

Molecular Cloning and Expression of CYP2J2, a Human Cytochrome P450 Arachidonic Acid Epoxygenase Highly Expressed in Heart*

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A cDNA encoding a human cytochrome P450 arachidonic acid epoxygenase was isolated from a human liver cDNA library. Sequence analysis revealed that this 1,876-base pair cDNA contained an open reading frame and encoded a new 502-amino acid protein designated CYP2J2. Blot hybridization analysis of RNA prepared from human tissues revealed that CYP2J2 was highly expressed in the heart. Recombinant CYP2J2 protein was prepared using the baculovirus expression system and purified to near electrophoretic homogeneity. The enzyme metabolized arachidonic acid predominantly via olefin epoxidation to all four regioisomeric *cis*-epoxyeicosatrienoic acids (catalytic turnover 65 pmol of product formed/nmol of cytochrome P450/min at 30 °C). Epoxidation of arachidonic acid by CYP2J2 at the 14,15-olefin was highly enantioselective for (14*R*,15*S*)-epoxyeicosatrienoic acid (76% optical purity). Immunoblotting of microsomal fractions prepared from human tissues using a polyclonal antibody raised against the recombinant hemoprotein confirmed primary expression of CYP2J2 protein in human heart. The *in vivo* significance of CYP2J2 was suggested by documenting the presence of epoxyeicosatrienoic acids in the human heart using gas chromatography/mass spectroscopy. Importantly, the chirality of CYP2J2 products matched that of the epoxyeicosatrienoic acid enantiomers present, *in vivo*, in human heart. We propose that CYP2J2 is one of the enzymes responsible for epoxidation of endogenous arachidonic acid pools in human heart and that epoxyeicosatrienoic acids may, therefore, play important functional roles in cardiac physiology.

The role of P450¹ in the NADPH-dependent epoxidation of arachidonic acid is well documented (1–3). The primary products formed are four regioisomeric *cis*-epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-EET) (1–3). Thus far, arachidonic acid epoxygenase activity has been demonstrated in microsomal fractions prepared from several organs including liver,

kidney, lung, and pituitary (4–8). Studies utilizing both purified and recombinant P450 enzymes have shown that (a) the epoxygenase reaction is enantioselective, (b) the reaction asymmetry is P450 enzyme specific, and (c) the predominant epoxygenase isoforms belong to the CYP2 gene family (4, 6, 9–13).² The chiral nature of endogenous EET pools in liver, kidney, lung, and plasma confirms the biosynthetic origin of these eicosanoids and documents an endogenous role for microsomal P450 in the bioactivation of arachidonic acid (6, 11, 14, 15).

The potential physiological significance of the epoxygenase reaction is highlighted by the fact that the EETs possess numerous biological activities including modulation of membrane ion fluxes, stimulation of peptide hormone release, and effects on airway smooth muscle (Refs. 6 and 16–19 and references therein). Recent studies demonstrating that (a) the rat renal epoxygenase is under regulatory control by dietary salt, (b) alterations of the rat renal epoxygenase induce hypertension in rats fed a high salt diet, and (c) urinary excretion of epoxygenase metabolites is increased during pregnancy-induced hypertension in humans have supported the hypothesis that P450-derived arachidonic acid metabolites may be involved in the pathophysiology of hypertension (5, 19–21). The EETs have also been shown to have other cardiovascular effects. For example, EETs cause renal artery vasoconstriction (14), cerebral, intestinal, and coronary artery vasodilation (22–25), inhibition of platelet aggregation (26), and cardiac myocyte shortening (27). Importantly, the EETs have been shown to exacerbate the response of the heart to ischemia and reperfusion (27).

P450 has been identified using spectral, immunologic, and/or monooxygenase activity assays in heart microsomal fractions from several vertebrate species including scup, rat, rabbit, and pig (28–31). In addition, aromatic hydrocarbons have been shown to induce P450 activity in scup, chick embryo, and rabbit heart (30, 32, 33). Despite these studies, the identity of the P450 isoforms present in heart tissues has not been reported. Furthermore, the function of this ubiquitous enzyme system in the heart remains unknown. In this report, we describe the cloning and cDNA-directed expression of a new human P450 arachidonic acid epoxygenase that is highly expressed in human heart. We also show that human heart contains substantial quantities of endogenous EETs and that the chirality of these EETs matches that of those produced by the recombinant enzyme.

EXPERIMENTAL PROCEDURES

Materials—[α -³²P]dATP, [γ -³²P]ATP, and [1-¹⁴C]arachidonic acid were purchased from DuPont NEN. Restriction enzymes, *Escherichia coli* polymerase I, and T4 polynucleotide kinase were purchased from New England Biolabs. Triphenylphosphine, α -bromo-2,3,4,5,6-pentafluorotoluene, *N,N*-diisopropylethylamine, *N,N*-dimethylformamide,

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U37143.

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¹ The abbreviations used are: P450, cytochrome P450; EET, *cis*-epoxyeicosatrienoic acid; DHET, *vic*-dihydroxyeicosatrienoic acid; HPLC, high performance liquid chromatography; PFB, pentafluorobenzyl; pfu, plaque-forming units; kb, kilobase.

² The cytochrome P450 nomenclature in Ref. 56 is used throughout this manuscript.

and diazald were purchased from Aldrich. All other chemicals and reagents were purchased from Sigma unless otherwise specified.

