ORIGINAL ARTICLE

The effect of uric acid on homocysteine-induced endothelial dysfunction in bovine aortic endothelial cells

Ivana Papezikova, Michaela Pekarova, Antonín Lojek, Lukas Kubala

Institute of Biophysics, Academy of Sciences of the Czech Republic, Brno, Czech Republic

Correspondence to: Lukáš Kubala PhD, Institute of Biophysics, v. v. i., Academy of Sciences of The Czech Republic, Královopolská 135, 612 65 Brno, Czech Republic
PHONE: +420-541 517 117, FAX: +420-541 211 293, E-MAIL: kubalal@ibp.cz

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Abstract

OBJECTIVES: Elevated plasma uric acid indicates an increased risk of cardiovascular diseases associated with endothelial dysfunction. However, the role of uric acid in the pathogenesis of endothelial dysfunction is still a matter of debate. It is not clear whether uric acid is a real causative risk factor, an inert marker, or even a protective molecule with respect to its antioxidant properties. We have studied the effect of uric acid on intact endothelial cells as well as cells with homocysteine-induced endothelial dysfunction.

DESIGN: Bovine aortic endothelial cells were treated with uric acid (100 – 600 μM) and homocysteine (100 μM) or with uric acid only. After 24 hours, the cells were stimulated with 1 μg/ml of calcium ionophore A23187, and nitric oxide (NO) production was measured electrochemically with the use of a NO-sensitive microelectrode. The expression of endothelial nitric oxide synthase (eNOS) and eNOS phosphorylation at Ser1179 was estimated with the use of Western blotting. Interaction between NO and uric acid was measured with a NO electrode. Superoxide generation was measured with the use of the fluorescence dye MitoSox Red.

RESULTS: Homocysteine strongly diminished A23187-induced NO release. 100 μM uric acid slightly restored NO production; higher concentrations were ineffective. Interestingly, a dose-dependent decrease of NO release was observed in the cells treated only with uric acid. Uric acid did not scavenge NO and did not change eNOS protein expression or phosphorylation at Ser1179, but dose-dependently increased superoxide production in A23187-stimulated cells.

CONCLUSION: In conclusion, uric acid decreased NO bioavailability and enhanced superoxide generation in A23187-stimulated bovine aortic endothelial cells.

INTRODUCTION

It has been known for many years that elevated plasma uric acid is strongly associated with increased cardiovascular risk (Hayden & Tyagi, 2004). Hyperuricaemia is a good predictor of cardiovascular events and hypertension development, as well as adverse outcome of heart failure and stroke (Reynolds, 2007). However, the role of uric acid in the pathogenesis of cardiovascular diseases is still a matter of debate. The potential harmful effect of uric acid contrasts with the fact that uric acid is one of the most important antioxidants in body fluids, which effectively scavenges a variety of reactive oxygen species (Becker et al., 1991). Unravelling the role of uric acid in the develop-

Abbreviations & units

BAEC	bovine aortic endothelial cells
DMEM	Dulbecco´s Modified Eagle Medium
eNOS	endothelial nitric oxide synthase
Hcy	homocysteine
UA	uric acid

ment of cardiovascular disease is complicated by the frequent presence of other risk factors as well as by the fact that uric acid production by xanthine oxidoreductase is accompanied by the generation of the superoxide radical, which is clearly involved in the pathogenesis of cardiovascular diseases. It is not clear, therefore, whether uric acid is a real causative risk factor, an inert marker, or even a protective molecule with respect to its antioxidant properties (Kannelis & Kang, 2005).

This work is focused on the effect of uric acid on nitric oxide (NO) production by endothelial cells. A decreased NO bioavailability is a hallmark of endothelial dysfunction, which is an early stage in the development of cardiovascular diseases. The effect of uric acid was studied on intact cells as well as on cells with homocysteine-induced endothelial dysfunction. This design was chosen to see the potentially adverse effect of uric acid (induction of endothelial dysfunction in intact cells) or beneficial effect (abolishing of homocysteine-induced endothelial dysfunction).

MATERIAL AND METHODS

Cell culture and treatment. BAEC (ECACC, UK) were cultivated in Bovine Endothelial Cell Growth Medium (ECACC, UK). For treatments, serum free DMEM (PAN, Germany) was used. Confluent cells at passage 4 – 7 were used for the experiments.

