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CYP2J Subfamily Cytochrome P450s in the Gastrointestinal Tract: Expression, Localization, and Potential Functional Significance

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SUMMARY

Our laboratory recently described a new human cytochrome P450 arachidonic acid epoxygenase (CYP2J2) and the corresponding rat homologue (CYP2J3), both of which were expressed in extrahepatic tissues. Northern analysis of RNA prepared from the human and rat intestine demonstrated that CYP2J2 and CYP2J3 mRNAs were expressed primarily in the small intestine and colon. In contrast, immunoblotting studies using a polyclonal antibody raised against recombinant CYP2J2 showed that CYP2J proteins were expressed throughout the gastrointestinal tract. Immunohistochemical staining of formalin-fixed, paraffin-embedded intestinal sections using anti-CYP2J2 IgG and avidin-biotin-peroxidase detection revealed that CYP2J proteins were present at high levels in nerve cells of autonomic ganglia, epithelial cells, intestinal smooth muscle cells, and vascular endothelium. The distribution of this immunoreactivity was confirmed by *in situ* hybridization using a CYP2J2-specific antisense RNA probe. Microsomal fractions prepared from human jejunum catalyzed the NADPH-dependent metabolism of arachidonic acid to epoxyeicosatrienoic acids as the principal reaction products. Direct evidence for the *in vivo* epoxidation of arachidonic acid by intestinal cytochrome P450 was provided by documenting, for the first time, the presence of epoxyeicosatrienoic acids in human jejunum by gas chromatography/mass spectrometry. We conclude that human and rat intestine contain an arachidonic acid epoxygenase belonging to the CYP2J subfamily that is localized to autonomic ganglion cells, epithelial cells, smooth muscle cells, and vascular endothelium. In addition to the known effects on intestinal vascular tone, we speculate that CYP2J products may be involved in the release of intestinal neuropeptides, control of intestinal motility, and/or modulation of intestinal fluid/electrolyte transport.

Intestinal P450s have been proposed to play important roles in the biotransformation of ingested xenobiotics and activation/detoxification of chemical carcinogens in this tissue (Refs. 1 and 2 and references therein). Reports that the intestinal P450 system is also capable of ω/ω-1 hydroxylation of arachidonic acid and prostaglandins has led to speculation that these enzymes may be involved in the generation of biologically active eicosanoids (3, 4). A number of P450 enzymes have been shown to be constitutively expressed in the gastrointestinal tract of humans, including members of the CYP1A, CYP2C, CYP2D, CYP2E, and CYP3A subfamilies (5–8).[1] In addition, CYP1A, CYP2B, CYP2E, and CYP3A subfamily P450s have been described in rat intestine (5, 8–11). These P450s occur at the highest concentrations in the duodenum, near the pylorus, and at decreasing concentrations distally, being low-undetectable in the colon (1, 5, 6, 11). Furthermore, immunohistochemical studies have demonstrated that enzyme expression is limited to the columnar epithelial cells of the villus with little or no expression in the crypts of Lieberkühn and in nonepithelial cells (7, 10, 11). Recently, Kikuta *et al*. described a new rabbit P450 (CYP2J1) that was exclusively expressed in the small intestine, but the function of that intestinal hemoprotein remains unknown (12).

AA can be oxidized by at least three distinct enzymatic pathways (13 and references therein). In the intestine, the

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[1] The P450 nomenclature detailed in Ref. 40 is used throughout this report.

ABBREVIATIONS: P450, cytochrome P450; EET, *cis*-epoxyeicosatrienoic acid; DHET, *vic*-dihydroxyeicosatrienoic acid; AA, arachidonic acid; 20-OH-AA, 20-hydroxyeicosatetraenoic acid; HPLC, high performance liquid chromatography; GC/MS, gas chromatography/mass spectrometry; PFB, pentafluorobenzyl; SDS, sodium dodecyl sulfate; SSC, standard saline citrate.

cyclooxygenase (e.g., prostaglandins, thromboxane, and prostacyclin) and lipoxygenase (e.g., leukotrienes and hydroxyeicosatetraenoic acids) products are differentially distributed and have been shown to have effects on intestinal motility, secretion, and blood flow (14). Although multiple P450 monooxygenases have been shown to catalyze the formation of EETs, mid-chain HETEs, and C19/C20 alcohols of AA (19-hydroxyeicosatetraenoic acid and 20-OH-AA) in extraintestinal tissues (13, 15, 16), little information is available about the metabolism of arachidonic acid by intestinal P450s or the biological actions of the resulting products. Macica *et al.* (3) reported that rabbit intestinal microsomal fractions metabolized AA to several regioisomeric HETEs and that 20-OH-AA caused dilation of isolated perfused mesenteric arteries. Proctor *et al.* (17) showed that several of the EET regioisomers caused a dose-dependent vasodilation of the rat intestinal microcirculation. The effects of EETs on peptide hormone release and fluid/electrolyte transport in extraintestinal tissues (13, 18, 19 and references therein) suggest that these AA metabolites may have similar functions in the gut. It is not known whether intestinal microsomal fractions can metabolize AA to EETs or these fatty acid epoxides are present *in vivo* in the intestine. Furthermore, the P450 enzyme or enzymes responsible for production of these bioactive eicosanoids in the gastrointestinal tract remain unknown.

Our laboratory has recently cloned a new human P450 cDNA (CYP2J2) (20) and a corresponding rat homologue (CYP2J3),[2] both of which were highly expressed in extrahepatic tissues. The recombinant rat and human CYP2J proteins were active in the metabolism of AA to EETs (20, 20a). The purpose of the current study was to (a) examine the expression and localization of CYP2J P450s in the gastrointestinal tract, (b) evaluate the metabolism of AA by human intestinal microsomal fractions, and (c) determine whether CYP2J products, the EETs, were produced *in vivo* in the intestine. Based on molecular, immunologic, and biochemical data, we show that (a) human and rat intestine contain an active AA epoxygenase belonging to the CYP2J subfamily that is localized to specific cell types throughout the gastrointestinal tract and (b) in addition to the cyclooxygenase and lipoxygenase pathways, the P450 epoxygenase pathway is an important member of the intestinal AA metabolic cascade.

Experimental Procedures

Materials. [α-^{32}P]dATP and [1-^{14}C]AA were purchased from Du Pont-New England Nuclear (Boston, MA). *Escherichia coli* polymerase I was purchased from New England Biolabs (Beverly, MA). Triphenylphosphine, α-bromo-2,3,4,5,6-pentafluorotoluene, *N*,*N*-diisopropylethylamine, *N*,*N*-dimethylformamide, and diazald were purchased from Aldrich Chemical (Milwaukee, WI). All other chemicals and reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise specified. Pathologically normal human esophagus, stomach, duodenum, ileum, jejunum, and colon were obtained through the Cooperative Human Tissue Network (National Disease Research Interchange, Philadelphia, PA) or from local tissue donors. Rat intestinal tissues were obtained from male Fischer 344 rats fed NIH 31 rodent chow (Agway, St. Mary, OH) *ad libitum* and killed by lethal CO_2 inhalation.

Isolation of total RNA and Northern analysis. Normal human and rat intestinal tissues were snap-frozen in liquid nitrogen immediately after collection and stored at −80° until use. Total RNA was extracted using the guanidinium thiocyanate/cesium chloride density gradient centrifugation method as previously described (21). For Northern analysis, total RNA (30 μg) was denatured and electrophoresed in 1.2% agarose gels containing 0.2 M formaldehyde. After capillary-pressure transfer to GeneScreen Plus nylon membranes (New England Nuclear), the blots were hybridized with either the 1.9-kb CYP2J2 cDNA probe (human) or the 1.8-kb CYP2J3 cDNA probe (rat), both of which were labeled by nick translation using *E. coli* polymerase I and [α-^{32}P]dATP (20, 20a). Hybridizations were performed at 42° in 50% formamide containing 1 M NaCl, 1% (w/v) SDS, 10% (w/v) dextran sulfate, and 0.1 mg/ml heat-denatured salmon sperm DNA. After exposure, the radiolabeled probes were removed by boiling, and the blots were hybridized to a cDNA probe encoding human γ-actin. RNA loading was also assessed by comparing the densities of the 28S and 18S rRNA bands on ethidium bromide-stained gels by scanning densitometry.

Protein immunoblotting and immunohistochemistry. Microsomal fractions were prepared from frozen normal human and rat intestinal tissues by differential centrifugation at 4° as previously described (16). For some experiments, rats were pretreated with either phenobarbital (80 mg/kg/day intraperitoneally for 3 days followed by the addition of 0.05% phenobarbital sodium salt to drinking water for 10 days), β-naphthoflavone (40 mg/kg/day intraperitoneally for 4 days), clofibrate (250 mg/kg/day intraperitoneally for 4 days), or acetone (1% in drinking water for 7 days). Microsomal fractions prepared from human lymphoblastic cells transfected with cDNAs to human CYP1A1, CYP2A6, CYP2E1, CYP2B6, CYP2D6, CYP2C9, and CYP2C19 were purchased from Gentest (Woburn, MA). Cell lysates prepared from *Sf9* cells infected with recombinant CYP2C8 baculovirus were used as a source of human CYP2C8 (22). Polyclonal anti-human CYP2J2 IgG was raised in New Zealand White rabbits against the purified, recombinant CYP2J2 protein and affinity purified as previously described (20). For immunoblotting, microsomal fractions, cell lysates, or partially purified, recombinant CYP2J2 were electrophoresed in SDS-10% (w/v) polyacrylamide gels (80 × 80 × 1 mm), and the resolved proteins were transferred electrophoretically onto nitrocellulose membranes. Membranes were immunoblotted using rabbit anti-human CYP2J2 IgG, goat anti-rabbit IgG conjugated to horseradish peroxidase (BioRad, Richmond, CA), and the ECL Western Blotting Detection System (Amersham Life Sciences, Buckinghamshire, UK) as previously described (20). Neither preimmune IgG nor rabbit nonimmune IgG (Biogenex Laboratories, San Ramon, CA) significantly cross-reacted with microsomal fractions prepared from human or rat tissues. Antibodies to rat CYP1A1, CYP2B1, CYP2E1, and CYP4A1 were purchased from Gentest and used according to manufacturer's instructions.

