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α-Ketoacids Stimulate Rat Renal Cysteine Conjugate β-Lyase Activity and Potentiate the Cytotoxicity of S-(1,2-Dichlorovinyl)-L-cysteine

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SUMMARY

Renal cysteine conjugate β-lyase (β-lyase) catalyzes the bioactivation of nephrotoxic cysteine *S*-conjugates. β-Lyase activity is present in both renal cytosolic and mitochondrial fractions, and, although the cytosolic β-lyase is identical to glutamine transaminase K, the mitochondrial β-lyase has not been characterized. Because β-lyase is a pyridoxal phosphate (PLP)-dependent enzyme, pyridoxamine phosphate (PMP) formation may ocur during the metabolism of cysteine *S*-conjugates. In this study, the effects of α-ketoacids, which may convert the PMP form of the enzyme to the pyridoxal phosphate form, on the metabolism and cytotoxicity of cysteine *S*-conjugates were examined; the PMP enzyme is catalytically inactive in β-elimination reactions, but is catalytically active in transamination reactions. Both α-keto-γ-methiolbutyrate (KMB) and α-ketobutyrate enhanced the metabolism of *S*-(2-benzothiazolyl)-L-cysteine (BTC) to 2-mercaptobenzothiazole by rat renal cytosol or mitochondria. KMB and phenylpyruvate potentiated both the cytotoxicity of *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC) in isolated rat renal proximal tubular cells and the inhibition of mitochondrial respiration produced by DCVC. These results are consistent with the formation of PMP during the renal cytosolic or mitochondrial metabolism of cysteine *S*-conjugates. Mitochondrial β-lyase was previously localized in the outer membrane. To examine whether β-lyase activity is present in mitoplasts, but in the PMP form, the effects of KMB on the metabolism of BTC to 2-mercaptobenzothiazole and on the DCVC-induced inhibition of state 3 respiration in mitoplasts were studied. The majority of the mitochondrial β-lyase activity was present in the outer membrane, and the specific activity of the outer membrane β-lyase was greater than that of the mitoplast β-lyase. KMB produced equivalent stimulation of β-lyase activity in intact mitochondria, in mitochondrial outer membranes, and in mitoplasts and potentiated DCVC-induced inhibition of respiration in intact mitochondria, but not in mitoplasts. These results provide additional evidence for the central role of β-lyase in the bioactivation of nephrotoxic cysteine *S*-conjugates.

The hypothesis (1) that the nephrotoxicity of several halogenated alkenes is attributable to glutathione *S*-conjugate formation followed by metabolism of the glutathione conjugates to the corresponding cysteine *S*-conjugates, which are activated by renal cysteine conjugate β-lyase (β-lyase), has been validated. For example, hepatic microsomal and cytosolic glutathione *S*-transferases catalyze glutathione *S*-conjugate formation with chlorotrifluoroethylene (2), tetrafluoroethylene (3), and hexachloro-1,3-butadiene (4), and these *S*-conjugates, along with the synthetic (5) and, perhaps, biosynthetic (6) *S*-conjugate, *S*-(1,2-dichlorovinyl)glutathione, are all nephrotoxic. The metabolism of the glutathione *S*-conjugates to the corresponding cysteine *S*-conjugates is required for the expression of toxicity (5, 7, 8). The cysteine *S*-conjugates are metabolized by β-lyase to pyruvate, ammonia, and sulfur-containing metabolites (3, 8, 9) that may partially decompose to yield hydrogen sulfide (10). A role for renal β-lyase in cysteine *S*-conjugate-induced nephrotoxicity has been established through studies that correlate subcellular and suborganelle distribution of β-lyase activity and toxicity (11) and through the use of β-lyase inhibitors and α-methyl and homocysteine analogues of nephrotoxic cysteine conjugates (5, 7, 12).

β-Lyase activity is present in both the cytosolic and mitochondrial fractions of rat kidney (5, 11, 13, 14). The renal cytosolic β-lyase is identical to glutamine transaminase K (15). Renal mitochondrial β-lyase activity is apparently present in outer membrane (11) and matrix (16) preparations.

