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Biochemical Pharmacology 66 (2003) 2029–2038
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Pharmacologic or genetic ablation of maleylacetoacetate isomerase increases levels of toxic tyrosine catabolites in rodents

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Received 30 April 2003; accepted 24 July 2003

Abstract

Dichloroacetate (DCA) is both an environmental contaminant and an investigational drug for diseases involving perturbed mitochondrial energetics. DCA is biotransformed to glyoxylate by maleylacetoacetate isomerase (MAAI). Previous studies have shown that DCA decreases MAAI activity in rat liver in a time- and dose-dependent manner and may target the protein for degradation *in vivo*. We now report that the MAAI protein is depleted in a time- and dose-dependent manner in the livers of Sprague–Dawley rats exposed to DCA. This decrease in protein expression is not mirrored by a decrease in the steady-state levels of MAAI mRNA, indicating that the depletion is exclusively a post-transcriptional event. We also investigated the pharmacokinetics of DCA in the recently developed MAAI knockout (MAAI-KO) mouse. MAAI-KO mice maintain high plasma and urine drug concentrations and do not biotransform DCA to monochloroacetate to a significant extent. Therefore, no alternative pathways for DCA clearance appear to exist in mice other than by MAAI-mediated biotransformation. DCA-naïve MAAI-KO mice accumulate very high levels of the tyrosine catabolites maleylacetone and succinylacetone, and DCA exposure did not significantly increase the levels of these compounds. MAAI-KO mice also have high levels of fumarylacetone and normal levels of fumarate. These results demonstrate that pharmacologic or genetic ablation of MAAI cause potentially toxic concentrations of tyrosine intermediates to accumulate in mice and perhaps in other species.

Keywords: Dichloroacetate; Biotransformation; Toxicology; Glutathione transferases; Maleylacetoacetate isomerase; Gene knockout

1. Introduction

Animals catabolize phenylalanine and tyrosine by a multienzyme pathway that leads to formation of fumarate and acetoacetate (Fig. 1). Loss of function mutations in the terminal enzyme of this pathway, fumarylacetoacetate hydrolase, is the cause of hereditary tyrosinemia type I [1]. Absence of hydrolase activity leads to accumulation of intermediates of phenylalanine/tyrosine metabolism, such as maleylacetoacetate (MAA) and fumarylacetoacetate (FAA), which are considered to be hepato-toxins, and their ketone derivatives. Hereditary tyrosinemia type I is also associated with accumulation of succinylacetone (SA). SA inhibits an early step in heme synthesis, resulting in buildup of the heme precursor delta-aminolevulinate (δ-ALA). These perturbations in heme metabolism are considered to be responsible for the neuropathic complication of hereditary tyrosinemia type I.

The xenobiotic DCA is a by-product of water chlorination and thus is a ubiquitous environmental contaminant

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Abbreviations: DCA, dichloroacetate; MAAI, maleylacetoacetate isomerase; SD, Sprague–Dawley; mRNA, messenger ribonucleic acid; MAAI-KO, maleylacetoacetate isomerase knockout; SA, succinylacetone; δ-ALA, delta-aminolevulinate; CLA, congenital lactic acidosis; PDH, pyruvate dehydrogenase; GSTZ1, glutathione transferase zeta 1; MA, maleylacetone; HMG-CoA, β-hydroxy-β-methylglutaryl-CoA; NMR, nuclear magnetic resonance; PVDF, polyvinylidene fluoride; SSC, selenosemicarbazide; GC-MS, gas chromatography-mass spectrometry; SIM, selective ion monitoring; MCA, monochloroacetic acid; FA, fumarylacetone; MAA, maleylacetoacetate; FAA, fumarylacetoacetate.

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doi:10.1016/j.bcp.2003.07.002