For immunohistochemistry and *in situ* hybridization, rat and human intestinal tissues were fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin. Localization of CYP2J protein expression was investigated using the anti-CYP2J2 IgG (1:100 dilution) on serial sections (5–6 μm) of human and rat esophagus, stomach, small intestine, and colon. Slides were deparaffinized in xylene and hydrated through a graded series of ethanol to 1× Automation buffer (Biomeda, Burlingame, CA) washes. Endogenous peroxidase activity was blocked with 3% (v/v) hydrogen peroxide for 15 min. After rinsing in 1× Automation buffer, slides were microwave-treated, cooled, and blocked with normal goat serum, and the primary antibody was applied for 30 min. Both preimmune IgG and rabbit nonimmune IgG were used as the negative controls in place of the primary antibody. The bound primary antibody was visualized by avidin-biotin-peroxidase detection using the Vectastain Rabbit Elite Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions and with 3,3′-diaminobenzidine as the color-developing reagent. Slides were counterstained with Harris hematoxylin, dehydrated through a graded series of ethanol to xylene washes, and cover-slipped with Permount (Fisher, Springfield, NJ). Specific CYP2J2 immunohistochemical staining of the human intestinal sections was confirmed by adsorption of the anti-CYP2J2 IgG with a 100-fold molar excess of the

purified, recombinant CYP2J2 antigen in 140 mM NaCl, 4 mM KCl, and 10 mM sodium phosphate buffer, pH 7.4, at 4° for 18 hr.

Preparation of RNA probes and *in situ* hybridization. To generate RNA probes, two separate 150–160-bp CYP2J2 fragments (corresponding to nucleotides 992-1150 and nucleotides 1449–1600 of the published CYP2J2 cDNA sequence) (20) were amplified by PCR using gene-specific primers, the CYP2J2 cDNA template, and *AmpliTaq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) and cloned into the pCR II vector using reagents supplied in the Original TA Cloning Kit (InVitrogen, Carlsbad, CA). The identities and orientation of the inserts were corroborated by restriction enzyme digestion and sequence analysis on an Applied Biosystems (Norwalk, CT) Model 373 Automated Sequencer using the Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Cetus). The recombinant plasmids were linearized, and the antisense (sequence complementary to the CYP2J2 mRNA) and sense (sequence identical to the CYP2J2 mRNA) digoxigenin-labeled RNA probes were transcribed using the T7 or SP6 RNA polymerases, respectively. *In vitro* transcriptions were performed using a MEGAscript kit (Ambion, Austin, TX) following the manufacturer's recommendations for incorporation of digoxigenin. Transcript sizes were confirmed by gel electrophoresis, and probe concentrations were determined using a dot blot technique by comparing serial dilutions of probe with a control of known concentration.

Tissues were sectioned under RNase-free conditions and mounted onto positively charged slides. The sections were deparaffinized, rehydrated to 1× phosphate-buffered saline (1× = 10 mM KH_2PO_4, 100 mM Na_2HPO_4, 1.37 M NaCl, 270 mM KCl), and then treated sequentially with 0.2 M HCl, 0.3% (v/v) Triton X-100, and 10 μg/ml Proteinase K for 15 min at 37°. The enzyme was inactivated with glycine, and the tissues were acetylated. After equilibration in 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.4), a hybridization solution containing 50% formamide, 10% (w/v) dextran sulfate, 1× Denhardt's solution, 4× SSC, 10 mM dithiothreitol, 1 mg/ml yeast tRNA, 1 mg/ml denatured salmon sperm DNA, and 2 ng/μl concentration of the 992-1150 nucleotide digoxigenin-11-UTP-labeled antisense RNA probe was applied to each section. The slides were warmed to 75° for 15 min and then hybridized overnight at 42°. After hybridization, the slides were rinsed with 2× SSC and then washed with 2× SSC containing 50% formamide at 42°. Unbound RNA probe was degraded with RNase A and removed through several washes in 2× SSC and a single wash in 2× SSC containing 50% formamide at 42°. The hybridized probe was detected by incubating the sections overnight with an alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer-Mannheim Biochemicals, Indianapolis, IN) used at a 1:500 dilution. The colorimetric reaction was performed at room temperature using nitroblue tetrazolium salt, 5-bromo-4-chloro-3-indolyl phosphate (Boehringer-Mannheim), 10% polyvinyl alcohol, and 1 mM levamisole. After the chromogen reaction reached the desired intensity, slides were placed in a buffer containing 1 mM EDTA and 10 mM Tris·HCl, pH 8.1, and counterstained in Nuclear Fast Red (Vector Laboratories, Burlingame, CA). Controls for the specificity of the *in situ* hybridization reaction included (a) hybridization of tissue sections under identical conditions using the 1449–1600-nucleotide digoxigenin-labeled antisense RNA probe, (b) hybridization of tissue sections under identical conditions using the 992-1150-nucleotide and the 1449–1600-nucleotide digoxigenin-labeled sense RNA probes, (c) cohybridization of tissue sections with the 992-1150-nucleotide digoxigenin-labeled antisense RNA probe and 10-fold excess of unlabeled antisense probe, (d) omission of the antisense probe from the hybridization solution, and (e) omission of the anti-digoxigenin antibody from the detection step.

Microsomal incubations with AA. Microsomal fractions used for incubations with AA were prepared from fresh, pathologically normal human jejunum obtained from local tissue donors. Reaction mixtures containing 0.05 M Tris-Cl buffer, pH 7.5, 0.15 M KCl, 0.01 M $MgCl_2$, 8 mM sodium isocitrate, 0.5 IU/ml isocitrate dehydrogenase, 2.0–4.0 mg/ml microsomal protein, and [1-^{14}C]AA (25–55 μCi/μmol; 50–75 μM, final concentration) were constantly stirred at 30°. After temperature equilibration, NADPH (1 mM, final concentration) was added to initiate the reaction. At 30-min intervals, aliquots were withdrawn, and the reaction products were extracted into ethyl ether, dried under a nitrogen stream, and separated by reverse-phase HPLC on a 5-μm Microsorb C18 column (4.6 × 250 mm, Rainin Instruments, Woburn, MA) using the following solvent program: $CH_3CO_2H/H_2O/CH_3CN$ (0.1:49.95:49.95) isocratic conditions for 30 min, then a linear solvent gradient from $CH_3CO_2H/H_2O/CH_3CN$ (0.1:49.95:49.95) to $CH_3CO_2H/H_2O/CH_3CN$ (0.1:24.95:74.95) over 70 min, and then a linear solvent gradient from $CH_3CO_2H/H_2O/CH_3CN$ (0.1:24.95:74.95) to CH_3CO_2H/CH_3CN (0.1:99.9) over 25 min at 1 ml/min. Reaction products were quantified by on-line liquid scintillation using a Radiomatic Flo-One β-detector (Radiomatic Instruments, Tampa, FL) as previously described (23). In some experiments 0.2 mM 1,2-epoxy-3,3,3-trichloropropane (ETCP), a microsomal epoxide hydrolase inhibitor, was added to the incubation mixture just before initiation with NADPH.

Quantification of endogenous EETs in human jejunum. Methods used to quantify endogenous EETs present in human jejunum were similar to those used to quantify endogenous EETs in human heart (20). Briefly, freshly obtained tissues (0.5–1.0 g) were frozen in liquid nitrogen and immediately homogenized in 10–15 ml of 10 mM sodium phosphate buffer, pH 7.4, containing 140 mM NaCl, 4 mM KCl, and 1 mg/ml triphenylphosphine, a hydroperoxide-reducing agent. The homogenate was extracted twice, under acidic conditions, with two volumes of chloroform/methanol (2:1) and once more with an equal volume of chloroform, and the combined organic phases were evaporated in tubes containing mixtures of [1-^{14}C]8,9-, 11,12-, and 14,15-EET (55–57 μCi/μmol, 80 ng each) internal standards. Saponification to recover phospholipid-bound EETs was followed by SiO_2 column purification. The eluent, containing a mixture of radiolabeled internal standards and total endogenous EETs, was resolved into individual regioisomers and enantiomers by HPLC as previously described (24). For analysis, aliquots of individual EET-PFBs were dissolved in dodecane and analyzed by GC/MS on a Kratos Concept ISQ mass spectrometer (Kratos Analytical, Ramsey, NJ) operating under negative-ion chemical ionization conditions, at 5.3 keV accelerating potential, at a mass resolution of 1200, and using methane as a bath gas. Quantifications were made by selected ion monitoring of m/z 319 (loss of PFB from endogenous EET-PFB) and m/z 321 (loss of PFB from [1-^{14}C]EET-PFB internal standard). The EET-PFB/[1-^{14}C]EET-PFB ratios were calculated from the integrated values of the corresponding ion current intensities.

