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COMPARATIVE CYTOTOXICITY OF ANGIOTENSIN-CONVERTING ENZYME INHIBITORS IN CULTURED RAT HEPATOCYTES*

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Abstract—Captopril and enalapril, angiotensin-converting enzyme inhibitors (ACEIs), have been associated with idiosyncratic hepatotoxicity. Such drug reactions may be caused by the formation of reactive metabolites by cytochrome P450 isozymes, which can then cause direct or immune-mediated toxicity. Previously, we have demonstrated that enalapril cytotoxicity in primary cultures of rat hepatocytes was due, at least in part, to cytochrome P450-dependent metabolism, and that glutathione was involved in the detoxification process. In the present study, we extended our investigations into mechanisms of cytotoxicity, using rat hepatocyte cultures, to captopril and three recently marketed ACEIs: fosinopril, lisinopril and quinapril. After 24 hr of exposure to lisinopril or enalaprilat (the deesterified metabolite of enalapril), hepatocytes did not show any evidence of cytotoxicity, measured by lactate dehydrogenase leakage, even at 10 mM drug concentrations. The other ACEIs were toxic to the liver cells, with the rank order of toxicity as quinapril (LC_{50} = 0.28 mM) > fosinopril (LC_{50} = 0.4 mM) > enalapril (LC_{50} = 2.0 mM) > captopril (LC_{50} = 20 mM). *In vivo* pretreatment of rats with pregnenolone-16α-carbonitrile to induce isozymes of the P450 3A subfamily significantly enhanced the cytotoxicities of quinapril, fosinopril and enalapril but did not affect captopril cytotoxicity. Pretreatment with P450 inducers selective for other isozyme subfamilies (ethanol, β-naphthoflavone and phenobarbital) did not alter the *in vitro* toxicity of any of the ACEIs. Co-incubation with SKF525-A (15 μM) or troleandomycin (0.1 mM) reduced the hepatocidal toxicities of quinapril, fosinopril and enalapril. Preincubation with buthionine sulfoximine (2 mM) enhanced the cytotoxicities of quinapril, fosinopril, enalapril and captopril. The results of this study indicate that like enalapril, quinapril and fosinopril can also undergo P450 3A-dependent bioactivation and require maintenance of glutathione status for detoxification, and that captopril causes cytotoxicity independent of cytochrome P450 metabolism.

Key words: angiotensin-converting enzyme inhibitors, rat hepatocytes, cytotoxicity, cytochrome P450, glutathione

Captopril (CP)‡ and enalapril maleate (EN) were the first orally active angiotensin-converting enzyme inhibitors (ACEIs) to become commercially available for treating hypertension. During recent years, the number of ACEIs being developed and becoming available for clinical use has increased markedly. This is due to their effectiveness in the therapy of both hypertension and congestive heart failure, and because they are generally well-tolerated drugs. Adverse effects most commonly associated with these drugs include hypotension, cough and rash [1–3]. There have been comparatively few reports of hepatotoxicity. Although rare, the severity of some cases [4–6] has drawn attention to this adverse effect. The mechanisms of liver injury are not known. Some investigators have suggested that CP-associated toxicities may be mediated by the presence of a sulfhydryl group [7, 8]. Subsequent reports of hepatotoxicity in patients receiving EN [9–14] suggested that other mechanisms must be involved in EN-associated liver injury since EN does not contain a sulfhydryl group. We have shown previously that the cytotoxicity of EN in primary cultures of rat hepatocytes was mediated, at least in part, by pregnenolone-16α-carbonitrile (PCN)-inducible cytochrome P450 species (P450 3A1 and 3A2), and that reduced glutathione (GSH) was involved in detoxification [15, 16]. These *in vitro* findings were substantiated subsequently by *in vivo* studies in rats in which the acute hepatotoxicity produced by single high doses of EN was enhanced by pretreatment with PCN or GSH-depleting agents, and was reduced by administration of the cytochrome P450 inhibitor, cobalt protoporphyrin, or the GSH precursor, L-2-oxothiazolidine-4-carboxylic acid [17].

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‡ Abbreviations: ACEI, angiotensin-converting enzyme inhibitor; BSO, buthionine sulfoximine; CP, captopril; EN, enalapril maleate; ET, enalaprilat; FOS, fosinopril sodium; LDH, lactate dehydrogenase; LIS, lisinopril dihydrate; PB, sodium phenobarbital; PCN, pregnenolone-16α-carbonitrile; QUIN, quinapril hydrochloride; SKF525-A, 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride; TAO, troleandomycin; αNF, α-naphthoflavone; and BNF, β-naphthoflavone.

