-old pigs by collagenase treatment and were cultured as previously reported (5). Cells were used at passages 3–5.

Detection of NO production by PAEC using the fluorescent indicator DAF-FM diacetate. Control PAEC and PAEC treated with UA (0, 5.0, or 7.5 mg/dl, 24 h) were first washed with HBSS and then incubated with 5 μ M 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) diacetate dissolved in HBSS for 30 min at 37°C in darkness. After incubation PAEC were washed to remove excess fluorescent probe. Fresh HBSS containing 0.1 mM L-arginine without or with UA (5.0 or 7.5 mg/dl) was added to PAEC, and cells were incubated for an additional 20 min to allow complete deesterification of the intracellular diacetate. After this procedure, direct visualization of NO production by PAEC was assessed using

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a laser-scanning confocal microscope with excitation and emission maxima at 495 and 515 nm, respectively. Within the cytoplasm of several (5–6) cells in the field, regions of interest (ROI) were randomly chosen and the intensities of fluorescence in the ROI were quantified every 60 s using LSM 510 software for the Carl Zeiss Laser Scanning Microscope. On the fifth minute from the beginning of fluorescence measurements, bradykinin (BK, the final concentration of 2 μ M) was added to cells to stimulate NO production. The average DAF-FM fluorescence in ROI at the beginning of measurements was taken as F_0 , and all following points were expressed as a ratio of the average DAF-FM fluorescence at the particular time point (F) to F_0 .

cGMP measurements. Measurements of cGMP levels in PAEC were performed by the acetylation enzyme immunoassay (EIA) protocol on 100 μ l of ethanol cellular extracts using cGMP EIA Biotrak system (Amersham Biosciences). Details of preparing ethanol extracts from PAEC for cGMP measurements have been reported by us (15).

Measurement of L-arginine uptake by PAEC. L-Arginine uptake was measured using L-[3 H]arginine (a mixture of 50 μ M unlabeled L-arginine plus L-[3 H]arginine, 10 μ Ci/ml) according to the method reported by us (36). Briefly, PAEC grown in 24-well plates were washed with 0.5 ml of a buffer of the following composition (in mM): 140 LiCl, 5 KCl, 2 Na_2HPO_4 , 1.2 MgSO_4 , 2.5 CaCl_2 , 11 glucose, and 10 HEPES-Tris (pH 7.4, 37°C, LiCl-Dulbecco solution) and then incubated with L-[3 H]arginine in LiCl-Dulbecco solution containing different concentrations of UA for 1 min. The incubation was stopped by washing cells four times with 2 ml of ice-cold LiCl-Dulbecco solution. After solubilization of the cells, radioactivity was quantitated by liquid scintillation spectrometry.

Determination of eNOS activity in the total membrane fraction from PAEC. Control PAEC and PAEC treated with UA were scraped and homogenized in buffer A (50 mM Tris·HCl, pH 7.4, containing 0.1 mM EDTA and EGTA, 1 mM PMSF, and 1 μ g/ml leupeptin). The homogenates were centrifuged at 100,000 g for 60 min at 4°C, and the total membrane fraction pellet was resuspended in buffer B (buffer A + 2.5 mM CaCl_2). eNOS activity in the total membrane fraction was determined by monitoring the formation of L-[3 H]citrulline from L-[3 H]arginine in buffer A with the addition of UA, 1 mM NADPH, 100 nM calmodulin, 10 μ M BH_4 , and 5 μ M L-arginine containing purified L-[3 H]arginine for 30 min at 37°C. The measurement of L-[3 H]citrulline formation was performed as previously described (27).

Western blot analysis of eNOS and arginase expressions in cell lysates. PAEC were washed twice with ice-cold PBS without calcium or magnesium and scraped in lysis buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 0.4% deoxycholate, and 60 mM octylglucoside) containing protease and phosphatase inhibitor cocktails (Calbiochem). The protocols for Western blot analysis and for quantification of the density of the bands have been reported by us (15). For the analysis, anti-eNOS (BD Transduction Laboratories) and anti-arginase I or II (Santa Cruz Biotechnology) antibodies were used.

Arginase assays. Arginase activity in intact PAEC was determined by measuring urea contents in the culture media using α -isonitrosopropiophenone (9% in absolute ethanol) as previously described (8). Cells grown in 100-mm dishes were incubated in 7 ml of serum-free RPMI with or without UA, and three aliquots of culture media (each 350 μ l) were taken for measuring urea content. The amount of urea produced was used as an index of arginase activity. For testing the direct effects of UA on arginase, cell lysates were used. To prepare PAEC lysates for arginase assay, PAEC were grown to confluence in 100-mm dishes, rinsed twice with ice-cold HBSS, and then scraped into 0.5 ml of lysis buffer containing 50 mM Tris·HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM EGTA, and protease inhibitors [inhibitor cocktail Set III (Calbiochem)]. PAEC were lysed by sonication for 30 s. Cell lysates were incubated with L-arginine (0.5 M; pH 9.7) with or without UA (7.5

mg/dl) at 37°C for 60 min. The hydrolysis reaction of L-arginine by arginase was stopped by adding 750 μ l of an acid solution mixture ($\text{H}_2\text{SO}_4\text{-H}_3\text{PO}_4\text{-H}_2\text{O}$, 1:3:7), and urea produced was determined using α -isonitrosopropiophenone.

