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Acivicin-induced alterations in renal and hepatic glutathione concentrations and in γ-glutamyltransferase activities

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Abstract

γ-Glutamyltransferase (γ-GT) catalyzes the hydrolysis of glutathione, glutathione *S*-conjugates, and γ-substituted L-glutamate derivatives. Acivicin is an irreversible inhibitor of γ-GT that has been used to study the role of γ-GT in glutathione homeostasis and glutathione-dependent bioactivation reactions. The present studies were undertaken because of reported conflicting effects of acivicin on the nephrotoxicity of some haloalkenes that undergo glutathione-dependent bioactivation. The objective of this study was to test the hypothesis that acivicin may alter renal glutathione concentrations; acivicin-induced changes in renal glutathione concentrations may alter the susceptibility of the kidney to the nephrotoxic effects of haloalkenes. Hence, diurnal and acivicin-induced changes in renal and hepatic glutathione concentrations along with renal and hepatic γ-GT activities were investigated. The previously observed diurnal variations in hepatic glutathione concentrations in fed rats were confirmed, but no diurnal variations were observed in renal glutathione concentrations or in renal or hepatic γ-GT activities. Renal and hepatic glutathione concentrations and γ-GT activities were measured in tissue homogenates from rats given 0, 0.1, or 0.2 mmol acivicin/kg (i.p.) and killed 0, 2, 4, 8, 12, or 24 hr later. Renal glutathione concentrations were increased above control values in acivicin-treated rats, whereas acivicin had no effect on hepatic glutathione concentrations. Renal γ-GT activities decreased within 2 hr after giving acivicin and remained decreased for 24 hr. Acivicin had no effect on hepatic γ-GT activities, except at 24 hr after treatment when values in acivicin-treated rats were elevated compared with controls. Although the present studies do not afford an explanation of the mechanism whereby acivicin increases the nephrotoxicity of some haloalkenes, they do indicate that acivicin is not a reliable probe to investigate the role of γ-GT in haloalkene-induced nephrotoxicity.

Keywords: Glutathione; γ-Glutamyltransferase; Acivicin; Haloalkenes; Glutathione *S*-conjugates; Mercapturic acid pathway; β-Lyase; 2-(Fluoromethoxy)-1,1,3,3,3-pentafluoro-1-propene; Compound A; Sevoflurane; Hexachloro-1,3-butadiene

1. Introduction

γ-GT (EC 2.3.2.2) catalyses the hydrolysis or transfer of the γ-glutamyl group of glutathione, glutathione *S*-conjugates, and γ-substituted L-glutamate derivatives [1,2]. Acivicin [(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid] is an irreversible inhibitor of γ-GT [3] that is used to explore the role of γ-GT in glutathione homeostasis and glutathione *S*-conjugate biotransformation and bioactivation.

Although glutathione is a well-established cytoprotective agent, glutathione-dependent bioactivation reactions are well known [4]. The selective nephrotoxicity of a range of haloalkenes is attributable to the cysteine conjugate β-lyase pathway, which involves glutathione *S*-conjugate formation, γ-GT- and dipeptidase-catalyzed hydrolysis of the glutathione *S*-conjugates to the corresponding cysteine *S*-conjugates, active uptake of the cysteine *S*-conjugates by renal amino acid transporters, and bioactivation of the cysteine *S*-conjugates by cysteine conjugate β-lyase (for a review, see [5]).

With haloalkenes that undergo β-lyase-dependent bioactivation, acivicin would be expected to block toxicity. Acivicin inhibits the nephrotoxicity of *S*-(1,2-dichlorovinyl)glutathione *in vivo* and *in vitro* [6,7], but increases the nephrotoxicity of hexachloro-1,3-butadiene in rats [8] but

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Abbreviations: γ-GT, γ-glutamyltransferase; Compound A, 2-(fluoromethoxy)-1,1,3,3,3-pentafluoro-1-propene.

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not in mice [9]. Acivicin also increases the nephrotoxicity of Compound A, a degradation product of the anesthetic sevoflurane, *in vivo* [10–13].

