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In vivo evidence that Agxt2 can regulate plasma levels of dimethylarginines in mice

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ABSTRACT

Elevated plasma concentrations of the asymmetric (ADMA) and symmetric (SDMA) dimethylarginine have repeatedly been linked to adverse cardiovascular clinical outcomes. Both dimethylarginines can be degraded by alanine–glyoxylate aminotransferase 2 (Agxt2), which is also the key enzyme responsible for the degradation of endogenously formed β-aminoisobutyrate (BAIB). In the present study we wanted to investigate the effect of BAIB on Agxt2 expression and Agxt2-mediated metabolism of dimethylarginines. We infused BAIB or saline intraperitoneally for 7 days in C57/BL6 mice via minipumps. Expression of Agxt2 was determined in liver and kidney. The concentrations of BAIB, dimethylarginines and the Agxt2-specific ADMA metabolite α-keto-δ-(N(G),N(G)-dimethylguanidino)valeric acid (DMGV) was determined by LC–MS/MS in plasma and urine. As compared to controls systemic administration of BAIB increased plasma and urine BAIB levels by a factor of 26.5 ($p < 0.001$) and 25.8 ($p < 0.01$), respectively. BAIB infusion resulted in an increase of the plasma ADMA and SDMA concentrations of 27% and 31%, respectively, (both $p < 0.05$) and a 24% decrease of plasma DMGV levels ($p < 0.05$), while expression of Agxt2 was not different.

Our data demonstrate that BAIB can inhibit Agxt2-mediated metabolism of dimethylarginines and show for the first time that endogenous Agxt2 is involved in the regulation of systemic ADMA, SDMA and DMGV levels. The effect of BAIB excess on endogenous dimethylarginine levels may have direct clinical implications for humans with the relatively common genetic trait of hyper-β-aminoisobutyric aciduria.

1. Introduction

Numerous experimental as well as clinical studies have characterized endogenously formed methylarginines such as asymmetric (ADMA) and symmetric dimethylarginine (SDMA) as markers and possible mediators of adverse cardiovascular clinical outcomes [1–4]. ADMA and SDMA are formed, when proteins containing posttranslationally methylated arginine residues are degraded. ADMA has been characterized as a nitric oxide synthase (NOS) inhibitor, and both ADMA and SDMA may also interfere with the cellular transport of L-arginine [5,6]. Acute infusion of ADMA in humans causes (among others) endothelial dysfunction and an increase in blood pressure [7,8]. This makes the metabolism of methylarginines an interesting target for pharmacological interventions. So far, the dimethylarginine dimethylaminohydrolases (DDAH1 and DDAH2), which degrade ADMA (but not SDMA), have been proposed as the primary targets for possible modification of ADMA levels [9,10]. In 1987 Ogawa et al. [11] demonstrated in rats that a significant proportion of ADMA and SDMA is metabolized by alanine–glyoxylate aminotransferase 2 (Agxt2), but only recently, the interest in a possible alternative pathway involving the human AGXT2 has been revived. Rodionov et al. [12] could show in 2010 that overexpression of AGXT2 in mice lowers the ADMA concentrations in tissues and ameliorates some of the pathophysiological effects of ADMA. This work suggests that pharmacological augmentation of AGXT2 expression and/or activity might promise therapeutic approach for treatment of the ADMA-mediated pathological conditions.

In addition to dimethylarginines AGXT2 has also been shown to metabolize β-aminoisobutyrate, while its polymorphisms have been linked to the metabolic trait hyper-β-aminoisobutyric acidu-

Abbreviations: ADMA, asymmetric dimethylarginine; AGXT2, alanine–glyoxylate aminotransferase 2; BAIB, beta-aminoisobutyrate; DDAH, dimethylarginine dimethylaminohydrolase; DMGV, α-keto-δ-(N(G),N(G)-dimethylguanidino)valeric acid; SDMA, symmetric dimethylarginine.

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ria [13–15], which is relatively common in some populations. However, high excretion of BAIB aside, data regarding the biochemical and clinical correlates of this condition remain rather limited [16,17].