Electrochemical measurement of NO production, and protein concentration in the cell lysates. The cells were cultivated in collagen-coated dishes (TPP, Sweden). When the cells reached confluence, the medium was changed for serum-free DMEM, and the cells were pre-treated with 100, 300 or 600 μM of sterile filtered uric acid (Sigma, Germany). After 2 hours, 100 μM of DL-homocysteine (Sigma, Germany) was added. After 24 h the cells were washed, the medium was changed and a NO sensitive microelectrode (Innovative Instruments, USA) was immersed into the medium. After stabilization of the signal, the cells were stimulated with 1 μg/ml of A23187 and NO production was followed up for 5 minutes. After the measurement, the cells were lysed for Western blot analysis with a SDS lysing buffer adjusted with protease inhibitor cocktail and PhosphoStop (Roche). The protein levels in the cell lysates were measured with the use of BCA protein assay (Pierce).

Cell viability. BAEC were treated with uric acid and homocysteine and stimulated with A23187, as described above. Cell viability was measured luminometrically with the use of the ATP Cell Viability test (BioThema, Sweden), as described by Lojek *et al.* (2008).

Uric acid reactivity with NO. A NO electrode was immersed in 100, 300 or 600 μM uric acid dissolved in DMEM. The solutions were mixed continuously, and the temperature was kept at 37°C. After the NO electrode background signal was stable and reached a plateau, distilled water saturated with NO gas was injected into the solution, reaching a final concentration of 1.74 μM of NO. After the injection, rapid signal increase followed. The resulting curves were compared to the curve obtained after NO injection to uric acid–free DMEM.

Uric acid interaction with NO electrode. The NO electrode was stabilized in 9.5 ml of serum free DMEM. Then 0.5 ml of 6 mM uric acid was injected into the vial. The signal change was followed up and compared to the change after the injection of 0.5 ml of DMEM.

Expression of eNOS, phosphorylation at Ser1179. Western blot analysis was performed in a standard manner. For determining the total eNOS and phospho-eNOS, polyclonal rabbit anti-eNOS and anti-phospho eNOS antibodies (Cell Signalling, 1:500) and donkey anti-rabbit IgG horseradish peroxidase –conjugated antibody (GE Healthcare, 1:3000) were used. The immunoreactive bands were detected, and the results were evaluated, as described previously by Papežíková *et al.* (2008).

Superoxide generation. Superoxide generation was measured with the use of fluorescent probe MitoSox Red. The cells were cultivated on collagen-coated 6 well plates and treated with uric acid and homocysteine as described above. After 24 hours, the cells were washed twice and incubated with 5 μM MitoSox Red in serum-free DMEM for 10 minutes. Then the cells were stimulated with A23187. After 20 minutes, the cells were washed and detached with trypsine/EDTA. Cell suspension was pipetted onto a fluorimetric plate and fluorescence was measured with excitation/emission wavelenghts set on 510/592 nm respectively.

Statistical analysis. Data are reported as mean ± SEM of three independent experiments. Data were evaluated with the use of Student t-test, and statistical significance was assumed when $p<0.05$.

RESULTS

Our results show that homocysteine strongly decreased A23187-stimulated NO release, (***Figure 1***) without a significant effect on cell viability (data not shown). Treatment with uric acid did not prevent a decrease in NO production. Only 100 μM concentration, which corresponds to the lower limit of the human physiological range, had a slightly protective effect. In the cells treated only with uric acid, a dose-dependent decrease in NO release was observed (***Figure 2***). The experiments in chemical systems revealed neither uric acid interference with NO-sensitive microelectrode (data not shown), nor direct NO scavenging by uric acid (***Figure 3***). Neither homocysteine nor uric acid had a significant effect on eNOS protein expression or phosphorylation

