0026-895X/92/061020-07$03.00/0

MOLECULAR PHARMACOLOGY, 42:1020-1026

Immunochemical Identity of the 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin- and β-Naphthoflavone-Induced Cytochrome P-450 Arachidonic Acid Epoxygenases in Chick Embryo Liver: Distinction from the ω-Hydroxylase and the Phenobarbital-Induced Epoxygenase

AKIO KANETOSHI,[1] A. MICHELE WARD, BRIAN K. MAY, and ARLEEN B. RIFKIND

Departments of Pharmacology (A.K., A.M.W., A.B.R.) and Medicine (A.B.R.), Cornell University Medical College, New York, New York 10021, and the Department of Biochemistry, University of Adelaide, Adelaide, South Australia (B.K.M.)

Received June 2, 1992; Accepted September 22, 1992

SUMMARY

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), β-naphthoflavone (βNF), and phenobarbital (PB) cause marked induction of cytochrome P-450 (P-450)-mediated arachidonic acid metabolism in chick embryo liver. We show here that the P-450 arachidonic acid epoxygenases induced by TCDD and βNF are immunochemically indistinguishable from each other and unrelated to the arachidonic acid epoxygenase induced by PB. On Western blots, IgG from an antiserum against βNF_{AA}, a 55-kDa P-450 arachidonic acid epoxygenase purified from βNF-treated chick embryo liver, immunoreacted selectively and to the same extent with a 55-kDa band in liver microsomes from chick embryos treated with TCDD or βNF. It failed to react with proteins from untreated, solvent-treated, or PB-treated embryos on immunoblots or to immunoinhibit PB-induced arachidonic acid metabolism. Anti-βNF_{AA} IgG immunoinhibited all arachidonic acid metabolism by reconstituted βNF_{AA} and formation of arachidonic epoxides (EETs) and monohydroxylated derivatives (HETEs) by microsomes from TCDD- and βNF-treated livers; it did not inhibit ω-hydroxylation. In contrast, IgG from an antiserum against the major PB-induced chicken P-450s, 2H1 and 2H2, immunoreacted with two major PB-induced P-450s, of 48 and 49 kDa, on Western blots. It also immunoinhibited formation of EETs and HETEs by PB-treated microsomes entirely and ω-hydroxylation by 50%. It failed to react with TCDD- or βNF-induced P-450s on Western blots or to immunoinhibit TCDD- or βNF-induced arachidonic acid metabolism. Because other P-450s with which anti-βNF_{AA} and anti-PB IgG cross-reacted were inactive in arachidonic acid epoxygenation, the findings are consistent with βNF_{AA} being principally responsible for the epoxygenation induced by TCDD and βNF and 2H1 and/or 2H2 being responsible for epoxygenation induced by PB. Further, the P-450 arachidonate ω-hydroxylase and the epoxygenase in livers of TCDD- or βNF-treated embryos are immunochemically unrelated, whereas those in livers of PB-treated embryos may be partly related.

Both *Ah* receptor-dependent P-450 inducers, such as the environmental toxin TCDD and βNF, and an *Ah* receptor-independent inducer, PB, increase P-450-mediated arachidonic acid metabolism in chick embryo liver (1). Although TCDD was 10^6 times more potent than PB and 2×10^5 times more potent than βNF (1), at sufficiently high doses both classes of inducers achieved comparable degrees of induction, increasing formation of arachidonic acid epoxides (EETs) and HETEs by 10-fold or more.

Recent studies with reconstituted P-450s indicated that different P-450s catalyze the enhanced arachidonic acid metabolism in response to βNF and PB treatment (2). The highest arachidonic acid epoxygenase activity among P-450s in livers from βNF-treated chick embryos was exhibited by a 55-kDa P-450 (βNF_{AA}); this P-450 was purified. The highest epoxygenase activity among P-450s from PB-treated livers was found in partially purified fractions containing principally the major PB-induced chicken P-450s 2H1 and 2H2 (3), based on amino-terminal amino acid sequence data.

In this paper, we present immunochemical evidence that βNF_{AA} is primarily responsible for the induced arachidonic acid metabolism in microsomes from both TCDD- and βNF-treated chick embryo livers and that the P-450 that catalyzes the

This work was supported by United States Public Health Service Grant ES-03606.

[1] Present address: Hokkaido Institute of Public Health, Sapporo, 060, Japan.

ABBREVIATIONS: *Ah*, aryl hydrocarbon; BSA, bovine serum albumin; EET, epoxyeicosatrienoic acid; HETE, monohydroxyeicosatetraenoic acid, βNF, β-naphthoflavone; PB, phenobarbital; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; GC/MS, gas chromatography/mass spectrometry; P-450, cytochrome P-450; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

induced arachidonic acid epoxygenation in PB-treated livers is immunochemically unrelated to βNF_{AA}. Further, the P-450s from TCDD-treated livers catalyzing ω-hydroxylation and epoxygenation of arachidonic acid are shown to be immunochemically unrelated, whereas those from PB-treated livers may be partially related.

Experimental Procedures

Materials. Sources of materials were as follows: acrylamide, *N,N'*-methylene-bisacrylamide, and SDS, Bio-Rad Laboratories (Richmond, CA); nitrocellulose membranes (0.45 μm), Hoefer Scientific Instruments (San Francisco, CA); DE-52 cellulose, Whatman BioSystems Ltd. (Kent, England); BSA (fraction V), anti-rabbit IgG (whole molecule) peroxidase-linked goat IgG, molecular weight markers, NADPH, and Tween 20, Sigma Chemical Co. (St. Louis, MO); [1-^{14}C]arachidonic acid (specific activity, 52.0 mCi/mmol), NEN Research Products (Boston, MA); unlabeled arachidonic acid, Nu-Check Prep (Elysian, MN); βNF, Aldrich Chemical Co., Inc. (Milwaukee, WI); and HPLC-grade organic solvents, VWR Scientific (Piscataway, NJ). TCDD was a gift from the Monsanto Chemical Co. (St. Louis, MO) and sodium PB from Dr. Michiko Okamoto (Cornell University Medical College, New York, NY).