Hence, although there is considerable evidence for the β-lyase-dependent bioactivation of many haloalkenes, the failure of acivicin to block the nephrotoxicity of some haloalkenes, notably Compound A, has led to the contention that their nephrotoxicity is not attributable to β-lyase-dependent bioactivation in rats [10,11].

The objective of the present studies was to test the hypothesis that acivicin may alter renal glutathione homeostasis. Such alterations in renal glutathione concentrations may concomitantly alter the susceptibility of the kidney to the nephrotoxicity of some haloalkene-derived glutathione *S*-conjugates. Accordingly, we examined the time- and dose-dependent effect of acivicin on hepatic and renal glutathione concentrations and γ-GT activities in rats. The results showed that acivicin decreased renal γ-GT activities, but increased renal glutathione concentrations.

2. Materials and methods

2.1. Materials

Acivicin, glutathione, 5,5′-dithiobis-(2-nitrobenzoic acid), trichloroacetic acid, L-γ-glutamyl-*p*-nitroanilide hydrochloride, *N*-glycylglycine hydrochloride, and *p*-nitroaniline were purchased from Sigma-Aldrich Chemical Co. All other reagents were obtained from commercial sources.

2.2. Animal treatments

Male, Fischer 344 rats (175–200 g, Charles River) were used. The rats were housed three per cage in the Vivarium of the University of Rochester under conditions of controlled temperature and humidity. The rats were kept in a room with a 12-hr light/12-hr dark cycle and were allowed free access to food and water.

Rats were given 0, 0.1, or 0.2 mmol acivicin/kg (i.p.) dissolved in 3 mL of normal saline; control rats were given saline alone. The rats were treated at 9.00 a.m. EST in all experiments. The rats were anesthetized with ether and decapitated 0, 2, 4, 8, 12, and 24 hr after treatment. The livers and kidneys were immediately removed and rinsed with ice-cold 300 mM sucrose/10 mM Tris/10 mM $MgCl_2$ buffer (pH 7.25). Samples of liver (400 mg) and kidney (400 mg) were collected, placed in 5 volumes of buffer, and homogenized. A 2-mL sample of the homogenate was immediately mixed with 2.5 mL of 0.02 M EDTA solution and 0.5 mL of 20% trichloroacetic acid solution. The mixture was centrifuged at 10,000 g for 15 min at 4°, and the supernatant was decanted and stored at 4° until analyzed for glutathione concentrations. The remainder of the homogenate was immediately frozen at −80° and subsequently analyzed for γ-GT activity.

2.3. Analyses

Hepatic and renal glutathione concentrations were quantified with Ellman's reagent [14]. Briefly, 1 mL of the cold supernatant from the acidified homogenate was mixed with 2 mL of 0.4 M Tris–HCl buffer (pH 8.9) and 50 μL of 0.01 M 5,5′-dithiobis-(2-nitrobenzoic acid) dissolved in methanol. After 15 min, the absorbance at 410 nm was measured with a 96-well microplate reader (MR 5000, Dynatech Laboratories Inc.). Glutathione concentrations were determined from a standard curve prepared with known concentrations of glutathione dissolved in 0.02 M EDTA solution (pH 7.25).

Hepatic and renal γ-GT activities were determined by the method of Orlowski and Meister [15]. The liver homogenate was used directly, and the kidney homogenate was diluted 100-fold in 300 mM sucrose/10 mM Tris/10 mM $MgCl_2$ buffer (pH 7.25). A sample (100 μL) of the homogenate was added to a solution containing 100 μL of 5 mM L-γ-glutamyl-*p*-nitroanilide, 100 μL of 20 mM *N*-glycylglycine HCl, and 700 μL of 10 mM Tris/10 mM $MgCl_2$ buffer (pH 9.0). The mixture was incubated for 5 min in a water bath at 37°. The reaction was stopped by addition of 150 μL of 50% trichloroacetic acid solution. The mixture was centrifuged at 13,000 g for 15 min, and 50 μL of 5 N NaOH was added. Samples were transferred to a 96-well plate reader, and the absorbance was measured at 410 nm. The concentration of *p*-nitroaniline formed was determined from a standard curve prepared with known concentrations of *p*-nitroaniline dissolved in 1 mL Tris/$MgCl_2$ buffer (pH 9.0), 150 μL 50% TCA, and 50 μL 5 N NaOH.