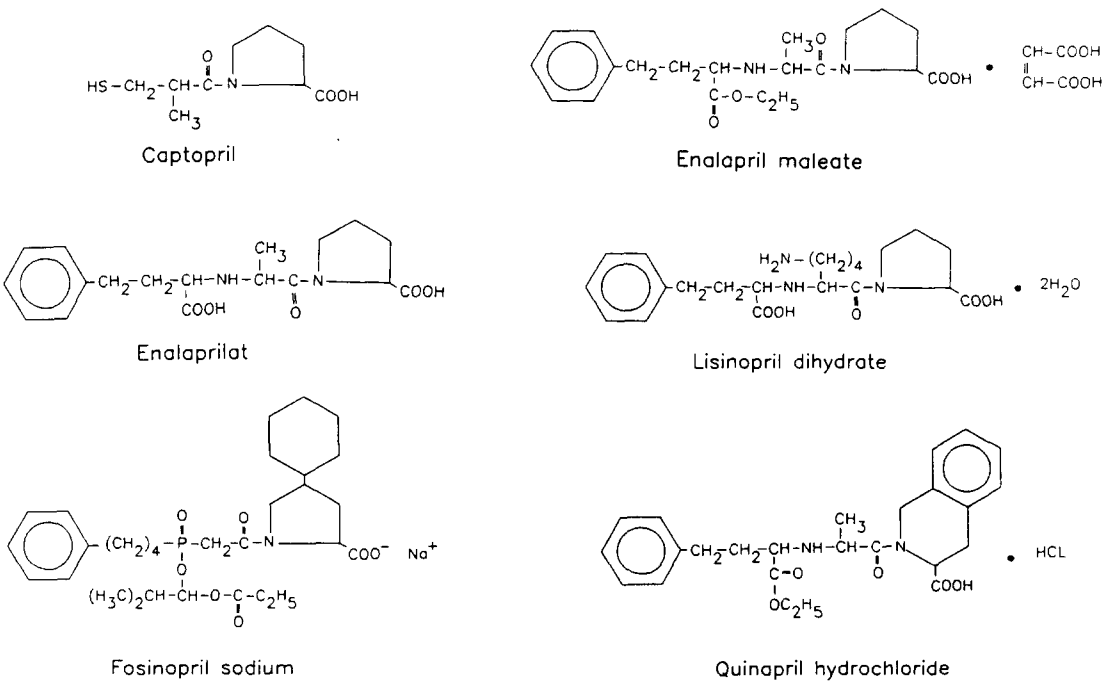

Fig. 1. Structural formulae of the ACEIs investigated.

The newer ACEIs, which have been marketed recently in Canada, are lisinopril, fosinopril and quinapril. All are structurally related to CP and EN. Their structural formulae are shown in Fig. 1. EN is a prodrug requiring hydrolysis to form the active drug enalaprilat (ET), while lisinopril is the lysine analogue of ET. Fosinopril contains a phosphinic group in the position corresponding to the α-amino group of EN. Like EN, fosinopril is converted by deesterification to the active drug, fosinoprilat. Quinapril has a quinoline moiety replacing the proline ring of EN. Quinapril also is hydrolyzed after absorption to the active diacid quinaprilat. Unlike EN and fosinopril, both quinapril and its diacid have pharmacological activity, although the potency of quinapril is lower than that of quinaprilat [18]. Since the mechanism for hepatotoxicity of EN may be related to the formation of an unknown cytotoxic metabolite [15–17], it was of interest to evaluate the cytotoxicity of other ACEIs in primary cultures of rat hepatocytes, and to determine whether cytochrome P450-dependent metabolism has a role in their cytotoxicity. Furthermore, it was hypothesized that the chemical relatedness of different ACEIs may provide insight into structural requirements for cytotoxicity towards hepatocytes, which possibly may be related to clinical liver toxicity. We report here on a comparative study of the cytotoxicity of CP, EN, ET, fosinopril, lisinopril and quinapril in rat hepatocyte cultures.

MATERIALS AND METHODS

Chemicals. Lisinopril dihydrate (LIS) and EN were gifts from Merck Frosst Canada Inc. (Pointe-Claire, Quebec). ET was from Merck, Sharp & Dohme International (Rahway, NJ). Fosinopril sodium (FOS) was donated by Bristol-Myers Squibb Canada Inc. (Saint-Laurent, Quebec). Quinapril hydrochloride (QUIN) was provided by Warner-Lambert Canada Inc. (Scarborough, Ontario). 2 - Diethylaminoethyl - 2,2 - diphenylvalerate hydrochloride (SKF525-A) was a gift from Smith, Kline & French (Toronto, Ontario). Troleandomycin (TAO) was provided by Pfizer Canada Inc. (Kirkland, Quebec). CP, α-naphthoflavone (αNF), β-naphthoflavone (βNF), pregnenolone-16α-carbonitrile (PCN) and DL-buthionine-[*S,R*]-sulfoximine (BSO) were purchased from the Sigma Chemical Co. (St. Louis, MO). Sodium phenobarbital (PB) was obtained from BDH Chemicals (Toronto, Ontario). All other chemicals and reagents were purchased from regular chemical suppliers.

