Enhanced Postischemic Functional Recovery in CYP2J2 Transgenic Hearts Involves Mitochondrial ATP-Sensitive K⁺ Channels and p42/p44 MAPK Pathway

John Seubert,* Baichun Yang,* J. Alyce Bradbury, Joan Graves, Laura M. Degraff, Scott Gabel, Rebecca Gooch, Julie Foley, John Newman, Lan Mao, Howard A. Rockman, Bruce D. Hammock, Elizabeth Murphy, Darryl C. Zeldin

Abstract—Human CYP2J2 is abundant in heart and active in the biosynthesis of epoxyeicosatrienoic acids (EETs); however, the functional role of this P450 and its eicosanoid products in the heart remains unknown. Transgenic mice with cardiomyocyte-specific overexpression of CYP2J2 were generated. CYP2J2 transgenic (Tr) mice have normal heart anatomy and basal contractile function. CYP2J2 Tr hearts have improved recovery of left ventricular developed pressure (LVDP) compared with wild-type (WT) hearts after 20 minutes ischemia and 40 minutes reperfusion. Perfusion with the selective P450 epoxygenase inhibitor N-methylsulphonyl-6-(2-proparglyoxyphenyl)hexanamide (MS-PPOH) for 20 minutes before ischemia results in reduced postischemic LVDP recovery in WT hearts and abolishes the improved postischemic LVDP recovery in CYP2J2 Tr hearts. Perfusion with the ATP-sensitive K⁺ channel (K_{ATP}) inhibitor glibenclamide (GLIB) or the mitochondrial K_{ATP} (mitoK_{ATP}) inhibitor 5-hydroxydecanoate (5-HD) for 20 minutes before ischemia abolishes the cardioprotective effects of CYP2J2 overexpression. Flavoprotein fluorescence, a marker of mitoK_{ATP} activity, is higher in cardiomyocytes from CYP2J2 Tr versus WT mice. Moreover, CYP2J2-derived EETs (1 to 5 μmol/L) increase flavoprotein fluorescence in WT cardiomyocytes. CYP2J2 Tr mice exhibit increased expression of phospho-p42/p44 mitogen-activated protein kinase (MAPK) after ischemia, and addition of the p42/p44 MAPK kinase (MEK) inhibitor PD98059 during reperfusion abolishes the cardioprotective effects of CYP2J2 overexpression. Together, these data suggest that CYP2J2-derived metabolites are cardioprotective after ischemia, and the mechanism for this cardioprotection involves activation of mitoK_{ATP} and p42/p44 MAPK. (Circ Res. 2004;95:506-514.)

Key Words: arachidonic acid • cytochrome P450 • eicosanoid • ischemia/reperfusion • mitoK_{ATP} channel • MAPK

Cytochrome P450 epoxygenases metabolize arachidonic acid (AA) to epoxyeicosatrienoic acids (EETs), which are converted to dihydroxyeicosatrienoic acids (DHETs) by epoxide hydrolases.¹ These P450-derived eicosanoids possess potent biological effects in extracardiac tissues.¹ In contrast, less is known about P450 epoxygenases and their eicosanoid products in the heart. Multiple P450s are expressed in heart tissue.¹³ Among these, CYP2J2 is unique in that it is primarily expressed in the heart, abundant in cardiomyocytes, and active in the biosynthesis of EETs.³⁴ The recent identification of a functionally relevant polymorphism in the CYP2J2 gene that is associated with cardiovascular disease risk in humans supports the clinical relevance of this pathway.⁵⁶ Increased EET biosynthesis in stenosed coronary arteries and during cardiac ischemia/reperfusion suggests the hypothesis that EETs may serve a protective mechanism in the ischemic myocardium.⁷⁸ Both EETs and DHETs have potent vasodilatory effects in the coronary circulation.⁹¹⁰ Indeed, the P450 epoxygenase metabolites are leading candidates for endothelial-derived hyperpolarizing factor, the nitric oxide synthase– and cyclooxygenase-independent vasodilator that hyperpolarizes vascular smooth muscle cells by opening C₅⁻activated K⁺ channels.⁹¹⁰ The EETs have been shown to activate p42/p44 MAPK in coronary vascular endothelial and smooth muscle cells.¹¹¹² The EETs also directly affect cardiomyocyte function. EETs shorten the cardiac action potential, inhibit cardiac Na⁺ channels, and activate cardiac K_{ATP}.¹³¹⁵ In general, coronary vasodilation, inhibition of Na⁺ channels, activation of K_{ATP}, shortening of the action potential, and activation of p42/p44 MAPK confer cardioprotection during ischemia/reperfusion; however, studies on the influence of P450 epoxygenase products on postischemic recovery of heart contractile function have produced conflicting re-
sults. Moreover, CYP2J2 metabolizes linoleic acid (LA) to epoxysteracdeenolic acids (EpOMEs), which depress cardiac function.

To examine the cardiac effects of P450-derived eicosanoids under basal conditions and during ischemia/reperfusion, we used the cardiomyocyte-specific α-myosin heavy chain (αMHC) promoter to overexpress the human CYP2J2 cDNA in a transgenic model. CYP2J2 Tr mice have normal heart anatomy and basal contractile function, but exhibit improved posts ischemic recovery of left ventricular function. Moreover, our data suggest that the mechanism for this cardioprotection by CYP2J2 metabolites involves activation of mitoKATP and p42/p44 MAPK.

Materials and Methods

For an expanded Materials and Method section, see the online data supplement available at http://circres.ahajournals.org.

Transgenic Mice

The CYP2J2 cDNA (GenBank U37143) was cloned into the vector pBOS-αMHC-hGH, a generous gift from Dr. Jeffrey Robbins (University of Cincinnati, Ohio). This vector contains the αMHC promoter to drive cardiomyocyte-specific expression of the transgene and human growth hormone (hGH)/polyA sequences to enhance transgene mRNA stability. The linearized transgene was microinjected into pronuclei of single cell C57BL/6NTac mouse embryos (Taconic, Germantown, NY), which were implanted into pseudopregnant mice. Founders were identified by a combination of PCR and Southern blotting of genomic DNAs. All studies were approved by the NIEHS Animal Care and Use Committee.