Method of measurement of arginase gene expression by RT-PCR. Total RNA was isolated with TRIzol reagent (Life Technologies, Rockville, MD), cleaned with RNeasy Minelute Cleanup Kit (Qiagen), and treated with DNase I (Ambion). Purified RNA (2 μ g) was converted to cDNA with SuperScript III reverse Transcriptase (Invitrogen) with Oligo d(T)₂₀ primers in a total volume of 20 μ l. Real-time PCR was conducted using Applied Biosystems Power SYBR Green PCR master mix on an ABI Prism 7500 sequence detection system. PCR primers for porcine arginase I (accession number: AY039112), arginase II (accession number: NM009705),

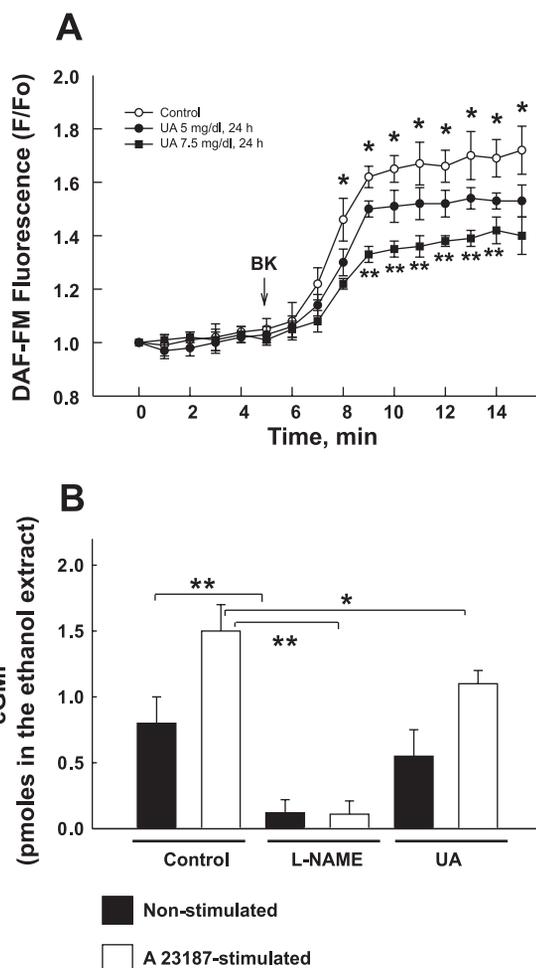


Fig. 1. Effect of uric acid (UA) on stimulated nitric oxide (NO) production (A) and cGMP accumulation (B) in pulmonary arterial endothelial cells (PAEC). A: PAEC were incubated in the absence (control) or presence of UA (5 or 7.5 mg/dl) in culture media for 24 h. After the incubation, NO detection using a fluorescent probe 5-methylamino-2,7-difluoro fluorescein (DAF-FM) was performed as described in EXPERIMENTAL PROCEDURES. Each point represents the mean DAF-FM fluorescence \pm SD at each particular time point. * P < 0.05 control vs. UA 5 mg/dl; ** P < 0.05 UA 7.5 mg/dl vs. UA 5 mg/dl. B: PAEC grown in 6-well plates were incubated with 200 μ M nitro-L-arginine methyl ester (L-NAME) for 1 h or 7.5 mg/dl UA for 24 h. In the end of the treatment period, the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (0.3 mM) was added to cells and 15 min later PAEC were stimulated with A23187 (10 μ M) for 10 min. In the end of the stimulation, PAEC were washed with PBS and scraped in 0.3 ml of 65% ethanol to get the ethanol extract. Extracted cGMP was determined using a cGMP detection kit. Bars are the means of 2 experiments made in duplicate. * P < 0.05; ** P < 0.01.

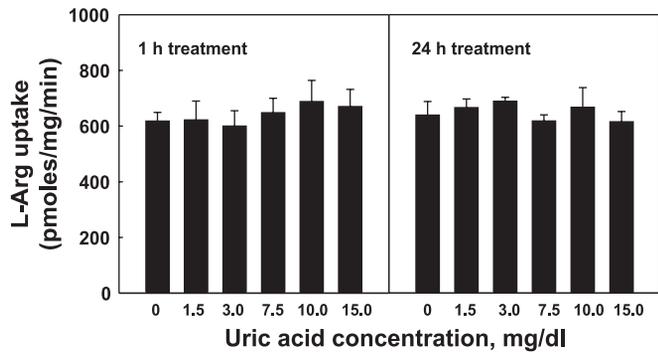


Fig. 2. Effects of UA on L-arginine uptake by PAEC. Cultured PAEC were incubated with different concentrations of UA dissolved in serum-free RPMI. After incubation, Na-independent L-[³H]arginine transport was assayed as described in EXPERIMENTAL PROCEDURES. Results are the means ± SE of 3 experiments with 4 replicates in each experiment.

and β-actin (accession number: U07786) were as follows: arginase I forward 5'-TGAAAGATTACGGGGACCTG-3', reverse: 5'-CTTTTCCCACAGACCTTGGGA-3' (product size 87 bp) arginase II forward 5'-CACCCCTCACCACTTCATCT-3' (product size 97 bp), reverse 5'-GAAAATCCTGGGAGTTGTGG-3'; β-actin forward 5'-GGACCTGACCGACTACCTCA-3', reverse 5'-GCGACGTAGCAGACTTTC-3' (product size 111 bp). All samples were run in triplicate. Melting curve analysis for arginase I, arginase II, and β-actin amplification products indicated one specific product for each and no primer-dimer formation. For negative controls, the same RNA preparations were used with the omission of the reverse-transcriptase step to confirm the absence of DNA contamination. Relative gene expression of arginase I and arginase II was estimated by ABI Prism 7500 software with β-actin mRNA used as an internal reference.