Isolation of RNA, Synthesis and Screening of the cDNA Libraries—Normal human tissues obtained through the Cooperative Human Tissue Network (National Disease Research Interchange, Philadelphia) were rapidly frozen in liquid nitrogen and maintained at -80°C for up to 6 months prior to use. Rabbit tissues were obtained from male New Zealand White rabbits sacrificed by lethal intravenous injection of sodium pentobarbital. Poly(A)⁺ mRNA was prepared by the guanidium thiocyanate/oligo(dT)-cellulose method using total RNA extraction and mRNA purification kits supplied by Pharmacia Biotech Inc. Oligo(dT)-primed Uni-Zap cDNA libraries were synthesized from human kidney and liver poly(A)⁺ mRNA using a Lambda Zap-cDNA synthesis kit obtained from Stratagene following the manufacturer's instructions. The human kidney cDNA library had an amplified titre of 3.6×10^9 pfu/ml and an average insert size of 1.5 kb. The human liver cDNA library had an amplified titre of 4.8×10^9 pfu/ml and an average insert size of 2.0 kb. The human kidney library was screened with a 1.6-kb cDNA fragment containing parts of the published sequence of human CYP2C10, including the untranslated 3'-end regions (34, 35). Approximately 5×10^6 phage were plated, employing XL-1 Blue *E. coli* as host, at a density of 10^4 pfu/plate. Nucleic acid hybridizations were done at 57°C in 0.9 M NaCl containing 0.05 M $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (pH 7.0), 0.5% SDS, 0.01 M EDTA, $5 \times$ Denhart's solution, and 0.1 mg of heat-denatured salmon sperm DNA/ml. Approximately 140 duplicate positive clones were identified, of which 35 clones, selected at random, were plaque purified and rescued into pBluescript SK(+) (Stratagene). Plasmid DNAs were replicated in DH5 α competent *E. coli* (Invitrogen) grown in Luria's Broth containing 0.1 mg of ampicillin/ml and isolated using a Qiagen plasmid purification kit (Qiagen Inc., Chatsworth, CA). Insert sizes were determined by agarose electrophoresis after *Eco*RI and *Xho*I digestion of the purified plasmids. The pBluescript cDNA inserts were partially sequenced by the dideoxy chain termination method using Sequenase Version 2.0 (U. S. Biochemical Corp.) and the T3 and T7 oligonucleotide primers. Nucleotide sequences were analyzed by searching GenBank and EMBL data bases utilizing GCG software (Genetics Computer Group, Inc., Madison, WI). One of the duplicate positive clones (clone DZ1, 0.9-kb cDNA insert) contained a new sequence, which shared 50–60% identity with several human and rodent CYP2 family P450 cDNAs and 83% identity with a rabbit P450, CYP2J1 (36). Nucleic acid blot hybridization analysis using total RNA prepared from human liver, kidney, and lung demonstrated that RNA transcripts that hybridized to the cDNA insert contained within this clone were more abundant in human liver as compared with human kidney or lung. As a result, this new human cDNA fragment, which was deemed too short to code for a functional P450 protein, was used as a probe to screen a human liver library to isolate the corresponding full-length cDNA. Approximately 5×10^5 plaques were screened, and 25 of 52 duplicate positive clones were plaque purified, rescued into pBluescript SK(+), and characterized. Five of the clones contained nucleotide sequences that were identical and shared homology with several human and rodent CYP2 family P450s. One of these clones (clone SW2-14, 1.9 kb) was completely sequenced, utilizing a total of 16 oligonucleotide primers (20–25 nucleotides each) that spanned the entire length of the sense and antisense cDNA strands. The entire sequence was confirmed on an Applied Biosystems model 373A DNA sequencer (Perkin-Elmer Corp.) using a PRISM Ready Reaction DyeDeoxy Terminator Cycle sequencing kit (Perkin-Elmer Corp.). Oligonucleotides were synthesized using an Applied Biosystems DNA/RNA synthesizer (Perkin-Elmer Corp.) and purified using G-25 Sephadex columns (Pharmacia Biotech Inc.).

Nucleic Acid Blot Hybridization Analysis—Total RNA (20 μg) prepared from human tissues 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 the cloned 1.9-kb SW2-14 cDNA insert. Poly(A)⁺ mRNA (5 μg) prepared from rabbit tissues was electrophoresed, transferred to nylon membranes, and hybridized with either the 1.9-kb SW2-14 cDNA insert or with the following rabbit CYP2J1 sequence-specific oligonucleotide probe (5'-GCTGTAATTTTGATCCAGTTTCCTCAGAGGCTAATTTCTCTGATATTTCC-3'), complementary to residues 1615–1664 of the rabbit CYP2J1 cDNA (36). Hybridizations were performed at 42°C in 50% formamide containing 1 M NaCl, 1% (w/v) SDS, 10% (w/v) dextran sulfate, and 0.1 mg of heat-denatured salmon sperm DNA/ml. Double-stranded cDNA probes were labeled by nick translation using *E. coli* polymerase I and [γ - ^{32}P]dATP. Oligonucleotide probes were end labeled using T4 polynucleotide kinase and [γ - ^{32}P]ATP.

Heterologous Expression and Purification of Recombinant CYP2J2—

High yield expression of the protein encoded by the cloned 1.9-kb SW-14 cDNA insert (human CYP2J2)³ was accomplished using the MAXBAC baculovirus expression system (Invitrogen) following the manufacturer's instructions. Briefly, cultured SF9 insect cells were cotransfected with a pBlueBacIII transfer vector containing the CYP2J2 cDNA and wild-type baculovirus DNA in a cationic liposome. Recombinant viruses were purified, and the presence of a CYP2J2 cDNA insert was corroborated by polymerase chain reaction analysis. Cultured SF9 cells grown in spinner flasks at a density of $1.5\text{--}2 \times 10^6$ cells/ml were then infected with high titre CYP2J2 recombinant baculovirus stock in the presence of 5 μM hemin, and cells expressing recombinant CYP2J2 were harvested 72 h after infection, washed once with phosphate-buffered saline, and lysed in 0.1 M sodium phosphate (pH 7.4) containing 20% (v/v) glycerol, 1% (w/v) sodium cholate, 0.1 μM EDTA, and 0.1 μM dithiothreitol. Insoluble protein was removed by centrifugation for 1 h at $100,000 \times g$, and the P450 content of the resulting crude cell lysate was determined spectrally according to the method of Omura and Sato (37) using a Shimadzu UV-3000 dual-wavelength/double-beam spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD).

