NOSs (nitric oxide synthases) catalyse the oxidation of L-arginine to L-citrulline and nitric oxide via the intermediate NOHA (N\textsuperscript{ω}-hydroxy-L-arginine). 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. It has also been shown that NOHA is reduced by microsomes and mitochondria to L-arginine. In the present study, we show for the first time that both human isoforms of the newly identified mARC (mitochondrial amidoxime reducing component) enhance the rate of reduction of NOHA, in the presence of NADH cytochrome b\textsubscript{5} reductase and cytochrome b\textsubscript{5}, by 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 nitric oxide biosynthesis, considering a mitochondrial NOS has been identified. Moreover, this reduction is not restricted to NOHA since the analogous arginase inhibitor NHAM (N\textsuperscript{δ}-hydroxy-N\textsuperscript{ω}-methyl-L-arginine) is also reduced by this system.

Key words: cytochrome b\textsubscript{5}, mitochondrial amidoxime reducing component (mARC), benzamidoxime, Mo cofactor sulfurase C-terminal-domain-containing (MOSC), nitric oxide, N\textsuperscript{ω}-hydroxy-L-arginine (NOHA).

INTRODUCTION

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

Moreover, it has become apparent that NOHA itself is a regulator of NO formation, since it can be liberated from the active site of NOSs [9,10] and acts as a potent inhibitor of arginases (EC 3.5.3.1) [11]. Arginases 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 utilization, such as the oxidation of NOHA to NO by haemoproteins [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 non-specific reductive pathway is in particular involved in the detoxification of xenobiotics [20,21]. In mitochondria the enzyme system responsible consists of NADH cyt cyt (cytochrome) b\textsubscript{5} reductase, cyt b\textsubscript{5} and a third enzyme. This third component has been identified in pig liver mitochondria and represents a novel Mo (molybdenum)-containing enzyme called mARC (mitochondrial amidoxime reducing component) [22]. The human genome harbours two genes encoding for two mARC proteins, which we designated mARC\textsubscript{1} and mARC\textsubscript{2}. hmARC\textsubscript{1} (human mARC\textsubscript{1}) has been shown to be capable of catalysing the activation of N-hydroxylated prodrugs [23], whereas recombinant expression and characterization of human mARC\textsubscript{2} (hmARC\textsubscript{2}) is described for the first time in the present study.

Abbreviations used: cyt, cytochrome; ICP-MS, inductively coupled plasma-MS; mARC, mitochondrial amidoxime reducing component; hmARC, human mARC; Moco, Mo cofactor; MOSC, Moco sulfurase C-terminal-domain-containing; MPT, molybdopterin; NHAM, N\textsuperscript{ω}-hydroxy-N\textsuperscript{δ}-methyl-L-arginine; NOHA, N\textsuperscript{ω}-hydroxy-L-arginine; NOS, nitric oxide synthase; mNOS, mitochondrial NOS; OMV, outer membrane vesicle; o-PA, o-phthalaldehyde; RP, reversed phase; RSD, relative standard deviation.

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Although several authors have described the existence of a mtNOS (mitochondrial NOS), which seems to be a splice variant of nNOS (neuronal NOS) and be implicated in cell respiration [24], there is still debate about its existence and function. Nevertheless, the question arose as to 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 also examined a possible reduction of NHAM (Nω-hydroxy-Nδ-methyl-L-arginine), an analogous potent inhibitor of arginase, in order to show that mARCs can be considered to generally be capable of reducing NOHAs [25]. The present study investigated mitochondria, mitochondrial outer membranes, mARC purified from pig liver and heterologously expressed hmARC1 and hmARC2 for their capacity to reduce the aforementioned derivatives.

EXPERIMENTAL

Chemicals

All substances were commercially available and were obtained from Sigma–Aldrich, Fluka, Merck or Roth unless otherwise stated. NOHA was purchased from Cayman chemicals and NHAM was synthesized as described previously [26].