Animals and pretreatments. Male Fischer rats (175–200 g) were obtained from Charles River (St. Constant, Quebec). They were cared for in accordance with the principles contained in the *Guide to the Care and Use of Experimental Animals* as prepared by the Canadian Council on Animal Care. The enzyme inducers βNF, PB and PCN were administered to rats as described previously [15]. Ethanol (EtOH) was given to rats as a 15% (v/v) solution in drinking water for 3 days to induce P450 2E1 [19]. The EtOH treatment was accompanied by an approximately 10% reduction in body weight as has been observed previously [19]. The EtOH-pretreated rats were killed on day 4.

Hepatocyte isolation and culture. Hepatocytes were isolated by collagenase perfusion of the liver in anesthetized rats as described in detail previously [15]. Initial cell viability, determined by trypan blue exclusion, was: control, 83 ± 6%; EtOH-induced, 81 ± 1%; βNF-induced, 85 ± 3%; PB-induced, 82 ± 6%; and PCN-induced, 87 ± 5%. Cells were

seeded into 25-cm^2 collagen-coated culture flasks at a density of 10^6 viable hepatocytes in 5 mL of Williams' E medium supplemented with 5% fetal bovine serum. Two hours after seeding, the medium was replaced with fresh medium to remove unattached cells. Attachment efficiency, determined by lactate dehydrogenase (LDH) activity, was: control, 74 ± 10%; EtOH-induced, 81 ± 5%; βNF-induced, 79 ± 2%; PB-induced, 75 ± 11%; and PCN-induced, 71 ± 8%.

ACEIs, at various concentrations, were incubated with the hepatocyte monolayers for 20 hr at 37°C in 95% air/5% CO_2. BSO (2 mM), αNF (10 μM), SKF525-A (15 μM) and TAO (0.1 mM) were added after the 2-hr attachment and allowed to incubate for 1 hr before the addition of the ACEIs. These concentrations of BSO and P450 inhibitors have been shown previously by ourselves [15–16] and other investigators [20–21] to be effective in rat hepatocyte primary cultures. Flasks with BSO contained 0.4% dimethyl sulfoxide, and flasks with αNF contained 0.1% dimethyl sulfoxide; at these concentrations dimethyl sulfoxide did not affect cell viability.

Cytotoxicity measurement. The amount of LDH activity released into the medium at 20 hr was determined as described previously [15]. For each flask, cytotoxicity was expressed as LDH activity in the medium as a percentage of total LDH activity for that flask.

Statistical analysis. Results are expressed as means ± SEM. Statistical significance between con-

