Toxicology and Applied Pharmacology **168,** 268–279 (2000)
doi:10.1006/taap.2000.9053, available online at http://www.idealibrary.com on IDEAL®

Analysis of the Cytotoxic Properties of Linoleic Acid Metabolites Produced by Renal and Hepatic P450s

Jeffery H. Moran,* Lex A. Mitchell,* J. Alyce Bradbury,† Wei Qu,† Darryl C. Zeldin,†
Rick G. Schnellmann,* and David F. Grant*,[1]

*Department of Pharmacology and Toxicology, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205; and
†Division of Intramural Research, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709

Received May 18, 2000; accepted August 23, 2000

Analysis of the Cytotoxic Properties of Linoleic Acid Metabolites Produced by Renal and Hepatic P450s. Moran, J. H., Mitchell, L. A., Bradbury, J. A., Qu, W., Zeldin, D. C., Schnellmann, R. G., and Grant, D. F. (2000). *Toxicol. Appl. Pharmacol.* **168, 268–279.**

Cytochrome P450 epoxidation of linoleic acid produces biologically active metabolites which have been associated with many pathological conditions that often lead to acute renal failure. In the present study, we evaluated the ability of specific cytochrome P450s to produce linoleic acid monoepoxides. We then tested the cytotoxic properties of linoleic acid, linoleic acid monoepoxides, and corresponding diols in a rabbit renal proximal tubule model. CYP1A2, CYP2E1, CYP2J2, CYP2J3, CYP2J5, and CYP2J9 metabolized linoleic acid at rates comparable to arachidonic acid and produced linoleic acid monoepoxides as major products. Cytotoxicity studies showed that linoleic acid, linoleic acid monoepoxides, and corresponding diols are toxic at pathologically relevant concentrations (100–500 μM). Concentration-dependent studies showed that linoleic acid and linoleic acid monoepoxides are the most toxic and induce mitochondrial dysfunction prior to cell death. Cytoprotectants known to block cell death associated with mitochondrial dysfunction and oxidative stress did not prevent cell death induced by linoleic acid and linoleic acid monoepoxides. This study shows that P450s in the CYP1 and CYP2 gene families metabolize linoleic acid to linoleic acid monoepoxides and that the monoepoxides, as well as linoleic acid, disrupt mitochondrial function without causing oxidative stress. © 2000 Academic Press

P450[2] epoxidation of AA and LA is well established and associated with the CYP2 gene family (Bylund *et al.,* 1998a,b; Capdevila *et al.,* 1991; Oliw *et al.,* 1996; Zeldin *et al.,* 1995). The primary AA and LA epoxidation products formed are *cis*-epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-EET) and *cis*-epoxyoctadecenoic acids (9,10- and 12,13-EOA), respectively. Bylund *et al.* (1998a,b) demonstrated that recombinant CYP1A2, CYP2C8, CYP2C9, CYP2C19, and CYP3A4 metabolize LA and form EOA, DHOA, and HODE metabolites, suggesting that several P450s are capable of metabolizing LA and forming similar products. CYP2J2 is a human isozyme that is abundant in kidney and active in the metabolism of AA to EETs (Wu *et al.,* 1996). Rat CYP2J3 and mouse CYP2J5 and CYP2J9 are expressed also in kidney and are active in AA metabolism (Ma *et al.,* 1999; Wu *et al.,* 1996, 1997). Each of the CYP2Js are catalytically distinct enzymes and produce mixtures of EETs and HETEs (Ma *et al.,* 1999; Wu *et al.,* 1996, 1997). However, it is not known whether these enzymes metabolize linoleic acid.

While there are many studies demonstrating the biological relevance of P450-derived AA products (Hoebel *et al.,* 1998; McGiff and Carroll, 1991; McGiff *et al.,* 1991, 1993; Quilley *et al.,* 1997), the physiological and pathological significance of 9,10-EOA and 12,13-EOA are not known. These LA metabolites are produced *in vivo* during specific pathological events. For example, Kosaka *et al.* (1994) showed that 9,10-EOA and 12,13-EOA can reach high concentrations (>100 μM) in the serum of patients with significant burns, and Ozawa *et al.* (1988) isolated these metabolites from the lungs of rats exposed to toxic concentrations of oxygen. 9,10-EOA and 12,13-EOA have been associated with adult respiratory distress syndrome, increased mortality of severely burned patients, systemic shock, and have been shown to induce cardiac failure in dogs (Fukushima *et al.,* 1988; Kosaka *et al.,* 1994; Ozawa *et al.,* 1988; Sugiyama *et al.,* 1987). Patients with severe burns or adult respiratory distress syndrome often die from multiple organ failure. Since, in many of these cases, there is renal failure due to acute tubular necrosis, it is important to under-

[1] To whom correspondence should be addressed at: Department of Pharmacology and Toxicology, University of Arkansas for Medical Sciences, 4301 West Markham, Slot 638, Little Rock, AR 72205. Telephone: (501) 686-8878. Fax: (501) 686-8970. E-mail: grantdavidf@exchange.uams.edu.

[2] Abbreviations used: P450, cytochrome P450; AA, arachidonic acid; LA, linoleic acid; EET, *cis*-epoxyeicosatrienoic acid; EOA, *cis*-epoxyoctadecenoic acid; DHOA, dihydroxyoctadecenoic acid; HODE, hydroxyoctadecdienoic acid; HETE, hydroxyeicosatetraenoic acid; sEH, soluble epoxide hydrolase; RPT, renal proximal tubules; CYPOR, cytochrome P450 oxidoreductase; NP-HPLC, normal-phase high-pressure liquid chromatography; RP-HPLC, reverse-phase high-pressure liquid chromatography; DMSO, dimethyl sulfoxide; GLY, glycine; DEF, deferoxamine; DPPD, *N,N′*-diphenyl-1,4-phenyl-enediamine; LDH, lactate dehydrogenase; SE, standard error; ANOVA, analysis of variance.

0041-008X/00 $35.00

stand the biological activities of 9,10-EOA and 12,13-EOA in kidney.

Recent *in vitro* studies have used the methyl ester derivatives of linoleic acid, EOAs, and DHOAs. These studies have suggested an sEH-dependent pathway for the toxicity induced by 9,10- and 12,13-EOA (Moghaddam *et al.*, 1997; Moran *et al.*, 1997; Stimers *et al.*, 1999). This mechanism of activation leads to the formation of the vicinal diols via sEH hydrolysis. Sf-21 cells expressing human or mouse sEH were shown to be susceptible to methyl-EOA and methyl-DHOA; however, control Sf-21 cells (expressing β-galactosidase) were only susceptible to methyl-DHOA mediated toxicity (Moghaddam *et al.*, 1997). Moran *et al.* (1997) showed that approximately 50% cellular lysis occurred when RPT were exposed to a racemic mixture of methyl-DHOA, but not methyl-EOA and methyl-LA. RPT exhibited mitochondrial dysfunction and inhibition of active Na^+ transport prior to cell death. The toxicological effects seen in these studies, however, may be nonspecific due to the high concentrations utilized (1.0 mM) and may not be a true reflection of what occurs *in vivo* because methyl esters rather than free acids were used.

Understanding how these biologically active compounds are produced and what role they have in normal physiological and pathological processes could lead to new therapeutic strategies for patients with severe burns or suffering from multiple organ failure. The current study was designed to further characterize P450 isozymes which metabolize LA and to address some of the controversies in the literature concerning which LA metabolites produce toxicity. We demonstrate that linoleic acid epoxides are major products of CYP1A2, CYP2E1, CYP2J2, CYP2J3, CYP2J5, and CYP2J9 metabolism, and our toxicity studies show that LA, LA-EOAs, and LA-DHOAs are all toxic in the rabbit RPT model. We describe mitochondrial effects and determine relative toxicity of these metabolites. We show for the first time that free-acid LA metabolites have a different toxicity profile than the methyl ester LA metabolites in rabbit RPT.

MATERIALS AND METHODS

Materials. [1-^{14}C]linoleic acid was purchased from Amersham (Piscataway, NJ). 9,10-EOA, 12,13-EOA, 9-HODE, and 13-HODE standards were purchased from Cayman Chemical Co. (Ann Arbor, MI) or synthesized in our laboratory. Recombinant CYP1A2, CYP2E1, and CYP4A11 were purchased from Gentest (Woburn, MA) and CYP2J2, CYP2J3, CYP2J5, and CYP2J9 were expressed in Sf9 cells using a baculovirus heterologous expression system, as described (Ma *et al.*, 1999; Wu *et al.*, 1996, 1997). All recombinant cytochromes P450 were coexpressed with CYPOR. Unless otherwise specified, all other chemicals and reagents were purchased from either Sigma Chemical Co. (St. Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI) and were reagent grade.

