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Biochemical Journal Immediate Publication. Published on 29 Oct 2010 as manuscript BJ20100960

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Short title: NOHA reduction by mitochondrial amidoxime reducing component

Nitric oxide synthases (NOSs) catalyse the oxidation of L-arginine to L-citrulline and nitric oxide via the intermediate N^{ω}-hydroxy-L-arginine (NOHA). This intermediate is rapidly converted further but to a small extent can also be liberated from the active site of NOSs and act as a transportable precursor of nitric oxide or potent physiological inhibitor of arginases. Thus, its formation is of enormous importance for the nitric oxide generating system. Meanwhile, it has been shown that NOHA is reduced by microsomes and mitochondria to L-arginine. In this study, we show for the first time that both human isoforms of the newly identified mitochondrial amidoxime reducing component (mARC) enhance reduction-rates of NOHA in the presence of NADH cytochrome b_5 reductase and cytochrome b_5 more than 500-fold. Consequently, these results provide the first hints that mARC might be involved in mitochondrial NOHA reduction and could be of physiological significance in affecting endogenous nitric oxide levels. Possibly, this reduction represents another regulative mechanism in the complex regulation of NO biosynthesis considering the existence of a recently identified mitochondrial NOS. Moreover, this reduction is not restricted to NOHA since the analogous arginase inhibitor N^{ω}-hydroxy-N^{δ}-methyl-L-arginine (NHAM) is reduced as well.

Key words: Nitric oxide, N^{ω}-hydroxy-L-arginine (NOHA), mitochondrial amidoxime reducing component (mARC), benzamidoxime, cytochrome b_5, MOSC1, MOSC2

INTRODUCTION

Nitric oxide (NO) is formed endogenously during a two-step oxidation by the action of nitric oxide synthases (NOSs, EC 1.14.13.39) from L-arginine via the intermediate N^{ω}-hydroxy-L-arginine (NOHA) [1]. NO is a physiological mediator with versatile functions such as the maintenance of vascular homeostasis, neuronal signaling and inhibition of tumor cell growth. Furthermore, it prevents atherosclerotic events and serves as a cytotoxic agent in immune defense [2, 3]. An impaired NO availability results in hypertonia and cardiovascular and erectile dysfunction. However, NO overproduction can lead to a number of severe diseases as well, e.g. migraine, septic shock or ischemia [4, 5]. Thus, a balanced regulation of NO formation is vital. Meanwhile, several regulative mechanisms have been identified ensuring a well-adjusted NO-biosynthesis: First, NOS isoenzymes are physiologically inhibited by endogenously formed N^{ω}-methylated L-arginine derivatives such as asymmetric N^{ω},N^{ω}-dimethyl-L-arginine and N^{ω}-monomethyl-L-arginine [6, 7]. These compounds, which are derived from the proteolysis of methylated arginine residues on various proteins, are degraded by dimethylarginine dimethylaminohydrolase (DDAH, EC 3.5.3.18) to L-citrulline and either dimethylamine or methylamine [8].

Moreover, NOHA itself becomes apparent as a regulator of NO formation since it can be liberated from the active site of NOSs [9, 10] and act as a potent inhibitor of arginases (EC 3.5.3.1) [11]. Arginases

Abbreviations used: cyt b_5, cytochrome b_5; cyt b_5 reductase, NADH cytochrome b_5 reductase; mARC, mitochondrial amidoxime reducing component; mtNOS, mitochondrial NOS; NHAM, N^{ω}-hydroxy-N^{δ}-methyl-L-arginine; NOHA, N^{ω}-hydroxy-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase, OMV, outer membrane vesicles.

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and NOSs are the predominant enzymes in L-arginine metabolism and compete for their common substrate L-arginine [12, 13]. Hence, arginase inhibition leads to an augmented substrate pool for NOSs and therefore enhances NO formation [14, 15]. In addition, NOHA has been hypothesized to act as a transportable precursor of NO since several authors reported NOS-independent pathways for NOHA such as the oxidation of NOHA to NO by hemoproteins [16-18]. For an overview of the central position of NOHA in the regulation of NO biosynthesis see Figure 1.

Another metabolic pathway affecting NO biosynthesis seems to be the physiological reduction of NOHA to L-arginine. This reduction has already been demonstrated in an earlier study with microsomal and mitochondrial enzyme sources [19].

This nonspecific reductive pathway is in particular involved in the detoxification of xenobiotics [20, 21]. In mitochondria the responsible enzyme system consists of NADH cytochrome b_5 reductase (cyt b_5 reductase), cytochrome b_5 (cyt b_5) and a third enzyme. This third component has recently been identified in pig liver mitochondria and represents a novel molybdenum containing enzyme called mitochondrial amidoxime reducing component (mARC) [22]. The human genome harbors two genes encoding for two mARC proteins, which we designated mARC1 and mARC2. Meanwhile, human mARC1 (hmARC1) has been shown to be capable of catalysing the activation of *N*-hydroxylated prodrugs [23], whereas recombinant expression and characterization of human mARC2 (hmARC2) is described for the first time within this study.

Recently, a mitochondrial NOS (mtNOS) has been identified, which seems to be implicated in cell respiration [24]. However, although several authors described the existence of mtNOS, which seems to be a splice variant of nNOS, there is still a matter of debate about the existence and function of mtNOS. Nevertheless, the question arose whether the newly identified mARC homologues are involved in the mitochondrial reduction of NOHA to L-arginine, since this reductive pathway might affect physiological NO formation. In addition, we examined a possible reduction of N^{ω}-hydroxy-N^{δ}-methyl-L-arginine (NHAM), an analogous potent inhibitor of arginase, in order to show that mARCs can be considered to generally be capable of reducing N^{ω}-hydroxy-L-arginines [25]. The present study investigated mitochondria, mitochondrial outer membrane, mARC purified from pig liver and heterologously expressed human mARC1 and mARC2 for their capacity to reduce the aforementioned derivatives.

