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N^{δ}-Methylated L-arginine derivatives and their effects on the nitric oxide generating system

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Abstract—So far N^{δ}-methyl-L-arginine (MA) is only detected in yeast cells. Assuming that MA also exists in mammalians we examined possible physiological effects of N^{δ}-methylated L-arginine derivatives on the nitric oxide generating system, that is, nitric oxide synthase (NOS), arginase and dimethylarginine dimethylaminohydrolase (DDAH). N^{δ}-methyl-L-citrulline (MC) turned out to be a weak non-specific inhibitor of nitric oxide synthases. Moreover, MA is hydroxylated by all human NOS isoforms to N^{ω}-hydroxy-N^{δ}-methyl-L-arginine (NHAM) but not converted further. This hydroxylated intermediate, however, was detected to be a potent inhibitor of bovine liver arginase with a K_i of 17.1 ± 2.2 μM.

1. Introduction

Nitric oxide (NO) is endogenously formed from L-arginine via nitric oxide synthases (NOSs) and is a potent vasodilatator, which regulates vascular tone, protects against atherosclerotic events and acts as a neuromediator with many physiological functions, including the formation of memory and modulation of pain.[1,2] Therefore, NO levels must be strictly regulated. Insufficient NO availability can be associated with hypertension,[3] coronary heart disease,[4] heart failure, and myocardial infarction as well as erectile dysfunction.[5] The predominant enzymes in this regulation process are nitric oxide synthases (NOSs) and arginases.[6] Activity of NOSs is physiologically regulated by endogenously produced N^{ω}-methylated L-arginines such as asymmetric dimethyl-L-arginine (ADMA) and monomethyl-L-arginine (NMMA). These compounds are degraded by dimethylarginine dimethylaminohydrolase (DDAH) to L-citrulline and dimethylamine or methylamine.[7]

Besides the physiological regulation of NOS isoenzyme activity by N^{ω}-methylated L-arginines, a regulatory mechanism between arginase and NOS becomes more and more apparent.[6] The intermediate in NOS catalysis N^{ω}-hydroxy-L-arginine (NOHA) is a strong inhibitor of arginase.[8] These main metabolic pathways for L-arginine compete for their common substrate. Essentially, arginase inhibition leads to increased NO levels by elevating the substrate pool of L-arginine for NOSs and thereby provides an option to indirectly affect NO-deficient conditions. Moreover, an age-related upregulation of arginase activity has been observed for mice as well as for humans and seems to be implicated in the pathophysiology of vascular dysfunction,[9] hypertension,[10] and atherosclerosis.[11] Accordingly, arginase inhibition presents a new promising pharmaceutical strategy in indirectly modulating NO levels.

Moreover, recent studies showed that modulation of DDAH activity could be another mechanism to indirectly affect NO formation by elevating or lowering N^{ω}-methylated L-arginine levels.[12]

In our studies we examined the effects of a methylation at the N^{δ}-position of L-arginine derivatives (Fig. 1). So far N^{δ}-methyl-L-arginine (MA) is only detected in yeast cells.[13] The enzyme catalyzing this reaction was identified in 1999.[14] However, further evidence of any physiological significance of MA has not yet been reported. This might be due to the fact that MA is not detected properly because of its very similar structure to L-NMMA whose concentration is assumed to be much

Keywords: L-Arginine; Dimethylarginine dimethylaminohydrolase; Nitric oxide synthase; Arginase; Nitric oxide.
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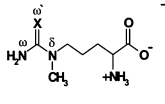
	NH_2^+	N^{δ}-methyl-L-arginine (MA)
	O	N^{δ}-methyl-L-citrulline (MC)
	NOH	N^{ω}-hydroxy-N^{δ}-methyl-L-arginine (NHAM)

Figure 1. N^{δ}-Methylated L-arginine derivatives.

higher. Accordingly, we guess that the physiological existence of N^{δ}-methylated L-arginines cannot be ruled out. In addition, since there is such a great similarity to the physiological substrates a pharmaceutical usability of these compounds is conceivable. Hence, we examined the influence of MA and its presumed metabolites of NOS catalysis on the predominant enzymes involved in regulation of NO levels. Synthesis of the herein examined N^{δ}-methylated L-arginine derivatives will be published elsewhere by Schade et al[15].

