Hyperuricemia Attenuates Aortic Nitric Oxide Generation, through Inhibition of Arginine Transport, in Rats

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Abstract

Objectives: Hyperuricemia provokes endothelial dysfunction (ECD). Decreased endothelial nitric oxide synthase (eNOS) activity is an important source of ECD. Cationic amino acid transporter-1 (CAT-1) is the specific arginine transporter for eNOS. We hypothesize that hyperuricemia inhibits arginine uptake. **Methods:** Experiments were performed in freshly harvested aortas from untreated animals and rats fed with oxonic acid (hyperuricemia), and compared to hyperuricemic rats treated with either allopurinol, benzbromarone or arginine. **Results:** Arginine transport was significantly decreased in hyperuricemia. Benzbromarone and arginine prevented the decrease in arginine transport in hyperuricemic rats while allopurinol did not. Arginine transport was significantly decreased in control rats treated with allopurinol. Blood pressure response to acetylcholine was significantly attenuated in hyperuricemic rats, an effect which was prevented in all other experimental groups. L-NAME inhibitable cGMP response to carbamyl-choline was significantly decreased in hyperuricemic rats and this was completely prevented by both benzbromarone and arginine, while allopurinol

partially prevented the aforementioned phenomenon. Hyperuricemia induced a significant increase in protein nitration that was prevented by benzbromarone, allopurinol, and arginine. Protein abundance of CAT-1, PKC α , and phosphorylated PKC α remained unchanged in all experimental groups. **Conclusions:** In hyperuricemia, the decrease in aortic eNOS activity is predominantly the result of attenuated arginine uptake.

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Introduction

Uric acid, a product of purine metabolism, is degraded in most mammals by the enzyme urate oxidase. Two mutations which occurred in early hominoid evolution rendered the uricase gene nonfunctional [1]. As a consequence, humans have higher serum uric acid levels compared with most mammals. Several conditions such as high-protein diet, alcohol consumption, high cell turnover, and renal failure, can result in high uric acid levels [2]. Cumulative evidence indicates that hyperuricemia is associated with cardiovascular morbidity. Experimental hyperuricemia in rodents resulted in the development of hypertension and kidney disease [3, 4]. Hyperuricemia is frequently found in patients with obesity, insulin resis-

tence, and hyperlipidemia [5]. Subjects with elevated serum uric acid levels are at high risk for developing hypertension and kidney disease [6, 7] and lowering serum uric acid in hyperuricemic individuals improved endothelial function [8]. Traditionally, uric acid has not been given an active role in the pathologic process underlying these conditions and it was even suggested that high serum uric acid levels may be protective for cardiac, vascular, and neural tissues as a powerful antioxidant [9].

Endothelial cell dysfunction (ECD) is a common precursor and denominator in patients with cardiovascular diseases. This syndrome is currently interpreted as encompassing disturbances in several functions: the barrier function of vascular endothelium, its impaired anti-thrombogenic properties, blunted angiogenic capacity, inappropriate regulation of vascular smooth muscle tonicity, proliferative capacity, and migratory properties [10]. Accumulated evidence suggests that many of these abnormalities are linked to the capacity of the constitutive, Ca^{2+} /calmodulin-sensitive nitric oxide synthase (eNOS) to generate adequate quantities of NO [11]. The administration of L-arginine (the sole precursor for NO synthesis) to hyperuricemic rats lowered arterial blood pressure and increased NOS-1 expression in the macula densa [4]. Uric acid infusion to healthy volunteers resulted in impaired acetylcholine-induced vasodilation in the forearm, suggesting that hyperuricemia exerts its deleterious effect on the endothelium via NO inhibition [12]. The mechanism by which uric acid attenuates NO generation is unknown. It was recently shown that uric acid activates NADPH oxidase resulting in increased production of reactive oxygen species, leading to decreased bioavailability of NO and increased protein nitration [9]. Our interest in this subject focuses on the role of intra-cellular arginine availability in governing eNOS activity, thus affecting endothelial function. Among several transport systems which mediate L-arginine uptake (γ^+ , $\text{b}^{0,+}$, $\text{B}^{0,+}$, and γ^+ L), system γ^+ is widely expressed and considered to be a major arginine transporter in most tissues and cells. Encoded by the cationic amino acid transporters CAT-1, CAT-2 and CAT-3, system γ^+ is characterized by high affinity for cationic amino acids, sodium independence, and stimulation of transport by substrate on the opposite (trans) side of the membrane [13, 14]. We hypothesize that delivery of transported arginine to membrane-bound eNOS, selectively by the cationic amino acid transporter-1 (CAT-1) rather than intra- or extra-cellular arginine concentration, is the predominant factor governing eNOS activity. Indeed, we have previously shown in several different animal models characterized