Vasorelaxation of porcine PAS. Porcine PAS (2.5–3 mm diameter × 3–4 mm length) were isolated from the lungs of 6- to 7-mo-old pigs. Vasorelaxation of PAS was evaluated as previously described (11, 24). Briefly, PAS suspended in individual organ

chambers (Radnoti Four-Unit Tissue Bath System) with 5 ml Earl's solution oxygenated with 95% O₂-5% CO₂ at 37°C. After 1-h equilibration at a resting force of 1.5 g, the vascular integrity of the PAS was assessed by monitoring 0.5 μM U-46619 (a thromboxane A₂ mimetic)-mediated PAS contraction and ACh (5 μM)-mediated vasodilation, respectively. Once vascular integrity was confirmed, PAS were washed several times and then incubated with UA (7.5 mg/dl) or (S)-(2-boronoethyl)-L-cystein (BEC, an arginase inhibitor; 100 μM) or UA and BEC together in an organ bath chamber for 60 min. Stable contraction was induced by 0.5 μM U-46619 for 10 min before ACh-induced vasorelaxation was tested. The vascular tensions were continuously monitored with an isometric force transducer (Harvard Apparatus, Holliston, MA).

Statistics. Data are presented as means ± SE. Statistically significant differences in mean values were tested by ANOVA. If ANOVA indicated significant differences, the data were further analyzed with a post hoc Tukey-HSD test. Differences were considered statistically significant if *P* < 0.05.

RESULTS

UA decreases NO production and inhibits accumulation of intracellular cGMP in PAEC. We tested the effects of UA on production of NO by cultured PAEC using the fluorescent probe DAF-FM (12). DAF-FM can detect real-time changes in intracellular NO following stimulation of NO production in endothelial cells with agents such as ACh, bradykinin, or calcium ionophores. Figure 1A demonstrates that UA causes a dose-dependent decrease in the NO production in cultured PAEC stimulated by 2 μM BK.

To confirm the observation that UA inhibits stimulated synthesis of NO in PAEC, we measured intracellular concentrations of the NO second messenger cGMP (22) in cells treated with UA. Figure 1B demonstrates that UA significantly decreases accumulation of cGMP in PAEC stimulated by the calcium-ionophore A23187 (10 μM). Accumulation of cGMP