In 1990 Ueno et al. [18] reported that intraperitoneal injections of BAIB in rats induced BAIB aminotransferase activity of Agxt2 in the liver (measured by using radiolabeled BAIB as substrate). So far, it remains unresolved whether the increase in Agxt2 activity resulted from induction of Agxt2 expression or from a direct stimulating effect on the enzyme activity. It also remains to be elucidated, if the observed effect of BAIB infusion extends to other species and other substrates of Agxt2 such as ADMA and SDMA. The goal of the current study was to test the hypothesis that systemic administration of BAIB regulates Agxt2-mediated metabolism of dimethylarginines *in vivo*.

2. Materials and methods

2.1. Chemicals

[$^2H_7$]-Labeled ADMA hydrochloride und [$^2H_7$] labeled L-arginine hydrochloride were obtained from EURISO-TOP (Saint-Aubin, France). L-NMMA monoacetate, ADMA dihydrochloride and SDMA dihydrochloride were purchased from Enzo Life Sciences GmbH (Lörrach, Germany). L-Arginine was obtained from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Acetonitrile hypergrade for LC–MS and SUPRAPUR® formic acid (98%) was purchased from Merck (Darmstadt, Germany), Water-Baker Analyzed LC–MS-Reagent from Mallinckrodt Baker B.V. (Deventer, Netherland).

2.2. Infusions of D,L-BAIB in mice via minipumps

The study was carried out following the requirements of the National Act on the Use of Experimental Animals (Germany) and was approved by the University and State Animal Welfare Committees. D,L-β-aminoisobutyrate (D,L-BAIB, Sigma Aldrich, Munich, Germany) was infused in 10 C57/BL6 male mice for 7 days using intraperitoneally implanted osmotic minipumps (Alzet, Charles River, Germany) at the rate 0.125 mg/g/day (diluted in saline, infusion rate – 1 μl/hour). The control group (10 mice) received the minipumps with saline. One mouse in the control group died during the course of the experiment due to the reasons unrelated to the study procedures. 24-h-urine was collected in metabolic cages during the last day of D,L-BAIB infusion and stored at −80 °C. After 7 days of infusion mice were killed and blood was collected by cardiac puncture into EDTA containing tubes (final concentration 5 mmol/L). Plasma was separated by centrifugation and stored at −80 °C. The samples of liver and kidney for investigation of gene expression by mRNA and immunoblot analysis were collected, immediately flash-frozen and stored at −80 °C.

2.3. Immunoblot analysis

Isolated tissue samples of mice and samples of human embryonic kidney (HEK293) cells overexpressing human AGXT2 protein (as positive control for immunoblot analysis) were homogenized in ice-cold 0.2% SDS solution containing a protease inhibitor mixture (Mini-complete Protease Inhibitor Cocktail Tablets; Roche Diagnostics-Applied Science, Mannheim, Germany). Protein concentrations were determined using a standard assay (BCA Protein Assay Reagent; Rockford, USA) according to manufacturer's instructions.

For immunoblot analysis, HEK cell lysates (10 μg of total protein) and tissue homogenates (50 μg of total protein) from liver and kidney of mice were prepared and diluted with Laemmli buffer (62 mM Tris–HCl, 2% SDS, 10% glycerol, 0.01% bromphenol blue, and 0.4 mM dithiothreitol). After incubation at 95 °C for 5 min proteins were separated by SDS–PAGE under reducing conditions on 10% polyacrylamide gels and transferred to nitrocellulose membranes (Protran Nitrocellulose Transfer Membrane; Whatman, Dassel, Germany) using a tank blotting system from Bio-Rad (Munich, Germany). Membranes were probed with a 3 μg/ml custom made rabbit polyclonal antibody (Eurogentec, Seraing, Belgium) raised against following amino acid sequence of human AGXT2: KPRMPPCDFMPERYQS (75% identical to murine Agxt2 and showing sufficient and specific cross reactivity with murine Agxt2). After incubation at 4 °C over night a horseradish peroxidase-conjugated goat-anti-rabbit antibody (Sigma Aldrich, Munich, Germany) was used as secondary antibody at a dilution of 1:10,000. Immunoreactive bands were visualized using ECL Western Blotting Detection Reagents from Amersham (GE Healthcare, Buckinghamshire, UK) and a Chemidoc XRS imaging system (Bio-Rad, Munich, Germany). To control sample loading, membranes were incubated for 30 min with Restore Western Blot Stripping Buffer (Pierce, Rockford, USA) at 37 °C and after washing reprobed with a mouse monoclonal anti-human β-actin antibody (Sigma Aldrich, Munich, Germany) at a dilution of 1:500. As secondary antibody a horseradish peroxidase-conjugated goat-anti-mouse antibody (Dianova, Hamburg, Germany) was used at a dilution of 1:10,000.

