Molecular Cloning, Expression, and Functional Significance of a Cytochrome P450 Highly Expressed in Rat Heart Myocytes

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A cDNA encoding a P450 monooxygenase was amplified from reverse transcribed rat heart and liver total RNA by polymerase chain reaction using primers based on the 5’- and 3’-end sequences of two rat pseudogenes, CYP2J3P1 and CYP2J3P2. Sequence analysis revealed that this 1,778-base pair cDNA contained an open reading frame and encoded a new 502 amino acid protein designated CYP2J3. Based on the deduced amino acid sequence, CYP2J3 was approximately 70% homologous to both human CYP2J2 and rabbit CYP2J1. Recombinant CYP2J3 protein was co-expressed with NADPH-cytochrome P450 oxidoreductase in Sf9 insect cells using a baculovirus expression system. Microsomal fractions of CYP2J3/NADPH-cytochrome P450 oxidoreductase-transfected cells metabolized arachidonic acid to 14,15-, 11,12-, and 8,9-epoxyeicosatrienoic acids and 19-hydroxyeicosatetraenoic acid as the principal reaction products (catalytic turnover, 0.2 nmol of product/nmol of cytochrome P450/min at 37 °C). Immunoblotting of microsomal fractions prepared from rat tissues using a polyclonal antibody raised against recombinant CYP2J2 that cross-reacted with CYP2J3 but not with other known rat P450s demonstrated abundant expression of CYP2J3 protein in heart and liver. Immunohistochemical staining of formalin-fixed paraffin-embedded rat heart tissue sections using the anti-CYP2J2 IgG and avidin-biotin-peroxidase detection localized expression of CYP2J3 protein in heart and liver. Immunohistochemical staining of formalin-fixed paraffin-embedded rat heart tissue sections using the anti-CYP2J2 IgG and avidin-biotin-peroxidase detection localized expression of CYP2J3 protein in heart and liver.

Cytochromes P450 (P450s) catalyze the NADPH-dependent oxidation of arachidonic acid to several unique eicosanoids in several species including humans (1–3). The primary products formed are four regioisomeric cis-epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-EET), six midchain cis-trans-conjugated dienols (5-, 8-, 9-, 11-, 12-, and 15-HETE), and ω/ω-1-alcohols of arachidonic acid (19-OH-AA and 20-OH-AA) (1–3). A particular interest in the epoxygenase reaction has developed, in part, because the EETs have been shown to be endogenous constituents of numerous tissues (4–8) and because of the potent biological activities attributed to the EETs and their hydration products, the vic-dihydrinoxyeicosatrienoic acids (1–3, 9, 10). Recent studies demonstrating that (a) the rat renal epoxygenase is under regulatory control by dietary salt, (b) experimental or genetic alterations of the renal epoxygenase induce hypertension in rats fed a high salt diet, (c) urinary excretion of epoxygenase metabolites is increased during pregnancy-induced hypertension in humans, and (d) EETs modulate vascular tone in the several tissues have supported the hypothesis that P450-derived arachidonic acid metabolites may be involved in the pathophysiology of hypertension (6, 9, 11–17).

Several investigators have provided spectral and/or immunologic evidence for the constitutive expression of P450 monooxygenases in scup, rat, rabbit, and pig heart (18–21). In addition, aromatic hydrocarbons have been shown to induce P450 activity in scup, chick embryo, and rabbit heart (20, 22, 23). Recently, our group reported the cDNA cloning and heterologous expression of a new human P450 (CYP2J2) that was highly and constitutively expressed in the heart (8). The recombinant CYP2J2 protein was active in the regio- and stereoselective epoxidation of arachidonic acid (8). Importantly, the chirality of CYP2J2 products matched that of the EET enantiomers present in vivo in human heart, suggesting that CYP2J2 was one of the predominant enzymes responsible for the epoxidation of endogenous cardiac arachidonic acid pools (8). Despite these investigations, the regulation and functional significance of CYP2J2 in the heart remains to be determined.

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of this hemoprotein and the epoxygenase metabolites that it produces in the heart remain unknown.

Studies on the importance and functional role of the human cardiac P450 eicosanoygenase(s) in human heart physiology and pathophysiology have not been possible because of the limited availability of fresh, histologically normal and abnormal human heart tissues. The presence of an animal model would greatly facilitate these investigations but would first require a detailed knowledge of the molecular and catalytic properties of the enzyme(s) involved and access to specific probes to study the regulation of the relevant enzyme(s) at the gene and/or protein level. In this report, we describe the cloning and cDNA-directed expression of a rat P450 that is highly expressed in cardiac myocytes and metabolizes arachidonic acid to EETs and 19-OH-AA. We also utilize an isolated-perfused rat heart model to demonstrate that one of these eicosanoid products (12,12-EET) improves functional recovery following prolonged global cardiac ischemia.