Treatment of chick embryos and preparation of microsomes. Embryonated chicken eggs (White Leghorn strain) were obtained from H n'R Poultry Farms (Cochecton, NY) and were incubated at 37°. Sixteen-day-old embryos were treated with βNF (6.7 mg in 0.1 ml of dimethylsulfoxide), TCDD [1 nmol (322 ng) in 0.005 ml of dioxane], or sodium PB (20 mg in 0.4 ml of distilled water) by injection through a hole in the shell into the fluids surrounding the embryo. The doses used maximally induced total chick embryo liver P-450 (4, 5). Controls were untreated or received solvent alone. After 48 hr microsomes were prepared as described previously (4, 5). Usually, livers receiving the same treatment were pooled (10–12 eggs/group).

Sources of anti-βNF_{AA} IgG and anti-PB IgG. Polyclonal antibodies were raised in rabbits (Pocono Rabbit Farms, Canadensis, PA) against βNF_{AA}, a 55-kDa P-450 purified from microsomes from livers of βNF-treated chick embryos as previously described in detail (2). Two rabbits were each injected intradermally with 100 μg of βNF_{AA} in complete Freund's adjuvant. Boosters were given subcutaneously in incomplete Freund's adjuvant, 5 μg/week for the first 2 weeks and then 20 μg biweekly. Preimmune serum was obtained before injection and immune serum was obtained at biweekly intervals from 6 weeks after the first immunization. Antisera titers were screened by immunoblotting against the antigen. IgG was prepared from the highest titer antisera (9 weeks after immunization) by ammonium sulfate precipitation and DE52 cellulose chromatography (6). IgG from both rabbits gave essentially the same results. Anti-PB IgG was prepared in the same manner from a rabbit antiserum against a broad band at about 50 kDa from liver microsomes from 17-day-old chick embryos that had been treated for 48 hr with 3 mg of allylisopropylacetamide (7). This drug, like PB, induces formation of P-450s 2H1 and 2H2 (3).

SDS-PAGE and immunoblotting. Discontinuous SDS-PAGE (8) was performed on slab gels (14.5 cm^2 × 1.0 mm) at 20 mA for a distance of 10 cm from the stacking gel (2 cm); 3% and 7.5% acrylamide were in the stacking and separating gels, respectively. Molecular weight markers included phosphorylase *b* (97,400), BSA (66,000), pyruvate kinase (chicken muscle) (58,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), and carbonic anhydrase (29,000). Protein staining was with Coomassie blue.

Western immunoblotting was carried out by the method of Towbin *et al.* (9). Proteins were transferred from SDS-polyacrylamide gels to nitrocellulose membranes at 30 V for 16 hr at 4°. Nitrocellulose membranes were incubated successively with 3% BSA in 10 mM Tris·HCl, pH 7.4, in 0.9% NaCl for 1 hr at room temperature to block nonspecific binding of IgG, with either the anti-βNF_{AA} IgG at 2.5 μg/ml or the anti-PB IgG at 10 μg/ml for 1 hr at room temperature, and then with anti-rabbit IgG (whole molecule) peroxidase-linked goat IgG (1/1000 dilution) for 1 hr at room temperature, each in the Tris·HCl saline solution containing 3% BSA. Optimal first and second antibody concentrations were established in preliminary experiments.

Nitrocellulose membranes were stained for protein with 0.1% amido black 10B in 25% isopropanol/10% (v/v) acetic acid in distilled water for 1–2 min (modification of mixture found in Ref. 10) and were destained in several changes of 25% isopropanol/10% acetic acid followed by distilled water. Immunoblots were developed in 30 ml of 0.03% 4-chloro-1-naphthol/0.03% hydrogen peroxide in 50 mM Tris·HCl, pH 7.6 (11).

Immunoquantitation. Immunoblots were scanned with an LKB UltroScan II laser densitometer (Pharmacia LKB Biotechnology, Piscataway, NJ), and band absorbance × band area was measured. Standard curves were constructed with increasing quantities of βNF_{AA}. Immunoquantitation data were derived from experiments where the densitometry value for the band of interest was within the linear range of the calibration curve.

Immunoinhibition of arachidonic acid metabolism. Chick embryo liver microsomes or the purified βNF_{AA} were preincubated with various concentrations of preimmune or immune IgG (15 min, 37°) before addition of other constituents of the reaction mixtures. Arachidonic acid metabolism was measured as described previously (2), by the method of Capdevila *et al.* (12), and metabolites were identified by retention times and in addition by coelution of standards for HETEs and ω-arachidonic acid and by GC/MS for EETs. TCDD and βNF-treated microsomes and βNF_{AA} generated a peak that eluted just after ω-OH-arachidonic acid. As this peak was not definitively identified, it is called X. Although ω-OH-arachidonic acid and were incompletely resolved, they were detected as two peaks by the radioactive flow detector. The cpm detected for each of the peaks by the flow detector were used as the basis of the measurement of the amounts of these metabolites. All assays were done under conditions where product formation was a linear function of enzyme concentration and incubation time. Reaction mixtures for assays in microsomes contained 30 μM [1-^{14}C]arachidonic acid, microsomes, and 1 mM NADPH in 0.1 M potassium phosphate (pH 7.4). Reaction mixtures were incubated at 37° for 5 min. For assays in reconstituted systems 0.125 nmol of βNF_{AA}, 0.125 nmol of NADPH-cytochrome *c* (P-450) reductase, and 10 μg of dilaurylphosphatidylcholine (sonicated before use) were substituted for microsomes and preincubated at room temperature for 5 min before addition of arachidonic acid. Reactions were started with 2 mM NADPH and were incubated for 1 min at 37°. All reaction mixtures were 0.25 ml. Reactions were stopped with 0.1 ml of glacial acetic acid. Products were extracted twice in 3 ml of ethyl acetate with 0.005% butylated hydroxytoluene. The pooled organic phases were dried under N_2 gas and resuspended in 0.11 ml of absolute ethanol. Aliquots of 0.02–0.05 ml were analyzed in duplicate by reverse phase HPLC, using a Pharmacia LKB Biotechnology system equipped with a μBondapac C_{18} column (3.9 × 300 mm) (Waters Associates, Milford, MA) and a Flow-One Beta model HS radioactivity flow monitor (Packard Instrument Co., Downers Grove, IL). A linear solvent gradient of 50% acetonitrile in H_2O to 100% acetonitrile, both containing 0.1% acetic acid, at a flow rate of 1 ml/min was used.