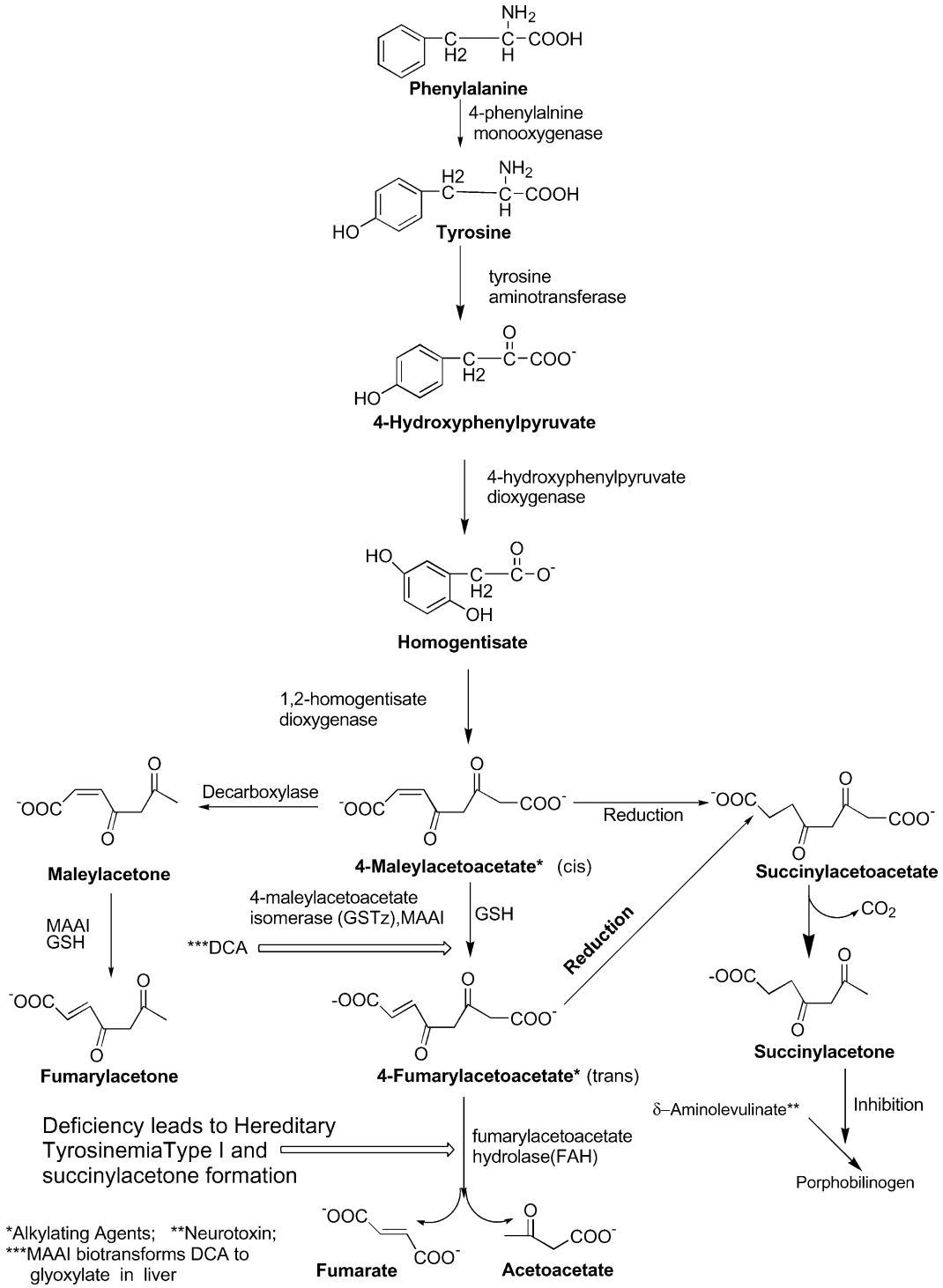

Fig. 1. Tyrosine catabolic pathway and postulated scheme for perturbation by DCA. Tyrosine (and phenylalanine) breaks down to fumarate and acetoacetate *via* a series of enzyme-catalyzed steps in the liver. DCA depletes MAAI, the penultimate enzyme in the pathway, causing the buildup of MA, FA, and SA. SA is a known inhibitor of δ-aminolevulinate dehydratase, causing buildup of δ-ALA and inhibition of heme biosynthesis.

[2]. However, it is also an investigational drug for the treatment of diseases of mitochondrial energetics, including congenital forms of lactic acidosis (CLA) [3–5]. DCA facilitates both cellular lactate removal and energy production by activating carbon flux through the mitochondrial pyruvate dehydrogenase (PDH) complex, mainly by inhibiting PDH-kinase and maintaining PDH in its unphosphorylated, catalytically active state [3]. DCA is hepatocarcinogenic in rodents at doses 10,000-fold higher than environmental levels [6–11]. Although clinical trials do not show evidence of carcinogenicity in humans [12,13], reversible peripheral neuropathy and mild hepato-toxicity

may be common side effects of chronic DCA exposure in humans, at doses of 25 mg/kg/day or greater [3,14].[2]

DCA is biotransformed to glyoxylate in the liver by the cytosolic enzyme glutathione transferase zeta 1 (GSTZ1) [15]. GSTZ1 is identical to MAAI, the penultimate enzyme in the phenylalanine/tyrosine catabolic pathway [16]. We reported earlier that DCA exposure decreases MAAI activity in rat hepatic cytosol, and that rats chronically exposed to DCA accumulate significant amounts of maleylacetone (MA) in their urine [17,18]. Recent studies from two groups have shown that a combination of DCA and GSH irreversibly inactivates rodent and human MAAI *in vitro* [15,18], suggesting that enzyme inactivation and subsequent degradation of the modified protein may decrease both protein expression and MAAI activity *in vivo*. These discoveries have relevance for understanding both DCA toxicology and the unusual kinetics of DCA biotransformation [3,19].