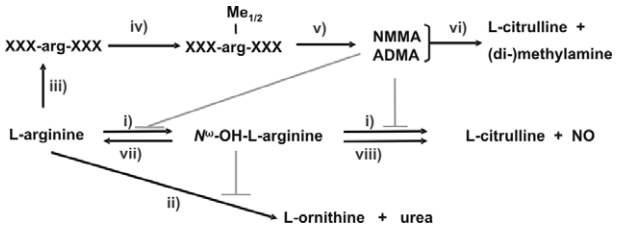
Besides these pathways in the NO generating system with high substrate specificity, a metabolism by cytochrome P450 (CYP450), which exhibits less substrate specificity, could be of physiological importance. *N*-Hydroxylations are known biotransformations of the CYP450 enzyme system but have not yet been described for L-arginine to NOHA, whereas for NOHA to L-citrulline and NO.[16] Additionally, a converse *N*-reduction of NOHA by microsomal enzymes to L-arginine was shown recently by Clement et al.[17]

All pathways studied are summarized in Scheme 1. Except the *N*-hydroxylation of MA to NHAM by iNOS[18] none of these pathways have been studied before.

2. Results and discussion

2.1. Inhibition of NOSs

As illustrated in the Introduction N^{ω}-methylated L-arginines are strong inhibitors of NOSs whereas not much is known about the effects of N^{δ}-methylated L-arginine derivatives. Thus, we investigated the inhibitory effects of MA, NHAM, and MC on human NOS isoenzymes. Nitric oxide formation was measured colorimetrically via the Griess assay.[19]



Scheme 1. Overview on L-arginine metabolism. NMMA and ADMA inhibit NOS activity, whereas N^{ω}-OH-L-arginine is a potent inhibitor of arginase. (i) NOSs; (ii) arginase; (iii) protein biosynthesis; (iv) protein arginine methyltransferase (PRMT); (v) protein degradation; (vi) DDAH; (vii) microsomal reduction; (viii) cytochrome P450. Steps (i), (ii), (vi), (vii), and (viii) were studied with the N^{δ}-methylated derivatives.

Table 1. Determined IC_{50} values ± SD (mM)

Compound	eNOS	iNOS	nNOS
MA	>10	>10	>10
NHAM	4.0 ± 0.6	5.0 ± 2.3	7.9 ± 0.6
MC	2.0 ± 0.1	2.4 ± 0.4	3.0 ± 0.9

Values are means of three experiments each comprising three incubations in seven different concentrations (0, 0.5, 1.0, 2.5, 5.0, 7.5, and 10 mM).

All tested compounds are very weak inhibitors of all three NOS isoforms (Table 1). MC shows the highest potency. This effect is significant when compared to NHAM for eNOS ($p = 0.026$) and nNOS ($p = 0.004$) whereas not for iNOS ($p = 0.209$).

Determined IC_{50} values indicate no selectivity for one isoenzyme unlike other known L-arginine-based NOS inhibitors like N^{ω}-propyl-L-arginine which possesses significant nNOS selectivity.[20]

Moreover, K_i values for other L-arginine analogs such as NMMA or L-thiocitrulline are determined to be in the low micromolar range[21,22] and many other substance classes (e.g., pyridine derivatives, amidines or pteridines) with higher inhibitory potency are already well-known.[23–25] Finally, if MA was a physiological regulator of NOS activity, the inhibitory concentrations would rather not be in the millimolar range but much lower. Concluding from these results, a physiological function for N^{δ}-methylated arginine derivatives as inhibitors of NOSs seems to be rather unlikely.

2.2. Metabolism by NOSs

A conversion of the herein described amino acids by the action of NOSs was investigated to study whether they bear a physiological significance or they can be used as NO-donors. Both MA and NHAM were tested as substrates for all isoforms of NOSs in concentrations up to 10 mM.

These investigations were already carried out for iNOS. It was demonstrated that MA is hydroxylated to NHAM but is not converted any further.[18] Our studies clearly show that all human NOS isoforms are capable to hydroxylate MA. iNOS exhibits much less specific activity than nNOS and eNOS (Fig. 2).

Specific turnover rates for MA are lower in comparison to those obtained with L-arginine as substrate. nNOS achieves about 35% of the activity of L-arginine conversion, iNOS which appears to be the slowest isoenzyme achieves about 16% (Fig. 3).

Accordingly, the first step of NOS catalysis occurred, whereas the second step to the urea derivative analogous to L-arginine catalysis could not be detected. This could be due to the fact that NHAM was formed in amounts that would not deliver detectable quantities of MC. This question was addressed with incubations of NHAM as the substrate in concentrations of 1 mM.