Other procedures. P-450 was measured by the carbon monoxide-plus sodium dithionite-reduced versus reduced difference spectrum, using an extinction coefficient of 91 mM^{-1} cm^{-1} for the difference in absorbance at 490 and 450 nm (13). Rat liver microsomal NADPH-cytochrome *c* (P-450) reductase was purified by the method of Yasukochi and Masters (14) from liver microsomes of PB-treated male Sprague-Dawley rats, as reported previously (2). Protein was measured by the method of Lowry *et al.* (15). ED_{50} values for immunoinhibition were determined by a median effect plot of the data using a least squares transformation by a computer program by J. Chou and T. C. Chou (Elsevier-Biosoft, Cambridge, UK).

Results

Immunodetection by anti-βNF_{AA} IgG and anti-PB IgG of P-450s in liver microsomes from chick embryos treated with βNF, TCDD, or PB. SDS-PAGE analysis of liver microsomes from chick embryos treated with βNF or TCDD showed increased protein staining of a band at 55 kDa (Fig. 1A). PB treatment (Fig. 1A) resulted in increased bands mainly at 48 and 49 kDa. There was also a smaller increase in a 53-kDa band. On immunoblots, anti-βNF_{AA} IgG (Fig. 1B) reacted with a single 55-kDa band in microsomes from βNF- and TCDD-treated chick embryos, corresponding to the band detected by protein staining. The anti-βNF_{AA} IgG did not immunoreact with any protein in microsomes from livers of control or PB-treated embryos (Fig. 1B).

Anti-PB IgG (Fig. 1C) mainly recognized bands at 48 and 49 kDa (the former more than the latter), corresponding to the major protein bands that were increased in microsomes from PB-treated embryos. These bands were also detected faintly in microsomes from control but not from βNF- or TCDD-treated embryos. Anti-PB IgG also faintly recognized a 53-kDa band that corresponded to the 53-kDa band that was also increased in microsomes from PB-treated embryos (see Fig. 1A); it also cross-reacted with a 52-kDa band in all the microsomes.

Immunodetection of βNF_{AA} by anti-βNF_{AA} IgG. The antigen βNF_{AA} was detected by anti-βNF_{AA} IgG visually at 0.05 pmol/lane and by laser densitometry at 0.02 pmol/lane (Fig. 2). Using the immunodensitometry values for βNF_{AA} to construct a standard curve, the amount of immunoreactive material in microsomes from TCDD- and βNF-treated chick embryos was measured. In three independent experiments, the immunoreactive material in microsomes from TCDD- and βNF-treated embryos gave essentially the same immunodensitometry measurements (mean densitometry units, 4.7 for TCDD- and 5.0 for βNF-treated microsomes at 3.7 and 3.8 nmol of P-450/lane, respectively). These findings indicated that anti-βNF_{AA} IgG reacted with the same amount of P-450 in microsomes from embryos treated with either agent. The result is consistent with βNF_{AA} being present to the same extent after TCDD or βNF treatment. The specific amount of βNF_{AA} in the microsomes could not, however, be immunoquantitated using the βNF_{AA} standard curve because the induced 55-kDa band in βNF- and TCDD-treated microsomes contains, in addition to βNF_{AA}, a 54.5-kDa P-450 that is inactive as an arachidonic acid epoxygenase but is active in aryl hydrocarbon hydroxylation (2) and is immunologically cross-reactive with βNF_{AA} (16).

Immunoinhibition by anti-βNF_{AA} IgG of arachidonic acid metabolism by βNF_{AA}. The effect of anti-βNF_{AA} IgG on arachidonic acid metabolism by reconstituted purified βNF_{AA} is shown in Fig. 3. Anti-βNF_{AA} IgG inhibited all of the P-450-mediated metabolism by its antigen. Its ED_{50} values for inhibition of arachidonic acid metabolism ranged from 0.3 to 0.5 mg of IgG/nmol of P-450 for the different products.

Immunoinhibition by anti-βNF_{AA} IgG of arachidonic acid metabolism in microsomes from TCDD- and βNF-treated chick embryo livers and failure of anti-PB IgG to immunoinhibit TCDD-induced arachidonic acid metabolism. Fig. 4 shows representative reverse phase HPLC chromatograms, demonstrating the effect of anti-βNF_{AA} IgG on arachidonic acid metabolism by liver microsomes from TCDD-treated chick embryos. The concentration dependence of the effects of anti-βNF_{AA} IgG are shown in Fig. 5 (*top*). The antibody virtually eliminated formation of EETs, HETEs, and X. It did not, however, affect formation of ω-OH-arachidonic