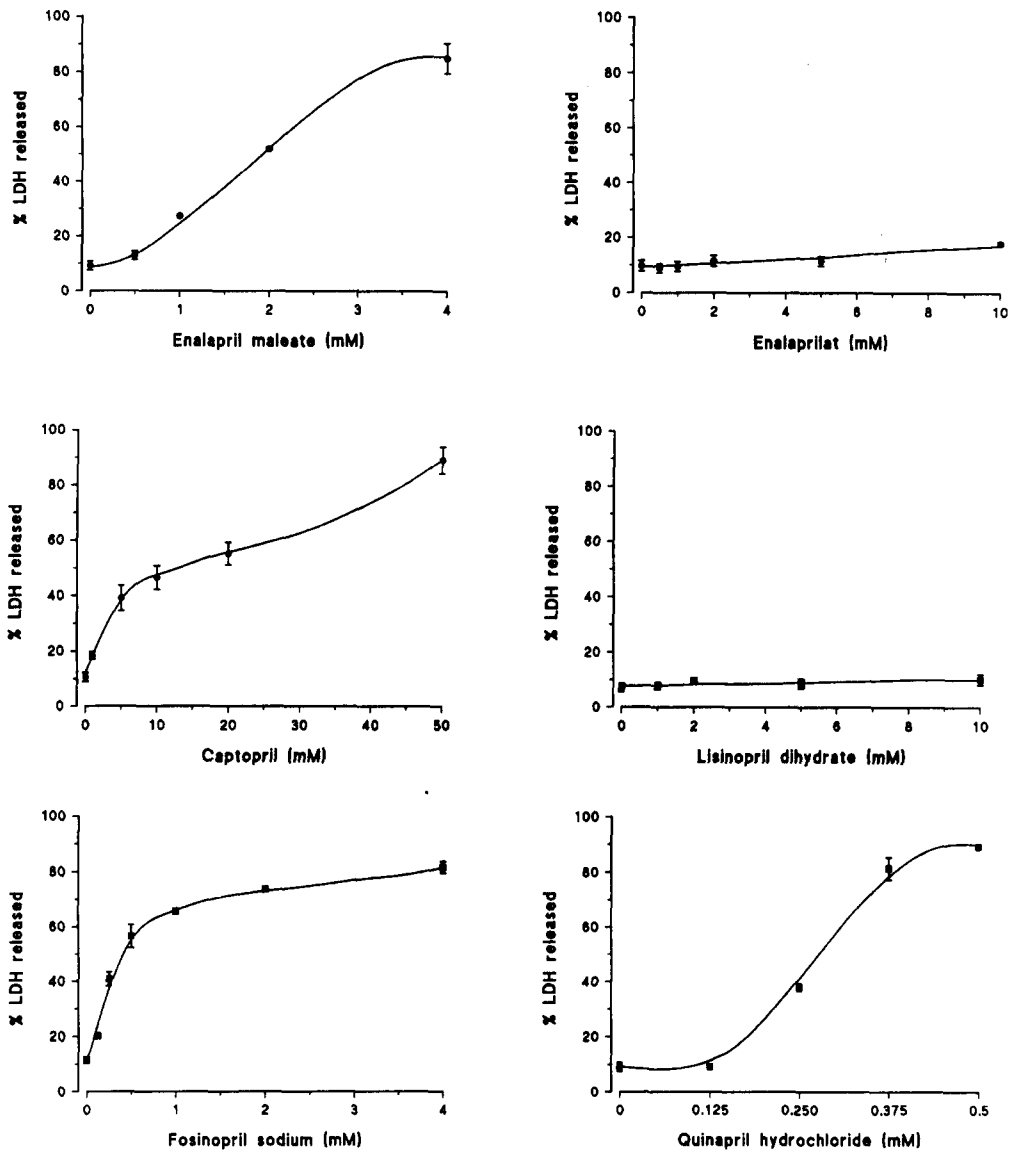

Fig. 2. Cytotoxic concentration–response curves for the six ACEIs evaluated. Cytotoxicity was measured by the percentage of LDH released into the medium during 20 hr in culture. Data are means from 3–4 individual experiments. Vertical bars represent ± SEM; if no bar is shown, the SEM was smaller than the symbol.

Table 1. Effects of cytochrome P450 inducers on the cytotoxicities of ACEIs

ACEI	(mM)	Percent LDH released into medium at 20 hr*				
		None	PB	Inducer βNF	PCN	EtOH
CP	4.0	40.38 ± 5.14	30.92 ± 2.19	30.57 ± 1.76	32.00 ± 3.91	39.59 ± 1.03
	20.0	49.78 ± 7.14	45.20 ± 2.90	49.07 ± 8.20	42.43 ± 3.11	45.86 ± 7.50
	50.0	78.21 ± 7.31	92.99 ± 0.58	89.07 ± 5.27	95.54 ± 0.62	63.73 ± 5.68
EN	1.0	26.67 ± 1.24	17.07 ± 2.83†	12.93 ± 1.07‡	72.78 ± 6.38‡	24.35 ± 4.92
	2.0	39.20 ± 4.03	38.17 ± 4.21	29.33 ± 5.43	94.56 ± 0.17§	32.32 ± 8.77
	3.0	60.74 ± 6.88	66.47 ± 3.08	60.53 ± 8.80	95.34 ± 0.10‡	53.09 ± 9.53
FOS	0.125	27.37 ± 9.28	21.16 ± 7.42	26.77 ± 4.69	72.57 ± 7.45†	21.78 ± 10.73
	0.400	52.89 ± 7.06	44.34 ± 6.49	35.88 ± 6.61	89.86 ± 1.81‡	57.95 ± 8.12
	2.000	69.22 ± 1.07	65.04 ± 7.46	59.04 ± 2.84†	93.09 ± 0.41§	69.77 ± 5.12
CP	0.200	19.08 ± 1.61	12.95 ± 2.60	17.78 ± 1.45	53.58 ± 3.26§	11.28 ± 0.80
	0.275	29.94 ± 4.36	21.03 ± 3.28	26.57 ± 2.24	72.36 ± 4.40‡	19.97 ± 4.84
	0.375	51.38 ± 3.97	35.31 ± 2.17†	50.29 ± 1.32	81.84 ± 1.79‡	43.08 ± 4.99

* Values are means ± SEM of 3–4 separate experiments.

