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# Promiscuous activity of arginine:glycine amidinotransferase is responsible for the synthesis of the novel cardiovascular risk factor homoarginine

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## ARTICLE INFO

## ABSTRACT

Low plasma homoarginine has emerged as a risk marker for cardiovascular disease. We exploited cells of a patient with a rare inborn error of metabolism to explore potential pathways of homoarginine synthesis, using stable isotopes and mass spectrometry. Control lymphoblasts, as opposed to lymphoblasts from an arginine:glycine amidinotransferase (AGAT)-deficient patient, were able to synthesize homoarginine from arginine and lysine. In contrast, in a patient with a deficiency of the urea cycle enzyme argininosuccinate synthase, plasma homoarginine was not decreased. We conclude that promiscuous activity of AGAT, a key enzyme in creatine synthesis, plays a pivotal role in homoarginine synthesis.

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## 1. Introduction

Homoarginine, a homolog of arginine, is often measured simultaneously with arginine and asymmetric dimethylarginine (ADMA), one of the methylated forms of arginine [1,2]. ADMA is an endogenous inhibitor of the production of nitric oxide from arginine by nitric oxide synthase (NOS) [3,4] and elevated ADMA is associated with cardiovascular disease [5,6]. Homoarginine differs from arginine by the presence of an additional methylene group in the carbon chain. Because of this structural similarity, homoarginine may act as a competing substrate or inhibitor of enzymes that use arginine as substrate. Homoarginine has indeed been shown to act as substrate for NOS [7,8], and may thus increase nitric oxide production. However, compared to arginine, the $K_m$ value of homoarginine is much higher, reflecting a lower catalytic efficiency of NOS using homoarginine as substrate [7,8]. In addition, homoarginine, by competing with arginine for cellular uptake, may limit the amount of intracellular arginine [9]. Therefore, despite homoarginine being a substrate for NOS, production of nitric oxide may be reduced at a high homoarginine to arginine ratio. Taken together, homoarginine may affect the cardiovascular system by either enhancing or limiting nitric oxide production. In addition, homoarginine, being an inhibitor of liver and bone alkaline phosphatase [10], may also be relevant to bone metabolism.

Low serum homoarginine has recently emerged as a potential new risk marker for stroke [11] and cardiovascular mortality [12–14], and in a study among female nursing home patients, low serum homoarginine was associated with high bone turnover [15]. To increase our understanding of the role of homoarginine in cardiovascular and other diseases, it is important to unravel the metabolic pathways involved in homoarginine synthesis and degradation in humans.

Evidence for in vivo synthesis of homoarginine from lysine was first reported in rats, where ^{14}C-labeled homoarginine was measured in liver and kidney after injection of ^{14}C-labeled lysine [16]. In humans, an increase in urinary homoarginine after oral lysine administration has been demonstrated in adults [16] as well as children [17]. The metabolic routes responsible for homoarginine synthesis from lysine in vivo have not yet been unraveled completely. Fig. 1 shows a schematic representation of the two pathways that have been suggested in literature most often. The first pathway uses arginine:glycine amidinotransferase (AGAT; EC 2.1.4.1.). The primary role of AGAT is the production of guanidino acetic acid (GAA) from arginine and glycine in the first step of the creatine-synthesis pathway [18]. This makes AGAT an important enzyme in maintenance of energy metabolism in different tissues, including the myocardium [19]. When AGAT uses lysine instead of glycine as amidino-acceptor, homoarginine is formed instead of GAA [20,21]. The second pathway for homoarginine synthesis that has been suggested in literature uses the urea-cycle enzymes [22,23], in which instead of ornithine, lysine is used by ornithine