Synthesis and purification of LA metabolites. The monoepoxides of LA were synthesized using previously reported methods (Rudolph *et al.*, 1997). Briefly, LA was dissolved in methylene chloride and incubated at room temperature for 30 min in the presence of methyltrioxorhenium (0.5 mol%), pyridine (12 mol%), and hydrogen peroxide (1.5 mol equivs). The reaction was terminated by the addition of a catalytic amount of manganese (IV) oxide (1–5 mg) and extracted using methylene chloride. The synthesized monoepoxides were silica purified using a mobile phase consisting of methylene chloride (94.9%)/ethyl acetate (5%)/acetic acid (0.1%). The silica-purified monoepoxides were either hydrolyzed to corresponding vicinal diols at room temperature for 1.5 h using $HClO_4$ (1.25%) or purified by NP-HPLC. The NP-HPLC mobile phase for the purification of the monoepoxides consisted of hexane (98.9%)/2-propanol (1%)/acetic acid (0.1%). The vicinal diols were purified also by NP-HPLC using a mobile phase consisting of hexane (95.9%)/2-propanol (4%)/acetic acid (0.1%). Purified 9,10-EOA and 12,13-EOA had the same retention time (approximately 8.9 and 7.6 min, respectively) as authentic standards (Cayman Chemical Co.) when separated on a silica Rainin Microsorb column (25 cm $\times$ 4.6 mm) using a flow rate of 1 mL/min. Using the NP-HPLC system described above, the elution times for 9,10-DHOA and 12,13-DHOA were approximately 13.1 and 10.0 min, respectively. Detection of the epoxides and diols was achieved by UV detection at 202 nm. All purified isomers were dried under nitrogen and stored at -20°C until assayed. Purified compounds routinely had $<1\%$ contamination of the other isomer (as assayed by HPLC) and had the predicted molecular weights when analyzed by positive and negative electrospray ionization mass spectroscopy (Mass Consortium, San Diego, CA).

Incubations of recombinant cytochrome P450s with LA. Reaction mixtures containing Tris–Cl buffer (50 mM, pH 7.5), KCl (150 mM), $MgCl_2$ (10 mM), sodium isocitrate (8 mM), 0.5 IU/mL isocitrate dehydrogenase, varying concentrations of P450 (0.10–0.45 μM), NADPH (1 mM), and [1-^{14}C]LA (55 μCi/μmol; 50–100 μM final concentration) were constantly stirred at 37°C for 10–180 min. All reactions were initiated with the addition of NADPH after temperature equilibration. At different time points, aliquots were withdrawn, extracted with ethyl ether over a saturated KCl aqueous buffer, and analyzed using both NP-HPLC and RP-HPLC. Instrumentation utilized for product analysis was the same as described by Ma *et al.* (1999). The mobile phases used for NP-HPLC and RP-HPLC separations consisted of hexane (98.6%)/2-propanol (1.3%)/acetic acid (0.1%) and acetonitrile (49–100%)/water (0.0–49.9%)/acetic acid (0.1%), respectively. For RP-HPLC analysis, the acetonitrile concentration increased linearly from 15–70 min. Both NP-HPLC and RP-HPLC separations were done at a constant flow rate (1 mL/min) and ambient temperature. Product profiles were based on the retention times of authentic standards purchased from Cayman Chemical Co. or synthesized in our laboratory.

RPT isolation and incubations. RPT were isolated and purified from 1.5–2.0 kg female New Zealand White rabbits (Myrtle's Rabbitry, Thompson Station, TN) by the Percoll method of Rodeheaver *et al.* (1990). RPT were suspended in an incubation buffer containing (in mM) alanine, 1; dextrose, 5; heptanoate, 2; lactate, 4; malate, 5; NaCl, 115; $NaHCO_3$, 15; KCl, 5; NaH_2PO_4, 2; $MgSO_4$, 1; $CaCl_2$, 1; HEPES, 10 (pH 7.4, 295 mOsm/kg). All RPT suspensions contained approximately 1 mg cellular protein/mL and were incubated under normoxia (95% air/5% CO_2) or hypoxia (95% N_2/5% CO_2) at 37°C in a gyrating water bath (185 rpm).

Following a 15-min preincubation, DMSO (diluent, 0.05%), LA (100–500 μM), 9,10- and 12,13-EOA (100–500 μM), or 9,10- and 12,13-DHOA (100–500 μM) were added directly to RPT suspensions and incubated for various times (1–180 min). Addition of LA (500 μM) did not significantly alter the pH of the RPT suspension (data not shown). Mechanistic studies included the simultaneous addition of GLY (2 mM), DEF (500 μM), or DPPD (2 μM), and incubations continued for 3 h before assessment of cell death, as determined by lactate dehydrogenase release (see below). For all other experiments, aliquots were removed at indicated times for assessment of cell death, mitochondrial function, and active ion transport.

RPT LA metabolism studies were performed by adding a trace amount of [1-^{14}C]LA (2×10^6 cpm/10 mg cellular protein) with unlabeled LA (500 μM). An aliquot was removed and counted before extraction with ethyl ether to determine extraction efficiency. Both media and RPT were extracted three times with ethyl ether and the metabolic products were evaporated to dryness under N_2 and resolved using the RP-HPLC system described above.

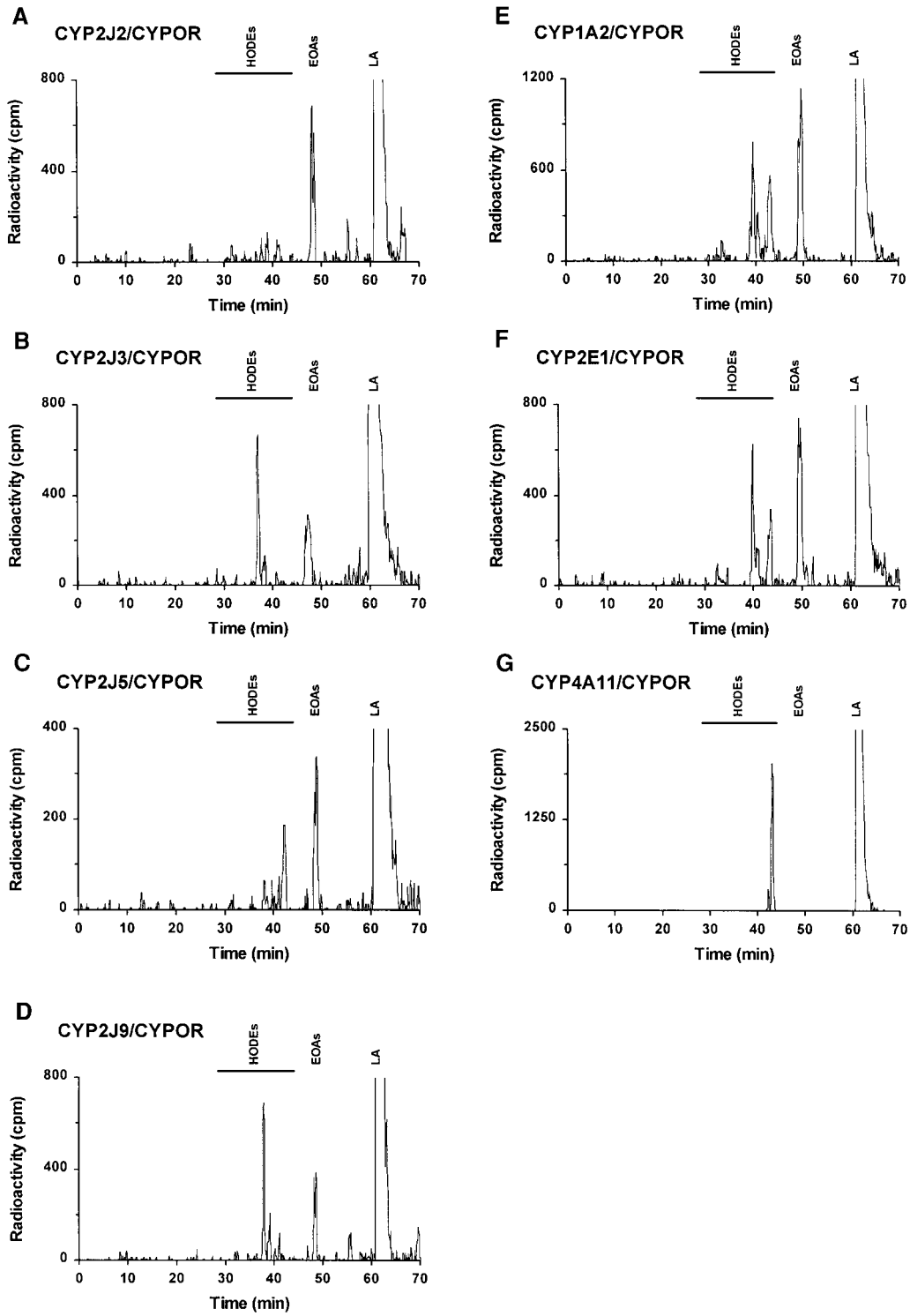

FIG. 1. RP-HPLC chromatographs of the organic soluble metabolites generated during incubation of Sf9 insect cell microsomes containing recombinant (A) CYP2J2, (B) CYP2J3, (C) CYP2J5, (D) CYP2J9, (E) CYP1A2, (F) CYP2E1, and (G) CYP4A11 and CYPOR with [^{14}C]LA. Chromatograms are representative of three to nine experiments. Incubation times varied from 10–180 min. The metabolites were separated into four major classes, LA, EOAs, HODEs, and DHOAs. Retention times are based on authentic standards and are approximately 61 min (LA), 49 min (9,10-EOA), 48 min (12,13-EOA), 38 min (9-HODE and 13-HODE), 23 min (9,10-DHOA), and 22 min (12,13-DHOA). Some unidentified metabolites were formed that elute in the HODE region (B–G). Incubation in the absence of NADPH and isocitrate dehydrogenase or with control Sf9 microsomes with [^{14}C]LA produced negligible metabolism and no specific products (data not shown).

