

## Enhanced Postischemic Functional Recovery in CYP2J2 Transgenic Hearts Involves Mitochondrial ATP-Sensitive K<sup>+</sup> 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-propargyloxyphenyl)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<sup>+</sup> channel (K<sub>ATP</sub>) inhibitor glibenclamide (GLIB) or the mitochondrial K<sub>ATP</sub> (mitoK<sub>ATP</sub>) inhibitor 5-hydroxydecanoate (5-HD) for 20 minutes before ischemia abolishes the cardioprotective effects of CYP2J2 overexpression. Flavoprotein fluorescence, a marker of mitoK<sub>ATP</sub> 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<sub>ATP</sub> and p42/p44 MAPK. (*Circ Res.* 2004;95:506-514.)

**Key Words:** arachidonic acid ■ cytochrome P450 ■ eicosanoid ■ ischemia/reperfusion ■ mitoK<sub>ATP</sub> channel ■ MAPK

Cytochrome P450 epoxygenases metabolize arachidonic acid (AA) to epoxyeicosatrienoic acids (EETs), which are converted to dihydroxyeicosatrienoic acids (DHETs) by epoxide hydrolases.<sup>1</sup> These P450-derived eicosanoids possess potent biological effects in extracardiac tissues.<sup>1</sup> In contrast, less is known about P450 epoxygenases and their eicosanoid products in the heart. Multiple P450s are expressed in heart tissue.<sup>1-3</sup> Among these, CYP2J2 is unique in that it is primarily expressed in the heart, abundant in cardiomyocytes, and active in the biosynthesis of EETs.<sup>3,4</sup> 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.<sup>5,6</sup>

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.<sup>7,8</sup> Both EETs and DHETs have potent vasodilatory effects in the coronary circulation.<sup>9,10</sup> 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 Ca<sup>2+</sup>-activated K<sup>+</sup> channels.<sup>9,10</sup> The EETs have been shown to activate p42/p44 MAPK in coronary vascular endothelial and smooth muscle cells.<sup>11,12</sup> The EETs also directly affect cardiomyocyte function. EETs shorten the cardiac action potential, inhibit cardiac Na<sup>+</sup> channels, and activate cardiac K<sub>ATP</sub>.<sup>13-15</sup> In general, coronary vasodilation, inhibition of Na<sup>+</sup> channels, activation of K<sub>ATP</sub>, 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-

Original received February 19, 2004; revision received June 16, 2004; accepted July 7, 2004.

From the Division of Intramural Research (J.S., B.Y., J.A.B., J.G., L.M.D., S.G., R.G., J.F., E.M., D.C.Z.), NIEHS/NIH, Research Triangle Park, NC; the Department of Entomology and Cancer Research Center (J.N., B.D.H.), University of California, Davis; and the Department of Medicine (L.M., H.A.R., D.C.Z.), Duke University Medical Center, Durham, NC.

\*Both authors contributed equally to this work.

Correspondence to Darryl C. Zeldin, MD, National Institute of Environmental Health Sciences, 111 T.W. Alexander Dr, Research Triangle Park, NC 27709. E-mail zeldin@niehs.nih.gov

© 2004 American Heart Association, Inc.

*Circulation Research* is available at <http://www.circresaha.org>

DOI: 10.1161/01.RES.0000139436.89654.c8

sults.<sup>3,16,17</sup> Moreover, CYP2J2 metabolizes linoleic acid (LA) to epoxyoctadecenoic acids (EpOMEs), which depress cardiac function.<sup>18,19</sup>

To examine the cardiac effects of P450-derived eicosanoids under basal conditions and during ischemia/reperfusion, we used the cardiomyocyte-specific  $\alpha$ -myosin heavy chain ( $\alpha$ MHC) 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 postischemic recovery of left ventricular function. Moreover, our data suggest that the mechanism for this cardioprotection by CYP2J2 metabolites involves activation of mitoK<sub>ATP</sub> 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 pBS- $\alpha$ MHC-hGH, a generous gift from Dr Jeffrey Robbins (University of Cincinnati, Ohio). This vector contains the  $\alpha$ MHC promoter to drive cardiomyocyte-specific expression of the transgene and human growth hormone (hGH)/polyA sequences to enhance transgene mRNA stability.<sup>20</sup> 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.<sup>6</sup> All studies were approved by the NIEHS Animal Care and Use Committee.