Northern Analysis, Immunoblotting, and Immunohistochemistry

Northern blotting was performed as described. Polyclonal antibodies against recombinant human CYP2J2 (anti-CYP2J2rec) and against CYP2J2-specific peptides HMDQNFGNRPVT (anti-CYP2J2pep1) and RESMPYTVNAHEVRQMGNIIPLN (anti-CYP2J2pep3) were prepared as described. Immunoblotting was performed as described. Control studies showed that anti-CYP2J2pep1 and anti-CYP2J2pep3 are immunospecific for CYP2J2, whereas anti-CYP2J2rec cross-reacts with mouse CYP2J isoforms. Immunohistochemical staining of formalin-fixed, paraffin-embedded heart sections was performed as described.

Fatty Acid Metabolism

Heart microsomal fractions were incubated with [1-14C]AA and products were analyzed by HPLC as described. Epoxygenase activity was calculated as the rate of EETs+DHETs produced per mg protein/min. Cardiomyocytes were isolated from neonatal CYP2J2 Tr and WT hearts as described and cultured on 1% gelatin-coated plates in Dulbecco modified Eagle’s medium containing 10% fetal bovine serum. Cardiomyocyte cell culture media was analyzed for epoxy and dihydroxy fatty acid derivatives of AA and LA using established HPLC/MS/MS methods.

Transthoracic Echocardiography and Assessment of Heart Anatomy

Two-dimensional M-mode echocardiography was performed using an HDI-5000 echocardiograph as described. CYP2J2 Tr mice (25 to 30 g, 4 to 6 months) and WT littermate controls were then euthanized, and hearts removed, dissected, weighed, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin/eosin for histological examination.

Isolated-Perfused Hearts

Hearts were perfused in the Langendorff mode as described. Hearts from all four CYP2J2 Tr lines and age/sex-matched WT littermate controls were perfused in a retrograde fashion at constant pressure (90 cmH2O) with continuously aerated (95% O2/5% CO2) Krebs-Henseleit buffer at 37°C. Hearts were perfused for 20 minutes (stabilization), then subjected to 20 minutes global no-flow ischemia, followed by 40 minutes reperfusion. For some experiments, hearts were stabilized for 20 minutes, then perfused with either the selective P450 epoxygenase inhibitor MS-PPOH (50 μmol/L), 11,12-EET (1 μmol/L), the sarcolemmal KATP (sarcKATP) and mitoKATP inhibitor GLIB (20 μmol/L), the selective mitoKATP inhibitor 5-HD (100 μmol/L), the KATP opener pinacidil (PIN, 100 μmol/L) or vehicle for 20 minutes, then subjected to 20 minutes ischemia and 40 minutes reperfusion. In other experiments, the MEK inhibitor PD98059 (10 μmol/L) or vehicle was administered during the 40 minutes reperfusion period. Recovery of contractile function was taken as LVDP at 40 minutes reperfusion expressed as a percentage of preischemic LVDP.

Flavoprotein Fluorescence

Cardiomyocytes were isolated from hearts of adult CYP2J2 Tr and WT mice and plated onto Laminin-coated dishes in M199 media as described. Endogenous flavoprotein fluorescence was used as a marker of mitoKATP activity. Fluorescence was excited by the 488-nm line of a krypton-argon laser, emission was recorded at 568 nm, and confocal images were taken on a Model 410 laser scanning confocal microscope (Carl Zeiss Inc, Thornwood, NY). Emitted fluorescence was assessed in CYP2J2 Tr and WT cardiomyocytes at baseline and after treatment with sodium cyanide (NaCN, 2 mmol/L), 2,4-dinitrophenol (DNP, 0.2 mmol/L), PIN (100 μmol/L), or vehicle for 20 minutes, then cultured for 20 minutes ischemia and 40 minutes reperfusion. In some experiments, changes in fluorescence were recorded in WT cardiomyocytes treated with either 14,15-EET (1 to 5 μmol/L), 11,12-EET (1 μmol/L), or vehicle. Changes in fluorescence were expressed as percentage change relative to baseline levels.

p42/p44 MAPK Expression and Activation

The expression of total and phospho-p42/p44 MAPK was determined in hearts at different times during the ischemia/reperfusion protocol. Individual hearts from either CYP2J2 Tr or WT mice were frozen after 20 minutes of perfusion, 10 minutes or 20 minutes of ischemia, and 10 minutes or 40 minutes of reperfusion. Protein from the 10 000g supernatant of individual hearts was resolved on SDS-polyacrylamide gels, transferred to nitrocellulose membranes and immunoblotted with antibodies to p42/p44 MAPK, phospho-p42/p44 MAPK (Cell Signaling Technology, Inc), or actin C-11 (Santa Cruz Biotechnology). Relative band intensities, expressed in arbitrary units of phospho-p42/p44 MAPK to total p42/p44 MAPK, were assessed by densitometry using a ChemiImager 4000 System (Alpha Innotech Corp).

Statistical Analysis

Data were analyzed by investigators who were blinded to genotype and treatment group assignment. Values are expressed as mean±SE. Data were analyzed by ANOVA or Student’s t test using SYSTAT software (SYSTAT Inc.). Values were considered significantly different if P<0.05.