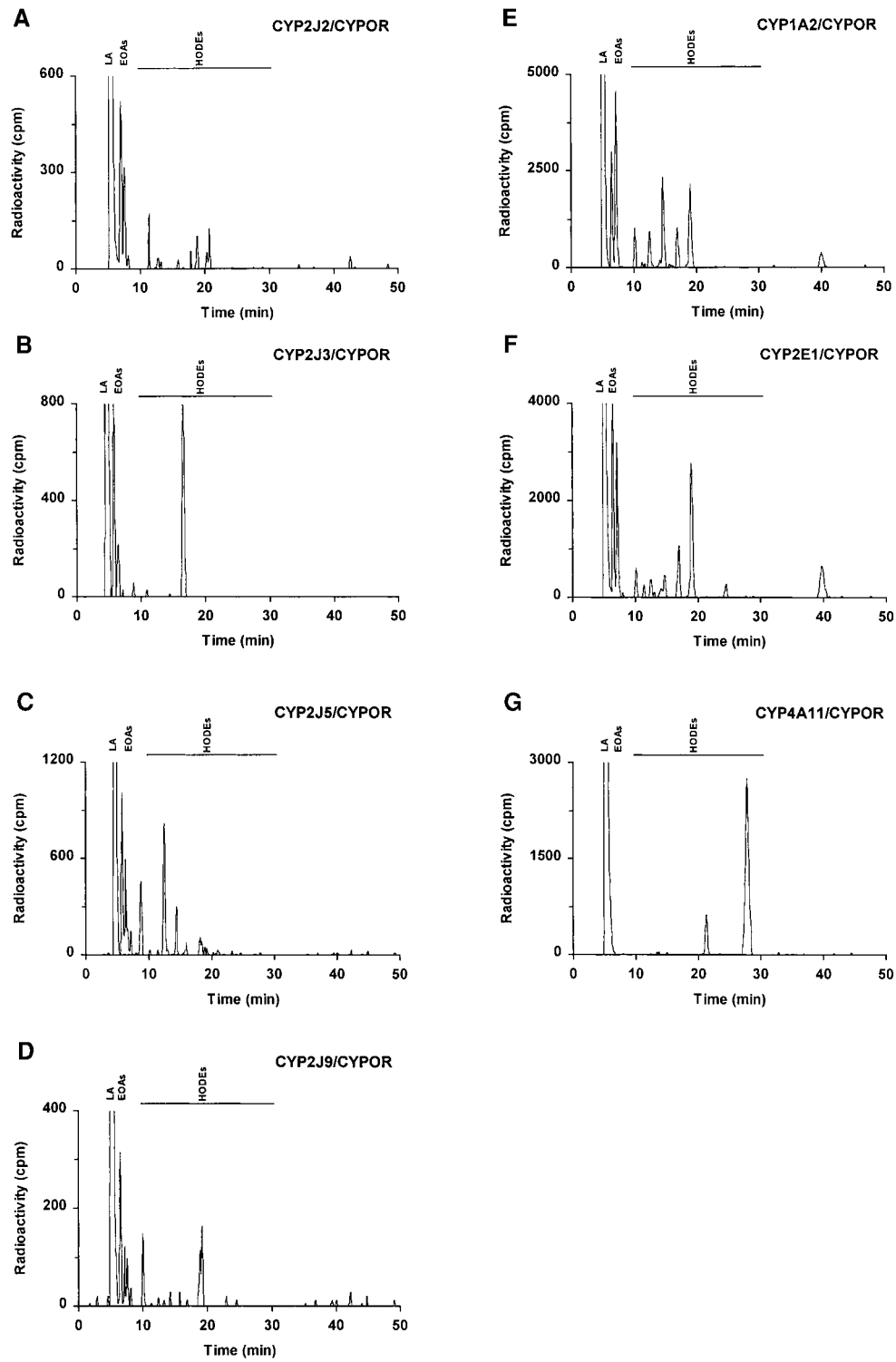

FIG. 2. NP-HPLC chromatograms of the organic soluble metabolites generated during incubation of Sf9 insect cell microsomes containing recombinant (A) CYP2J2, (B) CYP2J3, (C) CYP2J5, (D) CYP2J9, (E) CYP1A2, (F) CYP2E1, and (G) CYP4A11 and CYPOR with [^{14}C]LA. Incubation times varied from 10 to 180 min. The metabolites were separated into four major classes, LA, EOAs, HODEs, and DHOAs. Retention times are based on authentic standards and are approximately 5 min (LA), 7 min (12,13-EOA), 8 min (9,10-EOA), 14 min (13-HODE), and 23 min (9-HODE). Some unidentified metabolites were formed that elute in the HODE region (B–G).

TABLE 1
LA Metabolism by Sf9 Microsomes Coexpressing Recombinant Cytochrome P450s with CYPOR

	% HODEs	% EOAs	% 9,10-EOA	% 12,13-EOA	Rate pmol product/ (pmol P450 · min)
CYP1A2	54	46	61	39	5259 ± 449^{A}
CYP2E1	35	65	44	56	2643 ± 501^{B}
CYP2J2	ND	100	41	59	158 ± 36^{C}
CYP2J3	62	38	37	63	158 ± 22^{C}
CYP2J5	35	65	46	54	252 ± 52^{C}
CYP2J9	63	37	25	75	80 ± 34^{C}
CYP4A11	100	ND	ND	ND	208 ± 33^{C}

Note. Sf9 microsomes coexpressing the indicated recombinant P450 with CYPOR were incubated as described under Materials and Methods. Product profiles were determined from chromatograms seen in Fig. 2 and rate determinations are representative of three to seven experiments. Rate values are represented as means ± SE. Means with different superscripts are statistically different ($p < 0.05$), and some metabolites were not detected (ND).

Oxygen consumption. Oxygen consumption of RPT suspensions was measured using a Clark-type oxygen probe (Schnellmann, 1994). After basal oxygen consumption was determined, ouabain (100 μM), a specific inhibitor of Na^+/K^+-ATPase, was added to the RPT suspension to measure the ouabain-insensitive oxygen consumption. Ouabain-sensitive oxygen consumption, oxygen consumption associated with active Na^+ transport, was determined by taking the difference between basal and ouabain-insensitive oxygen consumption.

Biochemical assays. Cell death was measured by the release of LDH (Moran and Schnellmann, 1996). Protein content was determined as previously described (Bradford, 1976) or by using the BCA method as described by Pierce (Rockford, IL). P450 content was determined spectrally as described by Omura and Sato (1964) using a Shimadzu UV-3000 dual-wavelength/double-beam spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD).

Statistics. Data are presented as means ± SE and were analyzed by ANOVA. Multiple means were tested for significance using Student–Newman–Keuls test and a p value of <0.05.

RESULTS

Recombinant P450 metabolism of LA. Microsomal fractions prepared from Sf9 insect cells coexpressing CYP1A2, CYP2J2, CYP2J3, CYP2J5, CYP2J9, CYP2E1, or CYP4A11 and CYPOR metabolized LA to EOAs and HODEs as the principle products. We identified these metabolites by comparing RP-HPLC (Fig. 1) and NP-HPLC (Fig. 2) properties with those of authentic standards. None of the observed metabolites were formed in the absence of NADPH and isocitrate dehydrogenase or by using uninfected Sf9 insect cell microsomes. Based on the chromatographs in Figs. 1–2, we conclude that CYP2J2 and CYP4A11 have predominantly LA epoxygenase and LA hydroxylase activity, respectively. In contrast, CYP1A2, CYP2E1, CYP2J3, CYP2J5, and CYP2J9 have both LA epoxygenase and LA hydroxylase activities. CYP2J9 exhibited some regiospecificity and metabolized LA to predominantly 12,13-EOA (Table 1). None of the other P450s tested showed regiospecific epoxidation. The rates of LA metabolism reported in this study are comparable to previous reports of AA metabolism rates by various P450s (Hoch *et al.,* 2000; Ma *et al.,* 1999; Oliw *et al.,* 1996; Wu *et al.,* 1996, 1997) and LA metabolism rates by CYP2C P450s (Bylund *et al.,* 1998a,b; Draper and Hammock, 2000; Oliw *et al.,* 1996). Metabolic rates, percentage epoxidation vs. hydroxylation, and regiospecific epoxidation data are summarized in Table 1.