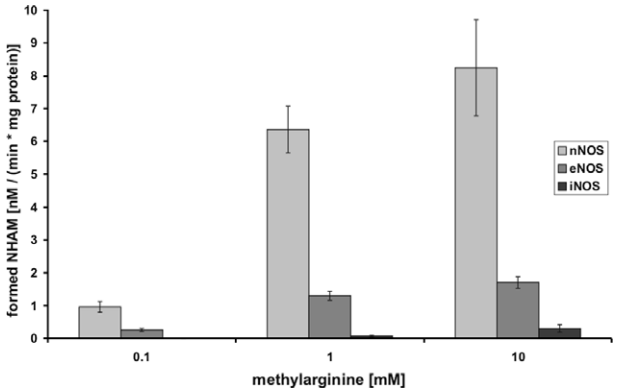


Figure 2. Hydroxylation of N^{δ}-methyl-L-arginine by NOS isoforms.

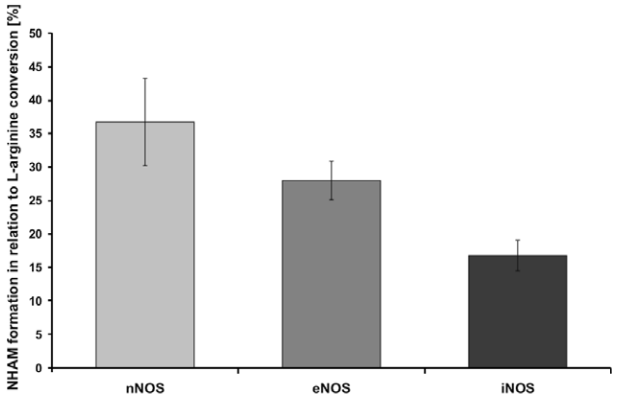


Figure 3. N^{δ}-Methyl-L-arginine hydroxylation relative to L-arginine catalysis.

For all incubations with NHAM MC could only be detected in traces, whereas a new metabolite N^{δ}-cyano-N^{δ}-methyl-L-ornithine (CMO) was detected. The identity of this compound was verified via LC/MS. The formation of cyanamides is already known as studies with various *N*-hydroxyarginine analogs showed.[26] Since CMO could also be monitored in samples without NOS isoforms comparisons between incubations of 1 mM NHAM with and without NOSs were performed in order to clarify whether the cyanamide formation is enzymatic. Overall, detected total amounts of cyanamide were very low (data not shown). Slightly more CMO could be detected in samples without NOSs, suggesting a non-enzymatic conversion.

An enzymatic formation of MC by the action of NOSs appeared rather unlikely, considering that the formed amount is negligibly low relative to CMO and a hydrolysis of the cyanamide to MC could be observed by investigating CMO stability. Thus, the second step in NOS catalysis did not occur at all.

Taken together substrate and inhibition studies, N^{δ}-methylation seems to impair affinity to the active site of NOSs. This is most likely due to the loss of one hydrogen bond from the N^{δ}-hydrogen to Glu. This hydrogen bond is of great importance for substrate linkage as shown with substrates such as L-canavanine or L-indospicine which are also not capable of forming this H-bond and therefore exhibit less affinity to NOSs.[27] Furthermore, this glutamate appears to be essential for NOS activity as Chen et al.[28] showed for eNOS via replacing it by Leu or Gln. These modified enzymes were not able to produce nitric oxide from L-arginine.

Thus, a physiological formation of NHAM is thinkable provided that MA exists in sufficient concentrations. Furthermore, N^{δ}-methylation of L-arginine derivatives provides no opportunity in the development of a new lead structure for NOS modulators. A pharmaceutical utilization for the tested N^{δ}-methylated compounds can be excluded.

2.3. Studies with arginase

An effect of N^{δ}-methylated L-arginine derivatives on this metabolism pathway has not yet been investigated. The modulation of arginase activity by these compounds is interesting because arginase and NOS compete for the same substrate, that is L-arginine, and inhibition of arginase could therefore be a mechanism to increase the NO biosynthesis.

Our studies showed that MA is neither a substrate nor an inhibitor of bovine liver arginase.

In contrast, NHAM has a strong inhibitory potency with an IC_{50} value of 123 μM and a K_i of 17.1 ± 2.2 μM (Fig. 4). Furthermore, the observed data clearly revealed a competitive inhibition mechanism which is known for related arginine derivatives (data not shown).

NOHA is known as an inhibitor of arginase[29] and it is noteworthy that the K_i of NOHA for bovine liver arginase was determined to be 30–150 μM.[29,30] Accordingly, NHAM possesses at least the same inhibitory potency as NOHA. Thus, methylation at the N^{δ}-position might improve the affinity to bovine liver arginase. K_i determination was carried out at pH of 9.4, the pH optimum of arginase. Some inhibitors of arginase show a lower potency at a pH of 7.4 compared to 9.4, for example the most potent arginase inhibitors, the boronic acid derivatives.[31] But for NOHA a relevant change in potency does not occur by changing the pH from 9.0 to 7.4.[30] Because of the similar structure one can expect that the K_i for NHAM at pH 7.4 should not significantly differ.

