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Role of dimethylarginine dimethylaminohydrolase activity in regulation of tissue and plasma concentrations of asymmetric dimethylarginine in an animal model of prolonged critical illness

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ABSTRACT

High plasma concentrations of asymmetric dimethylarginine (ADMA), an endogenous nitric oxide synthase inhibitor, are associated with adverse outcome in critically ill patients. Asymmetric dimethylarginine is released within cells during proteolysis of methylated proteins and is either degraded by dimethylarginine dimethylaminohydrolase (DDAH) or exported to the circulation via cationic amino acid transporters. We aimed to establish the role of DDAH activity in the regulation of tissue and plasma concentrations of ADMA. In 33 critically ill rabbits, we measured DDAH activity in kidney, liver, heart, and skeletal muscle and related these values to concentrations of ADMA in these tissues and in the circulation. Both DDAH activity and ADMA concentration were highest in kidney and lowest in skeletal muscle, with intermediate values for liver and heart. Whereas ADMA content was significantly correlated between tissues (r = 0.40-0.78), DDAH activity was not. Significant inverse associations between DDAH activity and ADMA content were only observed in heart and liver. Plasma ADMA was significantly associated with ADMA in the liver (r = 0.41), but not in the other tissues. In a multivariable regression model, DDAH activities in muscle, kidney, and liver, but not in heart, were negatively associated with plasma ADMA concentration, together explaining approximately 50% of its variation. In critical illness, plasma ADMA poorly reflects intracellular ADMA. Furthermore, tissue DDAH activity is a stronger predictor of plasma ADMA than of intracellular ADMA, indicating that, compared with DDAH activity, generation of ADMA and cationic amino acid transporter–mediated exchange may be more important regulators of intracellular ADMA.

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1. Introduction

Nitric oxide (NO), produced from arginine by NO synthase (NOS), plays an important role in the cardiovascular system by regulating vascular tone, reducing the interaction of white blood cells and platelets with the endothelium, and inhibiting proliferation of vascular smooth muscle cells [1]. Asymmetric dimethylarginine (ADMA) is an endogenously produced inhibitor of NOS [2]; and several prospective studies have shown that elevated plasma concentrations of ADMA, independent of traditional risk factors, predict cardiovascular events and mortality [3]. Especially in critically ill patients, ADMA levels may rise to high levels; and plasma ADMA is a strong independent predictor of adverse outcome [3,4].

The metabolism of ADMA and its exchange between different organ systems via the plasma compartment are illustrated in Fig. 1. Asymmetric dimethylarginine is released within cells upon proteolysis of proteins that have been posttranslationally methylated at arginine residues by protein arginine methyltranferases. Cytosolic ADMA is either degraded by 1 of the 2 isoforms of dimethylarginine dimethylaminohydrolase (DDAH) or exported via cationic amino acid transporters (CAT) to the circulation, from where it is taken up by other cells or excreted by the kidneys [5]. It has been estimated that whole-body production of ADMA in humans is around 300 μmol/d, of which more than 80% is metabolized by DDAH [6]. There is strong evidence that both type 1 and 2 isoforms of DDAH are of critical importance in the metabolism of ADMA [7]. Experiments in which either DDAH-1 or DDAH-2 was overexpressed in transgenic mice led to decreased plasma levels of ADMA [8,9]. Conversely, reduction of DDAH activity by deleting the gene or inhibiting its transcription with small inhibitory RNAs led to increased plasma ADMA levels [10-12]. Next to DDAH activity, also the activities of protein arginine methyltranferases and proteolytic enzymes, and CAT-mediated exchange between intra- and extracellular ADMA play a role in the regulation of intracellular ADMA levels, although the relative contributions of these processes are currently not known [13].

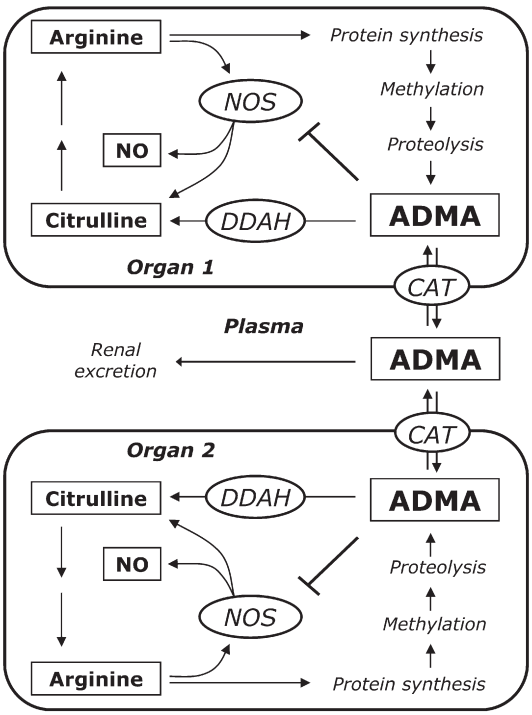

Fig. 1 – Metabolism and transport of ADMA. Free ADMA is generated during proteolysis of proteins that have been methylated at arginine residues. Intracellular ADMA, which is an inhibitor of NOS, is actively degraded by DDAH or exported from the cell to the plasma compartment via CAT. The plasma concentration of ADMA reflects a dynamic equilibrium and is the net result of release into and uptake from the plasma compartment by liver, kidney, heart, muscle, and other organs. In addition, a relatively small fraction of ADMA is cleared from the plasma by renal excretion.