The catalytic mechanism of PLP-dependent enzymes that catalyze β-elimination reactions is well known (17) and involves, after Schiff base or aldimine formation, proton abstrac-

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ABBREVIATIONS: PLP, pyridoxal phosphate; BTC, *S*-(2-benzothiazolyl)-L-cysteine; DCVC, *S*-(1,2-dichlorovinyl)-L-cysteine; KMB, α-keto-γ-methiolbutyrate; PMP, pyridoxamine phosphate.

tion from the α-carbon, a β-elimination reaction, and hydrolysis of the enzyme-bound eneamine to yield an α-ketoacid and ammonia (Fig. 1, pathway *a*). Transamination may result in transfer of the amino group to enzyme-bound PLP to form PMP during the metabolism of α-amino acids (18), including cysteine *S*-conjugates (Fig. 1, pathway *b*). PMP formation may be an important mechanism for the regulation of β-lyase activity, because the aldehyde form PLP is required for the catalysis of β-elimination reactions, even though the aldehyde exists as an internal aldimine with an ε-amino group of a lysine residue.

In this study, the effects of α-ketoacids, which serve as amino group acceptors, on renal β-lyase activity and cysteine *S*-conjugate-induced cytotoxicity were investigated. Evidence that α-ketoacids enhance the renal metabolism of BTC to 2-mercaptobenzothiazole and potentiate the cytotoxicity of DCVC is presented.

Materials and Methods

Collagenase (type IV), KMB, phenylpyruvate, digitonin (recrystallized twice from hot ethanol), and α-ketobutyrate sodium were purchased from Sigma Chemical Co. (St. Louis, MO). DCVC and BTC were synthesized by published procedures (13, 19).

Male Fischer 344 rats (175–225 g; Charles River Breeding Laboratories, Wilmington, MA) were killed by decapitation; renal cytosolic and mitochondrial fractions were prepared as previously described (13) and were used as the enzyme source without further purification. Protein concentrations were determined according to the method of Lowry *et al.* (20).

β-Lyase activity. Renal cytosolic and mitochondrial β-lyase activities with BTC (0.1 mM) as the substrate were determined in the presence and absence of KMB (0.1–5 mM) by measuring the formation of 2-mercaptobenzothiazole (13). KMB does not interfere with the assay of 2-mercaptobenzothiazole, but interference by phenylpyruvate prevented its use in BTC metabolism studies.

Cytotoxicity studies. Isolated kidney cells were prepared by the collagenase perfusion method of Jones *et al.* (21). Cell viability and concentration were estimated in the presence of 0.2% (w/v) trypan blue in a hemacytometer. Cell viability was typically 85–95%, and cell yield was 30–50 × 10^6 cells/rat.

Mitochondrial toxicity studies. Isolated mitochondria from rat kidney cortex were prepared by the method of Johnson and Lardy (22), except that this buffer was used: 225 mM sucrose, 10 mM potassium phosphate (pH 7.4), 5 mM $MgCl_2$, 20 mM KCl, and 20 mM triethanolamine hydrochloride (pH 7.4). Oxygen consumption was measured polarographically with a Clark-type electrode at 28°. The electrode was calibrated with air-saturated buffer, and zero oxygen concentration was obtained by addition of sodium sulfite. State 3, i.e., ADP-stimulated, respiration was measured according to the method of Estabrook (23) by addition of 3.3 mM succinate and 0.3 mM ADP to 1–2 mg of mitochondrial protein. The respiratory control ratio (state 3/state 4) for mitochondria was 4.7 ± 0.8 (n = 3), indicating tight coupling.

Mitoplast toxicity studies. Rat kidney mitochondria, isolated as described above, were fractionated by the digitonin method of Schnaitman and Greenawalt (24); the detailed procedure has been published (11). State 3 respiration in mitoplasts was measured as described above. The respiratory control ratio for mitoplasts was 3.5 ± 0.3 (n = 3).

Statistics. Statistical analyses were determined by analysis of variance with an MDCSTAT computer program (Micro Data Collection, Novato, CA). A level of $p < 0.05$ was chosen for acceptance or rejection of the null hypothesis.

Results

Stimulation of renal β-lyase activity. KMB (0.1–5 mM) produced a concentration-dependent increase in renal cytosolic and mitochondrial β-lyase activities (Fig. 2). With both kidney mitochondria and cytosol, addition of 5 mM KMB to the incubation mixtures resulted in an increase of more than 200% of control rates in the metabolism of BTC to 2-mercaptobenzothiazole. α-Ketobutyrate (5 mM) stimulated renal cytosolic and mitochondrial β-lyase activity by 165 ± 11 and 141 ± 3% (mean ± SE, n = 3), respectively.

