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Research Article

L-arginine regulates asymmetric dimethylarginine metabolism by inhibiting dimethylarginine dimethylaminohydrolase activity in hepatic (HepG2) cells

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Abstract. An increase in circulating asymmetric dimethylarginine (ADMA) and a decreased L-arginine/ADMA ratio are associated with reduced endothelial nitric oxide (NO) production and increased risk of vascular disease. We explored relations between ADMA, L-arginine and dimethylarginine dimethylaminohydrolase (DDAH) in liver (HepG2) cells. DDAH is the principle enzyme for the metabolism of ADMA. HepG2 cells metabolised 44.8 nmol/h of ADMA per 3.6×10^6 cells in the absence of L-arginine. The metabolism of ADMA at physiological (1 μmol/l, $p < 0.01$) and at pathological (100 μmol/l, $p < 0.01$) levels was inhibited dose-dependently by L-arginine (0–400 μmol/l) in cultured HepG2 cells and increased intracellular ADMA ($p = 0.039$). L-arginine competitively inhibited DDAH enzyme activity to $5.6 \pm 2.0\%$ of the untreated level ($p < 0.01$). We conclude that L-arginine regulates ADMA metabolism dose-dependently by competing for DDAH thus maintaining the metabolic balance of L-arginine and ADMA, and endothelial NO homeostasis.

Keywords. Asymmetric dimethylarginine, dimethylarginine dimethylaminohydrolase, L-arginine, HepG2, nitric oxide.

Asymmetric dimethylarginine (ADMA) is formed by the methylation of L-arginine residues in protein and is released during proteolysis. It competes with L-arginine for all three isoforms of nitric oxide (NO) synthase (eNOS, iNOS, and nNOS); L-arginine is the sole substrate of NOS [1–3]. The effects of ADMA on NO production and on basal vascular tone establish that it is an important contributor to endothelial cell dysfunction. An elevated plasma level of ADMA is reported to be an independent vascular disease risk factor and is associated with increased carotid intima thickness, increased cardiac mortality [4, 5], hypertension, myocardial infarction and stroke [6].

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Free ADMA is the product of protein turnover and is constantly produced by all cells. Most ADMA is metabolised by dimethylarginine dimethylaminohydrolase (DDAH) [7] and 10% of ADMA is excreted by the kidney [8]. The crystal structure of DDAH shows that it belongs to a structural superfamily of enzymes that have arginine or substituted arginine as a substrate, which suggests that L-arginine might be a competitive inhibitor of DDAH [9, 10]. ADMA also shares the y+ transport system with L-arginine [11] and L-arginine could therefore affect ADMA metabolism by competing for DDAH and the y+ transport system. If this hypothesis is correct, an elevated plasma level of L-arginine would increase the plasma level of ADMA. Circulating L-arginine is also essential for NO production and plasma levels of L-arginine influ-

ence the endothelial production of NO. Plasma levels of L-arginine vary depending on dietary intake and minor increases in plasma levels of ADMA have been shown to affect endothelial cell NO production and basal vascular tone and arterial blood flow [9]. This metabolic balance of L-arginine and ADMA appears essential for the homeostasis of endothelial NO production.

The regulation of arginine homeostasis involves dietary intake, arginine synthesis in the kidney and its utilisation by arginases and other arginine catabolic enzymes [12]. Whole-body arginine homeostasis is achieved largely by changes in the rate at which L-arginine is catabolised in relation to the exogenous dietary supply of arginine [12]. There are two isforms of arginase. Arginase I is found in the cytosol, participates in the urea cycle and is expressed at high levels in the liver [13]. A deficiency in arginase I in humans results clinically in argininaemia [14]. Arginase II is localised to mitochondria and is highly expressed in kidney and prostate. No human disease is known to be associated with a deficiency of arginase II. The liver plays an important role in the regulation of plasma arginine concentrations. It takes up large amounts of arginine from the hepatic circulation [15] and liver failure is associated with high plasma levels of L-arginine [15, 16].

Most circulating ADMA is metabolised in the liver and kidney. ADMA is mainly metabolised to L-citrulline by DDAH I which is highly expressed in liver and kidney [8, 15, 17], and a small amount of ADMA is also metabolised by aminotransferase in hepatic cells and excreted by the kidney [18]. In both liver and kidney failures there are increased plasma levels of ADMA, and liver transplantation in patients with liver failure reduces the plasma ADMA levels [17]. The hepatic cell line, HepG2, has a highly expressed y+ transport system on its membrane and could therefore be used to assess the effect of L-arginine on the metabolism of ADMA [19]. In the present study, we investigated hepatocyte-based metabolic relationships between L-arginine and ADMA as they have implications for the maintenance of a balanced circulating L-arginine to ADMA ratio and vascular NO production.