Toxicity of LA, LA monoepoxides, and LA diols to RPT. To examine the toxicity of LA, LA monoepoxides, and LA diols, RPT were exposed to different concentrations (100–500 μM) of LA, 9,10-EOA, 12,13-EOA, 9,10-DHOA, and 12,13-DHOA for 1–180 min. Control RPT cellular LDH release is approximately 10% following 3 h of exposure to DMSO in this model (Fig. 3). All tested compounds induced LDH release in a time- and concentration-dependent manner (Fig. 3). 12,13-EOA (500 μM) produced very rapid LDH release. Approximately 30% LDH release occurred within 30 min and continued to increase in a time-dependent manner for 3 h. However, at later time points LA appeared to be the most toxic and induced approximately 75% LDH release at 3 h. 9,10-EOA (500 μM), 12,13-EOA (500 μM), 9,10-DHOA (500 μM), and 12,13-DHOA (500 μM) were equally potent at 3 h and induced approximately 50% LDH release. Concentration-dependent studies suggested that LA, 9,10-EOA, and 12,13-EOA were the most potent of the tested compounds. Approximately 20% LDH release occurred with 3 h of exposure to LA (100 μM), 9,10-EOA (100 μM), and 12,13-EOA (100 μM), and approximately 60 and 45% LDH release occurred with 3 h of exposure to LA (333 μM) and the LA monoepoxides (333 μM), respectively (Fig. 3). Interestingly, RPT exposed to 9,10- and 12,13-DHOA (100–333 μM) for 3 h showed no statistical difference from control LDH release values.

Role of mitochondrial dysfunction in the toxicity of LA, LA monoepoxides, and LA diols. To determine whether mitochondria are the target of these fatty acids, RPT basal, ouabain-sensitive, and ouabain-insensitive respiration rates were measured. RPT basal respiration rates decreased approximately 50% (Fig. 4) after a 15-min exposure to 9,10-EOA (500 μM) and 12,13-EOA (500 μM). RPT basal respiration rates after a 15-min exposure to LA (500 μM), 9,10-DHOA (500 μM), and

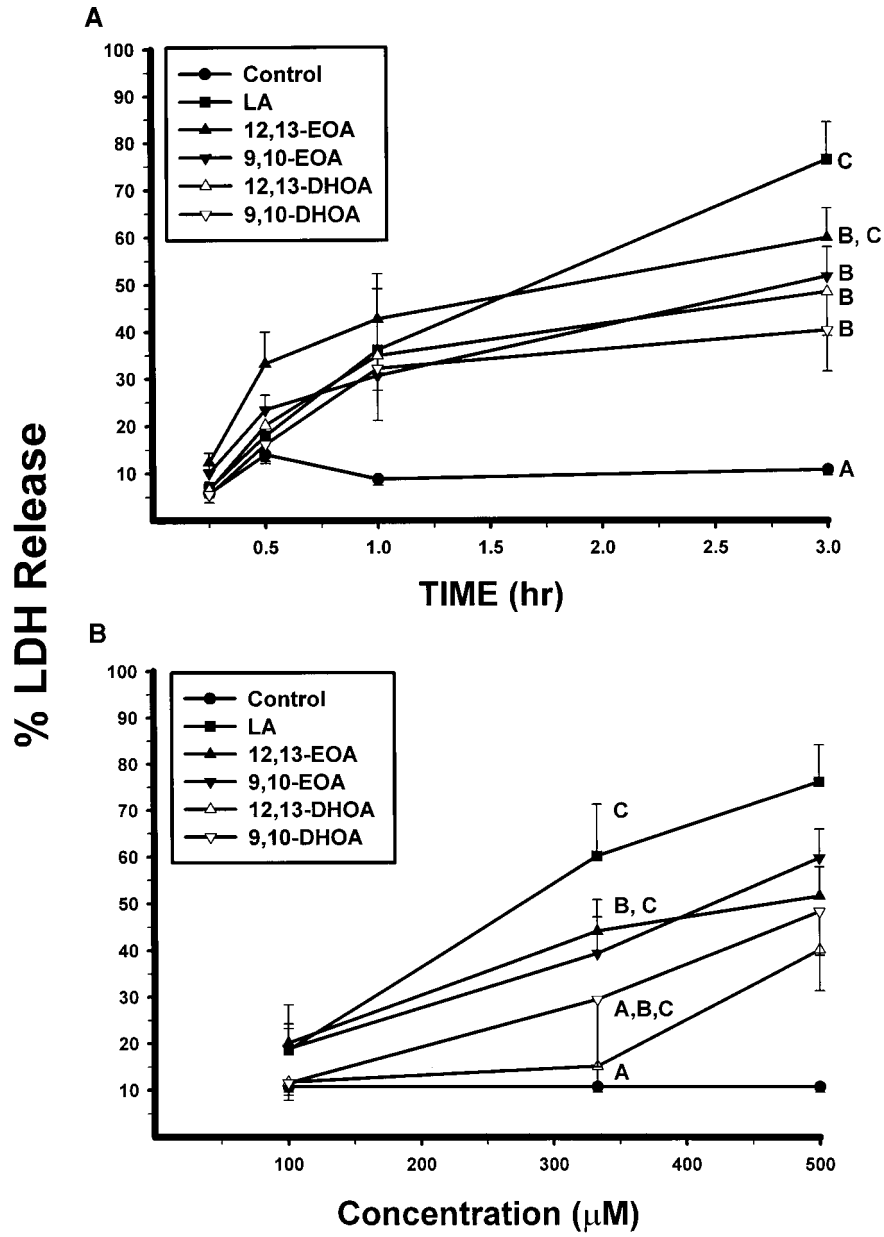

FIG. 3. (A) Time-dependent effects of LA (500 μM), 9,10-EOA (500 μM), 12,13-EOA (500 μM), 9,10-DHOA (500 μM), and 12,13-DHOA (500 μM) on RPT LDH release. (B) Concentration-dependent effects of LA, 9,10-EOA, 12,13-EOA, 9,10-DHOA, and 12,13-DHOA on RPT LDH release after 3 h of exposure. Data are represented as means ± SE (n = 3–19). Means with different superscripts are statistically different ($p < 0.05$).

12,13-DHOA were not statistically different from diluent-treated RPT.

Basal oxygen consumption can be divided into ouabain-sensitive oxygen consumption and ouabain-insensitive oxygen consumption. Ouabain-sensitive oxygen consumption is a direct result of Na^+/K^+ activity and is a marker of active Na^+ transport. Mitochondrial uncoupling results in a rapid increase in ouabain-insensitive oxygen consumption with a subsequent decrease (Schnellmann, 1994). LA (500 μM), 9,10-EOA (500 μM), and 12,13-EOA (500 μM) significantly decreased ouabain-sensitive oxygen consumption (53–67%) after 15 min of exposure (Fig. 4), whereas, 9,10-DHOA (500 μM) and 12,13-DHOA (500 μM) had minimal effects on ouabain-sensitive oxygen consumption. Ouabain-insensitive oxygen consumption was not affected after RPT were exposed to 9,10-DHOA (500 μM) and 12,13-DHOA (500 μM) for 15 min, but a 15-min exposure to LA (500 μM) produced a statistically significant increase in ouabain-insensitive oxygen consumption when compared to controls (Fig. 4). With exposure to 9,10-EOA (500 μM) and 12,13-EOA (500 μM), ouabain-insensitive oxygen consumption statistically increased within 1 min (Fig. 5) and, after 15 min, started decreasing (Fig. 4). The