Potentiation of DCVC-induced cytotoxicity. Renal cytosolic β-lyase activity is a catalytic property of glutamine transaminase K (15). Because KMB and phenylpyruvate are the two most active substrates for glutamine transaminase K (25), they were used to examine effects of α-ketoacids on DCVC-induced cytotoxicity in isolated, rat kidney proximal tubular cells (Fig. 3). Addition of 5 mM KMB (Fig. 3A) or 5

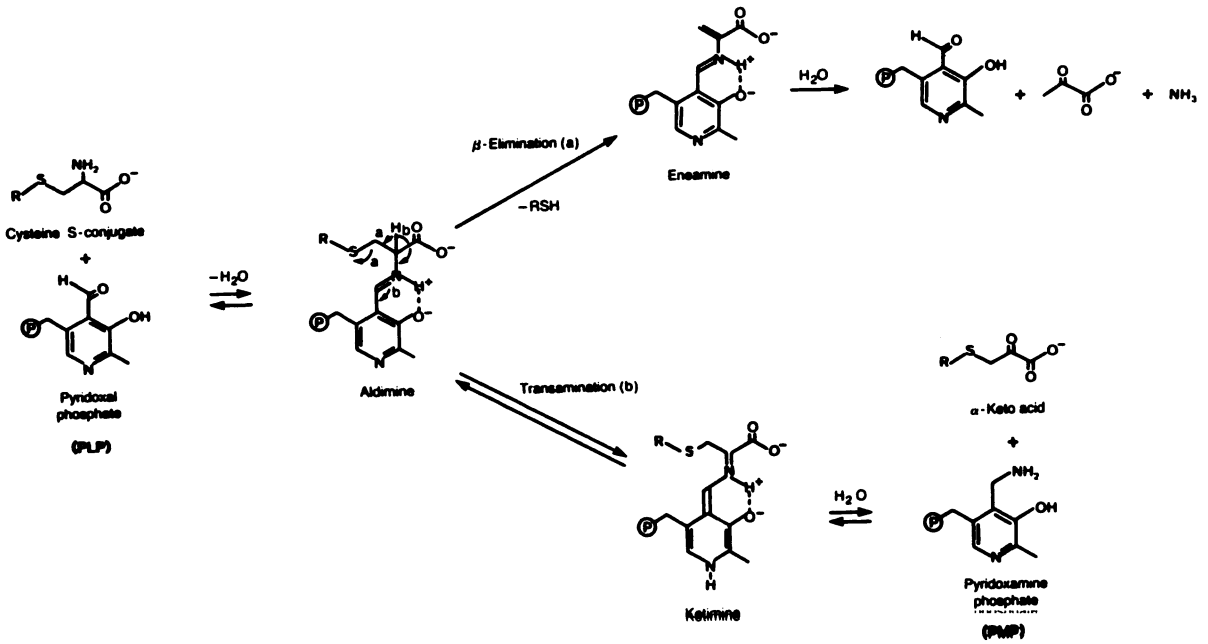

Fig. 1. Postulated mechanism for the metabolism of cysteine *S*-conjugates by renal β-lyase.