For purification of recombinant CYP2J2, the P450 and sodium cholate concentrations of the crude cell lysate were adjusted to 1 μM and 0.4%, respectively. The resulting suspension was loaded by gravity at room temperature onto a $3 \times 5\text{-cm}$ ω -aminooctyl-agarose (Sigma) column equilibrated with 0.1 M potassium phosphate (pH 7.4) containing 20% (v/v) glycerol, 0.1 μM EDTA, 0.1 μM dithiothreitol, and 0.4% (w/v) sodium cholate (buffer A). The column was washed with 4 column volumes of buffer A, and the bound CYP2J2 was eluted with buffer A containing 0.4% (v/v) Emulgen 911 (Kao Chemical Co., Tokyo). After dialysis versus 200 volumes of 10 mM Tris-Cl buffer (pH 7.4) containing 20% (v/v) glycerol, 0.1% (w/v) sodium cholate, 0.1 μM EDTA, and 0.1 μM dithiothreitol (buffer B), the CYP2J2 sample was loaded onto a $1 \times 5\text{-cm}$ hydroxylapatite (Bio-Rad) column equilibrated with buffer B containing 0.4% (v/v) Emulgen 911. The column was washed with 6 column volumes of equilibration buffer, 4 column volumes of 0.04 M sodium phosphate (pH 7.4) containing 20% (v/v) glycerol, 0.1 μM EDTA, 0.1 μM dithiothreitol, 0.1% (w/v) sodium cholate, and 0.4% (v/v) Emulgen 911, and the bound CYP2J2 was eluted with 0.1 M sodium phosphate (pH 7.4) containing 20% (v/v) glycerol, 0.1 μM EDTA, 0.1 μM dithiothreitol, 0.1% (w/v) sodium cholate, and 0.4% (v/v) Emulgen 911 and dialyzed versus 200 volumes of buffer B. Alternatively, the recombinant P450 was eluted using a stepwise gradient from 0.02 to 0.1 M sodium phosphate containing buffer. To remove free Emulgen 911, dialyzed CYP2J2 was loaded onto a second hydroxylapatite column equilibrated with buffer B and washed with 20–30 column volumes of buffer B (until the eluent absorbance at 280 nm reached a constant minimum). The CYP2J2 was eluted with 0.2 M sodium phosphate (pH 7.4) containing 20% (v/v) glycerol, 0.1 μM EDTA, 0.1 μM dithiothreitol, and 0.1% (w/v) sodium cholate, dialyzed versus 400 column volumes of cholate-free buffer B, and concentrated using a Centricon-30 microconcentrator (Amicon). Recombinant CYP2J2 was subjected to N-terminal amino acid analysis and molecular mass determination by mass spectrometry. For N-terminal amino acid analysis (38), partially purified CYP2J2 was electrophoresed on SDS, 10% (w/v) polyacrylamide slab gels ($200 \times 200 \times 1$ mm), electroblotted onto Immobilon-P polyvinylidene difluoride membranes (Millipore), stained with Coomassie Brilliant Blue R-250 (Bio-Rad), and sequenced directly in the membrane after removal of the protein stain using an Applied Biosystems 475A protein sequencer (Perkin-Elmer Corp.). Cycle yields were calculated by comparison with internal standards. For molecular mass determination, recombinant CYP2J2 was analyzed on a PerSeptive Biosystems Voyager RP matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (PerSeptive Biosystems, Houston, TX) using bovine serum albumin (M+H and M+2H) as an external mass calibrant and α -cyano-4-hydroxycinnamic acid as a matrix.

Production and Isolation of Polyclonal Antibody, Protein Immunoblotting—Polyclonal antibodies against human CYP2J2 were prepared by immunizing two New Zealand White rabbits with partially purified CYP2J2 protein in Freund's complete adjuvant as described (39). Booster doses of immunogen were administered at 4–6-week intervals in Freund's incomplete adjuvant. Pre-immune serum, collected from the rabbits prior to immunization, did not cross-react with CYP2J2 or with microsomal fractions prepared from human tissues. Immune serum from both rabbits gave identical patterns on immunoblots of human

³ The new sequence that is reported in this paper was submitted to the Committee on Standardized Cytochrome P450 Nomenclature and has been designated CYP2J2.

microsomal fractions. IgG fractions were isolated from the immune serum of one of the rabbits using an ImmunoPure IgG purification kit (Pierce). IgG was affinity purified by passage over an SF9 cell lysate column prepared using the AminoLink immobilization kit (Pierce). Microsomal fractions were prepared from normal human tissues by differential centrifugation at 4 °C as described previously (40) and stored at -80 °C until use. Microsomal fractions prepared from human lymphoblast cells transfected with cDNAs to human CYP1A1, CYP2A6, CYP2E1, CYP2B6, CYP2D6, CYP2C9, and CYP2C19 were purchased from GENTEST. Cell lysates prepared from SF9 cells infected with recombinant CYP2C8 baculovirus were used as a source of human CYP2C8 (10). For immunoblotting, microsomal fractions, cell lysates, and/or partially purified CYP2J2 were electrophoresed in SDS-10% (w/v) polyacrylamide gels (80 × 80 × 1 mm) and the resolved proteins transferred electrophoretically onto nitrocellulose membranes. Membranes were immunoblotted using affinity-purified rabbit-anti human CYP2J2 IgG, goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad), and the ECL Western blotting detection system (Amersham Life Sciences, Buckinghamshire, United Kingdom).

Reconstitution of Recombinant CYP2J2 Activity, Product Characterization—Purified rat liver NADPH-P450 reductase was a gift from Dr. Masahiko Negishi (NIEHS, National Institutes of Health). The catalytic activity of CYP2J2 toward arachidonic acid was determined by mixing purified, recombinant CYP2J2 with NADPH-P450 reductase (1 μ M each, final concentration) in the presence of sonicated L- α -dilauroyl-sn-glycero-3-phosphocholine (50 μ g/ml, final concentration). After 20 min at room temperature, the enzyme mixture was diluted to the final reaction volume with 0.05 M Tris-Cl buffer (pH 7.5) containing 0.15 M KCl, 0.01 M MgCl₂, 8 mM sodium isocitrate, and 0.5 IU of isocitrate dehydrogenase/ml and equilibrated at 30 °C for 2 min prior to the addition of [1-¹⁴C]arachidonic acid (25–55 μ Ci/ μ mol, 100 μ M, final concentration). Reactions were initiated with NADPH (1 mM, final concentration) and continued at 30 °C with constant mixing. At different time points, samples of the reaction mixture were extracted into ethyl ether, dried under a nitrogen stream, resolved by reversed-phase HPLC, and quantified by on-line liquid scintillation using a Radiomatic Flo-One β -detector (Radiomatic Instruments, Tampa, FL) (40). For chiral analysis, the EETs were collected from the HPLC eluent, derivatized to corresponding EET-PFB or EET-methyl esters, purified by normal-phase HPLC, resolved into the corresponding antipodes by chiral-phase HPLC, and quantified by liquid scintillation as described previously (41, 42). The benzphetamine *N*-demethylase activity of recombinant CYP2J2 was assessed using the same purified enzyme preparation under identical reaction conditions but employing benzphetamine (2 mM, final concentration) as the substrate. The reaction product (formaldehyde) was quantified according to the method of Nash (43). The sensitivity of the formaldehyde colorimetric assay was 10⁻⁶ M.