†–§ Significantly different from noninduced hepatocytes by Student's unpaired *t*-test; † $P < 0.05$, ‡ $P < 0.01$ and § $P < 0.01$.

trol and enzyme-induced hepatocytes was tested by the unpaired Student's *t*-test. Statistically significant effects of SKF525-A, TAO, αNF or BSO were tested by the paired Student's *t*-test. A P value of <0.05 was considered to be statistically significant.

RESULTS

Cytotoxicity evaluation. After a 20-hr incubation period, determination of LDH release into the culture medium demonstrated that EN, CP, FOS and QUIN were cytotoxic towards rat hepatocytes (Fig. 2). ET and LIS were not toxic, even at concentrations as high as 10 mM. QUIN was the most toxic of the six ACEIs tested. Its cytotoxic concentration range, determined from the sigmoidal curve, was 0.15 to 0.5 mM with the concentration producing 50% LDH release (LC_{50}) about 0.275 mM. FOS was the next most cytotoxic ACEI, with a toxic range of approximately 0.25 to 2.0 mM and an LC_{50} of 0.4 mM. EN was cytotoxic at 0.5 to 4.0 mM with an LC_{50} of about 2 mM. CP was considerably less toxic than the other three cytotoxic ACEIs: its cytotoxic concentration range was 1–50 mM with LC_{50} of approximately 20 mM. The cytotoxic concentration–response curve for CP appeared biphasic; the reason for this is unknown.

Effects of cytochrome P450 inducers. The effects of *in vivo* pretreatment with PB, βNF, PCN and EtOH, selective inducers of cytochrome P450 subfamilies, on the cytotoxicities of the ACEIs were evaluated (Table 1). Only PCN induction produced a consistent enhancement of the toxicity of EN, FOS and QUIN. CP toxicity was not affected by PCN induction. PCN itself had no effect on the viability of the hepatocyte cultures. None of the inducers altered the nontoxicity of exposure to ET or LIS (data not shown).

Effects of cytochrome P450 inhibitors. The P450 inhibitors, SKF525-A, TAO, and αNF were evaluated for their effects on ACEI cytotoxicity in control and PCN-induced hepatocytes. αNF did not influence the toxicity of any of the ACEIs tested (data not shown).

The toxic effect of CP was not modified by SKF525-A or TAO except in PCN-induced hepatocytes at the highest concentration, 50 mM, where both inhibitors demonstrated a modest protective effect (Fig. 3, a and b).

In control hepatocyte cultures. EN toxicity was reduced by TAO but was not affected by SKF525-A (Fig. 3c). In PCN-induced cells, both SKF525-A and TAO diminished the cytotoxicity of EN, with TAO having a significantly greater effect than SKF525-A at 2.0 mM EN ($P < 0.05$ by paired Student's *t*-test, Fig. 3d).

FOS toxicity at 2.0 mM was reduced by SKF525-A and TAO in control hepatocytes (Fig. 3e). This concentration and the next lower concentration, 0.4 mM, produced nearly total cell death in PCN-induced cultures. Consequently, the effects of SKF525-A and TAO had to be discerned at a lower FOS concentration. Both P450 inhibitors significantly reduced the degree of cytotoxicity of FOS at 0.125 mM in PCN-induced hepatocytes (Fig. 3f).

In control hepatocytes, SKF525-A and TAO did not demonstrate statistically significant modification of QUIN toxicity, although there appeared to be a trend towards a protective effect of SKF525-A (Fig. 3g). In PCN-induced hepatocyte cultures, this protective effect of SKF525A was statistically significant, and TAO showed a possible trend, although statistically insignificant, towards reducing QUIN toxicity (Fig. 3h).

Effect of GSH depletion by BSO. A 1-hr preincubation of the hepatocyte monolayers with 2 mM BSO resulted in greater than 40% reduction of cellular GSH concentration [determined by 5,5′-dithio-bis-(2-nitrobenzoic acid)-reactive nonprotein sulfhydryls]. This pretreatment of the cells enhanced their sensitivity to the cytotoxic actions of CP, EN, FOS and QUIN (Fig. 4). The effect was greatest for EN: a concentration of 1.0 mM EN was associated with only slight toxicity but BSO pretreatment resulted in greater than 90% loss of cell viability.