by endothelial dysfunction and decreased eNOS activity that CAT-1 activity is attenuated [15–18]. The predominant mechanism resulting in diminished CAT-1 activity in all these experiments was post-translational modulation of CAT-1 by protein kinase C α (PKC α) [15, 17, 18]. Using an experimental model of mild hyperuricemia, the experiments reported herein were designed initially to challenge again the hypothesis that hyperuricemia promotes endothelial dysfunction and to explore whether alterations in aortic arginine uptake by CAT-1 is the mechanism responsible for attenuated eNOS activity in these rats. We have also tried to elucidate a molecular mechanism to explain these observations.

Methods

Materials

All standard reagents were obtained from Sigma Chemical (St. Louis, Mo., USA), unless indicated otherwise. L-[H^3] arginine was supplied by Perkin Elmer Life and Analytical Sciences (Boston, Mass., USA).

All animal experiments described in this study were conducted in accord with the protocol approved by the institutional committee on ethics in animal experiments. Studies were performed using male Wistar rats weighing 200–250 g. Subsequently, rats were segregated into 7 groups.

- Group 1 (control): untreated rats, were allowed free access to regular rat chow and tap water.
- Group 2 (hyperuricemia): rats were fed, by gavage, with oxonic acid 750 mg/kg b.w./day for 4 weeks.
- Group 3 (allopurinol): normal rats were treated with allopurinol 150 mg/l in the drinking water starting from day 1.
- Group 4 (hyperuricemia + allopurinol): hyperuricemic rats (as in group 2) were given allopurinol (as in group 3).
- Group 5 (benzbromarone): normal rats were treated with the uricosuric agent benzbromarone (15 mg/kg/day in the drinking water starting from day 1).
- Group 6 (hyperuricemia + benzbromarone): hyperuricemic animals (as in group 2) were treated with benzbromarone (as in group 5).
- Group 7 (hyperuricemia + L-arginine): hyperuricemic rats (as in group 2) were given L-arginine 3% in the drinking water starting from day 1.

Additional animals were given i.p. injections of α -tocopherol, 90 mg/kg b.w. every other day or castor oil as a vehicle starting from day 1. At the end of the experiment (week 4), the animals were euthanized using CO_2 to allow harvesting of tissues and collection of blood from the aorta for determination of uric acid levels.

L-Arginine Uptake by Aortic Rings

Uptake of radiolabeled L-arginine in the rat aorta was measured according to previously described methods [16]. Immediately after sacrifice, the aorta was carefully excised from the left renal artery to the aortic valve ring and placed in ice-cold HEPES. The vessels were dissected free from adherent connective tissue

and cut into rings (length 3 to 4 mm). Each segment was cut longitudinally in half. To determine arginine transport, aortic segments of each experimental group were incubated and shaken for 10 min in HEPES buffer at pH 7.4, 37°C. L-[H³] arginine and L-arginine, in a final concentration of 1 mM, were added to a total volume of 2 ml for additional 1 min. The duration of 1 min was chosen since it was within the linear portion of uptake curves (data not shown). Transport was terminated by rapidly washing the aortic rings with ice-cold PBS buffer (4 times, 3 ml/tube). The rings were then dried and solubilized in 1 ml of 0.5% SDS in 0.5 N NaOH. 700 µl of the lysate were used to monitor radioactivity, by liquid scintillation spectrometry (Betamatic, Kontron). The remaining 300 µl were used for protein content determination by the Lowry method (Lowry assay kit, Sigma). To correct for non-specific uptake or cell membrane binding, additional studies were performed in which aortic segments were incubated with 10 mM unlabeled arginine in HEPES buffer, and the associated radioactivity was subtracted from each data point. Results are expressed as mean $\pm$ SE of at least 5 different animals.