Materials and methods.

Materials. L-arginine, ADMA, L-monomethylarginine (L-MMA), L-N-nitro-L-arginine methyl ester hydrochloride (L-NAME), *S*-nitroso-*N*-acetylpenicillamine (SNAP), L-norvaline and 3-mercaptopropionic acid were purchased from Sigma Aldrich (Sydney, Australia). Acetonitrile and o-phthaldialdehyde (OPA) were purchased from Merck (Kilsyth VIC Australia). Dulbecco's minimal essential medium (DMEM containing 400 µmol/l of L-arginine) and custom-made L-arginine-free DMEM, fetal bovine serum, L-glutamine and antibiotics were purchased from Invitrogen (Melbourne, Australia).

Cell culture. HepG2, a hepatoma cell line, was grown in DMEM supplemented with 10% fetal calf serum, 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 g/ml streptomycin to reach confluence and there were approximately 3.6×10^6 cells in a 25-cm^2 flask. All the cells were kept at 37 °C with 5% CO_2/atmosphere.

L-arginine and ADMA assays. We measured the concentrations of L-arginine and ADMA in the cell culture medium and cell lysate by high-performance liquid chromatography (HPLC) as described by Teerlink et al. [20]. Briefly, 0.2 ml cell culture medium or cell lysate was mixed with 0.1 ml of a 40 µmol/l solution of the internal standard L-MMA and 0.7 ml phosphate-buffered saline (PBS). This mixture was applied to Strata-X-C solid-phase extraction cartridges (Phenomenex Pty Ltd, Sydney, Australia) for extraction of basic amino acids. The amino acids were derivatised with OPA reagent containing 3-mercaptopropionic acid. The derivatives were separated by isocratic reversed-phase chromatography on a Waters Symmetry C18 column (3.9×150 mm; 5-µm particle size). The intra- and inter-assay coefficients of variation for measurements of L-arginine and ADMA were <5%, respectively.

Nitrate and nitrite assay in the cell culture medium. The cell culture medium was deproteinated by filtering through a 30-kDa molecular weight cut-off filter (Millipore, Sydney, Australia). Nitrate in the cell culture medium was reduced to nitrite with the nitrate reductase supplied in the nitrate/nitrite colorimetric assay kit (Cayman Chemical, Michigan USA). Nitrite levels were estimated using the Griess reagent and absorbance was read at 543 nm.

DDAH activity assay. DDAH activity in cell lysates was estimated by measuring ADMA degradation as described previously [21]. At the end of the experiments, the cells were lysed in lysis buffer [0.1 M Na_2HPO_4 containing 10 µg/ml of each protease inhibitor (aprotinin, leupeptin and pepstatin) pH 6.5] for 1 h on ice. The cell lysate was centrifuged at 50,000 g to separate soluble material from insoluble cell debris [7, 22]. The total protein concentration of the cell lysate was measured by the Bradford method (Bio-Rad Laboratories, Sydney, Australia). The cell lysate was divided into aliquots of 200 µl. To each aliquot, 2 µl of a 1 mmol/l ADMA solution was added (final concentration 10 µmol/l). 30% sulfurosalicylic acid (SSA) was added to one of the aliquots to inactivate DDAH to determine the baseline concentration of ADMA. The other aliquots were incubated at 37 °C for 30 min, then 30% SSA was added to stop the reaction. The level of ADMA in each aliquot was measured as described above. DDAH activity was calculated from the percentage of ADMA degradation.

DDAH I and II mRNA quantitation. The DDAH enzyme has two isoforms, DDAH I and DDAH II. DDAH I is the major isoform expressed in hepatocytes. Total RNA was extracted from the cultured cells using the TRIzol reagent (Invitrogen, Carslbad, Calif.). 1 μg of RNA was then reverse-transcribed using RevertAid (Fermentas International Inc., Burlington ON Canada). Quantitative real-time PCR experiments were performed with a Corbette Research RotorGene 3000A using SYBR green fluorescence. The forward primer for DDAH I was 5′-ACGAGGTGCTGAAATCTTGT-3′, and the reverse primer was 5′-CGGTGGTCACTCATCTGTTG-3′. The forward primer for DDAH II was 5′-AGTCTGTGTGGATGGGATG-3′, the reverse primer was 5′-CCTCAGGTGGCAGTTCTAG-3′. Measurements were normalised against the 18S rRNA gene as a housekeeping gene.