Both generation and DDAH-mediated metabolism of ADMA as well as inhibition of NOS activity by ADMA are intracellular processes; but most studies report on plasma ADMA levels, based on the underlying assumption that the concentration of ADMA in plasma accurately reflects intracellular ADMA levels. Although there are strong indications that, under pathological conditions, intracellular ADMA is elevated and may reach levels sufficient to inhibit NOS function [14], reports on the relation between plasma and intracellular ADMA levels are scarce.

Hyperglycemia caused by insulin resistance is a common metabolic disorder during critical illness and is associated with complications. Normalization of blood glucose levels with intensive insulin therapy reduced morbidity and mortality among critically ill patients in the surgical intensive care unit [15]. We observed that intensive insulin therapy prevented the early rise in plasma ADMA levels in these patients, suggesting that modulation of ADMA metabolism may contribute to the beneficial effects of intensive insulin therapy [16]. Using an animal model of prolonged critical illness, in which plasma insulin and glucose levels were independently manipulated, we were able to show that normoglycemia rather than glycemia-independent actions of insulin maintained physiological ADMA levels by preserving DDAH activity [17].

The aim of the present study was to investigate in detail the relation between plasma and intracellular concentrations of ADMA and the role of tissue DDAH activity in the regulation of ADMA concentration in both compartments in this animal model of prolonged critical illness.

2. Methods

2.1. Animal model

Animals were treated according to the *Principles of Laboratory Animal Care* formulated by the US National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the US National Institutes of Health. The protocol was approved by the local ethics committee for animal research. In brief, in male, adult New Zealand white rabbits, endogenous insulin deficiency was induced by alloxan; and critical illness was brought about by a third-degree burn injury. Insulin was administered via continuous

fixed-speed intravenous infusion to receive either low or high physiologic plasma insulin levels. On each insulin level, blood glucose was manipulated by adjusting the speed of a supplementary intravenous glucose infusion to receive either normoglycemia or hyperglycemia. This resulted in a design with 4 experimental arms: normal insulin levels and normoglycemia (n = 8), high insulin levels and normoglycemia (n = 8), normal insulin levels and hyperglycemia (n = 9), and high insulin levels and hyperglycemia (n = 8). Seven days post–burn injury, blood was collected and animals were killed to allow harvesting of tissue samples of myocardium, skeletal muscle (quadriceps femoris), kidney, and liver. The entire procedure has been described in more detail in a previous publication [17]. The analyses described in the current article are based on pooled data from the 4 experimental groups.

2.2. *Tissue workup and measurement of DDAH activity*

Frozen tissue samples were homogenized in 4 vol (4 mL/g wet weight of tissue) of ice-cold sodium phosphate buffer (100 mmol/L; pH 6.5) containing protease inhibitors (Complete protease inhibitor cocktail from Roche Applied Science, Mannheim, Germany) with an OMNI-2000 homogenizer (OMNI International, Waterbury, CT). Immediately after homogenization, a part of the homogenate was mixed with an equal volume of 1.2 mol/L perchloric acid; and after centrifugation (10 minutes at 2000*g* at 4°C), the supernatant was stored at 4°C until analysis of ADMA. The remainder of the homogenate was used for determination of DDAH activity by measurement of citrulline formation during incubation with excess of ADMA [18]. After a dual centrifugation procedure (10 minutes at 2000*g* followed by 30 minutes at 10 000*g*, both at 4°C) to remove cellular debris, 160 μL of the supernatant was mixed with 240 μL of a 4-mmol/L ADMA solution in sodium phosphate buffer (100 mmol/L; pH 6.5). Before and after incubation for 2 hours at 37°C, the reaction was stopped; and proteins were precipitated by transferring 200 μL of the incubation mixture to vials containing 8 mg sulfosalicylic acid. After vortex mixing, the vials were centrifuged (10 minutes at 3000*g* at 4°C); and citrulline was measured in the clear supernatant with high-performance liquid chromatography as previously described [19]. The increase in citrulline concentration during incubation was used to calculate DDAH activity (expressed as nanomoles per minute per gram wet weight of tissue). The within-assay coefficient of variation was less than 5%. To ensure the validity of the assay procedure, several control experiments were performed. When incubation was performed in the absence of either tissue homogenate or exogenous ADMA, no change of citrulline concentration was observed, demonstrating that citrulline formation indeed reflects DDAH activity. Upon incubation of tissue homogenates with excess exogenous citrulline (final concentration of 100 μmol/L), no decrease in citrulline concentration was observed, showing that metabolic enzymes that could potentially interfere with the DDAH assay by consuming the generated citrulline were not active under the assay conditions. To improve measurement precision in tissues with low DDAH activity, such as skeletal muscle, a relatively long 2-hour incubation time was used. Potentially, this might lead to deviation from assay linearity, especially in tissues with high DDAH activity, such as kidney. However, in none of the tissues examined in this study was deviation from linearity observed using incubation times up to 3 hours, as illustrated in Fig. 2 for kidney and liver.

2.3. *Measurement of ADMA*

Before analysis, the supernatants obtained after deproteinization of tissue homogenates with perchloric acid were neutralized by adding 400 μL of 0.5 mol/L Na_2HPO_4 to 200 μL of the supernatant. The concentration of ADMA in neutralized tissue extracts and plasma was determined using high-performance liquid chromatography as previously described [20] with modified chromatographic separation conditions [21]. Intra- and interassay coefficients of variation were 1.5% and 3.0%, respectively.