The exact molecular mechanism for DCA-induced depletion of MAAI protein and activity *in vivo* remains uncertain, although it is possible that irreversible modification by the drug may play a central role. DCA has been shown to affect gene expression of multiple cellular proteins, including maleic enzyme [20], HMG-CoA reductase [21,22], stearoyl-CoA desaturase, alpha-1 protease inhibitor, cytochrome b5, and carboxylesterase [23]. We therefore investigated the steady-state transcript levels of MAAI mRNA to determine whether there was a genetic basis for drug-induced MAAI protein depletion. Furthermore, it is not known whether blockade of MAAI diverts DCA into alternate pathways of biotransformation. Studies in humans exposed to [^{13}C]DCA indicate that the compound may undergo reductive dehalogenation to [^{13}C]monochloroacetate [24]. We therefore used the recently developed MAAI-KO mouse model to test the hypothesis that genetic ablation of MAAI prevents DCA biotransformation and leads to accumulation of potentially toxic metabolites of tyrosine, similar to or greater than that seen with pharmacologic ablation of the enzyme.

2. Materials and methods

2.1. Chemicals

Na[1,2-^{13}C]DCA was purchased from Cambridge Isotope Laboratories, and unlabeled Na-DCA from TCI America. All other chemicals used were of the purest grade available from Sigma-Aldrich Corp., or Fisher Scientific.

[^{14}C]DCA, specific activity 55.5 mCi/mmol, was purchased from American Radiolabeled Chemicals. MA and fumarylacetone (FA) were prepared by the method of Fowler and Seltzer [25]. MA was generated within 2 days of use by hydrolysis of its synthetic precursor, 4-acetonylidenebut-2-ene-4-olide. The 4-acetonylidenebut-2-ene-4-olide was synthesized from the reaction of equimolar amounts of maleic anhydride and isopropenyl acetate in the presence of aluminum chloride. The structure was verified by proton nuclear magnetic resonance (NMR) and was as reported in the literature. Portions of the 4-acetonylidenebut-2-ene-4-olide were hydrolyzed with sodium hydroxide to produce MA, then neutralized with HCl and the MA extracted into methylene chloride. The structure of the MA was confirmed by proton NMR. FA was prepared by heating an acid (pH 1) solution of MA at 50° for 5 min, cooling and extracting with methylene chloride. The structure of FA was verified by comparing its proton NMR with that reported in the literature [25].

2.2. Animals and dosing

2.2.1. Rats

Male Sprague–Dawley (SD) rats (250–300 g) were acclimatized for 1 week before experimentation. During dosing periods and control days, the rats were kept in metabolism cages (Nalgene Metabolic Cages) and urine was collected over 24 hr during the study days. DCA, at doses of 4, 12.5, 50, 200, and 1000 mg/kg, was administered by oral gavage in the morning for 1 or 5 days. Four animals were used for each dose. Twenty-four hours after the last dose, animals were sacrificed and the livers were removed to generate cytosol preparations or to extract total RNA.

2.2.2. Mice

The creation and phenotypic characterization of mice bearing a homozygous knockout of the MAAI gene (MAAI-KO) are described by Fernandez-Canon *et al.* [26]. These animals completely lack MAAI mRNA and protein but breed normally and do not exhibit any overt abnormalities unless stressed with a high tyrosine diet [26]. The knockout mice and wild-type littermate controls were exposed to a single dose of 50 mg/kg DCA (0.3 mL/20 g mouse) by oral gavage for $t = 0$, 15, 30, 45, 60, 90 min and 2 hr. For the knockout mice, two longer time points at 4 and 6 hr post-dosing were included. Five to seven mice were dosed at each time point and urine and blood were collected to measure tyrosine catabolites.

2.2.3. Western immunoblotting

Rat liver cytosol was prepared from freshly isolated liver by differential centrifugation, as previously reported [18]. Samples of rat liver cytosol, 10–40 ug, were treated with SDS and applied to discontinuous gels (4% stacking gel, 12% running gel), as described by Laemmli [27]. After electrophoretic separation, the proteins were transferred to PVDF membranes at 40 V overnight at 4°. Membranes were incubated with primary anti-MAAI chicken polyclonal antibody at 1:40,000, incubated with secondary anti-chicken donkey antisera (Research Diagnostics Inc.) at 1:50,000, and MAAI-specific bands were detected using

[2] Unpublished observations.

enhanced chemiluminescence kits (ECL-Plus, Amersham Biosciences). Autoradiograms were scanned and bands were quantitated using densitometry software. Steady-state protein levels were expressed as percentage of control by setting the level in the control samples to 100%.

2.2.4. Northern analysis

Total RNA was purified from 1 g liver slices using the RNA Midikit (QIAGEN), according to manufacturer's instructions. After spectroscopic quantitation, 20 μg of total RNA from each sample was applied onto 1.2% formaldehyde–agarose gels, transferred to Nylon N+ (Amersham Biosciences) paper, and probed with an MAAI probe radiolabeled by random priming (Amersham Biosciences). A 50% formamide–SSC hybridization format was used for pre-hybridization and hybridization. Blots were normalized by re-probing with a rat 28S rRNA probe after stripping and pre-hybridization. Blot quantitation was done using a PhosphorImager (Amersham Biosciences) and RNA steady-state levels were expressed as percentage of control by setting the level of the control to 100%.