EXPERIMENTAL PROCEDURES

Isolation of RNA, Synthesis and Screening of the cDNA Library, and Cloning of CYP2J3 by Polymerase Chain Reaction—Normal rat tissues were obtained from male Fisher 344 rats fed NIH 31 rodent chow (Agway, St. Mary, OH) ad libitum and sacrificed by lethal CO2 inhalation. Total RNA and poly(A)+ mRNA were prepared by the guanidium thiocyanate/oligo(dT)-cellulose method using total RNA extraction and mRNA purification kits supplied by Pharmacia Biotech Inc. Northern analysis of total RNA prepared from rat tissues using a 1.9-kb cDNA fragment containing the entire published sequence of human CYP2J2, including the untranslated 3'-end regions (8) demonstrated a 1.8-1.9-kb transcript that was most abundant in rat liver as compared with other rat tissues. As a result, we screened an oligo(dT)-primed Uni-Zap cDNA library, synthesized from rat liver poly(A)+ mRNA using a Lambda Zap-cDNA synthesis kit obtained from Stratagene (Stratagene, La Jolla, CA), with the 1.9-kb CYP2J2 cDNA probe. Nucleic acid hybridizations were done at 57 °C in 0.9 M NaCl containing 0.05 M NaH2PO4·Na2HPO4 (pH 7.0), 0.5% SDS, 0.01 M EDTA, 5 × Denhardt's solution, and 0.1 mg of heat-denatured salmon sperm DNA/ml. Approximatively 48 duplicate positive clones were identified of which 12 clones, selected at random, were plaque-purified and rescued into pBluescript plasmid DNA vectors containing the protein encoded by the cloned 1.8-kb cDNA insert (CYP2J3). One of these clones (clone SW9-1, designated CYP2J3) was completely sequenced by the dideoxy chain termination method using total RNA extraction and cDNA probes using the Ready-To-Go DNA labeling system (Amersham Corp.) and purified using G-25 Sephadex columns (Pharmacia).

Northern Blot Hybridization Analysis—Total RNA (20 μg) prepared from rat heart, lung, kidney, liver, stomach, and small intestine was denatured and electrophoresed in 1.2% agarose gels containing 0.2 M formaldehyde. After capillary pressure transfer to GeneScreen Plus nylon membranes (DuPont NEN), the blots were hybridized with either the 1.9-kb CYP2J2 cDNA insert or with the 1.8-kb SW9-1 cDNA insert. Hybridizations were performed at 42 °C in 50% formamide containing 1 × NaCl, 1% (w/v) SDS, 10% (w/v) dextran sulfate, and 0.1 mg heat-denatured salmon sperm DNA/ml. The cDNA probes were labeled with α-32P-dATP using the Megaprime DNA labeling system (Amersham Corp.).

Heterologous Expression of Recombinant CYP2J3—Co-expression of the protein encoded by the cloned 1.8-kb SW9-1 cDNA insert (CYP2J3) and human CYP2J3-P450 cDNA inserted in SbfI site of pAcUW51-CYPOR expression vector was accomplished using the pAcUW51-CYPOR shuttle vector (kindly provided by Dr. Cosette Serabjit-Singh, Glaxo Research Institute, Research Triangle Park, NC (25) and the BaculoGold Baculovirus Expression System (Pharmingen, San Diego, CA). Briefly, the CYP2J3 cDNA was released from the pCR™II cloning vector by digestion with SpeI and XbaI and then ligated with the pAcUW51-CYPOR vector that was linearized by digestion with XbaI. In the resulting expression vector (pAcUW51-CYPOR-CYP2J3), the expression of CYP2J3 was controlled by the p10 promoter, while the expression of CYP2J3 was independently controlled by the polyhedrin promoter. Cultured SF9 insect cells were co-transfected with the pAcUW51-CYPOR-CYP2J3 expression vector and linear wild-type BaculoGold viral DNA in a CaCl2 solution. Recombinant viruses were purified, and the presence of a CYP2J3 cDNA insert was corroborated by PCR analysis. Cultured SF9 cells grown in spinner flasks at a density of 1.5–2 × 106 cells/ml were then infected with high titer CYP2J3/CYPOR recombinant baculovirus stock in the presence of 500 μM δ-aminolevulinic acid. Cells co-expressing recombinant CYP2J3 and CYPOR were harvested 72 h after infection, washed twice with phosphate-buffered saline, and then lysed in 0.1 M sodium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, 1% (v/v) sodium cholate, 0.1 M EDTA, and 0.1 μM dithiothreitol (8) or used to prepare microsomal fractions by differential centrifugation at 4 °C as described previously (4). P450 content was determined spectrophotically according to the method of Omura and Sato (26) using a Shimadzu UV-3000 dual-wavelength/double-beam spectrophotometer (Shimadzu Scientific Instruments, Co., Ltd.). The recombinant P450 CYP2J3 was purified to the passage of the crude cell lysate over an α-aminomethylagarose (Sigma) column equilibrated with 0.1 M potassium phosphate (pH 7.4) containing 20% (v/v) glycerol, 0.1 M EDTA, 0.1 μM dithiothreitol, and 0.4%
was blocked with 3% (v/v) hydrogen peroxide for 15 min. After rinsing, endogenous peroxidase activity was blocked using 3% (v/v) hydrogen peroxide for 15 min. After rinsing, 1:200 dilution of rabbit anti-human CYP2J2 IgG was used according to the manufacturer’s instructions. Microsomal fractions prepared from human lymphoblast cells transfected with the cDNAs to rat CYP2A1 and CYP2E1 were also purchased from GENTEST. Purified preparations of rat CYP1A1, CYP2B1/CYP2B2, and CYP2C13 were a gift from Dr. Joyce Goldstein (NIAMS, National Institutes of Health). Partially purified, recombinant rat CYP2C11 and CYP2C23 were generously provided by Dr. Jorge Capdevila (Vanderbilt University, Nashville, TN). For immunoblotting, microsomal fractions or partially purified, recombinant P450s 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 (Bio-Rad), and the ECL Western blotting Detection System (Amersham) as described (8). Protein determinations were performed according to the method of Bradford (32).