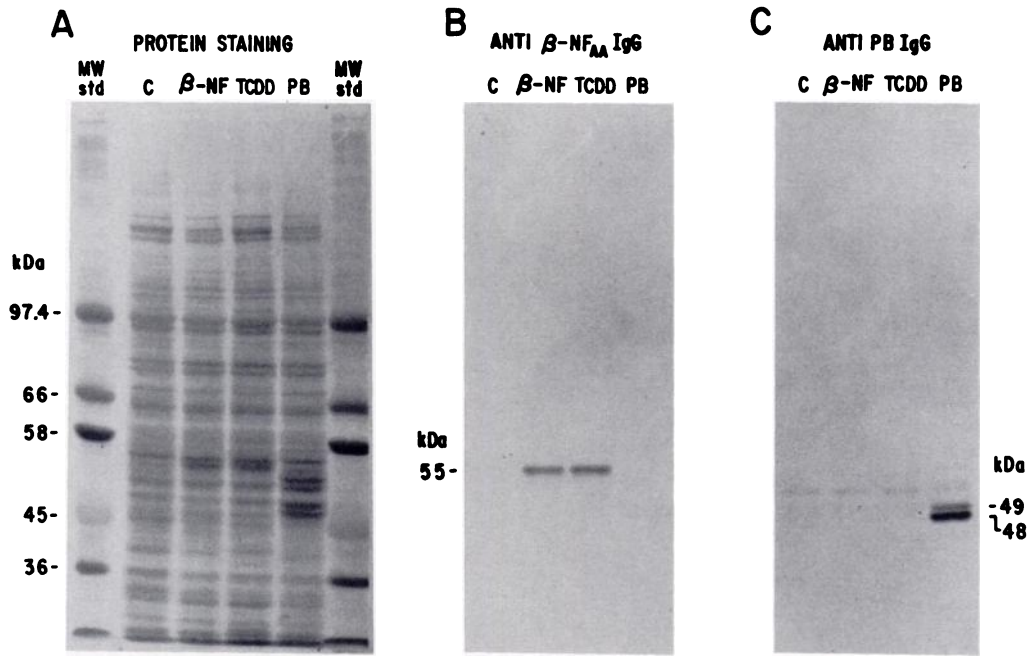

Fig. 1. Nitrocellulose transfers of proteins from chick embryo liver microsomes separated by SDS-PAGE, stained for protein (A) or immunoblotted with anti-βNF_{AA} IgG (B) or anti-PB IgG (C). Liver microsomes from 18-day-old chick embryos that were untreated (controls) (*C*) or were treated for 48 hr with either βNF (6.7 mg in 0.1 ml of dimethylsulfoxide), TCDD (322 ng in 0.005 ml of dioxane), or sodium PB (20 mg in 0.4 ml of water) were analyzed by SDS-PAGE (7.5% separating gel) and the proteins were transferred to nitrocellulose as described in Experimental Procedures. Microsomal P-450 contents (nmol/mg protein) were 0.29 for control, 0.76 for βNF-treated, 0.70 for TCDD-treated, and 1.56 for PB-treated microsomes. A, Stained with amido black 10B (30 μg of microsomal protein/lane); B, immunoblotted with anti-βNF_{AA} IgG (5 μg of microsomal protein/lane); C, immunoblotted with anti-PB IgG (5 μg of microsomal protein/lane).

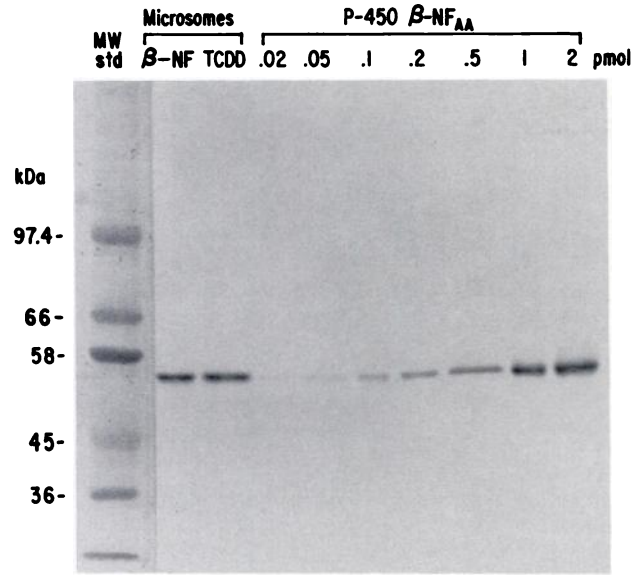

Fig. 2. Immunoquantitation of βNF_{AA}. Microsomes from chick embryos treated with βNF (3.8 pmol of P-450/lane) or TCDD (3.7 pmol of P-450/lane) or pure antigen (βNF_{AA}, 0.02–2 pmol/lane) were subjected to SDS-PAGE on a 7.5% gel, transferred to nitrocellulose, and immunoblotted with anti-βNF_{AA} IgG as described in Experimental Procedures. The immunoblots were scanned with a laser densitometer. A standard curve was constructed by plotting absorbance for each band × the band area as a function of βNF_{AA} concentration. The immunodensitometry values for βNF- and TCDD-treated microsomes fell within the linear portion of the standard curve.

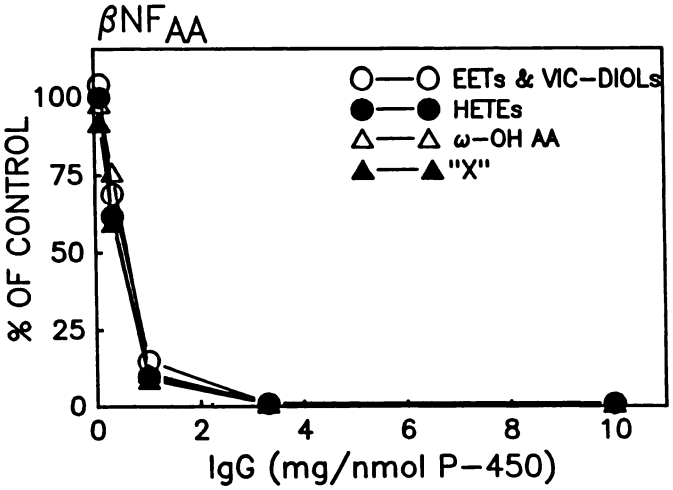

Fig. 3. Inhibition by anti-βNF_{AA} IgG of arachidonic acid metabolism by reconstituted βNF_{AA}. βNF_{AA} was preincubated without IgG, with preimmune IgG, or with anti-βNF_{AA} IgG for 15 min at room temperature. Arachidonic acid metabolism was assayed as described in Experimental Procedures. Preincubation with or without preimmune IgG did not affect product formation. Turnover numbers (nmol of product/nmol of P-450/min, mean ± standard error, three experiments) for product formation by βNF_{AA} in the absence of immune IgG in the experiment shown were 10.22 ± 0.39 for EETs, 1.56 ± 0.10 for HETEs, 1.31 ± 0.11 for X, 1.66 ± 0.19 for ω-OH-arachidonic acid, and 0.93 ± 0.22 for vicinal diols (secondary products of EETs). The ED_{50} values for immunoinhibition by anti-βNF_{AA} IgG (mg of IgG/nmol of P-450) were 0.4 for EETs and vicinal diols, 0.5 for HETEs, 0.3 for X, and 0.4 for ω-OH-arachidonic acid.

acid, indicating that different P-450s catalyze formation of ω-OH-arachidonic acid and of the other P-450 products in microsomes from livers of TCDD-treated embryos. The ED_{50} values (mg of IgG/nmol of P-450 for 50% inhibition) for immunoinhibition by anti-βNF_{AA} IgG of TCDD-induced arachidonate products ranged from 1.1 to 1.7.