Other methods. EETs were prepared by total chemical synthesis according to published procedures (25). [1-^{14}C]EET internal standards were synthesized from [1-^{14}C]AA (55–57 μCi/μmol) by nonselective epoxidation as previously described (26). DHET and [1-^{14}C]-DHET internal standards were prepared by chemical hydration of EETs and [1-^{14}C]EETs as previously described (27). All synthetic EETs and DHETs were purified by reverse-phase HPLC (23). Methylations were performed using an ethereal solution of diazomethane. PFB esters were formed by reaction with pentafluorobenzyl bromide as described (24). Protein determinations were performed according to the method of Bradford (28).

Results

Expression and distribution of CYP2J mRNA and protein in the gastrointestinal tract by Northern analysis and protein immunoblotting. Blot hybridization of total RNA extracted from human intestinal tissues under high stringency conditions using the radiolabeled CYP2J2 cDNA probe produced a single strong 1.9-kb band in ileum and a weaker band in jejunum and colon, demonstrating that CYP2J2 message was primarily expressed in the distal small intestine and at lower levels in the mid small intestine and

colon (Fig. 1). Transcripts were not detectable in stomach or duodenum, indicating that CYP2J2 mRNA expression was below the limits of detection of Northern analysis in these portions of the human intestinal tract (Fig. 1). Northern analysis of total RNA extracted from rat intestinal tissues using the radiolabeled CYP2J3 cDNA probe produced a strong 1.8-kb band in jejunum, demonstrating that CYP2J3 message was primarily expressed in the mid small intestine (Fig. 2). The 1.8-kb transcript was also present, albeit at markedly lower levels, in the duodenum, ileum, and colon but was not detectable in the esophagus or stomach, indicating that CYP2J3 mRNA expression was low in these portions of the rat intestinal tract (Fig. 2). The CYP2J3 cDNA also hybridized with a 2.4-kb transcript in rat jejunum and a 3.6-kb transcript in rat duodenum, jejunum, ileum, and colon (Fig. 2). The identity of these larger transcripts remain unknown, but they may represent alternate splice variants of CYP2J3 or new rat P450s that share nucleic acid sequence homology with CYP2J3.

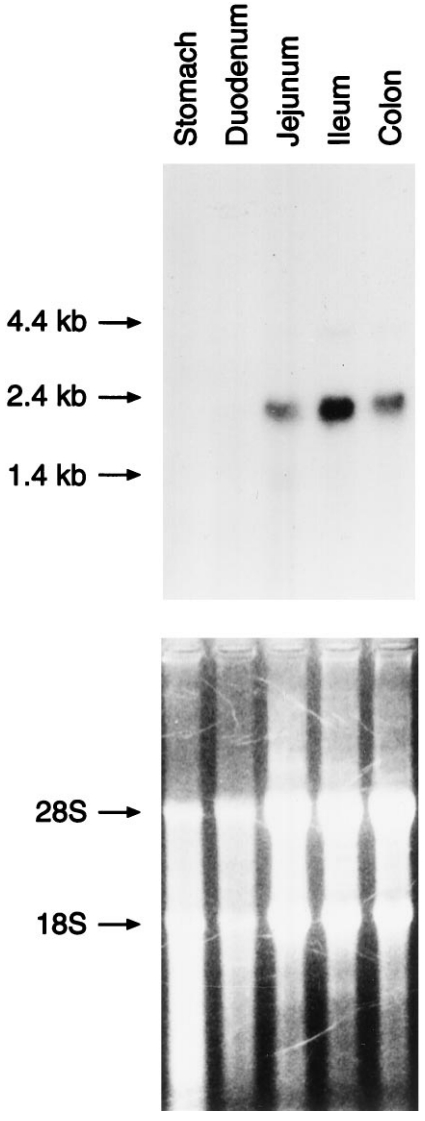

Fig. 1. Northern blot analysis of total RNA extracted from human intestinal tissues. Total RNA (30 μg) isolated from human stomach, duodenum, jejunum, ileum, and colon was denatured, electrophoresed in a 1.2% agarose gel containing 0.2 M formaldehyde, transferred to a nylon membrane, and blot hybridized with a radiolabeled CYP2J2 cDNA probe as described in Experimental Procedures. *Top,* autoradiograph of blot after 48-hr exposure time. *Bottom,* ethidium bromide-stained gel before transfer.

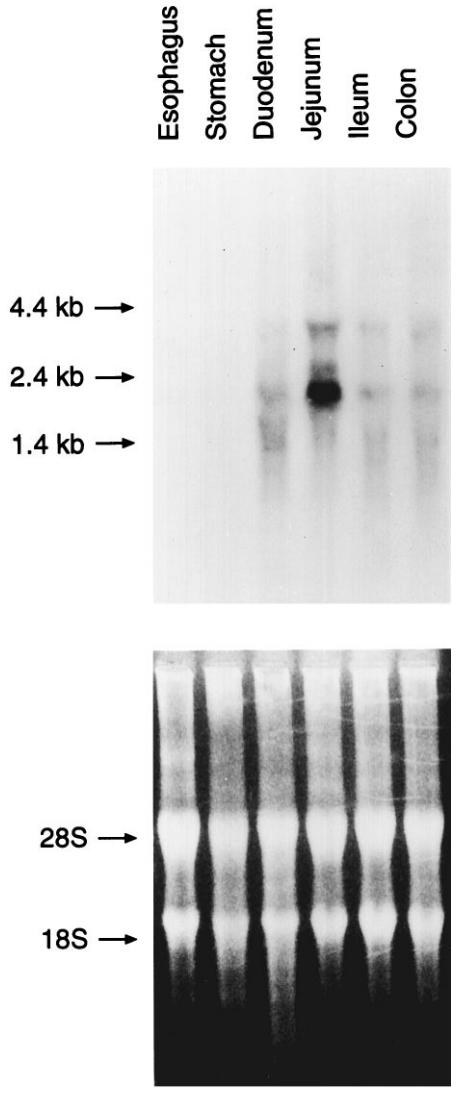

Fig. 2. Northern blot analysis of total RNA extracted from rat intestinal tissues. Total RNA (30 μg) isolated from rat esophagus, stomach, duodenum, jejunum, ileum, and colon was denatured, electrophoresed in a 1.2% agarose gel containing 0.2 M formaldehyde, transferred to a nylon membrane, and blot hybridized with a radiolabeled CYP2J3 cDNA probe as described in Experimental Procedures. *Top,* autoradiograph of blot after 48-hr exposure time. *Bottom,* ethidium bromide-stained gel before transfer.

In contrast to the Northern analysis results, immunoblotting studies using polyclonal antibodies raised against recombinant CYP2J2 demonstrated that CYP2J proteins were abundantly expressed throughout the entire human and rat gastrointestinal tract. As illustrated in Fig. 3, *bottom*, anti-CYP2J2 IgG immunoreacted with an electrophoretically distinct band at ~56 kDa in microsomal fractions prepared from human esophagus, stomach, duodenum, jejunum, ileum, and colon. Unlike other human intestinal P450s (1, 5, 6), the expression levels for the anti-CYP2J2 immunoreactive protein were highest in the esophagus and slightly lower but relatively constant throughout the remainder of the human gastrointestinal tract (Fig. 3, *bottom*). Control experiments demonstrated that the anti-CYP2J2 IgG immunoreacted