Western blotting. After treating the cells with L-arginine, they were lysed in protein lysis buffer for 1 h on ice [22]. After measuring total protein concentrations, equal amounts of protein were loaded and separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto nitrocellulose membranes. The blots were blocked with 2% dried milk and incubated for 2 h with goat polyconal anti-mouse DDAH I (Calbiochem-Novabiochem Sydney Australia) at concentrations of 1 : 100. After washing, blots were incubated with peroxidase-conjugated antibodies for two additional hours at concentrations of 1 : 2000 (anti-goat) with detection by ECL Western blotting reagents (Amersham Bioscience, Rydalmere NSW Australia).

Statistical analysis. The data are presented as means ± SD. All experiments were performed in duplicate and then repeated twice to confirm the results. We tested the differences between groups using an independent Student's t test. We used an ANOVA test to compare the means of more than two groups. Dose-dependent inhibition of L-arginine on the DDAH was analysed with ANOVA followed by post hoc tests correcting for multiple testing. $p < 0.05$ was considered statistically significant. Statistical analyses were performed using SPSS (SPSS 12.0 for Windows).

Results

Stability of L-arginine and ADMA in the cell culture medium. ADMA was not detected in DMEM or in the custom-made L-arginine-free DMEM, but about 5 μmol/l of ADMA was identified in the pure fetal calf serum. We assessed the stability of L-arginine and ADMA in the DMEM in the absence of cells after incubation for 72 h at 37 °C in 5% CO_2/atmosphere. At 0 and 72 h, the L- arginine concentrations were 398.75 and 398.22 μmol/l, and ADMA levels were 0.85 and 0.87 μmol/l, respectively. There was no degradation of L-arginine or ADMA during an incubation period of 72 h in the cell culture medium in the absence of cells at 37 °C. The maximum incubation period we used in our experiments was less than 72 h.

Metabolism of L-arginine and ADMA in HepG2 cells. We measured the metabolism of L-arginine and ADMA in 4 ml cell culture medium in a 25-cm^2 flask. The cells grown to confluence were used for the following study. HepG2 cells actively metabolised exogenous L-arginine and ADMA. The 400 μmol/l of L-arginine in the DMEM was metabolised to 195 ± 7.5 μmol/l after 8 h incubation. This is equivalent to a catabolic rate of 102 nmol/h per 3.6×10^6 cells. 100 μmol/l of ADMA was metabolised to 11.8 ± 0.02 μmol/l in 8 h by HepG2 cells in the absence of L-arginine. The catabolic rate of ADMA was approximately 44.8 nmol/h.

L-arginine inhibited ADMA metabolism dose-dependently. We explored the effect of L-arginine on ADMA metabolism in the HepG2 cells. The custom-made L-arginine-free cell culture medium was used for the following experiments. The effects of different dosages of L-arginine (0, 50, 100, 200 and 400 μmol/l) on the metabolism of ADMA (1 and 100 μmol/l) is shown in Figure 1. The L-arginine (≥50 μmol/l) significantly inhibited the metabolism of ADMA (1 and 100 μmol/l) during an 8-h incubation period. The L-arginine inhibited the metabolism of ADMA dose-dependently (ANOVA, ADMA 1 μmol/l, $p < 0.01$; ADMA 100 μmol/l, $p < 0.01$).

L-arginine increased intracellular ADMA levels. We also studied the metabolism of ADMA in the HepG2 cells. We first established that the 3.6×10^6 HepG2 cells were able to metabolise approximately 100 μmol/l of L-arginine in 4 ml cell culture medium every 3 h. The supplementation of 100 μmol/l of L-arginine every 3 h into DMEM maintained the levels of L-arginine in the cell culture medium at approximately 400 μmol/l during the experimental period. The metabolism of 1 μmol/l of ADMA in the HepG2 cells was significantly inhibited by the continuous L-arginine supplement (control vs L-arginine: 0.28 ± 0.03 vs 1.12 ± 0.05 μmol/l, $p < 0.001$) after 24 h of incubation. As shown in Figure 2, the L-arginine significantly increased the intracellular levels of ADMA from 0.7 ± 0.01 to 1.29 ± 0.08 nmol/mg ($p = 0.03$) after 24 h of incubation.

Interactions between L-arginine and ADMA metabolism. We then explored the effect of L-arginine on the metabolism of ADMA by inhibiting the metabolism of L-arginine with the arginase inhibitor, L-norvaline, as described previously [23]. With 400 μmol/l of L-arginine and 1 μmol/l of ADMA in the cell culture medium, after 12 h incubation, L-norvaline (20 mM) significantly in-