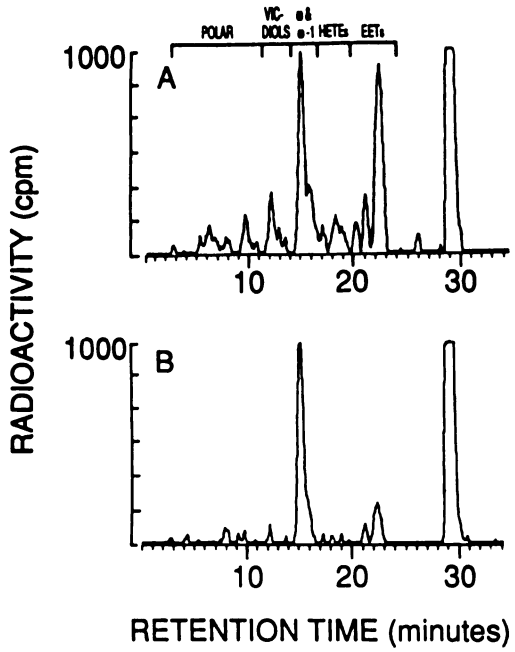

Fig. 4. Reverse phase HPLC chromatograms showing immunoinhibition by anti-βNF_{AA} IgG of TCDD-induced arachidonic acid metabolism. Liver microsomes (0.68 nmol of P-450/mg of protein) from chick embryos treated for 48 hr with 1 nmol (322 ng) of TCDD were preincubated for 15 min at room temperature with preimmune IgG (A) or anti-βNF_{AA} IgG (B) at 10 mg of IgG/nmol of P-450. Arachidonic acid metabolism, product extraction, and reverse phase HPLC were performed as described in Experimental Procedures. The peak eluting at 29–30 min in this and all other chromatograms shown is arachidonic acid. Arachidonic acid product formation in the sample incubated with the preimmune serum (nmol of product formed/mg of protein/min; means of duplicate determinations) was 0.82 for EETs, 0.17 for HETEs, 0.18 for X, 0.57 for ω-OH-arachidonic, and 0.27 for vicinal diols. The samples shown in A and B contained 126,160 and 128,088 cpm of ^{14}C, respectively.

Fig. 5 (*bottom*) shows that, in contrast to the marked inhibitory effect of anti-βNF_{AA} IgG, anti-PB IgG, an antibody against the major PB-induced P-450, did not inhibit arachidonic acid metabolism in microsomes from TCDD-treated embryos. Fig. 6 shows that anti-βNF_{AA} IgG had the same inhibitory effects on microsomal arachidonic acid metabolism after βNF treatment as it had after TCDD treatment. Its ED_{50} values for immunoinhibition of arachidonic acid metabolites ranged from 1.2 to 1.7, about the same range as the ED_{50} values for inhibition of the same products in microsomes from TCDD-treated embryos, evidence that anti-βNF_{AA} IgG was equally effective against P-450s induced by TCDD or βNF.

Immunoinhibition by anti-PB IgG of arachidonic acid metabolism in microsomes from PB-treated chick embryo livers and failure of anti-βNF_{AA} IgG to immunoinhibit PB-induced arachidonic acid metabolism. The HPLC chromatograms in Fig. 7 show that 10 mg of anti-PB IgG/nmol of P-450 completely abolished the enhanced formation of arachidonic acid EETs and HETEs in microsomes from PB-treated chick embryos but only partially inhibited the formation of ω-OH-arachidonic acid. Fig. 8 (*bottom*) shows the concentration dependence of the effect of anti-PB IgG on arachidonic acid metabolism in microsomes from PB-treated embryos. Whereas this antibody totally inhibited EET and HETE formation, it maximally inhibited ω-hydroxylation by

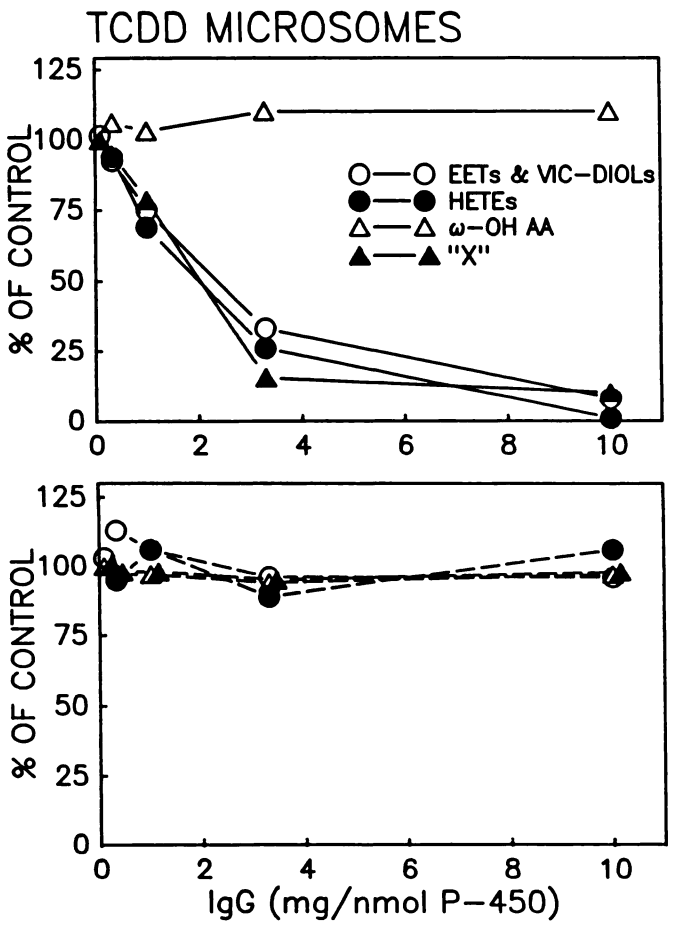

Fig. 5. Concentration-dependent effects of anti-βNF_{AA} IgG (*top*) and anti-PB P-450 IgG (*bottom*) on P-450-mediated arachidonic acid metabolism by liver microsomes from TCDD-treated chick embryos. Chick embryos were treated with TCDD as described in the legend to Fig. 4. Microsomes were preincubated for 15 min at room temperature without IgG or with preimmune or immune IgG in the concentrations shown on the *abscissa*. Then arachidonic acid metabolism was measured as described in Experimental Procedures. Preincubation and preimmune IgG had no effect. Mean values for product formation (nmol of product/mg of protein/min, mean ± standard error, nine experiments) in the absence of immune IgG were 0.87 ± 0.13 for EETs and vicinal diols (combined to reflect total epoxygenase activity), 0.15 ± 0.03 for HETEs, 0.09 ± 0.02 for X, and 0.40 ± 0.02 for ω-OH-arachidonic acid. ED_{50} values for immunoinhibition of arachidonic acid metabolites in TCDD-treated microsomes by anti-βNF_{AA} IgG (nmol of IgG/nmol of P-450) were 1.8 for EETs and vicinal diols, 1.1 for HETEs, and 1.6 for X.