Results

Development and Initial Characterization of Transgenic Mice

Six germline founder mice were generated by microinjection of the transgenic construct (Figure 1a) into single cell mouse embryos. Transgene-positive pups were identified by PCR (Figure 1b) and Southern blotting (Figure 1c) of genomic
DNA. Northern analysis with the CYP2J2 cDNA probe (Figure 1d) and immunoblotting with three CYP2J2 antibodies (Figure 1e) revealed that four of the founder lines had abundant cardiac expression of the transgene. Densitometry of immunoblots performed with the anti-CYP2J2rec antibody revealed a ~3-fold increase in CYP2J protein expression in CYP2J2 Tr versus WT hearts. The transgene was expressed in a cardiac-specific manner (Figure 1f) and immunohistochemical staining with two CYP2J2-selective antibodies demonstrated that transgene expression occurred primarily in cardiomyocytes (Figure 1g). Although there was some inter-animal variability in the magnitude of CYP2J2 overexpression, we did not observe any consistent or significant differences in CYP2J2 levels among transgenic offspring from founder lines Tr2, Tr3, Tr5, and Tr7. Therefore, all subsequent studies used heterozygous CYP2J2 Tr progeny of each of these overexpressing lines and age/sex-matched WT littermate controls.

Heart Anatomy and Baseline Function

The Table summarizes anatomic and functional characteristics in CYP2J2 Tr and WT hearts. There were no significant differences between the two groups in heart or individual chamber weights, echocardiographic dimensions or fractional shortening, heart rate, or hemodynamic parameters under basal conditions. Histological assessment of hematoxylin/eosin-stained sections revealed no pathology. These data indicate that CYP2J2 Tr hearts are anatomically and functionally normal at baseline.

Fatty Acid Metabolism

Microsomes from CYP2J2 Tr hearts exhibited ~3-fold higher AA epoxygenase activity than microsomes from WT hearts (Figure 2a), indicating increased capacity for cardiac EET biosynthesis with CYP2J2 overexpression and confirming that the overexpressed P450 was catalytically active. To further assess fatty acid metabolism from endogenous lipid pools, we measured levels of 11 different P450 epoxygenase–derived products of AA and LA in culture media from isolated CYP2J2 Tr and WT cardiomyocytes by HPLC/MS/MS. EETs levels in culture media were generally low and were not significantly different between CYP2J2 Tr and WT cardiomyocytes (Figure 2b). Importantly, CYP2J2 Tr cardiomyocytes released significantly more 14,15-DHET, 11,12-DHET, and 8,9-DHET (stable metabolites of 14,15-EET, 11,12-EET, and 8,9-EET) into culture media than did WT cardiomyocytes.
Cardiac Parameters in CYP2J2 Tr and WT Mice

<table>
<thead>
<tr>
<th></th>
<th>WT (n=8–25)</th>
<th>CYP2J2 Tr (n=8–25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>26.4±1.1</td>
<td>27.2±1.0</td>
</tr>
<tr>
<td>Heart weight, mg</td>
<td>108.5±6.0</td>
<td>111.9±3.6</td>
</tr>
<tr>
<td>Heart/body wt, mg/g</td>
<td>4.1±0.2</td>
<td>4.1±0.1</td>
</tr>
<tr>
<td>Left ventricle free wall weight, mg</td>
<td>80.9±5.2</td>
<td>84.4±2.7</td>
</tr>
<tr>
<td>Right ventricle free wall weight, mg</td>
<td>21.5±0.8</td>
<td>20.7±1.0</td>
</tr>
<tr>
<td>Left atrium weight, mg</td>
<td>3.0±0.2</td>
<td>3.4±0.1</td>
</tr>
<tr>
<td>Right atrium weight, mg</td>
<td>3.1±0.3</td>
<td>3.4±0.2</td>
</tr>
<tr>
<td>Left ventricular end-diastolic dimension, mm</td>
<td>3.3±0.1</td>
<td>3.1±0.1</td>
</tr>
<tr>
<td>Left ventricular end-systolic dimension, mm</td>
<td>1.6±0.1</td>
<td>1.5±0.1</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>51.6±2.0</td>
<td>52.3±3.0</td>
</tr>
<tr>
<td>Septal wall thickness, mm</td>
<td>0.67±0.03</td>
<td>0.61±0.01</td>
</tr>
<tr>
<td>Posterior wall thickness, mm</td>
<td>0.67±0.03</td>
<td>0.61±0.02</td>
</tr>
<tr>
<td>HR conscious, bpm</td>
<td>684±12</td>
<td>666±16</td>
</tr>
<tr>
<td>Velocity of circumferential fiber shortening, HR corrected, circ/sec</td>
<td>13.4±0.8</td>
<td>13.3±0.9</td>
</tr>
<tr>
<td>LVDP, cmH2O (Baseline)</td>
<td>129±8</td>
<td>128±10</td>
</tr>
<tr>
<td>LVEDP, cmH2O (Baseline)</td>
<td>7.2±0.6</td>
<td>6.5±0.4</td>
</tr>
<tr>
<td>Rate of contraction, dP/dtmax, cmH2O/sec (Baseline)</td>
<td>3825±217</td>
<td>3930±294</td>
</tr>
<tr>
<td>Rate of relaxation, −dP/dtmin, cmH2O/sec (Baseline)</td>
<td>−3095±169</td>
<td>−3185±243</td>
</tr>
<tr>
<td>HR perfused, bpm (Baseline)</td>
<td>360±15</td>
<td>370±18</td>
</tr>
<tr>
<td>Rate-pressure product, LVDP×HR, cmH2O/min (Baseline)</td>
<td>46130±3022</td>
<td>48758±5020</td>
</tr>
<tr>
<td>LVDP, cmH2O (R40)</td>
<td>27±3</td>
<td>47±6*</td>
</tr>
<tr>
<td>LVEDP, cmH2O (R40)</td>
<td>78±3</td>
<td>62±6*</td>
</tr>
<tr>
<td>Rate of contraction, dP/dtmax, cmH2O/sec (R40)</td>
<td>1039±168</td>
<td>1904±302*</td>
</tr>
<tr>
<td>Rate of relaxation, −dP/dtmin, cmH2O/sec (R40)</td>
<td>−764±112</td>
<td>−1435±302*</td>
</tr>
<tr>
<td>HR, perfused, bpm (R40)</td>
<td>291±13</td>
<td>346±14*</td>
</tr>
<tr>
<td>Rate-pressure product, LVDP×HR, cmH2O/min (R40)</td>
<td>8147±931</td>
<td>16501±2450*</td>
</tr>
</tbody>
</table>

Values are mean±SE.