2.4. *Statistical analyses*

Data are presented as mean ± standard deviation or median and interquartile range (P_{25}-P_{75}). Because the concentration of ADMA in tissues had a right-skewed distribution, statistical analyses were performed after natural logarithmic transformation. Univariate relations between variables were investigated using Pearson correlation analysis. Because the plasma concentration of ADMA is the net result of the release into and uptake from the plasma compartment by various organs (Fig. 1), we used multivariable regression models to assess the individual contributions of these organs. The relation between tissue levels of ADMA and the concentration of ADMA in plasma was explored by multivariable linear regression analysis with plasma ADMA as dependent variable and tissue content of ADMA in kidney, liver, heart, and muscle as independent explanatory

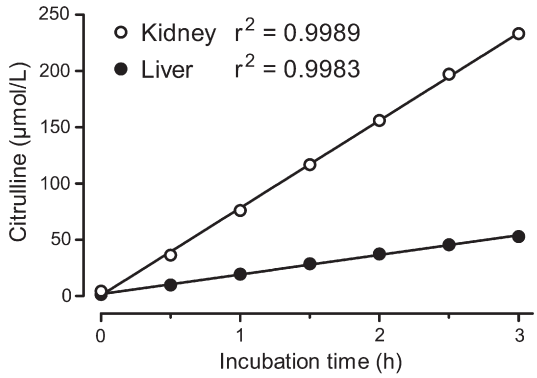

Fig. 2 – Linearity of citrulline formation by DDAH activity. Homogenates of kidney and liver were incubated at 37°C in the presence of 2.4 mmol/L ADMA. After incubation, proteins were precipitated; and the concentration of citrulline in the supernatant was measured. Data points represent the means of 3 replicate incubations of a single tissue preparation. The coefficient of variation was less than 3% at each time point. No significant deviation from linearity was observed for incubation times up to 3 hours. Calculated DDAH activities were 31.59 ± 0.43 and 3.75 ± 0.04 nmol/(min g) for the kidney and liver sample, respectively.

variables. A similar multivariable linear regression model was used to explore the relation between tissue DDAH activity and plasma ADMA. Regression coefficients were expressed as change in plasma ADMA concentration in micromoles per liter per 1–standard deviation increase of the independent explanatory variables. To obtain sufficient statistical power, the main analyses were performed on pooled data from all experimental groups. To assess whether treatment allocation altered the overall predictive value of the models or altered the strengths of the associations between the explanatory variables and plasma ADMA concentration, we added treatment modality (high or low glucose and high or low insulin, both added as dummy [0/1] variables) to the models. A two-sided P value < .05 was considered to indicate statistical significance. Statistical analyses were performed using SPSS 18.0 (SPSS, Chicago, IL).

3. Results

Mean plasma concentration of ADMA was 0.85 ± 0.27 μmol/L. Concentrations of ADMA and DDAH activity in tissue homogenates are summarized in Table 1. Of all tissues investigated, the highest mean DDAH activity was observed in kidney. Mean DDAH activities were approximately 3- to 4-fold lower in heart and liver than in kidney, and the lowest activity was observed in muscle tissue. A similar pattern was observed for the tissue content of ADMA, with a 6-fold higher level in kidney compared with muscle and intermediate levels in heart and liver.

3.1. *Relation between tissue ADMA and tissue DDAH activity*

Despite the rather large differences between ADMA concentrations in various organs, correlation analysis revealed that the ADMA concentrations were positively and significantly associated between all organs. Particularly strong relations were found among organs with a relatively high ADMA content, that is, kidney, liver, and heart (r = 0.65-0.78, all P < .001) and also between kidney and muscle (r = 0.61, P = .002), whereas the relations between muscle ADMA and ADMA in liver and heart were weaker but still significant (r = 0.44, P = .024 and r = 0.40, P = .045, respectively).

Table 1 – Tissue DDAH activity and ADMA content, and their relation

	DDAH activity (nmol/[min g])	ADMA concentration (nmol/g)	Correlation [a]	
			r	P value
Kidney	30.62 ± 8.48	3.00 (2.74-4.71)	–0.079	.70
Heart	11.19 ± 3.67	0.69 (0.58-0.92)	–0.385	.039
Liver	7.36 ± 2.45	1.09 (0.71-1.62)	–0.411	.027
Muscle	2.33 ± 0.63	0.49 (0.35-0.83)	0.022	.92

Data are presented as mean ± SD or median (P_{25}-P_{75}).
[a] Pearson correlation between DDAH activity and logarithmically transformed ADMA.

In contrast to the tissue content of ADMA, DDAH activities in the various organs were not significantly related. Correlation coefficients varied from r = –0.30 (P = .12) for the relation between DDAH activity in kidney and heart to r = 0.20 (P = .29) for the relation between DDAH activity in kidney and liver.

Because DDAH hydrolyzes ADMA, a negative association between DDAH activity and ADMA content at the organ level was anticipated. However, these associations (shown in Table 1) were only significant in heart (r = –0.385, P = .039, Fig. 3A) and liver (r = –0.411, P = .027, Fig. 3B).

3.2. *Relation between plasma ADMA and tissue ADMA*

Next, we examined whether the concentration of ADMA in plasma was associated with tissue levels of ADMA. Plasma ADMA was only significantly associated with ADMA in liver (r = 0.406, P = .029, Fig. 4) and not in the other tissues (all P > .2). To assess the independent predictive value of tissue ADMA levels to the plasma ADMA concentration, a multivariable linear regression model was built with plasma ADMA as dependent variable and tissue ADMA levels as independent predictor variables. In this model, the association between ADMA in the liver and in plasma was slightly attenuated and lost significance (P = .061); and the ADMA