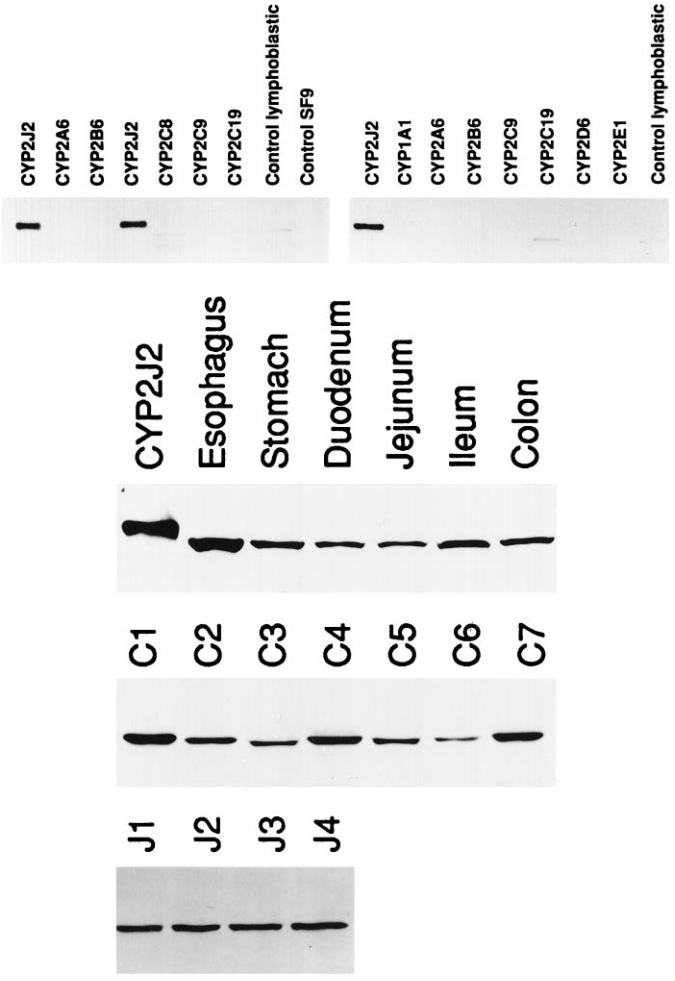

Fig. 3. Intestinal distribution of human CYP2J protein by immunoblotting. *Top*, purified recombinant CYP2J2; microsomal fractions prepared from human lymphoblastic cells transfected with the cDNAs to human CYP1A1, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, or CYP2E1; or microsomal fractions prepared from *Sf9* cells infected with CYP2C8 baculovirus (1 pmol of P450/lane) were electrophoresed on SDS-10% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with the anti-CYP2J2 IgG as described in Experimental Procedures. *Bottom*, *uppermost lanes*, recombinant CYP2J2 (0.25 pmol/lane) or microsomal fractions prepared from human esophagus, stomach, duodenum, jejunum, ileum, and colon (40 μg of microsomal protein/lane) were electrophoresed on SDS-10% polyacrylamide gels, and the resolved proteins were transferred to nitrocellulose, immunoblotted with affinity purified rabbit anti-human CYP2J2 IgG and goat anti-rabbit IgG conjugated to horseradish peroxidase, and visualized using the ECL detection system and autoradiography as described in Experimental Procedures. *C1–C7*, microsomal fractions prepared from seven different human colons were electrophoresed, transferred to nitrocellulose, and immunoblotted with the anti-CYP2J2 IgG as described in Experimental Procedures. *J1–J4*, microsomal fractions prepared from four different human jejunums were electrophoresed, transferred to nitrocellulose, and immunoblotted with the anti-CYP2J2 IgG as described in Experimental Procedures.

with recombinant CYP2J2 but did not cross-react with other previously described human CYP1 and CYP2 family P450s, including CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP2E1 (Fig. 3, *top*). As shown in Fig. 4, anti-CYP2J2 IgG immunoreacted with a single 58-kDa protein band in microsomal fractions prepared from rat esophagus, stomach, duodenum, jejunum, ileum, and colon. Pretreatment of animals with phenobarbital,

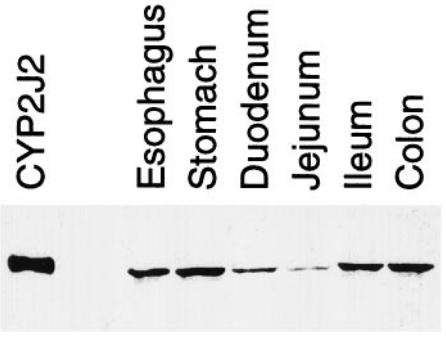

Fig. 4. Intestinal distribution of rat CYP2J protein by immunoblotting. Recombinant CYP2J2 (0.25 pmol/lane) or microsomal fractions prepared from rat esophagus, stomach, duodenum, jejunum, ileum, and colon (40 μg of microsomal protein/lane) were electrophoresed on SDS-10% polyacrylamide gels, and the resolved proteins were transferred to nitrocellulose, immunoblotted with affinity purified rabbit anti-human CYP2J2 IgG and goat anti-rabbit IgG conjugated to horseradish peroxidase, and visualized using the ECL detection system and autoradiography as described in Experimental Procedures.

β-naphthoflavone, clofibrate, or acetone induced CYP2B, CYP1A, CYP4A, and CYP2E subfamily P450s, respectively, in rat liver but had no effect on the rat intestinal expression of CYP2J protein (data not shown). Control experiments demonstrated that the anti-CYP2J2 IgG immunoreacted with recombinant rat CYP2J3 but did not cross-react with other previously described rat CYP1 and CYP2 family P450s, including CYP1A1, CYP2A1, CYP2B1, CYP2B2, CYP2C11, CYP2C13, CYP2C23, and CYP2E1 (20a).

To evaluate interindividual differences in expression of CYP2J protein in the human intestine, we performed immunoblotting on microsomal fractions prepared from an additional four human jejunum and an additional seven human colon specimens. As shown in Fig. 3, *bottom*, although there was some interindividual variation in the expression of CYP2J protein in human colon tissues, there was remarkably little interindividual variation in the expression of CYP2J protein in human jejunum specimens. There was little interanimal variability in expression of CYP2J protein in rat intestinal tissues (data not shown). Based on these data, we conclude that (a) CYP2J proteins are abundantly expressed throughout the entire human and rat gastrointestinal tract, (b) there is relatively low interindividual or interanimal differences in expression of CYP2J proteins in the intestine, and (c) CYP2J2 and CYP2J3 mRNA levels do not correlate well with CYP2J protein levels in these intestinal tissues.

Localization of intestinal CYP2J proteins by immunohistochemistry. To determine the distribution of CYP2J proteins within the intestine, we stained formalin-fixed, paraffin-embedded human and rat intestinal sections with the anti-CYP2J2 antibody. In both humans and rats, anti-CYP2J2 immunostaining was present throughout the entire gastrointestinal tract and localized to autonomic ganglion cells, epithelial cells, smooth muscle cells of the muscularis layer, and endothelium lining the blood vessels (Figs. 5–8).

In the rat esophagus, strong positive staining was noted in the squamous epithelium and in autonomic ganglion cells, less intense staining was present in esophageal smooth muscle cells and in vascular endothelium, and submucosal connective tissue did not stain (Fig. 5, A and C). Both preimmune IgG (Fig. 5, B and D) and rabbit nonimmune IgG (data not shown) produced negative staining throughout the rat

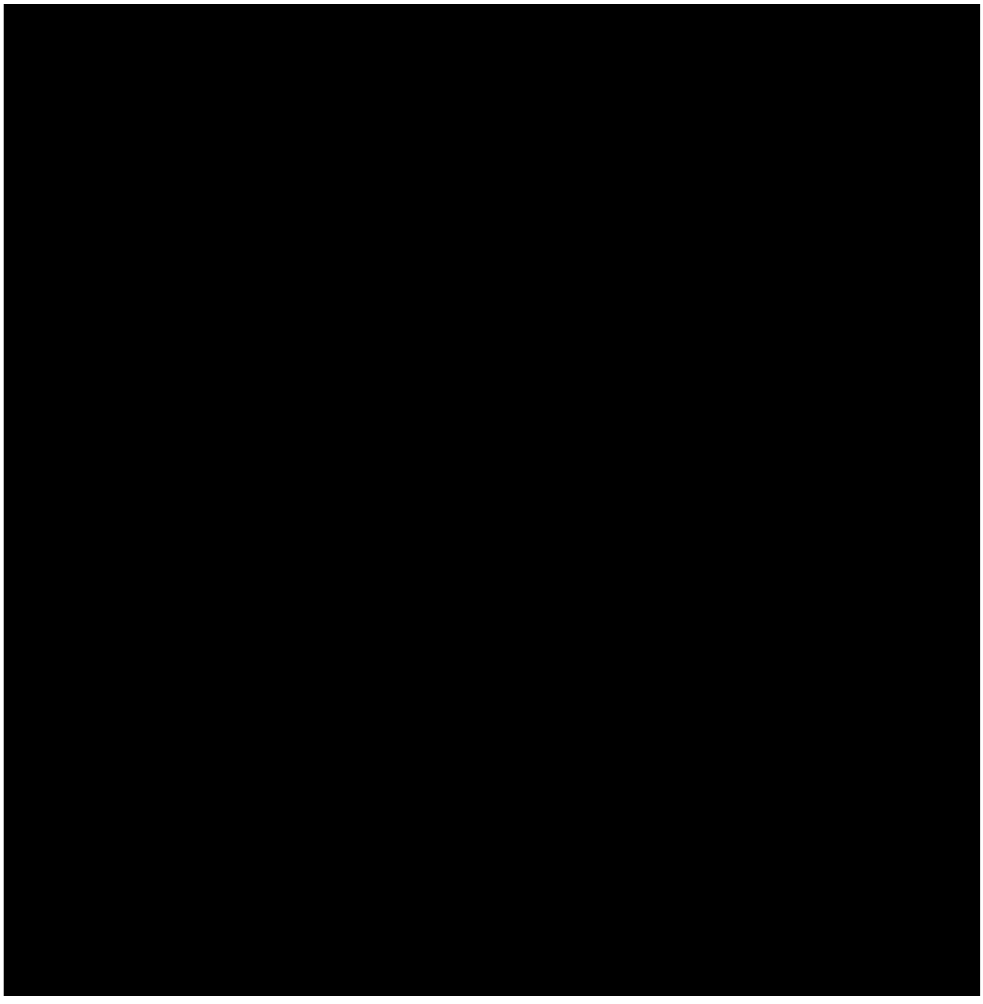

Fig. 5. Immunohistochemical localization of CYP2J protein in esophagus. Adjacent sections of rat (A–D) or human (E and F) esophagus immunostained with either rabbit anti-human CYP2J2 IgG (A, C, and E) or preimmune IgG (B, D, and F). *Arrow*, strong positive staining in a nerve cell of the autonomic ganglion. Magnification: 50× (A and B), 100× (C and D), and 25× (E and F).

esophagus. A similar staining pattern was observed in human esophagus (Fig. 5, E and F). Furthermore, adsorption of anti-CYP2J2 IgG with excess purified recombinant CYP2J2 abolished the positive reaction, thus demonstrating the specificity of the esophageal immunostaining for the CYP2J2 protein (data not shown).