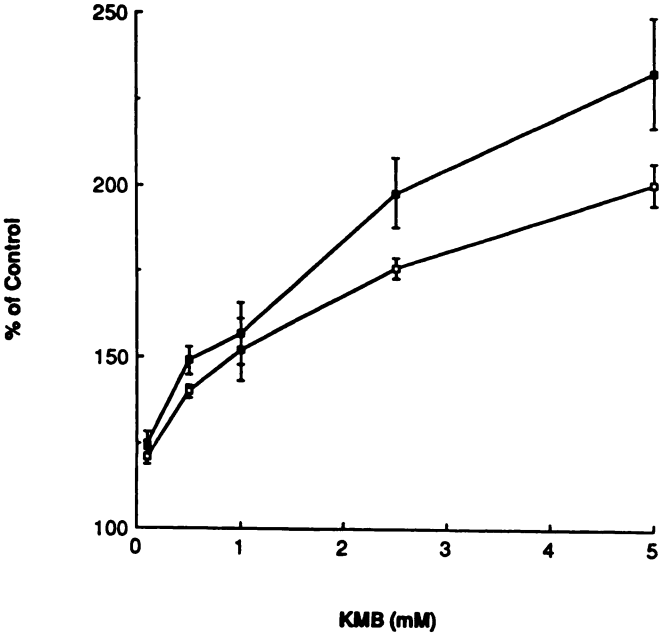

Fig. 2. Stimulation of renal metabolism of BTC to 2-mercaptobenzothiazole by KMB. Rat renal mitochondria and cytosol (1–1.5 mg of protein) were incubated for 6 min at 37° with BTC (0.1 mM) with either no addition (control) or with KMB (0.1–5 mM). The enzymatic reactions were terminated by addition of trichloroacetic acid, and the amounts of 2-mercaptobenzothiazole in the acid supernatant were determined. Results are the means of values obtained with four kidney cytosol (■) and mitochondria (□) preparations and are expressed as percentage of control. Velocities obtained with control cytosol and mitochondria were 1.2 ± 0.2 and 2.5 ± 0.1 nmol of product/min/mg of protein (mean ± SE, n = 3), respectively.

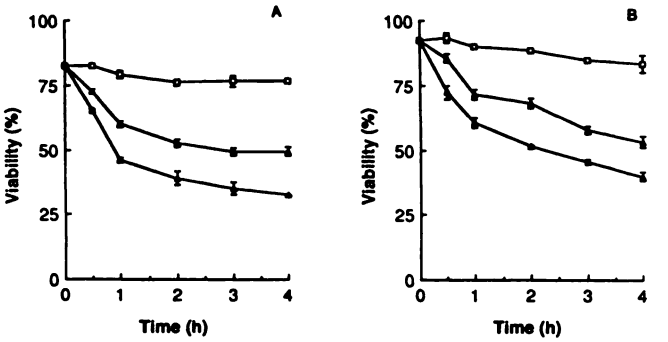

Fig. 3. Potentiation of DCVC-induced cytotoxicity by KMB (A) and phenylpyruvate (B) in isolated, rat kidney proximal tubular cells. Cell viability was measured by trypan blue exclusion. A. Cells (1 × 10^6/ml) were incubated at 37° with either no addition (□), addition of 0.1 mM DCVC (■), or addition of 0.1 mM DCVC and 5 mM KMB (△). B. Cells (1 × 10^6/ml) were incubated at 37° with no addition (□), addition of 0.1 mM DCVC (▲), or addition of 0.1 mM DCVC and 5 mM phenylpyruvate (△). Results are the means ± standard errors of three preparations. Loss of cell viability in the presence of DCVC and KMB or DCVC and phenylpyruvate was significantly greater ($p < 0.05$) than in the presence of DCVC alone at all times.

mM phenylpyruvate (Fig. 3B) potentiated DCVC-induced losses of cell viability at all times examined; the addition of either α-ketoacid alone had no effect on cell viability (data not shown).

DCVC-induced inhibition of state 3 mitochondrial respiration was also potentiated by the addition of α-ketoacids (Fig. 4). After 2 hr of incubation, oxygen consumption was inhibited by 39% with 0.1 mM DCVC alone and by 68% and 61% when both DCVC and KMB and both DCVC and phenylpyruvate,

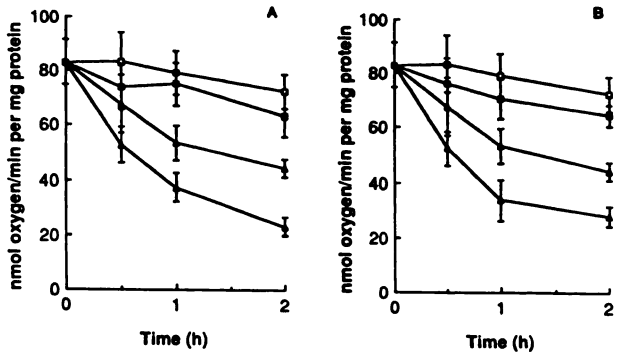

Fig. 4. Potentiation of DCVC-induced mitochondrial toxicity by KMB (A) and phenylpyruvate (B) in rat kidney mitochondria. Oxygen consumption (state 3 respiration) was measured in isolated mitochondria at 28° as described in Materials and Methods. Results are the means ± standard errors of three preparations. A. Control (□), + 5 mM KMB (■), + 0.1 mM DCVC (▲), + 0.1 mM DCVC + 5 mM KMB (△). B. Control (□), + 5 mM phenylpyruvate (■), + 0.1 mM DCVC (▲), + 0.1 mM DCVC + 5 mM phenylpyruvate (△). Rates of state 3 respiration in the presence of DCVC and KMB or DCVC and phenylpyruvate were significantly lower ($p < 0.05$) than in the presence of DCVC alone, with α-ketoacid-treated mitochondria as controls, at 2 hr.

respectively, were added. Addition of α-ketoacids alone had a small effect on mitochondrial respiration.