Preparation of subcellular fractions

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

Purification of native mARC (from pig liver)

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

SDS/PAGE

SDS/PAGE was carried out according to the method of Laemmli using a separation gel containing 12.5 % polyacrylamide [29]. Silver staining was performed according to the manufacturer’s protocol (Silver Staining Kit, Protein Plus One, GE Healthcare). Standards and samples were pretreated with 2-mercaptoethanol for 5 min at 100°C.

Immunoblot analysis

Immunoblot analysis was performed by blotting protein fractions separated by SDS/PAGE (12.5 % gels) with a primary polyclonal antibody raised against hmARC2 [anti-MOSC (Moco sulfurase C-terminal-domain-containing) 2 antibody, 1:10000 dilution; Sigma–Aldrich]. The secondary horseradish peroxidase-conjugated anti-(rabbit Ig) (Sigma–Aldrich) was used at a 1:10000 dilution and chemiluminescence was detected using an ECL (enhanced chemiluminescence) system (ECL Plus Western blotting detection system; GE Healthcare).

Determination of protein concentration

Protein concentration was determined according to the manufacturer’s protocol [BCA (bicinchoninic acid) protein assay kit combined with the protein assay preparation reagent set; Pierce].

FormA-dephospho analysis

Moco (Mo cofactor) and its Mo-free precursor MPT (molybdopterin) bound to the OMV-fraction and the purified mARC-enriched fraction was detected and quantified as described by Johnson 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 PCR using specific primers for amplification of mARC1 (mARC1-forward, 5'-ATATATGGATTCCATGGGCGCCGCTTCCCGG-3' and mARC1-reverse, 5'-AAATTTAGCTTTTACTGGCCCAGGTACAAG-3') and mARC2 (mARC2-forward, 5'-ATATATGATTCATGGGCGCTTCCATGGG-3' and mARC2-reverse, 5'-ATATTTAGCTTTTAACCCTCGGATACACAGGGTC-3') deduced from GenBank® entries NM_022746 and
NM_017898. Using this procedure full-length open reading frames of 1011 and 1005 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 N-termini of mARC1 and mARC2 was achieved by a second PCR using the full-length cDNA as a template and primer mARC1-N-del (5'-ATATATGATCCATCGCAGGAGTAGAGCAGTGGCG-3') or primer mARC2-N-del (5'-ATA-TATGGATCATGACAGGGACCCGTGCGGCGAAG-3') in the presence of the respective reverse primer as given above. Simultaneously, restriction sites for BamHI and HindIII 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 an N-terminal His6-tag. The sequences of the introduced cDNAs, encoding proteins of 286 (mARC1) and 285 (mARC2) amino acids, was verified by sequencing.

Expression and purification of recombinant hmARC1 and hmARC2

Routine protein expression of hmARC proteins was performed in freshly transformed Escherichia coli TP1000 cells [31]. Cells were grown aerobically in Luria–Bertani medium in the presence of 100 μg/ml ampicillin at 22°C to a D600 of 0.1 before induction with 15–30 μM IPTG (isopropyl-β-D-thiogalactopyranoside) and addition of 1 mM sodium molybdate. After induction, cells were grown for a further 20 h at 22°C. Cells were harvested by centrifugation (12000 g for 10 min) and stored at −70°C until use. Cell lysis was achieved by several passages through a French press cell following by sonication for 5 min on ice. After centrifugation, His6-tagged proteins were purified on a nickel-nitrilotriacetic acid superflow matrix (Qiagen) under native conditions at 4°C 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 cyt b5

Recombinant human cyt b5 was purchased from MoBiTec.

Recombinant human NADH cyt b5 reductase (isoform 2)

Expression of C-terminally truncated human cyt b5 reductase isoform 2 (GenBank® accession number NP_015565) from expression plasmid pQE80 (Qiagen) in E. coli DL41 cells and purification of the resulting recombinant protein was performed according to the method described by Kurian et al. [32].