Quantitation of Endogenous EETs in Human Heart—Methods used to quantify endogenous EETs present in human heart were similar to those used to quantify EETs in rat liver (11) and rabbit lung (6). Briefly, pathologically normal human heart tissues (1.5–2.0 g) were frozen in liquid nitrogen and immediately 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 μ Ci/ μ mol, 30 ng each). Saponification to recover phospholipid-bound EETs was followed by silica 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 described (11, 41, 42). For analysis, aliquots of individual EET-PFB esters were dissolved in dodecane and analyzed by gas chromatography/mass spectroscopy on a Kratos Concept ISQ mass spectrometer (Kratos Analytical, Inc. 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 at *m/z* 319 (loss of PFB from endogenous EET-PFB) and *m/z* 321 (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.

Other Methods—The EET internal standards were synthesized from [1-¹⁴C]arachidonic acid (55–57 μ Ci/ μ mol) by nonselective epoxidation as described previously (44). Racemic and enantiomerically pure EETs were prepared by total chemical synthesis according to published procedures (45–48). Methylations were performed using an ethereal solution of diazomethane (49). PFB esters were formed by reaction with

pentafluorobenzyl bromide as described (11). Protein determinations were performed according to the method of Bradford (50).

RESULTS AND DISCUSSION

Molecular Cloning of Human CYP2J2—Although there has been extensive studies of the rat and rabbit P450 arachidonic acid epoxygenases (4–6, 9, 11–13, 17–19), substantially less is known about the human enzyme system. In humans, EETs have been detected in the kidney (8), urine (21), reproductive tissue (51), and endothelium (52, 53). Laniado-Schwartzman and co-workers (54) have purified a P450 isoform (P450-AA) from human liver and demonstrated that it catalyzed the epoxidation of arachidonic acid. More recently, several purified and recombinant human P450s of the CYP2C subfamily have been shown to metabolize arachidonic acid with a high degree of regio- and enantiofacial selectivity (10, 55). Importantly, pregnancy-induced hypertension in humans results in significant increases in the urinary excretion of several epoxygenase metabolites, suggesting a role for this enzymatic pathway in human disease (21).

Studies on the importance and functional role of the human P450 arachidonic acid epoxygenases in human physiology and pathophysiology required a detailed knowledge of the molecular and catalytic properties of the enzymes involved and access to biospecific probes to study the regulation of the relevant enzymes at the gene and/or protein level. With these goals in mind, we screened a human liver library with a 0.9-kb human cDNA fragment that shared 83% identity with the previously cloned rabbit P450, CYP2J1 (36). Five of the clones contained nucleotide sequences that were identical. One of these clones (clone SW2-14, 1.9 kb) was selected for further study.

Complete nucleic acid sequence analysis of clone SW2-14 revealed that the cDNA was 1876 nucleotides long, contained an open reading frame between nucleotides 6 and 1511 flanked by initiation (ATG) and termination (TAA) codons, and contained a short 5'-end untranslated region and a 364-nucleotide 3'-end untranslated region with a polyadenylation tail (Fig. 1). The cDNA encoded a 502-amino acid protein that had a derived molecular mass of 57,653 Da. The deduced amino acid sequence for SW2-14 contained a putative heme binding peptide (FSIGKRA^uCLGEOLA^u), with the underlined conserved residues and the invariant cysteine at position 448 (Fig. 1). A comparison of the SW2-14 nucleotide sequence with those of other human P450s indicated that the extent of similarity was limited. Thus, human CYP1A2, CYP2A6, CYP2B6, and CYP2C10 exhibited 43, 48, 48, and 50% nucleic acid sequence identity with SW2-14, respectively. The differences in nucleotide sequence between SW2-14 and other human P450s were randomly distributed along the entire length of the cDNA. In contrast, SW2-14 was 83% identical to rabbit CYP2J1 with the highest variability occurring in the 3'-end untranslated region. Comparison of the deduced amino acid sequence encoded by SW2-14 with those of other P450s demonstrated the following: (a) 19–30% sequence identity with hemoproteins belonging to the CYP1, CYP3, CYP4, CYP5, and CYP6 families; (b) 40–46% sequence identity to hemoproteins belonging to the CYP2 family; and (c) 80% sequence identity to rabbit CYP2J1. Furthermore, amino acid alignment of the protein encoded by SW2-14 with that of rabbit CYP2J1 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, the human hemoprotein has been designated CYP2J2 (56).