Cardiomyocytes (Figure 2b). These data are consistent with the known regiochemistry of olefin epoxidation by CYP2J2 and the presence of an active epoxide hydrolase in mouse cardiomyocytes. In contrast, there were no significant differences between the two genotypes in the levels of EpOMEs or dihydroxyoctadecenoic acids (DHOMEs) released into culture media (Figure 2b).

Cardiac Performance After Ischemia/Reperfusion in Perfused Hearts

CYP2J2 Tr hearts had normal baseline contractile function, measured either as LVDP (Figure 3a; Table) or rate-pressure product (RPP=LVDP×HR) (Figure 3b; Table). Compared with WT, CYP2J2 Tr isolated-perfused hearts had significantly improved postischemic recovery of left ventricular function. The improved function was evident within 10 minutes of reperfusion and persisted throughout the recovery period (Figure 3a). At 40 minutes reflow, LVDP recovery was significantly higher in CYP2J2 Tr (37±4%) versus WT hearts (22±2%, P<0.01) (Figure 3a; Table). Similarly, RPP recovered significantly better in CYP2J2 Tr versus WT hearts (33±4% versus 18±3%, respectively; P<0.005) (Figure 3b; Table). Consistent with the improved postsischemic contractile function, left ventricular end-diastolic pressure (LVEDP) at 40 minutes reflow was significantly lower in CYP2J2 Tr (62±6 cmH2O) versus WT hearts (78±3 cmH2O; P<0.05) (Table). There were no differences between CYP2J2 Tr and WT hearts in time-to-onset of ischemic contracture (8.3±1.0 minutes versus 9.8±0.8 minutes, respectively; P=0.20) or maximal ischemic contracture (104±8 cmH2O versus 88±7 cmH2O, respectively; P=0.14). The improved postsischemic functional recovery was independently confirmed in each of
mediated cardioprotection, we conducted experiments in the four overexpressing lines. When analyzed separately by line, postischemic LVDP recovery in WT and CYP2J2 Tr littermates was 25±5% and 46±4% from line Tr2 (P=0.01), 23±5% and 38±5% from line Tr3 (P=0.04), 20±3% and 31±3% from line Tr5 (P=0.05), and 17±4% and 35±6% from line Tr7 (P=0.05).

To determine whether the effects of CYP2J2 overexpression were mediated by a P450 epoxygenase metabolite, we conducted experiments in the presence of MS-PPOH. This epoxygenase inhibitor caused a small but significant reduction in postischemic recovery in WT mice; recovery of LVDP at 40 minutes reflow was 23±2% in the absence of MS-PPOH and 13±4% in the presence of MS-PPOH (P<0.005) (Figure 3c). Importantly, MS-PPOH completely abolished the improved postischemic recovery in CYP2J2 Tr mice; recovery of the LVDP at 40 minutes reflow was 37±3% in the absence of MS-PPOH and 14±3% in the presence of MS-PPOH (P<0.005) (Figure 3c). Thus, percent LVDP recovery was comparable in the two genotypes after treatment with MS-PPOH. Interestingly, perfusion with physiologically relevant concentrations of 11,12-EET improved postischemic recovery in WT hearts (Figure 3d). Together, these data suggest that the cardioprotective effects of CYP2J2 overexpression are mediated by a P450 epoxygenase metabolite.

Role of KATP in Postischemic Functional Recovery
To determine whether KATP was involved in CYP2J2-mediated cardioprotection, we conducted experiments in the presence of the sarcKATP and mitoKATP inhibitor GLIB or the selective mitoKATP inhibitor 5-HD. Neither GLIB nor 5-HD had a significant effect on baseline LVDP. Perfusion with either GLIB or 5-HD for 20 minutes before ischemia resulted in a small but significant reduction in postischemic LVDP recovery in WT hearts (Figure 4). Interestingly, both inhibitors completely abolished the improved postischemic functional recovery in CYP2J2 Tr hearts (Figure 4). Thus, percent LVDP recovery at 40 minutes reperfusion was comparable in the two genotypes after treatment with either GLIB or 5-HD (Figure 4). Moreover, the KATP opener PIN improved postischemic functional recovery to a greater degree in WT compared with CYP2J2 Tr hearts such that percent LVDP recovery was comparable in the two genotypes after treatment with PIN (Figure 4). These data suggest the involvement of KATP in the cardioprotective effect of CYP2J2 overexpression.

Endogenous Flavoprotein Fluorescence
To further investigate the effect of CYP2J2 overexpression on mitoKATP activity, we measured endogenous flavoprotein fluorescence, an index of mitochondrial redox state.35,36

Figure 2. Fatty acid metabolism in CYP2J2 Tr and WT hearts. a, AA epoxygenase activity is enhanced in cardiac microsomes from CYP2J2 Tr animals. Values shown are mean±SE; n=4 pools, 4 to 6 hearts/pool for each group; P<0.05 vs WT. Oxylinipid levels in culture media from CYP2J2 Tr and WT cardiomyocytes. Values shown are mean±SE; n=7 to 10 per group; P<0.05 vs WT.