For immunohistochemistry, rat heart tissues were fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin. Localization of CYP2J3 protein expression was investigated using the anti-CYP2J3 IgG (1:200 dilution) on serial sections (5–6 μm) of rat atrium and ventricle. Slides were deparaffinized in xylene and hydrated through a graded series of ethanol to 1 × automatic buffer (1 × AB) (Biomedica, Burlington, CA) washes. Endogenous peroxidase activity was blocked with 3% (v/v) hydrogen peroxide for 15 min. After rinsing in 1 × AB, slides were microwave-treated, cooled, and blocked with normal goat serum, and the primary antibody was applied for 30 min. Preimmune rabbit IgG was used as the negative control in place of the primary antibody. The bound primary antibody was visualized by avin-biotin-peroxidase detection using the Vectastain Rabbit Elite kit (Vector Laboratories, Burlington, CA) according to the manufacturer’s instructions and using 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 covered-slipped with Permpermant™ (Fisher).

Quantification of Endogenous EETs in Rat Heart—Methods used to quantify endogenous EETs present in rat heart were similar to those used to quantify EETs in rat liver (5) and human heart (8). Briefly, heart tissues were frozen in liquid nitrogen and homogenized in 15 ml of phosphate-buffered saline containing triphenylphosphine (5–10 mg). 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 8,9-, 11,12-, and 14,15-[1-14C]EET internal standards (55–57 μCi/μmol, 30 ng each). Saponification to recover phospholipid-bound EETs was followed by silica column purification. The eluent, a mixture of radiolabeled internal standards and total endogenous EETs, was resolved into individual peaks by HPLC and analyzed by selected ion monitoring at m/z 219 (loss of PFB from endogenous EET-PFB) and m/z 241 (loss of PFB from [1-14C]EET-PFB internal standard). The EET-PFB-[1-14C]EET-PFB ratios were calculated from the integrated values of the corresponding ion current intensities.

Isolated Perfused Rat Heart Preparation—Male Sprague Dawley rats (250–320 g) were anesthetized with an intraperitoneal injection of sodium pentobarbital (25 mg) and anticoagulated with heparin (200 units intravenously). Hearts were excised, aortas were cannulated, and retrograde perfusions were begun under constant pressure (90 cm of H₂O) as described previously (33–35). Typical flow rates were 10–15 ml/min. The perfusate was a Kreb-Henseleit buffer containing 2.0 mM CaCl₂, 5.4 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 1.25 mM CaCl₂, 25 mM NaHCO₃, and 11 mM glucose, continuously aerated with humidified 95% O₂, 5% CO₂ and maintained at 37 °C. For assessment of contractile function, a balloon-tipped polyethylene catheter inserted via the left atrium through the mitral valve into the left ventricle was connected to a Statham P23d pressure transducer. Isovolumetric LVDP was measured throughout the experiment. The balloon was routinely inflated to an end diastolic pressure of 5–10 cm of H₂O. Total ischemia was created by cross-clamping the perfusate inflow line. To minimize subendocardial "no-reflow" at the end of the ischemic period, the left ventricular balloon was collapsed immediately prior to reperfusion. After a few minutes of reperfusion, the balloon was reinfated to an end diastolic pressure of 5–10 cm of H₂O to assess recovery of contractile function. Using this procedure, nearly complete restitution of coronary flow and creatine phosphate was achieved during the reflow period, indicating that subendocardial no-reflow was minimal.