In a previous study employing digitonin-fractionated mitochondria, β-lyase activity was localized to the mitochondrial outer membrane (11). Moreover, nephrotoxic cysteine *S*-conjugates, which inhibit state 3 respiration in intact kidney mitochondria, did not inhibit respiration in kidney mitoplasts, demonstrating a correlation between the suborganelle localization of bioactivation and toxicity. To investigate whether β-lyase activity is present in mitoplasts, but in the PMP form, the effect of KMB on BTC metabolism was studied (Table 1). As shown previously (11), the majority (>60%) of the mitochondrial β-lyase activity with BTC as substrate was recovered in the outer membrane fraction, and the specific activity of the outer membrane β-lyase was greater than that of the mitoplast β-lyase. Addition of KMB produced similar increases in β-lyase activity in intact mitochondria, in mitochondrial outer membranes, and in mitoplasts. Thus, even in the presence of KMB, most of the mitochondrial β-lyase activity was found in the outer membrane fraction. Similarly, as was found previously (11), DCVC did not have a significant effect on the rate of mitoplast state 3 respiration (Fig. 5). Addition of 5 mM KMB with DCVC did not have a significant effect on mitoplast respiration compared to DCVC alone.

Discussion

The present study shows that α-ketoacids stimulate the renal cytosolic and mitochondrial metabolism of BTC to 2-mercaptobenzothiazole and potentiate the cytotoxicity of DCVC. The magnitude of the stimulation of both the renal cytosolic and mitochondrial metabolism of BTC to 2-mercaptobenzothiazole by KMB was concentration dependent. Moreover, KMB was more potent than α-ketobutyrate in stimulating β-lyase activity; this is the expected result, because renal cytosolic β-lyase is identical with renal cytosolic glutamine transaminase K (15) and because glutamine transaminase K and β-lyase are much more active with KMB than with α-ketobutyrate as the substrate (15, 25).

TABLE 1

Effect of α-keto-γ-methiolbutyrate on S-(2-benzothiazolyl)-L-cysteine metabolism in renal mitochondria

Rat kidney mitochondria were fractionated with digitonin, and the β-lyase activity in outer membrane and mitoplast fractions was measured as described in Materials and Methods. Total activity was calculated by multiplying the specific activity by the amount of protein in each fraction. Activity is expressed as 2-mercaptobenzothiazole formation; 1 milliunit (mU) = 1 nmol/min. Results are the mean ± standard errors of four determinations.

Addition	Intact mitochondria		Outer membrane		Mitoplasts	
	Specific activity	Total activity	Specific activity	Total activity	Specific activity	Total activity
	mU/mg	*mU*	*mU/mg*	*mU*	*mU/mg*	*mU*
1 mM BTC	12.4 ± 2.7	638 ± 71	31.7 ± 4.1	453 ± 72	16.2 ± 1.9	273 ± 33
1 mM BTC + 5 mM KMB	18.9 ± 3.4	991 ± 81	56.4 ± 12.6	741 ± 47	25.0 ± 1.9	422 ± 34
% stimulation by KMB	55		64		55	

