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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