Assessment of cGMP Generation

Aortic tissue cGMP generation was determined by ELISA. Isolated aortic rings were incubated in HEPES buffer in which a phosphodiesterase inhibitor (3-isobutyl-1-methyl-xanthine 1 mM) was included to inhibit cGMP degradation. The suspension was then aliquoted (50 µl per tube) and incubated and shaken at 37°C for 10 min, with and without 0.5 mM of the NOS antagonist nitro-L-arginine methylester (L-NAME), after which they were subjected to carbamyl choline (100 mM, from Sigma), a selective eNOS agonist for additional 5 min. Following incubation the samples were rapidly frozen and then homogenized in trichloroacetate 5% at 4°C. The precipitate was removed by centrifugation (3,000 rpm, 10 min) and trichloroacetate was ether extracted. Residual ether was removed by heating the samples for 5 min at 70°C. The samples were then processed for measurements of cGMP by ELISA (R&D Systems). The difference between c-GMP values with and without incubation with L-NAME was used as an index of eNOS activity. Each experiment was repeated 6 times.

Protein Quantification by Western Blotting

Aortic CAT-1, PKC α , phosphorylated PKC α , and nitrotyrosine protein expression were determined by immunoblotting.

Excised aortas were separately placed in ice-cold PBS lysis buffer (pH 7.4), containing protease inhibitors (1 mM phenyl-methylsulfonyl fluoride, 4.5 µM leupeptin, and 5 µM aprotinin; ICN Biomedicals), 0.01% Triton X-100 and 0.1% SDS, then mechanically homogenized and left on ice for 45 min. Homogenates were subsequently centrifuged (13,000 rpm for 10 min, at 4°C). Cell lysates were stored in aliquots at -70 °C. A membrane fraction was obtained by adding to the pellet an equal volume of lysis buffer supplemented by Tween-20 (0.25%) to solubilize. The protein content of each sample was determined by the method of Lowry. Equal amounts of protein (30 µg) were prepared in sample buffer (2% SDS, 0.01% bromophenol blue, 25% glycerol, 0.0625 M Tris HCL, pH 6.8, 5% mercaptoethanol) and analyzed on a 7.5% SDS-PAGE gel. The gel was transferred onto Hybond ECL nitrocellulose membranes (Amersham Biosciences), and blocked in PBS-T containing 5% non-fat dried milk, at room temperature. Membranes were then incubated with polyclonal rabbit anti-rat CAT-1 antibodies, 1:500 (synthesized by Dr. O. Leitner, Weiz-

mann Institute, Rehovot, Israel), mouse anti-rat PKC α and phosphorylated PKC α antibodies or monoclonal mouse anti-rat nitrotyrosine antibodies (Santa Cruz Biotechnology) for 1 h at room temperature, washed and incubated with secondary HRP-conjugated goat anti-rabbit antibody (1:10,000) in PBS-T for 1 h. Membranes were subsequently washed 3 times, for 5 min each, in PBS-T. Membranes were then stripped and reprobed with monoclonal anti- β -actin antibodies as an internal control. The reactive bands corresponding to CAT-1, PKC α , and nitrotyrosine were detected by enhanced chemiluminescence (Kodak X-OMAT AR film) and quantified by densitometry (n = 4 different experiments).

Plasma ADMA and SDMA Measurement by High-Performance Liquid Chromatography

Samples were diluted and applied to a preconditioned chlorobenzoic acid solid-phase extraction cartridge. After elution with methanol:ammonia, the dried extracts were derivatized and injected onto a C18 high-performance liquid chromatography column (25 $\times$ 4.6 mm). ADMA and SDMA were quantified by reference to their fluorescence emissions. Data were acquired onto a HP Chemstation[®] and peak areas determined. The intra- and inter-assay coefficients of variability were 3.1 and 3.6%, respectively, for plasma ADMA and 2.6 and 3.1% for plasma SDMA.

Blood Pressure Monitoring

Blood pressure response to systemic administration of acetylcholine was measured in anesthetized animals (i.p. ketalar 8 mg/100 g b.w. and xylazine 0.25 mg/100 g b.w.) as previously described [19]. In brief, the right femoral artery was cannulated to measure arterial blood pressure (HP 1290 C Universal Quartz transducer and a Mennen Med recorder) and the right jugular vein was cannulated for the infusion of acetylcholine 7 µg/kg/min for 4 min. Three measurements of blood pressure were recorded at 2-min intervals, 30 min after completion of surgical procedures and following the administration of acetylcholine. The average values were used for comparison between groups.