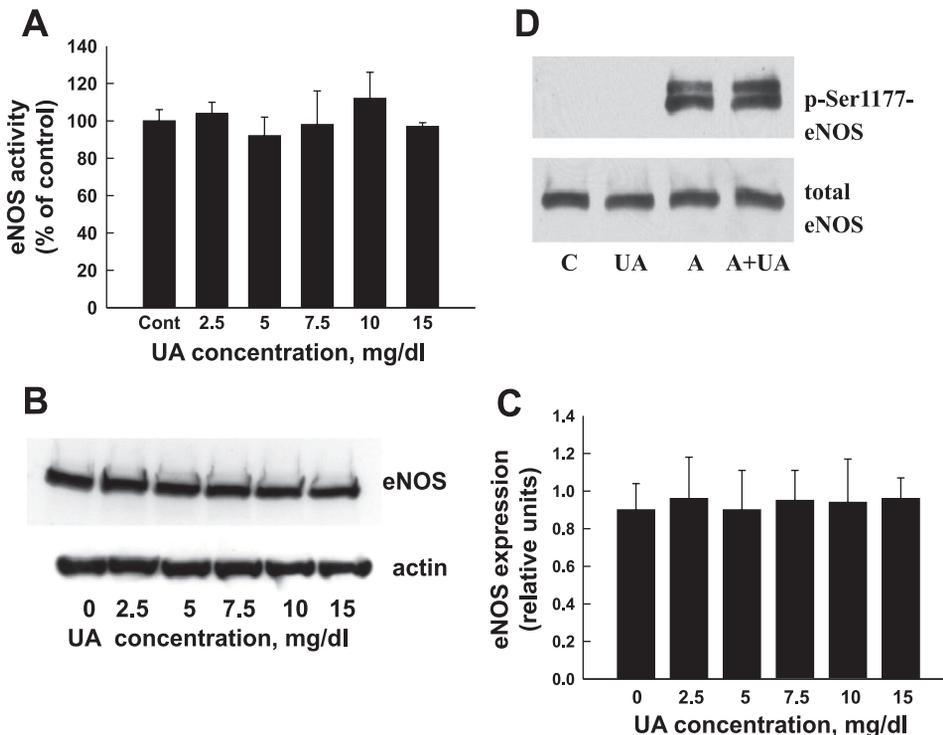


Fig. 3. Effects of UA on activity and expression of endothelial NO synthase (eNOS) in PAEC. A: cultured PAEC grown in 100-mm Petri dishes were incubated with different concentrations of UA dissolved in serum-free RPMI for 24 h. After the incubation, the total membrane fraction was isolated from PAEC, and eNOS activity was determined as described in EXPERIMENTAL PROCEDURES. B and C: Western blot analysis of eNOS contents was performed in cell lysates isolated from PAEC using anti-eNOS antibody. Western blot images from one of the experiments (B) as well as densitometric analysis of the data from all experiments (three) (means ± SE) are shown (C). The levels of eNOS expression are normalized to the actin levels in the same lysate samples. D: Western blot analysis of phospho-Ser¹¹⁷⁷-eNOS in PAEC stimulated with A23187 (10 μM). PAEC were grown on 100-mm dishes in the absence (Control, C) or presence of UA (7.5 mg/dl) for 24 h. After incubation with UA, culture medium was removed, and cells were stimulated with A23187 (A) dissolved in Hanks' balanced salt solution for 5 min. Western blot analysis of phospho-Ser¹¹⁷⁷-eNOS and total eNOS contents was performed in cell lysates using corresponding anti-eNOS antibody.

was completely blocked in stimulated and nonstimulated PAEC after preliminary incubation of cells for 1 h with L-NAME (200 μ M), suggesting that cGMP accumulation is NO dependent. Taken together with the DAF-FM data, these observations indicate that UA decreases NO production by PAEC.

Uric acid does not affect L-arginine transport and eNOS activity in PAEC. To investigate the mechanisms of reduced NO production in PAEC treated with UA, we tested the effects of UA on L-arginine transport and on eNOS activity and expression in PAEC. Cultured PAEC were incubated for 1 or 24 h with different concentrations of UA. After incubation, transport of L-[3 H]arginine was assayed. We found (Fig. 2) that neither short-term (1 h) treatment nor 24-h treatment of cultured PAEC with different concentrations of UA changed L-arginine uptake by PAEC.

Similarly, we did not observe changes in eNOS activity in the total membrane fraction isolated from PAEC treated with different concentrations of UA for 24 h (Fig. 3A) nor changes in eNOS expression in PAEC lysates (Fig. 3, B and C). We also tested the effects of UA on stimulated phosphorylation of eNOS. Ionophore A23187 (10 μ M) induced phosphorylation of eNOS at Ser¹¹⁷⁷ (Fig. 3D), and this response to A23187 was not changed in PAEC treated with UA (7.5 mg/dl) for 24 h (Fig. 3D). These data demonstrate that UA does not directly affect eNOS activity and expression under stimulated and nonstimulated conditions.

UA increases arginase activity in endothelial cells. To test the concept that UA affects arginase activity, which in turn might diminish L-arginine availability for NO production, we studied changes in urea production by cultured PAEC incubated with UA. For this, we measured urea content in culture media, and the amount of urea produced was used as an index of arginase activity. We found that PAEC treated with UA (5.0 or 7.5 mg/dl) produced significantly more urea than control cells in a dose-dependent manner (Fig. 4A). To determine whether the major source of urea in culture media was from the L-arginine-arginase enzymatic reaction, we measured urea production by PAEC grown in the presence of different amino acids in the culture media (Fig. 4B). In the presence of 10 mM L-lysine, which decreases cationic amino acid transporter-mediated L-arginine delivery in PAEC and substitutes for L-arginine inside the cells, urea production by PAEC was significantly reduced, whereas in the presence of 10 mM L-arginine (instead of normal 1 mM in the RPMI 1640 media) urea production was significantly increased. Amino acids that do not interfere with L-arginine uptake via cationic amino acid transporters such as 10 mM leucine or 10 mM alanine did not change urea production by PAEC. These data indicate that PAEC produce urea mainly through the L-arginine-arginase enzymatic reaction.

Possible mechanisms of UA-induced arginase activation were evaluated in experiments using a concentration of 7.5 mg/dl of UA. Recent studies have shown that two isoforms of arginase exist, arginase I and II, which differ in tissue and intracellular localization (21). According to studies from our laboratory and others (19, 30), arginase II is the major isoform of arginase expressed in endothelial cells. RT-PCR analysis demonstrated that UA did not alter gene expression of arginase II in PAEC (Fig. 5A). Incubation of PAEC with UA (7.5 mg/dl) for 24 h also did not change arginase II protein expression in