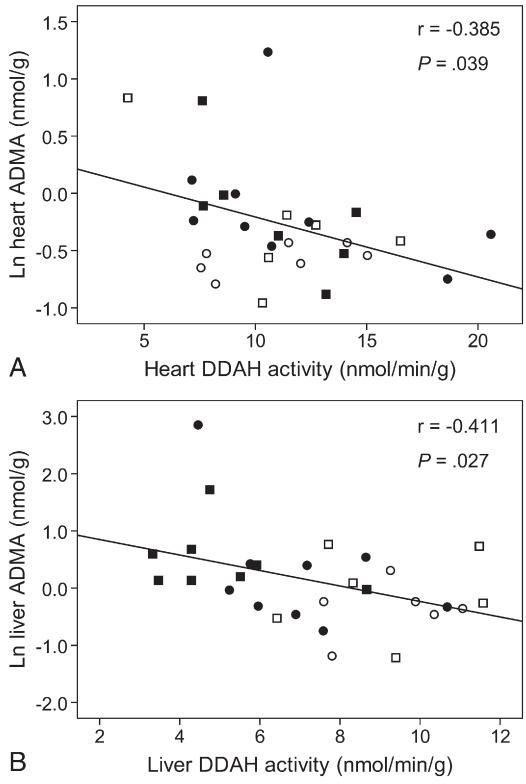

Fig. 3 – Relation between ADMA content and DDAH activity in heart (A) and liver (B). The natural logarithm of the measured ADMA content is plotted. Strengths of the associations are indicated by Pearson correlation coefficients. Treatment allocation: normal insulin (circles), high insulin (squares), normoglycemia (open symbols), and hyperglycemia (filled symbols).

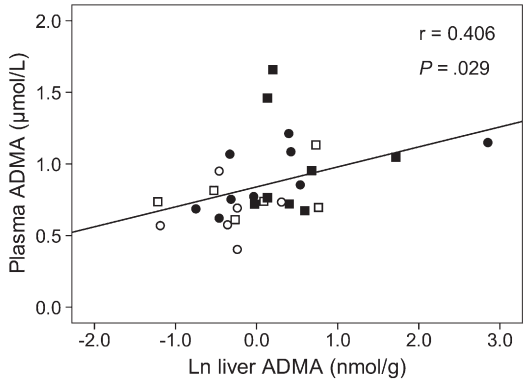

Fig. 4 – Relation between plasma ADMA and liver ADMA. The natural logarithm of the liver ADMA content is plotted. Strength of the association is indicated by Pearson correlation coefficient. Treatment allocation: normal insulin (circles), high insulin (squares), normoglycemia (open symbols), and hyperglycemia (filled symbols).

content of none of the other tissues contributed significantly to the model (all $P > .5$).

3.3. *Relation between plasma ADMA and tissue DDAH activity*

Finally, we examined the associations between the ADMA concentration in plasma and DDAH activity in the various tissues. There were a significant negative relation between plasma ADMA and DDAH activity in the kidney ($r = –0.501$, $P = .006$, Fig. 5) and weaker inverse associations with DDAH activity in liver ($r = –0.334$, $P = .077$) and muscle ($r = –0.317$, $P = .11$). A weak positive association between plasma ADMA concentration and DDAH activity in the heart was observed ($r = 0.283$, $P = .14$). To assess the independent contributions of tissue DDAH activities to plasma ADMA, a multivariable linear regression model was built (Table 2). This model revealed independent and significant associations of DDAH activities

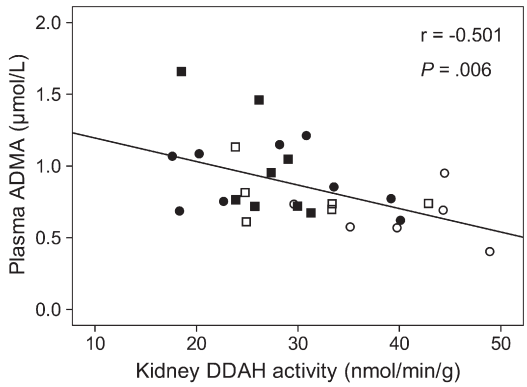

Fig. 5 – Relation between plasma ADMA and kidney DDAH activity. Strength of the association is indicated by Pearson correlation coefficient. Treatment allocation: normal insulin (circles), high insulin (squares), normoglycemia (open symbols), and hyperglycemia (filled symbols).

Table 2 – Multivariable linear regression model for plasma ADMA with DDAH activity in liver, kidney, heart, and muscle as independent predictor variables

Independent variables	Regression coefficient [a]	95% Confidence interval	*P* value
Muscle DDAH activity	–0.118	–0.206 to –0.029	.011
Liver DDAH activity	–0.098	–0.184 to –0.011	.028
Kidney DDAH activity	–0.097	–0.185 to –0.008	.033
Heart DDAH activity	0.066	–0.021 to 0.154	.13

R^2 model = 0.49; $P = .002$.

[a] Regression coefficients are expressed as change in plasma ADMA (micromoles per liter) per 1–standard deviation increase of the organ DDAH activity.

in kidney, liver, and muscle with the plasma ADMA concentration. The strongest association was observed for muscle DDAH activity with a regression coefficient of –0.118, indicating that an increase of DDAH activity in skeletal muscle by 1 standard deviation is associated with a decrease of 0.118 μmol/L plasma ADMA. The contributions from DDAH activities in liver and kidney to the model were almost equal (–0.098 and –0.097 μmol/L ADMA per standard deviation, respectively) and slightly less than the contribution of muscle DDAH activity. The DDAH activity in heart did not significantly contribute to the model. Overall, this model accounted for 49% of the variability in plasma ADMA concentrations, with a *P* value for the whole model of .002. The addition of treatment modality (high glucose or high insulin entered as dummy variables) to the model neither altered the associations between organ DDAH activity and plasma ADMA concentration nor improved the predictive value of the model.