FAD content determination

The FAD content of cyt b5 reductase was determined in 50 mM phosphate buffer, pH 7.4, containing 20% (w/v) glycerol, 0.1 mM DTT (dithiothreitol) and 1 mM sodium/EDTA by an Illustra NAP-25 column (GE Healthcare). Protein sample aliquots (200 μl) were denatured by heating for 10 min at 100°C, spiked with 5 ng of indium for internal standardization, and diluted to 2 ml with 2% (v/v) sub-boiled nitric acid. The resulting suspension was filtrated using a 30 kDa centrifuge filter (Millipore), and the clear filtrate was used for subsequent analysis by ICP-MS. This sample solution was manually introduced into a 7500cs ICP-MS instrument (Agilent Technologies) 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 Mo isotopes were monitored during the analysis, but only 98/78 was found to be free from interferences and, hence, was 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 (relative standard deviation; 1 σ). The limit of detection was 0.5 ng/ml Mo as calculated from the original protein solution prior to dilution. All sample preparatory work was done under class 100 clean-room conditions. Further details of the calibration strategy and

In vitro reduction assays

Incubations were carried out under aerobic conditions at 37°C 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 pre-incubation period of 3 min at 37°C the reaction was initiated by addition of NADH and terminated after 15 min by addition of 150 μl of methanol. Precipitated proteins were sedimented by centrifugation (12 000 g for 10 min) and the supernatant was analysed by HPLC. Incubation mixtures with native mARC (pig liver) consisted of 1 μg of the mARC-enriched fraction (which also contained cyt b5 and cyt b5 reductase). Incubation mixtures with recombinant hmARC1 or hmARC2 consisted of 200 pmol of cyt b5 20 pmol of NADH cyt b5 reductase and 10 μg of Mo enzyme. Incubation mixtures with the subcellular fractions (pig liver) contained 56 μg of mitochondria or 6 μg of OMVs 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% DMSO) and 5 mM MgCl2. After a pre-incubation period of 3 min the reaction was initiated by the addition of malate.

The apparent kinetic parameters Km and Vmax were calculated using non-linear 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 the method described by 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).

ICP-MS (inductively coupled plasma-MS)

The buffer of the mARC proteins was exchanged to 50 mM potassium phosphate buffer, pH 7.4, containing 20% (w/v) glycerol, 0.1 mM DTT (dithiothreitol) and 1 mM sodium/EDTA by an Illuistra NAP-25 column (GE Healthcare). Protein sample aliquots (200 μl) were denatured by heating for 10 min at 100°C, spiked with 5 ng of indium for internal standardization, and diluted to 2 ml with 2% (v/v) sub-boiled nitric acid. The resulting suspension was filtrated using a 30 kDa centrifuge filter (Millipore), and the clear filtrate was used for subsequent analysis by ICP-MS. This sample solution was manually introduced into a 7500cs ICP-MS instrument (Agilent Technologies) 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 Mo isotopes were monitored during the analysis, but only 98/78 was found to be free from interferences and, hence, was 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 (relative standard deviation; 1 σ). The limit of detection was 0.5 ng/ml Mo as calculated from the original protein solution prior to dilution. All sample preparatory work was done under class 100 clean-room conditions. Further details of the calibration strategy and
Mo analysis in organic matrices have been published previously [36–38].

HPLC method for the separation of NOHA and L-arginine, and NHAM and N\textsuperscript{2}-methyl-L-arginine

Amino acids were analysed by RP (reversed phase)–HPLC using o-PA (o-phthalaldehyde) precolumn derivatization. Metabolites were separated on a NovaPak RP-18 (4 mm × 250 mm) 4 μm column with a Phenomenex C\textsubscript{18} 4 mm × 3.0 mm guard column, and a Waters 717plus autosampler, a Waters 600 controller and a Waters 474 scanning fluorescence detector, set at λ\textsubscript{exc} of 338 nm and λ\textsubscript{em} of 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 2 min at room temperature (20–25°C) before injection. o-PA reagent was prepared as described previously [39].