EXPERIMENTAL

Chemicals

All substances were commercially available (Aldrich, Fluka, Merck, Roth) unless otherwise stated. NOHA was purchased from Cayman chemicals, NHAM was synthesized as described [26].

Preparation of subcellular fractions

Mitochondria were prepared from pig liver as previously described [27]. The outer membrane vesicle (OMV) fraction was purified similar to a method described by de Kroon *et al.* [28].

Purification of native mARC (pig liver)

A mARC-enriched protein fraction was purified from the OMV-fraction by ion-exchange chromatography on DEAE-52 cellulose according to a procedure described previously [22].

SDS-PAGE

SDS-PAGE was carried out by the method of Laemmli using a separation gel containing 12.5% polyacrylamide [29]. Silver staining was performed according to the manufacturer's directions (Silver Staining Kit, Protein Plus One, GE Healthcare). Standards and samples were pretreated with ß-mercaptoethanol for 5 min at 100℃.

Immunoblot analysis

Immunoblot analysis was performed by gel-blotting protein fractions to 12.5% SDS-PAGE using a primary polyclonal antibody raised against hmARC2 (MOSC2 antibody, Sigma) (1:10.000 dilution). The secondary horseradish peroxidase-conjugated anti-rabbit Ig (Sigma) was used in a 1:10.000 dilution and chemiluminescence was detected using the ECL system (ECL Plus Western Blotting Detection System, GE Healthcare).

Determination of protein concentration

Protein concentration was determined according to the manufacturer's directions (BCA protein assay kit combined with Protein Assay Preparation Reagent Set, Pierce).

FormA-dephospho analysis

Molybdenum cofactor (Moco) and its molybdenum-free precursor molybdopterin (MPT) bound to the OMV-fraction and the purified mARC-enriched fraction was detected and quantified as described in detail in Havemeyer *et al.* [30].

Cloning of human mARC1 and mARC2 cDNAs

Total RNA prepared from human HepG2 cells by using the E.Z.N.A. Total RNA kit (Peqlab) was reverse transcribed with AMV-reverse transcriptase (Promega) and oligo-d(T) primer according to standard procedures. cDNAs of mARC1 and mARC2 were obtained by subsequent polymerase chain reaction using specific primers for amplification of mARC1 (mARC1_forward: 5'-ATA TAT GGA TCC ATG GGC GCC GCC GGC TCC TCC GCG-3' and mARC1-reverse: 5'-AAA TTT AAG CTT TTA CTG GCC CAG CAG GTA CAC AGG-3') and mARC2 (mARC2_forward: 5'-ATA TAT GGA TCC ATG GGC GCT TCC AGC TCC TCC GCG-3' and mARC2_reverse: 5'-ATA ATT AAG CTT CTA CAC CAT CCG ATA CAC AGG GTC-3') deduced from GenBank entries NM_022746 and NM_017898. By this procedure full-length open reading frames of 1011 and 1105 nucleotides were obtained encoding for proteins of 337 and 335 amino acids for mARC1 and mARC2, respectively. Removal of putative mitochondrial targeting sequences at the NH_2-termini of mARC1 and mARC2 was achieved by a second polymerase chain reaction using the full-length cDNAs as template and primer mARC1_N-del (5'-ATA TAT GGA TCC ATG CAG CAG GTG GGC ACA GTG GCG-3') or primer mARC2_N-del (5'-ATA TAT GGA TCC ATG CAG CAG GTG GGC ACC GTG GCG AAG-3') in the presence of the respective reverse-primer as given above. Simultaneously, restriction sites for *Bam*HI and *Hind*III were introduced at the respective 5'- and 3'-ends, which enabled cloning of both mARC cDNAs into the pQE80 expression plasmid (Qiagen) downstream from a sequence encoding six NH_2-terminal histidine residues. Correctness of the introduced cDNAs that now encode proteins of 286 (mARC1) and 285 (mARC2) amino acids was confirmed by sequencing.

Expression and purification of recombinant human mARC1 and mARC2

Routine protein expression of mARC proteins was performed in freshly transformed *E. coli* TP1000 cells [31]. Cells were grown aerobically in Luria Bertani medium in the presence of 100 µg/mL ampicillin at 22℃ to an OD_{600} = 0.1 before induction with 15-30 µM isopropyl-β-D-thiogalactopyranoside and addition of 1 mM sodium molybdate. After induction, cells were grown for further 20 h at 22℃. Cells were harvested by centrifugation and stored at –70℃ until use. Cell lysis was achieved by several passages through a French press cell followed by sonication for 5 min on ice. After centrifugation, 6x histidine-tagged proteins were purified on a nickel-nitrilotriacetic acid superflow matrix (Qiagen) under native conditions at 4℃ according to the manufacturer's instructions. Expression of hmARC1 and hmARC2 in *E. coli* RK5206 and RK5204 cells was performed likewise. Eluted fractions were analysed by SDS-PAGE. To ensure that Moco, the essential prosthetic group for mARC1 and mARC2, was bound to the proteins expressed in *E. coli* TP1000, proteins were subjected to analysis of FormA-dephospho as described previously [22].

Recombinant human cytochrome b_5

Recombinant human cytochrome b_5 was purchased from MoBiTec.

Expression and purification of recombinant human NADH cytochrome b_5 reductase (Isoform 2)

Expression of *C*-terminally truncated human cytochrome b_5 reductase isoform 2 (GenPept accession NP_015565) from expression plasmid pQE80 (Qiagen) in *E. coli* DL41 cells and purification of the resulting recombinant protein was performed according to Kurian *et al.* [32].

FAD content determination

FAD content of cyt b_5 reductase was determined in 50 mM phosphate buffer pH 7.0. Samples were heated in the dark for 10 min at 100℃ and the protein sedimented by centrifugation. The supernatant was measured at 450 nm and the concentration calculated according to Whitby [33].