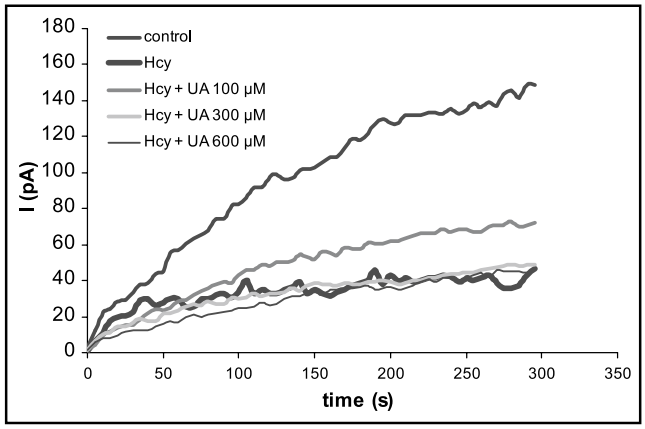

Figure 1. NO production measured electrochemically. Kinetic curves.

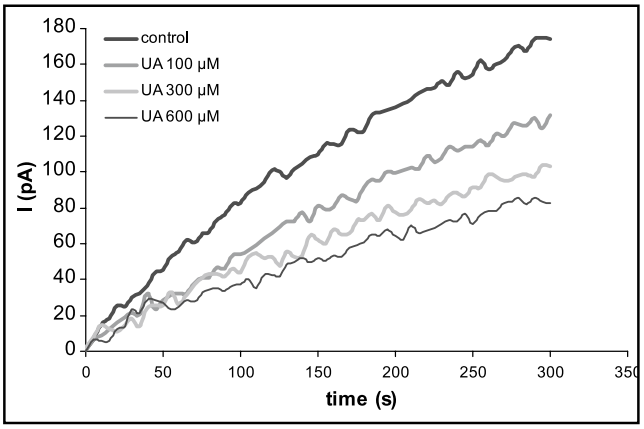

Figure 2. NO production measured electrochemically. Kinetic curves.

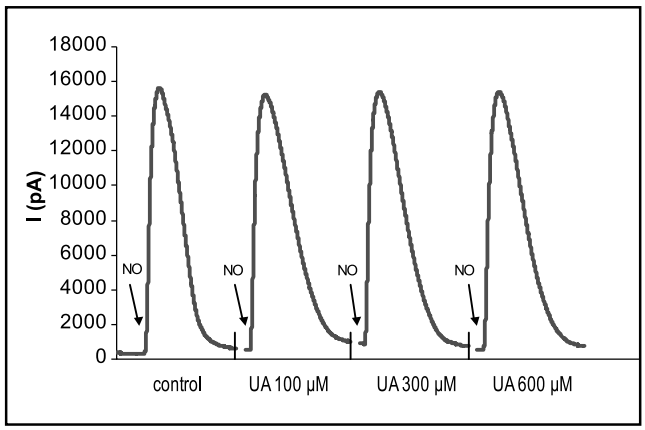

Figure 3. The effect of uric acid on NO-dependent signal detected electrochemically.

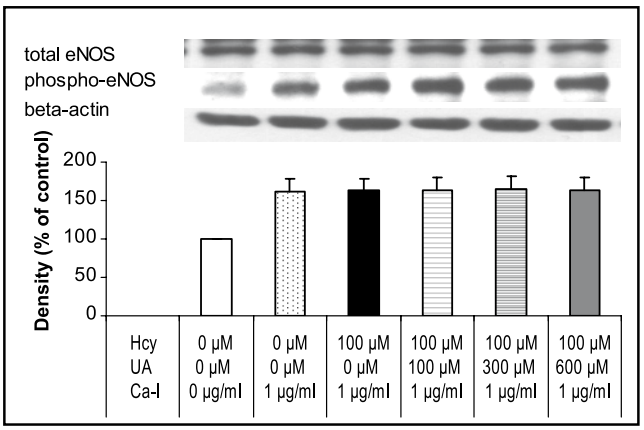

Figure 4. Expression of eNOS and phosphorylation at Ser1179. Density (phospho eNOS) and representative pictures.