Protein concentrations were determined by the method of Bradford with bovine serum albumin as the standard [16]. A 10-μL sample of the homogenate was diluted 100-fold with deionized water, and 100 μL of the mixture was mixed with 2.5 mL of 20% Bradford's reagent (Bio-Rad Laboratories). The absorbance of the mixture was determined at 595 nm.

Table 1
Diurnal variations in renal and hepatic glutathione concentrations in rats[a]

Time (hr)	Renal glutathione concentration	Hepatic glutathione concentration
0	13.8 ± 2.0	28.6 ± 6.3
2	16.2 ± 4.3	28.8 ± 5.9
4	14.7 ± 5.0	26.8 ± 9.0
8	16.1 ± 5.0	16.8 ± 3.0[b]
12	15.9 ± 7.3	17.5 ± 5.1[b]
24	15.7 ± 4.8	37.8 ± 5.9[c]

[a] Rats were housed and fed as shown in Section 2, and renal and hepatic glutathione concentrations were measured at the indicated times (0 hr = 9.00 a.m. EST). Glutathione concentrations are expressed as μmol/mg protein; data are shown as means ± SD, N = 9. One-way ANOVA with the Bonferroni post-test.

[b] Significantly different from values at 0, 2, 4, and 24 hr.

[c] Significantly different from values at 0, 4, 8, and 12 hr.

Table 2
Diurnal variations in renal and hepatic γ-GT activities in rats[a]

Time (hr)	Renal γ-GT activities	Hepatic γ-GT activities
0	923 ± 264[b]	0.60 ± 0.6[b]
2	955 ± 420	0.23 ± 0.09
4	1071 ± 237	0.21 ± 0.09
8	1287 ± 159	0.33 ± 0.33
12	945 ± 204	0.23 ± 0.15
24	929 ± 201	0.76 ± 0.84

[a] Rats were housed and fed as shown in Section 2, and renal and hepatic γ-GT activities were measured at the indicated times (0 hr = 9.00 a.m. EST). γ-GT activities are expressed as μmol/min/mg protein; data are shown as means ± SD, N = 9.

[b] One-way ANOVA with the Bonferroni post-test showed no significant time-dependent differences within each experimental group.

2.4. Statistical analyses

The data were analyzed by one-way ANOVA with Bonferroni post-test (GraphPad Software) for diurnal variations in glutathione concentrations and γ-GT activities (Tables 1 and 2) and by two-way ANOVA with Bonferroni post-test for the effect of acivicin on glutathione concentrations and γ-GT activities (Figs. 1 and 2). A level of $P < 0.05$ was used for acceptance or rejection of the null hypothesis.