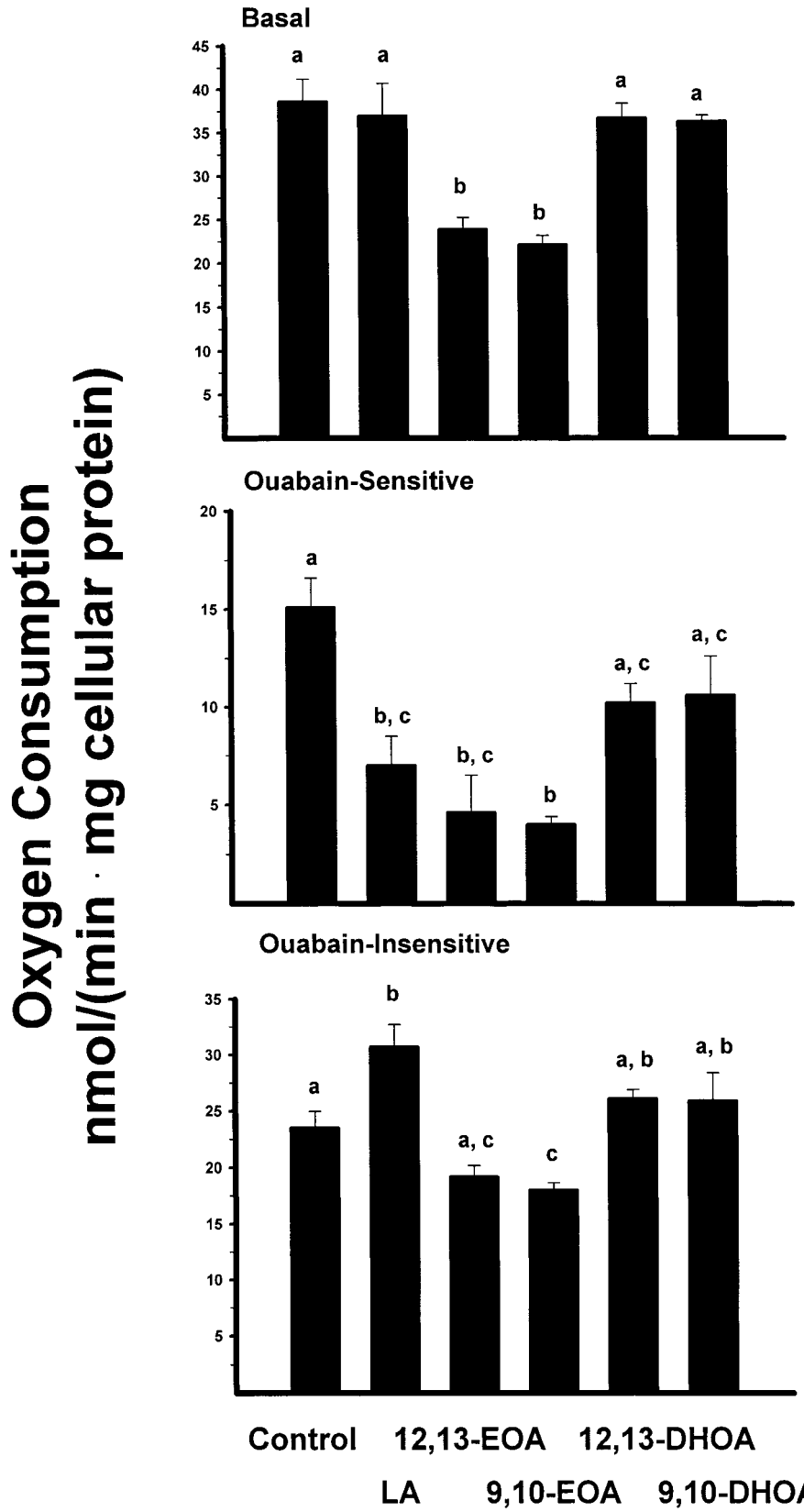

FIG. 4. The effect on RPT basal, ouabain-sensitive, and ouabain-insensitive oxygen consumption after a 15-min exposure to LA (500 μM), 9,10-EOA (500 μM), 12,13-EOA (500 μM), 9,10-DHOA (500 μM), and 12,13-DHOA (500 μM). Data are represented as means ± SE (n = 3–7). Means with different superscripts are statistically different ($p < 0.05$).

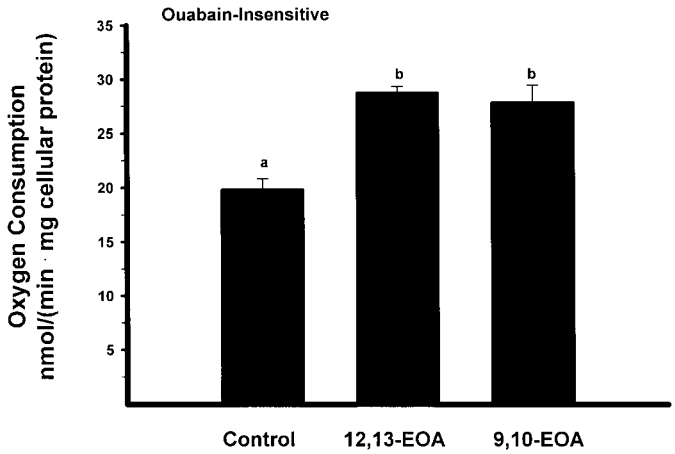

FIG. 5. The effect on ouabain-insensitive oxygen consumption after a 1-min exposure to 9,10-EOA (500 μM) and 12,13-EOA (500 μM). Data are represented as means ± SE (n = 3–4). Means with different superscripts are statistically different ($p < 0.05$).

decrease in ouabain-insensitive oxygen consumption after 15 min was only statistically significant for the 9,10-EOA (500 μM) treatment.

Affects of GLY, DEF, and DPPD on LA and LA monoepoxides-mediated toxicity. Various cytoprotectants were investigated to determine whether oxidative stress has a role in LA and LA monoepoxide mediated toxicity. GLY (2 mM), DEF (500 μM), and DPPD (2 μM) did not protect RPT exposed to LA (500 μM), 9,10-EOA (500 μM), and 12,13-EOA (500 μM) for 3 h from LDH release (Fig. 6). The concentrations used for these studies have been shown previously to protect against LDH release in the presence of mitochondrial toxicants (Miller *et al.,* 1994; Waters and Schnellmann, 1998) and chemical oxidants (Groves *et al.,* 1991; Schnellmann, 1988, 1991).

RPT metabolism of [^{14}C]-LA. To determine whether RPT metabolize LA, RPT were exposed to LA (500 μM) with a trace amount of [1-^{14}C]LA for 1 h and the metabolic products identified by HPLC. RPT metabolized LA approximately to 41% EOA/DHOA and 59% HODE metabolites (Fig. 7) at a rate of 51 pmol metabolites formed/(mg protein · min). In so far as DHOA formation must be preceded by EOA formation, the chromatogram in Fig. 7 demonstrates that rabbit RPT have LA epoxygenase and epoxide hydrolase activity.

DISCUSSION

P450 metabolism of AA is well documented and known to form many biologically active compounds (Carroll *et al.,* 1993; Force *et al.,* 1991; Harris *et al.,* 1990; Henrich *et al.,* 1990; Hirt *et al.,* 1989; Katoh *et al.,* 1991; Ma *et al.,* 1999; Madhun *et al.,* 1991; McGiff, 1991; McGiff *et al.,* 1993; Romero *et al.,* 1991; Sakairi *et al.,* 1995; Zou *et al.,* 1996). However, little is known about the overall importance of P450 metabolism of LA and about the relevance of LA-derived lipid mediators in kidney physiology/pathology. CYP2J2, CYP2J3, CYP2J5, CYP2J9, and CYP4A11 are highly expressed in kidney (Imaoka *et al.,* 1993; Ma *et al.,* 1999; Wu *et al.,* 1996, 1997) and metabolize LA at slower rates when compared to liver P450s, CYP1A2, and CYP2E1 (Duescher and Elfarra, 1994; Lieber, 1997; Nakajima *et al.,* 1994). Consistent with AA metabolism (Hoch *et al.,* 2000; Imaoka *et al.,* 1993; Ma *et al.,* 1999; Powell *et al.,* 1996; Wu *et al.,* 1996, 1997), the human P450s CYP2J2 and CYP4A11 exhibit primarily LA epoxygenase and LA hydroxylase activity, respectively. Interestingly, this study shows that CYP2E1 is a major LA epoxygenase; whereas, with AA, it exhibits primarily ω and ω-1 hydroxylase activity (Laethem *et al.,* 1993).

Analogous with EETs, LA monoepoxides have been shown to have biological activities such as causing vasodilation (Ishizaki *et al.,* 1995). LA monoepoxides also have been correlated with many pathological conditions (Fukushima *et al.,* 1988; Hanaki *et al.,* 1991; Hayakawa *et al.,* 1990; Ishizaki *et al.,* 1995; Sugiyama *et al.,* 1987). Despite many studies trying to elucidate the mechanism of LA-mediated toxicity, there remains controversy over the identity of the toxic intermediates and the enzymes involved in their production. 9,10- and 12,13-EOA have been correlated with the death of patients suffering from severe burns, adult respiratory distress syndrome, and systemic shock (Fukushima *et al.,* 1988; Kosaka *et al.,* 1994; Ozawa *et al.,* 1988; Sugiyama *et al.,* 1987). Ozawa *et al.* (1986) first showed 9,10-EOA to be a hepatic toxin in rat and suggested that the toxicity is due to the uncoupling of mitochondria. Sakai *et al.* (1995) subsequently showed that 9,10-EOA is also a rat lung toxin and inhibits mitochondrial respiration without uncoupling. Moran *et al.* (1997) and Moghaddam *et al.* (1997) suggested that the different mechanisms could be attributed to the ability of the organs to metabolize LA to LA epoxides and LA diols. Both studies suggested a sEH-dependent pathway and showed that methyl-DHOAs are toxic to rabbit RPT and Sf21 cells. Moghaddam *et al.* (1997) also reported similar results with free-acid derivatives in Sf21 cells. Consistent with rat hepatic studies, Moran *et al.* (1997) showed that methyl-DHOAs uncouple mitochondria in rabbit RPT prior to cell death. We, therefore, hypothesized that, like methyl-DHOAs, only 9,10-DHOA and 12,13-DHOA would induce mitochondrial dysfunction with subsequent cell death in rabbit RPT. This study demonstrates that LA, 9,10-EOA, 12,13-EOA, 9,10-DHOA, and 12,13-DHOA are all toxic at pathologically relevant concentrations (Kosaka *et al.,* 1994) and that LA and LA monoepoxides are the most potent. However, 9,10-DHOA and 12,13-DHOA are more potent than the methyl ester derivatives (Moran *et al.,* 1997). These data suggest that the response to this class of compounds is dependent on the chemical form (free acid vs. methyl ester) and the model used.