about 50%. The ED_{50} values for inhibition of P-450-generated arachidonic acid products by anti-PB IgG after PB treatment ranged from 0.2 to 0.4. Fig. 8 (*top*) shows that anti-βNF_{AA} IgG did not affect arachidonic acid metabolism in microsomes from PB-treated livers, demonstrating the selectivity of anti-PB IgG for PB-induced arachidonic acid metabolism.

Discussion

This paper shows that the P-450-mediated arachidonic acid epoxygenases induced by TCDD and βNF in chick embryo liver are immunochemically indistinguishable from each other and immunochemically distinct from P-450 epoxygenase induced by PB. These conclusions are based on qualitative and quantitative data. Western blots showed that anti-βNF_{AA} IgG, an antibody against a 55-kDa arachidonic acid epoxygenase puri-

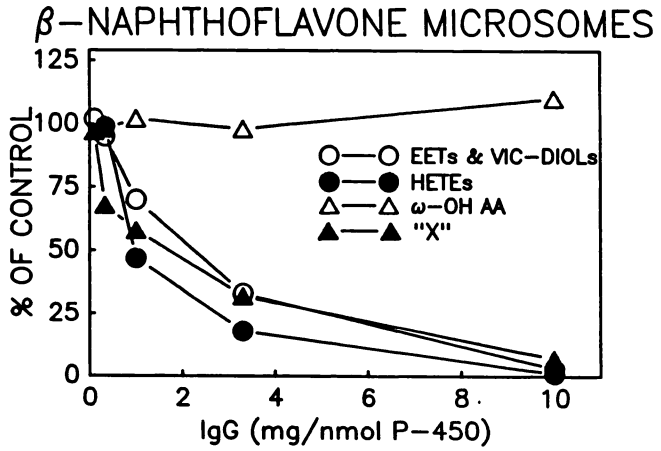

Fig. 6. Concentration-dependent immunoinhibition by anti-βNF_{AA} IgG of P-450-mediated arachidonic acid metabolism by microsomes from βNF-treated chick embryos. The procedures were the same as described in the legend to Fig. 5, except that the embryos were treated for 48 hr with 6.7 mg of βNF in 0.1 ml of dimethylsulfoxide. Mean values for product formation (nmol of product/mg of protein/min, mean ± standard error, six experiments) in the absence of immune IgG were 0.73 ± 0.03 for EETs and vicinal diols, 0.08 ± 0.01 for HETEs, 0.08 ± 0.01 for X, and 0.4 ± 0.02 for ω-OH-arachidonic acid. The ED_{50} values for immunoinhibition of arachidonic acid metabolites in βNF-treated microsomes by anti-βNF_{AA} IgG were 1.7 for EETs and vicinal diols, 1.4 for HETEs, and 1.2 for X.

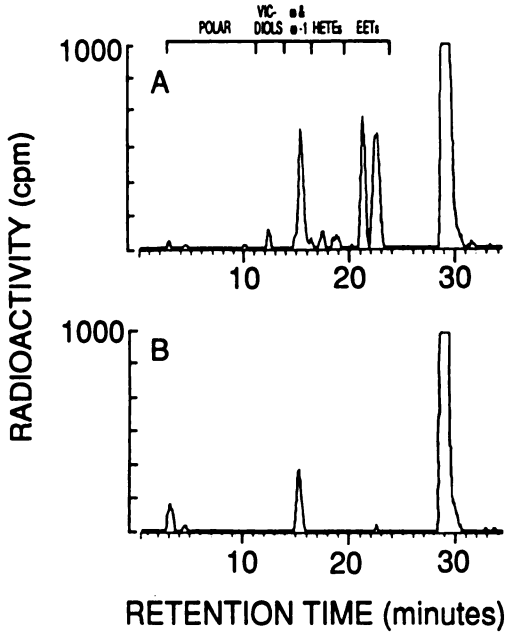

Fig. 7. Reverse phase HPLC chromatograms showing immunoinhibition by anti-PB IgG of PB-induced arachidonic acid metabolism. Liver microsomes (1.37 nmol of P-450/mg of microsomal protein) from chick embryos 48 hr after treatment with 20 mg of sodium PB were preincubated for 15 min at room temperature without IgG (A) or with anti-PB IgG (B) at 10 mg of IgG/nmol of P-450. Arachidonic acid metabolism, product extraction, and resolution by reverse phase HPLC were performed as described in Experimental Procedures. Arachidonic acid products formed in the absence of immune serum (nmol of product/mg of protein/min, means of duplicate determinations) for the sample shown were 0.74 for EETs, 0.09 for HETEs, 0.27 for ω-OH-arachidonic acid, and 0.05 for vicinal diols. The samples shown in A and B contained 197,609 and 131,023 cpm of ^{14}C, respectively.