Protein expression of Agxt2 in liver and kidney of mice was determined by densitometric analysis (Quantity One Software, Bio-Rad, Munich, Germany) and normalized to protein content of β-actin.

2.4. Expression of Agxt2 mRNA in liver and kidney

Total RNA from tissue samples was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Afterwards, first strand synthesis was accomplished via iScript Select cDNA Synthesis Kit (Bio-Rad, Munich, Germany). To determine the expression of *Agxt2* in samples, a real-time PCR technique was carried out on a LightCycler 2 System (Roche Diagnostics-Applied Science, Mannheim, Germany). LightCycler FastStart DNA Master^{PLUS} SYBR Green I Reagents (Roche Diagnostics-Applied Science, Mannheim, Germany) and following primer pairs were used for PCR: *Agxt2* (forward 5'-CTTCGGGACGAATTTG ATATCG-3' and reverse 5'-TCTTACTTAGCTCTTCTCTCCAT-3') and *β-actin* (forward 5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3' and reverse 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3'). Relative expression of *Agxt2* was normalized to *β-actin* expression as a housekeeping gene. To amplify PCR fragments, DNA was first denaturated for 10 min at 95 °C, followed by 40 cycles of denaturation for 10 s at 95 °C, annealing for 10 s at 64 °C and extending for 30 s at 72 °C. Finally, a melting curve analysis was performed.

For quantification of gene expression, a plasmid containing a 287 bp fragment of *Agxt2* was used as standard. The corresponding fragment was amplified from cDNA of mouse liver with the primer pair above. Correctness of the inserted fragment was verified by sequencing (AGOWA, Berlin, Germany), before.

2.5. Measurement of substrate levels via LC–MS/MS

ADMA and SDMA were measured by HPLC–MS/MS (Agilent 1100 HPLC System [Agilent Technologies, Waldbronn, Germany]; API 4000, Applied Biosystems, Darmstadt, Germany) as previously described with minor modification [19].

BAIB in plasma and urine of mice were determined by HPLC–MS/MS (Agilent 1100 HPLC System [Agilent Technologies, Waldbronn, Germany] with an EC 250/2 Nucleodur HILIC column [Machery-Nagel, Düren, Germany]; API 4000, Applied Biosystems, Darmstadt, Germany). Samples of 20 μL of urine or plasma were

precipitated by adding 50 μL (β-AIB-d6, 1 μg/mL in acetonitrile) or 100 μL (β-AIB-d6, 50 ng/mL in acetonitrile) internal standard solution, respectively. The isotope labeled BAIB standard was a kind gift of Ute Hofmann (IKP Stuttgart) [20]. After centrifugation the supernatant of the plasma sample was directly injected. The supernatant of the urine sample was diluted 1:50 with mobile phase (80% acetonitrile, 20% water, 0.5 g/L ammonium formate; pH 4) first. The mass transitions were *m*/*z* 104.0 to 85.9 for BAIB and *m*/*z* 110.0 to 91.8 for β-AIB-d6. The calibration ranged from 0.2 to 2.0 μmol/L BAIB in plasma and 1–300 μmol/L BAIB in urine. Samples with higher concentrations were diluted with dialysed plasma or synthetic urine in the ratio of 1:10 before sample preparation.