Nucleic Acid Blot Hybridization Analysis of Human RNA—To ascertain the relative organ abundance of CYP2J2, total RNA isolated from various human tissues was blot hybridized under high stringency conditions with the full-length

able expression in other rabbit tissues including colon and liver. Expression of CYP2J1 in rabbit heart was not reported. To confirm that rabbit CYP2J1 and human CYP2J2 had different tissue-specific distributions, we performed nucleic acid blot hybridization analysis on rabbit mRNA. Using both a sequence-specific oligonucleotide probe to rabbit CYP2J1 and the

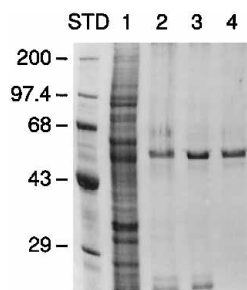


FIG. 3. **SDS-polyacrylamide gel electrophoresis of fractions obtained during the purification of recombinant CYP2J2 from insect cell lysates.** Fractions obtained during the purification of recombinant CYP2J2 from insect cell lysates (6–8 pmol of P450/lane) were electrophoresed on SDS, 10% polyacrylamide gels as described under “Experimental Procedures.” Gels were stained for 2 h in a 10% solution of Coomassie Brilliant Blue R250 dye and destained in 0.7% acetic acid containing 10% methanol. *STD*, molecular weight standards in Da; *lane 1*, CYP2J2-infected *SF9* cell lysate; *lane 2*, aminooctyl-agarose purified CYP2J2; *lane 3*, hydroxylapatite-purified CYP2J2; *lane 4*, sodium phosphate stepwise gradient (0.02–0.1 M) hydroxylapatite-purified CYP2J2.

human CYP2J2 cDNA probe, we detected mRNA transcript levels, albeit at low levels, in rabbit liver, lung, and kidney. The expression of CYP2J1 in rabbit heart was detectable but was clearly lower than in other rabbit tissues (data not shown). Based on these data, we conclude that rabbit CYP2J1 and human CYP2J2 have different tissue-specific distributions and that only the human CYP2J2 cDNA is predominately expressed in the heart.

Heterologous Expression and Purification of Recombinant Human CYP2J2—Several systems have been used to express mammalian P450s including yeast, bacteria, COS cells, vaccinia virus, and baculovirus (57–61). We chose the baculovirus system because it consistently gives high expression levels of recombinant protein and it requires minimal manipulation of the cloned cDNA (57). To express the protein encoded by the CYP2J2 cDNA, we cotransfected *SF9* insect cells with the pBlueBacIII transfer vector containing the cloned CYP2J2 insert and with wild-type baculovirus DNA. The resulting recombinant virus was plaque purified and utilized for the high titre infection of *SF9* cells grown in the presence of 5 μ M hemin as described previously (10, 57). Using these conditions, the level of expression of recombinant CYP2J2 was 100–150 nmol of P450/liter of infected *SF9* cells.

For purification of recombinant CYP2J2, infected *SF9* cells were lysed in the presence of 1% sodium cholate, and the crude protein lysate (specific content, 0.33 nmol of P450/mg of protein) was loaded onto an ω -aminooctyl-agarose column as described under “Experimental Procedures.” The large majority of the recombinant protein remained bound to the ω -aminooctyl-agarose, while most insect proteins eluted during sample loading and washing with the column equilibration buffer. The CYP2J2 was eluted as a single brown band after addition of 0.4% Emulgen 911 to the washing buffer. As shown in Fig. 3, this simple chromatographic step produced a substantially purified protein in 85% yield. Following dialysis, the ω -aminooctyl-agarose purified protein was loaded onto a hydroxylapatite column, washed with a buffer containing 0.04 M sodium phosphate, and the recombinant P450 eluted as a narrow band with a 0.10 M sodium phosphate-containing buffer affording a slightly more purified protein in 35–40% overall yield (Fig. 3). Alternatively, the recombinant P450 was eluted using a stepwise gradient from 0.02 to 0.1 M sodium phosphate-containing buffer affording a protein that was nearly electrophoretically pure in 30% overall yield (Fig. 3). The purified protein was dialyzed, passed over a second hydroxylapatite column to re-

move free Emulgen 911, dialyzed against detergent-free buffer, and concentrated. The resulting protein had a specific content of 7.14 nmol of P450/mg of protein and was obtained in 10% overall yield. Purified CYP2J2 migrated as a discrete band on SDS-polyacrylamide gels with an estimated molecular mass of 57,000 Da (Fig. 3). Based on the P450 specific content, recombinant CYP2J2 was estimated to be approximately 40% pure. The molecular mass of the purified, recombinant protein was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry to confirm that the protein expressed was, indeed, recombinant CYP2J2. The experimentally obtained molecular mass (57,666 Da) was within 0.02% of the derived molecular mass (57,653 Da). We further confirmed that the recombinant protein was identical with CYP2J2 by performing N-terminal amino acid sequence analysis on the purified protein. The experimentally obtained amino acid sequence (MLAAMGSLAAALWAVVHPT) was identical to the amino acid sequence predicted from the nucleic acid sequence of clone SW2–14 (Fig. 1).

Metabolism of Arachidonic Acid by Recombinant CYP2J2—Previous studies utilizing purified and/or recombinant hemoproteins have demonstrated that several members of the CYP2 gene family are capable of epoxidizing arachidonic acid (4, 6, 9, 10, 12, 13, 55). To reconstitute CYP2J2 activity and to ascertain the catalytic properties of the recombinant hemoprotein, we incubated purified CYP2J2 with NADPH-P450 reductase and arachidonic acid. As shown in Fig. 4, CYP2J2 metabolized arachidonic acid (catalytic turnover: 65 pmol of product formed/nmol of P450/min at 30 °C) generating all four regioisomeric EETs and 5,6-DHET as the main products (76% of the total reaction products). Insofar as 5,6-DHET formation must be preceded by 5,6-EET formation (62, 63), the chromatograms in Fig. 4 demonstrate that recombinant CYP2J2 is an arachidonic acid epoxygenase. We identified each of these metabolites by comparing their HPLC properties with those of authentic standards and by gas chromatography/mass spectroscopy (40). None of these metabolites were formed in the absence of NADPH or in the absence of NADPH-P450 reductase showing that the reaction was P450 mediated (Fig. 4). The catalytic turnover of recombinant CYP2J2 is similar to that of CYP2B4 but lower than that of other CYP2 family arachidonic acid epoxygenases (6, 10, 12, 13, 55).