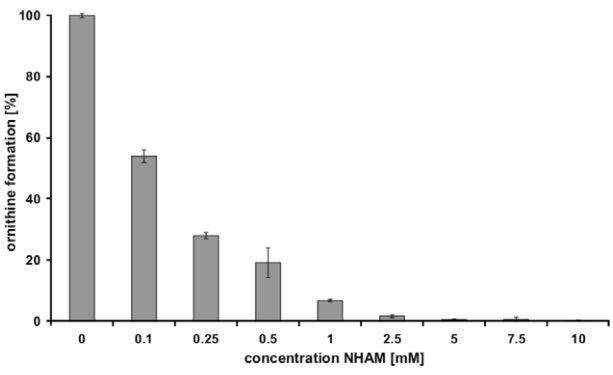


Figure 4. Arginase inhibition by N^{ω}-hydroxy-N^{δ}-methyl-L-arginine.

In summary, NHAM is a potent inhibitor of bovine liver arginase with a K_i in the same range as NOHA. Consequently, NHAM formed by NOSs could represent an endogenously regulator of arginase activity as it is already known for NOHA. Moreover, a pharmacological utilization is conceivable and NHAM might be a new pharmaceutical lead for arginase inhibitors, bearing potential for further structural modifications. In contrast to NOHA, NHAM is not converted by NOSs and therefore presents a better inhibitor of arginase.

2.4. Inhibition of human DDAH-1

The endogenously formed NOS inhibitors ADMA and NMMA are metabolized to L-citrulline and methylamines by the action of DDAH. Thus, DDAH-1 represents another enzyme involved in the physiological regulation of NO levels. Therefore, MA, NHAM, and MC were tested as inhibitors of recombinant human DDAH-1 in concentrations up to 10 mM.

None of these compounds appreciably affected DDAH-1 activity (IC_{50} values >5 mM). Therefore, a physiological modulation of DDAH-1 activity by N^{δ}-methylated L-arginine derivatives as well as a pharmaceutical usability can be excluded.

2.5. NHAM reduction by pig liver microsomes and mitochondria

It is known that microsomes are capable of reducing *N*-hydroxyguanidines such as NOHA,[17] which prompted us to examine the metabolism of NHAM. Assays were performed with pig liver microsomes and mitochondria as enzyme sources from the pig proved to be a good model for this reductive pathway.[32] Incubations were carried out at pH 6.3, the pH optimum for reduction,[17] and at a physiological pH of 7.4. In all assays the reduction to MA could be observed. Specific activity for mitochondria was determined to be 3.29 ± 0.22 nM MA min^{-1}[mg protein]$^{-1}$ (pH 6.3) and is about 3-fold higher than that for microsomes (1.03 ± 0.09 nM MA min^{-1}[mg protein]$^{-1}$). At a physiological pH of 7.4 specific activity was negligibly lower (Fig. 5).

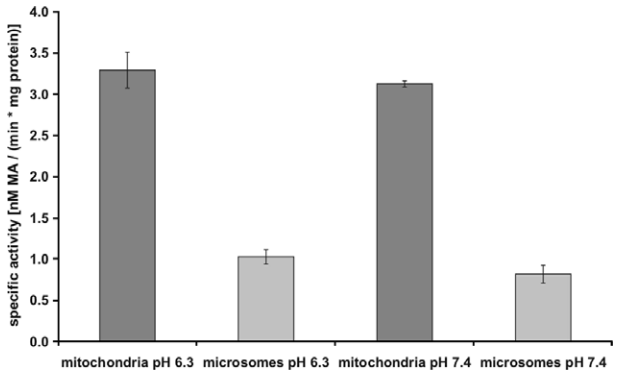


Figure 5. Reduction of N^{ω}-hydroxy-N^{δ}-methyl-L-arginine to N^{δ}-methyl-L-arginine by pig liver microsomes and mitochondria (performed at pH 6.3 and pH 7.4).

2.6. Oxidation studies with cytochrome P450

A transformation of NOHA to citrulline by rat liver microsomes has already been described.[16] Rat P450 enzymes of the subfamily 3A are responsible because induction of this enzyme subfamily by dexamethasone leads to a 7-fold increase in L-citrulline formation.[33]

Therefore, we tested rat liver microsomes containing 0.05 nM P450 after pretreatment and non-pretreatment of the rats with dexamethasone. HPLC analysis revealed the formation of CMO in these samples. Addition of CMO showed a concentration dependent increase of this signal. No further metabolites were observed, so that a conversion of NHAM to MC by cytochrome P450 could be ruled out. Consequently, in contrast to the conversion of NOHA by P450, NHAM is not metabolized to the corresponding urea derivative. In incubations without NADPH or protein no CMO could be detected. In incubations with MA and rat liver microsomes no oxidation of MA to NHAM could be observed.