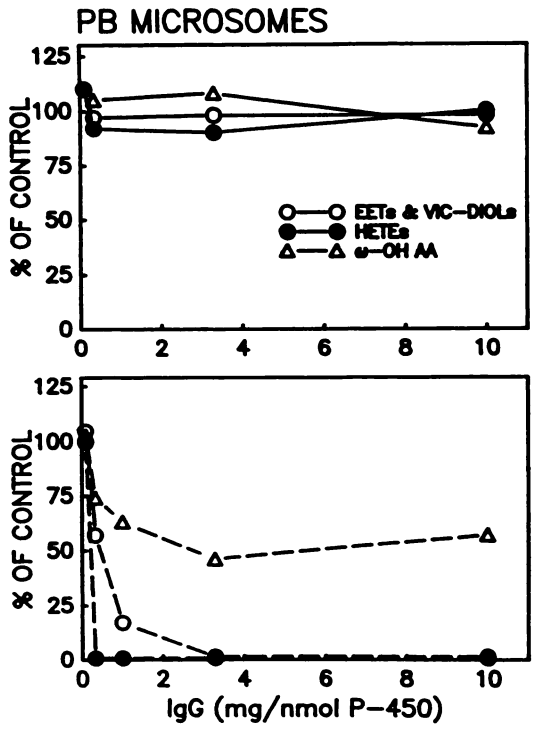

Fig. 8. Concentration-dependent effects of anti-βNF_{AA} IgG (*top*) and anti-PB IgG (*bottom*) on arachidonic acid metabolism by liver microsomes from PB-treated chick embryos. Treatment and experimental procedures were the same as described in the legend for Fig. 7. Mean values for product formation (nmol of product/mg of protein/min, mean ± standard error, five experiments) in the absence of immune IgG were 1.44 ± 0.26 for EETs and vicinal diols, 0.15 ± 0.05 for HETEs, and 0.67 ± 0.15 for ω-OH-arachidonic acid. The ED_{50} values for immunoinhibition by anti-PB IgG were 0.4 for EETs and vicinal diols, 0.2 for HETEs, and 0.4 for ω-OH-arachidonic acid (normalizing to 100% maximum inhibition for the latter).

fied from βNF-treated livers, recognized only bands at 55 kDa in both TCDD- and βNF-treated microsomes, in both to the same degree. In addition, anti-βNF_{AA} IgG immunoinhibited arachidonic acid epoxygenase activity in both TCDD- and βNF-treated microsomes, with almost the same ED_{50} values (1.7 and 1.8 mg of IgG/nmol of P-450, respectively), but it did not inhibit epoxygenase activity in PB-treated microsomes. In contrast, anti-PB IgG, an antibody against PB-induced P-450 that recognized PB-induced protein bands at 48 and 49 kDa but not the TCDD- and βNF-induced P-450 band, immunoinhibited induced arachidonic acid epoxygenation by PB- but not by TCDD- or βNF-treated microsomes. Thus, βNF_{AA} and the PB-induced epoxygenases are immunochemically unrelated to each other.

Although anti-βNF_{AA} IgG cross-reacted with a 54.5-kDa P-450 as well as with βNF_{AA}, the 54.5-kDa P-450, in contrast to βNF_{AA}, exhibited scanty epoxygenase activity (2). That observation and the finding reported here that anti-βNF_{AA} IgG nearly eliminated epoxygenase activity induced by TCDD and βNF suggest that βNF_{AA} is the major epoxygenase in livers of embryos treated with those agents. On the same line of reasoning, the 48- and 49-kDa P-450s, the major P-450s induced by PB, appear to be the primary catalysts of the epoxygenase activity induced by PB. Those P-450s had the highest reconstituted epoxygenase activity among P-450s from livers of PB-treated embryos (2), and an antibody against those P-450s eliminated the PB-induced epoxygenase activity. It is unlikely that P-450s in the 52- and 53-kDa bands with which the anti-PB IgG cross-reacted contribute much to the PB-induced epoxygenase activity because the 52-kDa band was also present in control microsomes, which are inactive in epoxygenation, and reconstituted P-450 fractions containing the 52- and 53-kDa bands and not the 48- and 49-kDa bands had low epoxygenase activity (2).

Usually, P-450s formed in response to *Ah* receptor-dependent and -independent P-450 inducers show preferential activity toward different substrates. The ability of both TCDD- and PB-induced P-450s to effect arachidonic acid epoxygenation, as shown here for chick embryo microsomes, is unusual. It does not reflect a general peculiarity of chick embryo P-450s, because they, like rodent P-450s, show the typical selective increases of aryl hydrocarbon hydroxylase and 7-ethoxyresorufin deethylase activities in response to treatment with *Ah* receptor ligands and of aminopyrine demethylase in response to PB (2) that are exhibited by other species. These findings show that, atypically, a P-450 substrate can be metabolized equieffectively by different P-450s induced by *Ah* receptor-dependent and -independent inducers. The fact that in this instance the substrate is arachidonic acid, a molecule with potentially large impact on signal transduction events, makes the finding of particular biological and pharmacological interest.

The close ED_{50} values for immunoinhibition of TCDD- and βNF-induced EETs, HETEs, and X by anti-βNF_{AA} IgG and for immunoinhibition of PB-induced EETs and HETEs by anti-PB IgG suggest that for each of the inducers the same inducer-specific P-450 catalyzes the formation of those metabolites. The same conclusion does not apply to ω-OH-arachidonic acid, which responded differently from the other metabolites to the antibodies. The different responses of EETs and ω-OH-arachidonic acid to the antibodies is consistent with prior evidence that EETs and ω-OH-arachidonic acid exhibited different rates of formation and different sensitivities to inhibitors in microsomes from embryos treated with TCDD and also those treated with PB (1). The resistance of ω-OH-arachidonic acid in microsomes from TCDD- or βNF-treated embryos to immunoinhibition by anti-βNF_{AA} IgG indicates that different P-450s in livers from embryos treated with these inducers catalyze formation of ω-OH-arachidonic acid, compared with the other P-450-generated products. The partial inhibition, to the extent of 50%, of ω-OH-arachidonic acid formation in livers from PB-treated embryos suggests that half of the ω-hydroxylation occurring after PB treatment could be catalyzed by the P-450 effecting EET and HETE formation or by an immunologically related isoform. The latter is more consistent with our prior finding that P-450 fractions from livers of PB-treated embryos containing the 48- and 49-kDa P-450s were most active in EET formation and exhibited epoxygenase activity almost exclusively (2), whereas the fraction most active in ω-hydroxylation contained mainly a 53-kDa P-450 (2), and anti-PB IgG cross-reacted slightly with a 53-kDa band in livers from PB-treated embryos.

Microsomes from untreated chick embryos metabolize arachidonic acid mainly to ω-OH-arachidonic acid (1, 2). They make only small amounts of EETs and little or no detectable ω-1-OH-arachidonic acid before treatment with P-450 inducers. In contrast, liver microsomes from untreated rats generate EETs and HETEs as well as ω- and ω-1-OH arachidonic acid (12, 17). Whereas PB also increased arachidonic acid epoxy-

genation in rat liver, βNF treatment decreased arachidonic acid epoxygenation (17) although it increased formation of 16–19-hydroxylated arachidonic acid (18). The differences in constitutive arachidonic acid metabolism and the marked induction of P-450 arachidonic acid epoxygenation by TCDD and βNF in chick embryo liver appear to reflect a species difference from rat liver (12, 17).