We examined several potential mechanisms for the toxicity induced by LA and LA monoepoxides and diols. The neutral

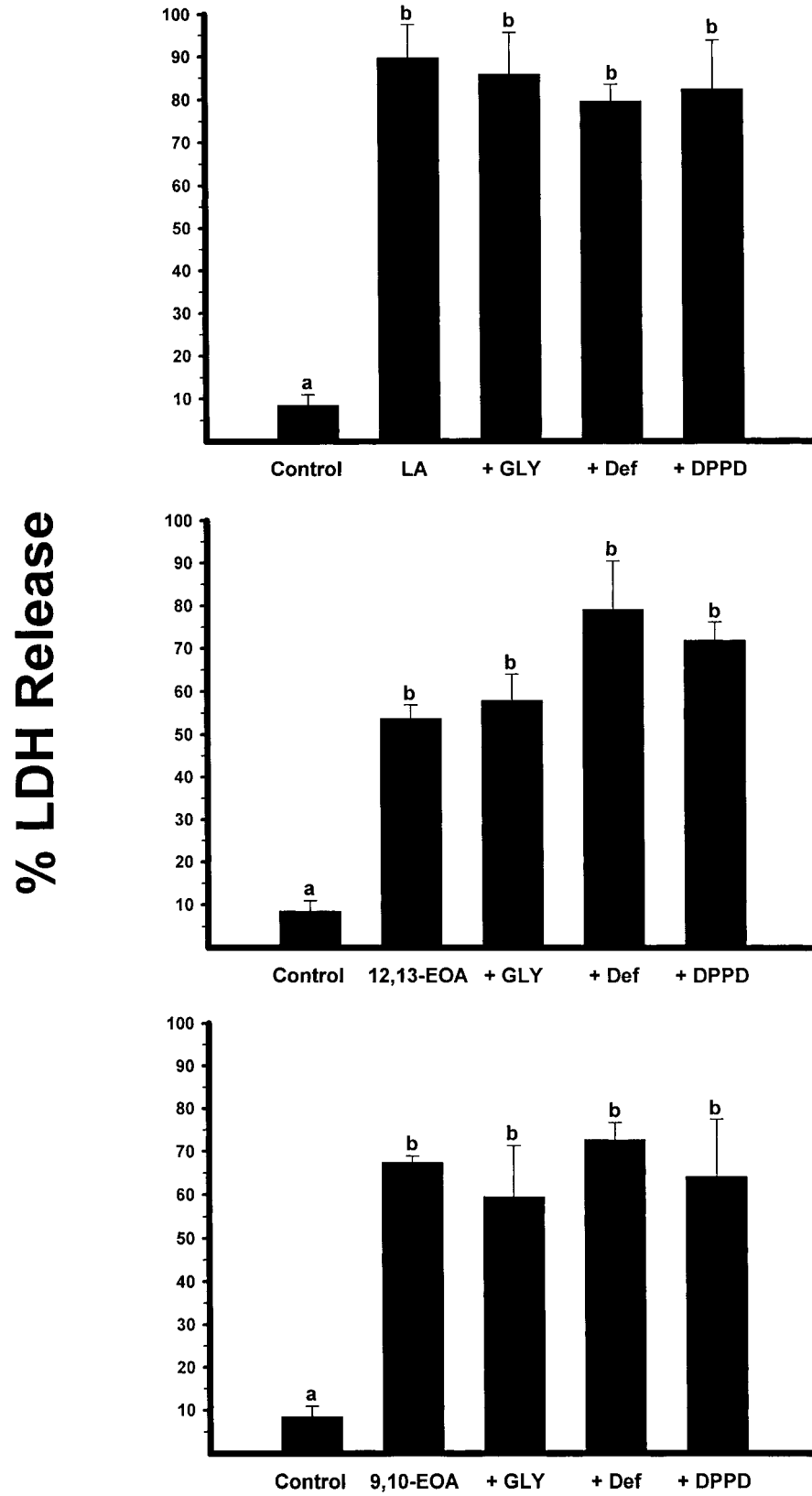

FIG. 6. The effect of the neutral amino acid GLY (2 mM), the iron chelator DEF (500 μM), and the antioxidant DPPD (2 μM) on RPT LDH release when exposed to LA (500 μM), 9,10-EOA (500 μM), and 12,13-EOA (500 μM) for 3 h. Data are represented as means $\pm$ SE ($n = 3$). Means with different superscripts are statistically different ($p < 0.05$).

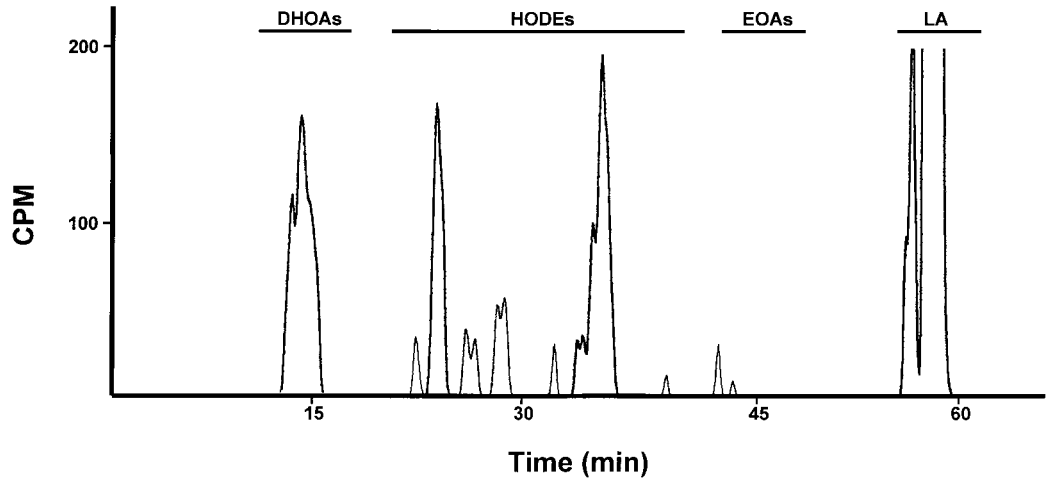

FIG. 7. RP-HPLC chromatogram showing the RPT metabolic products after 1 h of incubation with LA (500 μM) with a trace amount of [^{14}C]LA. Both media and RPT were extracted and total metabolism represented $<$1%. The three major products at 15, 25, and 32 min had retention times similar to DHOA and HODE authentic standards.

amino acid GLY, the iron chelator DEF, and the antioxidant DPPD have been shown to protect RPT from oxidative injury induced by chemical oxidants (Groves *et al.*, 1991; Schnellmann, 1988, 1991) and other mitochondrial toxicants (Miller *et al.*, 1994; Waters and Schnellmann, 1998). These cytoprotectants did not have any effect on RPT cell death when exposed to LA, 9,10-EOA, and 12,13-EOA, suggesting the toxicity is not due to oxidative stress. Our data suggest that the toxicity of these compounds is a direct result of disruption of mitochondrial function. The inability of GLY to protect against these mitochondrial toxins suggests that the mechanism of toxicity is different from model mitochondrial toxicants, antimycin A, rotenone, and hypoxia, which are commonly used to study acute renal failure.