About 5-fold higher concentrations of CMO could be observed in incubations with dexamethasone induced rat liver microsomes. This also indicates an involvement of the P450 subfamily 3A in the catalysis of NHAM. A metabolism of non-physiological *N*-hydroxyguanidines by P450 to the corresponding urea or cyanamide derivative and nitric oxide has been observed for several derivatives, for example, *N*-hydroxydebrisoquine or *N*-(4-chlorophenyl)-*N*′-hydroxyguanidine.[34–36]

Kinetic determination for dexamethasone induced rat liver microsomes led to a specific formation rate of 4.89 ± 0.55 nM CMO min^{-1}[nM cytochrome P450]$^{-1}$ which is about 5-fold higher than that of non-induced rat liver microsomes with a specific activity of 0.99 ± 0.15 nM CMO min^{-1}[nM cytochrome P450]$^{-1}$.

Addition of SOD completely inhibited the formation of CMO which indicates a superoxide anion mediated mechanism. To clarify this mechanism, NHAM was incubated in the absence of NADPH with 10 mM potassium superoxide with or without protein. All samples showed a formation of CMO. Small amounts of MC could be detected, which is most likely due to the hydrolytic instability of the cyanamide. Accordingly, the oxidase activity is involved in the catalysis of this reaction. This is explained by the 'uncoupling mechanism' of P450 isoenzymes leading to superoxide anions and hydrogen peroxide.[37] In summary, these findings indicate a mechanism mediated by superoxide anions and exclude the involvement of the monooxygenase activity of P450.

In contrast to small cyanamide amounts formed in incubations with NOS isoenzymes, the formation of CMO by P450 is about 200- to 1000-fold higher. Our investigations showed a Michaelis–Menten kinetic with a K_m of 60.42 μM and a V_{max} of 5.03 nM CMO min^{-1}[nM cytochrome P450]$^{-1}$. A linear substrate conversion could be observed up to the concentration of 0.2 mM. Substrate saturation occurred at 1.0 mM NHAM.

3. Conclusions

N^{δ}-Methylated L-arginine derivatives are weak inhibitors or, respectively, bad substrates for NOSs, probably due to the loss of the δ-*N* hydrogen bond. They possess no isoenzyme selectivity. Thus, a postulated physiological role as analogs of the endogenous N^{ω}-methylated arginines NMMA and ADMA seems unlikely as well as a pharmacological usability as modulators of NOSs.

In contrast, N^{ω}-hydroxy-N^{δ}-methyl-L-arginine is a potent inhibitor of bovine liver arginase, an attractive target for the treatment of NO-deficient conditions. The affinity to the active site of arginase appears to be improved by N^{δ}-methylation as the determined K_i value is lower than that for NOHA. This should be more enlightened by X-ray crystallography. Provided that NHAM is formed endogenously by NOSs, a physiological modulation in NO bioavailability is conceivable since NOHA, which exhibits a comparable K_i to NHAM, is meanwhile well accepted as physiological effector of NO biosynthesis.[38] Furthermore, NHAM might be a new pharmaceutical lead, bearing potential for further structural modifications including prodrug design. In addition, a methylene group reduced derivative of NHAM could be of great interest since N^{ω}-hydroxy-nor-L-arginine is known as a much more potent inhibitor of arginases than NOHA.[39]

Activity of human DDAH-1 is not affected by N^{δ}-methylated L-arginine derivatives. Thus, a pharmacological as well as a physiological function in this context can certainly be excluded.

Moreover, the metabolism of NHAM by cytochrome P450 was investigated in order to rate the physiological relevance and the drugability of this compound. The reduction of NHAM to MA could limit the effects of NHAM on arginase. In contrast, the conversion of NHAM to CMO by cytochrome P450 should not lower physiological concentrations considering that superoxide anions are rapidly catalyzed by ubiquitarily present SOD.

4. Experimental

4.1. General

All starting materials were commercially available (Aldrich, Fluka, Merck, Roth). (6*R*)-5,6,7,8-Tetrahydro-L-biopterin dihydrochloride (H_4B), calmodulin (bovine brain), and arginase (bovine liver) were from Alexis Biochemicals. Human recombinant nitric oxide synthase isoenzymes were purchased from either Alexis Biochemicals or Prof. Dr. B. Mayer (Karl-Franzens-University, Graz, Austria).