### Northern Analysis, Immunoblotting, and Immunohistochemistry

Northern blotting was performed as described.<sup>4</sup> Polyclonal antibodies against recombinant human CYP2J2 (anti-CYP2J2rec) and against CYP2J2-specific peptides HMDQNFGRPVTPMR (anti-CYP2J2pep1) and RESMPYTNNAVIHEVQRMGNIPLN (anti-CYP2J2pep3) were prepared as described.<sup>4,6</sup> Immunoblotting was performed as described.<sup>6</sup> Control studies showed that anti-CYP2J2pep1 and anti-CYP2J2pep3 are immunospecific for CYP2J2, whereas anti-CYP2J2rec cross-reacts with mouse CYP2J2 isoforms. Immunohistochemical staining of formalin-fixed, paraffin-embedded heart sections was performed as described.<sup>3</sup>

### Fatty Acid Metabolism

Heart microsomal fractions were incubated with [<sup>14</sup>C]AA and products were analyzed by HPLC as described.<sup>4,6</sup> 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<sup>21</sup> 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.<sup>22</sup>

### Transthoracic Echocardiography and Assessment of Heart Anatomy

Two-dimensional M-mode echocardiography was performed using an HDI-5000 echocardiograph as described.<sup>23</sup> 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.<sup>24</sup> Hearts from all four CYP2J2 Tr lines and age/sex-matched WT littermate controls were perfused in a retrograde fashion at constant pressure (90 cmH<sub>2</sub>O) with continuously aerated (95% O<sub>2</sub>/5% CO<sub>2</sub>) Krebs-Henseleit buffer at 37°C. Hearts were perfused for 40 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  $\mu$ mol/L), 11,12-EET (1  $\mu$ mol/L), the sarcolemmal K<sub>ATP</sub> (sarcK<sub>ATP</sub>) and mitoK<sub>ATP</sub> inhibitor GLIB (20  $\mu$ mol/L), the selective mitoK<sub>ATP</sub> inhibitor 5-HD (100  $\mu$ mol/L), the K<sub>ATP</sub> opener pinacidil (PIN, 100  $\mu$ mol/L) or vehicle for 20 minutes, then subjected to 20 minutes ischemia and 40 minutes reperfusion. In other experiments, the MEK inhibitor PD98059 (10  $\mu$ mol/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.<sup>16</sup> Endogenous flavoprotein fluorescence was used as a marker of mitoK<sub>ATP</sub> activity.<sup>25,26</sup> 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  $\mu$ mol/L), or 5-HD (100  $\mu$ mol/L). Measurements were taken from 55 to 60 cells from 4 to 7 individual animals of each genotype and intensities were quantified relative to background levels. In some experiments, changes in fluorescence were recorded in WT cardiomyocytes treated with either 14,15-EET (1 to 5  $\mu$ mol/L), 11,12-EET (1  $\mu$ mol/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).