2.2.5. Effect of MA on MAAI activity

Samples of dialyzed rat hepatic cytosol were incubated with 0.1 M HEPES, pH 7.6, and 0.1 or 0.4 mM MA in the presence or absence of 1 mM GSH for 0 or 30 min. Portions of the incubation mixture were immediately taken for assay of MAAI activity with 0.2 mM DCA as substrate, supplemented with additional GSH, as described previously [18]. The remainder was dialyzed overnight against two changes of 0.1 M HEPES buffer, pH 7.6, containing 1 mM GSH to remove MA, and re-assayed for MAAI activity with 0.2 mM DCA.

2.2.6. Mass spectrometric analysis of DCA and tyrosine metabolites

DCA levels in urine were measured as described previously [19]. We also developed a gas chromatography-mass spectrometry (GC-MS) method to quantitate tyrosine metabolites [28]. In brief, a plasma or urine sample (100 μL) was combined with 100 μL of 2-oxohexanoic acid [internal standard (IS) in water; 100 μg/mL] and 500 μL of 14% boron trifluoride in methanol in a 16 mm × 100 mm glass culture tube and the mixture was heated at 115° for 12 min on a heating block. The tubes were removed and allowed to cool to room temperature and 1 mL of water and 1 mL of methylene chloride were added. The sample was vortexed vigorously for 2 min, centrifuged to separate the layers, and the lower methylene chloride layer was transferred to an autosampler vial for analysis. Two microliters of each extract was injected into a Hewlett Packard 5890/5972 GC-MS system equipped with an HP-WAX column (0.25 mm i.d. × 30 M 0.15 um film thickness). The mass spectrometer was operated in an electron impact (EI) mode using SIM. Three unique ions were monitored for each analyte. The mass spectrometer was calibrated by preparing five separate standard solutions of the analytes that bracket the concentration levels anticipated in the samples. These solutions were derivitized and analyzed using the same procedure as describe above for the samples. A calibration curve was created by plotting the measured normalized area (compound area/IS area) vs. standard concentration.

The concentrations of the analytes in each collected animal sample were determined from the calibration curves and the values were plotted vs. time. The area under the curve (AUC) (ug/mL hr) was measured for each individual animal (data not shown) from this graph and the values were averaged for each analyte.

3. Statistical analyses

Means and standard deviations were calculated using Microsoft Excel software (Microsoft). Statistical tests of significance (one-way ANOVA) were performed utilizing MINITAB release 13.0 statistical software (Minitab, Inc.).

4. Results

4.1. DCA depletes MAAI protein in rat liver cytosol

SD rats were exposed to various doses of DCA by oral gavage for 1 or 5 days and steady-state levels of MAAI in liver cytosol were measured by Western blotting (Fig. 2A). A 1-day exposure to 200 mg/kg DCA caused a 2.5-fold reduction in protein level compared to control (one-way ANOVA, $P < 0.0001$; data not shown). Repeated dosing for 5 days decreased enzyme levels for all doses (one-way ANOVA, $P < 0.02$ for the 4 mg dose, $P < 0.0001$ for the 12.5, 50, and 200 mg doses; data not shown). A time- and dose-dependent decrease in the steady-state levels of the MAAI protein occurred after the 5-day exposure. Thus, after five consecutive doses of 200 mg/kg/day, the mean MAAI protein level was only 63% of control (data not shown). The decrease in protein steady-state levels was highly correlated ($r = 0.98$) with the decrease in MAAI activity, as measured by the biotransformation of DCA to glyoxylate [18].

4.2. DCA does not affect steady-state RNA levels of MAAI in rat liver

The mechanism of DCA's inactivation of MAAI, proposed by Tzeng *et al.* [29], could completely underlie the *in vivo* mechanism for the depletion of the protein. However, it is important to rule out a possible effect of DCA on the gene expression of MAAI, as DCA has been shown to alter the expression of a number of genes in rodents [20,23]. To investigate this possibility, we measured the steady-state levels of MAAI mRNA in the same livers used for the analysis of MAAI protein levels discussed above. Figure 2B and C shows that DCA exposure does not decrease the