Cyt b_5 reductase activity assay

Cyt b_5 reductase activity was determined by a modification of the ferricyanide reduction assay [34].

***In vitro* reduction assays**

Incubations were carried out under aerobic conditions at 37℃ in a shaking water bath. Incubation mixtures contained 0.5 mM substrate and 1.0 mM NADH in a total volume of 150 µl 20 mM MES

buffer, pH 6.0 or 100 mM potassium phosphate buffer, pH 6.0. After a preincubation period of 3 minutes at 37℃ the reaction was initiated by add ition of NADH and terminated after 15 minutes by addition of 150 µL methanol. Precipitated proteins were sedimented by centrifugation and the supernatant was analysed by HPLC. Incubation mixtures with native mARC (pig liver) consisted of 1 µg mARC-enriched fraction (which also contained cytochrome b_5 and cytochrome b_5 reductase). Incubation mixtures with recombinant hmARC1 or hmARC2 consisted of 200 pmol cytochrome b_5, 20 pmol NADH cytochrome b_5 reductase, and 10 µg molybdenum enzyme. Incubation mixtures of subcellular fractions (pig liver) contained 56 µg mitochondria or 6 µg OMV, respectively.
In alternative procedures NADH was omitted and replaced by a NADH-generating system consisting of 10 mM malate, 2 mM ADP, 2 mM NAD^+, 2.25 µM rotenone (dissolved in 0.1% (m/v) DMSO) and 5 mM $MgCl_2$. After a preincubation period of 3 minutes the reaction was initiated by addition of malate.
Apparent kinetic parameters K_m and V_{max} were calculated using nonlinear regression analysis (Sigma Plot 5.0; SPSS Science).

Organelle integrity

The integrity of the inner mitochondrial membrane was assessed by measuring the latency of malate dehydrogenase according to Iturbe-Ormaetxe et al. [35]. Latency of enzymes was determined with and without 0.02% Triton X-100. Latency (%) was calculated as [(activity with triton)-(activity without triton)]/(activity with triton).

Inductively coupled plasma-mass spectrometry (ICP-MS)
Buffer of mARC proteins was exchanged to 50 mM potassium phosphate buffer pH 7.4 containing 20% (m/v) glycerol, 0.1 mM DTT and 1 mM Na_2EDTA by an Illustra NAP-25 column (GE Healthcare). Protein sample aliquots with a volume of 200 µL were denatured by heating for 10 min at 100℃, spiked with 5 ng indium for internal standardisation, and diluted to 2 mL with 2% (v/v) subboiled nitric acid. The resulting suspension was filtrated using a 30 kDa centrifuge filter (Amicon Ultra – 0.5 Ultracel-PL membrane, Millipore), and the clear filtrate was used for subsequent analysis by ICP-MS. This sample solution was manually introduced into a 7500cs (Agilent Technologies) ICP-MS instrument via a free-aspirating PFA micro-nebulizer with a sample uptake of 200 µL/min, and special attention was paid to sample uptake and wash-out times. All natural molybdenum isotopes were monitored during the analysis but only 98 m/z was found to be free from interferences and, hence, used for quantification. Calibration solutions were prepared from single-element stock solutions (Alpha Aesar). Procedural blanks as well as sample replicates and international certified reference materials (NIST 1643e, BIR-1) were measured along with the unknown samples for the assessment of analytical precision and accuracy. The reproducibility as estimated from replicate measurements was better than 1% RSD (1sigma). The limit of detection is 0.5 ng/mL Mo as calculated for the original protein solution prior to dilution. All sample prep work was done under class 100 clean-room conditions. Further details of the calibration strategy and molybdenum analysis in organic matrices have been published previously [36-38].

HPLC method for the separation of NOHA and L-arginine, alternatively NHAM and N^{δ}-methyl-L-arginine

Amino acids were analysed by RP-HPLC using *o*-PA precolumn derivatization. Metabolites were separated on a NovaPak RP_{18} (4 × 250 mm) 4 µm with a Phenomenex C18, 4 × 3.0 mm guard column, autosampler Waters 717plus, Waters 600 Controller and a Waters 474 scanning fluorescence detector, set at λ_{ex}: 338 nm, λ_{em}: 425 nm. For derivatization the autosampler was set to mix 14 µL of *o*-PA reagent with 20 µL of sample and allowed to react for two minutes at room temperature before injection. *o*-PA reagent was prepared as described previously [39].
Elution was carried out isocratically with 10 mM potassium phosphate buffer (pH 4.65) (75%), methanol (15%), and acetonitrile (10%). The flow-rate was kept at 1 mL/min.
Characteristic retention times [min]: NOHA, 9.1 ± 0.2; L-arginine, 10.0 ± 0.2; NHAM, 10.8 ± 0.1; N^{δ}-methyl-L-arginine, 12.2 ± 0.2.

HPLC method for the separation of benzamidoxime and benzamidine

HPLC analysis were performed on a LiChrospher 60, RP-select B, (4 × 125 mm, 5 µm) with a LiChrospher 60, RP-select B, (4 × 4.0 mm) guard column, Waters e2695 XC Separations Modul, Waters 2998 Photodiode Array Detector.

Elution was carried out isocratically with 20 mM potassium phosphate buffer / 0.1% trifluoroacetic acid (pH 7.5) (65%), and acetonitrile (35%). The flow-rate was kept at 1 mL/min. Characteristic retention times [min]: benzamidoxime, 1.8 ± 0.1; benzamidine, 3.8 ± 0.1.