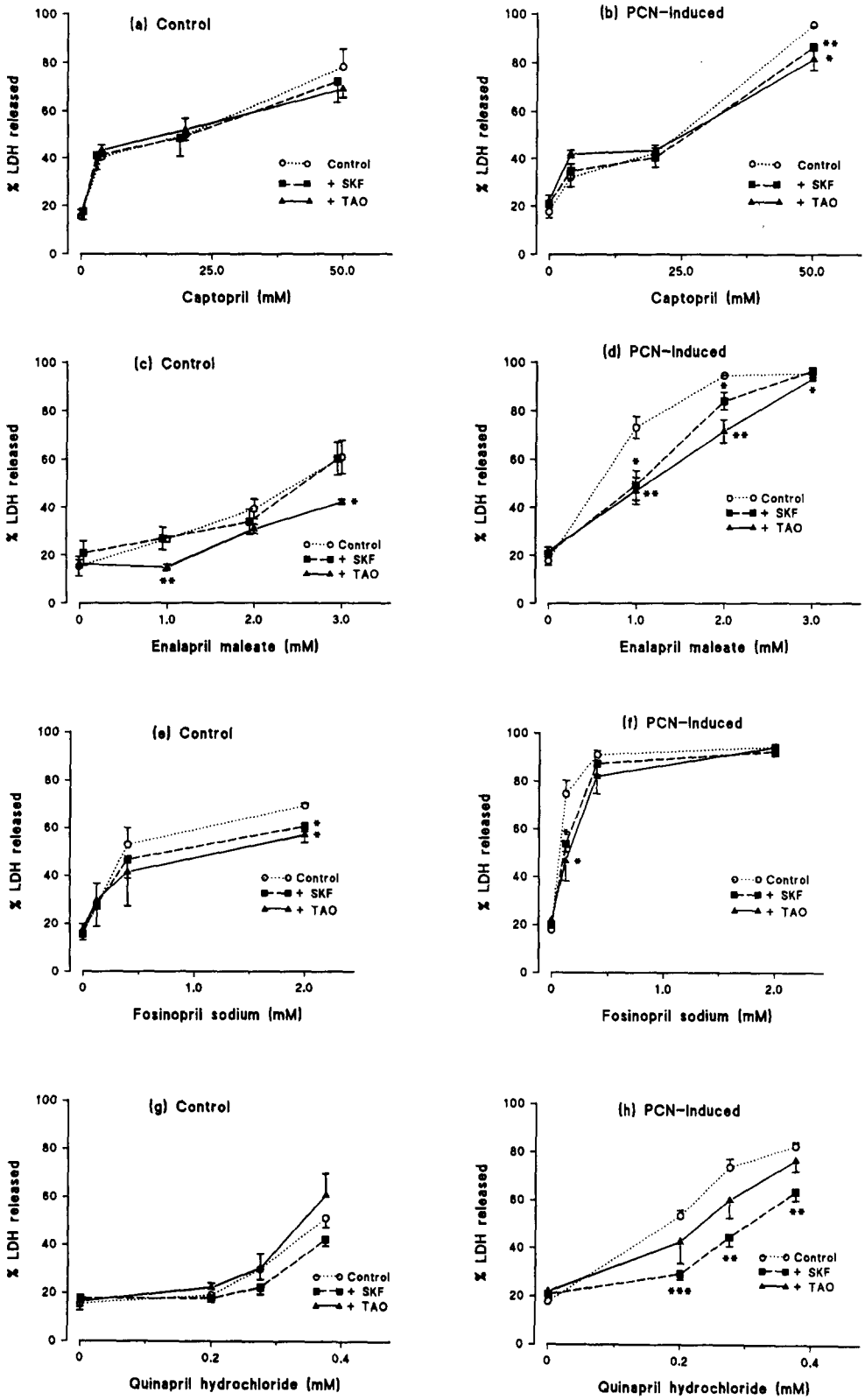

Fig. 3. Effects of the cytochrome P450 inhibitors SKF525-A (SKF) and TAO on the cytotoxicity of ACEIs. Primary cultures of hepatocytes, isolated from control and PCN-pretreated rats, were incubated with SKF525-A (15 μM) or TAO (0.1 mM) for 1 hr prior to the addition of the ACEI. The percentage of LDH released into the medium was determined after 20 hr of incubation with the ACEI. Data are means ± SEM from 3–4 individual experiments. Key: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with culture without inhibitor (paired Student's t-test).