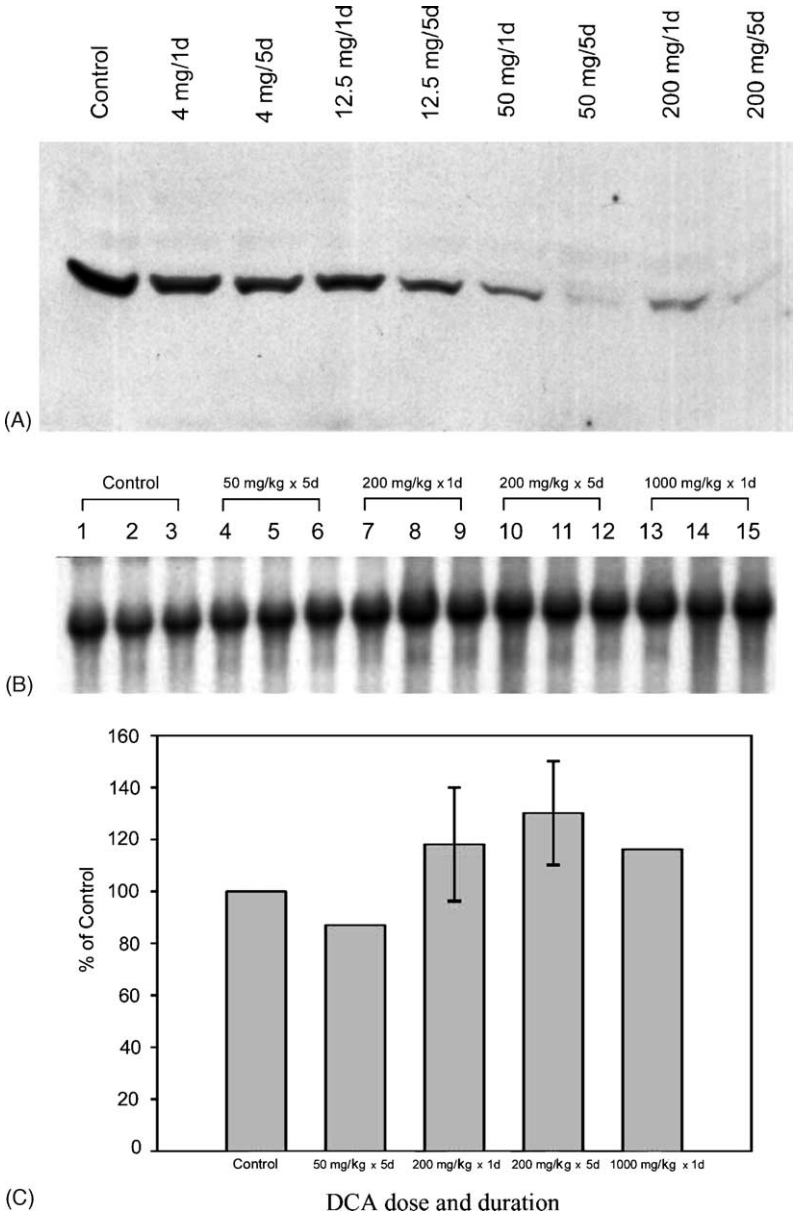

Fig. 2. DCA depletes MAAI protein in a dose- and time-dependent manner but does not alter the steady-state mRNA levels of MAAI. (A) Representative Western blot of liver MAAI in SD rats dosed with 4, 12.5, 50, or 200 mg DCA by oral gavage for 1 or 5 days. (B) Detection of MAAI mRNA by Northern blot of total liver RNA, using a random-primed probe derived from cloned MAAI cDNA. (C) PhosphorImager quantitation of the Northern blot shown in blot B. Quantitations are normalized to 28S rRNA and expressed as a percent of control, setting the level in the control to 100%.

steady-state transcript levels of MAAI, even at very high doses of DCA (1 g/kg). Therefore, MAAI protein depletion by DCA appears to be entirely a post-transcriptional event.

4.3. DCA clearance is reduced in the absence of MAAI

Knockout mice accumulated very high levels of [^{13}C]DCA in the urine (Fig. 3A), compared to undetectable levels in the urine of wild-type mice (not shown). In fact, urinary DCA concentrations steadily increased in MAAI-KO mice and did not plateau during the 6-hr observation period (Fig. 3A). Figure 3B shows the levels of [^{13}C]DCA and its metabolites [^{13}C]glyoxylate and [^{13}C]oxalate in urine. Knockout mice had markedly lower oxalate levels, consistent with reduced biotransformation of DCA. In the absence of the MAAI enzyme, alternative pathways for DCA clearance, e.g. by its conversion to monochloroacetic acid (MCA), might be activated in the MAAI-KO mice. However, we found no appreciable MCA in the urine of knockout mice exposed to DCA (data not shown).

4.4. MAAI-KO mice accumulate high levels of toxic tyrosine catabolites

We showed earlier that rats exposed to DCA demonstrate a dose- and time-dependent accumulation of MA in the urine [18]. MAAI-KO mice should also accumulate high levels of tyrosine intermediates upstream of MAAI.