Elution was carried out isocratically with 75% 10 mM potassium phosphate buffer, pH 4.65, 15% methanol and 10% acetonitrile (by vol.). The flow-rate was kept at 1 ml/min.

Characteristic retention times were: NOHA, 9.1 ± 0.2 min; L-arginine, 10.0 ± 0.2 min; NHAM, 10.8 ± 0.1 min; and N\textsuperscript{2}-methyl-L-arginine, 12.2 ± 0.2 min.

HPLC method for the separation of benzamidoxime and benzamidine

HPLC analysis were performed on a LiChrospher 60, RP-select B (4 mm × 125 mm) 5 μm column with a LiChrospher 60, RP-select B (4 mm × 4 mm) guard column, Waters e2695 Xc separation module and Waters 2998 photodiode array detector.

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

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 OMV fraction of pig liver mitochondria (Figures 2 and 3). This mARC-enriched enzyme fraction was capable of reducing our model compound benzamidoxime without the addition of cyt b\textsubscript{5} reductase and cyt b\textsubscript{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 (results not shown). The cyt b\textsubscript{5} content was 0.38 ± 0.03 nmol·mg\textsuperscript{-1} of protein (n = 7) and the cyt b\textsubscript{5} reductase activity was 29.8 ± 6.5 μmol·min\textsuperscript{-1}·mg\textsuperscript{-1} of total protein (n = 22).

This purified protein fraction showed an N-reductive activity of 728 ± 63 nmol of benzamidine·min\textsuperscript{-1}·mg\textsuperscript{-1} of total protein. Thus amidoxime reductase activity was enriched 40-fold in comparison with the porcine mitochondria and 2.6-fold by comparison with the OMV fraction (results 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 investigated further using benzamidoxime as a model substrate. Since intact inner mitochondrial membranes are 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 were able to reduce benzamidoxime upon the addition of exogenous NADH (see Table 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 (results not shown). These results 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 confirmed further by using incubation mixtures with mitochondria containing malate, ADP, rotenone and NAD\textsuperscript{+} instead of 1 mM
NADH. The resulting N-reductive activity (4.1 ± 0.8 nmol of benzamidine·min⁻¹·mg⁻¹ of total protein) decreased approx. 80% in comparison with incubation mixtures containing exogenous NADH (25.0 ± 9.9 nmol of benzamidine·min⁻¹·mg⁻¹ of total protein) instead of the NADH-generating system. The residual activity of the mitochondria detected 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% of the 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 resulted in a 150% increase of N-reductive activity (6.3 ± 0.4 nmol of benzamidine·min⁻¹·mg⁻¹ of total protein) using endogenously formed NADH.

Expression and characterization of recombinant hmARC1 and hmARC2 with benzamidoxime

Both recombinantly expressed proteins hmARC1 and hmARC2 were obtained in adequate purity (Figure 4) and, owing to expression in the *E. coli* strain TP1000, which accumulates the eukaryotic form of Moco, were loaded with Moco. hmARC1 and hmARC2 were 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 were found. On the basis of ICP-MS analysis, the average Mo content for both proteins turned out to be 0.3 mol of Mo·mol⁻¹ of protein. Moreover, both proteins were capable of reducing the model substrate benzamidoxime to benzamidine in a reconstituted enzyme system with cyt b₅ and cyt b₅ reductase. The reaction was investigated further 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 b₅ and cyt b₅ reductase led to benzamidine production to a moderate extent. Reduction rates were increased 15-fold for hmARC1 and 300-fold for hmARC2 when compared with incubations lacking mARC isoforms. In addition, the reduction was dependent on the presence of NADH.