The regio- and stereochemical composition of EETs produced by recombinant CYP2J2 is shown in Table I. Metabolism of arachidonic acid by the hemoprotein was only moderately regioselective with epoxidation occurring preferentially at the 14,15-olefin (37% of total EET products) and less often at the 11,12-, 8,9-, and 5,6-olefins (18, 24, and 21% of total EET products, respectively). Thus, CYP2J2 is less regioselective than rabbit CYP2C2 and human CYP2C8, which generate only 14,15- and 11,12-EETs (10, 13, 55). The regioselectivity of CYP2J2 also is different from (a) rat CYP2C23, which generates primarily 11,12-EET (9, 12); (b) rabbit CYP2B4, which has a unique preference for the 5,6-olefin (6); and (c) human CYP2C9 and CYP2C10, which do not generate significant quantities of 5,6-EET (10, 55). Epoxidation by CYP2J2 at the 14,15-olefin was highly enantioselective for (14*R*,15*S*)-EET (ratio of antipodes 3:1) (Table I). In contrast, epoxidation at the 11,12- and 8,9-olefins was non-enantioselective and thus generated racemic EETs (Table I). The stereoselectivity of epoxidation at the 5,6-olefin could not be evaluated because 5,6-EET underwent rapid, chemical hydration to 5,6-DHET and the δ -lactone of 5,6-DHET. The stereoselectivity of CYP2J2 is different than that previously reported for several purified and recombinant rodent P450 epoxygenases including CYP1A1, CYP2B1, CYP2C11, and CYP2C23 (4, 9). The stereoselectivity

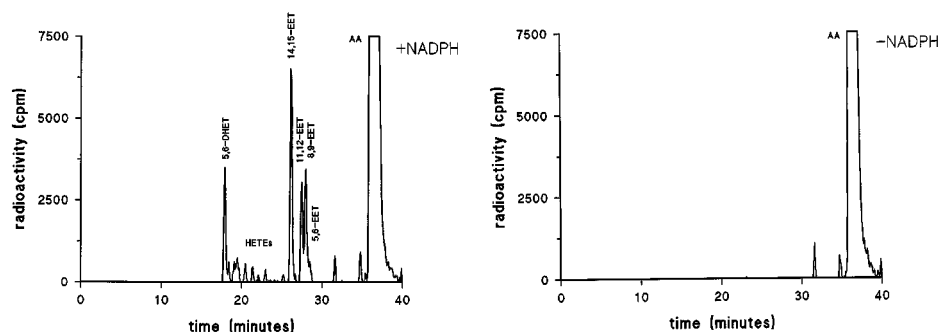


FIG. 4. **Reversed-phase HPLC chromatogram of the organic soluble metabolites generated during incubation of purified, recombinant CYP2J2 with [14 C]arachidonic acid.** Purified, recombinant CYP2J2 and NADPH-P450 reductase (1 μ M final concentration, each) were reconstituted in the presence of L- α -dilauroyl-*sn*-glycero-3-phosphocholine (50 μ g/ml) and incubated at 30 $^{\circ}$ C with [14 C]arachidonic acid (100 μ M, final concentration) with/without NADPH (1 mM, final concentration). After 1 h, the reaction products were extracted and resolved by reversed-phase HPLC as described under "Experimental Procedures." Peak identifications were made by comparisons of the HPLC properties of individual peaks with those of authentic standards and by gas chromatography/mass spectroscopy. Ordinate, radioactivity in cpm; abscissa, time in min. Left panel, incubation with NADPH; right panel, incubation without NADPH.

TABLE I

Regio- and stereochemical composition of EETs produced by recombinant CYP2J2

The arachidonic acid epoxygenase activity of recombinant CYP2J2 was reconstituted in the presence of NADPH-P450 reductase, dilaurylphosphatidylcholine, and NADPH as described under "Experimental procedures." After 1 h, the reaction products were extracted into ethyl ether and resolved by reversed-phase HPLC. The distribution value for 5,6-EET included product isolated as 5,6-DHET and the δ -lactone of 5,6-DHET. For chiral analysis, the EETs were derivatized to corresponding EET-PFB or EET-methyl esters, purified by normal phase HPLC, and resolved into the corresponding antipodes by chiral phase HPLC as described under "Experimental Procedures." Values shown are averages of at least four different experiments with S.E. <5% of the mean. ND, not determined.

Regioisomer	Distribution	Enantioselectivity	
		<i>R,S</i>	<i>S,R</i>
	% total		
14,15-EET	37	76	24
11,12-EET	18	49	51
8,9-EET	24	47	53
5,6-EET	21	ND	ND

of CYP2J2 is also different from that reported for the human epoxygenases CYP2C8, CYP2C9, and CYP2C10 (10, 55).

Kikuta and co-workers (36) have previously reported that rabbit CYP2J1 rapidly catalyzed the *N*-demethylation of benzphetamine to formaldehyde. The same group also found that CYP2J1 did not catalyze lauric acid or arachidonic acid ω -oxidation.⁴ To confirm that rabbit CYP2J1 and human CYP2J2 had different substrate specificities, we incubated CYP2J2 with benzphetamine in the presence of NADPH-P450 reductase and NADPH. Despite multiple attempts and prolonged incubation times, we were unable to demonstrate significant enzymatic metabolism of benzphetamine by recombinant CYP2J2. Based on these data, we conclude that rabbit CYP2J1 and human CYP2J2 have different enzymological properties.

Expression of CYP2J2 Protein in Human Tissues by Immunoblotting—The tissue-specific expression of CYP2J2 was confirmed by protein immunoblotting using polyclonal antibodies raised against the purified, recombinant hemoprotein. As illustrated in Fig. 5, anti-CYP2J2 IgG immunoreacted with an electrophoretically distinct band at approximately 56,000 Da in microsomal fractions prepared from human heart. Anti-CYP2J2 IgG also produced a discrete band, albeit much less intense, with microsomal fractions prepared from human lung, kidney, jejunum, and skeletal muscle but did not react with microsomal fractions prepared from human aorta or vena cava

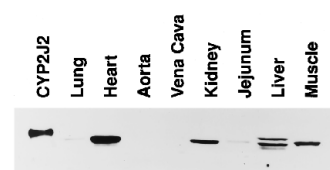


FIG. 5. **Tissue-specific expression of CYP2J2 by protein immunoblotting.** Purified recombinant CYP2J2 (0.25 pmol/lane) or microsomal fractions prepared from human lung, heart, aorta, vena cava, kidney, jejunum, liver, and skeletal muscle (30 μ g of microsomal protein/lane) were electrophoresed on SDS, 10% polyacrylamide gels, and the resolved proteins were transferred to nitrocellulose membranes as described under "Experimental Procedures." Membranes were immunoblotted using affinity-purified rabbit anti-human CYP2J2 IgG and goat anti-rabbit IgG conjugated to horseradish peroxidase. The immunoreactive proteins were visualized using the ECL detection system and autoradiography. Lane 1, purified, recombinant CYP2J2; lane 2, lung; lane 3, heart; lane 4, aorta; lane 5, vena cava; lane 6, kidney; lane 7, jejunum; lane 8, liver; lane 9, skeletal muscle.