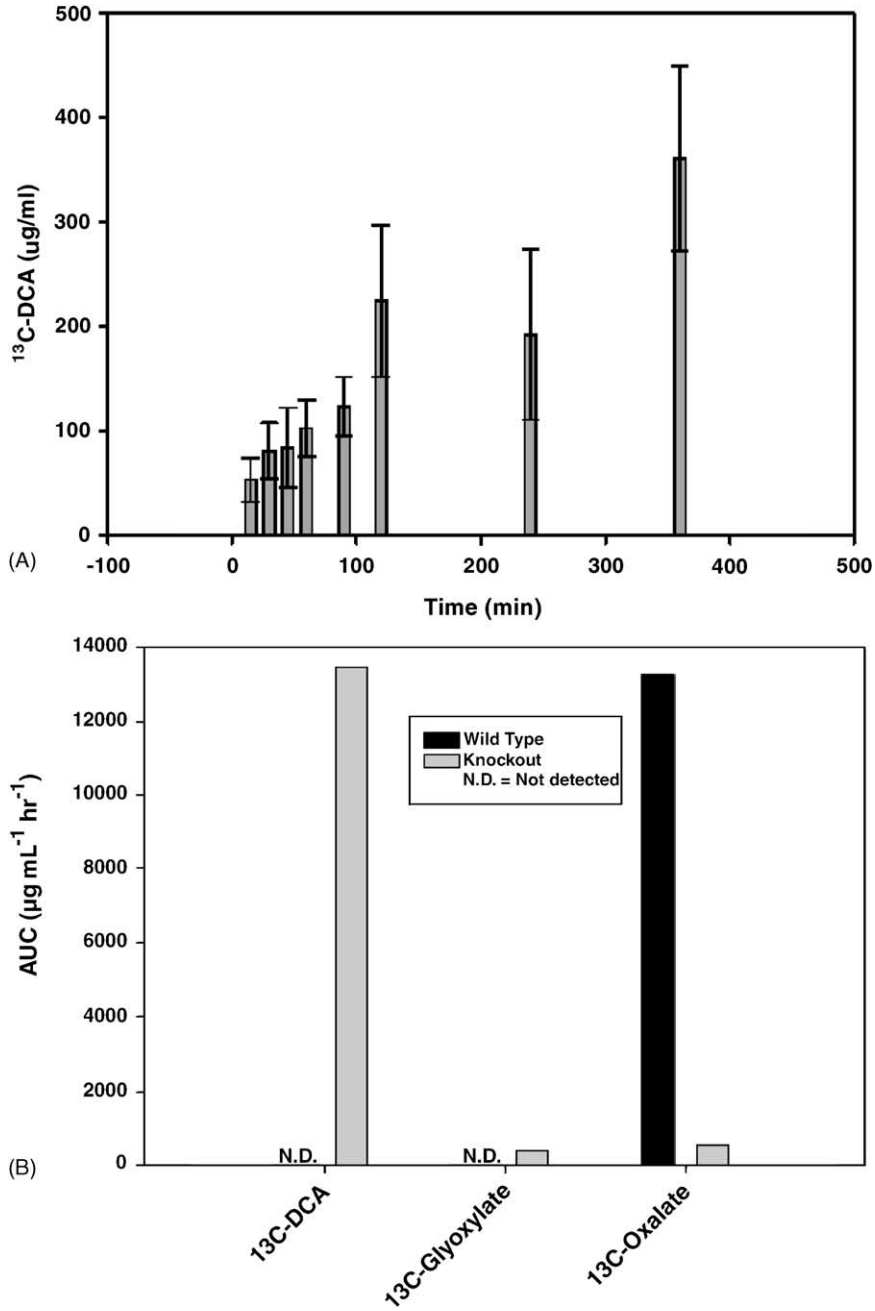

Fig. 3. DCA metabolism in MAAI-KO and littermate control mice. (A) [^{13}C]DCA was administered by oral gavage to MAAI-KO and littermate control mice. Urinary DCA was measured by GC-MS. Urinary [^{13}C]DCA levels steadily increased in the MAAI-KO mice over the 6-hr observation period and did not plateau, whereas [^{13}C]DCA was undetectable in wild-type littermate controls. (B) The biotransformation products of DCA, [^{13}C]glyoxylate and [^{13}C]oxalate, are detected at low levels in MAAI-KO mice. Note the high levels of [^{13}C]oxalate and undetectable [^{13}C]DCA in the wild-type mice, demonstrating rapid metabolism of [^{13}C]DCA. AUC, cumulative area under the curve for all animals at a time point. N.D., not detected.

To investigate this possibility, we measured the levels of MA, FA, SA, and fumarate in the urine of wild-type and knockout mice by GC-MS (Fig. 4). Knockout mice had very high urinary concentrations of MA (∼130-fold), compared to wild-type animals and high concentrations of SA that was undetectable in the urine of wild-type animals. The concentrations of these intermediates were high even in DCA-naïve mice (zero time of DCA dosing) and there was no significant difference in MA and SA levels after 6 hr of DCA exposure (one-way ANOVA, $P > 0.3$). Knockout animals also had markedly high levels of FA compared to wild-type mice, but urinary fumarate concentrations in the two groups were similar.

4.5. MA inhibits MAAI activity

Addition of MA to rat hepatic cytosol inhibited MAAI activity with DCA as substrate (Table 1). Incubation of MA with cytosol for 30 min before assaying activity resulted in a greater inhibition than that observed when MA and DCA