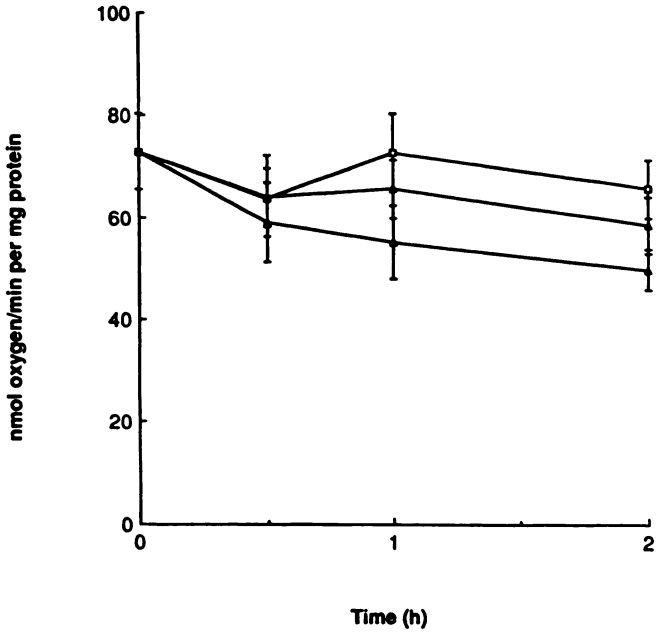

Fig. 5. Effect of DCVC and KMB on state 3 respiration in rat kidney mitoplasts. Oxygen consumption was measured at 28°, as described in Materials and Methods. Results are the means ± standard errors of three preparations. Control (□), + 1 mM DCVC (▲), + 1 mM DCVC + 5 mM KMB (△). Rates of state 3 respiration in the presence of DCVC and KMB were not significantly different ($p > 0.05$) from those in the presence of DCVC alone, and rates of state 3 respiration in the presence of DCVC or DCVC and KMB were not significantly different ($p > 0.05$) from control incubations.

The α-ketoacid-induced increase in the rate of BTC metabolism is consistent with some of the β-lyase being present in the PMP form or conversion of the enzyme to the PMP form, which does not catalyze a β-elimination reaction, during the metabolism of BTC (Fig. 1), or both. The finding that *S*-(1,2-dichlorovinyl)-3-mercaptopyruvate is a metabolite of DCVC (15) may indicate that PMP formation occurs during β-lyase-catalyzed cysteine conjugate metabolism, although L-amino acid oxidase may also catalyze the formation of the ketoacid. Addition of the amino group acceptor KMB facilitates the regeneration of PLP, which can accept another molecule of the cysteine *S*-conjugate and catalyze the β-elimination reaction (Fig. 1, reversal of pathway *b*). Similarly, pyruvate stimulates DCVC metabolism in LLC-PK1 cells (26), and α-ketobutyrate stimulates the renal metabolism of *S*-(2-benzothiazolyl)-L-homocysteine to 2-mercaptobenzothiazole (12) and potentiates the cytotoxicity of *S*-(1,2-dichlorovinyl)-L-homocysteine (27). Hence, the metabolism of nephrotoxic *S*-conjugates by the PLP-dependent β-lyase may be regulated by α-ketoacids, which facilitate the conversion of the PMP form of the enzyme to the PLP form (Fig. 1).

The stimulatory effects of α-ketoacids on renal β-lyase activity are reflected in the potentiation of DCVC-induced losses of cell viability and inhibition of mitochondrial state 3 respiration. Both KMB and phenylpyruvate enhanced the cytotoxicity of DCVC in isolated kidney cells and potentiated the inhibition of mitochondrial respiration produced by DCVC. These results are supportive of a role for the β-lyase in the cellular and mitochondrial toxicity of DCVC. Again, the results indicate that some of the cytosolic and mitochondrial β-lyase is present in the PMP form or that PMP formation occurs as a side reaction during the β-elimination reaction, or both, and that addition of α-ketoacids regenerates the PLP form, allowing enhanced metabolism and increased expression of toxicity.

Previous studies showed that the most of the mitochondrial β-lyase activity was present in the mitochondrial outer membrane, although activity was detected in the matrix (11); moreover, cysteine *S*-conjugates that require activation by the β-lyase were toxic to mitochondria, but not to mitoplasts, indicating that bioactivation occurred in the mitochondrial outer membrane. Robbins and Stevens (16) reported the purification of renal β-lyase from the mitochondrial matrix. This report and the observation that α-ketoacids stimulate mitochondrial β-lyase activity raised the possibility that the β-lyase was present in the mitochondrial matrix but largely in the PMP form. Addition of KMB stimulated the low amount of activity present in both the mitoplast fraction and the outer membrane by similar degrees, so that, even in the presence of an α-ketoacid, most of the mitochondrial β-lyase activity was found in the outer membrane. Accordingly, DCVC did not produce significant inhibition of state 3 respiration in renal mitoplasts, and the toxicity of DCVC was not significantly enhanced by KMB. These results show that, although β-lyase activity is present in the mitochondrial matrix, most of the mitochondrial β-lyase activity is located in the outer membrane, and that the outer membrane β-lyase is responsible for the bioactivation of DCVC.

The results presented above provide evidence that the activity of β-lyase in kidney cells and mitochondria is determined by the ratio of PLP/PMP forms of the enzyme and that α-ketoacids, by promoting the regeneration of the PLP form of the enzyme, increase both metabolism and toxicity of cysteine *S*-conjugates.

In summary, the present study provides further evidence for the role of renal β-lyase in DCVC-induced nephrotoxicity and insight into the regulation of β-lyase activity. Further studies on the distribution, characterization, and regulation of renal β-lyase are warranted.

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