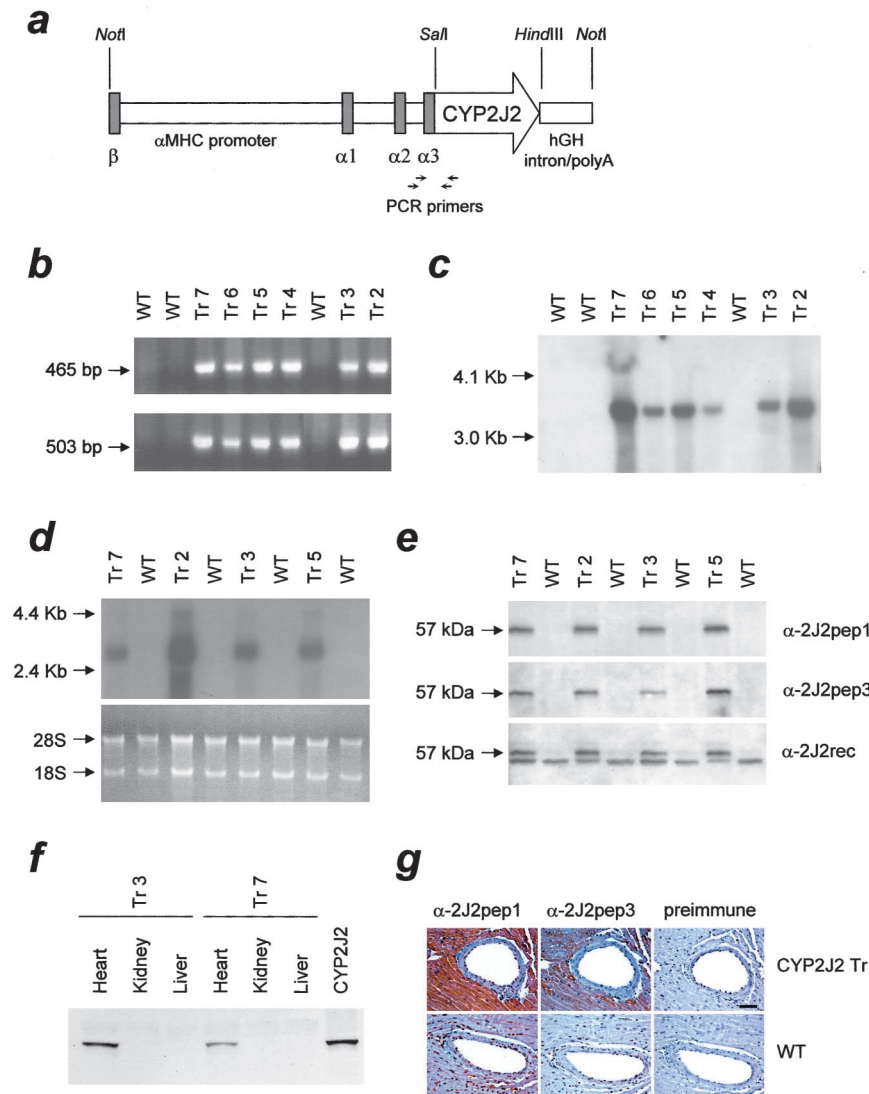
### Statistical Analysis

Data were analyzed by investigators who were blinded to genotype and treatment group assignment. Values are expressed as mean  $\pm$  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



**Figure 1.** Generation, identification, and initial characterization of CYP2J2 transgenic mice. a, Schematic representation of the transgenic construct which contains the  $\alpha$ MHC promoter, CYP2J2 cDNA, and hGH intron/polyA sequences. Positions of PCR primers used to detect the transgene are shown. b, PCR identification of germline CYP2J2 transgenic founders. c, Southern blot demonstrating presence of the transgene in the founders. d, Northern blot showing presence of CYP2J2 transcripts in hearts from four founder lines (Tr2, Tr3, Tr5, and Tr7). Bottom panel shows ethidium bromide stained gel to assess loading. e, Immunoblots demonstrating overexpression of CYP2J2 protein in hearts from four founder lines. Anti-CYP2J2pep1 and anti-CYP2J2pep3 are specific for CYP2J2; anti-CYP2J2rec cross-reacts with murine cardiac CYP2J (bottom band). f, Immunoblot probed with anti-CYP2J2pep1 illustrating cardiac-specific expression of the transgene in two founder lines. g, Immunohistochemistry demonstrating abundant CYP2J2 expression in cardiomyocytes of CYP2J2 transgenic hearts. Transgene expression was low-undetectable in coronary vascular smooth muscle and endothelial cells.

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 an  $\approx 3$ -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  $\approx 3$ -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