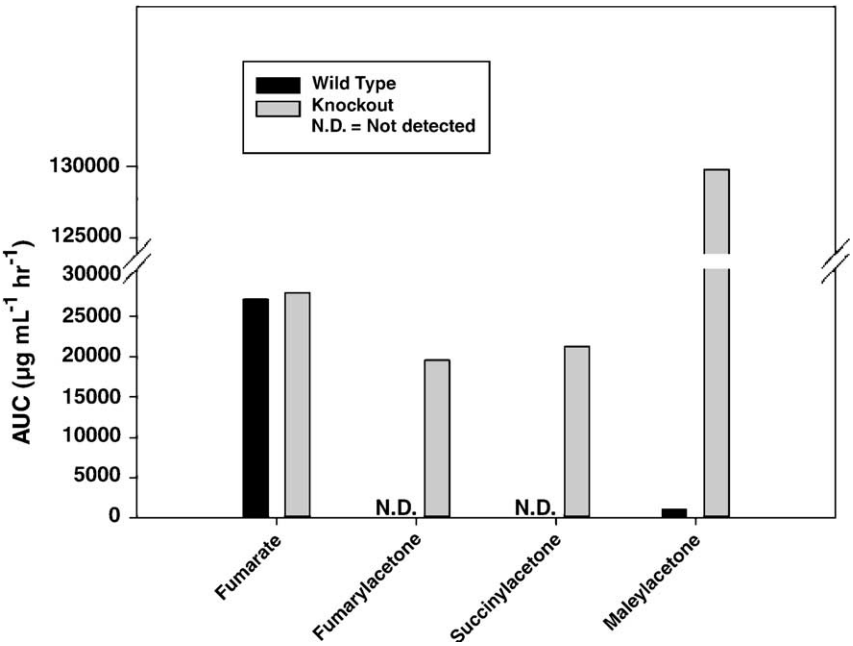

Fig. 4. Tyrosine catabolism is severely perturbed in MAAI-KO mice. MAAI-KO mice accumulate high urinary concentrations of FA, SA, and MA, compared to undetectable (FA and SA) or very low (MA) levels in littermate control mice. N.D., not detected.

Table 1
Effect of *in vitro* MA treatment on rat hepatic cytosolic MAAI activity with DCA as substrate

Treatment of cytosol	% control activity
No addition[a]	100
0.1 mM MA + GSH, assayed immediately	63.3 ± 4.8
0.1 mM MA + GSH, assayed after 30-min incubation	50.6 ± 0.5
0.1 mM MA, no GSH, assayed after 30-min incubation	36.2 ± 2.3
0.4 mM MA + GSH, assayed after 30-min incubation	38.1 ± 5.2
0.4 mM MA, no GSH, assayed after 30-min incubation	4.81 ± 0.7
0.4 mM MA + GSH, incubated for 30 min, dialyzed and assayed	53.6 ± 18.3
0.4 mM MA, no GSH, incubated for 30 min, dialyzed and assayed	24.5 ± 8.8

Values shown are mean ± SD (N = 3). All activities are significantly lower than control, $P < 0.01$. Removal of MA by dialysis did not restore the activity of cytosol.

[a] Activities from controls were set at 100%.

were added to incubation vials at the same time. The effect of MA was increased by conducting the initial incubation with MA in the absence of GSH. Extensive dialysis of cytosol that had been incubated with MA, with or without GSH, restored only part of the MAAI activity.

5. Discussion

Repeated treatment with DCA significantly prolongs its plasma elimination half-life in both rodents [17] and humans [19,30]. *In vitro* studies with hepatic cytosol derived from DCA-treated rats have shown that DCA inhibits MAAI activity in both SD [17] and Fischer 344 rats [31], and a single 1 g/kg dose completely eliminates enzyme activity [18]. This effect on MAAI activity appears to be directly correlated with DCA-induced loss of immunoreactive MAAI protein in liver cytosol of male Fischer 344 rats [31] and young (6–10 weeks) B6C3F1 mice [32]. In this report, we demonstrate a dose- and time-dependent depletion of the MAAI protein in liver cytosol of SD rats exposed to DCA (4–1000 mg/kg dose range). A number of recent *in vitro* studies suggest that a combination of DCA and GSH irreversibly inactivate MAAI by covalently modifying the protein and targeting it for degradation [29,31]. A cysteine to alanine mutation at position 16 in GSTZ1 has been shown to be resistant to both DCA-induced inactivation and to labeling by [^{35}S]GSH, indicating a role for this cysteine in adduct formation [33]. Lantum *et al*. [34] recently reported the alkylation and inactivation of MAAI by MA and FA in the absence of GSH, indicating that these compounds are substrate and product inhibitors of MAAI.

The effect of MA on DCA biotransformation by MAAI appears to be due to a combination of reversible and irreversible interactions of MA with the MAAI enzyme.

As shown in previous studies, addition of MA directly to incubation mixtures resulted in inhibition of MAAI activity [18]. Treatment of cytosol with MA for 30 min before assay resulted in a greater inhibition of MAAI activity, and removal of the MA by dialysis did not fully restore this activity, as would be expected if MA were acting only as a competitive substrate. When MA was incubated with cytosol in the absence of GSH, the inhibition was greater, suggesting that MA reacts with nucleophilic sites on the MAAI enzyme. Such interactions have been suggested by prior studies of human MAAI [34]. We have demonstrated that DCA-treated rats excrete more MA than untreated rats. Thus, it is possible that MA and its precursor, MAA, contribute to the destruction of MAAI observed in DCA-treated animals. However, it is presently unknown whether concentrations of MA in cells exposed to DCA are sufficient to exert such an action on the isomerase.