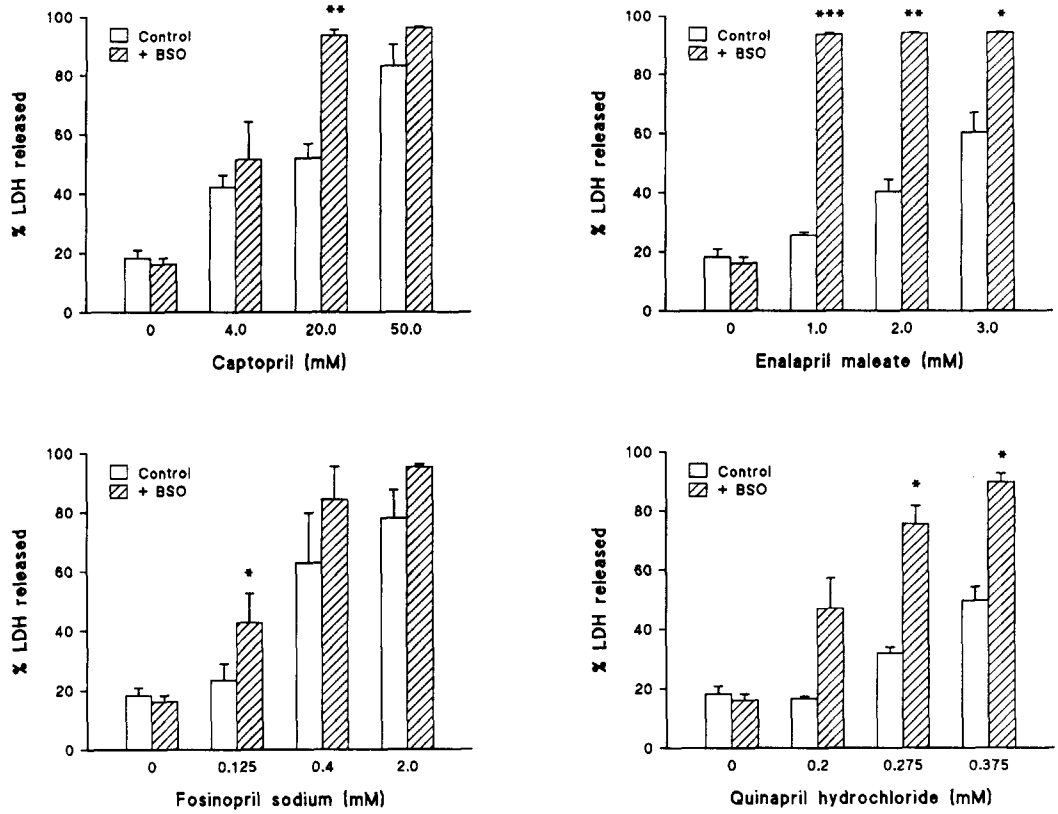

Fig. 4. Effect of BSO depletion of GSH on the cytotoxicity of ACEIs. Primary cultures were incubated with 2 mM BSO for 1 hr prior to the addition of the ACEI. The percentage of LDH released into the medium was determined after 20 hr of incubation with the ACEI. Data are means ± SEM from 3 individual experiments. Key: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with culture without BSO (paired Student's *t*-test).

DISCUSSION

The results of this study provide indirect evidence that the cytotoxicity of FLOS and QUIN in rat hepatocyte cultures is also mediated, at least partially, by P450 3A-dependent metabolism, as had been demonstrated previously for EN [15]. Cytotoxicity of these drugs was enhanced by *in vivo* pretreatment with PCN, a selective inducer of P450 3A1 and 3A2 isozymes in the rat [22, 23]. None of the other P450 inducers employed: PB (predominantly 2B1 and 2B2 induction) [24], βNF (1A1 and 1A2 induction) [23] and EtOH (2E1 induction) [23], had any effect on the cytotoxicity of the ACEIs. Furthermore, the toxicities of EN, FOS and QUIN were diminished by the classical non-selective P450 inhibitor, SKF525-A, and the 3A-specific inhibitor, TAO [25], with some suggestion of a greater effect with TAO. The 1A1, 1A2-selective inhibitor αNF [26] had no effect. The observation of a P450 3A-mediated enhancement of FOS and QUIN cytotoxicity was somewhat surprising since P450-mediated biotransformation does not play an evident major role in the metabolism of these ACEIs. In humans, FOS is completely hydrolyzed to the pharmacologically active diacid, fosinoprilat, which is the major metabolite in plasma, urine and feces. However, appreciable quantities of *p*-hydroxyfosinoprilat have also been detected [27, 28]. Formation of *p*-hydroxyfosinoprilat would be catalyzed by cytochrome P450; however, it is unlikely that this would be the cytotoxic chemical entity. Generally, highly reactive and cytotoxic metabolites are short-lived and/or rapidly bound to cellular macromolecules, and therefore unlikely to be detected in appreciable quantities in plasma or urine. However, it is not known whether the *p*-hydroxy metabolite is formed by rat hepatocytes and, if so, whether it is cytotoxic.

After absorption, QUIN also undergoes rapid deesterification to the active metabolite, quinalaprilat. Other than quinalaprilat, two diketopiperazine analogues have been identified in human urine following the administration of QUIN [29]. Diketopiperazine derivatives of other ACEIs also have been reported [30, 31]; these are believed to result from non-enzymatic cyclization characteristic of dipeptides. Therefore, the postulated P450-mediated cytotoxic metabolites of FOS and QUIN are unknown, as is also the case for EN [15–17].