RESULTS

Characterization of the purified mARC-enriched fraction with benzamidoxime

A purified protein fraction enriched with native mARC protein was obtained by ion-exchange chromatography of the solubilized outer membrane vesicle (OMV) fraction of pig liver mitochondria (Fig. 2 and 3). This mARC-enriched enzyme fraction was capable of reducing our model compound benzamidoxime without the addition of cyt b_5 reductase and cyt b_5 since it was contaminated with these electron transfer proteins. Thus, the addition of these proteins to the purified fraction did not enhance conversion rates (data not shown). The cyt b_5 content was 0.38 ± 0.03 nmol/mg (n = 7), and cyt b_5 reductase activity was determined as 29.8 ± 6.5 µmol * min^{-1} * mg^{-1} (n = 22).
This purified fraction showed a *N*-reductive activity of 728 ± 63 nmol benzamidine * min^{-1} * mg^{-1} total protein. Thus, amidoxime reductase activity was enriched 40–fold by comparison to the porcine mitochondria and 2.6–fold by comparison to the OMV fraction (data not shown). Chemical detection of FormA-dephospho, the oxidation product of Moco [40] showed a 2.3-fold enrichment of Moco which is in good accordance with the described elevation of the benzamidoxime reductase activity.
In addition, the *N*-reductive activity of the purified mitochondria was further specified using benzamidoxime as model substrate. Since intact inner mitochondrial membrane is impermeable to NADH, exogenous NADH from the medium freely diffuses to the intermembranous space of mitochondria, but cannot enter the matrix. In accordance with the postulated localization of the *N*-reductive system on the outer mitochondrial membrane [22, 41], purified mitochondria are able to reduce benzamidoxime by the addition of exogenous NADH (Tab. 2). No reduction of the substrate was detected, when NADH was omitted. Furthermore, the reduction could not be inhibited by the respiratory-chain inhibitor rotenone.
The *N*-reductive activity was not altered significantly when fractioned mitochondria (mechanically disrupted using a potter-Elvehjem homogenizer or lysolecithin-treated according to Bernheim [42]) were used for incubation studies (data not shown). This supported the assumption, that the *N*-reductive enzyme system is located on the outer mitochondrial membrane.
The mARC-containing enzyme system must therefore be inaccessible to endogenously generated NADH. This was proven by incubation mixtures with mitochondria containing malate, ADP, rotenone and NAD^+ instead of 1 mM NADH. The resulting *N*-reductive activity (4.1 ± 0.8 nmol benzamidine * min^{-1} * mg^{-1}) decreased about 80% in comparison with incubation mixtures containing exogenous NADH (25.0 ± 9.9 nmol benzamidine * min^{-1} * mg^{-1}) instead of the NADH-generating system. The detected residual activity of the mitochondria can be explained by the incomplete integrity of the purified mitochondria: Measuring the latency of malate dehydrogenase in purified mitochondria with or without Triton X-100 indicates that approx. 40% inner membranes of purified mitochondria are damaged. Therefore, endogenously generated NADH is able to access the *N*-reductive enzyme system in isolated mitochondria. In consequence, we could demonstrate that further disruption of the inner membrane by mechanical disruption results in a 150% increase of *N*-reductive activity (6.3 ± 0.4 nmol benzamidine * min^{-1} * mg^{-1}) using endogenously formed NADH.

Expression and characterization of recombinant human mARC1 and mARC2 with benzamidoxime

Both recombinantly expressed proteins hmARC1 and hmARC2 were obtained in adequate purity (Fig. 4) and due to expression in the *E. coli* strain TP1000, which accumulates the eukaryotic form of the molybdenum cofactor (Moco), were loaded with Moco. hmARC1 and hmARC2 have been subjected to FormA-dephospho analysis, which allows the common quantification of bound Moco and its metal-free precursor MPT. In fact, for both mARC proteins high saturation levels with MPT/Moco have been found. Based on ICP-MS analysis, the average Mo content for both proteins turned out to be 0.3 mol Mo/mol protein. Moreover, both proteins were capable of reducing the model substrate benzamidoxime to benzamidine in a reconstituted enzyme system with cyt b_5 and cyt b_5 reductase. The reaction was further specified by omitting single components. Reduction rates were highest for the complete enzyme system and intensely reduced in the absence of one of the proteins (Table 1). Incubations with only cyt b_5 and cyt b_5 reductase led to benzamidine in a moderate extent. Reduction

rates were increased 15-fold for hmARC1 and 300-fold for hmARC2 when compared to incubations lacking mARC isoforms. In addition, the reduction was dependent on the presence of NADH.
The reduction followed Michaelis-Menten kinetics which is displayed for both hmARC isoforms in Figure 5. Determined kinetic parameters for the reduction of benzamidoxime are K_m = 180 ± 5 µM and V_{max} = 34.2 ± 2.4 nmol * min^{-1} * mg^{-1} total protein for recombinant hmARC1 and K_m = 0.83 ± 0.17 mM and V_{max} = 307 ± 22 nmol * min^{-1} * mg^{-1} total protein for recombinant hmARC2, respectively. Control incubations with mARC proteins that were either binding molybdopterin, a molybdenum-free precursor of Moco, or had no pterin bound due to expression in *E. coli* RK5206 and RK5204, respectively, showed no reductive activity indicating that the molybdenum site is essential for catalytic activity.

Reduction of N^{ω}-hydroxy-L-arginine by mitochondria, OMV, and purified mARC-enriched fraction

In order to examine whether the newly identified mARC proteins are involved in the reduction of NOHA to L-arginine, incubations with mitochondria, mitochondrial OMV and a purified mARC-enriched fraction have been carried out. These incubations confirmed that NOHA is reduced to L-arginine by pig liver mitochondria as previously reported [19]. However, the main finding of this experiment is that specific reduction rates increase from mitochondria over OMV (10-fold increase) to mARC enriched fractions (40-fold increase, specific activity 481 nmol * min^{-1} * mg^{-1}) (Table 2), which can be correlated with the enrichment of the amidoxime reductase activity (see above). Since the purified mARC enriched fraction already contained cyt b_5 and cyt b_5 reductase, addition of these proteins to the purified fraction did not enhance detected conversion rates (data not shown).
Accordingly, these data indicate that NOHA is reduced to L-arginine by the described enzyme system consisting of mARC, cyt b_5 and cyt b_5 reductase.