The reduction followed Michaelis–Menten kinetics, as shown for both hmARC isoforms in Figure 5. The kinetic parameters determined for the reduction of benzamidoxime were $K_m = 180 ± 5$ μM and $V_{max} = 34.2 ± 2.4$ nmol·min⁻¹·mg⁻¹ of total protein for recombinant hmARC1 and $K_m = 0.83 ± 0.17$ mM and $V_{max} = 307 ± 22$ nmol·min⁻¹·mg⁻¹·min⁻¹ of total protein for recombinant hmARC2 respectively. Control incubations with mARC proteins that either bound MPT, the Mo-free precursor of Moco, or had no pterin bound, due to expression in *E. coli* RK5206 and RK5204 respectively, had no reductive activity indicating that the Mo site is essential for catalytic activity.

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

A complete incubation mixture consisted of 10 μg of hmARC, 200 pmol of cyt b₅, 20 pmol of cyt b₅ reductase, 1 mM NADH and 0.5 mM benzamidoxime in 150 μl of 20 mM Mes buffer, pH 6.0. Results are means ± S.D. for three different incubations each analysed twice via HPLC.

<table>
<thead>
<tr>
<th>mARC isoform</th>
<th>Composition of system</th>
<th>Specific activity (nmol·min⁻¹·mg⁻¹ of total protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant hmARC1</td>
<td>Complete</td>
<td>34.3 ± 0.0</td>
</tr>
<tr>
<td>Without cyt b₅</td>
<td>15.4 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>Without cyt b₅ reductase</td>
<td>1.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Without hmARC1</td>
<td>2.2 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Only hmARC1</td>
<td>2.2 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Without NADH</td>
<td>0.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Recombinant hmARC2</td>
<td>Complete</td>
<td>90.7 ± 5.6</td>
</tr>
<tr>
<td>Without cyt b₅</td>
<td>0.9 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Without cyt b₅ reductase</td>
<td>1.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Without hmARC2</td>
<td>0.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Only hmARC2</td>
<td>0.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Without NADH</td>
<td>0.1 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

**Reduction of NOHA by mitochondria, OMV and the 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 purified mARC-enriched fractions were carried out. These incubations confirmed that NOHA is reduced to L-arginine by pig liver mitochondria as reported previously [19]. However, we found that the specific reduction rates increased from mitochondria to OMV (a 10-fold increase) to mARC-enriched fractions (40-fold increase, specific activity of 481 nmol·min⁻¹·mg⁻¹·min⁻¹ of protein) (Table 2), which correlates with the enrichment of the amidoxime reductase activity (see above). Since the purified mARC-enriched fraction already contained cyt b₅ and cyt b₅ reductase, addition of these proteins to the purified fraction did not enhance the conversion rates (results not shown).

Accordingly, these results indicate that NOHA is reduced to L-arginine by the described enzyme system consisting of mARC, cyt b₅ and cyt b₅ reductase.

Figure 4  SDS/PAGE analysis of recombinant hmARC1 and hmARC2

After expression in *E. coli* and purification by affinity chromatography 20 μg each of hmARC1 and hmARC2 subjected to SDS/PAGE (12 % gels) and subsequently stained with Coomassie Brilliant Blue. Masses are indicated in kDa on the left-hand side.
The enzyme activity of the hmARC isoforms was analysed as a function of the benzanidoxime concentration. The curves were calculated from the Michaelis–Menten equation fitted to the data points and the parameters $V_{\text{max}}$ and $K_m$ calculated.