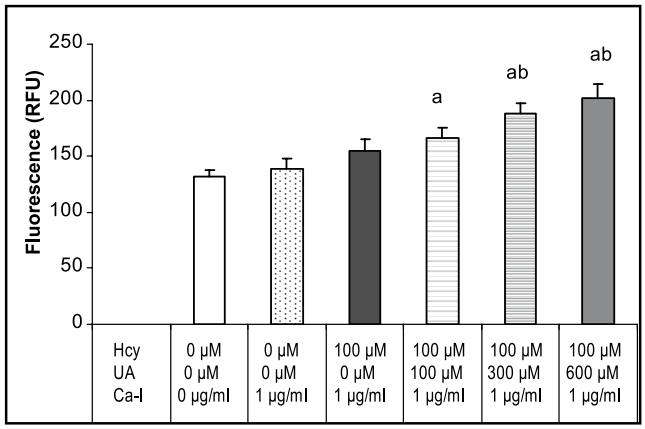

Figure 5. Superoxide production in A23187-stimulated BAEC. Statistically significant changes from control are marked with a, significant changes from Hcy-treated group are marked with b.

at activation site Ser1179 (***Figure 4***). Dose-dependently, uric acid enhanced superoxide generation in A23187-stimulated cells (***Figure 5***).

DISCUSSION

It was shown previously that homocysteine-induced endothelial dysfunction was associated with an increased oxidative stress and could be abolished by treatment with antioxidants (Ramaswami et al., 2004, Zhang et al., 2007). In our study, uric acid did not significantly prevent the development of endothelial dysfunction, despite its antioxidant properties. This was not surprising in light of finding that uric acid induced endothelial dysfunction in intact cells. Thus, the main question that arose involved the mechanism of decreasing NO levels by uric acid. To explain this effect, we tested uric acid interaction with a NO electrode, as uric acid is an electrochemically active compound that can be a source of interference in amperometrical measurements. After excluding this possibility, NO scavenging and inactivation by uric acid was considered. Reaction between uric acid and NO was documented by Suzuki (2006) and Gersch *et al.* (2008). However, high NO concentrations irrelevant for endothelial cells were used in both studies. Our results show that 100 - 600 µM uric acid did not inactivate NO, when NO was present at micromolar concentrations.

Impaired NO bioavailability is a consequence of decreased NO production, increased NO inactivation, or a combination of both (Chatterjee & Catravas, 2008). The production of endothelium-derived NO by eNOS is regulated by multiple mechanisms which involve eNOS protein expression and posttranslational modifications, phosphorylation/dephosphorylation of activatory/inhibitory sites and the presence of substrate and cofactors. (Govers & Rabelink, 2001). Our results show that neither homocysteine nor uric acid affected eNOS expression. A disturbance in eNOS activation by uric acid is implausible as well. One of the mechanisms regulating eNOS activity is a multi-site phosphorylation of specific serine and threonine residues on the enzyme (Mount *et al.* 2007). In this study, phosphorylation of Ser1179, the most important regulatory phosphorylation site, was evaluated and no significant changes were observed in any of A23187-stimulated groups. However, unchanged phosphorylation of Ser1179 does not necessarily mean unaffected NO production. When functional eNOS is depleted of cofactors or L-arginine and eNOS uncoupling occurs, NO synthesis cannot be completed, and the activated enzyme releases the superoxide radical instead of NO. This has three important consequences – a decrease in NO production, NO inactivation by superoxide, and a generation of peroxynitrite, a highly reactive molecule that is able to attack a variety of biologically important targets. Our results show that uric acid dose-dependently enhanced superoxide production in A23187-stimulated cells. Although this work does not provide evidence that uncoupled eNOS was a source of the detected superoxide, it is known that NO bioavailability is strongly dependent on the presence of superoxide, regardless of its origin. If superoxide is present at the same time and site as NO, it reacts with NO in a diffusion-controlled rate, giving rise to peroxynitrite. As mentioned above, uric acid is a potent antioxidant, which reacts with a variety of reactive oxygen/nitrogen species, giving rise to less reactive and less harmful products. However, uric acid virtually does not react with superoxide (Becker et al., 1991, Papežíková *et al.* 2007). Thus, uric acid could not prevent the reaction between superoxide and NO. At best, it could react with secondarily generated peroxynitrite and peroxynitrite-derived oxidants and prevent oxidative injury. These results can at least partially explain the paradoxical link between classical antioxidant uric acid and an increased risk of cardiovascular diseases associated with endothelial dysfunction.

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