4. Discussion

To gain more insight into the role of tissue DDAH activity in the regulation of intracellular and plasma ADMA levels, we analyzed data from a rabbit model of critical illness, in which ADMA metabolism was modulated by independently manipulating plasma glucose and insulin levels. The most salient findings of our study are as follows: (1) DDAH activities in muscle, kidney, and liver, but not in heart, were inversely and independently associated with plasma ADMA and together explained approximately 50% of the variation in the concentration of plasma ADMA. (2) At the organ level, DDAH activity was only inversely associated with ADMA in the heart and liver, but not with ADMA in kidney and muscle, suggesting that, in the latter organs, DDAH activity is not a major determinant of intracellular ADMA levels. (3) Although ADMA levels in the various organs were strongly correlated, plasma ADMA was only significantly associated with ADMA in the liver and not in the other organs, indicating that plasma ADMA is a poor indicator of tissue levels of ADMA.

Over the past years, both type 1 and 2 isoforms of DDAH have emerged as critical regulators of NO bioavailability [7].

Overexpression of either DDAH-1 or DDAH-2 in transgenic mice led to decreased plasma ADMA levels [8,9], whereas reduction of DDAH activity by deleting the gene or by inhibiting its transcription with small inhibitory RNAs led to increased plasma ADMA levels [10-12]. Overall, the data from our rabbit model confirm that DDAH is a major player in the regulation of plasma ADMA levels. The plasma concentration of ADMA was negatively associated with DDAH activity in muscle, kidney, and liver; and multivariable linear regression analysis confirmed that DDAH activities in these tissues all independently contributed to the plasma level of ADMA, together explaining 49% of its variation. The major contribution of renal DDAH activity to this regression model is in line with previous studies showing that the kidneys play a prominent role in the clearance of ADMA. By measurement of arteriovenous concentration differences, net renal extraction of ADMA has been demonstrated in humans and rats [22,23]. The kidney clears ADMA from the circulation both by degradation via DDAH and by urinary excretion. It should be noted that urinary ADMA excretion differs between species, being prominent in healthy humans [2,24,25] and mice [25], but almost absent in rats [24,25] and rabbits [26]. Therefore, in the present rabbit model, renal DDAH activity was probably the sole route of renal ADMA clearance. However, there is evidence that, in healthy humans, urinary excretion constitutes only a minor pathway for elimination of ADMA [6]. In accord with this notion, plasma ADMA levels are already elevated during early stages of chronic kidney disease, that is, before the decline of glomerular filtration rate [27,28].

The contribution of hepatic DDAH activity to regulation of plasma ADMA levels is in agreement with previous studies showing that, next to the kidney, the liver also plays an important role in ADMA metabolism [3,29,30]. Hepatic clearance of ADMA from the circulation, estimated from measurement of blood flow and arteriovenous concentration differences across the liver, was shown to be only slightly lower than renal clearance in both humans and rats [31,32]. Plasma ADMA levels are increased in patients with liver cirrhosis [33], alcoholic hepatitis [34], and acute liver failure [35] and have also been shown to increase after a major hepatic resection [36]. Moreover, plasma levels of ADMA in patients with liver failure decrease upon successful liver transplantation [37,38].

Although DDAH activity in skeletal muscle was much lower than in the other tissues investigated, it was a significant and independent predictor of plasma ADMA concentration; and its contribution to the multivariable regression model was even slightly higher than the contributions of kidney and liver. The most likely explanation for this surprising finding is that the total mass of skeletal muscle is far higher than the mass of kidney or liver. Already in 1979, Lou [39] described that urinary excretion of ADMA, but not symmetric dimethylarginine (SDMA), was strongly elevated in children with muscular dystrophy, which is consistent with an important role of muscular DDAH in the regulation of circulatory ADMA. Diminished clearance of circulating ADMA by skeletal muscle tissue might help explain the elevation of plasma ADMA levels associated with insulin resistance and type 2 diabetes mellitus reported in a number of studies [40-43]. Insulin has been shown to stimulate uptake of glucose in skeletal muscle by inducing vasodilation [44]. A blunted vasodilatory response to insulin in insulin-resistant individuals may reduce uptake not only of glucose but also of ADMA. Because insulin-induced vasodilation is partly NO-dependent [44], increased ADMA levels may then exacerbate the impairment of the vasodilatory response.

Despite the fact that DDAH activity in the heart was intermediate between activities in kidney and liver and approximately 5-fold higher than in skeletal muscle, cardiac DDAH activity was not a significant predictor of plasma ADMA. Conversely, DDAH activity in the heart showed a significant negative association with its ADMA content. This suggests that cardiac DDAH is very important for regulation of ADMA levels at the local level, but plays no major role in the regulation of circulatory ADMA. This is consistent with results from several studies in humans and animals. Myocardial proteome analysis in dogs revealed that expression of DDAH is related to local perfusion, with high expression of DDAH and low ADMA levels in areas of low local flow [45]. Decreased myocardial DDAH expression and/or activity was found in humans with coronary heart disease and in dogs with congestive heart failure or atrial fibrillation [46-48].

There are several indications that DDAH activity is not a major determinant of ADMA content at the organ level. First, differences in mean ADMA levels between organs changed in parallel with DDAH activity; for example, the highest DDAH activity and ADMA levels were observed in the kidney, whereas in muscle, both DDAH activity and ADMA were lower than in the other tissues. Second, although we observed a strong correlation between the ADMA content of various tissues, DDAH activities were not significantly correlated. Finally, DDAH activity was not related to ADMA content of muscle and kidney; and the inverse associations between DDAH activity and ADMA content of heart and liver were significant but not very strong (r = ~0.4), indicating that, in these tissues, variation in DDAH activity explains approximately 16% (r^2) of the variation in ADMA content.