The Agxt2-specific ADMA metabolite DMGV was measured in plasma and urine of mice using a LC–MS/MS method as previously described [21].

2.6. Generation of a HEK293 cell line stably expressing human AGXT2

Human embryonic kidney (HEK293) cells were stably transfected (as previously described [5]) with the expression vector pcDNA3.1(+) containing full-length cDNA of human *AGXT2*. For amplification of the fragment following primer pair was used: forward 5'-TGAGTGGGAGAAATGACTCTAAT-3' and reverse 5'-CTGACAATGTTACTTAGCTCTTC-3'.

2.7. Statistical analysis

Comparisons of Axgt2 substrates and DMGV levels in plasma and urine of mice, as well as comparisons of Agxt2 expression in liver and kidney of mice were performed using the 2-tailed Student's *t* test. Statistical significance was defined as a p value < 0.05. Values are reported as mean ± S.E. For the calculations, the Prism 5 software (ver. 5.00 for Windows; GraphPad Software, San Diego, CA) was used.

3. Results

3.1. Effect of D,L-BAIB infusion on D,L-BAIB concentrations

D,L-BAIB concentrations in plasma and urine were measured using LC–MS/MS after 7 days of intraperitoneal D,L-BAIB infusion (Fig. 1). Plasma levels were significantly increased in the mice treated with D,L-BAIB as compared to the mice treated with saline (3.50 ± 0.93 μmol/L vs. 0.13 ± 0.01 μmol/L; $^{**}p < 0.01$). The mice treated with D,L-BAIB also excreted higher amounts of D,L-BAIB in urine compared to the mice from the control group (88.7 ± 6.5 μmol/L vs. 3.3 ± 0.1 μmol/L; $^{***}p < 0.001$).

3.2. Effect of D,L-BAIB infusion on Agxt2 expression

The effect of D,L-BAIB infusion on *Agxt2* mRNA expression in tissues was estimated using real-time RT PCR. Results (Fig. 2) indicated no significant alteration ($p > 0.05$) of *Agxt2* mRNA expression in kidney and liver of mice injected with D,L-BAIB compared to control group. The *Agxt2* mRNA expression was normalized to the *β-actin* expression.

Agxt2 protein expression in tissues of both groups was determined by immunoblot analysis (Fig. 3A). Agxt2 was detected on SDS–PAGE as a single band of about 50 kDa using the polyclonal anti-AGXT2 antibody. Lysates of HEK cells overexpressing human AGXT2 (about 52 kDa) were used as positive control. The membranes were also probed with anti-β-actin antibody, to verify proper sample loading and to perform densitometric analysis. The results (Fig. 3B) revealed no significant alteration of Agxt2 protein levels after D,L-BAIB administration compared to the control group ($p > 0.05$).

3.3. Effect of D,L-BAIB infusion on DMGV and dimethylarginine concentrations

We measured the plasma and urine concentrations of the Agxt2-specific ADMA metabolite DMGV using LC–MS/MS in order to assess the effect of D,L-BAIB infusion on Agxt2 activity towards ADMA. Plasma levels of DMGV were significantly decreased in mice treated with D,L-BAIB (Fig. 4A) as compared to the control group (0.17 ± 0.02 μmol/L vs. 0.21 ± 0.01 μmol/L; $^{*}p < 0.05$), while the urine levels stayed unchanged (data not shown). D,L-BAIB infusion resultedalso in a significant increase in both ADMA (0.57 ± 0.04 μmol/L vs. 0.45 ± 0.03 μmol/L in controls; $^{*}p < 0.05$) and SDMA (0.16 ± 0.01 μmol/L vs. 0.20 ± 0.02 μmol/L; $^{*}p < 0.05$) levels in plasma shown in Fig. 4B and C. The urine levels of dimethylarginines were not significantly different (data not shown).