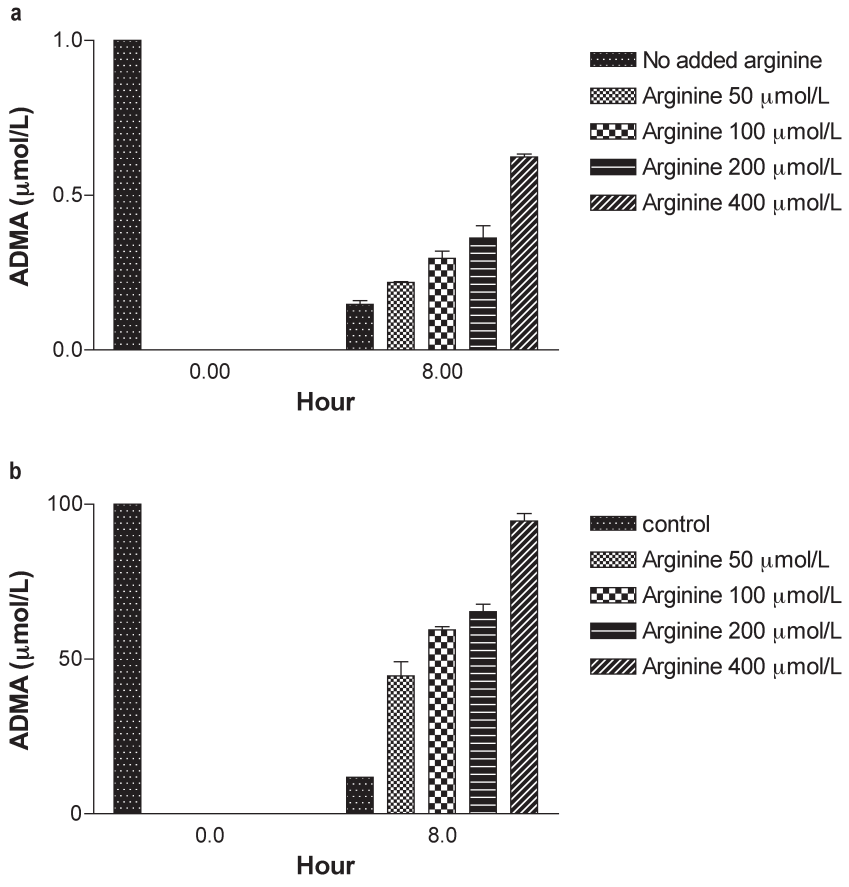

Figure 1. In custom made, L-arginine-free cell culture medium, L-arginine was added into the medium at concentrations of 0, 50, 100, 200 and 400 μmol/l with two different concentrations of ADMA 1 μmol/l (*a*) and 100 μmol/l (*b*). L-arginine inhibited metabolism of both concentrations of ADMA dose-dependently.

hibited the metabolism of L-arginine (control vs L-norvaline, 97.99 ± 1.42 vs 146.41 ± 11.02 μmol/l, $p < 0.05$). The metabolism of ADMA was also inhibited (control vs L-norvaline, 0.47 ± 0.03 vs 1.0 ± 0.02 μmol/l, $p < 0.05$).

The effect of NO on ADMA metabolism. L-arginine is the sole substrate of NOS, and it is possible that increased NO production induced by L-arginine supplement is responsible for the reduced ADMA metabolism. We therefore investigated this possibility using the NO donor SNAP (100 mM stock in DMSO). SNAP was added into the cell culture medium (DMEM containing 400 μmol/l of L-arginine with 10% fetal calf serum) at a final concentration of 1 mmol/l. The vehicle DMSO was also added into the cell culture medium in the same amounts as used for the SNAP. Neither the SNAP nor the vehicle altered the metabolism of ADMA (control vs SNAP: 0.87 ± 0.01 vs 0.81 ± 0.04 μmol/l, $p = 0.29$ at 12 h and 0.08 ± 0.002 vs 0.12 ± 0.02 μmol/l, $p = 0.43$ at 24 h). These findings suggest that increased NO production is not responsible for the L-arginine-induced inhibition of ADMA metabolism.

Inhibition of DDAH enzyme activity by L-arginine. We explored the effect of L-arginine on DDAH activity in HepG2 cell lysates. ADMA (0.5, 1.0, 2, 4 and 8 μmol/l) was added into the cell lysate with or without 100 μmol/l of L-arginine and levels measured after incubation at 37 °C for only 15 min. As shown in Figure 3a, L-arginine significantly inhibited the metabolism of ADMA (see legend for p values). The amounts of ADMA (0.5, 1, 2, 4 and 8 μmol/l) metabolised by DDAH in the cell lysates with and without 100 μmol/l L-arginine depended on the concentrations of added ADMA (ANOVA, $p < 0.01$, the amounts of ADMA metabolised between different dosages of added ADMA were all significantly different with post hoc tests, all $p < 0.05$). This suggests a competitive inhibition of DDAH enzyme activity by L-arginine and ADMA.