The liver excepted, our data do not support the concept that plasma ADMA closely reflects intracellular ADMA levels. The concentration of ADMA in the circulation was not significantly associated with ADMA levels in heart, kidney, and muscle. The high blood content of the liver and the fact that the hepatic sinusoids are lined by a relatively permeable fenestrated endothelium may explain the positive association between ADMA in the liver and the circulation. However, this association was attenuated and lost significance in the multivariable regression model. We have previously shown that, in this rabbit model, plasma levels of SDMA closely reflect intracellular SDMA levels in heart, kidney, and liver [17]. This strong association between plasma and tissue levels of SDMA possibly reflects efficient CAT-mediated exchange of SDMA between both compartments. Although transport by CAT is probably equally effective for ADMA as for SDMA, extensive intracellular degradation of the former by DDAH may attenuate the association between intra- and extracellular ADMA. Different behavior of ADMA and SDMA has previously been demonstrated in animal and human models of acute inflammation. Induction of endotoxemia by injection of lipopolysaccharide into rats lowered plasma levels of both arginine and ADMA,

whereas plasma levels of SDMA increased [23]. In patients undergoing elective knee arthroplasty, the postoperative systemic inflammatory response was accompanied by a transient decrease of plasma ADMA, whereas the concentration of SDMA remained unchanged [49]. Possibly, tissue-specific increases in CAT activity provoked by inflammatory stimuli contribute to the differential behavior between both methylated arginines [13].

It is striking that DDAH activities in liver, kidney, and muscle together explained a large portion of the variation in circulatory ADMA levels; but in the latter 2 organs, DDAH activity was not significantly related to intracellular ADMA levels. It should be noted that the pool of circulatory ADMA is much smaller than the intracellular ADMA pools. This implies that DDAH-mediated degradation of a small amount of circulatory ADMA can strongly lower plasma ADMA levels, whereas a similar relative reduction of intracellular ADMA requires degradation of much larger amounts of ADMA. Another potential explanation is that ADMA from the plasma compartment has better access to DDAH compared with intracellular ADMA. Immunostaining of rat mesenteric vessels for DDAH-2 was positive in cytoplasmic vesicles and the apical membrane of endothelial cells [12]. The presence of DDAH-2 in the plasma membrane may provide ADMA from the plasma compartment, possibly after CAT-mediated cellular uptake, with direct access to cellular DDAH, explaining preferential metabolism of circulatory ADMA. Endothelial NOS and CAT-1 are both located in caveolae in the plasma membrane [50], providing a mechanism for direct delivery of plasma arginine to NOS and thereby explaining the "arginine paradox," that is, the observation that extracellular rather than intracellular arginine concentrations are critical to NO production [50,51]. Close proximity of DDAH to, or association of DDAH with, the caveolar CAT-NOS complex would place together all key players involved in NO production within the microenvironment of the plasma membrane. This would explain why DDAH has a larger impact on plasma ADMA than on intracellular ADMA. In addition, this model implies that plasma ADMA rather than intracellular ADMA is critical in regulating NOS activity, consistent with many studies that have found plasma ADMA to be an independent predictor of cardiovascular morbidity and mortality [3].

Some limitations of the present study deserve comment. First, this study was designed to investigate the modulation of arginine and ADMA levels by glycemic control [17]. For the present analysis, animals from all treatment groups were pooled because the number of animals per group was too small to allow separate analysis. However, the result of the multivariable regression model was not altered if treatment allocation was included as additional predictor variable. Moreover, for the present purpose, the pooling of treatment groups was advantageous because the inclusion of ill animals treated with different regimens ensured a fairly wide range of DDAH activities and ADMA levels, thereby increasing the likelihood of detecting significant associations with a low number of observations. Second, we acknowledge that regulation of the DDAH-ADMA system and the response of this system to metabolic changes during critical illness may differ between species; and therefore, the results of this study in rabbits cannot be directly extrapolated to humans. A third limitation is that ADMA content and DDAH activity were measured in tissue homogenates; and therefore, we obtained no information on intraorgan heterogeneity of ADMA content and DDAH activity, which is very prominent in the kidney [7,25] and possibly in other organs as well. Fourth, measurement of DDAH activity does not discriminate between both isoforms of DDAH. Recent evidence has indicated that the effect of DDAH-1 on endothelial NO production is largely ADMA dependent, whereas the effect of DDAH-2 is mainly ADMA independent [52]. Finally, it is very likely that other organs that were not evaluated in the present study, such as the lung [53,54], also contribute to the generation and metabolism of ADMA.

In conclusion, the results from this animal model of prolonged critical illness show that DDAH activities in several organs in concert determine plasma levels of ADMA, confirming that DDAH is an important player in the regulation of circulatory ADMA. Novel findings include the contribution of muscle DDAH as a potential determinant of plasma ADMA levels and the fact that tissue DDAH activity is a stronger predictor of circulatory ADMA than of intracellular ADMA levels. The latter finding indicates that, compared with DDAH activity, the generation of ADMA by protein methylation/proteolysis and CAT-mediated exchange between intracellular and extracellular ADMA may be more important regulators of intracellular ADMA levels.

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Conflict of Interest

The authors declare that there is no conflict of interest associated with this manuscript.