Statistical Analysis

Data are presented as mean $\pm$ SE. One-way analysis of variance (ANOVA) was conducted for comparison between groups. Post-hoc analysis using LSD algorithm was performed to allocate the source of significance.

Results

Uric Acid Levels

Four weeks of feeding with oxonic acid significantly increased plasma uric acid levels compared to control rats (1.7 ± 0.2 vs. 1.1 ± 0.1 mg/dl, $p < 0.05$, $n = 5$). While co-administration of either allopurinol or benzbromarone completely prevented this elevation. Arginine had no effect on serum uric acid levels (fig. 1).

Aortic Arginine Transport

The first set of experiments was designed to explore a possible effect of hyperuricemia on aortic arginine trans-

Fig. 1. Serum uric acid levels in the various experimental groups. Results shown are mean $\pm$ SEM. * $p < 0.05$ vs. controls (n = 5 animals per group). CTL = Controls; OX = hyperuricemia; ALL = allopurinol; OX + ALL = oxonic acid and allopurinol; BENZ = benzbromarone; OX + BENZ = oxonic acid and benzbromarone; OX + ARG = oxonic acid and L-arginine.

Fig. 3. Arterial pressure obtained in anesthetized animals before and after intravenous administration of acetylcholine. Results shown are mean $\pm$ SEM. * $p < 0.05$ vs. controls (n = 6). See figure 1 for list of abbreviations.

Fig. 2. Uptake of radiolabeled arginine (L-[H³] arginine) by freshly harvested aortic rings from the various experimental groups. Data are presented as the mean $\pm$ SEM of 8 different rats. * $p < 0.05$ vs. control; # $p < 0.05$ vs. OX. See figure 1 for list of abbreviations.

port and the impact of normalizing serum uric acid levels by either inhibiting its synthesis (allopurinol) or enhancing renal uric acid excretion (benzbromarone). When compared to normo-uricemic rats, hyperuricemia induced a significant decrease in aortic arginine uptake.

The administration of benzbromarone and arginine to oxonic acid treated rats completely prevented this decrease in arginine transport. In contrast, allopurinol failed to restore normal arginine uptake velocities in hyperuricemic animals. Interestingly, allopurinol attenuated arginine transport even in healthy animals, whereas benzbromarone had no such effect when administered to controls (fig. 2).

Effects of Arginine Transport on Endothelial Function

The decrease in systemic blood pressure following the administration of acetylcholine was significantly attenuated in hyperuricemic rats compared to controls (96 ± 4 vs. 85 ± 3 mm Hg, $p < 0.05$), while no differences were observed between control rats and the remaining experimental groups (fig. 3). To further explore the effect of decreased arginine uptake on the endothelium in hyperuricemic rats, we measured cGMP generation following stimulation with carbamyl choline, a selective eNOS agonist with and without the co-administration of L-NAME (NOS inhibitor), and used the difference between these values as an index of aortic eNOS activity. Carbamyl choline-stimulated cGMP levels were significantly decreased in hyperuricemic rats as compared to control animals. The co-administration of benzbromarone and arginine prevented the aforementioned finding. Allopurinol significantly attenuated the decrease in cGMP generation;

Fig. 4. **a** cGMP generation by freshly harvested aortic rings following stimulation with carbamyl choline (CCh), an eNOS agonist with and without co-incubation with L-NAME (NOS inhibitor). Results shown are mean $\pm$ SEM of duplicate determinations from 6 separate experiments. **b** The absolute increase in cGMP generation mediated by nitric oxide synthesis [CCh – (CCh + L-NAME)] (NO-mediated CCh responsiveness). * $p < 0.05$ vs. control. # $p < 0.05$ vs. OX. See figure 1 for list of abbreviations.

however, cGMP levels remained lower than control. When administered to control animals, neither allopurinol nor benzbromarone had an effect on cGMP generation (fig. 4a, b).

We employed Western blotting to evaluate protein nitration as an index of peroxynitrite generation. Figure 5 depicts an electrophoretic profile of nitrotyrosine-modified proteins in aortic rings harvested from the different experimental groups. The extent of nitrotyrosine formation was significantly increased in hyperuricemic rats in comparison to control animals. Allopurinol, benzbromarone, and arginine administration completely prevented the aforementioned finding. Allopurinol and benzbromarone had no effect on protein nitration in control rats.