We also explored the effect of D-arginine on the metabolism of 1 μmol/l of ADMA in HepG2 cell lysate. L-arginine or D-arginine (100 μmol/l) was added into the cell lysate separately. The cell lysate without adding L-arginine or D-arginine was used as a positive control and its DDAH activity taken as 100%. The cell lysate incubated at 100 °C for 30 min to inactivate the enzyme activity was

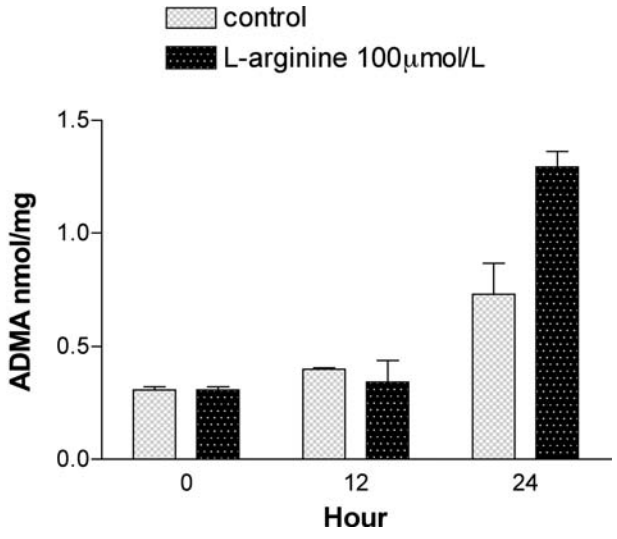

Figure 2. After 24 h of incubation, the intracellular level of ADMA measured in the cell lysate was significantly higher in the cells supplemented with 100 μmol/l of L-arginine every 3 hours (1.29 ± 0.08 vs 0.7 ± 0.01 nmol/mg protein, p = 0.039).

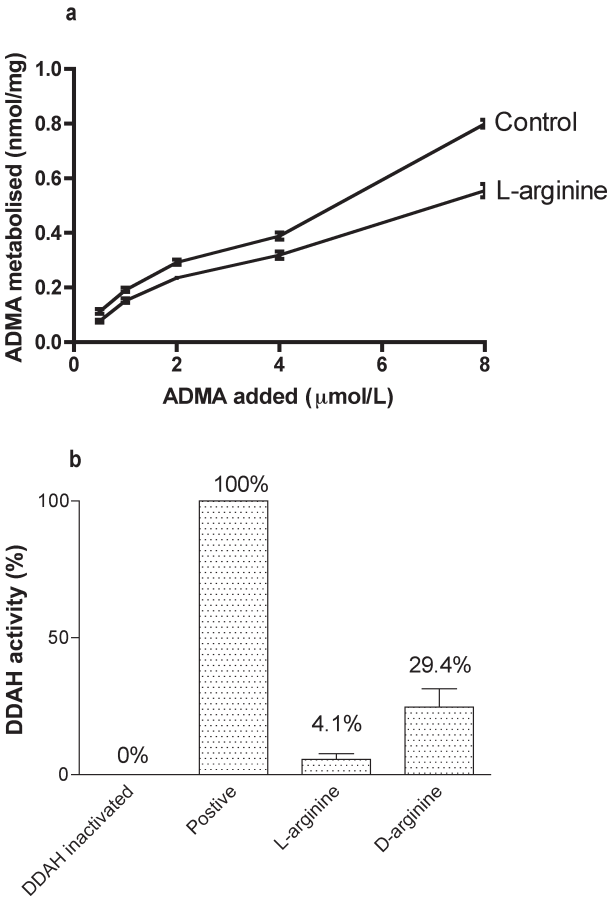

Figure 3. (*a*) ADMA was added into 200 μl of cell lysate (total protein concentration, 3.47 ± 0.32 mg/ml) with and without 100 μmol/l L-arginine and incubated at 37 °C for 15 min. L-arginine significantly inhibited ADMA metabolism with all concentrations of ADMA added into the cell lysate (p = 0.044, 0.042, 0.021, 0.033 and 0.006 for ADMA 0.5, 1, 2, 4 and 8 μmol/l, respectively). The amounts of ADMA (0.5, 1, 2, 4, 8 μmol/l) metabolised by DDAH in the cell lysates both with and without 100 μmol/l L-arginine were positively correlated with the concentrations of added ADMA ($p < 0.05$). (*b*) Activity of DDAH in the HepG2 cells treated with L-arginine and D-arginine. L-arginine and D-arginine inhibited DDAH activity in the cell lysate compared with the positive control (5.6 ± 2.0% vs 100%, p = 0.01 for L-arginine; 24.7 ± 3.6% vs 100%, p = 0.04 for D-arginine). The inhibition effect of D-arginine on DDAH was weaker than that of L-arginine (24.7 ± 3.6% vs 5.6 ± 2.0%, p = 0.03).

used as the negative control and its DDAH activity was taken as 0. As shown in Figure 3b, the L-arginine significantly reduced DDAH activity to 5.6 ± 2.0% (p = 0.01), while the D-arginine reduced DDAH activity to 24.7 ± 3.6% (p = 0.04). Thus, there was a higher efficacy for the inhibition of DDAH activity with the L-arginine than with the D-arginine at 100 μmol/l (p = 0.03).