## Cardiac Parameters in CYP2J2 Tr and WT Mice

|   | WT (n=8–25) | CYP2J2 Tr (n=8–25) |
|---|-------------|--------------------|
| Body weight, g  | 26.4±1.1    | 27.2±1.0           |
| Heart weight, mg  | 108.5±6.0   | 111.9±3.6          |
| Heart/body wt, mg/g   | 4.1±0.2     | 4.1±0.1            |
| Left ventricle free wall weight, mg   | 80.9±5.2    | 84.4±2.7           |
| Right ventricle free wall weight, mg  | 21.5±0.8    | 20.7±1.0           |
| Left atrium weight, mg  | 3.0±0.2     | 3.4±0.1            |
| Right atrium weight, mg   | 3.1±0.3     | 3.4±0.2            |
| Left ventricular end-diastolic dimension, mm                                  | 3.3±0.1     | 3.1±0.1            |
| Left ventricular end-systolic dimension, mm                                   | 1.6±0.1     | 1.5±0.1            |
| Fractional shortening, %  | 51.6±2.0    | 52.3±3.0           |
| Septal wall thickness, mm   | 0.67±0.03   | 0.61±0.01          |
| Posterior wall thickness, mm  | 0.67±0.03   | 0.61±0.02          |
| HR conscious, bpm   | 684±12      | 666±16             |
| Velocity of circumferential fiber shortening, HR corrected, circ/sec          | 13.4±0.8    | 13.3±0.9           |
| LVDP, cmH <sub>2</sub> O (Baseline)   | 129±8       | 128±10             |
| LVEDP, cmH <sub>2</sub> O (Baseline)  | 7.2±0.6     | 6.5±0.4            |
| Rate of contraction, dP/dt <sub>max</sub> , cmH <sub>2</sub> O/sec (Baseline) | 3825±217    | 3930±294           |
| Rate of relaxation, -dP/dt <sub>min</sub> , cmH <sub>2</sub> O/sec (Baseline) | -3095±169   | -3185±243          |
| HR perfused, bpm (Baseline)   | 360±15      | 370±18             |
| Rate-pressure product, LVDP×HR, cmH <sub>2</sub> O/min (Baseline)             | 46130±3022  | 48758±5020         |
| LVDP, cmH <sub>2</sub> O (R40)  | 27±3        | 47±6*              |
| LVEDP, cmH <sub>2</sub> O (R40)   | 78±3        | 62±6*              |
| Rate of contraction, dP/dt <sub>max</sub> , cmH <sub>2</sub> O/sec (R40)      | 1039±168    | 1904±302*          |
| Rate of relaxation, -dP/dt <sub>min</sub> , cmH <sub>2</sub> O/sec (R40)      | -764±112    | -1435±302*         |
| HR, perfused, bpm (R40)   | 291±13      | 346±14*            |
| Rate-pressure product, LVDP×HR, cmH <sub>2</sub> O/min (R40)                  | 8147±931    | 16501±2450*        |

Values are mean±SE.

Body and heart weights were determined using an analytical balance; dimensions and percent fractional shortening were assessed by transthoracic echocardiography in conscious mice; hemodynamic parameters were measured in isolated-perfused hearts. There were no significant differences in any of these parameters between CYP2J2 Tr and WT mice at baseline. Significant differences were observed in hemodynamic parameters after 20 minutes ischemia and 40 minutes reperfusion (R40). \* $P<0.05$  vs WT.

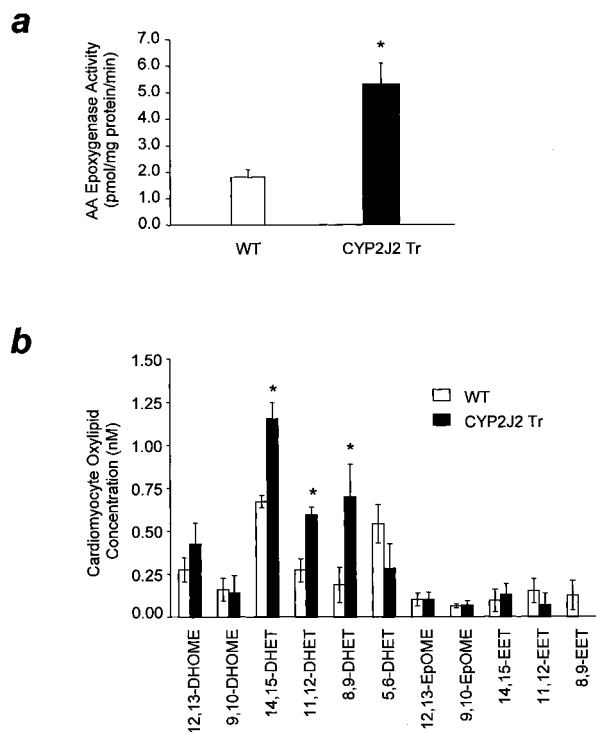
HR indicates heart rate; LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure.