Regulation of Aortic CAT-1 in Hyperuricemia

To explore a possible role for CAT-1 in arginine traffic in hyperuricemia, we next examined CAT-1 protein levels. CAT-1 protein was identified as ~90 kDa, in aortic tissue. We found that CAT-1 abundance was unchanged in aortas harvested from hyperuricemic rats and was not affected by treating the animals with either allopurinol or benzbromarone (fig. 6a, b). In order to evaluate a possible posttranslational mechanism for CAT-1 inactivation in hyperuricemia, we performed Western blotting for PKC α , a classical isoform of PKC that regulates CAT-1 activity. The signal of the membrane fraction of PKC α ,

as well as the abundance of its activated form phosphorylated PKC α (p-PKC α), remained unchanged in all experimental groups (fig. 7a–d). In order to challenge the hypothesis that, in hyperuricemia, upregulation of PKC α provokes a decline in arginine transport, rats were treated with α -tocopherol, a known inhibitor of PKC activity. Tocopherol administration had no effect on arginine transport in all experimental groups (fig. 8). Finally, the putative effect of the 2 endogenous N-dimethylated L-arginine derivatives, L-ADMA and L-SDMA, on arginine uptake in the current study was examined. We found that serum levels of these 2 arginine analogues did not differ in all experimental groups (data not shown).

Discussion

The present study demonstrates that aortic arginine uptake is attenuated in hyperuricemic rats, leading to decreased eNOS activity. These data support the notion that uric acid, rather than being a surrogate marker, is actually an active player in the pathogenesis of endothelial dysfunction. We also introduce a novel mechanism to explain this phenomenon. It has been previously shown that the consequences of decreased arginine transport are far beyond a mere substrate depletion. NOS enzymes contain 4 redox-active prosthetic groups (FAD, FMN, heme,

Fig. 5. a Western blot analysis of nitrotyrosine-modified protein expression in aortic rings freshly harvested from the various experimental groups. These blots are representative of 4 different experiments. **b** Densitometric analysis of nitrotyrosine-modified protein contents of the experiments shown in **a**. Each bar represents the mean of the relative density units $\pm$ SE from 4 different experiments. * $p < 0.05$ vs. the corresponding control; # $p < 0.05$ vs. the corresponding OX. See figure 1 for list of abbreviations.

Fig. 6. a Representative Western blot analysis showing regulation of CAT-1 protein level in freshly harvested aortic rings. **b** Densitometric analysis of aortic CAT-1 contents from the various experimental groups. Each bar represents the mean of the relative density units $\pm$ SE from 4 different experiments. See figure 1 for list of abbreviations.

Fig. 7. **a** Representative Western blot analysis showing regulation of PKCα protein level in freshly harvested aortic rings. **b** Densitometric analysis of PKCα contents of the same experiments shown in **a**. Each bar represents the mean $\pm$ SE from 4 different experiments. **c** Representative Western blot analysis showing regulation of phosphorylated PKCα

protein level in freshly harvested aortic rings from the same experimental groups. **d** The relative IOD ratios of phosphorylated PKCα to PKCα in freshly harvested aortic rings from the same experimental groups. Each bar represents the mean $\pm$ SE from 4 different rats. Data from control are normalized to 1. See figure 1 for list of abbreviations.

Fig. 8. Effect of α -tocopherol, a PKC α inhibitor (90 mg/kg b.w. every other day for 4 weeks) or castor oil as a vehicle, on arginine transport in freshly harvested aortic rings. Data are presented as the mean $\pm$ SEM of at least 5 different experiments. See figure 1 for list of abbreviations.