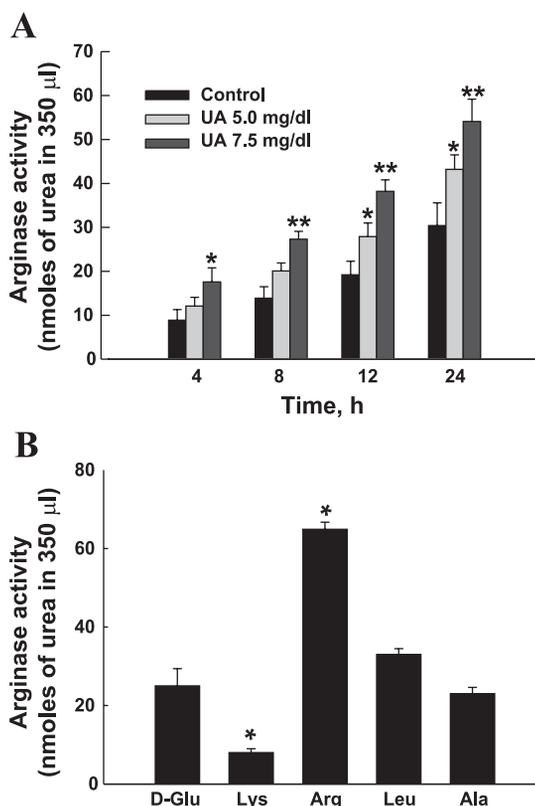


Fig. 4. Effects of UA and amino acids on urea production by PAEC. A: effects of UA on urea production by PAEC. Culture medium (350 μ l) was taken for urea measurements at 4, 8, 12, and 24 h after the beginning of incubation with UA (5.0 or 7.5 mg/dl). The values of urea contents in the culture media at 0 time points (at the beginning of cell incubation with UA) were subtracted from values determined after 4, 8, 12, or 24 h incubation. Data are from 3 experiments made in duplicates. * $P < 0.05$ vs. control (without UA); ** $P < 0.01$ vs. corresponding controls and UA 5.0 mg/dl. B: PAEC were grown in 100-mm dishes in serum-free RPMI media (7 ml/dish) with addition of 10 mM D-glucose (as a control) or amino acids (10 mM L-lysine, 10 mM L-arginine, 10 mM L-leucine, or 10 mM L-alanine). After incubation for 24 h, samples of media were taken for analysis of urea contents. Data are from 2 experiments made in triplicate. * $P < 0.01$ vs. control.

PAEC (Fig. 5B), suggesting that UA (or its metabolites) are able to stimulate arginase II activity directly without changing enzyme expression. To test the possibility that UA stimulates arginase II activity directly, we determined arginase activity in PAEC homogenates in the presence and absence of UA in the reaction mixture. We found that in the presence of UA (5.0 and 7.5 mg/dl), arginase activity in cell homogenates was significantly higher compared with the reaction mixture without UA (Fig. 5C). Next, we determined how UA affected the Michaelis-Menten kinetics of arginase activity in these cell homogenates. For this, we measured urea production by cell homogenates at different concentrations of L-arginine (0.28–35.7 mM) in the absence or the presence of UA (7.5 mg/dl) in the incubation mixture. Analysis of data using a Lineweaver-Burk plot (Fig. 5D) demonstrates that UA increases the affinity of arginase for L-arginine in homogenates of PAEC ($K_m = 1.1 \pm 0.1$ mM in control vs. 0.6 ± 0.1 mM in the presence of UA; $P < 0.01$) without affecting significantly maximal velocity (V_{max} , 543 ± 100 vs. 452 ± 130 nmol \cdot mg $^{-1}\cdot$ h $^{-1}$ in the presence of UA; $P > 0.05$). To test whether the effects of UA are specific for endothelial