### Table 2  
**In vitro reduction of NOHA by mitochondrial preparations**

See the Experimental section for details of how the assays were performed. Results are means $\pm$ S.D. for three different incubations each analysed twice via HPLC.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme source</th>
<th>Specific activity (nmol $\cdot$ min$^{-1}$ $\cdot$ mg$^{-1}$ of total protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOHA</td>
<td>Mitochondria</td>
<td>$11.7 \pm 0.9$</td>
</tr>
<tr>
<td>NOHA</td>
<td>OMV</td>
<td>$107.0 \pm 10.1$</td>
</tr>
<tr>
<td>NOHA</td>
<td>Purified mARC</td>
<td>$481.1 \pm 91.0$</td>
</tr>
</tbody>
</table>

### Reduction of NOHA by recombinant hmARC proteins

The involvement of mARC in the reduction of NOHA was verified further by using recombinantly expressed enzyme sources. The applied hmARC1 and hmARC2 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 $39.1 \pm 1.4$ nmol $\cdot$ min$^{-1}$ $\cdot$ mg$^{-1}$ of total protein for hmARC1 and $56.8 \pm 5.0$ nmol $\cdot$ min$^{-1}$ $\cdot$ mg$^{-1}$ of total protein for hmARC2 (Table 3). The reduction followed Michaelis–Menten kinetics and kinetic parameters for the reduction of NOHA were $K_m = 86 \pm 13$ $\mu$M and $V_{\text{max}} = 55.5 \pm 1.7$ nmol $\cdot$ min$^{-1}$ $\cdot$ mg$^{-1}$ of total protein for hmARC1 and $K_m = 3.0 \pm 0.3$ mM and $V_{\text{max}} = 373 \pm 20$ nmol $\cdot$ min$^{-1}$ $\cdot$ mg$^{-1}$ of total protein for hmARC2. Additionally, incubations were performed omitting one component at a time. As expected, incubations containing cyt $b_5$, cyt $b_5$ reductase and hmARC showed the highest activity, whereas incubations lacking hmARC showed a significant lower activity, demonstrating the involvement of hmARC in this reductive pathway (Table 3). Comparisons of the activities of the complete enzyme system with the incubations lacking hmARC enzymes showed that reduction rates were increased 30-fold by hmARC1 and more than 500-fold by hmARC2. Another important observation was that the activity of the complete incubation mixture was 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 described previously [22,23].

Reduction of NHAM

It has been shown previously that the N-reductive enzyme system under investigation possesses a broad substrate specificity [23]. Therefore the question arose as to whether hmARCs are capable of also reducing other NOHAs. Thus we examined the reduction of NHAM, a known potent arginase inhibitor, by the complete enzyme system and by omitting single components. As shown in Table 3, NHAM is reduced to N̄-methyl-L-arginine with highest reduction rates seen 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 $K_m = 272 ± 60 \, \mu M$ and $V_{\text{max}} = 43.1 ± 2.8 \, \text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of total protein for hmARC1 and $K_m = 3.7 ± 0.4 \, \text{mM}$ and $V_{\text{max}} = 36.5 ± 2.3 \, \text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of total protein for hmARC2. In summary, we conclude from these results that this enzyme system can be generally considered to be capable of reducing NOHAs.

DISCUSSION

Initial studies on the Mo-containing enzyme mARC, purified from mammalian liver mitochondria, had shown that the enzyme was capable of reducing amidoxime structures to the corresponding amidines [24]. The complete reductive enzyme system represented a three-component complex consisting of cyt $b_5$ reductase, cyt $b_5$, and mARC as the catalytic part [22].

The human genome encodes two homologous mARC proteins, hmARC1 and hmARC2, designated as MOSC1 and MOSC2 in the databases. Previous studies have described the reduction of several N-hydroxylated prodrugs by the recombiant full-length hmARC1 in a reconstituted enzyme system together with cyt $b_5$ and its reductase. In the present study both hmARC1 and hmARC2 were expressed in their soluble forms, without their N-terminal putative mitochondrial targeting sequences, since initial experiments showed an increased activity for these proteins (results 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 were considerable differences in activity; both the $K_m$ and $V_{\text{max}}$ for hmARC2 are one order of magnitude above those of hmARC1 (Table 1 and Figure 5).