Reduction of N^{ω}-hydroxy-L-arginine by recombinant human mARC proteins

The involvement of mARC in the reduction of NOHA was further verified by using recombinantly expressed enzyme sources. The applied human homologue mARC1 and mARC2 enzymes were *N*-terminally truncated to remove the putative mitochondrial targeting signals. Incubations with the complete reconstituted enzyme system consisting of recombinant human cyt b_5, human cyt b_5 reductase and recombinant hmARC1 or hmARC2, respectively, led to the formation of L-arginine with a specific activity of 39.1 ± 1.4 nmol * min^{-1} * mg^{-1} total protein for hmARC1 and 56.8 ± 5.0 nmol * min^{-1} * mg^{-1} total protein for hmARC2 (Table 3). The reduction followed Michaelis-Menten kinetics and kinetic parameters for the reduction of NOHA were determined as K_m = 86 ± 13 µM and V_{max} = 55.5 ± 1.7 nmol * min^{-1} * mg^{-1} total protein for hmARC1 and K_m = 3.0 ± 0.3 mM and V_{max} = 373 ± 20 nmol * min^{-1} * mg^{-1} total protein for hmARC2, respectively. Additionally, incubations were performed omitting one component at a time. As expected, incubations containing cyt b_5, cyt b_5 reductase and hmARC showed highest activity, whereas incubations lacking hmARC showed a significant lower activity, demonstrating the involvement of hmARC in this reductive pathway (Table 3). When comparing the activities of the complete enzyme system with incubations lacking hmARC enzymes reduction rates were increased 30-fold by hmARC1 and more than 500-fold by hmARC2. Another important observation of this study is that the activity of the complete incubation mixture is significantly higher than the sum of activities of the separate components. Thus, the increase in turnover rate cannot just be traced back to the addition of two independent reductive enzyme systems, since these enzymes seem to potentiate their activities as has been already described [22, 23].

Reduction of N^{ω}-hydroxy-N^{δ}-methyl-L-arginine

As already known, this *N*-reductive enzyme system possesses a broad substrate specifity [23]. Therefore, the question arose whether hmARCs are capable of reducing other N^{ω}-hydroxy-L-arginines as well. Thus, we examined the reduction of NHAM, a known potent arginase inhibitor, by the complete enzyme system and by omitting single components. As demonstrated in Table 3, NHAM is reduced to N^{δ}-methyl-L-arginine with highest reduction rates for the complete system. As for NOHA, in the absence of hmARC isoforms the turnover rates drop to less than 3% of the activity of the complete system. Further studies revealed Michaelis-Menten type kinetics with K_m = 272 ± 60 µM and V_{max} = 43.1 ± 2.8 nmol * min^{-1} * mg^{-1} total protein for hmARC1 and K_m = 3.7 ± 0.4 mM and V_{max} = 36.5 ± 2.3 nmol * min^{-1} * mg^{-1} total protein for hmARC2. In summary, concluding from these results this enzyme system can be generally considered to be capable of reducing N^{ω}-hydroxy-L-arginines.

Biochemical Journal Immediate Publication. Published on 29 Oct 2010 as manuscript BJ20100960

DISCUSSION

A hitherto unknown molybdenum-containing enzyme has been discovered in previous studies [22]. It was named “mitochondrial amidoxime reducing component” (mARC), because initial studies revealed that this enzyme purified from mammalian liver mitochondria is capable of reducing amidoxime structures to the corresponding amidines. This reductive enzyme system represents a three component complex consisting of cyt b_5 reductase, cyt b_5, and mARC as the catalytic part [22].

The human genome codes for two homologuous mARC-proteins, hmARC1 and hmARC2, designated as MOSC1 and MOSC2 in the databases. Former studies described the reduction of several *N*-hydroxylated prodrugs by the recombinant full-length hmARC1 in a reconstituted enzyme system together with cyt b_5 and its reductase. In this study both hmARC1 and hmARC2 were expressed in their soluble forms without their putative mitochondrial targeting sequences at the NH_2-termini since initial experiments showed an increased activity for these proteins (data not shown).

By use of the proteins expressed in *E.coli* TP1000 we demonstrated that both recombinant mARC proteins bind Moco and are able to reduce the *N*-hydroxylated model substrate benzamidoxime to benzamidine in a reconstituted enzyme system together with cyt b_5 and cyt b_5 reductase. However, there are considerable differences in activity since both K_m and V_{max} for hmARC2 are one order of magnitude above those of hmARC1 (Table 1, Fig. 5).

A mARC enriched fraction was purified from the outer membrane of pig liver mitochondria as previously described (Fig. 2) [22]. Immuno analysis with a commercially available human anti-mARC2 antibody detected porcine mARC in the partially purified fraction (Fig. 3). However, it is unclear if the human antibody is able to distinguish porcine mARC1 and mARC2. MALDI-TOF results of our former purification demonstrated that a mARC2 homologuous protein rather than a mARC1-like protein is localized at the outer mitochondrial membrane.

So far, this enzyme system is known to reduce *N*-hydroxylated xenobiotics. In particular, we investigated the activation of *N*-hydroxylated prodrugs to the active form with this constituted enzyme system [23]. Physiologically, this reductive enzyme system is assumed to be involved in detoxification processes. However, the question arose whether this newly identified enzyme fulfils other physiological functions, although no physiological substrate has been identified so far. Therefore, we supposed that NOHA, the intermediate of NO biosynthesis, might be reduced to L-arginine by this enzyme system and thus might represent another physiological regulatory mechanism in the complex regulation of NO formation.