Effects of L-arginine on DDAH expression. We measured DDAH I and II mRNA levels in HepG2 cells cultured in DMEM containing 400 μmol/l of L-arginine with supplements of 100 μmol/l of L-arginine every 3 h. We used the HepG2 cells cultured in L-arginine-free medium as the control. The L-arginine did not have a significant effect on DDAH I mRNA levels (DDAH I/18S ratio, control vs L-arginine, 6.08 ± 1.27 vs 6.1 ± 0.33 at 12 h, 5.23 ± 0.43 vs 5.37 ± 0.39 at 24 h, 4.13 ± 0.02 vs 4.61 ± 0.49 for 36 h, 4.19 ± 0.1 vs 4.27 ± 0.96 at 48 h, all $p > 0.05$). DDAH II mRNA expression was also not affected by the continuous L-arginine supplementation (DDAH II/18S ratio, control vs L-arginine, 4.4 ± 0.91 vs 4.8 ± 0.25 at 12 h, 6.7 ± 0.55 vs 5.5 ± 0.3 at 24 h, 6.75 ± 0.35 vs 6.21 ± 0.1 for 36 h, 5.26 ± 0.2 vs 5.74 ± 0.73 at 48 h, all $p > 0.05$).

We also quantified DDAH I protein levels with Western blotting. As shown in Figure 4, the protein levels of DDAH I in HepG2 cells progressively increased in both HepG2 cells cultured in L-arginine-free medium and in medium with the addition of L-arginine. The addition of L-arginine to the cell culture medium significantly increased the protein levels of DDAH I compared with those in L-arginine-free medium at 12 ($p < 0.01$) and 36 h ($p < 0.01$). Our findings indicate that while L-arginine did not affect the mRNA levels of DDAH I and II, it did increase the protein levels of DDAH I, suggesting a post-transcriptional effect.

Effects of D-arginine on ADMA metabolism. The inhibition of L-arginine on ADMA metabolism could be solely due to L-arginine competing for y+ transport and increased ADMA synthesis. D-arginine is actively transported into cells and does not share the y+ transport system with L-arginine and ADMA due to the system's

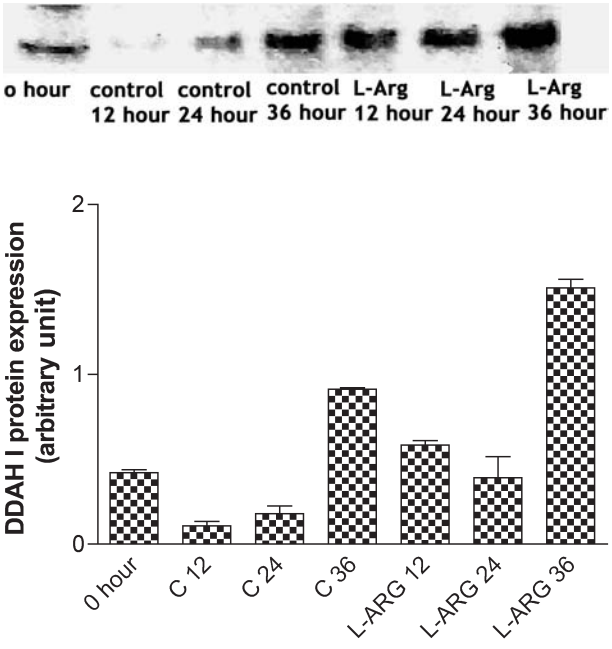

Figure 4. Protein expression of DDAH I at 12, 24 and 36 h in HepG2 cells cultured in L-arginine-free medium and in medium with 400 µmol/l of L-arginine together with 100 µmol/l supplementation of L-arginine every 3 h. The L-arginine significantly increased the expression of DDAH I at 12 h ($p < 0.01$) and at 36 h ($p < 0.01$).