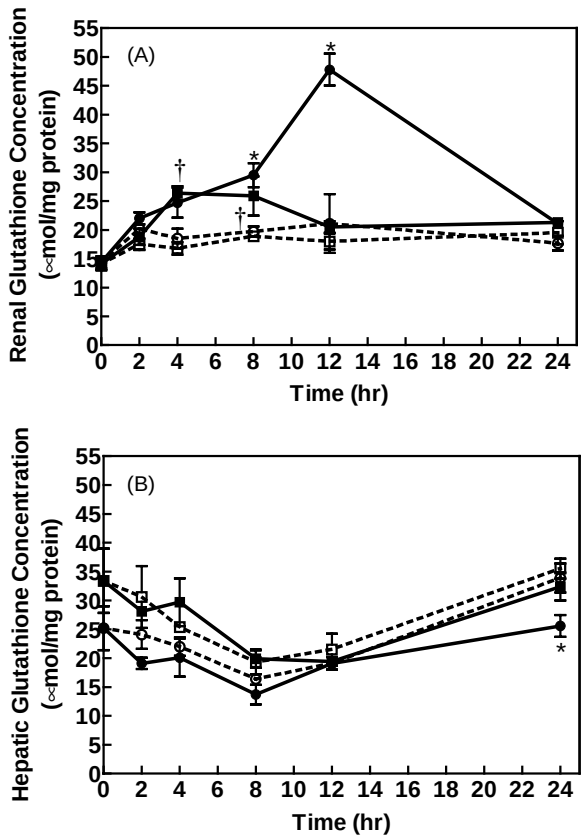

Fig. 1. Dose- and time-dependent effects of acivicin on renal (A) and hepatic (B) glutathione concentrations. Rats were given 0.1 or 0.2 mmol acivicin/kg, and glutathione concentrations were measured at the indicated times (0 hr = 9.00 a.m. EST), as described in Section 2. Glutathione concentrations are expressed as μmol/mg protein; data are shown as means ± SD, N = 3. (□) Control (0.1 mmol acivicin/kg); (■) treated (0.1 mmol acivicin/kg); (○) control (0.2 mmol acivicin/kg); (●) treated (0.2 mmol acivicin/kg). Two-way ANOVA with the Bonferroni post-test: (A) $^{\dagger}P < 0.05$, 0.1 mmol acivicin/kg compared with respective control group at 4 and 8 hr; $^{*}P < 0.05$, 0.2 mmol acivicin/kg compared with respective control group at 8 and 12 hr. (B) $^{*}P < 0.05$, 0.2 mmol acivicin/kg compared with respective control group at 24 hr.

3. Results

3.1. Diurnal variations in renal and hepatic glutathione concentrations

No diurnal variations in renal glutathione concentrations were observed (Table 1). A decrease in hepatic glutathione concentrations at 8 and 12 hr compared with 0, 2, 4, or 24 hr was observed, and the value at 24 hr was significantly different than the values at 0, 2, 4, 8, and 12 hr. (Table 1). These changes in hepatic glutathione concentrations were not reflected in renal glutathione concentrations.

3.2. Diurnal variations in renal and hepatic γ-GT activities

No significant diurnal variations in renal or hepatic γ-GT activities were observed, but renal γ-GT activities were much higher than hepatic γ-GT activities (Table 2).

3.3. Effect of acivicin on renal and hepatic glutathione concentrations

In acivicin-treated rats, a significant increase in renal glutathione concentrations was seen at 4 and 8 hr after treatment in rats given 0.1 mmol acivicin/kg and at 8 and 12 hr after treatment in rats given 0.2 mmol acivicin/kg (Fig. 1A). Acivicin had no effect on hepatic glutathione concentrations (Fig. 1B). Acivicin (0.05 mmol/kg) did not alter renal or hepatic glutathione concentrations (data not shown).

3.4. Effect of acivicin on renal and hepatic γ-GT activities

Acivicin (0.1 and 0.2 mmol/kg) caused significant decreases in renal γ-GT activities within 2 hr of treatment that persisted for 24 hr (Fig. 2A). Acivicin failed to alter hepatic γ-GT activities, except in rats given 0.2 mmol acivicin/kg where a significant increase in γ-GT activities was observed 24 hr after treatment (Fig. 2B).

4. Discussion

Cellular glutathione concentrations are an important determinant of cellular response to injury [17,18], and the toxicity of xenobiotics is modulated by the selective modification of tissue glutathione concentrations [2]. Hence, the present studies were designed to test the hypothesis that acivicin may alter renal and hepatic glutathione concentrations. Accordingly, the dose- and time-dependent effects