Within our experiments we were able to show the reduction of this putative physiological substrate by the characterized molybdenum-containing enzyme system, for the first time. We demonstrated that a purified mARC-enriched fraction, which additionally contained cyt b_5 and its reductase, was able to reduce physiologically occurring NOHA (Table 2). The specific activity of this reduction is high (481 nmol * min^{-1} * mg^{-1}) considering the turn-over rates for L-arginine by NOSs stated in the range of 10 µmol * min^{-1} * mg^{-1} to 30 nmol * min^{-1} * mg^{-1} for purified NOS [43, 44]. However, a high specific activity alone does not prove a physiological impact on NO levels as long as we do not know the expression levels of mARC. At present our knowledge about localization and expression of native mARC is still relatively restricted. Nevertheless, first hints point to a prevailing expression of mARCs at least in mitochondria since the specific activity for NOHA reduction in mitochondria (10 nmol * min^{-1} * mg^{-1} protein) is about four orders of magnitude above NOS activity when compared to basal NOS activity in cardiac mitochondria, which is stated with 2 pmol * min^{-1} * mg^{-1} protein [45].

The potential involvement of the described mARC-containing enzyme system in the reduction of NOHA was further examined by using recombinant enzyme sources which also catalysed this conversion in a reconstituted enzyme system (Table 3). Our experiments showed highest activity for the complete three component system and lower activity in incubations with only the two component enzyme system without hmARCs. Addition of hmARC1 augments the activity more than 30-fold compared to incubations lacking the molybdenum enzyme, whereas activity is even more increased by hmARC2 (about 500-fold).

In general, conversion rates of the native enzyme system in the purified mARC-enriched fraction were about 10-fold higher than the conversion rates of the recombinant reconstituted enzyme system. Because different mARC sources (cDNA-expressed versus purified from pig liver) were used, the rates obtained are dependent on several aspects that influence the absolute enzyme activity. Perhaps, truncation of mARC enzymes may have an effect on the efficiency. It can be assumed that the enzymes must interact in a highly specific manner to allow effective electron transfer from NADH via cyt b_5 reductase and cyt b_5 to the Moco of mARC and that the hydrophobic domains ensure an optimal complex formation of the enzymes. Therefore, it is not surprising that the truncated recombinant mARC homologues are less active than the native full-length protein purified from pig liver. Nevertheless, identification of the roles of hydrophobic domains as well as of the optimal stoichiometry

of the three enzymes affecting conversion rates will require further studies. Additionally, lower activity of recombinantly expressed membrane bound enzymes is a common problem, which is also known from recombinant NOSs [46].

Nevertheless, our results clearly demonstrate that the described mARC-containing enzyme system might fulfil a physiological function in the reduction of NOHA to L-arginine. NOHA represents the intermediate in NOS-catalysis to yield NO. It can be liberated from the active site of NOS and act as a transportable precursor of NO since NOS-independent pathways for NOHA, such as the oxidation of NOHA to NO by hemoproteins, have been reported [16-18]. Moreover, NOHA is known as an endogenous inhibitor of arginases and thus might physiologically enhance NO biosynthesis by elevating the substrate pool for NOSs. Consequently, the above mentioned mechanisms seem to be limited by the herein described reduction since it lowers endogenous NOHA concentrations and thus the significance of NOHA as transportable precursor of NO at least in this metabolic context is questionable.

We assume that hmARC2 is located at the outer mitochondrial membrane to protect the cell from excessive NO formation. In particular, hmARC2 is believed to lower cytosolic NOHA concentrations thereby protecting cells from high, possibly cytotoxic NO levels. This scenario would go along with our previous findings of mARC being involved in detoxication processes by reducing several *N*-hydroxylated xenobiotics that are assumed to be mutagenic and toxic. Furthermore, it is noteworthy that NOHA represents a potent physiological inhibitor of arginase. Since arginase I is a cytosolic enzyme, another physiological function of hmARC might be reducing NOHA levels to (1) ensure sufficient arginase activity required for a well-functioning urea cycle, or (2) to forward L-ornithine formation, which is essential for polyamine biosynthesis. Interestingly enough, there are no additional metabolic pathways for NOHA so far known other than its conversion to NO by NOSs. Possibly, the reduction of NOHA simply represents a recovery mechanism for L-arginine to feed other pathways such as protein and polyamine biosynthesis.

With regard to mtNOS, a physiological association between mtNOS and the mARC-containing enzyme system seems rather unlikely given that hmARC is located on the outer mitochondrial membrane [22, 41], whereas mtNOS is located on the inner mitochondrial membrane. However, contrary to our results, other authors identified mARC1 and 2 as inner mitochondrial membrane proteins in mouse [47].

More studies are needed to further elucidate a potential physiological function of mARCs on NO biosynthesis. Particularly, the influence of hmARC on NO biosynthesis in cell lines or animal models - ideally on the basis of mARC silencing/knockdown and mARC overexpression - should be addressed.

Besides the reduction of NOHA, the reconstituted recombinant mARC-containing enzyme system is also capable of reducing other N^{ω}-hydroxy-L-arginines, such as NHAM (Table 3). This non-physiological compound represents a potent arginase inhibitor, and in contrast to NOHA, is not converted by NOSs [25]. NHAM is reduced by all examined enzyme systems, however, at significantly lower turnover rates than those for NOHA (Table 3). Nevertheless, the herein presented results revealed that both mARC homologues are generally capable of reducing N^{ω}-hydroxyguanidine containing compounds.

In summary, our studies show for the first time the identification of a putative physiological substrate for hmARC. The herein described reduction of NOHA might be involved in the complex regulation of NO biosynthesis. Hitherto, the mARC-containing enzyme system was only known to be capable of reducing *N*-hydroxylated xenobiotics and was assumed to be involved in detoxication processes [22, 23]. Additionally, this enzyme system was utilised for the activation of prodrugs [23]. Whether mARC is involved in physiological NOHA reduction and is capable of physiologically affecting NO levels has to be supported by additional studies. We are currently working on cell-based experiments to investigate effects of mARC activity on NOS-dependent NO generation and arginase activity.