4. Discussion

In the present study systemic administration of BAIB in mice did not affect tissue Agxt2 expression, but led to decrease in plasma concentrations of the Agxt2-specific ADMA metabolite DMGV, strongly suggesting that BAIB competitively inhibited the activity of Agxt2 towards other substrates. Therefore, infusion of BAIB and subsequent inhibition of Agxt2 activity resulted in elevation of plasma concentrations of ADMA and SDMA, which have been linked to increased mortality in several clinical studies. Taken together the present data provide the first evidence that endogenous Agxt2 regulates systemic levels of dimethylarginines. As detailed

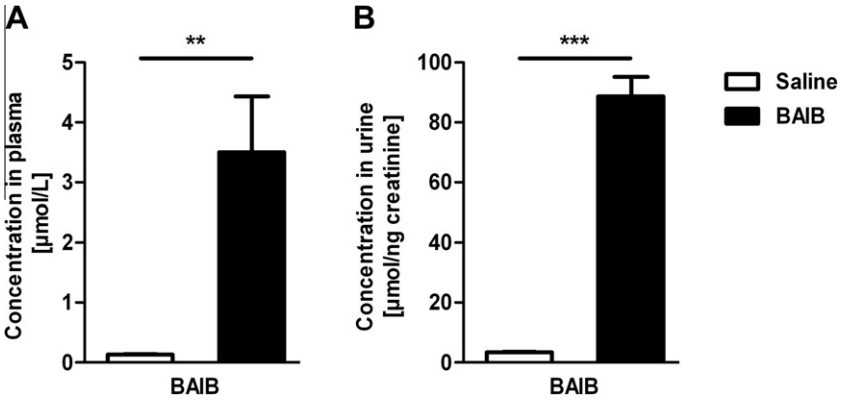

Fig. 1. D,L-BAIB concentrations of treated and untreated mice measured by LC–MS/MS. D,L-BAIB accumulated significantly in plasma (A; $^{**}p < 0.01$) and urine (B; $^{***}p < 0.001$) of mice treated with D,L-BAIB for 7 days ($n = 10$) compared to the control group ($n = 9$) treated with saline.

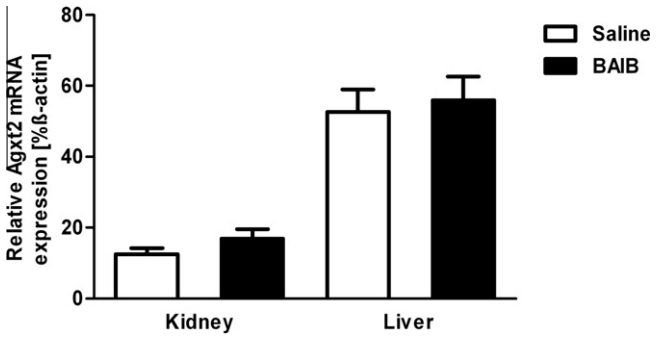

Fig. 2. Expression of *Agxt2* mRNA in tissues of mice determined via real-time RT PCR. Relative *Agxt2* mRNA expression was not significantly different ($p > 0.05$) in kidney and liver of mice treated with D,L-BAIB ($n = 10$) compared to the control group treated with saline ($n = 9$).