LIS, which was not cytotoxic in rat hepatocyte cultures, is not metabolized significantly [2]. ET, the pharmacologically active diacid metabolite of EN, also was not cytotoxic in cultures of rat hepatocytes. An inability of ET to enter hepatocytes has been suggested [32], which may account for the observed

absence of cytotoxicity. Since LIS also lacks the ester moiety which is associated with enhanced entry of EN into hepatocytes, it is likely that its nontoxicity in rat hepatocytes is related to an inability to enter the cells.

Once inside the hepatocyte, EN can be converted to ET by liver esterase. We believe that it is EN, rather than ET, which is responsible for the formation of a cytotoxic metabolite. When we incubated hepatocyte cultures in the presence of the esterase inhibitor, sodium fluoride, we observed a 33% enhancement of the cytotoxicity of 1 mM EN with 1 mM sodium fluoride, and a 50% enhancement of 2 mM EN toxicity with 2.5 mM sodium fluoride. This concentration of sodium fluoride was associated with partial inhibition of EN deesterification to ET, as determined by HPLC analysis. Complete inhibition was not possible due to the toxicity of sodium fluoride at concentrations higher than 2.5 mM.

In this study, we investigated the effect of depleting hepatocellular GSH by pretreatment with BSO, a potent and specific inhibitor of γ-glutamylcysteine synthetase, the rate-limiting enzyme in GSH biosynthesis [33]. BSO pretreatment augmented the cytotoxicity of CP, EN, FOS and QUIN. The importance of GSH in the detoxification of reactive metabolites has been well established [34]. Therefore, it might be presumed that this BSO effect, with regard to EN, FOS and QUIN, is related to the postulated P450-mediated formation of cytotoxic metabolites: In the absence of sufficient GSH to detoxify the reactive metabolites, toxicity occurs at lower concentrations of the drugs. With regard to CP, obviously a different mechanism must be involved since induction of cytochrome P450 isozymes by PCN or other chemical inducers did not influence the cytotoxic response to any appreciable degree.

The metabolism of CP includes the formation of disulfide and mixed disulfides with protein and endogenous thiols [35]. The sulfhydryl moiety of CP is responsible for the binding, via a covalent disulfide bond, to plasma proteins [36]. CP–plasma protein conjugates are sufficiently stable to induce CP-specific IgG antibody responses but can be dissociated by interaction with endogenous thiols such as GSH or cysteine [7, 36]. Thus, in our hepatocyte monolayers, the enhancement of cytotoxicity by BSO pretreatment may be due to increased free sulfhydryl groups of CP, available to bind to cellular components and produce toxicity.

The cytotoxic concentrations of the ACEIs determined in this study range from 100- to 1000-fold higher than therapeutic plasma levels. Therefore, it is clear that a direct cytotoxic mechanism is extremely unlikely to cause hepatotoxicity during normal therapeutic use of these drugs. The hepatotoxicity associated with CP may have an underlying immunological mechanism as a consequence of the ability of CP to act as a hapten [7]. Clinical observations of rash, fever and eosinophilia, frequently reported in patients with CP-induced liver abnormalities, support the view of an immunological mechanism [4, 37]. Since the rare hepatic injury associated with EN therapy may also have an immune basis [9, 11–13], it is conceivable that a reactive metabolite formed by cytochrome P450 biotransformation may act as a hapten. Although we cannot extrapolate from the *in vitro* rat data to humans, it is possible that P450 3A-dependent bioactivation of EN, FOS and QUIN may also occur in humans. It has been demonstrated that the P450 3A subfamily is highly conserved in mammalian species [22]. However, species differences have been noted in the regulation of expression of some members of the rat and human 3A subfamilies [38].

FOS, LIS and QUIN are newer ACEIs associated with only a few years of clinical experience. To date, there have not been any clinical reports of hepatotoxicity associated with FOS or QUIN, to our knowledge. By contrast, as of 1989, worldwide, excluding the United Kingdom, there were 164 cases of CP-associated liver dysfunction reported to the World Health Organization and 29 cases reported in the United Kingdom [5]. This may, at least in part, be a reflection of the much longer history of clinical use of CP, which was first released in 1977. In view of the evidence from this study suggesting a role for P450 bioactivation in the cytotoxicities of FOS and QUIN in rat hepatocytes, there may be potential for hapten-mediated immune reactions with these drugs. Future clinical experience will reveal whether this is the case. LIS, which has been marketed on a worldwide basis since 1988, has been asssociated with a single case of hepatotoxicity which initially manifested as a hypersensitivity reaction and developed into fulminant hepatitis [6]. Since our study provided no evidence for reactive metabolite formation from LIS, immune reactions to some ACEIs, on the other hand, may not depend on biotransformation.

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