ACKNOWLEDGEMENTS

We thank Sven Wichmann, Petra Köster, and Ulrike Westernströer for their excellent technical assistance.

FUNDINGS

Financial support by the Deutsche Forschungsgemeinschaft (DFG, Cl 56/9-1 and DFG, ME 1266/24-1) is greatly appreciated.

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Biochemical Journal Immediate Publication. Published on 29 Oct 2010 as manuscript BJ20100960

Table 1 ***In vitro*** **reduction of benzamidoxime by recombinantly expressed human mARC isoforms**

A complete incubation mixture consisted of 10 µg hmARC, 200 pmol cyt b_5, 20 pmol cyt b_5 reductase, 1 mM NADH, 0.5 mM benzamidoxime in 150 µL of 20 mM MES buffer pH 6.0. Data are means ± standard deviations of 3 different incubations each analysed twice via HPLC.

mARC isoform	Composition	Specific activity [nmol * min^{-1} * mg^{-1} total protein]
recombinant hmARC1	complete	34.3 ± 0.9
	- cyt b_5	15.4 ± 2.3
	- cyt b_5 reductase	1.6 ± 0.2
	- hmARC1	2.2 ± 0.4
	only hmARC1	2.2 ± 0.4
	- NADH	0.2 ± 0.1
recombinant hmARC2	complete	90.7 ± 5.6
	- cyt b_5	0.9 ± 0.1
	- cyt b_5 reductase	1.2 ± 0.2
	- hmARC2	0.3 ± 0.1
	only hmARC2	0.2 ± 0.1
	- NADH	0.1 ± 0.1

Table 2 ***In vitro*** **reduction of N^{ω}-hydroxy-L-arginine by mitochondrial preparations**

For experimental details see Experimental. Data are means ± standard deviations of 3 different incubations each analysed twice via HPLC.

Substrate	Enzyme source	Specific activity [nmol * min^{-1} * mg^{-1} total protein]
N^{ω}-hydroxy-L-arginine (NOHA)	mitochondria	11.7 ± 0.9
	OMV	107.0 ± 10.1
	purified mARC	481.1 ± 91.0

Table 3. ***In vitro*** **reduction of N^{ω}-hydroxy-L-arginines by recombinantly expressed human mARC isoforms**

A complete incubation mixture consisted of 10 µg mARC1 and mARC2, respectively, 200 pmol cyt b_5, 20 pmol cyt b_5 reductase, 1 mM NADH, 0.5 mM substrate in 150 µl of 20 mM MES buffer pH 6.0. Data are means ± standard deviations of 3 different incubations each analysed twice via HPLC.

mARC isoform	Composition	Specific activity [nmol * min^{-1} * mg^{-1} total protein]	
		NOHA reduction	NHAM reduction
recombinant hmARC1	complete	39.1 ± 1.4	20.6 ± 0.9
	- cyt b_5	11.5 ± 2.7	8.9 ± 0.2
	- cyt b_5 reductase	1.6 ± 0.3	0.7 ± 0.2
	- hmARC1	1.2 ± 1.1	0.5 ± 0.5
	only hmARC1	1.8 ± 0.4	1.6 ± 0.3
	- NADH	0.5 ± 0.5	0.1 ± 0.1
recombinant hmARC2	complete	56.8 ± 5.0	7.7 ± 0.4
	- cyt b_5	1.7 ± 1.7	0.2 ± 0.2
	- cyt b_5 reductase	1.0 ± 0.4	0.7 ± 0.3
	- hmARC2	0.1 ± 0.1	0.2 ± 0.2
	only hmARC2	0.0 ± 0.0	0.3 ± 0.3
	- NADH	0.1 ± 0.1	0.1 ± 0.1

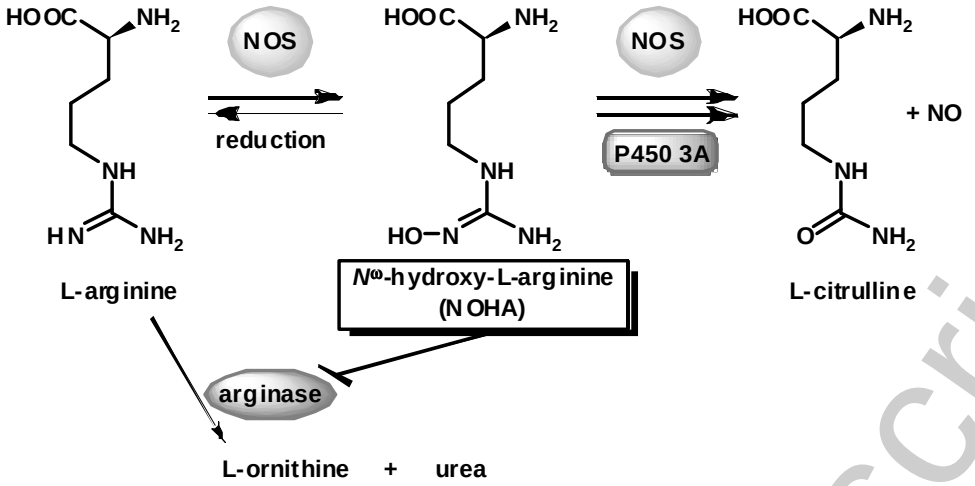

Figure 1
Overview on N^{ω}-hydroxy-L-arginine metabolism and its influence on NO biosynthesis. P450 3A, cytochrome P450 3A isoenzymes; NO, nitric oxide; NOS, nitric oxide synthase.