The increase in ouabain-insensitive oxygen consumption seen with exposures to LA and LA monoepoxides suggests uncoupling of mitochondria (Schnellmann, 1994) and is consistent with rat liver mitochondria studies (Ozawa *et al.*, 1986) and rabbit RPT studies using methyl-DHOAs (Moran *et al.*, 1997). The decrease in ouabain-sensitive oxygen consumption is likely due to the decrease in ATP secondary to mitochondrial uncoupling. The rate at which RPT metabolize LA (Fig. 7) suggests that the toxicity is due to the parent compound because at the observed rate, 51 pmol metabolites formed/(mg protein · min), the concentration of epoxide/diol formed in 1 h would be approximately 1 μM. However, intracellular formation of epoxide/diol (1 μM) may be adequate to induce mitochondrial dysfunction/cell death. Analysis of time course and mitochondrial function studies shows that EOAs inhibit mitochondrial function and induce cell death more rapidly than LA. The more water-soluble DHOAs are the least toxic when administered extracellularly, which may be a reflection of the ability of these compounds to cross the plasma membrane and exert toxic effects on mitochondria. Although these data suggest specific mitochondrial effects, we cannot rule out other nonspecific effects. Our toxicity data are very consistent with previous studies showing the uncoupling properties of LA in isolated mitochondria (Jarmuszkiewicz *et al.*, 2000; Pastore *et al.*, 2000). Future studies using isolated mitochondria from rabbit RPT will determine if the effects of these metabolites are exerted directly on mitochondria.

In summary, we have shown that P450s in the CYP1 and CYP2 gene families metabolize LA to 9,10-EOA and 12,13-EOA. Also, we found that LA, 9,10-EOA, 12,13-EOA, 9,10-DHOA, and 12,13-DHOA are cytotoxic compounds at high but pathologically relevant concentrations (Kosaka *et al.*, 1994) and that the free-acid forms are more toxic than the methyl esters (Moran *et al.*, 1997). The toxic effects of these compounds appear to be mediated through mitochondrial dysfunction with subsequent loss of ion transport and cell death, and based on previous reports, is similar to the mechanism of methyl-DHOAs in rabbit RPT (Moran *et al.*, 1997). However, we show important differences between our studies using free acids and those done previously with the methyl esters. We have found no evidence that oxidative stress plays a significant role in the toxicity of these compounds. The relative toxic potencies of these compounds are as follows: LA $\geq$ LA monoepoxides $>$ LA diols. Therefore, metabolism of LA monoepoxides via sEH to the vicinal LA diols may represent a detoxification rather than an activation pathway in rabbit RPT.

ACKNOWLEDGMENTS

The authors thank Anthony Jude, Jay Harriman, and Xiuli Liu for technical assistance, and Tom Eling and Joyce Goldstein for helpful comments during the preparation of this manuscript. This work was supported in part by the Division of Intramural Research, NIEHS/NIH, and NIH Grants GM56708 (D.F.G.) and ES09129 (R.G.S.). Portions of this work were presented at the Society of Toxicology annual meeting (2000).

REFERENCES

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254.

Bylund, J., Ericsson, J., and Oliw, E. H. (1998a). Analysis of cytochrome P450 metabolites of arachidonic and linoleic acids by liquid chromatography-mass spectrometry with ion trap MS. *Anal. Biochem.* **265,** 55–68.

Bylund, J., Kunz, T., Valmsen, K., and Oliw, E. H. (1998b). Cytochromes P450 with bisallylic hydroxylation activity on arachidonic and linoleic acids studied with human recombinant enzymes and with human and rat liver microsomes. *J. Pharmacol. Exp. Ther.* **284,** 51–60.

Capdevila, J. H., Dishman, E., Karara, A., and Falck, J. R. (1991). Cytochrome P450 arachidonic acid epoxygenase: Stereochemical characterization of epoxyeicosatrienoic acids. *Methods Enzymol.* **206,** 441–453.

Carroll, M. A., Balazy, M., Margiotta, P., Falck, J. R., and McGiff, J. C. (1993). Renal vasodilator activity of 5,6-epoxyeicosatrienoic acid depends upon conversion by cyclooxygenase and release of prostaglandins. *J. Biol. Chem.* **268,** 12260–12266.

Draper, A. J., and Hammock, B. D. (2000). Identification of CYP2C9 as a human liver microsomal linoleic acid epoxygenase. *Arch. Biochem. Biophys.* **376,** 199–205.

Duescher, R. J., and Elfarra, A. A. (1994). Human liver microsomes are efficient catalysts of 1,3-butadiene oxidation: Evidence for major roles by cytochromes P450 2A6 and 2E1. *Arch. Biochem. Biophys.* **311,** 342–349.

Force, T., Hyman, G., Hajjar, R., Sellmayer, A., and Bonventre, J. V. (1991). Noncyclooxygenase metabolites of arachidonic acid amplify the vasopressin-induced Ca^{2+} signal in glomerular mesangial cells by releasing Ca^{2+} from intracellular stores. *J. Biol. Chem.* **266,** 4295–4302.

Fukushima, A., Hayakawa, M., Sugiyama, S., Ajioka, M., Ito, T., Satake, T., and Ozawa, T. (1988). Cardiovascular effects of leukotoxin (9,10-epoxy-12-octadecenoate) and free fatty acids in dogs. *Cardiovasc. Res.* **22,** 213–218.

Groves, C. E., Lock, E. A., and Schnellmann, R. G. (1991). Role of lipid peroxidation in renal proximal tubule cell death induced by haloalkene cysteine conjugates. *Toxicol. Appl. Pharmacol.* **107,** 54–62.

Hanaki, Y., Kamiya, H., Ohno, M., Hayakawa, M., Sugiyama, S., and Ozawa, T. (1991). Leukotoxin, 9,10-epoxy-12-octadecenoate: A possible responsible factor in circulatory shock and disseminated intravascular coagulation. *Jpn. J. Med.* **30,** 224–228.

Harris, R. C., Homma, T., Jacobson, H. R., and Capdevila, J. (1990). Epoxyeicosatrienoic acids activate Na^+/H^+ exchange and are mitogenic in cultured rat glomerular mesangial cells. *J. Cell Physiol.* **144,** 429–437.

Hayakawa, M., Kosaka, K., Sugiyama, S., Yokoo, K., Aoyama, H., Izawa, Y., and Ozawa, T. (1990). Proposal of leukotoxin, 9,10-epoxy-12-octadecenoate, as a burn toxin. *Biochem. Int.* **21,** 573–579.

Henrich, W. L., Falck, J. R., and Campbell, W. B. (1990). Inhibition of renin release by 14,15-epoxyeicosatrienoic acid in renal cortical slices. *Am. J. Physiol.* **258,** E269–E274.

Hirt, D. L., Capdevila, J., Falck, J. R., Breyer, M. D., and Jacobson, H. R. (1989). Cytochrome P450 metabolites of arachidonic acid are potent inhibitors of vasopressin action on rabbit cortical collecting duct. *J. Clin. Invest.* **84,** 1805–1812.

Hoch, U., Zhang, Z., Kroetz, D. L., and Ortiz de Montellano, P. R. (2000). Structural determination of the substrate specificities and regioselectivities of the rat and human fatty acid omega-hydroxylases. *Arch. Biochem. Biophys.* **373,** 63–71.

Hoebel, B. G., Steyrer, E., and Graier, W. F. (1998). Origin and function of epoxyeicosatrienoic acids in vascular endothelial cells: More than just endothelium-derived hyperpolarizing factor? *Clin. Exp. Pharmacol. Physiol.* **25,** 826–830.

Imaoka, S., Ogawa, H., Kimura, S., and Gonzalez, F. J. (1993). Complete cDNA sequence and cDNA-directed expression of CYP4A11, a fatty acid omega-hydroxylase expressed in human kidney. *DNA Cell Biol.* **12,** 893–899.

Ishizaki, T., Takahashi, H., Ozawa, T., Chang, S. W., and Voelkel, N. F. (1995). Leukotoxin, 9,10-epoxy-12-octadecenoate causes pulmonary vasodilation in rats. *Am. J. Physiol.* **268,** L123–L128.

Jarmuszkiewicz, W., Almeida, A. M., Vercesi, A. E., Sluse, F. E., and Sluse-Goffart, C. M. (2000). Proton Re-uptake partitioning between uncoupling protein and ATP synthase during benzohydroxamic acid-resistant state 3 respiration in tomato fruit mitochondria. *J. Biol. Chem.* **275,** 13315–13320.

Katoh, T., Takahashi, K., Capdevila, J., Karara, A., Falck, J. R., Jacobson, H. R., and Badr, K. F. (1991). Glomerular stereospecific synthesis and hemodynamic actions of 8,9-epoxyeicosatrienoic acid in rat kidney. *Am. J. Physiol.* **261,** F578–F586.

Kosaka, K., Suzuki, K., Hayakawa, M., Sugiyama, S., and Ozawa, T. (1994). Leukotoxin, a linoleate epoxide: Its implication in the late death of patients with extensive burns. *Mol. Cell Biochem.* **139,** 141–148.

Laethem, R. M., Balazy, M., Falck, J. R., Laethem, C. L., and Koop, D. R. (1993). Formation of 19(S)-, 19(R)-, and 18(R)-hydroxyeicosatetraenoic acids by alcohol-inducible cytochrome P450 2E1. *J. Biol. Chem.* **268,** 12912–12918.

Lieber, C. S. (1997). Cytochrome P-4502E1: Its physiological and pathological role. *Physiol. Rev.* **77,** 517–544.