In the human stomach, CYP2J2 immunostaining was most prominent in the nerve cells of the autonomic ganglion and in chief cells lining the lower third of the gastric glands (Fig. 6, A, C, and E). Less intense staining was noted in the surface columnar epithelial cells, epithelium lining the gastric pits, parietal cells of the gastric glands, endothelium lining the small blood vessels, and muscularis smooth muscle cells (Fig. 6, A, C, and E). Connective tissue cells and inflammatory cells of the lamina propria did not stain (Fig. 6, A, C, and E). Both preimmune IgG (Fig. 6, B, D, and F) and rabbit nonimmune IgG (data not shown) produced negative staining throughout the human stomach. Furthermore, adsorption of anti-CYP2J2 IgG with excess purified recombinant CYP2J2 abolished the positive reaction, thus demonstrating the specificity of the gastric immunostaining for the CYP2J2 protein (Fig. 6J). A similar staining pattern was observed in rat glandular stomach (Fig. 6, G, H, and I).

In the rat small intestine, positive staining was most prominent in the autonomic ganglion cells and in the absorptive epithelial cells covering the villi and lining the crypts of Lieberkühn (Fig. 7, A and C). Less intense staining was present in the mucus-producing goblet cells, muscularis smooth muscle cells, and vascular endothelium (Fig. 7, A and C). Connective tissue cells and inflammatory cells of the lamina propria did not stain (Fig. 7, A and C). Both preimmune IgG (Fig. 7, B and D) and rabbit nonimmune IgG (data not shown) produced negative staining throughout the rat small intestine. A similar staining pattern was observed in human small intestine (Fig. 7, E and F). Furthermore, adsorption of anti-CYP2J2 IgG with excess purified recombinant CYP2J2 abolished the positive reaction, thus demonstrating the specificity of the small intestinal immunostaining for the CYP2J2 protein (data not shown).

In the human colon, strong positive staining was noted in the surface columnar epithelium and autonomic ganglion cells (Fig. 8, A, C, and E). Less intense staining was noted in the muscularis smooth muscle cells, goblet cells lining the crypts of Lieberkühn, and vascular endothelium (Fig. 8, A, C, and E). Connective tissue cells and inflammatory cells of the lamina propria did not stain (Fig. 8, A and C). Both preimmune IgG (Fig. 8, B, D, and F) and rabbit nonimmune IgG (data not shown) produced negative staining throughout the human colon. A similar staining pattern was observed in rat colon (Fig. 8, G and H).

Localization of intestinal CYP2J2 mRNA expression by *in situ* hybridization. *In situ* hybridization of formalin-fixed, paraffin-embedded human intestinal sections with the 992-1150-nucleotide digoxigenin-labeled antisense RNA

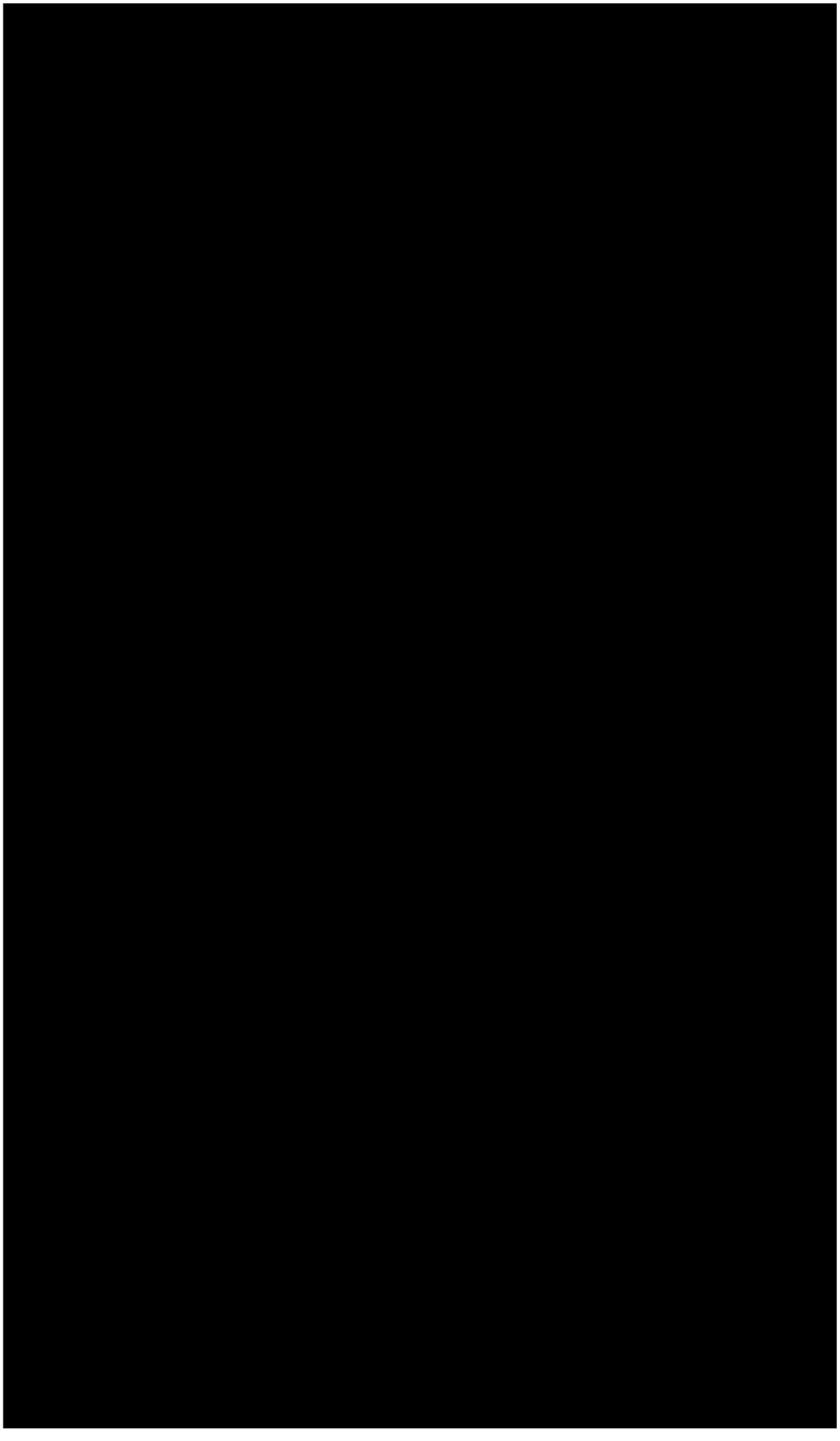

Fig. 6. Immunohistochemical localization of CYP2J protein in stomach. Adjacent sections of human (A–F, J) or rat (G–I) stomach immunostained with either rabbit anti-human CYP2J2 IgG (A, C, E, and G), preimmune IgG (B, D, F, and H), hematoxylin and eosin (I), or anti-human CYP2J2 IgG preincubated with a 100-fold molar excess of purified recombinant human CYP2J2 (J). *Closed arrows*, strong positive staining in nerve cells of the autonomic ganglion; *open arrow*, staining in vascular endothelial cells; *arrowheads*, positive staining in chief cells. Magnification: 25× (A, B, and J), 50× (C and D), and 100× (E–I).