The ability of a P-450 induced by *Ah* receptor ligands to increase production of biologically active products from an endogenous membrane constituent, arachidonic acid, in chick embryo liver indicates that enhanced arachidonic acid metabolism can be part of the pleiotropic response to the *Ah* receptor, at least in some species. P-450 arachidonic acid metabolites have been reported to have diverse effects including enhanced hormone release from pituitary and pancreas cells, changes in fluid balance and vasoactivity (reviewed in Ref. 12), and increased intracellular calcium and cell growth in renal mesangial cells (19). Several of these effects resemble changes caused by TCDD, i.e., changes in hormonal regulation, fluid balance, intracellular calcium, and cell growth (20, 21). The findings raise the interesting possibility that in some species, and in some tissues, P-450s induced by *Ah* receptor agonists such as TCDD may modulate or mediate some TCDD-induced changes in cellular homeostasis in part through the metabolism of endogenous substrates to biologically active metabolites.

Acknowledgments

We thank Dr. Jorge Capdevila, Vanderbilt University (Nashville, TN) for assistance with arachidonic acid metabolite identification and for performing the GC/MS analysis of the EET region peaks. We also thank Jason Orlinick for his assistance and helpful suggestions.

References

1. Rifkind, A. B., M. Gannon, and S. S. Gross. Arachidonic acid metabolism by dioxin-induced cytochrome P-450: a new hypothesis on the role of P-450 in dioxin toxicity. *Biochem. Biophys. Res. Commun.* **172**:1180–1188 (1990).
2. Nakai, K., A. M. Ward, M. Gannon, and A. B. Rifkind. β-Naphthoflavone induction of a cytochrome P-450 arachidonic acid epoxygenase in chick embryo liver distinct from the aryl hydrocarbon hydroxylase and from phenobarbital induced arachidonate epoxygenase. *J. Biol. Chem.* **267**:19503–19512 (1992).
3. Hobbs, A. A., L. A. Mattschoss, B. K. May, K. E. Williams, and W. H. Elliott. The cDNA and protein sequence of a phenobarbital-induced chicken cytochrome P-450. *J. Biol. Chem.* **261**:9444–9449 (1986).
4. Rifkind, A. B., S. Sassa, J. Reyes, and H. Muschick. Polychlorinated aromatic hydrocarbon lethality, mixed-function oxidase induction, and uroporphyrinogen decarboxylase inhibition in the chick embryo: dissociation of dose-response relationships. *Toxicol. Appl. Pharmacol.* **78**:268–279 (1985).
5. Rifkind, A. B., M. Troeger, and H. Muschick. Kinetic evidence for heterogeneous responsiveness of mixed function oxidase isozymes to inhibition and induction by allylisopropylacetamide in chick embryo liver. *J. Biol. Chem.* **257**:11717–11727 (1982).
6. Thomas, P. E., A. Y. H. Lu, D. Ryan, S. B. West, J. Kawalek, and W. Levin. Multiple forms of rat liver cytochrome P-450: immunochemical evidence with antibody against cytochrome P-448. *J. Biol. Chem.* **251**:1385–1391 (1976).
7. Brooker, J. D., G. Srivastava, I. A. Borthwick, B. K. May, and W. H. Elliott. Evidence that 2-allyl-2-isopropylacetamide, phenobarbital and 3,5-diethoxycarbonyl-1,4-dihydrocollidine induce the same cytochrome P_{450} mRNA in chick embryo liver. *Eur. J. Biochem.* **136**:327–332 (1983).
8. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–685 (1970).
9. Towbin, H., T. Staehelin, and J. Gordon. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354 (1979).
10. Gershoni, J. M., and G. E. Palade. Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to a positively charged membrane filter. *Anal. Biochem.* **124**:396–405 (1982).
11. Harlow, E., and D. Lane. *Antibodies: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1988).
12. Capdevila, J. H., J. R. Falck, E. Dishman, and A. Karara. Cytochrome P-450 arachidonate oxygenase. *Methods Enzymol.* **187**:385–394 (1990).
13. Omura, T., and R. Sato. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**:2370–2378 (1964).
14. Yasukochi, Y., and B. S. S. Masters. Some properties of a detergent-solubilized NADPH-cytochrome *c* (cytochrome P-450) reductase purified by biospecific affinity chromatography. *J. Biol. Chem.* **251**:5337–5344 (1976).
15. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
16. Kanetoshi, A., A. M. Ward, and A. B. Rifkind. Immunochemical separation of cytochrome P-450s metabolizing arachidonic acid (AA) in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and phenobarbital (PB)-induced livers. *FASEB J.* **5**:A1570 (1991).
17. Capdevila, J. H., A. Karara, D. J. Waxman, M. V. Martin, J. R. Falck, and F. P. Guengerich. Cytochrome P-450 enzyme-specific control of the regio- and enantiofacial selectivity of the microsomal arachidonic acid epoxygenase. *J. Biol. Chem.* **265**:10865–10871 (1990).
18. Falck, J. R., S. Lumin, I. Blair, E. Dishman, M. V. Martin, D. J. Waxman, F. P. Guengerich, and J. H. Capdevila. Cytochrome P-450-dependent oxidation of arachidonic acid to 16-, 17-, and 18-hydroxyeicosatetraenoic acids. *J. Biol. Chem.* **265**:10244–10249 (1990).
19. Force, T., G. Hyman, R. Hajjar, A. Sellmayer, and J. V. Bonaventre. 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**:3800–3807 (1991).
20. Poland, A., and J. C. Knutson. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. *Annu. Rev. Pharmacol. Toxicol.* **22**:517–54 (1982).
21. McConkey, D. J., P. Hartzell, S. K. Duddy, H. Hakansson, and S. Orrenius. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin kills immature thymocytes by Ca^{2+}-mediated endonuclease activation. *Science (Washington D. C.)* **242**:256–259 (1988).

Send reprint requests to: Arleen B. Rifkind, M.D., Department of Pharmacology, Cornell University Medical College, 1300 York Avenue, New York, NY 10021.