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

From previous studies this enzyme system was known to reduce N-hydroxylated xenobiotics; in particular, we had investigated the activation of N-hydroxylated prodrugs to the active form [23]. Physiologically, this reductive enzyme system was assumed to be involved in detoxification processes. However, the question arose as to whether this newly identified enzyme fulfills other physiological functions, although no physiological substrate had been thus far identified. 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.

Our present experiments showed the reduction of this putative physiological substrate by the characterized Mo-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 was high ($481 \, \text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of protein) considering the turnover rates for L-arginine by NOHAs stated to be in the range of $10 \, \mu M \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of protein to $30 \, \mu M \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of protein for purified NO [43,44]. However, a high specific activity alone does not prove a physiological impact on NO levels 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 of protein) is approximately four orders of magnitude above NOs activity when compared with the known basal NO activity in cardiac mitochondria, stated to be 2 pmol · min⁻¹ · mg⁻¹ of 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 catalyzed this conversion in a reconstituted enzyme system (Table 3). Our experiments showed the highest activity for the complete three component system and lower activity in incubations with only a two component enzyme system without hmARCs. Addition of hmARC1 augmented the activity more than 30-fold compared with incubations lacking the Mo enzyme, whereas activity was increased even more by hmARC2 (approx. 500-fold).

In general, the conversion rates of the native enzyme system in the purified mARC-enriched fraction were approx. 10-fold higher than the conversion rates of the recombinant reconstituted enzyme system. However, because different mARC sources (cDNA-expressed compared with purified from pig liver) were used, the rates obtained were dependent on several aspects that influence the absolute enzyme activity. For example, it is possible that truncation of mARC enzymes could have had 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 hydrophobic domains ensure an optimal complex formation of the enzymes. Therefore it is not surprising that the truncated recombinant mARC homologues were 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, a lower activity of recombinantly expressed membrane-bound enzymes is a common problem, and is also known to affect recombinant NOHAs [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 NO-mediated 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 haemoproteins, have been reported [16–18]. Moreover, NOHA
is known to be 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 a 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 be consistent with our previous findings of mARC being involved in detoxication processes by reducing several N-hydroxylated xenobiotics 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 (i) ensure a sufficient arginase activity, as required for a well-functioning urea cycle, or (ii) to promote L-ornithine formation, which is essential for polyamine biosynthesis. Interestingly enough, so far there are no additional metabolic pathways for NOHA known other than its conversion into 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 have identified mARC1 and mARC2 as inner mitochondrial membrane proteins in mouse [47].

More studies are needed to elucidate further 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 overexpression, should be addressed.

Besides the reduction of NOHA, the reconstituted recombinant mARC-containing enzyme system is also capable of reducing other NOHAs such as NAM (Table 3). This non-physiological compound represents a potent arginase inhibitor, and in contrast with NOHA, is not converted by NOSs [25]. NAM was reduced by all of the enzyme systems examined, however, at significantly lower turnover rates than those with NOHA (Table 3). Nevertheless, the results from the present study revealed that both mARC homologues are generally capable of reducing Nω-hydroxyguanidine-containing compounds.

In summary, the present study identifies for the first time 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 the detoxification processes [22,23]. Additionally, this enzyme system was utilized 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.

AUTHOR CONTRIBUTION

Jürgen Kotthaus wrote most of the manuscript and performed several in vitro assays. Bettina Wahl performed the recombinant enzyme expression of the hmARC isoforms. Antje Havemeyer performed the in vitro incubations with benzamidoxime substrate and wrote several paragraphs of the manuscript. Joscha Kotthaus performed in vitro incubations with NOHA substrate. Dennis Schade performed the chemical synthesis of NHAM. Dieter Garbe-Schönberg performed the ICP-MS Mo analysis. Ralf Mendel performed the recombinant enzyme expression of the hmARC isoforms and provided direction for co-workers. Florian Bitner performed the recombinant enzyme expression of the hmARC isoforms and wrote several paragraphs of the manuscript. Bernd Clement, as head of the laboratory, initiated these studies and supported the authors.

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