and BH₄) that can, in principle, transfer electrons to O₂. When arginine sources are depleted, a functional NOS may turn into a dysfunctional superoxide-generating enzyme, leading to accumulation of reactive oxygen species such as peroxynitrite [20, 21]. In other words, adequate

arginine supply is crucial for maintaining endothelial function by 2 different mechanisms: (1) allowing for sufficient NO generation, and (2) preventing superoxide synthesis by NOS. Since an increase in oxidative stress has been described in hyperuricemia [12], it is conceivable to hypothesize that decreased arginine transport contributes to the accumulation of reactive oxygen species in hyperuricemia. In order to test this hypothesis, using Western blotting, we estimated the extent of protein nitration, a marker for oxidation mediated by peroxynitrite. Indeed, we have found that hyperuricemic rats exhibit increased protein nitration and that normalizing serum uric acid concentration and administration of L-arginine prevented the decrease in arginine transport and attenuated protein nitration. In the aggregate, our data suggest that, in addition to a decrease in eNOS activity, as demonstrated by attenuated aortic cGMP generation and a decreased systemic blood pressure response to acetylcholine, the decrease in aortic arginine transport enhances peroxynitrite generation in hyperuricemic rats.

An intriguing finding was that the administration of allopurinol to hyperuricemic rats, despite correcting serum uric acid levels, did not restore arginine transport velocities. However, when allopurinol was given to control animals, it also inhibited arginine transport, suggesting a direct effect of allopurinol on the arginine transport system. In order to delineate between uric acid-lowering

effect of allopurinol from other unknown effects which could potentially impact arginine metabolism, we performed experiments using an uricosuric agent, benzbromarone. Indeed, decreasing serum uric acid levels via enhancement of its renal excretion restored arginine transport velocities. It is well known that allopurinol is not simply an inhibitor of uric acid generation. Allopurinol, while inhibiting xanthine oxidase, induces production of superoxide radicals during its conversion to oxypurinol. This effect may adversely influence arginine metabolism [22]. Taken together, our data strongly suggest that hyperuricemia attenuates aortic arginine uptake, and raise a question whether xanthine oxidase inhibition affects arginine metabolism through pathways which are not related to uric acid metabolism. Though confusing, the results of the experiments with allopurinol can be used to strengthen our hypothesis. The fact that allopurinol partially improved eNOS activity in spite of having no effect on arginine transport suggests that the decrease in eNOS activity in chronic hyperuricemia is multifactorial. Nevertheless, only by preventing the decrease in arginine transport was eNOS activity fully restored, emphasizing a crucial role for attenuated arginine transport in hyperuricemia-induced eNOS inactivation.

We have tried to elucidate a molecular mechanism to explain our findings. The fact that changes in arginine uptake due to hyperuricemia were not associated with changes in CAT-1 protein content strongly supports the notion that these events involve a post-translational CAT-1 modulation. We were therefore intrigued to explore a putative mechanism for the aforementioned finding. A possible involvement of PKC α in the regulation of L-arginine transport in different cell types has been discussed for the last several years [23, 24]. We have recently reported in 4 different experimental models characterized by diminished arginine transport, namely, hypercholester-

olemia, chronic renal failure, pregnancy, and aging in the male rat, a post-translational regulation of CAT-1 which was associated with upregulation of PKC α [15, 16, 18, 25]. We have decided to focus exclusively on the membrane fraction of PKC α since this is where the interaction between PKC α and CAT-1 occurs. In contrast to our previous observations, in the current set of experiments changes were neither found in the aortic membrane-bound fraction of PKC α nor in its activated form, phosphorylated PKC α . Moreover, inhibiting PKC activity by systemic application of α -tocopherol failed to improve arginine uptake velocities in hyperuricemic rats, which also supports the notion that in hyperuricemia, CAT-1 is not modulated by PKC α .

A different mechanism which can potentially interfere with L-arginine transport involves the 2 N-dimethylated L-arginine derivatives, L-ADMA and L-SDMA. Moreover, CAT proteins transport L-arginine analogues both into and out of cells, resulting in an exchange of cationic amino acids between both sides of the membranes. This implies that these L-arginine analogues not only compete with L-arginine for transport but are also capable of driving out the intracellular L-arginine [26]. However, serum levels of ADMA and SDMA did not differ in all experimental groups. Taken together, the 2 known mechanisms which were previously found to down-regulate CAT system activity through either post-translational modulation or competitive inhibition of the transporters do not seem to play a role in hyperuricemia.

In conclusion, hyperuricemia attenuates aortic arginine transport resulting in decreased eNOS activity and increase in peroxynitrite accumulation. These events can be prevented by systemic administration of L-arginine. The exact molecular mechanism by which hyperuricemia modulates CAT-1 activity still remains to be defined.

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