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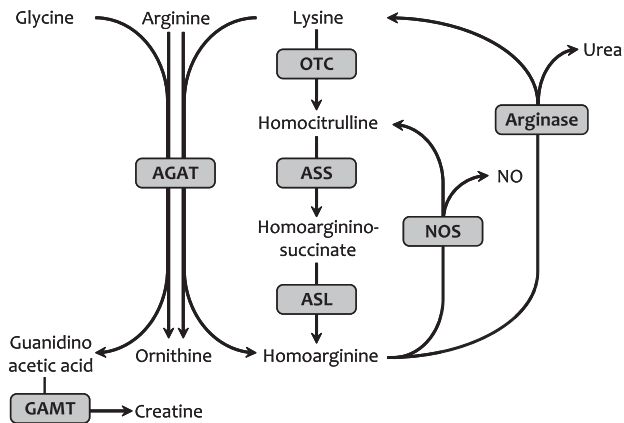
**Fig. 1.** Suggested metabolic routes for homoarginine formation and degradation. The main function of arginine:glycine amidinotransferase (AGAT) is the transfer of an amidino-group from arginine to glycine, resulting in formation of guanidino acetic acid, which is subsequently methylated by guanidinoacetate methyltransferase (GAMT) to form creatine. However, when AGAT uses lysine instead of glycine as acceptor of the amidino-group, homoarginine is formed. Another possible pathway for homoarginine synthesis is by the enzymes of the urea cycle. When lysine instead of ornithine is used as substrate by ornithine transcarbamoylase (OTC), homocitrulline is formed, which can be converted into homoargininosuccinate by argininosuccinate synthase (ASS) and subsequently into homoarginine by argininosuccinate lyase (ASL). Because of its structural similarity to arginine, homoarginine can be used as substrate by arginase, forming lysine and urea, or by nitric oxide synthase (NOS) forming nitric oxide (NO) and homocitrulline.

transcarbamoylase (OTC), resulting in formation of homocitrulline instead of citrulline. Next, homocitrulline is converted into homoargininosuccinate by argininosuccinate synthase (ASS; EC 6.3.4.5.), and subsequently into homoarginine by argininosuccinate lyase (ASL; EC 4.3.2.1.).

In the present study we have exploited rare inborn errors of metabolism to gain more insight in the de novo synthesis of homoarginine in humans, using stable isotopes and mass spectrometry. To this end, the capacity for homoarginine synthesis in lymphoblasts from a patient affected with AGAT-deficiency was compared to control lymphoblasts. Furthermore, the concentration of homoarginine in plasma of an ASS-deficient patient was compared to levels in age-matched controls.

## 2. Materials and methods

### 2.1. Chemicals and reagents

RPMI-medium, Earls balanced salt solution (EBSS), and Hanks balanced salt solution (HBSS) were obtained from Invitrogen, (Carlsbad, CA, USA). Potassium hydroxide, 70% perchloric acid, and L-glutamine were purchased at Merck (Darmstadt, Germany) and D-glucose at VWR International (Poole, England). Fetal bovine serum, 10% (FBS) was obtained from Bodinco (Alkmaar, The Netherlands) and bovine serum albumin (BSA) from Sigma (St. Louis, MO, USA). $^{15}N_2$-(guanidino)-arginine:HCl (98% $^{15}N_2$), $^{13}C_2–^{15}N$-glycine (98% $^{13}C_2$, 96–99% $^{15}N$), and $^{13}C_6$-L-lysine:2 HCl (98% $^{13}C_6$) were obtained from Eurisotop (Saint Aubin Cedex, France).

### 2.2. Patient samples

The previously described AGAT-deficient lymphoblasts with a homozygous pathogenic mutation in the *AGAT*-gene [24] were used. A control lymphoblastoid cell line was obtained from Centre d′ Etude du Polymorphisme Humain (Paris, France).

Plasma from a 4-day old male suffering from citrullinemia with a pathogenic mutation in the arginino succinate synthase (*ASS*)-gene, was available for the determination of amino acid levels, including homoarginine.

### 2.3. Lymphoblasts

Lymphoblasts were cultured in 50 ml RPMI-medium containing 10% FBS and 1% L-glutamine, until confluence was reached. After harvesting the cells by centrifuging 5 min at 571×g and 20 °C, the pellets were washed twice in 25 ml EBSS + 0.1% D-glucose. Next, intracellular amino acids were depleted essentially as previously described [25]. In brief, the washed cell pellets were incubated for 1 h at 37 °C in 35 ml EBSS supplemented with 0.1% (D-glucose and 0.25% BSA. After depletion, cells were harvested (5 min, 571×g, 20 °C), washed twice in 25 ml HBSS, and stored at −20 °C.

