Short Communication

Reduction of N-Hydroxy-sulfonamides, Including N-Hydroxy-valdecoxib, by the Molybdenum-Containing Enzyme mARC

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ABSTRACT:

Purification of the mitochondrial enzyme responsible for reduction of N-hydroxylated amidine prodrugs led to the identification of two newly discovered mammalian molybdenum-containing proteins, the mitochondrial amidoxime reducing components mARC-1 and mARC-2 (Gruenewald et al., 2008). These 35-kDa proteins represent a novel group of molybdenum proteins in eukaryotes as they form a molybdenum cofactor-dependent enzyme system consisting of three separate proteins (Havemeyer et al., 2006). Each mARC protein reduces N-hydroxylated compounds after reconstitution with the electron transport proteins cytochrome b₅ and b₆ reductase. In continuation of our drug metabolism investigations (Havemeyer et al., 2006; Gruenewald et al., 2008), we present data from reconstituted enzyme systems with recombinant human and native porcine enzymes showing the reduction of N-hydroxy-sulfonamides (sulfohydroxamic acids) to sulfonamides: the N-hydroxy-sulfonamide N-hydroxy-valdecoxib (N-hydroxy-4-[5-methyl-3-phenyl-4-isoxazolyl]-benzenesulfonamide) represents a novel cyclooxygenase (COX)-2 inhibitor and is therefore a drug candidate in the treatment of diseases associated with rheumatic inflammation, pain, and fever. It was synthesized as an analog of the known COX-2 inhibitor valdecoxib (4-[5-methyl-3-phenyl-4-isoxazolyl]-benzenesulfonamide) (Talley et al., 2000). N-Hydroxy-valdecoxib had low in vitro COX-2 activity but showed significant analgesic activity in vivo and a prolonged therapeutic effect compared with valdecoxib (Erđelyi et al., 2008). In this report, we demonstrate that N-hydroxy-valdecoxib is enzymatically reduced to its pharmacologically active metabolite valdecoxib. Thus, N-hydroxy-valdecoxib acts as prodrug that is activated by the molybdenum-containing enzyme mARC.

Introduction

The enzymatic processes involved in the N-oxidative pathway are very well understood, whereas reduction pathways have been investigated in depth. The reduction of nitrogen-containing groups like aromatic nitro compounds, hydroxamic acids, oximes, tertiary N-oxides, aza compounds, and N-hydroxyguanidines by the molybdenum hydroxylases aldehyde oxidase and xanthine oxidase have been described previously (Dambrova et al., 1998; Kitamura et al., 2006). However, the activities were often only described for in vitro investigations under the exclusion of oxygen. The in vivo relevance is thus questionable. Microsomal and mitochondrial reductions of hydroxylamines and amidoximes that are O₂-insensitive have been investigated by different groups (Kadlubar and Ziegler, 1974; Kurian et al., 2004, 2006; Andersson et al., 2005; Clement et al., 2005), and it is well accepted that cytochrome b₅ and its reductase are involved in the reduction activities mentioned. The membrane-bound forms of these electron transport proteins are located in the mitochondrial outer membrane and the endoplasmic reticulum (Borgese et al., 1993).

Investigation of the aerobic reduction of amidoxime structures led to the discovery of a hitherto unknown molybdenum-containing enzyme system (Havemeyer et al., 2006). It was named “mitochondrial amidoxime reducing component” (mARC), because initially N-reduction of amidoxime structures was studied with this enzyme purified from mammalian liver mitochondria. After recombinant expression of human mARC (Gruenewald et al., 2008), it became clear that besides sulfate oxidase, xanthine oxidoreductase and aldehyde oxidase, a fourth molybdenum-containing enzyme, exist. The human genome encodes for two homologous mARC proteins, mARC-1 and mARC-2 (designated as MOSC1 and MOSC2 in the databases). Both mARC homologs are able to reduce benzamidoxime as a model substrate in a reconstituted enzyme system together with NADH cytochrome b₅ and its reductase. We were able to demonstrate that this molybdenum-containing enzyme system is responsible for amidoxime/N-hydroxyguanidine prodrug reduction to the pharmacologically active drug (Gruenewald et al., 2008).

Although N-hydroxylated sulfonamides (sulfohydroxamic acids) have considerable potential to treat a variety of disorders, the reduction pathways have been largely ignored. For example, N-substituted sulfonamides could act as nitric oxide donors (Shirotta et al., 1999), carboxic anhydrase (Mincione et al., 1998), and tyrosinase inhibitors (Khan et al., 2005). The recent development of a selective cyclooxygenase (COX)-2 inhibitor with a better adverse event profile, using valdecoxib as a lead compound, led to the corresponding N-hydroxylated sulfonamide (Erđelyi et al., 2008). N-Hydroxy-valdecoxib is also a known metabolite of valdecoxib in humans (Yuan et al., 2002). Recent in vivo studies of analgesic and anti-inflammatory effects showed that the N-hydroxylated analog is not only more potent, it also has a prolonged activity.