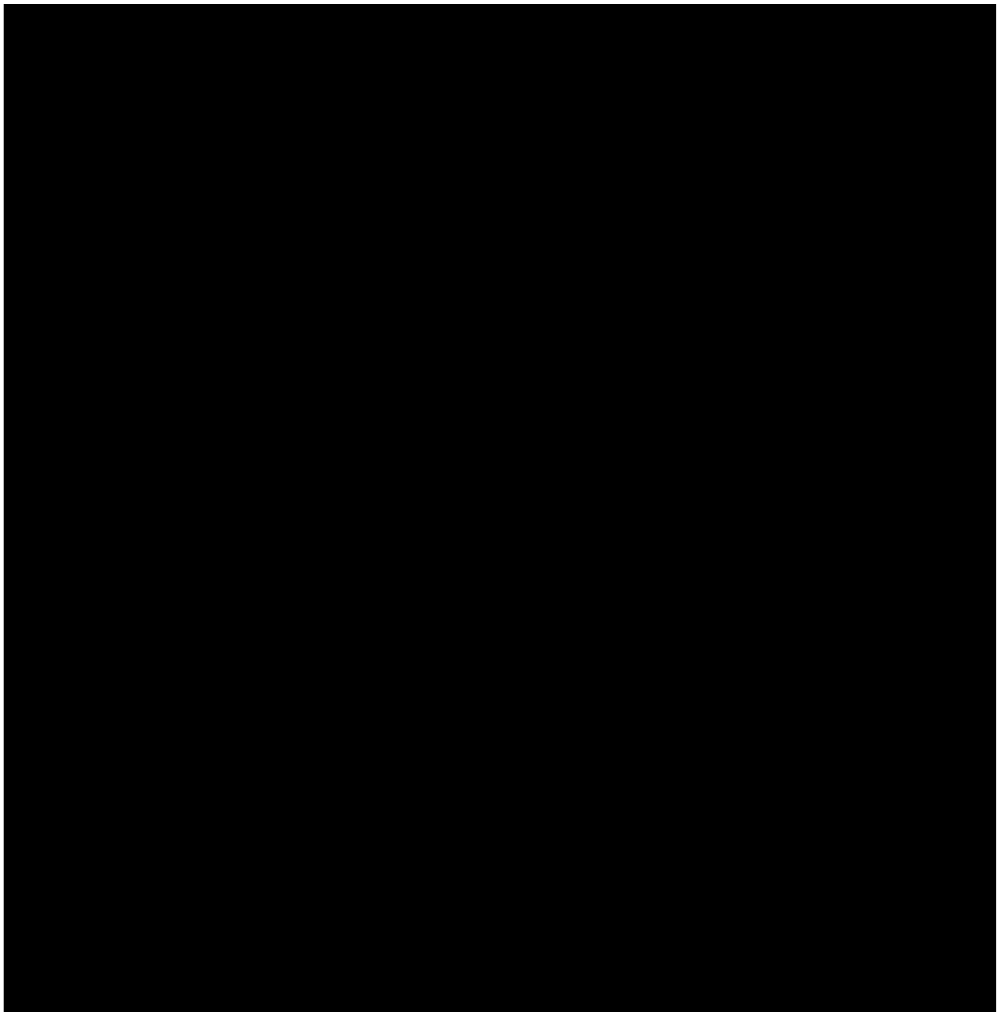

Fig. 7. Immunohistochemical localization of CYP2J protein in small intestine. Adjacent sections of rat (A–D) or human (E and F) small intestine immunostained with either rabbit anti-human CYP2J2 IgG (A, C, and E) or preimmune IgG (B, D, and F). *Arrows*, strong positive staining in nerve cells of the autonomic ganglion. Magnification: 25× (A and B), 100× (C and D), and 10× (E and F).

probe revealed that CYP2J2 mRNA expression was primarily localized to intestinal epithelial cells and nerve cells of the autonomic ganglion (Fig. 9, A, C, E, G, and I). CYP2J2 mRNA was also expressed, albeit at lower levels, in smooth muscle cells of the muscularis layer and endothelium lining small and large blood vessels but not in connective tissue cells (Fig. 9, A, C, E, G, and I). Hybridization of intestinal sections with the 992-1150-nucleotide digoxigenin-labeled sense RNA probe yielded no appreciable staining (Fig. 9, B, D, F, H, and J). Cohybridization of the digoxigenin-labeled antisense RNA probe with a 10-fold excess of unlabeled antisense RNA probe abolished all positive staining, thus demonstrating the specificity of the hybridization for the CYP2J2 mRNA. Omission of the probe or of the anti-digoxigenin antibody also abolished the positive staining. Identical results were obtained with the 1449–1600-nucleotide digoxigenin-labeled antisense and sense RNA probes (data not shown).

***In vitro* metabolism of AA by human intestinal microsomal fractions.** Incubations of human jejunum microsomal fractions with [1-^{14}C]AA in the presence of NADPH resulted in the formation of EETs, DHETs, mid-chain HETEs, and C19/C20 alcohols of AA (rate of metabolism, 10 pmol of product formed/mg of microsomal protein/min at 30°) (Fig. 10). We identified these metabolites by comparing their HPLC properties with those of authentic standards and by GC/MS (23). None of these metabolites were formed in the absence of NADPH, suggesting that the reaction was P450 mediated (data not shown). The predominant metabolites formed, accounting for~53% of the total products, were EETs and their hydration products, the DHETs (Fig. 10). The C19/C20 alcohols and mid-chain HETEs accounted for ~34% and ~13% of the total products, respectively (Fig. 10). No significant formation of prostaglandins or leukotrienes was observed under the incubation conditions described in Experimental Procedures. Incubations performed in the presence of the microsomal epoxide hydrolase inhibitor 1,2-epoxy-3,3,3-trichloropropane yielded significantly more EETs relative to DHETs; however, the overall metabolism of AA was reduced by >50% (data not shown). Insofar as DHET formation must be preceded by EET formation (27), the chromatogram in Fig. 10 demonstrates that human jejunum microsomes contain an active AA epoxygenase or epoxygenases. Regiochemical analysis of the epoxygenase metabolites formed revealed that epoxidation occurred most often at the 14,15-olefin (45% of the total) and less often at the 11,12- and 8,9-olefin (25% and 30% of the total, respectively). No detectable epoxidation occurred at the 5,6-olefin. Regiochemical analysis of the alcohols formed revealed that hydroxylation occurred most often at the C20 position (55% of the total) and less often at the C19 position (21% of the total).

Detection of EETs in human jejunum by GC/MS. Using a combination of HPLC and GC/MS, we detected substan-

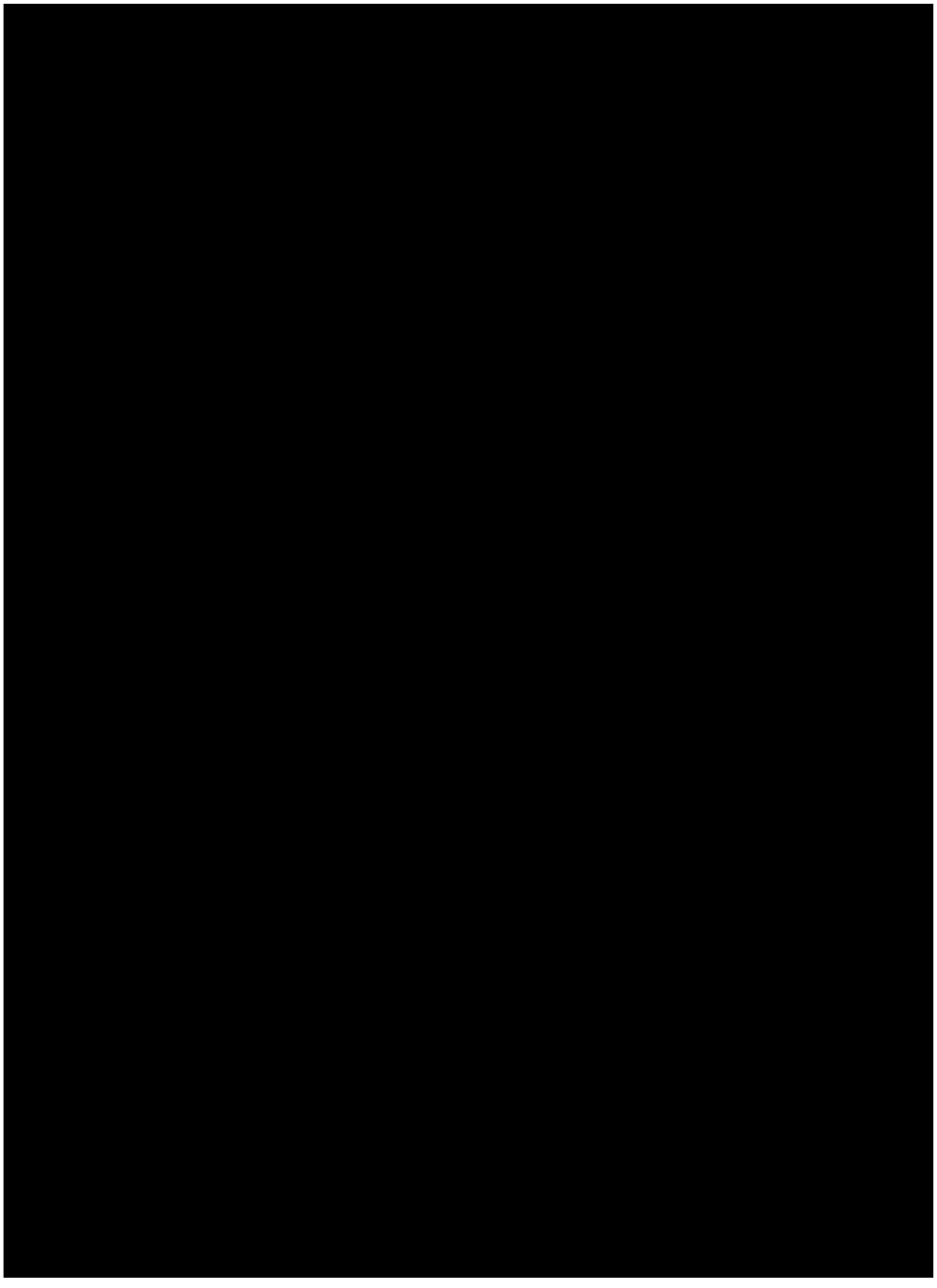

Fig. 8. Immunohistochemical localization of CYP2J protein in colon. Adjacent sections of human (A–F) or rat (G and H) colon immunostained with either rabbit anti-human CYP2J2 IgG (A, C, E, and G) or preimmune IgG (B, D, F, and H). *Closed arrows*, strong positive staining in nerve cells of the autonomic ganglion; *open arrows*, staining in vascular endothelial cells. Magnification: 25× (A and B), 50× (C, D, G, and H), and 100× (E and F).

tial amounts of EETs in human jejunum. As shown in Table 1, human jejunum contained ~40 ng of total EET/g of tissue. The 14,15-, 11,12-, and 8,9-EETs were each present in roughly equal amounts (31%, 36%, and 33% of the total, respectively) (Table 1). The labile 5,6-EET underwent extensive decomposition during the extraction and purification process used and therefore could not be quantified. Chiral analysis of human jejunum EETs revealed that the 14(*R*),15(*S*)-, 11(*R*),12(*S*)-, and 8(*S*),9(*R*)-EETs were the predominant antipodes (optical purity, 69%, 65%, and 63%, respectively) (Table 1). Because stereoselective formation of eicosanoids is a sufficient criterion to establish their enzymatic origin (24), we conclude, on the basis of the data in Table 1, that 14,15-, 11,12-, and 8,9-EET were produced *in vivo* by the human jejunum epoxygenase or epoxygenases.