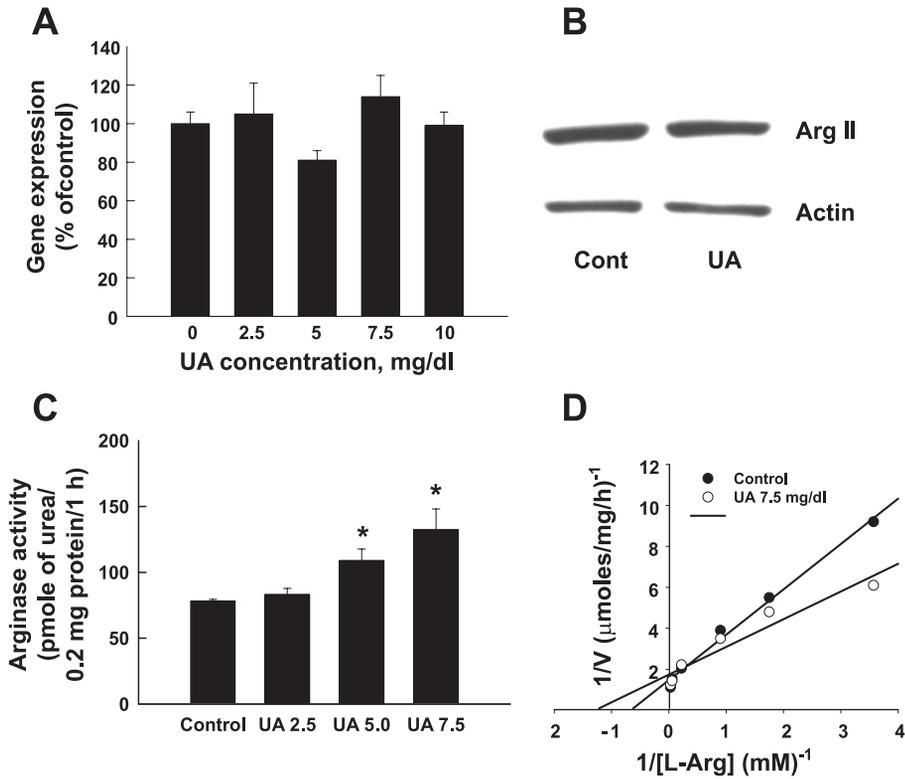


Fig. 5. Effects of UA on arginase expression and activity in PAEC. *A*: arginase II (Arg II) gene expression in PAEC measured by RT-PCR. Cultured PAEC were incubated with different concentrations of UA dissolved in serum-free RPMI for 24 h. Relative gene expression was estimated as described in EXPERIMENTAL PROCEDURES. Data are from 2 experiments run in triplicate. *B*: Western blot analysis of the UA effects on Arg II protein expression. PAEC were grown in 100-mm dishes containing 10 ml of serum-free RPMI without or with UA (7.5 mg/dl) for 24 h. After incubation, PAEC were collected in lysis buffer, and Western blot analysis was performed using anti-Arg II antibody (Santa Cruz Biotechnology). Typical images from 4 independent experiments are presented. *C*: effect of UA on arginase activity in PAEC lysates. Arginase activity in cell lysates was determined as described in EXPERIMENTAL PROCEDURES in the reaction media without (Control) or with UA (2.5, 5.0, or 7.5 mg/dl). Data are from 3 experiments made in duplicate. **P* < 0.05. *D*: analysis of the Michaelis-Menten (*K_m*) kinetics for arginase in PAEC lysates. Cell lysates were incubated with different concentrations of L-arginine in the absence (Control) or the presence of UA (7.5 mg/dl) in the incubation mixture for 1 h and then amounts of synthesized urea were measured. The parameters of maximal velocity (*V_{max}*) and *K_m* were evaluated using Lineweaver-Burk double-reciprocal plots. Data are from one typical experiment of 4 independent experiments are presented.

arginase II, we determined arginase activity in rat kidney and liver tissue lysates (the kidney expresses the arginase II isoform; the liver expresses the arginase I isoform) in the absence and the presence UA (7.5 mg/dl). We found that UA activated arginase activity in kidney and liver lysates (Fig.

6). Similar to the effects of UA on endothelial arginase II, UA-induced activation of arginase I in the rat liver homogenates and arginase II in the rat kidney homogenates were mediated through increased arginase affinity for L-arginine (Fig. 6, *C* and *D*).

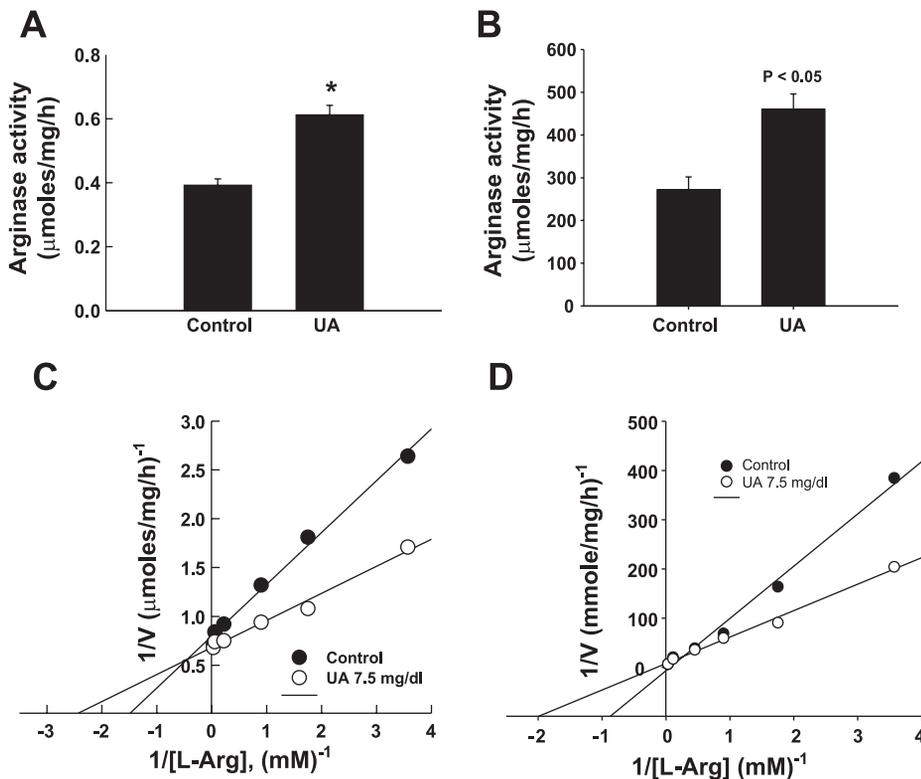


Fig. 6. Effects of UA on arginase activity in tissue homogenates of the rat kidney and liver. Tissue homogenates of kidney (*A*) and liver (*B*) were incubated with 125 mM L-arginine in the absence (Cont) or the presence of UA (7.5 mg/dl) in the incubation mixture. After 1-h incubation, urea produced was determined. Data are from 3 experiments made in duplicate. **P* < 0.05. *C* and *D*: analysis of *K_m* kinetics for arginases in rat tissue homogenates. Tissue homogenates of rat kidney (*C*) and liver (*D*) were incubated with different concentrations of L-arginine (0.28–35.7 mM) in the absence (Control) or the presence of UA (7.5 mg/dl) for 1 h. After the incubation, urea contents were measured. The parameters of *V_{max}* and *K_m* were determined using Lineweaver-Burk plots and demonstrated that UA increased arginase affinity for L-arginine. Data are from one typical experiment of 3 independent experiments are presented.