Changes in pH and high energy phosphate content were measured by 31P NMR. pH was measured using the shift difference between intracellular inorganic phosphate and creatine phosphate (33–35). Hearts were bathed in the perfusate to improve magnetic homogeneity. The perfusate was switched to phosphate-free Kreb-Henseleit buffer so that the inorganic phosphate content of the perfusate would be composed of only intracellular phosphate. 31P NMR spectra were obtained at 161.9 MHz on a Varian Unity Plus 400-MHz wide-bore NMR spectrometer with the variable temperature probe at 37.0 ± 0.5 °C. The sample was shimmed on the proton signal from the heart, and we routinely obtained a nonspinning line width at one-half height of ~0.2 ppm. Spectra were single-averaged over 5 min using a 2-s interval between scans with a point spacing of 70° (29 μs). The spectral width was ±5006 Hz, and 4096 data points were collected. The free induction decay was multiplied by an exponential function corresponding to 40-Hz line broadening before Fourier transformation.

To determine the effects of CYP2J3 products on the recovery of contractile function following global ischemia, hearts were initially perfused with Kreb-Henseleit buffer for 25 min, followed by 10 min of perfusion recirculating 100 ml of Kreb-Henseleit buffer containing 11,12-EET (0.1–5 μM final concentration), 14,15-EET (5 μM final concentration), 19-OH-AAA (5 μM final concentration), or vehicle alone (ethanol 0.1% final concentration), followed by 5 min of nonrecirculated perfusion without test compound or vehicle. The hearts were then subjected to 20 min of ischemia followed by 40 min of reperfusion. The duration of ischemia was chosen to provide a moderate degree of contractile dysfunction so that beneficial or detrimental effects of the eicosanoids could be detected. Experimental parameters (LVPD, pH, intracellular ATP) were monitored at baseline, during perfusion with the test compounds or vehicle, during the ischemic period, and during reflow.

Surgical Procedures—The [1-14C]EET internal standards were synthesized from [1-14C]arachidonic acid (55–57 μCi/μmol) by nonselective epoxidation as described previously (36). Racemic and enantiomerically pure EETs were prepared by total chemical synthesis according to published procedures (37–40). 4-vic-Dihydroxyeicosatrienoic acids and 1-14C-labeled 4-vic-dihydroxyeicosatrienoic acid were prepared by chemical hydration of individual EETs as described (41). HETEs and C-19-20 alcohols of arachidonic acid were synthesized as described previ-
ously (42). All synthetic compounds were purified by reverse phase HPLC (27). Methylation was performed using an ethereal solution of diazomethane (43). PFB esters were formed by reaction with pentafluorobenzyl bromide as described (5). Trimethylsilyl ethers were prepared using 25% (v/v) bis(trimethylsilyl)trifluoroacetamide in anhydrous pyridine (44).

**Statistical Methods—**

All values are expressed as mean ± S.E. Data were analyzed by analysis of variance using SYSTAT software (SYSTAT Inc., Evanston, IL). When F values indicated that a significant difference was present, Tukey’s HSD test for multiple comparisons was used. Values were considered significantly different if p was <0.05.

**RESULTS**

**Molecular Cloning of Rat CYP2J3—**PCR amplification of reverse transcribed RNA prepared from rat liver using primer pairs based on the 5'- and 3'-end sequences of CYP2J3P1 and CYP2J3P2 produced a single 1.8-kb band on agarose gels. An identical size band was obtained from PCR amplification of reverse transcribed rat heart RNA. The cDNAs contained in these bands were ligated into the pCR™II vector and characterized. Nine clones (five from rat heart, four from rat liver) contained identical nucleotide sequences. One of these clones (clone SW9-1) was selected for further study.

Complete nucleic acid sequence analysis of clone SW9-1 revealed that the cDNA was 1,778 nucleotides long, contained an open reading frame between nucleotides 10 and 1515 flanked by initiation (ATG) and termination (TGA) codons, and contained a short 5' -end untranslated region and a 263-nucleotide 3'-end untranslated region (Fig. 1). The cDNA encoded a 502-amino acid protein that had a derived molecular mass of 57,969 Da. The deduced amino acid sequence for the protein encoded by SW9-1 contained a putative heme binding peptide (FSMGKRALCQEQIWA) with the underlined conserved residues and the invariant cysteine at position 448 (Fig. 1). A comparison of the SW9-1 deduced amino acid sequence with those of other rat P450s indicated that the extent of similarity was limited. Thus, rat CYP1A1, CYP2B1, CYP2C7, CYP2D1, CYP2E1, CYP3A1, and CYP4A1 exhibited 36, 43, 38, 42, 41, 26, and 24% amino acid sequence identity with SW9-1, respectively. In contrast, the deduced amino acid sequence of SW9-1 was 72% identical to the rabbit CYP2J1 sequence (24) and 73% identical to the human CYP2J2 sequence (8). Furthermore, amino acid alignment of the protein encoded by SW9-1 with that of rabbit CYP2J1 and human CYP2J2 demonstrated that most of the differences represented conservative changes, i.e. replacement with residues with overall similar chemical properties. Based on the amino acid sequence homology with rabbit CYP2J1 and human CYP2J2, the rat hemoprotein has been designated CYP2J3 (45).