Discussion

The intestinal P450 system has long been thought to function primarily in the metabolism of exogenous compounds, including drugs and carcinogens (1, 2). Oxidation of these ingested chemicals can cause either activation or detoxification (1, 2). Over the past decade, there has been an increased awareness that this ubiquitous enzyme system may also be involved in the bioactivation of endogenous substrates such as AA (3, 4). In this report, we provide molecular and immu-

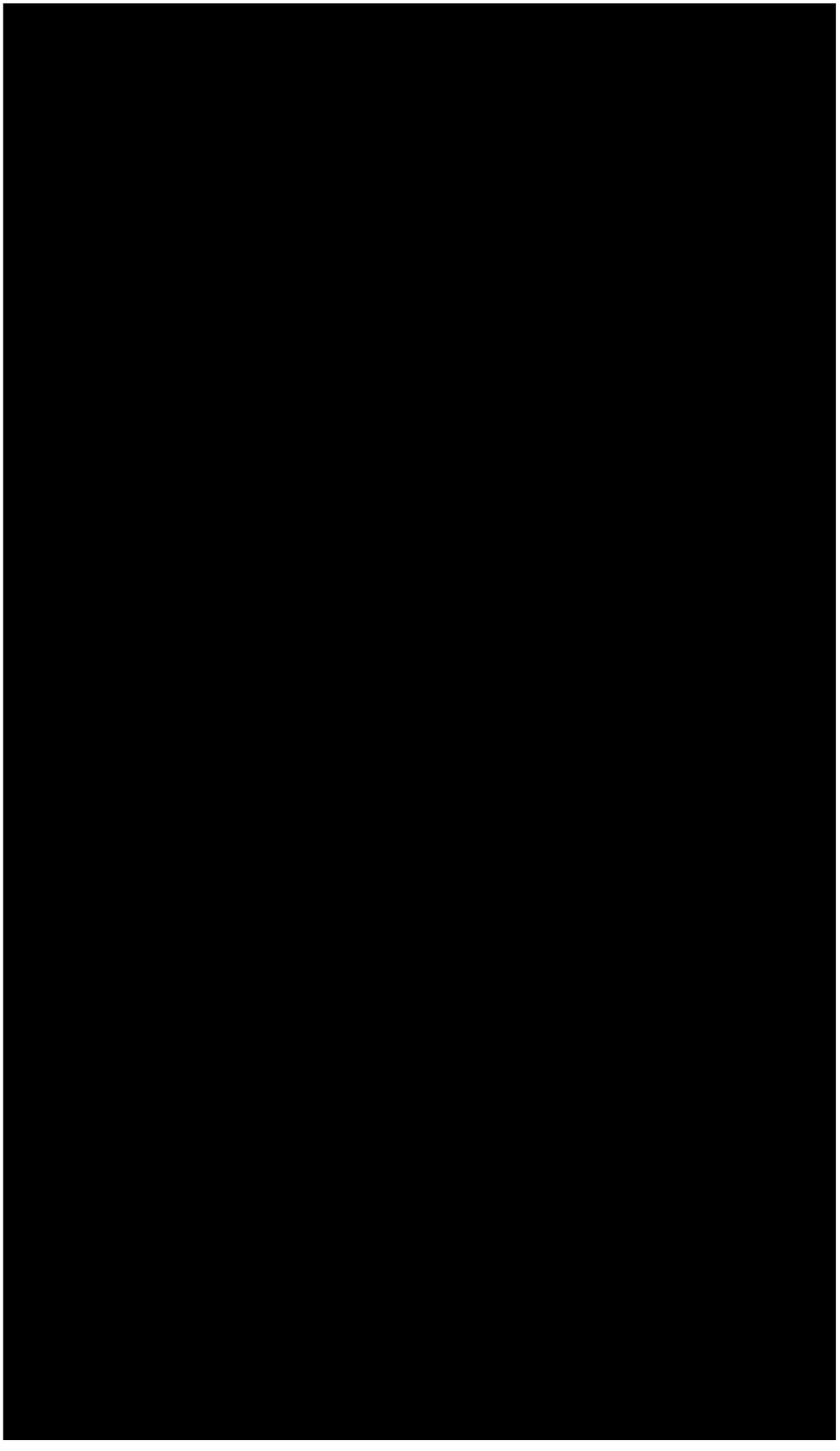

Fig. 9. Distribution of CYP2J2 mRNA in human intestine by in situ hybridization. *In situ* hybridization analysis of adjacent sections of human esophagus (A and B), stomach (C and D), small intestine (E–H), and colon (I and J) using an antisense (A, C, E, G, and I) or a sense (B, D, F, H, and J) digoxigenin-labeled CYP2J2 RNA probe. *Closed arrows*, strong positive staining in nerve cells of the autonomic ganglion. Magnification: 25× (A–D), 50× (E, F, I, and J), and 100× (G and H).

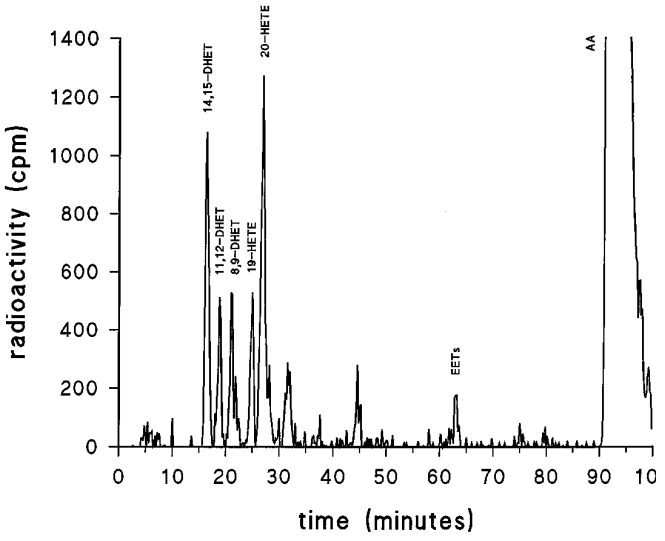

Fig. 10. Metabolism of AA by human jejunum microsomal fractions. Human jejunum microsomal fractions (2.0 mg/ml of microsomal protein) were incubated with [1-^{14}C]AA, NADPH, and an NADPH-regenerating system. After 30 min at 30°, the ethyl ether-soluble products were resolved by reverse-phase HPLC, and the eluent radioactivity was monitored with an on-line radioactive flow detector as described in Experimental Procedures. Metabolites were identified by comparing their HPLC retention time with those of authentic standards and by GC/MS. *HETE*, hydroxyeicosatetraenoic acid.

TABLE 1

Regiochemical and stereochemical composition of human jejunum EETs

The enantiomers of human jejunum 14,15-, 11,12-, and 8,9-EET were extracted, purified, and quantified as described in the text. Concentration values are averages of five determinations on different intestinal tissues with standard error of <15% of the mean.

Regioisomer	Concentration	Distribution	Enantioselectivity	
			R,S	*S,R*
	ng/g of jejunum	*% of total*		
14,15-EET	12	31	69	31
11,12-EET	14	36	65	35
8,9-EET	13	33	37	63
5,6-EET	N.D.	N.D.	N.D.	N.D.

N.D. = not determined.

nological evidence to show that CYP2J P450s are highly expressed throughout the entire gastrointestinal tract and that expression is localized to specific cell types within the intestine. Furthermore, we provide biochemical data to demonstrate that CYP2J products, the EETs, are produced *in vitro* by human intestinal microsomal fractions and are present *in vivo* in human intestine.

Previous studies have demonstrated the constitutive expression of a number of P450 monooxygenases in the rat and human gastrointestinal tract, including members of the CYP1A, CYP2B, CYP2C, CYP2D, CYP2E, and CYP3A subfamilies (5–11). Without exception, in both rats and humans, P450 expression levels were highest in the proximal small intestine and occurred at decreasing concentrations distally, being lowest in the ileum and undetectable in the colon (1, 5, 6, 11). In contrast, CYP2J protein expression is relatively constant throughout the entire gastrointestinal tract from esophagus to colon. In fact, CYP3A4 is the only other P450 that has been shown to be constitutively expressed, albeit at extremely low levels, in esophagus, stomach, or colon (5). A number of factors are known to alter the levels of expression of intestinal P450 genes, including enzyme induction by ingested xenobiotics and dietary factors (1, 8). In this regard, several investigators have reported relatively large interindividual variation in the expression of intestinal P450s (5, 6). The remarkably constant expression of human CYP2J protein in several different jejunum and colon specimens together with the fact that pretreatment with common P450 inducers does not alter the expression of rat CYP2J protein suggests that the CYP2J enzymes may be less susceptible to induction by these factors.