REFERENCES

[1] Moncada S, Higgs EA. The discovery of nitric oxide and its role in vascular biology. Br J Pharmacol 2006;147:S193-201.
[2] Vallance P, Leone A, Calver A, et al. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. Lancet 1992;339:572-5.
[3] Siroen MP, Teerlink T, Nijveldt RJ, et al. The clinical significance of asymmetric dimethylarginine. Annu Rev Nutr 2006;26:203-28.
[4] Nijveldt RJ, Teerlink T, Van der Hoven B, et al. Asymmetrical dimethylarginine (ADMA) in critically ill patients: high plasma ADMA concentration is an independent risk factor of ICU mortality. Clin Nutr 2003;22:23-30.
[5] Teerlink T. ADMA metabolism and clearance. Vasc Med 2005;10(Suppl 1):S73-81.
[6] Achan V, Broadhead M, Malaki M, et al. Asymmetric dimethylarginine causes hypertension and cardiac dysfunction in humans and is actively metabolized by dimethylarginine dimethylaminohydrolase. Arterioscler Thromb Vasc Biol 2003;23:1455-9.
[7] Palm F, Onozato ML, Luo Z, et al. Dimethylarginine dimethylaminohydrolase (DDAH): expression, regulation, and

function in the cardiovascular and renal systems. Am J Physiol Heart Circ Physiol 2007;293:H3227-45.
[8] Dayoub H, Achan V, Adimoolam S, et al. Dimethylarginine dimethylaminohydrolase regulates nitric oxide synthesis. Genetic and physiological evidence. Circulation 2003;108: 3042-7.
[9] Hasegawa K, Wakino S, Tatematsu S, et al. Role of asymmetric dimethylarginine in vascular injury in transgenic mice overexpressing dimethylarginine dimethylaminohydrolase 2. Circ Res 2007;101:e2-10.
[10] Leiper J, Nandi M, Torondel B, et al. Disruption of methylarginine metabolism impairs vascular homeostasis. Nat Med 2007;13:198-203.
[11] Hu X, Xu X, Zhu G, et al. Vascular endothelial-specific dimethylarginine dimethylaminohydrolase-1–deficient mice reveal that vascular endothelium plays an important role in removing asymmetric dimethylarginine. Circulation 2009;120:2222-9.
[12] Wang D, Gill PS, Chabrashvili T, et al. Isoform-specific regulation by NG,NG-dimethylarginine dimethylaminohydrolase of rat serum asymmetric dimethylarginine and vascular endothelium-derived relaxing factor/NO. Circ Res 2007;101:627-35.
[13] Teerlink T, Luo Z, Palm F, et al. Cellular ADMA: regulation and action. Pharmacol Res 2009;60:448-60.
[14] Cardounel AJ, Cui H, Samouilov A, et al. Evidence for the pathophysiological role of endogenous methylarginines in regulation of endothelial NO production and vascular function. J Biol Chem 2007;282:879-87.
[15] Van den Berghe G, Wouters P, Weekers F, et al. Intensive insulin therapy in the critically ill patients. N Engl J Med 2001;345:1359-67.
[16] Siroen MP, Van Leeuwen PA, Nijveldt RJ, et al. Modulation of asymmetric dimethylarginine in critically ill patients receiving intensive insulin treatment: a possible explanation of reduced morbidity and mortality? Crit Care Med 2005;33: 504-10.
[17] Ellger B, Richir MC, Van Leeuwen PA, et al. Glycemic control modulates arginine and asymmetrical-dimethylarginine levels during critical illness by preserving dimethylarginine-dimethylaminohydrolase activity. Endocrinology 2008;149: 3148-57.
[18] Siroen MP, Teerlink T, Bolte AC, et al. No compensatory upregulation of placental dimethylarginine dimethylaminohydrolase activity in preeclampsia. Gynecol Obstet Invest 2006;62:7-13.
[19] Teerlink T, Van Leeuwen PA, Houdijk AP. Plasma amino acids determined by liquid chromatography within 17 minutes. Clin Chem 1994;40:245-9.
[20] Teerlink T, Nijveldt RJ, De Jong S, et al. Determination of arginine, asymmetric dimethylarginine, and symmetric dimethylarginine in human plasma and other biological samples by high-performance liquid chromatography. Anal Biochem 2002;303:131-7.
[21] De Jong S, Teerlink T. Analysis of asymmetric dimethylarginine in plasma by HPLC using a monolithic column. Anal Biochem 2006;353:287-9.
[22] Nijveldt RJ, Van Leeuwen PA, Van Guldener C, et al. Net renal extraction of asymmetrical (ADMA) and symmetrical (SDMA) dimethylarginine in fasting humans. Nephrol Dial Transplant 2002;17:1999-2002.
[23] Nijveldt RJ, Teerlink T, Van Guldener C, et al. Handling of asymmetrical dimethylarginine and symmetrical dimethylarginine by the rat kidney under basal conditions and during endotoxaemia. Nephrol Dial Transplant 2003;18: 2542-50.
[24] Teerlink T. HPLC analysis of ADMA and other methylated L-arginine analogs in biological fluids. J Chromatogr B 2007;851: 21-9.
[25] Al Banchaabouchi M, Marescau B, Possemiers I, et al. NG,NG-dimethylarginine and NG,N'G-dimethylarginine in renal insufficiency. Pflügers Arch 2000;439:524-31.
[26] McDermott JR. Studies on the catabolism of Ng-methylarginine, Ng, Ng-dimethylarginine and Ng, Ng-dimethylarginine in the rabbit. Biochem J 1976;154:179-84.
[27] Kielstein JT, Böger RH, Bode-Böger SM, et al. Marked increase of asymmetric dimethylarginine in patients with incipient primary chronic renal disease. J Am Soc Nephrol 2002;13: 170-6.
[28] Wang D, Strandgaard S, Borresen ML, et al. Asymmetric dimethylarginine and lipid peroxidation products in early autosomal dominant polycystic kidney disease. Am J Kidney Dis 2008;51:184-91.
[29] Richir MC, Bouwman RH, Teerlink T, et al. The prominent role of the liver in the elimination of asymmetric dimethylarginine (ADMA) and the consequences of impaired hepatic function. JPEN J Parenter Enteral Nutr 2008;32:613-21.
[30] Wilcken DE, Sim AS, Wang J, et al. Asymmetric dimethylarginine (ADMA) in vascular, renal and hepatic disease and the regulatory role of L-arginine on its metabolism. Mol Genet Metab 2007;91:309-17.
[31] Siroen MP, Van der Sijp JR, Teerlink T, et al. The human liver clears both asymmetric and symmetric dimethylarginine. Hepatology 2005;41:559-65.
[32] Nijveldt RJ, Teerlink T, Siroen MP, et al. The liver is an important organ in the metabolism of asymmetrical dimethylarginine (ADMA). Clin Nutr 2003;22:17-22.
[33] Lluch P, Torondel B, Medina P, et al. Plasma concentrations of nitric oxide and asymmetric dimethylarginine in human alcoholic cirrhosis. J Hepatol 2004;41:55-9.
[34] Mookerjee RP, Malaki M, Davies NA, et al. Increasing dimethylarginine levels are associated with adverse clinical outcome in severe alcoholic hepatitis. Hepatology 2007;45: 62-71.
[35] Mookerjee RP, Dalton RN, Davies NA, et al. Inflammation is an important determinant of levels of the endogenous nitric oxide synthase inhibitor asymmetric dimethylarginine (ADMA) in acute liver failure. Liver Transpl 2007;13: 400-5.
[36] Nijveldt RJ, Teerlink T, Siroen MP, et al. Elevation of asymmetric dimethylarginine (ADMA) in patients developing hepatic failure after major hepatectomy. JPEN J Parenter Enteral Nutr 2004;28:382-7.
[37] Siroen MP, Warlé MC, Teerlink T, et al. The transplanted liver graft is capable of clearing asymmetric dimethylarginine. Liver Transpl 2004;10:1524-30.
[38] Becker T, Mevius I, de Vries DK, et al. The L-arginine/NO pathway in end-stage liver disease and during orthotopic liver and kidney transplantation: biological and analytical ramifications. Nitric Oxide 2009;20:61-7.
[39] Lou MF. Human muscular dystrophy: elevation of urinary dimethylarginines. Science 1979;203:668-70.
[40] Abbasi F, Asagmi T, Cooke JP, et al. Plasma concentrations of asymmetric dimethylarginine are increased in patients with type 2 diabetes mellitus. Am J Cardiol 2001;88:1201-3.
[41] Stühlinger MC, Abbasi F, Chu JW, et al. Relationship between insulin resistance and an endogenous nitric oxide synthase inhibitor. JAMA 2002;287:1420-6.
[42] Lin KY, Ito A, Asagami T, et al. Impaired nitric oxide synthase pathway in diabetes mellitus: role of asymmetric dimethylarginine and dimethylarginine dimethylaminohydrolase. Circulation 2002;106:987-92.
[43] Chan NN, Chan JC. Asymmetric dimethylarginine (ADMA): a potential link between endothelial dysfunction and cardiovascular diseases in insulin resistance syndrome? Diabetologia 2002;45:1609-16.