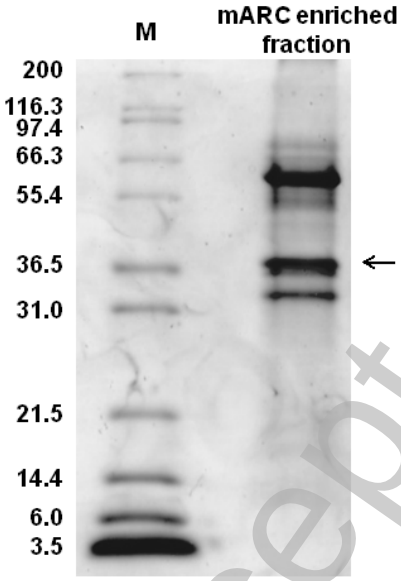

Figure 2
SDS-PAGE and Coomassie stain of purified mARC-enriched fraction (pig liver). mARC-enriched fraction (15 µg protein), molecular mass marker (Mark12 unstained standard, Invitrogen) (M, masses are indicated in kDa to the left of panel). mARC protein band is marked with an arrow.

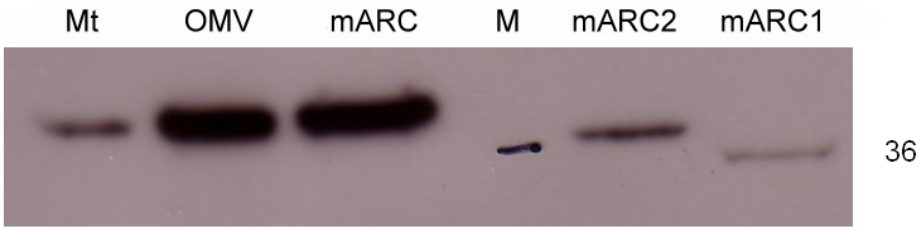

Figure 3
Immunoblot analysis of mARC-containing enzyme fractions. For immunoblot analysis anti-mARC2 antibody (anti-MOSC2, Sigma) was used as primary antibody. Pig liver mitochondria (Mt, 1.5 µg protein), OMV fraction (OMV, 1.5 µg protein), mARC-enriched fraction (mARC from pig liver, 1.5 µg protein), Molecular mass marker (See Blue Plus 2 Prestained, Invitrogen) (M, mass is indicated in kDa to the right of panel), recombinant hmARC2 (mARC2, 0.05 µg protein), recombinant hmARC1 (mARC1, 0.05 µg protein).

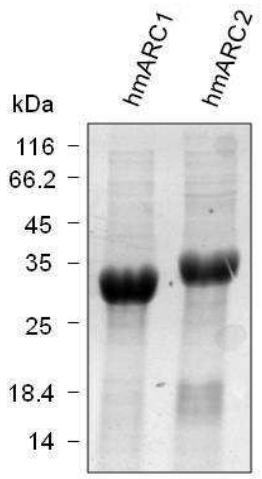

Figure 4
SDS PAGE analysis of recombinant hmARC1 and hmARC2. After expression in *E. coli* and purification by affinity chromatography 20 µg of hmARC1 and hmARC2, respectively, were electrophoresed on 12% SDS PAGE gels and subsequently stained with Coomassie Brilliant Blue (masses are indicated in kDa to the left of panel).

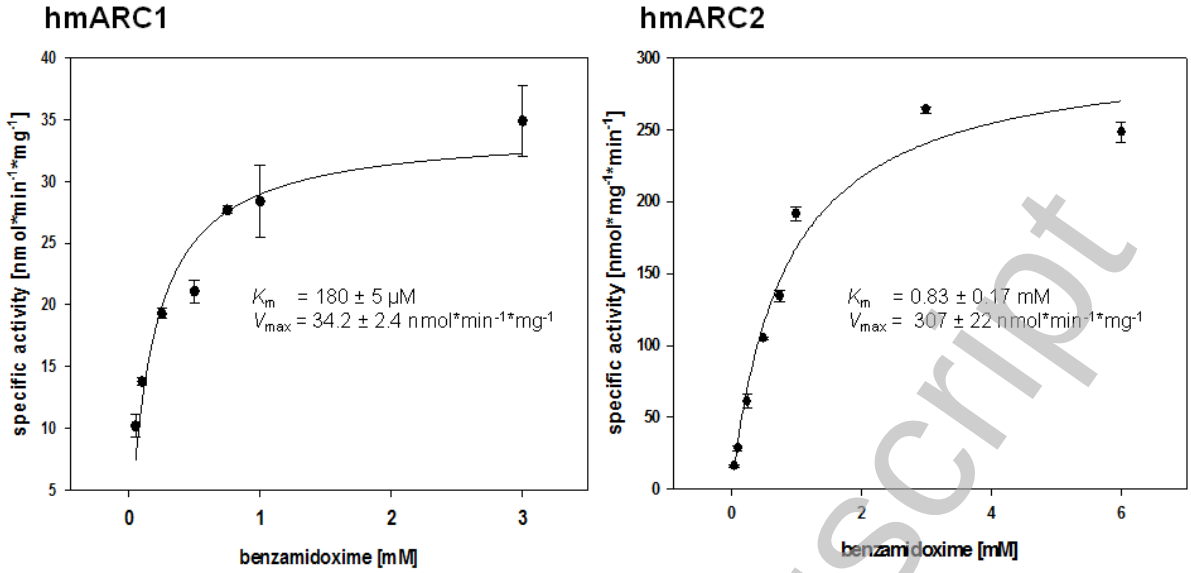

Figure 5

***N*-reductive enzyme activity of the recombinant reconstituted enzyme system containing hmARC isoforms depending on variations of benzamidoxime concentration.** Enzyme activity was analysed as a function of the benzamidoxime concentration. The curves were calculated from the Michaelis-Menten equation fitted to the data points; parameters V_{max} and K_m were calculated.