4.1.1. Inhibition of nitric oxide synthases. Enzymes for these studies were obtained from Alexis Biochemicals. Incubations were performed in assay buffer containing 50 mM triethanolamine, pH 7.0, 1 mM NADPH, 5 μM FMN, 5 μM FAD, 10 μM H_4B, 1 mM L-arginine, and 5 mM DTT. Assay buffer for incubations with eNOS and nNOS additively required 1 mM $CaCl_2$ and 20 μg/mL calmodulin for optimal activity. Reactions were initiated by adding the NOS preparation, followed by incubation in a final volume of 64 μL for 30 min at 37 °C. In order to remove excessive NADPH a solution of 16 μL lactate dehydrogenase (20 U/mL) and 1.6 mM sodium pyruvate were added. After incubation for 20 min at 37 °C reactions were terminated by adding 40 μL of ice-cold acetonitrile. Samples were centrifuged (8000*g*, 5 min) and formed NO_2^- was analyzed by colorimetric detection via Griess assay. A standard NOS assay consisted of three incubations containing either MA, MC or NHAM as inhibitor in seven different concentrations (0, 0.5, 1.0, 2.5, 5.0, 7.5, and 10 mM). Each assay was performed thrice.

4.1.2. Colorimetric detection of NO_2^- (Griess assay). Supernatants (100 μL) were added in microcuvettes to 24 μL of Griess reagent (sulfanilamide 5.4% and *N*-(1-naphthyl)-ethylenediamine 0.9% in 1 N HCl) and the absorption of the formed azo dye was measured at 543 nm against a blank containing all cofactors diluted in assay buffer (Varian Cary 50 UV–VIS spectrophotometer). Since excessive NADPH can cause low values by reducing the diazonium cation as well as the azo dye, it is necessary to remove NADPH using an additional incubation with lactate dehydrogenase and sodium pyruvate.

Incubations which contained no inhibitors were referred to 100%. IC_{50} values were calculated by determination of a linear equation.

4.1.3. Metabolism by nitric oxide synthases. Assays were performed according to the NOS assay described in Section 4.1.1 with following alterations: eNOS and nNOS were obtained from Prof. Dr. B. Mayer, buffer for nNOS additively contained 1 mM CHAPS, 10 mM 2-mercaptoethanol and 0.5 mM EDTA and no L-arginine. MA as substrate was applied in three different concentrations: 0.1, 1.0, and 10 mM while NHAM was applied at 1 and 10 mM. Samples were analyzed via analytical reversed phase HPLC.

4.1.4. Analytical reversed phase HPLC (NOS). Amino acids from NOS metabolism studies and NHAM reduction studies were analyzed by RP-HPLC using *o*-PA precolumn derivatization (according to a previously published method).[40] Metabolites were separated at 30 °C on a NovaPak RP_{18} (4 × 150 mm, VDS Optilab) 4 μm with a Phenomenex C18, 4 × 3.0 mm guard column, autosampler Waters 717plus, Waters 600 Controller, and a Waters 470 scanning fluorescence detector, set at λ_{ex}: 338 nm, λ_{em}: 425 nm. Gradient system: eluent A consisted of 86% 10 mM phosphate buffer (pH 4.65), 8% acetonitrile, 6% methanol; eluent B consisted of 40% acetonitrile, 30% methanol, 30% aqua bidest.

For derivatization the autosampler was set to mix 7 μL of *o*-PA reagent with 5 μL of sample and allowed to react for 1 min at room temperature before injection. *o*-PA reagent was prepared by solving 50 mg *o*-PA in

1 mL methanol followed by adding 9 mL of 0.2 M potassium borate buffer, pH 9.4, and 53 μL of 2-mercaptoethanol. Stability of CMO under the conditions of *o*-PA derivatization was confirmed by LC/MS.

The following elution conditions were used: flow-rate was kept at 1 mL/min; 0–2 min, isocratic with 100% eluent A; 2–3.5 min, linear gradient to 96% eluent A and 4% eluent B; 3.5–10 min, isocratic with 96% eluent A and 4% eluent B; 10–13 min, linear gradient to 93% eluent A and 7% eluent B; 13–22 min, isocratic with 93% eluent A and 7% eluent B; 22–24 min, linear gradient to 76% eluent A and 24% eluent B; 24–32 min, isocratic with 76% eluent A and 24% eluent B; 32–34 min linear gradient to 40% eluent A and 60% eluent B; 34–36 min isocratic with 40% eluent A and 60% eluent B; reequilibration was initiated by a 2-min linear gradient step to 100% eluent A; 38–46 min, reequilibration with 100% eluent A.