It is likely that protein inactivation/degradation could be the major mechanism for DCA-induced MAAI depletion *in vivo*. However, a simple model of DCA-based inactivation of the MAAI protein does not satisfactorily explain the protracted inhibition of MAAI activity, long after DCA is undetectable in the circulation, suggested by pharmacokinetic studies [3]. A washout period of weeks or even months may be required before DCA pharmacokinetics returns to levels observed in DCA-naïve human subjects [3,19]. These observations suggest that other mechanisms may operate in addition to direct inactivation of MAAI by DCA. Furthermore, prior studies have uncovered multiple effects of DCA on the expression of genes involved in lipid homeostasis and possibly in other metabolic pathways in humans and rodents [20–23,35–37]. Accordingly, we investigated whether the DCA-induced depletion of MAAI was associated with lower steady-state levels of liver MAAI mRNA. However, mRNA levels remained unchanged at all DCA exposure levels. Indeed, MAAI mRNA levels were similar to control even at the highest DCA dose of 1 g/kg body weight, which nearly completely abrogated MAAI activity [16]. Therefore, the dramatic effects of DCA exposure on MAAI protein levels appear to operate wholly at a post-transcriptional level. DCA could directly or indirectly affect translation of MAAI mRNA, perhaps by inhibiting gene/family-specific translation factors or protein kinases. Detailed studies on the recovery of MAAI protein after DCA dosing are required to test these postulates.

The finding that MAAI is depleted by DCA has important implications for understanding the toxicology of this xenobiotic, because of the central role of MAAI in the catabolism of phenylalanine and tyrosine (Fig. 1). Reduced MAAI activity could lead to the accumulation of MAA, MA, SA, and δ-ALA, leading to hepato-renal and neurological toxicity [1]. Indeed, administration of 200 mg DCA/kg/day for 5 days to SD rats increased urinary MA 4- to 8-fold [18]. DCA may also markedly elevate urinary δ-ALA in both rats and humans [38].

Homozygous MAAI-KO mice were recently created [39] to investigate the consequences of MAAI disruption on tyrosine catabolism. These mice do not display an overt phenotype after more than a year of life and breed normally, although they accumulate high levels of SA in the urine [39]. However, the animals are sensitive to diet-induced phenylalanine/tyrosine overload and display increased levels of oxidative stress markers. These findings suggest that MAAI-KO animals may have an increased predisposition to develop a carcinogenic phenotype later in life [39].

The knockout mice provide a model system to investigate both the pharmacokinetics of DCA and the biochemical perturbation of tyrosine catabolism in the complete absence of MAAI. When MAAI-KO mice were exposed to DCA at a dose of 50 mg/kg body weight, urinary DCA levels increased in these animals during the 6-hr observation period but monochloroacetate concentrations did not change. Subtle differences may exist in MCA levels between wild-type and knockout animals that are below our level of detection. However, high DCA levels in the knockout animals demonstrate that the monochloroacetate pathway does not contribute significantly to the plasma clearance of DCA. Thus, we conclude that no major alternative pathways for DCA clearance exist in the mouse, other than by MAAI-mediated dehalogenation to glyoxylate, although we cannot rule out minor routes of DCA catabolism that do not involve MAAI or accumulation of monochloroacetate. The small levels of oxalate formed in the knockout mice could be due to low-level, non-enzymatic conversion of DCA to glyoxylate mediated by GSH. Alternatively, oxalate formation may reflect the dehalogenation of DCA by other GSH transferases or enzymes that do not require GSH for activity.

DCA-naïve knockout mice accumulate very high levels of MA and SA, consistent with inhibition of the tyrosine catabolic pathway. DCA exposure did not significantly alter the level of MA or SA in the knockout animals. This indicates that DCA exerts its effects on tyrosine catabolism solely by its action on the MAAI protein. However, the knockout mice also accumulated markedly high urinary levels of FA compared to wild-type animals and had urinary levels of fumarate similar to those found in normal mice. Since MAAI-KO mice completely lack the MAAI protein, these results are consistent with the operation of a non-enzymatic, GSH-mediated bypass pathway for the isomerization of MAA, as suggested by Fernandez-Canon *et al*. [26]. Although this pathway would not be as efficient as the enzyme-mediated reaction [16,17], high concentrations of MAA in the knockout mice could lead to the high steady-state levels of MA, FA, and fumarate observed in our experiments.

In conclusion, pharmacologic or genetic ablation of MAAI leads to a dramatic perturbation of the tyrosine catabolic pathway. However, the operation of a non-enzymatic bypass for conversion of MAA to FAA probably

allows MAAI-KO mice to tolerate relatively high concentrations of tyrosine catabolites. DCA is not actively metabolized to either glyoxylate or monochloroacetate in the MAAI-KO mice and its plasma elimination is markedly prolonged.

Acknowledgments

This work was supported by ES 07355 and ES 07375 to Peter W. Stacpoole and DK 48252 to Markus Grompe from the National Institutes of Health. We thank A. Aller and C. Caputo for technical and editorial assistance, respectively.

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