Arginase inhibitor prevents the inhibitory effect of UA on stimulated production of cGMP and abolishes UA-induced inhibition of ACh-stimulated vasodilation of PAS. The arginase inhibitor BEC (100 μ M) blocked the UA-induced increase in urea production by PAEC (Fig. 7A). At the same time, BEC prevented the inhibitory effects of UA on cGMP production in PAEC stimulated by A23187 (Fig. 7B). To test whether arginase inhibition alters the physiological effects of UA, we studied vasorelaxation of porcine PAS treated with concentrations of UA corresponding to plasma concentrations observed in hyperuricemic patients. These experiments demonstrated that UA (7.5 mg/dl) inhibited ACh-induced vasorelaxation of porcine PAS (Fig. 7C). The arginase inhibitor BEC (100 μ M) abolished the inhibitory effects of UA on ACh-induced vasorelaxation of PAS (Fig. 7C).

DISCUSSION

The present experiments revealed that UA attenuated production of NO stimulated by BK or A23187 and production of its second messenger cGMP by cultured porcine PAEC. Similar effects of UA were reported in bovine aortic endothelial cells (14) and in human umbilical vein endothelial cells (13)

supporting the notion that UA has a widespread effect of inhibiting vascular endothelial NO production.

Endothelial cells generate NO from L-arginine via a constitutive eNOS (26), and it follows that the UA-induced reduction of NO production in PAEC is due to some impairment in the L-arginine-eNOS pathway. Our experiments with detection of L-arginine transport in UA-treated PAEC indicate that if changes in L-arginine availability are involved in the UA-induced reduction of NO production, they are not mediated through alterations in L-arginine uptake. We also did not observe changes in eNOS activity in the total membrane fraction isolated from PAEC treated with different concentrations of UA or changes in the total eNOS expression in PAEC lysates or changes in A23187-stimulated eNOS phosphorylation, demonstrating that UA does not directly affect eNOS activity and expression under stimulated and nonstimulated conditions. NO production by eNOS depends not only upon intracellular L-arginine availability but the availability of cofactors, in particular, tetrahydrobiopterin (20). It was shown that UA scavenging of peroxynitrite partly protected eNOS from enzymatic uncoupling (16), suggesting that UA should be beneficial for NO production by preventing oxidation of tetra-