ABBREVIATIONS: mARC, mitochondrial amidoxime reducing component; COX, cyclooxygenase; NOHBSA, N-hydroxy-benzenesulfonamide; NOHTSA, N-hydroxy-4-toluenesulfonamide; m.p., melting point; BzSA, benzenesulfonamide; TSA, 4-toluenesulfonamide; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; apo, apoprotein.
in comparison to valdecoxib. Contrary to expectations, in vitro COX-2 inhibitory potency of N-hydroxy-valdecoxib was low compared with valdecoxib (Erődy et al., 2008). These data suggest that N-hydroxy-valdecoxib is bioactivated by reduction in vivo. Thus, N-hydroxy-valdecoxib could serve as a prodrug (Fig. 1). If enzymatic metabolism is necessary for the pharmacological effect of valdecoxib, then it is necessary for further drug development to identify the reductive enzymes involved to be able to evaluate potential interspecies variability, genetic polymorphisms, and drug–drug interactions.

In the current study, the N-reductive substrate metabolism of N-hydroxy-sulfonamides in the recombinant and purified native porcine liver mARC-containing enzyme system is characterized by evaluating their kinetic parameters $K_{\text{m}}$ and $V_{\max}$. Furthermore, conversion rates of N-hydroxy-sulfonamide reduction by porcine liver mitochondria were compared with results obtained using purified native and recombinant enzymes.

In continuation of our drug metabolism studies, this report demonstrates that the described enzyme system is also able to reduce N-hydroxy-sulfonamides, such as N-hydroxy-valdecoxib, to their corresponding sulfonamides.

**Materials and Methods**

**Chemicals.** N-Hydroxy-benzenesulfonamide (NOHBSA) and N-hydroxy-4-toluenesulfonamide (NOHTSA) were synthesized according to the following procedure: 2.0 g (29 mmol) hydroxylamine-HCl was dissolved in refluxing methanol, and a solution of 0.6 g (26 mmol) sodium in 12.0 ml of ethanol was added. After cooling, the precipitated sodium chloride was filtered off, and in the remaining solution 1.7 g (10 mmol) of benzenesulfonyl chloride or 1.9 g (10 mmol) of toluenesulfonyl chloride was added. Most of the methanol was evaporated. The remainder was filtered, and the solution was evaporated to dryness in vacuo. The solid thus obtained was extracted three times with 5.0 ml of warm diethyl ether. Evaporation of the solvent afforded NOHBSA [melting point (m.p.) 120°C (Smith and Hein, 1960) or NOHTSA (m.p. 143–144°C) (Przybylski and Kuprysziwski, 1975). In the same way, the corresponding sulfonamides benzenesulfonamide (BzSA) and 4-toluenesulfonamide (TSA) were obtained: 1.0 ml of benzenesulfonyl chloride (8 mmol) or 1.5 g of toluenesulfonyl chloride (8 mmol) was heated with 5.0 ml of concentrated ammonia solution for 15 min. Upon dilution with water, the corresponding sulfonamides precipitated [BzSA, m.p. 153°C (Johnson et al., 1978) and TSA, m.p. 141–142°C (Mcfarland et al., 1987)]. The solid was recrystallized with ethanol and washed. N-Hydroxy-valdecoxib and valdecoxib were prepared according to the literature (Talley et al., 1996; Erdey et al., 2008). Benzamidine (N-hydroxy-benzene-carboximidamide) was synthesized from benzonitride and hydroxylamine as described previously (Kruger, 1885). All other chemicals were obtained from Merck KGaA (Darmstadt, Germany), Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany), or Promochem (Wesel, Germany).

**Preparation of Subcellular Fractions.** Mitochondria and microsomes were prepared from porcine liver (Hovius et al., 1990; Clement et al., 2005).

**Purification of Native Enzymes.** Cytochrome $b_5$ was purified from porcine liver microsomes as described previously (Tanguchi et al., 1984; Clement et al., 1997). NADH cytochrome $b_5$ reductase was purified from porcine liver microsomes, similar to the procedure described for the purification of NADH cytochrome P450 reductase (Tanguchi and Masters, 1976). MARC was purified from porcine liver mitochondria, as described previously (Havemeyer et al., 2006).