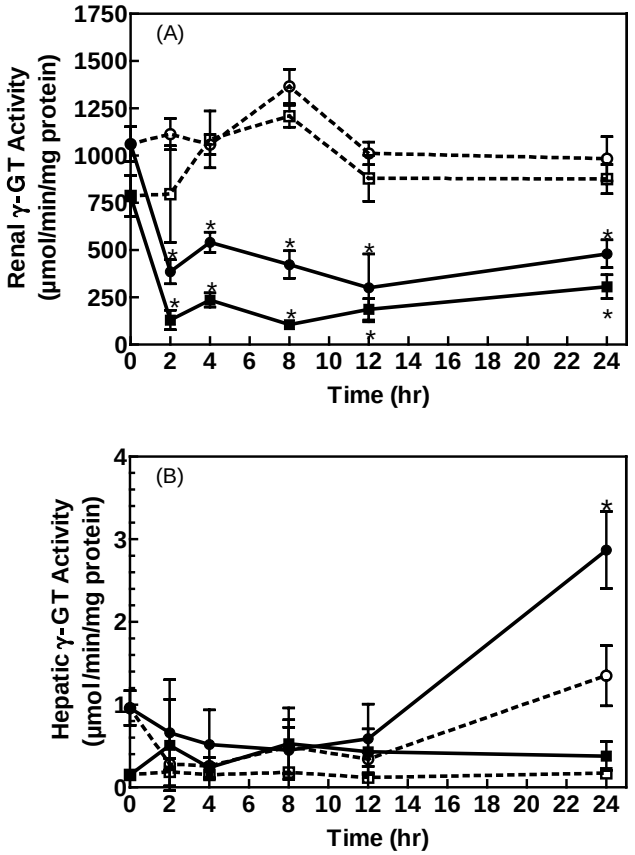

Fig. 2. Dose- and time-dependent effects of acivicin on renal (A) and hepatic (B) γ-GT activities. Rats were given 0.1 or 0.2 mmol acivicin/kg, and glutathione concentrations were measured at the indicated times (0 hr = 9.00 a.m. EST), as described in Section 2. γ-GT activities are expressed as μmol/min/mg protein; data are shown as means ± SD, N = 3. (□) Control (0.1 mmol acivicin/kg); (■) treated (0.1 mmol acivicin/kg); (○) control (0.2 mmol acivicin/kg); (●) treated (0.2 mmol acivicin/kg). Two-way ANOVA with the Bonferroni post-test: (A) $^{*}P < 0.05$, 0.1 or 0.2 mmol acivicin/kg compared with respective control group at 2, 4, 8, 12, and 12 hr. (B) $^{*}P < 0.05$, 0.2 mmol acivicin/kg compared with respective control group at 24 hr.

of acivicin on renal and hepatic glutathione concentrations and γ-GT activities were measured.

4.1. Diurnal variations in renal and hepatic glutathione concentrations

Previous studies have demonstrated a significant diurnal variation in hepatic glutathione concentrations in fed rats. Beck *et al.* [19] found that hepatic glutathione concentrations were lowest at 8.00 p.m. to midnight. Similarly, Jaeger *et al.* [20] found that hepatic glutathione concentrations were lowest at 7.00 p.m. to 10.00 p.m. Similar results have been reported in fasted rats [21]. Hence, the present results confirm these earlier studies on diurnal variations in hepatic glutathione concentrations in rats (Table 1). No diurnal variations in renal glutathione concentrations were observed in the present studies (Table 1); apparently diurnal variations in renal glutathione concentrations have not been investigated previously.

4.2. Diurnal variations in renal and hepatic γ-GT activities

No diurnal variations in renal or hepatic γ-GT activities were observed (Table 2). Apparently diurnal variations in renal and hepatic γ-GT activities have not been investigated previously. The data do show, however, that the diurnal variations in hepatic glutathione concentrations are not paralleled by changes in hepatic or renal γ-GT activities.

4.3. Effect of acivicin on renal and hepatic glutathione concentrations

Previous studies by Kramer *et al.* [22] showed increases in renal glutathione concentrations measured only at 16 hr after treatment in rats given 5 (0.03 mmol/kg) to 30 mg (0.17 mmol/kg) acivicin/kg. In the present studies, acivicin at doses of 0.1 and 0.2 mmol/kg increased renal glutathione concentrations at 4–12 hr (Fig. 1A). Acivicin failed to alter hepatic glutathione concentrations (Fig. 1B).