Figure 3. Postischemic recovery of left ventricular function in CYP2J2 Tr and WT mice. a, LVDP at baseline, at the end of ischemia, and after 5 to 40 minutes reperfusion (R5, R10, R20, R30, and R40) in WT and CYP2J2 Tr hearts. Values shown are mean±SE; n=25 per group; P<0.05 vs WT, b, RPP at baseline and 40 minutes reperfusion in WT and CYP2J2 Tr hearts. Values shown are mean±SE; n=25 per group; P<0.05 vs baseline of same genotype; †P<0.05 vs WT at 40 minutes reperfusion. c, Postischemic LVDP recovery at 40 minutes reperfusion expressed as percentage of baseline LVDP in hearts administered vehicle or MS-PPOH (50μmol/L) for 20 minutes before ischemia. Values represent mean±SE, n=10 to 11 per group; †P<0.05 vs WT; †P<0.05 vs vehicle control of same genotype. d, Postischemic LVDP recovery at 40 minutes reperfusion expressed as percentage of baseline LVDP in WT hearts administered vehicle or 11,12-EET (1μmol/L) for 20 minutes before ischemia. Values represent mean±SE; n=4 per group; P<0.05 vs vehicle.
Under basal conditions, CYP2J2 Tr cardiomyocytes exhibited increased flavoprotein fluorescence compared with WT cardiomyocytes (Figure 5a). Blinded, quantitative analysis of 55 to 60 cells from 4 individual animals of each genotype revealed a significantly higher relative fluorescent intensity in the CYP2J2 Tr mice (Figure 5b). Control experiments conducted with 5-HD (Figure 5a), PIN, NaCN, and DNP (data not shown) confirmed that the emitted fluorescence correlated well with changes in mitochondrial redox status and mitoK\textsubscript{ATP} activity. We also examined the effect of CYP2J2-derived metabolites on flavoprotein fluorescence in WT cardiomyocytes. Application of 14,15-EET (1 to 5 \text{\mu mol/L}), the major CYP2J2 product, resulted in a dose-dependent increase in flavoprotein fluorescence (Figure 5c). A similar effect was observed after application of 1 \text{\mu mol/L} 11,12-EET (Figure 5c). The effects of 14,15-EET and 11,12-EET were rapid and lasted up to 10 minutes (see online Movie, shown only for 11,12-EET, and available in the online data supplement). These findings indicate that CYP2J2-derived eicosanoids activate mitoK\textsubscript{ATP}. Together with the inhibitor studies, these data suggest that mitoK\textsubscript{ATP} activation is one mechanism for improved postischemic functional recovery in CYP2J2 Tr mice.

### Role of p42/p44: MAPK Activation in Postischemic Functional Recovery

To determine whether the MAPK signaling pathway was involved in the cardioprotective mechanism, we examined the phosphorylation status of p42/p44 MAPK in WT and CYP2J2 Tr hearts at baseline, during ischemia, and during reperfusion. There were no significant differences in the expression of phospho-p42/p44 MAPK between WT and CYP2J2 Tr hearts under basal conditions or during ischemia (Figure 6a). Interestingly, expression of phospho-p42/p44 MAPK was significantly higher in CYP2J2 Tr hearts compared with WT hearts at 10 minutes and 40 minutes of reperfusion (Figure 6a). Likewise, the ratio of phospho-p42/p44 MAPK to total p42/p44 MAPK expression was significantly greater in CYP2J2 Tr hearts than in WT hearts during reperfusion (Figure 6b). Activation of p42/p44 MAPK has been proposed to occur downstream of mitoK\textsubscript{ATP} opening\textsuperscript{27}; hence, we examined whether inhibition of mitoK\textsubscript{ATP} affects p42/p44 MAPK activation in our model. Notably, administration of 5-HD before ischemia failed to abolish the enhanced phosphorylation of p42/p44 MAPK in CYP2J2 Tr hearts during reperfusion (Figure 6b). To determine whether activation of the p42/p44 MAPK pathway was required for CYP2J2-mediated cardioprotection in CYP2J2 Tr hearts, we administered the MEK inhibitor PD98059 during the reperfusion period and examined the effect on LVDP recovery. Importantly, treatment with PD98059 had minimal effect on LVDP recovery in WT hearts but completely abolished the improved postischemic recovery of LVDP in CYP2J2 Tr hearts (Figure 6c). Thus, percent LVDP recovery was comparable in the two genotypes after treatment with PD98059. Together, these data suggest that activation of p42/p44
by guest on July 11, 2017 http://circres.ahajournals.org/ Downloaded from
cardiomyocyte function in vitro,3,13 documenting that these P450-derived eicosanoids can affect
progress in this area. Moreover, studies on the biological
P450 expression/activity on cardiac function has limited
the absence of an animal model to study the effects of altered
pharmacological tools to manipulate this pathway in vivo and
nous functions in the heart. However, the lack of specific
precise molecular composition of mitoK ATP remains un-
Among these, significant interest has focused on the role of
overexpression are mediated by a P450 epoxygenase
overexpression are mediated by a P450 epoxygenase
metabolite. The dis-
regarding the functional significance of P450s in the heart.
There has been considerable controversy in the literature
proved postischemic functional recovery in CYP2J2 Tr mice.
MAPK during reperfusion is another mechanism for
improved postischemic functional recovery in CYP2J2 Tr mice.

Discussion
There has been considerable controversy in the literature
regarding the functional significance of P450s in the heart.
Some studies have focused on the role of these enzymes in the
cardiac metabolism of drugs and xenochemicals.2 The discov-
er of CYP2J2 as a primarily cardiac P450 active in the
epoxidation of AA to EETs,4 together with recent studies
documenting that these P450-derived eicosanoids can affect
cardiomyocyte function in vitro,3,13–17 has led to the hy-
thesis that this enzyme may also have important endoge-
ous functions in the heart. However, the lack of specific
pharmacological tools to manipulate this pathway in vivo and
the absence of an animal model to study the effects of altered
P450 expression/activity on cardiac function has limited
progress in this area. Moreover, studies on the biological
effects of P450 metabolites in the heart have often produced
conflicting results. For example, EETs are reported to have
both positive16,17 and negative28 inotropic effects in the heart
under basal conditions. After ischemia/reperfusion, EETs are
reported to have both cardioprotective1 and cardiodepressant
effects.17 In light of these controversies, we developed a
transgenic model to study the effects of CYP2J2 overexpres-
ion on cardiac function. Our major finding is that the
CYP2J2 transgenic mice have normal heart anatomy and
basal contractile function, but exhibit improved postischemic
recovery of left ventricular function. Given that EET biosyn-
thesis is enhanced in stenosed coronary arteries8 and during
cardiac ischemia/reperfusion injury,7 these findings may also
have important therapeutic implications.