### 2.4. Enzyme assays in lymphoblasts

Upon analysis, lymphoblast pellets were thawed on ice. Each pellet was reconstituted in 1 ml 100 mmol/l $Na_2HPO_4$, pH = 7.5 and cells were lysed using an ultrasonic probe (Bandalin Sonopuls mini 20 with MS 1.5 titanium microtip) for 3 × 10 s at ∼0.250 kJ (=90% of maximum power). After centrifugation (5 min, 8800×g, 4 °C), 50 µl supernatant was used for the determination of protein content.

To determine whether AGAT is capable to synthesize homoarginine from arginine and lysine, a lymphoblast lysate sample containing 50 µg protein was incubated with 30 µl 100 mmol/l $^{15}N_2$-(guanidino)-arginine and 30 µl 160 mmol/l $^{13}C_6$-lysine. To reach a total volume of 200 µl, 100 mmol/l $Na_2HPO_4$, pH = 7.5 was added. At $t = 0$ and after 18 hours incubation at 37 °C, proteins were precipitated with 30 µl 4 mol/l perchloric acid, and the samples were neutralized with 20 µl 6 mol/l KOH. After centrifugation, the supernatant was used to measure the formation of $^{13}C_6–^{15}N_2$-homoarginine (mass transition 253–89) with an LC-MS/MS method [1]. In short, the internal standard $D_4$-homoarginine was added to the samples and the basic amino acids were extracted using solid phase extraction. After derivatization, the resulting butyl ester derivatives were measured with LC–MS/MS.

To confirm that AGAT is functioning properly, a second assay was done in which a lymphoblast lysate sample containing 50 µg protein was incubated with 30 µl 100 mmol/l $^{15}N_2$-(guanidino)-arginine and 30 µl 160 mmol/l $^{13}C_2–^{15}N$-glycine in a total volume of 200 µl. The formation of $^{13}C_2–^{15}N_3$-guanidino acetate was measured after 18 h incubation at 37 °C using GC–MS [26,27]. Additionally, lactate dehydrogenase (LDH; EC 1.1.1.27.) activity was determined to confirm viability of both the AGAT-deficient and the control cell line. Activity was determined using a spectrophotometric assay, which follows the accumulation of NADH in the presence of pyruvate [28].

### 2.5. Measurement of homoarginine in plasma

In a 4-day old ASS-deficient male patient, plasma homoarginine was determined simultaneously with arginine, monomethylarginine (MMA) and both asymmetric and symmetric dimethylarginine (ADMA and SDMA, respectively) using our recently validated HPLC method with tandem mass spectrometry detection [1]. The concentrations of these amino acids were compared to concentrations measured in plasma of 10 age-matched children without inborn or acquired disorders of amino acid metabolism.

## 3. Results

### 3.1. Homoarginine formation in lymphoblasts

Both the lysates of AGAT-deficient and the control cell lines showed normal LDH-activity, indicating proper quality of the lysates. During the 18-h incubation at 37 °C in the presence of

$^{15}N_2$-(guanidino)-arginine and $^{13}C_6$-lysine, control lymphoblasts produced 0.475 pmol $^{15}N_2$–$^{13}C_6$-homoarginine/min/mg protein (Fig. 2). Lysates from the AGAT-deficient lymphoblasts, however, did not show any detectable $^{15}N_2$–$^{13}C_6$-homoarginine formation. To confirm that the control lymphoblasts expressed functional AGAT, the production of $^{15}N_3$–$^{13}C_2$-GAA from $^{15}N_2$-(guanidino)-arginine and $^{15}N$–$^{13}C_2$-glycine was also measured. The lysates of the control lymphoblasts showed an activity of 27.6 pmol $^{15}N_3$–$^{13}C_2$-GAA/min/mg protein, which is in line with the previously reported reference range of 21–70 pmol $^{15}N_3$–$^{13}C_2$-GAA/min/mg protein [27]. In the AGAT-deficient lymphoblasts $^{15}N_3$–$^{13}C_2$-GAA formation was not detectable, confirming the absence of any residual AGAT-activity. Possible homoarginine formation from lysine catalyzed by the urea-cycle enzymes would lead to the formation of $^{13}C_6$-homoarginine instead of $^{15}N_2$–$^{13}C_6$-homoarginine, since $^{15}N_2$-(guanidino)-arginine is not involved. In lysates of both the AGAT-deficient as well as the control lymphoblasts formation of $^{13}C_6$-homoarginine was not detectable.