The mechanism whereby acivicin treatment leads to increased renal glutathione concentrations is not understood. Previous studies have shown, however, that acivicin-induced inhibition of γ-GT on the basolateral membrane of renal proximal tubular cells leads to an increased uptake of *S*-(1,2-dichlorovinyl)glutathione [23]. Similarly, acivicin treatment increases the nephrotoxicity of *S*-(2-chloroethyl)-glutathione, apparently by increasing the uptake of the intact glutathione *S*-conjugate [22]. The present studies showed that acivicin-induced inhibition of γ-GT is also associated with increases renal glutathione concentrations. Although the mechanism whereby acivicin increases renal glutathione concentration is not known, it is possible that the acivicin-induced decrease in glutathione degradation may result in increased luminal glutathione concentrations, which may lead to increased tissue glutathione concentrations.

4.4. Effect of acivicin on renal and hepatic γ-GT activities

Acivicin is a well-established, irreversible inhibitor of γ-GT [24,25], and the inhibition of γ-GT by acivicin was confirmed in the present studies (Fig. 2). Previous studies also showed that renal γ-GT activities are much higher than hepatic γ-GT activities [26], a finding that was also confirmed in the present studies.

4.5. What is the relationship between acivicin treatment and the enhanced nephrotoxicity of some haloalkenes?

Although the γ-GT-dependent mercapturic acid pathway is largely associated with the detoxication of xenobiotics, the glutathione-dependent toxicity of a range of compounds is also well known [4]. Several haloalkenes are selective nephrotoxins, and previous studies have elucidated the glutathione-, γ-GT-, and β-lyase-dependent bioactivation of haloalkenes to cytotoxic intermediates [5].

Acivicin has been used to investigate the role of γ-GT in the bioactivation of nephrotoxic haloalkenes and haloalkenes, but conflicting results have been reported. Acivicin increases the nephrotoxicity of *S*-(2-chloroethyl)glutathione [22], a direct-acting nephrotoxin, apparently by increasing the uptake of the intact glutathione *S*-conjugate in the kidney. Although a role for the β-lyase-dependent bioactivation of hexachloro-1,3-butadiene has been established [27,28], acivicin increases the nephrotoxicity of hexachloro-1,3-butadiene in rats [8] but inhibits its nephrotoxicity in mice [9]. In contrast, acivicin blocks the nephrotoxicity of *S*-(1,2-dichlorovinyl)glutathione in rats and its cytotoxicity in isolated rat renal proximal tubular cells [6,7]. Compound A is nephrotoxic in rats [29,30], and considerable evidence is available that supports the concept that Compound A undergoes glutathione- and β-lyase-dependent bioactivation [31–34]. Acivicin, however, increases the nephrotoxicity of Compound A *in vivo* [10–13]. The present studies do not, however, shed light on the mechanisms by which acivicin increases the nephrotoxicity of some haloalkenes.

The proposed mechanism for renal uptake of glutathione and glutathione *S*-conjugates synthesized in the liver is associated with γ-GT- and dipeptidase-catalyzed hydrolysis of glutathione and the uptake of cysteine or cysteine *S*-conjugates by the kidney [35], although intact glutathione or glutathione *S*-conjugates are taken up by the kidney or kidney cells when γ-GT is inhibited [22,23]. Hence, acivicin-induced inhibition of γ-GT may promote the uptake of intact glutathione or glutathione *S*-conjugates by the kidney, which would explain the present observations.

The present studies do not, however, provide an explanation for the observation that acivicin increases the nephrotoxicity of some haloalkenes that undergo glutathione- and β-lyase-dependent bioactivation. The present studies do demonstrate that acivicin is not a reliable probe for investigating the role of γ-GT in the bioactivation of nephrotoxic haloalkenes.

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