How does CYP2J2 overexpression produce beneficial car-
diac effects after ischemia? To address this question, we first
examined fatty acid metabolism in CYP2J2 Tr and WT
hearts. Microsomes from CYP2J2 Tr hearts had increased AA
epoxygenase activity compared with WT hearts, indicating
increased capacity for cardiac EET biosynthesis with CYP2J2
overexpression. Moreover, isolated CYP2J2 Tr cardiomyo-
cytes released more DHETs into culture media than did WT
cardiomyocytes. In contrast, there were no significant dif-
f erences between the two genotypes in the levels of LA
metabolites released. These data suggest that the preferred
substrate for CYP2J2 in the cardiomyocyte is AA rather than
LA. Next, we examined the effect of a selective P450
epoxygenase inhibitor on postischemic recovery of contrac-
tile function. We found that MS-PPOH caused a small but
significant reduction in postischemic LVDP recovery in WT
mice, suggesting a role for P450 epoxygenase metabolites in
mediating cardioprotection under normal conditions. Im-
portantly, MS-PPOH completely abolished the improved postis-
chemic LVDP recovery in CYP2J2 Tr mice, further suggest-
ing that the cardioprotective effects of CYP2J2 overexpres-
sion are mediated by a P450 epoxygenase metabolite.

Various cardioprotective mechanisms have been proposed
to explain enhanced functional recovery after ischemia.
Among these, significant interest has focused on the role of
KATP. Two pharmacologically distinct KATP types have been
identified in cardiomyocytes, sarcoKATP and mitoKATP.29
SarcoKATP is activated during cardiac ischemia when cytoplas-
ic ATP is depleted and affects membrane excitability.
Activation leads to shortening of the cardiac action potential
and reduced intracellular calcium overload.30,31 Several
sarcoKATP openers produce beneficial effects on the myocar-
dium in animal models of ischemia, and several sarcoKATP
inhibitors block ischemic preconditioning.30,31 Structurally,
cardiac sarcoKATP is composed of an octomeric complex of two
types of subunits (Kir6.2 and SUR2A). The EETs have been
shown to be potent activators of sarcoKATP by reducing channel
sensitivity to ATP; however, the exact site on the channel that
interacts with EETs remains enigmatic.13,14 Although the
decision molecular composition of mitoKATP remains un-
known, preliminary studies suggest the presence of a multi-
protein complex containing succinate dehydrogenase.32 Im-
portantly, recent pharmacological data indicate that selective
activation of mitoKATP confers cardioprotection after ische-
emia26,30,31; however, a role for P450 epoxygenase metabo-
lites in this process has not been investigated. In this regard,
we observed that the beneficial effects of CYP2J2 overexpression are abolished by 5-HD, which is selective for mitoK<sub>ATP</sub>. Interestingly, we also demonstrated increased flavoprotein fluorescence, a marker of mitochondrial redox status, in CYP2J2 Tr cardiomyocytes consistent with enhanced mitoK<sub>ATP</sub> activation with CYP2J2 overexpression. Moreover, treatment of WT cardiomyocytes with EETs increased flavoprotein fluorescence. Taken together, these data suggest that one mechanism for the cardioprotective effects of CYP2J2 overexpression could be activation of mitoK<sub>ATP</sub> by 5-HD. Although the precise pathways by which mitoK<sub>ATP</sub> activation confers cardioprotection remain unknown, potentially beneficial consequences of opening mitoK<sub>ATP</sub> include depolarization of the intramitochondrial membrane, transient swelling of the intramitochondrial space, enhanced respiration via the electron transport chain, reduced mitochondrial calcium overload, and altered production of reactive oxygen species.

Activation of p42/p44 MAPK has also been proposed to be cardioprotective after ischemia/reperfusion, although the precise mechanisms whereby p42/p44 MAPK activation confers cardioprotection remain unknown. Recent studies demonstrate that EETs activate the MAPK pathway in endothelial and vascular smooth muscle cells. The data presented herein demonstrate that p42/p44 MAPK activation is enhanced in CYP2J2 Tr hearts during reperfusion. We cannot rule out the possibility that at least some of the differences in phosphorylation of p42/p44 MAPK between CYP2J2 Tr and WT hearts during reperfusion were a result of protection rather than a cause of protection; however, inhibition of the MAPK pathway with a MEK inhibitor administered during reperfusion abolished the improved postischemic functional recovery in the CYP2J2 Tr animals. Together, these data suggest that another component of the cardioprotective mechanism in the CYP2J2 Tr mice involves activation of p42/p44 MAPK. We observed rapid activation of p42/p44 MAPK during early reperfusion in CYP2J2 Tr hearts, indicating that it occurred in response to the ischemic event. This is consistent with a recent report by Hausenloy and coworkers showing that activation of p42/p44 MAPK at reperfusion is essential for preconditioning-induced protection. In contrast, mitoK<sub>ATP</sub> activation was enhanced before ischemia in CYP2J2 Tr hearts. Gross and coworkers recently proposed that activation of p42/p44 MAPK occurs downstream of mitoK<sub>ATP</sub> opening. However, inhibition of mitoK<sub>ATP</sub> by 5-HD did not eliminate the differences in phosphorylation status of p42/p44 MAPK between CYP2J2 Tr and WT hearts. This suggests that the cardioprotective effect observed in the CYP2J2 Tr mice involves a parallel cascade of events involving both mitoK<sub>ATP</sub> and p42/p44 MAPK. The fact that inhibitors of either pathway completely abolish the improved postischemic functional recovery in CYP2J2 Tr mice suggest that activation of both are required for CYP2J2-mediated cardioprotection to occur. Figure 7 illustrates a schematic of the proposed mechanisms of cardioprotection in CYP2J2 Tr mice.