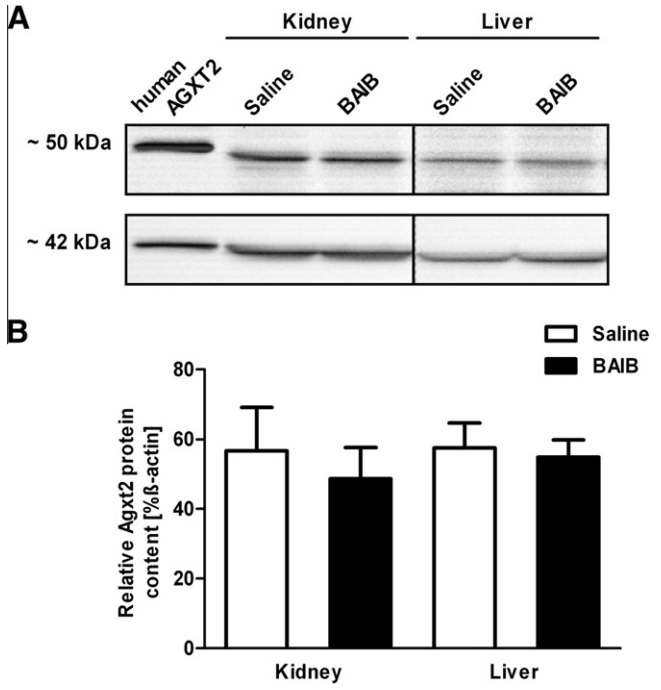

Fig. 3. Relative Agxt2 protein content in tissues of mice. Immunoblot analysis (A) was performed to determine relative Agxt2 protein content, which was not significantly different ($p > 0.05$) in kidney and liver of mice treated with D,L-BAIB ($n = 10$) compared to control group ($n = 9$) treated with saline (B).

further below, the effect of BAIB excess on endogenous dimethylarginine levels may have clinical implications for humans with the relatively common genetic trait of hyper-β-aminoisobutyric aciduria [16,17].

The key finding of the current study that systemic administration of BAIB resulted in downregulation of Agxt2 activity in relation to dimethylarginines appears to be in contrast with the data previously published by Ueno et al., who showed that infusion of BAIB could lead to upregulation, rather than downregulation of the Agxt2 activity towards BAIB in rat liver lysates [18]. The study by Ueno and colleagues, however, was different from the present study in several ways: Ueno et al. used a different animal species (rats) and administered BAIB using repetitive i.p. injections with potential tissue damage and large fluctuations in plasma BAIB concentrations rather than continuous infusion using minipumps. Furthermore, Ueno and colleagues measured Agxt2 activity towards BAIB *ex vivo* in the liver lysates at the alkaline conditions (pH 8.8) in the presence of excess of pyruvate, which serves as one of the two main amino acceptors for Agxt2, but is also a product of this enzyme in the reaction of amino group transfer from alanine to glyoxylate. Our goal on the other hand was to use plasma DMGV levels to assess the *in vivo* "net flux" of ADMA via the Agxt2 pathway thus focusing on a different substrate and completely different *in vivo* reaction settings.

Our finding that BAIB infusion downregulates Agxt2 activity towards dimethylarginines *in vivo* is in agreement with the original experiments by Kontani et al., who also showed that BAIB can competitively inhibit protein activity towards the Agxt2 substrate β-alanine [13]. In the same study Kontani and colleagues estimated the K_m value of Agxt2 for BAIB as 0.12 mM, which is 50-fold lower than the K_m value of Agxt2 for SDMA (6.4 mM). Taking together with the observation by Ogawa et al. [22] that Agxt2 has similar activity towards ADMA and SDMA, the estimation of the Agxt2 K_m values by Kontani and colleagues suggests that Agxt2 has higher affinity to BAIB as a substrate, than to both dimethylarginines, which is also consistent with inhibition of the Agxt2 activity towards ADMA and SDMA after systemic BAIB administration observed in the present work.

It was demonstrated in previous studies that systemic levels of dimethylarginines are predominantly regulated either at the level of enzymatic metabolism or at the level of renal excretion [7,11,23]. Any potential impairment of DDAH activity would selectively affect the levels of ADMA, but not of SDMA, which is different from what we observed. A potential effect of BAIB infusion on renal function would also not entirely explain our data, because in that case one would expect to see a much stronger increase in