4.1.5. Arginase assay. Arginase solution was prepared by dissolving 0.1 mg bovine liver arginase (specific activity 19.3 U/mg) in 10 mL of activation buffer (0.725 mM manganese(II)) and incubated for at least 3 h at 37 °C for reactivation.

Assays were performed by applying 100 μL of 10 mM L-arginine solution, pH 9.4, and 29.3 μL of the inhibitor solution. Reactions were started by adding 20.7 μL enzyme preparation. Thus, final concentrations were 6.67 mM of L-arginine and 100 μM of manganese(II). Incubations were performed at 37 °C for 30 min and terminated by adding 150 μL of stop solution (7 M urea in 0.25 M acetic acid). Afterwards, probes were centrifuged (8000*g*, 5 min) and analyzed by ion-pair HPLC. NHAM was applied as inhibitor in concentrations of 0.1, 0.5, 1.0, 2.5, 5.0, 7.5, and 10 mM. Formed amounts of L-ornithine were determined by ion-pair HPLC.

4.1.6. Ion-pair HPLC (arginase assay). Amino acids were derivatized with *o*-PA/2-mercaptoethanol using postcolumn derivatization because L-ornithine is capable of forming different adducts with *o*-PA complicating its quantification by precolumn derivatization.[41] Metabolites were separated on a LiChrospher 60 RP-select B column, 125 × 4 mm; guard column: LiChroCART 4 × 4 mm, LiChrospher 60 RP-select B, 5 μm. The HPLC system consisted of a Waters ERC-3512 degaser and a Waters autosampler WISP 710B equipped with two Waters 510 HPLC pumps and one Waters postcolumn pump Model 590. Fluorescence was measured with a Waters 470 scanning fluorescence detector set at λ_{ex}: 340 nm, λ_{em}: 455 nm. Gradient system: eluent A consisted of 5 mM sodium heptanesulfonate (pH 3); eluent B was methanol.

The postcolumn derivatization reagent was prepared by adding 480 mg *o*-PA (dissolved in 10 mL ethanol) to 2.4 L of buffer, pH 9.25, consisting of 0.133 M H_3BO_3 and 0.667 M NaOH. Finally, 1.2 mL 2-mercaptoethanol was added and the reagent was allowed to react for at least 90 min before use.

Following elution conditions were used: the flow-rate was kept at 1 mL/min; 0–10 min, isocratic with 100% eluent A; 10–11 min, linear gradient to 95% eluent A and 5% eluent B; 11–25 min, isocratic with 95% eluent A and 5% eluent B; 25–26 min, linear gradient to 100% eluent A; 26–30 min, reequilibration with 100% eluent. The reagent flow-rate was kept at 1 mL/min.

4.1.7. K_i-determination. For the K_i-determination of NHAM the *in vitro* arginase assay was modified as follows: concentration of L-arginine was varied from 0.5 to 20 mM (0.5, 1, 2, 5, and 20 mM) and incubated each with NHAM in five different concentrations (5, 15, 100, 500, and 1500 μM) and once without NHAM.

V_{max} and K_m were determined for each substrate concentration and K_i value calculated. For every substrate concentration V_{max} and K_m were determined with SigmaPlot 8.0 (SPSS Inc.) and the K_i calculated using Microsoft Excel.

4.1.8. Construction of human DDAH-1 expression plasmid. cDNA of human DDAH-1 was obtained from RZPD (Berlin, Germany) and PCR amplified with following primers: forward 5′-AAGGATCCATGGC CGGGCTCGGCCAC-3′ containing a BamH I restriction site (underlined) and reverse 5′-GGAAGCTTGC AGCTCAGGAGTCT-3′, that contained a HinD III restriction site (underlined) in conditions given below: an initial denaturation at 94 °C for 3 min, followed by 28 cycles of 94 °C for 30 s, 45 °C for 45 s, 72 °C for 3 min, and a final extension at 72 °C for 15 min. The PCR product was ligated into the expression vector pQE-30 (Qiagen) using BamH I and HinD III restriction sites.