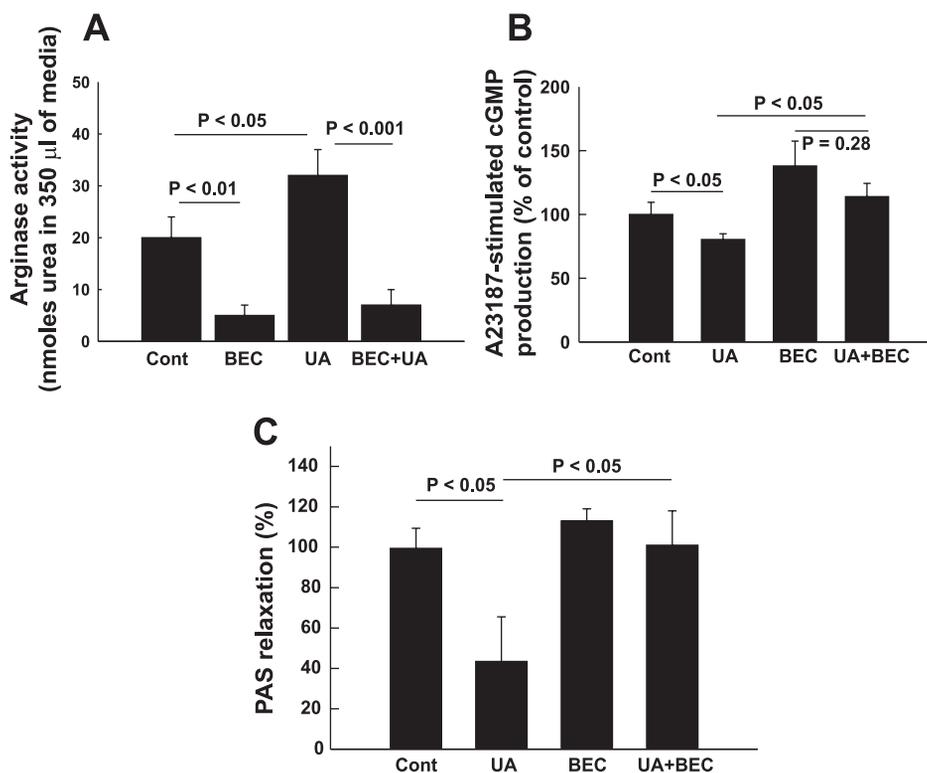


Fig. 7. Arginase inhibition prevents the UA effect on cGMP production in PAEC and on vasorelaxation of U-46619-contracted porcine PAS. A: an arginase inhibitor (S)-(2-boronoethyl)-L-cysteine (BEC) blocks UA-induced stimulation of urea production. PAEC were grown on 100-mm dishes in 7 ml of RPMI without (Control) or with an arginase inhibitor BEC (100 μ M), UA (7.5 mg/dl), or UA + BEC for 24 h. At the end of the incubation period, aliquots of culture media were taken for analysis of urea contents. Data are from 3 experiments made in duplicate. B: arginase inhibitor BEC prevents the UA-induced reduction of cGMP production by PAEC. PAEC grown on 60-mm dishes were treated without (Control) or with an arginase inhibitor BEC (100 μ M), UA (7.5 mg/dl), or UA + BEC for 1 h. In the end of the treatment period, the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (0.3 mM) was added to cells and 15 min later PAEC were stimulated with A23187 (10 μ M) for 15 min. In the end of the stimulation, PAEC were washed with PBS and scraped in 0.3 ml of 65% ethanol to get the ethanol extract. Contents of cGMP in 50 μ l of the ethanol extract were determined using a cGMP detection kit. Bars are the means \pm SE of 4 experiments made in duplicate. C: arginase inhibitor BEC prevents the UA effects on vasorelaxation of U-46619-contracted porcine PAS. PAS were washed and incubated without (Control) or with BEC (100 μ M), UA (7.5 mg/dl), or UA + BEC for 1 h in Earl's buffer at 37°C for 1 h. PAS were precontracted with a thromboxane mimetic U-46619 (0.5 μ M), and then the vasorelaxation responses to ACh were assessed in PAS incubated without (control) or with corresponding drugs. To standardize the data, the vascular tone of U-46619-contracted PAS was set to 0% relaxation. For each treatment, 4 separate PAS were used.

hydrobiopterin. The absence of activating effects of UA on the L-[³H]arginine to L-[³H]citrulline conversion by PAEC eNOS in the membrane fraction in our experiments can be explained by the fact that eNOS activity was performed in the absence of exogenous oxidants (such as peroxynitrites) and because tetrahydrobiopterin was included in the reaction mixture.

Before the discovery of L-arginine as the substrate for NO synthesis, it was known that L-arginine is metabolized by arginase in the L-arginine-urea cycle (21). Two isoforms of arginase exist, arginase I and II, which differ in tissue and intracellular localization. The dominant isoform of arginase in endothelial cells is arginase II (19, 30). Competing with eNOS for a common substrate, arginases have a potential to modulate NO production in endothelial cells, probably by changing the availability of intracellular L-arginine for eNOS (4, 7, 28).

Our experiments demonstrated that UA is able to modulate arginase activity in PAEC. Treatment of PAEC with UA did not affect arginase mRNA or protein expression, indicating that UA modifies the activity of existing arginase. What is more, the addition of UA to PAEC lysates stimulated urea production from L-arginine supporting the notion that UA stimulates arginase directly or through interaction with components present in cell lysates. The kinetic analysis of arginase activation by UA demonstrated that UA affects the affinity of arginase for L-arginine, suggesting that UA may modify the structure of the L-arginine binding site. This effect of UA was not specific for endothelial arginase. A similar increase in the affinity for L-arginine was observed in homogenates of rat liver (expressing arginase I) and kidney (expressing arginase II) after the addition of UA, indicating that there is no specificity in the UA action regarding different isoforms of arginase.

The mechanism(s) of arginase activation by UA is not clear and an evaluation of the potential mechanisms of UA-induced arginase activation and changes in the affinity of arginase for L-arginine is a goal of our future investigation. Independent of the mechanisms that might be involved, UA-induced arginase activation attenuates stimulated cGMP production by PAEC, and the arginase inhibitor BEC prevented the inhibitory effects of UA on cGMP production in PAEC. These observations confirm the data of others that endothelial arginase contributes to impaired NO signaling (4, 7, 28).

The fact that UA activates arginase may have an important physiological significance. Our experiments in isolated PAS have demonstrated that UA impairs pulmonary artery responses to ACh, and arginase inhibition recovers this impairment. In humans, pulmonary hypertension is commonly associated with hyperuricemia (3, 23, 31). Because of the ability of UA to activate endothelial arginase, increased plasma contents of UA in patients with pulmonary hypertension could potentially contribute to reduced local NO levels in the pulmonary vasculature that may have a role in the hemodynamic changes. In addition, it has been shown that arginase may regulate cell proliferation through participation in polyamine synthesis. The coproduct of arginase activity is L-ornithine, a substrate for ornithine decarboxylase, the initial enzyme in the polyamine biosynthetic pathway. Arginase activity might be a limiting factor for polyamine synthesis and cell proliferation in endothelial cells (17, 18) as well as in vascular smooth muscle cells (25, 21). Because of its ability to activate arginase, UA might induce accelerated proliferation of vascular smooth muscle cells. Together with impaired NO production by lung endothe-

lial cells, this acceleration might have a significant impact on obliteration of pulmonary blood vessels. This might explain why pulmonary hypertensive patients with hyperuricemia live dramatically shorter than patients without hyperuricemia (23, 33).

In conclusion, UA inhibits vasodilation in the lung by reducing NO production by endothelial cells. The decrease in NO production in lung endothelial cells by UA is mediated via the L-arginine-arginase enzymatic reaction. Inhibition of arginase attenuates the inhibitory effects of UA on NO production by lung endothelial cells. These studies support a role for UA in the altered NO metabolism that has been observed in pulmonary hypertension.

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DISCLOSURES

R. J. Johnson is listed as an inventor on pending patent applications on lowering uric acid in cardiorenal disease that have been filed by the University of Washington, University of Florida and Cell Systems. R. J. Johnson also is on the Scientific Board for Nephromics, which evaluates diagnostics for preeclampsia. Other authors do not have conflicts of interests.

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