### 3.2. Homoarginine in plasma of an ASS-deficient patient

As expected, the plasma arginine concentration in the ASS-deficient patient was very low, that is, 10.8 μmol/l, approximately sixfold lower than the mean concentration (62.0 ± 20.0 μmol/l) in the healthy age-matched control subjects. In contrast, the homoarginine concentration in the plasma of the ASS-deficient patient was approximately eightfold higher than in the age-matched controls (5.1 μmol/l versus 0.61 ± 0.40 μmol/l, respectively). Concentrations of the methylated arginines in the plasma of the ASS-deficient patient were similar to the concentrations measured in the control subjects (0.65 μmol/l versus 0.81 ± 0.16 μmol/l for ADMA, 0.85 μmol/l versus 0.78 ± 0.36 μmol/l for SDMA, and 0.078 μmol/l versus 0.109 ± 0.025 μmol/l for MMA).

## 4. Discussion

The main finding of this study is that *de novo* synthesis of homoarginine was not detectable in AGAT-deficient lymphoblasts, whereas in a control lymphoblast cell line detectable amounts of homoarginine were newly synthesized from arginine and lysine. A second key finding is that in plasma from an ASS-deficient patient the concentration of homoarginine was eightfold fold higher than in control subjects, as opposed to the decreased level that would be expected if the urea-cycle enzymes were mainly responsible for homoarginine synthesis. In creatine synthesis, the intermediate GAA is formed by AGAT-catalyzed transfer of an amidino-group between arginine and glycine. Substrate promiscuity of AGAT leads to transfer of the amidino-group from arginine to lysine, leading to conversion of the latter into homoarginine. Overall, our data show that this promiscuous activity of AGAT, rather than urea cycle enzymes, is likely responsible for *de novo* synthesis of homoarginine in humans.

Already in 1964 Ryan and Wells showed that labeled homoarginine was synthesized from labeled lysine in rats [16], and that in humans oral administration of lysine resulted in the urinary excretion of homoarginine and homocitrulline. Although this proved that homoarginine originates from lysine, the possible routes involved remained unclear. After it was shown that arginase is able to accept homoarginine as a substrate [29], the idea of an alternate urea-cycle was put forth [23]. Also the involvement of a transamidination reaction similar to that of AGAT was suggested by Ryan et al. [21]. In support of this notion, homoarginine producing plants contain a gene for an arginine:lysine amidinotransferase [20]. Amidinotranferases are well conserved within plants, but similarity decreases when compared to mammals. The specific amino acid binding domains, however, remain conserved [20].
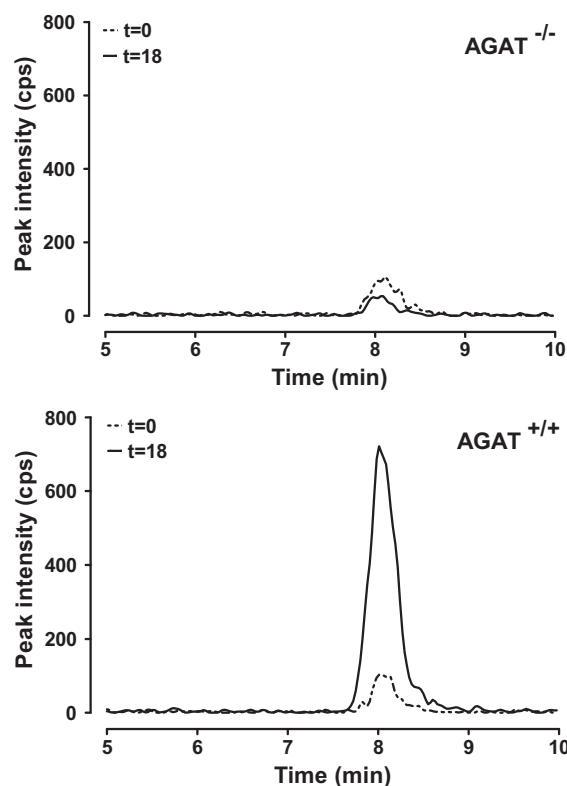