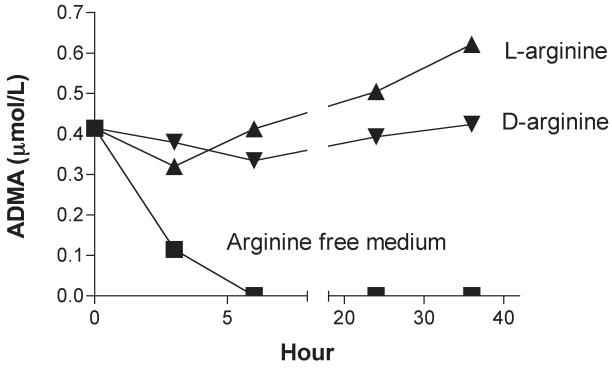

Figure 5. L-arginine and D-arginine (400 µmol/l) were added separately into the arginine-free cell culture medium. 100 µmol/l supplement of L-arginine or D-arginine/3 h was added for up to 36 h. The ADMA was metabolised by the HepG2 cells cultured in arginine-free medium. However, ADMA accumulated in the cell culture medium in the L-arginine- and D-arginine-treated HepG2 cells.

stereospecificity. The cells cannot use D-arginine to synthesise protein. Therefore, we compared the effects of D-arginine and L-arginine (400 µmol/l) on the metabolism of ADMA (1 µmol/l) in HepG2 cells. We also used ADMA metabolism in HepG2 cells cultured in arginine-free medium as the control. D-arginine or L-arginine at 100 µmol/l was added separately into the cell culture medium every 3 h for up to 24 h to maintain D-arginine or L-arginine at 400 µmol/l in the cell culture medium.

As shown in Figure 5, the metabolism of ADMA was also inhibited by D-arginine, and ADMA was released into the cell culture medium. In comparison, L-arginine also inhibited the metabolism of ADMA, but the ADMA level in the cell culture medium treated with L-arginine was higher than in that treated with D-arginine. The cells treated with L-arginine released 2.8 nmol of ADMA per 3.6×10^6 cells into the cell culture medium and the cells treated with D-arginine released 0.96 nmol of ADMA per 3.6×10^6 cells during 36 h of culture. This is much less than the amount of ADMA metabolised by HepG2 cells (44.8 nmol/h) in the absence of L-arginine and D-arginine. These results show that the effect of L-arginine on the metabolism of ADMA is not due to the increased turnover of ADMA. They also establish that L-arginine and D-arginine both inhibit intracellular DDAH activity, and that the L-arginine-mediated inhibition of DDAH is greater than that of D-arginine.

Discussion

Our study presents a novel metabolic model for the regulation of circulating ADMA levels. We show that L-arginine regulates physiological and pathological levels of ADMA metabolism in hepatocytes. For a given concentration of DDAH enzyme, the amount of ADMA metabolised was positively correlated with the concentration of added ADMA. The pattern was the same in the presence of 100 µmol/l of L-arginine but significantly less ADMA was metabolised. Our study suggests that L-arginine inhibits ADMA metabolism by competing for DDAH enzyme in HepG2 cells. This is consistent with a previous report that L-arginine is a competitive inhibitor of DDAH in the rat kidney [10].

Homeostasis of NO is critical for normal vascular function and production of NO is proportional to the plasma level of L-arginine. An increased plasma level of ADMA, even within the physiological range, is associated with decreased NO production [3, 24]. Our results suggest that plasma levels of ADMA are correlated with plasma levels of L-arginine in healthy subjects. Therefore NO production would remain relatively stable despite variations in the plasma levels of L-arginine. We also postulate that the L-arginine would also inhibit ADMA metabolism in vascular endothelial cells due to the similarity between DDAH I and DDAH II. Whilst ADMA has been considered a risk factor for vascular disease, our study suggests that ADMA has a pivotal physiological role in the regulation of NO production and that this is critical for normal vascular function.

The effect of L-arginine on ADMA metabolism in our experiments was not due to an increase in the intracellular

production of ADMA. With the continuous supplement of L-arginine and D-arginine in our experiments, HepG2 cells treated with L-arginine only released 0.96 nmol more ADMA than in the D-arginine-treated HepG2 cells. However, the same number of HepG2 cells could metabolise 44.8 nmol/h of ADMA. Therefore, the phenomenon we observed was not due to increased production of ADMA. The inability of DDAH to metabolise symmetric dimethylarginine is due to the presence of the symmetric dimethyl group on the guanidinium end which presents a steric and electrical impediment for the active site of DDAH; however, the optical difference between L-arginine and D-arginine occurs at the other end of the molecule. Thus, it is not surprising to find that D-arginine could also inhibit DDAH activity.

ADMA has previously been reported to compete with L-arginine for the y+ transport system [11]. However, if the inhibition effect of L-arginine on the metabolism of ADMA is due to competition for the y+ transport system, the plasma L-arginine would totally block the ADMA influx due to its 200-fold difference in concentration. This would not explain the correlation between plasma levels of L-arginine and ADMA [25]. Our studies found that with continuous L-arginine supplementation, HepG2 cells released ADMA to the cell culture medium rather than taking up the exogenous ADMA and that the L-arginine increased the intracellular level of ADMA. These findings establish that L-arginine competes with ADMA for intracellular DDAH. Moreover, our study showed that D-arginine also inhibited DDAH activity in the HepG2 cells and cell lysate. D-arginine has a similar structure to L-arginine but does not share the same transport system with L-arginine and ADMA.