**Heterologous Expression and Enzymatic Characterization of Recombinant CYP2J3—**Recombinant CYP2J3 protein was co-expressed with CYPOR in Sf9 insect cells using the baculovirus expression system according to previously described methods (8, 25, 46). Under the conditions outlined under “Experimental Procedures,” the level of expression of recombinant CYP2J3 was 5–10 nmol of P450/liter of infected Sf9 cells. Partial purification of recombinant CYP2J3 protein by single passage of baculovirus-infected Sf9 cell lysate over an O-aminooctyl-agarose column produced a protein that migrated as a prominent band with a molecular mass of approximately 58 kDa on Coomassie Brilliant Blue-stained SDS-polyacrylamide gels (Fig. 2). A higher molecular mass band at approximately 75–77 kDa corresponding to recombinant CYPOR was also present in the partially purified protein preparation (Fig. 2).

To reconstitute CYP2J3 activity and to ascertain the catalytic properties of the recombinant hemoprotein, we incubated microsomal fractions prepared from CYP2J3/CYPOR-transfected cells with arachidonic acid in the presence of NADPH and an NADPH-regenerating system. As shown in Fig. 3, CYP2J3 metabolized arachidonic acid to 14,15-, 11,12- and 8,9-EETs and 19-OH-AA as the principal reaction products (catalytic turnover, 0.2 nmol of product formed/nmol of P450/min at 37 °C). These metabolites were identified by comparing their HPLC properties with those of authentic standards and by GC/MS analysis. None of the metabolites were formed in the absence of NADPH showing that the reaction was P450-mediated.
Fig. 2. SDS-polyacrylamide gel electrophoresis of partially purified, recombinant CYP2J3. Recombinant CYP2J3 was expressed in Sf9 insect cells using the baculovirus expression system and partially purified by single passage over an a-amino-octyl agarose column. Ten microliters of the column eluent corresponding to 8 pmol of P450 was electrophoresed on an SDS-10% polyacrylamide gel as described under “Experimental Procedures.” The gel was stained for 2 h in a 10% solution of Coomassie Brilliant Blue R250 dye and destained in 0.7% acetic acid containing 10% methanol. Molecular masses are shown in kDa.

At (Fig. 3). Thus, based on the chromatogram in Fig. 3, we conclude that CYP2J3 is both an arachidonic acid epoxygenase and -1 hydroxylase. Regiochemical analysis of the EETs, which accounted for approximately half of the total reaction products, revealed a preference for epoxidation at the 14,15-olefin (41% of total EET products) (Table I). Epoxidation at the 11,12- and 8,9-olefins occurred less often (27 and 28% of total EET products, respectively), whereas epoxidation at the 5,6-olefin occurred only rarely (4% of total EET products) (Table I). Stereochemical analysis of CYP2J3-derived EETs revealed a slight preference for 14(S),15(R)-, 11(R),12(S)-, and 8(R),9(S)-EETs (optical purity, 57, 62, and 60%, respectively) (Table I).

Expression of CYP2J3 by Northern Analysis and Protein Immunoblotting—Blot hybridization of total RNA extracted from various rat tissues under high stringency conditions using the radiolabeled CYP2J3 cDNA probe produced three bands in rat liver: (a) a strong 1.8-kb band corresponding to the abundant liver CYP2J3 transcript; (b) a slightly less intense band at approximately 3.2 kb; and (c) a lower intensity band at approximately 4.4 kb (Fig. 4). The identity of the 3.2- and 4.4-kb transcripts remains unknown, but these larger transcripts may represent alternate splice variants of CYP2J3. In contrast, CYP2J3 transcripts were present at markedly lower levels in rat heart, lung, kidney, stomach, and small intestine (Fig. 4). Thus, based upon the Northern analysis, we conclude that (a) CYP2J3 mRNA is primarily expressed in rat liver and at lower levels in extrahepatic tissues and (b) two additional transcripts are observed in the liver and in extrahepatic tissues, the identities of which are unknown.