4.1.9. Expression and purification of His-tagged human DDAH-1. The expression plasmid was transferred into *Escherichia coli* BL21 cells. Typically, expression was carried out by inoculating 600 mL of LB medium containing 100 μg/mL ampicillin and 40 μg/mL kanamycin with 20 mL of oculant from a saturated overnight culture and shaking at 37 °C. When the culture reached an OD_{600} of 0.6, isopropyl-β-D-thiogalactopyranoside was added in a final concentration of 100 μM and expression was continued for an additional 5 h at 30 °C. The medium was centrifuged at 4500*g* at 4 °C for 10 min and the cells were stored at −80 °C or immediately resuspended in 10 mL of cell lysis buffer (20 mM K_2HPO_4, 150 mM NaCl, 2 mM 2-mercaptoethanol, 1 mM PMSF, and 1 mM benzamidine). Cells were disrupted with acid washed glass beads and the lysate centrifuged at 12000*g* for 10 min at 4 °C. Ni–NTA–agarose (150 μL) (Qiagen) was added to the supernatant and shaken for 90 min at 4 °C to allow optimal protein binding to the matrix. The suspension was packed into a column and washed with 2 mL washing solution (50 mM potassium phosphate buffer, pH 7.0, 300 mM NaCl, 0.5 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM benzamidine, 1 mM PMSF, and 10% glycerol) plus 10 mM imidazole. Washing was continued with 0.75 mL washing solution plus 20 mM imidazole and elution was carried out with 0.8 mL washing solution containing 100 mM imidazole.

4.1.10. Inhibition of human DDAH-1. A standard assay consisted of 2 μg recombinant DDAH-1 in 75 μL of 50 mM potassium phosphate buffer, pH 7.4, and L-NMMA in a final concentration of 200 μM. The inhibitor was applied in concentrations from 100 μM up to 10 mM. Incubations were performed at 37 °C for 30 min, reactions were stopped by adding 75 μl of ice-cold acetonitrile. Samples were centrifuged at 12000*g* for 10 min and then formed amounts of L-citrulline were analyzed by reversed phase HPLC using *o*-PA/2-mercaptoethanol precolumn derivatization.

4.1.11. Analytical reversed phase HPLC (DDAH). Analytics were similar to that used for NOSs except following altered elution conditions: 0–2 min, isocratic with 100% eluent A; 2–3.5 min, linear gradient to 90% eluent A and 10% eluent B; 3.5–12 min, isocratic with 90% eluent A and 10% eluent B; 12–16 min, linear gradient to 25% eluent A and 75% eluent B; 16–25 min, isocratic with 25% eluent A and 75% eluent B; 25–28 min, linear gradient to 100% eluent A; 28–35 min, reequilibration with 100% eluent A. Amounts of injected sample and derivatization reagent were doubled.

4.1.12. NHAM reduction by pig liver microsomes and mitochondria. Incubations were performed with 0.3 mg pig liver microsomes or mitochondria. NHAM was applied as substrate in a final concentration of 1 mM. Reactions were started by the addition of NADH in a final concentration of 1 mM. All substances were dissolved in 100 mM phosphate buffer, pH 7.4, respectively, pH 6.3, to a final volume of 250 μL. After 30 min incubation at 37 °C the reactions were terminated by adding 250 μL of ice-cold acetonitrile. Samples were centrifuged (8000*g*, 5 min) and 10 μL of the supernatant was analyzed by RP-HPLC using precolumn derivatization with *o*-PA.

4.1.13. Oxidation studies with cytochrome P450. NHAM was incubated with a microsomal liver homogenate of Wistar-rats after pretreatment and non-pretreatment with dexamethasone. Pretreatment was performed by daily intraperitoneal injection of 50 mg/kg dexamethasone (suspended in corn oil 50 mg/mL) over 4 days. Rats without pretreatment were treated by injection of the same volume corn oil.

Incubations were performed at 37 °C in a final volume of 100 μL. All components were dissolved in 50 mM phosphate buffer, pH 7.4, and applied as follows: NHAM in a final concentration of 1 mM, 1 mM $MgCl_2$, and rat liver microsomes with a content of 0.05 nM P450. Some incubations additively contained SOD (100–500 U) or potassium superoxide (10 mM). Reactions were initiated by the addition of 0.5 mM NADPH and incubated for 30 min at 37 °C. Reactions were stopped by adding 100 μL of ice-cold acetonitrile. Samples were centrifuged at 15000*g* (4 °C) and 10 μL of the supernatant was analyzed by ion-pair HPLC using postcolumn derivatization with *o*-PA/2-mercaptoethanol.

4.1.14. Ion-pair HPLC (CYP450 studies). HPLC separation was performed as described above for the arginase assays with the following alterations.

Elution conditions were: 0-8 min, isocratic with 100% eluent A, flow-rate 0.7 mL/min; 8–9 min, linear gradient to 98% eluent A and 2% eluent B, flow-rate 1.0 mL/min; 9–30 min, isocratic with 98% eluent A and 2% eluent B, flow-rate 1.0 mL/min; 30–31 min, linear gradient to 100% eluent A, flow-rate 0.7 mL/min; 31–40 min, reequilibration with 100% eluent A, flow-rate 0.7 mL/min.

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