**Expression and Purification of Recombinant Human mARC-1 and mARC-2.** Full-length open reading frames of human mARC1 and mARC2 cDNAs of 1011 and 1105 base pairs, respectively, were truncated by polymerase chain reaction to remove the coding sequence for the putative N-terminal mitochondrial targeting sequences (according to GenPept accession numbers NP_073583 and NP_060368). The resulting 286 (mARC-1) and 285 (mARC-2) amino acids encoding cDNAs were cloned into the pQE80 expression plasmid (Qiagen, Hilden, Germany), and proteins were expressed in Escherichia coli TP1000 cells (Palmer et al., 1996) and purified as described previously for full-length mARC-1 (Gruenewald et al., 2008). Expression and purification of the N-terminally truncated recombinant proteins are described by Kotthaus and coworkers (Ju. Kotthaus, B. Wahl, A. Havemeyer, Jo. Kotthaus, D. Schade, D. Garbe-Schönberg, R. Mendel, F. Bittner, and B. Clement, manuscript submitted for publication).

**Expression and Purification of Recombinant Human Cytochrome $b_5$ and Cytochrome $b_5$ Reductase.** Expression of C-terminally truncated human cytochrome $b_5$ (GenPept accession number NP_085056) and human cytochrome $b_5$ reductase isoform 2 (GenPept accession number NP_015565) from expression plasmid pQE80 (Qiagen) in E. coli DL41 cells and purification of the resulting recombinant protein were performed according to Kurian et al. (2004). For some incubation studies, commercial available cytochrome $b_5$ (MoBiTec GmbH, Gottingen, Germany) was used.

**Determination of Protein Concentration.** Protein concentrations were determined using a BCA protein assay Kit (Pierce, Rockford, IL) following the manufacturer’s instructions or using Ropi Quant solution (Roth, Karlsruhe, Germany) according to Bradford (1976).

**Enzyme Assays.** Cytochrome $b_5$ was estimated from the difference spectra between an oxidized and NADH reduced preparation (Estabrook and Werringer, 1978). NADH cytochrome $b_5$ reductase was determined by a modification of the ferricyanide reduction assay (Mihara and Sato, 1978).

**SDS-Polyacrylamide Gel Electrophoresis.** SDS-polyacrylamide gel electrophoresis (PAGE) was carried out using a separation gel containing 12% polyacrylamide (Laemmli, 1970). Silver staining was performed according to the manufacturer’s directions (Silver Staining Kit, Protein Plus One; GE Healthcare, Chalfont St. Giles, UK). Standards and samples were pretreated with β-mercaptoethanol for 5 min at 100°C.

**Molybdenum Cofactor Analysis.** To identify the molybdenum cofactor coordinated by the purified mARC enzyme (porcine liver), the nit-l reconstitution assay was used as described previously (Nason et al., 1971; Havemeyer et al., 2006).

**Incubations with N-Hydroxylated Substrates.** Incubations were carried out under aerobic conditions at 37°C in a shaking water bath. Incubation mixtures contained 0.5 or 2 mM substrate and 1.0 mM NADH in a total volume of 150 μl of potassium phosphate buffer (100 mM), pH 6.0 or 6.3.
After a preincubation period of 3 min at 37°C, the reaction was initiated by addition of NADH and terminated after 15 to 30 min by addition of acetoni- trile. Precipitated proteins were sedimented by centrifugation, and the super- natant was analyzed by high-performance liquid chromatography (HPLC). Incubation mixtures with native mARC (porcine liver) consisted of 200 pmol of cytochrome b₅ (porcine liver or recombinant protein), 0.05 units NADH cytochrome b₅ reductase (porcine liver), and 0.2 to 3 μg of molybdenum enzyme. Minor contaminations of sulfonamide in the substrate were subtracted for calculation of the enzymatic conversion rates.

Incubation mixtures with recombinant mARC-1 or mARC-2 consisted of 200 pmol of cytochrome b₅ (recombinant protein), 0.05 units NADH cytochrome b₅ reductase (porcine liver or recombinant protein), and 10 to 30 μg of molybdenum enzyme. Incubation mixtures of porcine liver mitochondria con- tained 50 μg of protein. Apparent kinetic parameters Kₘ and Vₚₜₚ were estimated using nonlinear regression analysis (Sigma Plot 5.0; SPSS Science, Chicago, IL).

HPLC Method for the Separation of N-Hydroxy-valdecoxib and Valde- coxib. Separation was carried out isocratically with 62% (v/v) acetonitrile and 38% (v/v) water, pH 3.2, using a Symmetry C18 column (5 μm, 4.6 × 250 mm; Waters, Milford, MA) with a security guard cartridge system (C18, 3 × 4 mm; Phenomenex, Torrance, CA) as precolumn at a flow rate of 1.0 ml/min. The effluent was monitored at 240 nm. The retention times were 20.0 ± 0.2 min (N-hydroxy-valdecoxib) and 17.6 ± 0.1 min (valde- coxib).