[44] Baron AD, Clark MG. Role of blood flow in the regulation of muscle glucose uptake. Annu Rev Nutr 1997;17:487-99.
[45] Laussmann T, Janosi RA, Fingas CD, et al. Myocardial proteome analysis reveals reduced NOS inhibition and enhanced glycolytic capacity in areas of low local blood flow. FASEB J 2002;16:628-30.
[46] Chen X, Niroomand F, Liu Z, et al. Expression of nitric oxide related enzymes in coronary heart disease. Basic Res Cardiol 2006;101:346-53.
[47] Chen Y, Li Y, Zhang P, et al. Dimethylarginine dimethylaminohydrolase and endothelial dysfunction in failing hearts. Am J Physiol Heart Circ Physiol 2005;289:H2212-9.
[48] Liu H, Qu X, Liang Z, et al. Variance of DDAH/PRMT/ADMA pathway in atrial fibrillation dogs. Biochem Biophys Res Commun 2008;377:884-8.
[49] Blackwell S, O'Reilly DS, Reid D, et al. Plasma dimethylarginines during the acute inflammatory response. Eur J Clin Invest 2011;41:635-41.
[50] McDonald KK, Zharikov S, Block ER, et al. A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric-oxide synthase may explain the "arginine paradox". J Biol Chem 1997;272:31213-6.
[51] Hardy TA, May JM. Coordinate regulation of L-arginine uptake and nitric oxide synthase activity in cultured endothelial cells. Free Radic Biol Med 2002;32:122-31.
[52] Pope AJ, Karuppiah K, Kearns PN, et al. Role of dimethylarginine dimethylaminohydrolases in the regulation of endothelial nitric oxide production. J Biol Chem 2009;51:35338-47.
[53] Millatt LJ, Whitley GS, Li D, et al. Evidence for dysregulation of dimethylarginine dimethylaminohydrolase I in chronic hypoxia-induced pulmonary hypertension. Circulation 2003;108:1493-8.
[54] Bulau P, Zakrzewicz D, Kitowska K, et al. Analysis of methylarginine metabolism in the cardiovascular system identifies the lung as a major source of ADMA. Am J Physiol Lung Cell Mol Physiol 2007;292:L18-24.