In summary, we used the αMHC promoter to overexpress human CYP2J2 in mouse heart. CYP2J2 Tr mice have normal basal heart anatomy and function, but exhibit improved postischemic recovery of left ventricular function. Moreover, our data suggest that the mechanism for the improved postischemic recovery in CYP2J2 Tr mice involves activation of mitoK<sub>ATP</sub> and p42/p44 MAPK. These studies are the first to document an endogenous role for this enzyme system in the heart and may have implications for the treatment of ischemic heart disease.

Acknowledgments

The work was supported by the NIEHS Division of Intramural Research, NIH HL61558 (H.A.R.), and NIH ES04710 and NIH ES04699 (B.D.H.). We acknowledge the NIH Transgenic Mouse Development Facility and Carl Pinkert (contracts NO1-HD-53229 and NO1-DE-12634) for assistance with mouse production. We also thank Jeff Reece of the NIEHS Confocal Microscopy Center and Jesse Degraff, James Clark, Tracy Demianenko, and Page Myers for assistance with various aspects of this work.

References


Enhanced Postischemic Functional Recovery in CYP2J2 Transgenic Hearts Involves Mitochondrial ATP-Sensitive K+ Channels and p42/p44 MAPK Pathway
John Seubert, Baichun Yang, J. Alyce Bradbury, Joan Graves, Laura M. Degraff, Scott Gabel, Rebecca Gooch, Julie Foley, John Newman, Lan Mao, Howard A. Rockman, Bruce D. Hammock, Elizabeth Murphy and Darryl C. Zeldin

Circ Res. 2004;95:506-514; originally published online July 15, 2004; doi: 10.1161/01.RES.0000139436.89654.c8

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/95/5/506

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2004/08/24/95.5.506.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
EXPANDED MATERIALS AND METHODS

**Transgenic Mice.** The coding region of the CYP2J2 cDNA (GenBank U37143) was cloned into the *SalI*-HindIII sites of the vector pBS-αMHC-hGH, a generous gift from Dr. Jeffrey Robbins (University of Cincinnati). This vector contains the αMHC promoter to drive cardiomyocyte-specific expression of the transgene and human growth hormone (hGH)/polyA sequences to enhance transgene mRNA stability. The linearized transgene was microinjected into pronuclei of single cell C57BL6/J mouse embryos which were implanted into pseudopregnant mice. Founders were identified by a combination of PCR and Southern blotting of genomic DNAs. PCR reactions utilized the following oligonucleotide primers (αMHCF1, 5’-GGCACTCTTAGCAAAACCTCAGG-3’; CYP2J2R1, 5’-AGCCAGTAATAAGAAGAACTGCAGA-3’; αMHCF2, 5’-TCTGACAGAGAAGCAGACTTTA-3’; CYP2J2R2, 5’-AAGATATGTTCTCGCATAGGGGTC-3’). Southern blotting was as described. All studies were approved by the NIEHS Animal Care and Use Committee.

**Northern Analysis, Immunoblotting, Immunohistochemistry.** For northern blots, total RNA was electrophoresed on denaturing agarose gels, transferred to HybondN™ membranes (Amersham Pharmacia Biotech), and hybridized with the radiolabeled CYP2J2 cDNA probe as described. Polyclonal antibodies against recombinant human CYP2J2 (anti-CYP2J2rec) and against the CYP2J2-specific peptides HMDQNFGNRPVTPMR (anti-CYP2J2pep1) and RESMPYTNAAHEVQRMGNIPLN (anti-CYP2J2pep3) were raised in rabbits as described. Control studies showed that anti-CYP2J2pep1 and anti-CYP2J2pep3 are immunospecific for CYP2J2, whereas anti-CYP2J2rec cross-reacts with mouse CYP2J isoforms.
but not with non-CYP2J P450s. Immunohistochemical staining of formalin-fixed, paraffin-embedded heart tissue sections was performed as described.4

**Fatty Acid Metabolism.** Heart microsomal fractions (2mg protein/ml) were incubated with [1-14C]AA (100µM) as described.3 Products were extracted and analyzed by high-performance liquid chromatography.2,3 Epoxygenase activity was calculated as the rate of EETs + DHETs produced/mg protein/min. Cardiomyocytes were isolated from neonatal CYP2J2 Tr and wild type (WT) hearts as described5 and cultured on 1% gelatin-coated plates at 37°C in Dulbecco’s modified Eagle’s medium (DMEM/F12) containing 10% fetal bovine serum under an atmosphere of 5% CO2/95% air for 24 hours. Cardiomyocyte cell culture media was extracted with ethyl acetate and samples were analyzed for epoxy and dihydroxy fatty acid derivatives of AA and LA using established HPLC/MS/MS methods.6

**Transthoracic Echocardiography, Assessment of Heart Anatomy.** Two-dimensional guided M-mode echocardiography was performed using an HDI 5000 echocardiograph as described.7 CYP2J2 Tr mice (male/female, 25-30g, 4-6mo) and WT littermate controls were then sacrificed, and hearts removed, dissected, and weighed. Hearts were fixed in 10% neutral buffered formalin, processed routinely, embedded in paraffin, sectioned (5-6µm), and stained with hematoxylin/eosin for histological examination.