HPLC Method for the Separation of NOHBSA and BzSA or NOHTSA and TSA. The separation was carried out isocratically with 5% (v/v) acetonitrile and 95% (v/v) 20 mM phosphate buffer (pH 4.0) (separation of NOHBSA and BzSA) or 10% (v/v) acetonitrile and 90% (v/v) 20 mM phosphate buffer (pH 4.0) (separation of NOHTSA and TSA) using a LiChroCART 125-4 HPLC Cartridge with LiChrospher RP-select B (5 μM) and a LiChroCART 4-4 guard column (Merck KGaA) as precolumn at a flow rate of 1.0 ml/min. The effluent was monitored at 220 or 226 nm. The retention times were 8.2 ± 0.5 min (NOHBSA), 11.4 ± 0.6 min (BzSA), 19.3 ± 0.4 min (NOHTSA), and 14.3 ± 0.3 min (TSA).

HPLC Method for the Separation of Benzamidoxime and Benzamide. The separation was carried out as described previously (Clement et al., 2005).

Results and Discussion

N-Hydroxy-valdecoxib is a novel COX-2 inhibitor in preclinical development. Although it shows only low COX-2 activity in vitro, it has good analgesic and anti-inflammatory potency in vivo (Erdélyi et al., 2008). Therefore, a simple in vitro assay in an early state of drug development was needed to confirm the presumed in vivo reduction of N-hydroxy-valdecoxib to its active sulfonamide structure (Fig. 1). In this report, we demonstrate the reduction of several N-hydroxy-sulfonamides (Fig. 2), including N-hydroxy-valdecoxib, to their corre- sponding sulfonamides by porcine liver mitochondria. The following Vₚₜ_max values were determined: 7.4 nmol sulfonamide/(min · mg mitochondrial protein) (reduction of N-hydroxy-valdecoxib, Kₘ = 0.6 mM), 15.6 nmol sulfonamide/(min · mg mitochondrial protein) (re- duction of NOHTSA, Kₘ = 1.1 mM), and 20.6 nmol sulfonamide/(min · mg mitochondrial protein) (reduction of NOHBSA, Kₘ = 2.4 mM).

In conclusion, the analgesic and anti-inflammatory properties of N-hydroxy-valdecoxib together with its weak in vitro effect (Erdélyi et al., 2008) could be explained by the reduction of N-hydroxy- valdecoxib to the pharmacologically active sulfonamide. However, the formation of valdecoxib after in vivo administration of its N-
complement the apo-NADPH nitrate reductase in crude extracts of the Neurospora crassa nit-1 mutant, which is deficient in Moco biosynthesis, thus reconstituting apoprotein (apo)-NADPH nitrate reductase activity. The purified mARC enzyme was able to reconstitute apo-NADPH nitrate reductase activity with a specific activity of 1.7 ± 0.2 μmol nitrite/(mg · min), confirming that this enzyme is a Moco-binding protein.

The reduction of N-hydroxy-valdecoxib to valdecoxib by native mARC (purified from porcine liver mitochondria) followed Michaelis-Menten kinetics with $K_m = 1.5$ mM and $V_{max} = 51.2$ mmol/min · mg total protein. Thus, the specific activity was 7-fold enriched in the purified system compared with porcine liver mitochondria, with similar Michaelis-Menten constants ($K_m$ values).

Involvement of mARC in N-hydroxy-sulfonamide reduction was further verified by using recombinant enzymes that also catalyzed this transformation (Tables 1 and 2). Either no or only very little activity was detected if the two-component enzyme system consisted of only cytochrome b5 and its reductase was incubated [maximum of 1.7 ± 0.5 mmol sulfonamide/(min · mg total protein)]. Regarding the $K_m$ values, no substrate specificity for N-hydroxy-sulfonamides with both mARC homologs could be detected, but efficiency of catalysis ($V_{max}/K_m$) is higher for mARC-1 than for mARC-2 (Table 2).

It is not surprising that conversion rates of the recombinant molybdenum enzymes were 2- to 30-fold lower than that of purified native mARC protein from porcine liver mitochondria (Table 1) or than that of mitochondria. These varying rate results may be due to the different origins of the mARC proteins (cDNA-expressed versus purified from mitochondria). These varying rate results may be due to the different origins of the mARC proteins (cDNA-expressed versus purified from mitochondria). These various rate results may be due to the different origins of the mARC proteins (cDNA-expressed versus purified from mitochondria).

In summary, sufficient in vitro metabolism of N-hydroxy-sulfonamides, including N-hydroxy-valdecoxib, could be detected using recombinant enzymes. Therefore, the current study confirms the usefulness of the described recombinant enzymes for obtaining in vitro data for drug metabolism (Tables 1 and 2).

The reduction of N-hydroxy-sulfonamides supports previous studies (Gruenewald et al., 2008) stating that mARC plays a key role in the reductive biotransformation of structurally diverse N-hydroxylated compounds. Thus, the molybdenum-containing enzyme mARC is a protein that should be added to the list of prominent drug-metabolizing enzymes.

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References


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