(Fig. 5). In contrast, anti-CYP2J2 IgG immunoreacted with three electrophoretically distinct bands in microsomal fractions prepared from human liver: (a) a predominant band electrophoretically similar to the CYP2J2 immunoreactive band observed in extrahepatic human tissues, (b) a lower mobility band of slightly lower intensity, and (c) a higher mobility band of least intensity. Control studies using microsomal fractions prepared from cells expressing recombinant human CYP1A1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP2E1 demonstrated that anti-CYP2J2 IgG did not cross-react with these human CYP1 and CYP2 family P450s (data not shown). Based on these data, we concluded that (a) CYP2J2 protein is highly expressed in human heart and at lower levels in extracardiac tissues, (b) in addition to CYP2J2 protein, human liver microsomal fractions contain two additional proteins that share immunochemical determinants with CYP2J2, and (c) the cross-reactive liver proteins appear to be different from previously characterized P450s of the CYP1A, CYP2A, CYP2B, CYP2C, CYP2D, and CYP2E subfamilies. Further work will be necessary to determine if human liver contains multiple CYP2J isoforms or if anti-CYP2J2 IgG detects other, previously uncharacterized, human liver P450s. All five duplicate positive clones isolated from the human liver cDNA library and characterized by partial nucleic acid sequence analysis and restriction enzyme digestion were identical, suggesting that if human liver contains multiple CYP2J isoforms, then clone SW2-14 encodes the most abundant one. To our knowledge, the expression of a P450 in skeletal muscle has not been reported.

With the exception of human kidney, CYP2J2 protein levels correlated well with CYP2J2 mRNA levels (Figs. 2 and 5). In

⁴ Y. Kikuta, personal communication.

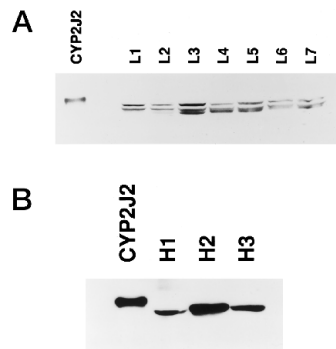


FIG. 6. Interindividual variation in expression of CYP2J2 protein in different human livers and hearts by protein immunoblotting. *Panel A*, purified, recombinant CYP2J2 or human liver microsomal fractions (50 μ g of microsomal protein/lane) prepared from seven different human livers (L1–L7) were electrophoresed on SDS, 10% polyacrylamide gels, and the resolved proteins were transferred to nitrocellulose membranes, immunoblotted with affinity-purified rabbit anti-human CYP2J2 IgG and goat anti-rabbit IgG conjugated to horseradish peroxidase, and detected using the ECL detection system as described under “Experimental Procedures.” *Panel B*, purified, recombinant CYP2J2 or human heart microsomal fractions (30 μ g of microsomal protein/lane) prepared from three different human hearts (H1–H3) were electrophoresed, transferred to nitrocellulose, and immunoblotted with rabbit anti-human CYP2J2 IgG as described under “Experimental Procedures.”

human kidney, CYP2J2 protein was expressed at moderate levels despite low mRNA expression. These results are particularly interesting given the potential relevance of the epoxygenase enzyme system to kidney salt and/or water metabolism and to the pathophysiology of human hypertension (5, 8–10, 14, 19–21). Other investigators have noted the lack of correlation between protein and mRNA levels for some human P450s and have proposed that translation rate and/or protein turnover may be important in determining human P450 hemoprotein levels (64). Fig. 5 also demonstrates that the purified, recombinant CYP2J2 protein produces an immunoreactive band that migrates with a slightly lower mobility (higher molecular mass) than the bands produced by endogenous CYP2J2 present in human tissues. We have determined that the molecular mass of the purified, recombinant CYP2J2 protein is nearly identical to that calculated from the amino acid sequence derived from the CYP2J2 cDNA. The differences in electrophoretic mobility between recombinant CYP2J2 and endogenous CYP2J2, although minor, suggest that (a) the endogenous hemoprotein is produced in a truncated form, (b) the endogenous protein is post-translationally modified, or (c) the clone that we isolated (clone SW2–14) encodes a P450 that shares antigenic determinants with a related, slightly lower molecular weight protein that is more abundant in human tissues and is predominately expressed in heart. Further investigation will be necessary to determine if these explanations, or others, can account for the minor differences in electrophoretic mobility between the endogenous and the recombinant proteins.

To evaluate interindividual differences in expression of CYP2J2 protein, we performed immunoblotting on microsomal fractions prepared from an additional seven human liver and three human heart specimens. Protein immunoblotting of the human liver microsomal fractions revealed remarkably low interindividual variation in the expression of CYP2J2 protein (*middle band*) in human liver tissue (Fig. 6A). In contrast, the expression of the cross-reactive liver proteins (*upper and lower bands*) were more variable (Fig. 6A). Thus, while some livers contained roughly equal amounts of CYP2J2 and the two cross-reactive proteins (e.g. L3–L7), others primarily expressed CYP2J2 and the lower mobility protein (*upper band*) (L1–L2).

TABLE II

Regio- and stereochemical composition of human heart EETs

The enantiomers of human heart 14,15-, 11,12-, and 8,9-EET were extracted, purified, and quantified as described under “Experimental Procedures.” Concentration values shown are averages of three determinations on different heart tissues with S.E. <15% of the mean. For enantioselectivity, S.E. <5% of the mean. ND, not determined.