Ma, J., Qu, W., Scarborough, P. E., Tomer, K. B., Moomaw, C. R., Maronpot, R., Davis, L. S., Breyer, M. D., and Zeldin, D. C. (1999). Molecular cloning, enzymatic characterization, developmental expression, and cellular localization of a mouse cytochrome P450 highly expressed in kidney. *J. Biol. Chem.* **274,** 17777–17788.

Madhun, Z. T., Goldthwait, D. A., McKay, D., Hopfer, U., and Douglas, J. G. (1991). An epoxygenase metabolite of arachidonic acid mediates angiotensin II-induced rises in cytosolic calcium in rabbit proximal tubule epithelial cells. *J. Clin. Invest.* **88,** 456–461.

McGiff, J. C. (1991). Cytochrome P-450 metabolism of arachidonic acid. *Annu. Rev. Pharmacol. Toxicol.* **31,** 339–369.

McGiff, J. C., and Carroll, M. A. (1991). Cytochrome P450-dependent arachidonate metabolites, renal function and blood pressure regulation. *Adv. Prostaglandin Thromboxane Leukotriene Res.* 675–682.

McGiff, J. C., Carroll, M. A., and Escalante, B. (1991). Arachidonate metabolites and kinins in blood pressure regulation. *Hypertension* **18,** III150–III157.

McGiff, J. C., Quilley, C. P., and Carroll, M. A. (1993). The contribution of cytochrome P450-dependent arachidonate metabolites to integrated renal function. *Steroids* **58,** 573–579.

Miller, G. W., Lock, E. A., and Schnellmann, R. G. (1994). Strychnine and glycine protect renal proximal tubules from various nephrotoxicants and act in the late phase of necrotic cell injury. *Toxicol. Appl. Pharmacol.* **125,** 192–197.

Moghaddam, M. F., Grant, D. F., Cheek, J. M., Greene, J. F., and Hammock, B. D. (1997). Bioactivation of leukotoxins to their toxic diols by epoxide hyrolase. *Nature Med.* **3,** 562–566.

Moran, J. H., and Schnellmann, R. G. (1996). A rapid beta-NADH-linked fluorescence assay for lactate dehydrogenase in cellular death. *J. Pharmacol. Toxicol. Meth.* **36,** 41–44.

Moran, J. H., Weise, R., Schnellmann, R. G., Freeman, J. P., and Grant, D. F. (1997). Cytotoxicity of linoleic acid diols to renal proximal tubular cells. *Toxicol. Appl. Pharmacol.* **146,** 53–59.

Nakajima, T., Elovaara, E., Gonzalez, F. J., Gelboin, H. V., Raunio, H., Pelkonen, O., Vainio, H., and Aoyama, T. (1994). Styrene metabolism by

cDNA-expressed human hepatic and pulmonary cytochromes P450. *Chem. Res. Toxicol.* **7,** 891–896.

Node, K., Huo, Y., Ruan, X., Yang, B., Spiecker, M., Ley, K., Zeldin, D. C., and Liao, J. K. (1999). Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. *Science* **285,** 1276–1279.

Oliw, E. H., Bylund, J., and Herman, C. (1996). Bisallylic hydroxylation and epoxidation of polyunsaturated fatty acids by cytochrome P450. *Lipids* **31,** 1003–1021.

Omura, T., and Sato, R. (1964). The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification, and properties. *J. Biol. Chem.* **239,** 2379–2385.

Ozawa, T., Hayakawa, M., Takamura, T., Sugiyama, S., Suzuki, K., Iwata, M., Taki, F., and Tomita, T. (1986). Biosynthesis of leukotoxin, 9,10-epoxy-12-octadecenoate, by leukocytes in lung lavages of rat after exposure to hyperoxia. *Biochem. Biophys. Res. Commun.* **134,** 1071–1078.

Ozawa, T., Sugiyama, S., Hayakawa, M., Satake, T., Taki, F., Iwata, M., and Taki, K. (1988). Existence of leukotoxin 9,10-epoxy-12-octadecenoate in lung lavages from rats breathing pure oxygen and from patients with the adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* **137,** 535–540.

Pastore, D., Fratianni, A., Di Pede, S., and Passarella, S. (2000). Effects of fatty acids, nucleotides and reactive oxygen species on durum wheat mitochondria. *FEBS Lett.* **470,** 88–92.

Powell, P. K., Wolf, I., and Lasker, J. M. (1996). Identification of CYP4A11 as the major lauric acid omega-hydroxylase in human liver microsomes. *Arch. Biochem. Biophys.* **335,** 219–226.

Quilley, J., Fulton, D., and McGiff, J. C. (1997). Hyperpolarizing factors. *Biochem. Pharmacol.* **54,** 1059–1070.

Rodeheaver, D. P., Aleo, M. D., and Schnellmann, R. G. (1990). Differences in enzymatic and mechanical isolated rabbit renal proximal tubules: Comparison in long-term incubation. *In Vitro Cell Dev. Biol.* **26,** 898–904.

Romero, M. F., Hopfer, U., Madhun, Z. T., Zhou, W., and Douglas, J. G. (1991). Angiotensin II actions in the rabbit proximal tubule. Angiotensin II mediated signaling mechanisms and electrolyte transport in the rabbit proximal tubule. *Ren. Physiol. Biochem.* **14,** 199–207.

Rudolph, J., Reddy, K. L., Chiang, J. P., and Sharpless, K. B. (1997). Highly efficient epoxidation of olefins using aqueous H_2O_2 and catalytic methyltrioxorhenium/pyridine: pyridine-mediated ligand acceleration. *J. Am. Chem. Soc.* **119,** 6189–6190.

Sakai, T., Ishizaki, T., Ohnishi, T., Sasaki, F., Ameshima, S., Nakai, T., Miyabo, S., Matsukawa, S., Hayakawa, M., and Ozawa, T. (1995). Leukotoxin, 9,10-epoxy-12-octadecenoate inhibits mitochondrial respiration of isolated perfused rat lung. *Am. J. Physiol.* **269,** L326–L331.

Sakairi, Y., Jacobson, H. R., Noland, T. D., Capdevila, J. H., Falck, J. R., and Breyer, M. D. (1995). 5,6-EET inhibits ion transport in collecting duct by stimulating endogenous prostaglandin synthesis. *Am. J. Physiol.* **268,** F931–F939.

Schnellmann, R. G. (1988). Mechanisms of *t*-butyl hydroperoxide-induced toxicity to rabbit renal proximal tubules. *Am. J. Physiol.* **255,** C28–C33.

Schnellmann, R. G. (1991). Intracellular calcium chelators and oxidant-induced renal proximal tubule cell death. *J. Biochem. Toxicol.* **6,** 299–303.

Schnellmann, R. G. (1994). Measurement of oxygen consumption. In *Methods in Toxicology, Part B, In Vitro Toxicity Indicators* (C. A. Tyson and J. M. Frazier, Eds.), pp. 128–139. Academic Press, San Diego.

Stimers, J. R., Dobretsov, M., Hastings, S. L., Jude, A. R., and Grant, D. F. (1999). Effects of linoleic acid metabolites on electrical activity in adult rat ventricular myocytes. *Biochim. Biophys. Acta* **1438,** 359–368.

Sugiyama, S., Hayakawa, M., Nagai, S., Ajioka, M., and Ozawa, T. (1987). Leukotoxin, 9,10-epoxy-12-octadecenoate, causes cardiac failure in dogs. *Life Sci.* **40,** 225–231.

Waters, S. L., and Schnellmann, R. G. (1998). Examination of the mechanisms of action of diverse cytoprotectants in renal cell death. *Toxicol. Pathol.* **26,** 58–63.

Wu, S., Chen, W., Murphy, E., Gabel, S., Tomer, K. B., Foley, J., Steenbergen, C., Falck, J. R., Moomaw, C. R., and Zeldin, D. C. (1997). Molecular cloning, expression, and functional significance of a cytochrome P450 highly expressed in rat heart myocytes. *J. Biol. Chem.* **272,** 12551–12559.

Wu, S., Moomaw, C. R., Tomer, K. B., Falck, J. R., and Zeldin, D. C. (1996). Molecular cloning and expression of CYP2J2, a human cytochrome P450 arachidonic acid epoxygenase highly expressed in heart. *J. Biol. Chem.* **271,** 3460–3468.

Zeldin, D. C., DuBois, R. N., Falck, J. R., and Capdevila, J. H. (1995). Molecular cloning, expression and characterization of an endogenous human cytochrome P450 arachidonic acid epoxygenase isoform. *Arch. Biochem. Biophys.* **322,** 76–86.

Zou, A. P., Fleming, J. T., Falck, J. R., Jacobs, E. R., Gebremedhin, D., Harder, D. R., and Roman, R. J. (1996). 20-HETE is an endogenous inhibitor of the large-conductance Ca(2+)- activated K+ channel in renal arterioles. *Am. J. Physiol.* **270,** R228–R237.