Western blots of microsomal fractions prepared from Sf9 insect cells infected with the recombinant CYP2J3 baculovirus stock using the polyclonal antibody prepared against purified, recombinant human CYP2J2 (8) showed a primary band at approximately 58 kDa indicating that the anti-CYP2J2 IgG did, in fact, cross-react with rat CYP2J3 (Fig. 5A). Control studies demonstrated that the anti-CYP2J2 IgG did not cross-react with the following CYP1 or CYP2 family rat P450s: CYP1A1, CYP2A1, CYP2B1, CYP2B2, CYP2C11, CYP2C13, CYP2C23, and CYP2E1 (Fig. 5B). To examine the tissue-specific expression of CYP2J3 protein, we performed immunoblotting of microsomal fractions prepared from various rat tissues. As illustrated in Fig. 5C, anti-CYP2J2 IgG immunoreacted with an electrophoretically distinct band at approximately 58 kDa in microsomal fractions prepared from rat heart and liver. Anti-CYP2J2 IgG also produced a discrete band, albeit much less intense, with microsomal fractions prepared from rat lung, kidney, stomach, small intestine, and colon but did not react with microsomal fractions prepared from rat brain (Fig. 5C). There was little interanimal variation in the tissue expression of CYP2J3 protein (data not shown). To evaluate the effect of known P450 inducers on the hepatic expression of CYP2J3, we pretreated animals with phenobarbital, -naphthoflavone, clofibrate, or acetone and then examined the expression of liver CYP2J3 by protein immunoblotting. As shown in Fig. 6A, pretreatment of animals with phenobarbital induced liver CYP2B1/CYP2B2 expression but had no effect on expression of CYP2J3. Similarly, pretreatment of animals with -naphthoflavone, clofibrate, or acetone to induce liver CYP1A1/CYP1A2, CYP4A1, and CYP2E1 did not alter liver CYP2J3 protein expression (Figs. 6B, 6C, and 6D). Based on these data, we concluded that (a) CYP2J3 protein is highly expressed in rat heart and liver and at lower levels in other rat tissues; (b) CYP2J3 mRNA levels do not correlate well with CYP2J3 protein levels; and (c) liver CYP2J3 protein expression is not significantly altered by the known P450 inducers.
TABLE I
Regio- and sterechemical composition of EETs produced by recombinant CYP2J3

| Regiosomer | Distribution | Enantioselectivity |
|------------|--------------|--------------------|
|            | % of total   | R,S                | S,R                |
| 14,15-EET  | 41           | 43                 | 57                 |
| 11,12-EET  | 27           | 62                 | 38                 |
| 8,9-EET    | 28           | 60                 | 40                 |
| 5,6-EET    | 4            | ND                 | ND                 |

* ND, not determined.

induced by phenobarbital, β-naphthoflavone, clofibrate, or acetone.

Localization of Cardiac CYP2J3 Expression by Immunohistochemistry—To determine the distribution of CYP2J3 protein within the heart, we stained formalin-fixed paraffin-embedded rat heart tissue sections using the anti-CYP2J2 IgG. As shown in Fig. 7, A and C, CYP2J3 immunoreactivity was abundantly present in both atrial and ventricular myocytes. Staining was also present, albeit less intense, in endothelial cells lining the endocardium, whereas subendocardial connective tissue did not stain (Fig. 7, A and C). Preimmune IgG produced negative staining throughout the rat heart, demonstrating the specificity of the immunostaining for CYP2J3 protein (Fig. 7, B and D).

To our knowledge, this is the first demonstration of expression of a P450 in cardiac myocytes.

Quantitation of Endogenous EETs in Rat Heart—Whereas in vitro studies are an important tool for the enzymatic characterization of metabolic pathways, they provide limited information with regard to the in vivo production and concentration of metabolized products. Using a combination of HPLC and GC/MS techniques, we detected substantial amounts of EETs in rat heart tissue. Rat heart contained 69 ± 7 ng of total EET/g of heart (range 52–103 ng of EET/g of heart, n = 8). The major EET regioisomers present in rat heart were 14,15-EET and 8,9-EET (33 and 39% of the total, respectively) followed by lower amounts of 11,12-EET (28% of the total). The labile 5,6-EET suffers extensive decomposition during the extraction and purification process used and therefore cannot be quantified.

The documentation of EETs as endogenous constituents of rat heart provided evidence supporting the in vivo metabolism of arachidonic acid by CYP2J3.