**Fig. 2.** Homoarginine formation in AGAT-deficient and control lymphoblasts. Chromatograms of $^{15}N_2$–$^{13}C_6$-homoarginine in lymphoblasts of an AGAT-deficient (AGAT$^{-/-}$) patient (upper panel) and a control (AGAT$^{+/+}$) cell line (lower panel). The closed line represents the amount of $^{15}N_2$–$^{13}C_6$-homoarginine present after an 18-hour incubation at 37 °C in the presence of $^{15}N_2$-(guanidino)-arginine and $^{13}C_6$-lysine, and the dotted line represents the baseline measurement.

Compared to the formation of GAA by AGAT in control lymphoblasts, the rate of homoarginine synthesis was about 60-fold lower at similar substrate concentrations. This is in line with homoarginine synthesis by transfer of an amidino-group from arginine to lysine being a secondary activity of AGAT, next to its main role in the synthesis of GAA from arginine and glycine. It should be noted that enzyme promiscuity is widespread, probably because the catalysis of multiple reactions offers evolutionary advantages [30]. There are several parallels between homoarginine and GAA in support of a common origin of both metabolites. First, mean levels of both homoarginine and GAA are higher in men compared to women [13,31]. Second, both compounds are mainly formed in the kidney where AGAT is located [16,18], and are significantly decreased in plasma of patients with end-stage renal disease [32].

The elevated homoarginine levels we measured in the ASS-deficient patient, were also reported in several other urea-cycle disorders [17,33]. Kato suggested that this elevation is the result of decreased degradation of lysine, increasing its availability as substrate for AGAT-catalyzed homoarginine synthesis [34]. Another possibility is downregulation of arginase as a compensatory reaction to the very low levels of arginine associated with ASS-deficiency. Assuming unaltered AGAT-catalyzed synthesis of homoarginine, a reduction of homoarginine degradation by arginase may then lead to elevated plasma levels.

It should be noted that although our data unequivocally implicate AGAT in homoarginine synthesis, a role of urea-cycle enzymes cannot be fully excluded. To be able to give a more definitive answer on the involvement of the urea-cycle enzymes in homoarginine synthesis, measurement of homoarginine synthesis in cell lines lacking one or more urea-cycle enzymes may be useful.

Additionally, it cannot be fully excluded that uptake from the diet contributes to normal endogenous homoarginine levels, since the grass pea, lentil and similar legumes, which are used for human consumption, contain a high amount of non-protein amino acids, including homoarginine [35–37]. More research is needed to evaluate to what extent dietary homoarginine contributes to plasma levels of homoarginine in humans.

As to whether high or low homoarginine levels are more beneficial with respect to cardiovascular disease, remains to be established. Low homoarginine levels have been associated with cardiovascular risk and mortality [12–14], possibly reflecting the local failing creatine metabolism in the myocardium [19], whereas high homoarginine levels may interfere with nitric oxide production, potentially leading to elevated blood pressure [9]. Research in the field of creatine metabolism has produced an extensive body of knowledge on the regulation of AGAT activity [18], which may also provide valuable clues to potential pharmaceutical or dietary strategies to alter homoarginine synthesis by targeting AGAT. Supplementation with creatine, for example, effectively reduces synthesis of GAA by feedback repression of AGAT and may therefore also reduce synthesis of homoarginine. In contrast to this expectation, in healthy volunteers receiving creatine supplementation that effectively reduced GAA levels, circulating homoarginine levels were increased by 35% [38]. This likely reflects involvement of secondary pathways, and shows that more research into this field is required.

In conclusion, our data show that promiscuous activity of AGAT, a key enzyme in the synthesis of creatine, plays a pivotal role in the synthesis of the novel cardiovascular risk factor homoarginine.

## 5. Disclosures

None.

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