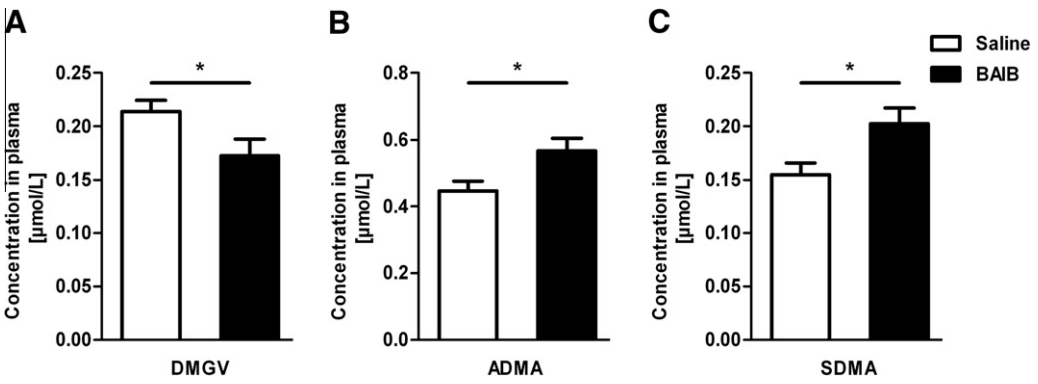

Fig. 4. Concentrations of DMGV and dimethylarginines in plasma after infusion of D,L-BAIB. Levels of DMGV (A) were significantly decreased in plasma of mice treated with D,L-BAIB ($n = 10$) compared to the control group ($n = 9$) treated with saline ($^*p < 0.05$). ADMA (B) and SDMA (C) accumulated significantly (both $^*p < 0.05$) in plasma of mice receiving D,L-BAIB infusion ($n = 10$) compared to control group ($n = 9$).

SDMA levels compared to ADMA, because SDMA is much more sensitive to the changes in renal function than ADMA [24,25]. We propose therefore that increase in plasma levels of the dimethylarginines was caused by competitive inhibition of the Agxt2 activity towards dimethylarginines by BAIB *in vivo*.

Moreover, in rats 16% of ADMA metabolism appears to be mediated by Agxt2 [11] and overexpression of human AGXT2 in mice reduces ADMA levels [12]. However, on the basis of several studies involving alternative enzymes [26–29], it was assumed that DDAH1 is the enzyme mainly responsible for elimination of ADMA in humans and mice, which suggested a minor role of AGXT2 in ADMA metabolism. Our data challenge this concept and suggest that at least in this model endogenous Agxt2 controls plasma levels of both ADMA and SDMA.

4.1. Implications of the present data for human diseases

The observation that at least in some conditions AGXT2 controls the systemic levels of ADMA and SDMA delivers a basis for clinical studies estimating the activity of AGXT2-mediated pathway for ADMA and SDMA metabolism in the diseases associated with elevated levels of both dimethylarginines. Taken together with the AGXT2 overexpression experiments [12], our work also suggests that upregulation of AGXT2 might be a perspective therapeutic approaches for treatment of the ADMA-(and SDMA)-mediated pathologies in humans.

Furthermore, the present experimental setting may actually offer a short term model for a human metabolic trait known since 1951 as hyper-β-aminoisobutyric aciduria [15], which is especially common in Asian populations with a prevalence of approximately 40% [16,17]. In patients with hyper-β-aminoisobutyric aciduria the AGXT2 enzyme appears to be functionally deficient. So far, little is known regarding the possible clinical consequences of hyper-β-aminoisobutyric aciduria [14], which has recently been linked to a SNP within the *AGXT2 gene*[14]. The increase of urinary BAIB excretion achieved by infusion of BAIB is compatible with the increase seen in humans homozygous for the *AGXT2* SNP rs37369. Moreover, in our experiments short term infusion of BAIB resulted in an increase of plasma ADMA and SDMA levels by 27% and 31%, respectively. This may be of relevance as epidemiological data from the Framingham Offspring Study indicate that in humans an elevation of plasma ADMA levels by 24% (approx. 1 standard derivation) is associated with a relative increase in total mortality of 21% [1]. In addition, recent studies show similar associations with mortality for SDMA [4]. Compensatory DDAH activity may largely attenuate the effects of impaired AGXT2 activity on ADMA levels in humans. In contrast, the effect of impaired AGXT2 activity on SDMA levels may be more profound due to the lack of alternative metabolizing enzymes. Therefore, a possible association of BAIB levels and clinical outcomes in patients with hyper-β-aminoisobutyric aciduria, mediated via increased plasma dimethylarginine levels, should be investigated.

In conclusion, the present data suggest that endogenous Agxt2 can regulate plasma levels of dimethylarginines and that elevation of BAIB can result in disturbed metabolism of ADMA and SDMA.

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