There was no inhibition of ADMA metabolism when the HepG2 cells were treated with the NO donor SNAP. This implies that the L-arginine-NO pathway is not involved in the inhibiting mechanism. Leiper et al. [26] found that an NO donor impaired DDAH II activity in endothelial cells by nitrosylation of the DDAH II enzyme. Knipp et al. [27] recently reported that the holo-form of DDAH I is resistant to nitrosylation whilst the apo-form is sensitive to nitrosylation, and DDAH II does not have the holo-form. Thus the difference between the study of Leipper et al. [26] and ours could relate to structural differences between DDAH I and II. The mechanism by which L-arginine increases the post-transcriptional expression of DDAH I needs to be explored.

The liver plays a crucial role in regulating circulating plasma ADMA levels in animals and humans because of its highly expressed DDAH [17, 18]. Kielstein et al. [28] and MacAllister et al. [29] found that regular dialysis in renal failure patients did not result in an immediate significant decrease in plasma ADMA. However, the ADMA level decreased gradually over a period of 5 h after the end of the dialysis session. Kielstein et al. [28] attributed this to ADMA protein binding. This could not be confirmed because the method used to measure the plasma level of ADMA measures free, and not free plus bound, ADMA. Our results suggest an explanation for their observations; haemodialysis causes depletion of plasma levels of L-arginine [28] and the reduced circulating level of L-arginine results in increased ADMA metabolism in liver cells. Our study could also explain the failure of L-arginine supplementation to improve endothelial cell function.

From our findings we propose an explanation for the L-arginine paradox. This is illustrated in Figure 6. Intracellular L-arginine is packed in vesicles located near mitochondria and is not freely accessible for NOS and arginase so that the supply of L-arginine to NOS depends on a constant influx of L-arginine. Although the Km of arginase is 1000 times higher than that of NOS, increased arginase expression has recently been shown to reduce

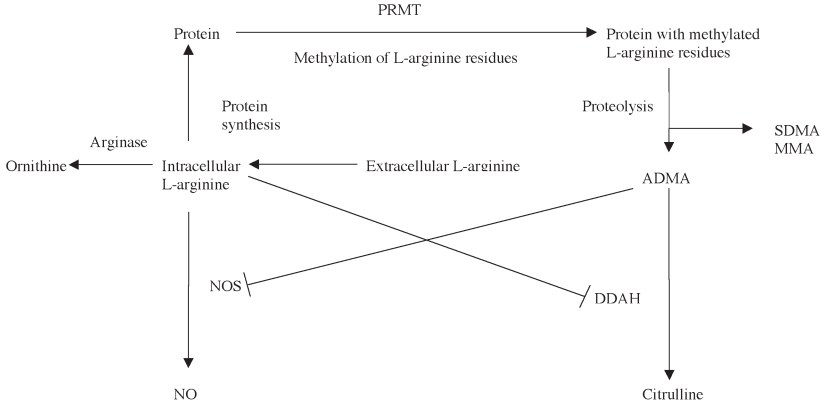

Figure 6. The mechanism we propose for the regulation of NO production. Intracellular levels of L-arginine and the NOS inhibitor ADMA determine the production of NO at a given expression of NOS. The availability of L-arginine for NOS is determined by combinations of the expression of arginase, the extracellular level of L-arginine, the expression of y+ transport and competition for y+ transport. Intracellular L-arginine regulates ADMA metabolism by competing for DDAH enzyme PRMT, protein arginine methyltransferases; MMA, monomethylarginine.

NO production [30]. Previous studies have also shown that hepatic arginase could metabolise much more than the normal amount of L-arginine influx into cells. Arginase competes with NOS for L-arginine so that only a proportion of the L-arginine influx is available for NOS in a limited time. In the present study, the influx of L-arginine regulated the metabolism of ADMA by competing with ADMA for DDAH. Thus, we suggest that the L-arginine paradox is due to arginase competition with NOS for L-arginine and to the inhibition of NOS by ADMA. In other words, there probably is no L-arginine paradox at all. First, NOS may not reach its maximum activity due to competition with arginase. Second, the circulating levels of ADMA are correlated with the circulating levels of L-arginine, and ADMA competes with L-arginine for NOS.

In conclusion, the present study provides new insights into the L-arginine-NO pathway and for therapeutic approaches involving L-arginine supplementation.

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