**Isolated-Perfused Hearts.** Hearts were perfused in the Langendorff mode as described.8 Briefly, hearts from all four CYP2J2 Tr lines and age/sex-matched WT littermate controls were cannulated and perfused in a retrograde fashion at constant pressure (90cmH2O) with continuously aerated (95%O2/5%CO2) Krebs-Henseleit buffer at 37°C. For assessment of cardiac function, a balloon-tipped catheter inserted into the left ventricle through the left atrium was connected to a pressure transducer. The intraventricular balloon pressure and volume were
adjusted to give an initial end-diastolic pressure of 10cmH₂O. A PowerLab system (AD
Instruments) was used to process data. Hearts were perfused with buffer for a 40min stabilization
period, then subjected to 20min global no-flow ischemia, followed by 40min reperfusion. For
some experiments, hearts were stabilized for 20min, then perfused with either the selective P450
epoxygenase inhibitor N-methylsulphonyl-6-(2-proparglyoxyphenyl) hexanamide (MS-PPOH,
50µM), synthetic 11,12-EET (1µM), the sarcolemmal Kₐₜₚ (sarcKₐₜₚ) and mitochondrial Kₐₜₚ
(mitoKₐₜₚ) channel inhibitor glibenclamide (GLIB, 20µM), the selective mitoKₐₜₚ channel
inhibitor 5-hydroxydecanoate (5-HD, 100µM), the Kₐₜₚ channel opener pinacidil (PIN, 100µM)
or vehicle for 20min, then subjected to 20min ischemia and 40min reperfusion. In other
experiments, the p42/p44 MAPK kinase (MEK) inhibitor PD98059 (10µM) or vehicle was
administered during the 40min reperfusion period. Recovery of contractile function was taken as
left ventricular developed pressure (LVDP) at the end of reperfusion expressed as a percentage
of preischemic LVDP.

Flavoprotein Fluorescence. Cardiomyocytes were isolated from the hearts of adult
CYP2J2 Tr and WT mice using previously published procedures. Briefly, hearts were excised,
mounted in a Langendorff apparatus, perfused with oxygenated 37°C Ca²⁺-free Tyrode’s solution
for 5min, and then perfused with Ca²⁺-free Tyrode’s solution containing collagenase type II (75
U/ml) (Worthington, LakeWood, NJ) for 8-10min at a flow rate of 2-5 ml/min. Hearts were then
minced and gently agitated in fresh Ca²⁺-free Tyrode’s solution at 37°C to separate cells.
Following centrifugation for 2min at 100g to remove debris, cardiomyocytes were plated onto
Laminin-coated dishes (30µg/ml) and incubated in M199 media (1.2mM Ca²⁺) at 37°C under an
atmosphere of 5% CO₂/95% air for 24 hours prior to analysis. Endogenous flavoprotein
fluorescence was used as a marker of mitochondrial redox state and mitoKₐₜₚ channel activity as
described. Fluorescence was excited by the 488nm line of a krypton-argon laser, emission was recorded at 568nm and confocal images were taken at 20X magnification at room temperature on a Model 410 laser scanning confocal microscope (Carl Zeiss Inc., Thornwood, NY). Emitted fluorescence was assessed in CYP2J2 Tr and WT cardiomyocytes at baseline and following treatment with sodium cyanide (NaCN, 2mM), 2,4-dinitrophenol (DNP, 0.2mM), PIN (100µM) or 5-HD (100µM). Measurements were taken from 55-60 cells from 4-7 individual animals of each genotype and intensities were quantified relative to background levels. In some experiments, changes in fluorescence were recorded in WT myocytes treated with either 14,15-EET (1-5µM), 11,12-EET (1µM) or vehicle. Changes in fluorescence were expressed as percentage change relative to baseline levels.

p42/p44 MAPK Expression and Activation. The expression of total p42/p44 MAPK and phospho-p42/p44 MAPK was determined in hearts at different times during the ischemia-reperfusion protocol. Individual hearts from either CYP2J2 Tr or WT mice were snap frozen in liquid nitrogen after 20min of perfusion (B20), 10min (I10) or 20min of ischemia (I20), and 10min (R10) or 40min of reperfusion (R40). Protein (50µg) from the 10,000g supernatant of individual hearts was resolved on 12% sodium dodecylsulfate-polyacrylamide gels, transferred to nitrocellulose membranes and immunoblotted with antibodies to either p42/p44 MAPK (1:200), phospho-p42/p44 MAPK (1:200) (Cell Signaling Technology, Inc., Beverly, MA), or actin C-11 (Santa Cruz Biotechnology, Santa Cruz, CA). Relative band intensities, expressed in arbitrary units of phospho-p42/p44 MAPK to total p42/p44 MAPK, were assessed by densitometry using a ChemiImager 4000 Imaging System (Alpha Innotech Corp., San Leandro, CA).
Statistical Analysis. Data were analyzed by investigators who were blinded to genotype and treatment group assignment. Values are expressed as mean ± SE. Data were analyzed by ANOVA or Student’s t-test using SYSTAT software (SYSTAT Inc.). Values were considered significantly different if $P<0.05$. 
LEGEND TO SUPPLEMENTAL VIDEO

Time-elapsed video of flavoprotein fluorescence, a marker of mitochondrial $K_{ATP}$ channel activity, in adult mouse ventricular myocytes. Cells were cultured for 24 h on laminin-coated dishes in M199 media at 37°C and mitochondrial redox status was assessed by flavoprotein fluorescence. Fluorescence was excited by the 488 nm line of a krypton-argon laser, emission was recorded at 568 nm and confocal images were taken at 20X magnification at room temperature on a Model 410 laser scanning confocal microscope (Carl Zeiss Inc., Thornwood, NY). Images of individual myocytes were taken at baseline and every 30 sec following treatment with either vehicle (ethanol) or 1 µM 11,12-EET for 12 min. Videos were created from confocal images using MetaMorph v6.1 software (Universal Imaging Corp., Downingtown, PA).
REFERENCES