Our data suggest that there is a discordance between intestinal CYP2J2 mRNA and protein levels. We previously reported that CYP2J2 mRNA levels do not correlate well with CYP2J2 protein levels in the kidney (20). Other investigators have noted the lack of correlation between human P450 protein and mRNA levels and have proposed that differences in protein translation rate and/or protein turnover may be important in determining human P450 hemoprotein levels (29). Alternatively, the yield of microsomes may be significantly greater from intestinal cells that express CYP2J2 (e.g., epithelial cells, smooth muscle cells), whereas RNA is likely to represent all intestinal cell types. It is notable that the anti-CYP2J2 antibody recognizes an electrophoretically distinct protein band in microsomal fractions prepared from human intestinal tissues but that the mobility of this protein differs slightly from that of the purified, recombinant CYP2J2 protein. These differences in the electrophoretic mobility, although minor, suggest that either the endogenous human hemoprotein is produced in a truncated form, the endogenous human protein is post-translationally modified, or CYP2J2 shares antigenic determinants with a related, slightly lower-molecular-weight protein that is more abundant in human intestinal tissues. The discordance between CYP2J2 mRNA and protein expression in human intestinal tissues, together with very recent evidence that both rat and mouse contain more than one CYP2J isoform,[2,3] suggest additional complexity of the human CYP2J subfamily. However, the following pieces of evidence support the contention that the immunoreactive protein observed in Western blots and by immunohistochemical staining of human intestinal tissue is CYP2J2: (a) the anti-CYP2J2 antibody strongly immunoreacts with CYP2J2 but does not cross-react with other known human CYP1 and CYP2 family P450s, (b) prestaining adsorption of anti-CYP2J2 IgG with excess purified recombinant CYP2J2 abolishes the positive immunohistochemical staining reaction, (c) *in situ* hybridization using two different CYP2J2-specific RNA probes localizes expression of CYP2J2 mRNA to the same intestinal cell types that express large amounts of CYP2J2 immunoreactive protein by immunohistochemistry, and (d) despite vigorous attempts by our research group and others to identify potentially related human CYP2J or CYP2J-like P450 isoforms, only a single human CYP2J enzyme (CYP2J2) has been described to date (20). In fact, we recently screened a human intestinal cDNA library with the full-length CYP2J2 cDNA probe, and all seven duplicate positive clones that were iso-

[2] J. Ma and D. C. Zeldin. Cloning, expression, and function of mouse CYP2J5 and CYP2J6. Manuscript in preparation.
[3] L. S. Kaminsky, personal communication.

lated and partially characterized contained sequences that were identical to human CYP2J2.[4] This suggests that if human intestine contains multiple CYP2J isoforms, then CYP2J2 is the most abundant one. Further investigation is necessary to determine whether human intestine also contains other antigenically related, previously uncharacterized P450s.

Several studies have been conducted to localize expression of P450s within the intestine by immunohistochemistry. Murray *et al*. (7) have shown that human CYP3A4 expression was confined to columnar epithelial cells of small intestinal villi and to mast cells within the lamina propria, with no staining in goblet cells, epithelial cells of the crypts of Lieberkühn, connective tissue, intestinal smooth muscle, or blood vessels. Rich *et al.* (10) reported that rat CYP2B1 and CYP2B2 were constitutively present only in intestinal epithelial cells, graded in quantity from the crypt to maximally at the tips of villi. Similarly, Shimizu *et al*. (11) detected rat CYP2E1 in intestinal villus epithelial cells. Pretreatment of rats with phenobarbital, β-naphthoflavone, or ethanol, respectively, induced CYP2B1/2, CYP1A1, and CYP2E1 in epithelial cells, in both the villi and crypts, but not in nonepithelial cells (10, 11, 30). In the current study, CYP2J2 immunoreactivity was present in intestinal epithelial cells as well as in nonepithelial cells, including, most prominently, nerve cells of the autonomic ganglia and, to a lesser extent, smooth muscle cells and vascular endothelium. The localization of CYP2J2 expression within the human intestine was confirmed by *in situ* hybridization using two CYP2J2-specific RNA probes. To our knowledge, this is the first demonstration of a P450 mRNA or protein in nonepithelial intestinal cells.

The cellular localization of CYP2J proteins may have important functional implications. For example, the localization of CYP2J proteins to the vascular endothelium has important implications given the well-documented effects of the EETs in controlling vascular tone in the intestine and extraintestinal tissues (17, 31, 32). The expression of CYP2J proteins in the absorptive epithelial cells of the small intestine and colon suggests a role for the EETs in fluid/electrolyte transport in the gut, especially given the known potent effects of epoxygenase products in controlling fluid and electrolyte transport in extraintestinal tissues (19). The expression of CYP2J proteins in chief cells lining the gastric glands together with the known potent effects of the EETs in stimulating peptide hormone secretion in extraintestinal tissues (33) suggests that the EETs may be involved in the release of pepsinogen in the stomach. The expression of CYP2J proteins in nerve cells of autonomic ganglia, in conjunction with the known effects of EETs in stimulating the release of somatostatin and other neuropeptides (18), suggests a role of these eicosanoids in mediating the effects of intestinal neurotransmitters and controlling gut motility. Further work will be necessary to better define the importance and functional significance of epoxygenase products in gastrointestinal physiology and pathophysiology.

Microsomal fractions prepared from human jejunum catalyzed the NADPH-dependent metabolism of AA to EETs and DHETs as the principle reaction products. The mid-chain HETEs and C19/C20 alcohols of AA were produced in lower amounts. The profile of epoxygenase metabolites formed during incubations of human jejunum microsomal fractions with AA was qualitatively similar to that previously reported for human liver and human kidney microsomal fractions (34–37). In contrast, the product profile of human intestinal microsomes was different from that reported for rabbit intestine, in which mid-chain HETEs and C19/C20 alcohols were the major products (3). Importantly, recombinant human CYP2J2 produces neither 19-hydroxyeicosatetraenoic acid nor 20-OH-AA (20), suggesting that human intestine probably contains more than one P450 enzyme that metabolizes AA. The identity of these other intestinal monooxygenases remains unknown; however, several of the P450s known to be expressed in the small intestine have been shown to metabolize AA to EETs and HETEs, including members of the CYP1A, CYP2B, CYP2C, and CYP2E subfamilies (15, 16, 35, 36).

Although *in vitro* studies are important for the enzymatic characterization of metabolic pathways, they provide limited information with respect to the *in vivo* production and concentration of the formed metabolites. The demonstration of EETs as endogenous constituents of human jejunum provided further evidence to support the *in vivo* intestinal cytochrome P450 metabolism of AA. To our knowledge, this is the first report documenting the presence of EETs in intestinal tissue. Compared with human liver, human kidney cortex, and human heart, human jejunum contained less total EET/g of tissue (~10%, ~12%, and ~66% of the total EET present in each of these tissues, respectively) (20, 34, 38). The regiochemical profile of human jejunum EETs was similar to that previously reported for human heart (20) but different from that reported for human liver and kidney cortex (34, 38). Stereoselective formation of eicosanoids is a sufficient criterion to establish their enzymatic origin (24). To confirm that the recovered EETs were, in fact, biosynthesized by the intestinal epoxygenase or epoxygenases, we performed chiral analysis and showed that the 14(*R*),15(*S*)-, 11(*R*),12(*S*)- and 8(*S*),9(*R*)-EETs were the predominant antipodes. The stereochemical profile of human jejunum EETs was quite similar to that previously reported for human liver (34). In contrast, the chirality of human jejunum EETs was different from that of human kidney cortex (38), in which racemic 8,9-EET was recovered, and human heart (20), which produces racemic 8,9- and 11,12-EETs. These findings have important implications given that some of the biological actions of the EETs are stereoselective (32, 39).

In summary, we provided molecular and immunological data that demonstrate that CYP2J mRNAs and proteins are abundantly expressed throughout the entire human and rat gastrointestinal tract from esophagus to colon. Furthermore, we use immunohistochemical staining and *in situ* hybridization to show that CYP2J2 mRNA and protein expression is localized to nerve cells of autonomic ganglia, intestinal epithelial cells, intestinal smooth muscle cells, and vascular endothelium. We report that CYP2J2 products, the EETs, are produced *in vitro* during incubations of human intestinal microsomal fractions with AA and are found *in vivo* in human intestine. We conclude that (a) in addition to the cyclooxygenase and lipoxygenase pathways, the P450 epoxygenase pathway is an important member of the human intestinal AA metabolic cascade, and (b) CYP2J enzymes are among the P450 epoxygenases that form the EETs in the gut. We spec-

[4] J. Ma and D. C. Zeldin, unpublished observations.

ulate that in addition to known effects on intestinal vascular tone, these fatty acid epoxides may have important physiological roles in the release of intestinal neuropeptides, control of intestinal motility, and/or modulation of intestinal fluid/electrolyte transport.

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