Functional Significance of CYP2J3 Products in the Heart—The abundant expression of CYP2J3 protein in rat heart myocytes suggested that some of the CYP2J3 products may play an important role in cardiac function. Using an isolated-perfused rat heart model, we first investigated whether several of the CYP2J3 products had effects on cardiac contractility under basal conditions. At base line, isolated-perfused rat hearts had an LVPD of 106 ± 9 mm Hg and normal pH, pO2, and intracellular ATP. Ten minutes of perfusion with a 5 μM final concentration of synthetic 11,12-EET, 14,15-EET, or 19-OH-AA had no significant effects on LVPD (data not shown). We also investigated whether these eicosanoids altered function following global cardiac ischemia. In the presence of vehicle alone (n = 7), 20 min of ischemia followed by 40 min of reperfusion resulted in recovery of only 44 ± 6% of preischemic contractile function (Fig. 8). The addition of synthetic 11,12-EET (5 μM final concentration) (n = 5) to the perfusate prior to global ischemia resulted in a 1.6-fold improvement in recovery of cardiac contractility (LVPD = 69 ± 5% of base line, p = 0.01 versus vehicle alone) (Fig. 8). In contrast, the addition of either synthetic 14,15-EET (n = 5) or synthetic 19-OH-AA (n = 4) (5 μM final concentration, each) to the perfusate prior to global ischemia had no demonstrable effect on recovery of cardiac contractility (LVPD, 54 ± 3 and 47 ± 7% of base line, respectively, p = not significant) (Fig. 8). At 1 μM final concentration, 11,12-EET caused a 1.3-fold improvement in cardiac contractility following global ischemia compared with vehicle alone (n = 6, p = 0.04). At a 0.1 μM final concentration, the effect of 11,12-EET (n = 4) on postischemic recovery of cardiac contractility was not significantly different from that of vehicle alone (LVPD, 60 ± 6 and 44 ± 6% of base line for 11,12-EET and vehicle alone, respectively, p = not significant). Thus, both 1 and 5 μM 11,12-EET caused a significant improvement in recovery of cardiac contractility following prolonged global ischemia. To our knowledge, this is the first demonstration of a beneficial effect of a P450 epoxygenase metabolite in the heart. The addition of 1 μM 11,12-EET had no significant effect on the decline in either intracellular pH or ATP during ischemia (data not shown). Control experiments using [1-14C]EET regioisomers demonstrated that the EETs remained fully miscible in the perfusion solution for the duration of the experiment.

DISCUSSION

Cytochromes P450 and/or their associated monooxygenase activities have been identified in microsomal fractions prepared from heart tissues of a number of species including scup, rat, rabbit, guinea pig, and pig (18–23); however, the identity and functional significance of the cardiac P450 isoform(s) have not been investigated. Recently, our group described a new human P450 (CYP2J2) that was active in the epoxidation of arachidonic acid to EETs and was highly and constitutively expressed in the heart (8). Studies on the regulation of this enzyme in the heart and on the functional significance of the EETs with respect to cardiac physiology and pathophysiology have been severely hampered by limited availability of normal and abnormal human heart tissue specimens and by the absence of biospecific probes necessary to examine the enzyme system in an appropriate animal model. Herein, we report the
cDNA cloning and cDNA-directed expression of a rat P450 (CYP2J3) that is highly expressed in the heart, predominately localized to atrial and ventricular cardiac myocytes, and active in the monoxygenation of arachidonic acid. Furthermore, we use an established isolated-perfused rat heart model to demonstrate that one of the CYP2J3 products, 11,12-EET, improves postischemic recovery of cardiac contractile function.

The recombinant CYP2J3 protein catalyzed the NADPH-dependent metabolism of arachidonic acid to all four regioisomeric EETs and 19-OH-AAA as the principal reaction products; hence, CYP2J3 is both an arachidonic acid epoxygenase and an arachidonic acid ω-1-hydroxylase. The CYP2J3 product profile is distinct from that previously reported for CYP2J1 and CYP2J2; therefore, the three CYP2J subfamily hemoproteins possess different enzymological properties (8, 24). CYP2J1 catalyzes the N-demethylation of benzphetamine to formaldehyde but has not been reported to metabolize arachidonic acid (8, 24). CYP2J2 catalyzes the epoxidation of arachidonic acid but does not produce 19-OH-AAA (8). Furthermore, the regio- and stereo- selective properties of EETs formed by recombinant CYP2J2 and CYP2J3 are different. Thus, while CYP2J2 favors epoxidation at the re,si face of the 14,15-olefin and produces racemic 11,12- and 8,9-EET (8), CYP2J3 displays a preference for 14(S),15(R)-, 11(R),12(S)-, and 8(R),9(S)-EET. The CYP2J3 product profile is also different from that previously reported for other rodent P450 epoxygenases including CYP1A1, CYP2B1, CYP2C11, and CYP2C23 (47, 48).