Regioisomer	Concentration	Distribution	Enantioselectivity	
			<i>R,S</i>	<i>S,R</i>
	ng/g heart	% total		
14,15-EET	23	39	63	37
11,12-EET	14	24	44	56
8,9-EET	22	37	43	57
5,6-EET	ND	ND	ND	ND

Protein immunoblotting of the human heart microsomal fractions revealed that, while CYP2J2 was expressed at high levels in each of the heart tissues, there was greater interindividual variation in CYP2J2 expression in human heart than in human liver (Fig. 6B). In a given individual, however, CYP2J2 expression was always significantly higher in the heart than in extracardiac tissues. Many factors are known to alter the levels of expression of human P450 genes including genetic polymorphism, enzyme induction, and/or inhibition and developmental factors (65–67). A number of investigators have reported large (3–115-fold) interindividual variation in expression of human P450s of the CYP1, CYP2, CYP3, and CYP4 gene families (64, 68–70).

Quantitation of Endogenous EETs in Human Heart—Whereas *in vitro* studies are an indispensable tool for the enzymatic characterization of metabolic pathways, they provide limited information with regard to the *in vivo* production and concentration of formed metabolites. The documentation of EETs as endogenous constituents of human heart provided evidence supporting the *in vivo* metabolism of arachidonic acid by CYP2J2. Using a combination of HPLC and gas chromatography/mass spectroscopy, we detected substantial amounts of EETs in human heart tissue. As shown in Table II, human heart contained approximately 60 ng of total EET per gram of heart. The major EET regioisomers present in human heart were 14,15-EET and 8,9-EET (39 and 37% of the total, respectively) followed by lower amounts of 11,12-EET (24% of the total) (Table II). The labile 5,6-EET suffers extensive decomposition during the extraction and purification process used and therefore cannot be quantified. Chiral analysis of human heart EETs revealed that, for the 14,15-EET regioisomer, (14*R*,15*S*)-EET was the major enantiomer recovered (optical purity, 63%) (Table II). In contrast, 11,12- and 8,9-EETs were recovered in nearly racemic mixtures (Table II). Importantly, the chirality of endogenous EETs recovered from human heart (Table II) matched the chirality of CYP2J2 products (Table I), suggesting that CYP2J2 was one of the predominant enzymes responsible for epoxidation of arachidonic acid pools in human heart. To our knowledge, this report is the first documentation of EETs as endogenous constituents of heart tissue. Previous work has demonstrated that both cyclooxygenase and lipoxygenase metabolites of arachidonic acid are produced in the heart and that these eicosanoids may participate in the myocardial injury that is associated with ischemia reperfusion (71–73). The EETs have been shown to have direct positive inotropic effects on cardiac myocytes, delay recovery from myocardial ischemia, and have potent effects of vascular smooth muscle tone (14, 22–25, 27). The EETs have also been shown to affect ion transport in multiple tissues including vascular smooth muscle cells and cardiac myocytes (19, 27, 74, 75). Thus, our results indicate that, as a member of the human heart arachidonic acid metabolic cascade, CYP2J2 plays a cen-

tral role in the biosynthesis of biologically active EETs and, consequently, may have important role(s) in cardiac function.

Compared with human kidney cortex, human heart contains approximately 5-fold less total EETs and has a distinctly different regio- and stereochemical profile (76). Thus, whereas both tissues favor epoxidation at the *re, si* face of the 14,15-olefin and produce racemic 8,9-EET, only human heart produces racemic 11,12-EET. The chirality of endogenous EETs recovered from human heart also differs from those isolated from rat liver and rabbit lung in which (14*R*,15*S*)-, (11*S*,12*R*)-, and (8*S*,9*R*)-EET were the predominant antipodes (6, 11). In fact, our data suggest that the regio- and stereochemical properties of the arachidonic acid epoxygenases are organism and tissue specific. These findings have important implications given that many of the biological actions of the EETs appear to be both regio- and stereoselective (6, 14, 26). Stereoselective formation of eicosanoids is a sufficient criterion to establish their enzymatic origin (11). Therefore, based on the data presented in Table II, we conclude that 14,15-EET was produced *in vivo* by the human heart epoxygenase. Although the racemic nature of endogenous human heart 11,12- and 8,9-EETs precludes a definitive statement regarding their biosynthetic origin, the fact that CYP2J2, an epoxygenase highly expressed in human heart, also produces racemic 11,12- and 8,9-EETs supports the contention that these eicosanoids are formed enzymatically.

The P450 monooxygenases have long been thought to function primarily in the metabolism of exogenous compounds including drugs and carcinogens (66, 67). Over the past 10–15 years, there has been an increased awareness that this ubiquitous enzyme system may also be involved in the bioactivation of endogenous substrates such as steroids and fatty acids (16–18). The documentation of P450 monooxygenases in vertebrate heart tissue (28–33), together with the known cardiovascular effects of P450 arachidonic acid epoxygenase metabolites (14, 22–25, 27, 74, 75), suggests that this enzyme system may play important functional roles in cardiac physiology and pathophysiology. We report here the cDNA cloning and cDNA-directed expression of CYP2J2, a new human P450 that is highly and constitutively expressed in heart. We demonstrate that the recombinant hemoprotein catalyzes the regio- and stereoselective epoxidation of arachidonic acid and show that the chirality of CYP2J2 products matches that of the enantiomers present *in vivo* in human heart. We conclude, therefore, that CYP2J2 is one of the predominant enzymes responsible for epoxidation of endogenous arachidonic acid pools in human heart and suggest that, in addition to the cyclooxygenase and lipoxygenase pathways, the P450 monooxygenase pathway is an important member of the cardiac arachidonic acid metabolic cascade. We speculate that epoxygenase metabolites may be important in maintaining cardiac homeostasis and that altered local concentration of these eicosanoids may lead to cardiac dysfunction. As pathologically normal human heart tissue becomes available, it will be important to (a) evaluate the metabolism of arachidonic acid by cardiac microsomal fractions, (b) localize expression of CYP2J2 within the heart by immunohistochemistry and *in situ* hybridization, and (c) further examine the role that the EETs may play in cardiac physiology.

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