Northern analysis demonstrated that CYP2J3 mRNA was expressed primarily in the liver and at lower levels in extrapulmonary tissues including the heart. In contrast, immunoblotting showed that CYP2J3 protein was present at high levels in both liver and heart and at lower levels in several other tissues including lung, kidney, and intestine. Several investigators have noted a lack of correlation between mRNA and protein levels for other P450 enzymes and have postulated that tissue-specific differences in protein translation rate and/or protein turnover may be important in determining P450 hemoprotein expression (49). A number of factors are known to alter the expression of P450 monoxygenases including enzyme induction by xenobiotics and dietary factors (5, 47, 50–54), enzyme induction by physiologically relevant manipulations (11), and enzyme suppression by cytokines (55, 56). The data showing lack of induction of liver CYP2J3 by several well characterized P450 inducers suggests that this enzyme may be less susceptible to induction by xenobiotics. Further work will be necessary to better define the intrinsic and extrinsic factors that regulate CYP2J3 gene expression.

Immunohistochemical studies revealed that CYP2J3 expression was highly enriched in atrial and ventricular myocytes and present at lower levels in other heart cells including endothelial cells. Previous work has documented the presence of CYPOR as well as CYP1A1, CYP2B, CYP3A, and CYP4B subfamily P450s in vascular smooth muscle cells and vascular endothelial cells (57–59). Other investigators have shown induction of CYP1A1 in vascular endothelial cells in several different scup tissues including the heart (23, 60). To our knowledge, however, the predominant expression of a P450...
tion was “Experimental Procedures.” Magnifica-
tical methods were as described under mune IgG (A and C) or preim-
mune IgG (B and D). Immunohistochem-
ical methods were as described under “Experimental Procedures.” Magnifica-
tion was ×20 (A and B) and ×40 (C and 
D).

FIG. 7. Immunohistochemical local-
ization of CYP2J3 in rat heart. Shown are photomicrographs of adjacent sections of rat atrium (A and B) and ventricle (C and D) immunostained with either anti-
human CYP2J2 IgG (A and C) or preim-
mune IgG (B and D). Immunohistochem-
ical methods were as described under “Experimental Procedures.” Magnifica-
tion was ×20 (A and B) and ×40 (C and 
D).

FIG. 8. Effects of CYP2J3 products on recovery of cardiac con-
tractility following global ischemia. Isolated rat hearts were per-
fused with Krebs-Henseleit buffer containing 11,12-EET, 19-OH-AA, 14,15-EET (5 μM final concentration each), or vehicle alone (ethanol, 0.1% final concentration) as described under “Experimental Proce-
dures.” The hearts were then subjected to 20 min of global ischemia fol-
lowed by 40 min of reperfusion. The LVDP during reperfusion is 
shown for each experimental group and reported as the percentage of initial LVDP ± S.E.

monooxygenase in cardiac myocytes has not been previously reported and appears to be a unique feature of CYP2J subfam-
ily P450s. The cellular localization of CYP2J3 in the heart 
suggests a potential functional role for CYP2J3 products in 
cardiac muscle cell physiology and/or pathophysiology. For ex-
ample, EETs and 19-OH-AA are reported to have effects on 
cellular K+ channels, an effect that would be expected to pro-
tect the heart against the functional consequences of global ischemia (14, 61, 63, 71). Consistent with the data in this study, 
Moffat and co-workers (72) found that EETs had no effect on 
contractility or coronary pressure in normoxic, perfused guinea 
pig hearts. However, in contrast to our data, Moffat et al. (72) 
observed that, at lower doses than those used in the current 
study, 11,12- and 5,6-EET delayed the recovery of cardiac con-
tractility or coronary pressure in normoxic, perfused guinea 
pig hearts. This discrepancy could be due to species differences (rat versus 
guinea pig) but more probably reflects differences in the sever-
ty of ischemia and the continued availability of glucose in the 
low flow model. Further work will be necessary to better define 
the mechanisms involved in the cardioprotective effects of 
arachidonic acid epoxygenase metabolites.

In summary, we report the cDNA cloning and cDNA-directed 
expression of CYP2J3, a rat P450 arachidonic acid mono-
oxygenase that is highly expressed in the heart and localized to 
atrial and ventricular myocytes. We further demonstrate that 
one of the CYP2J3 products, 11,12-EET, improves functional 
recovery following prolonged, global cardiac ischemia. We con-
clude that CYP2J3 is one of the predominant enzymes respon-
sible for oxidation of endogenous arachidonic acid pools in rat 
heart and that EETs may play important functional roles in the 
response of the heart to ischemia.

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Note Added in Proof—Since the submission of this manuscript, we became aware of a rat CYP2J4 cDNA recently cloned by Dr. Laurence S. Kaminsky and co-workers. We do not yet know whether the polyclonal anti-CYP2J2 Ig used in this paper cross-reacts with CYP234.

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Molecular Cloning, Expression, and Functional Significance of a Cytochrome P450 Highly Expressed in Rat Heart Myocytes
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