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.<sup>4</sup> 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 postischemic contractile function, left ventricular end-diastolic pressure (LVEDP) at 40 minutes reflow was significantly lower in CYP2J2 Tr (62±6 cmH<sub>2</sub>O) versus WT hearts (78±3 cmH<sub>2</sub>O;  $P<0.05$ ) (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 cmH<sub>2</sub>O versus 88±7 cmH<sub>2</sub>O, respectively;  $P=0.14$ ). The improved postischemic functional recovery was independently confirmed in each of



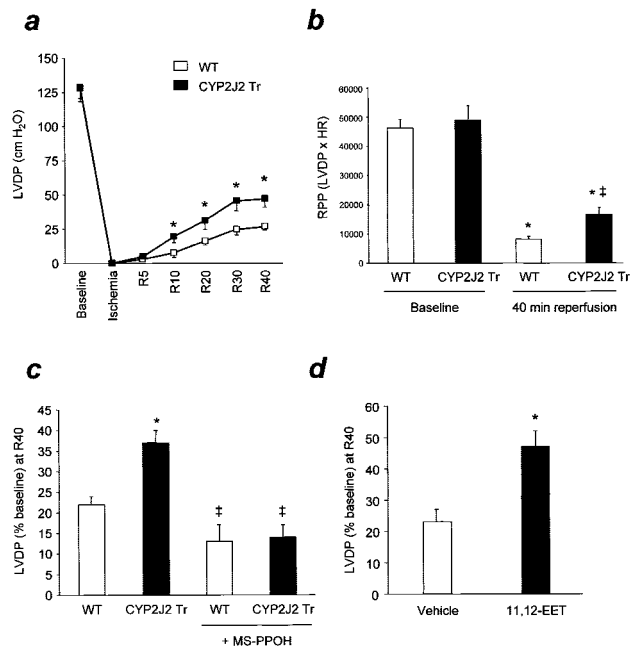
**Figure 2.** Fatty acid metabolism in CYP2J2 Tr and WT hearts. a, AA epoxygenase activity is enhanced in cardiac microsomes from CYP2J2 Tr animals. Values shown are mean $\pm$ SE; n=4 pools, 4 to 6 hearts/pool for each group; \* $P$ <0.05 vs WT. b, Oxylipid levels in culture media from CYP2J2 Tr and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

the four overexpressing lines. When analyzed separately by line, postischemic LVDP recovery in WT and CYP2J2 Tr littermates was 25 $\pm$ 5% and 46 $\pm$ 4% from line Tr2 ( $P$ =0.01), 23 $\pm$ 5% and 38 $\pm$ 5% from line Tr3 ( $P$ =0.04), 20 $\pm$ 3% and 31 $\pm$ 3% from line Tr5 ( $P$ =0.05), and 17 $\pm$ 4% and 35 $\pm$ 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 $\pm$ 2% in the absence of MS-PPOH and 13 $\pm$ 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 $\pm$ 3% in the absence of MS-PPOH and 14 $\pm$ 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 $K_{ATP}$ in Postischemic Functional Recovery

To determine whether  $K_{ATP}$  was involved in CYP2J2-mediated cardioprotection, we conducted experiments in the

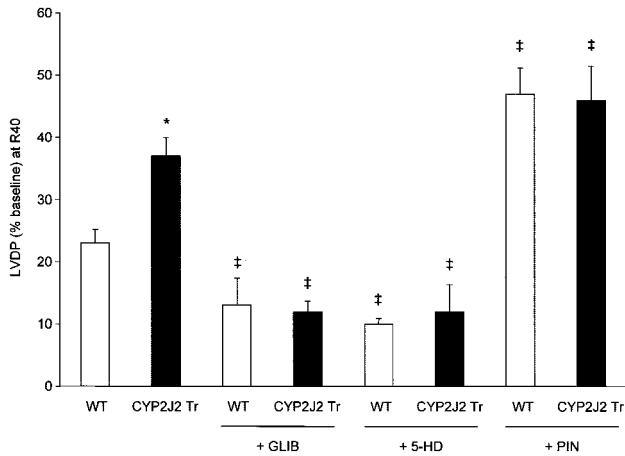


**Figure 3.** Postischemic recovery of left ventricular function in CYP2J2 Tr and WT mice. a, LVDP at baseline, at the end of ischemia, and after 5 to 40 minutes reperfusion (R5, R10, R20, R30, and R40) in WT and CYP2J2 Tr hearts. Values shown are mean $\pm$ SE; n=25 per group; \* $P$ <0.05 vs WT. b, RPP at baseline and 40 minutes reperfusion in WT and CYP2J2 Tr hearts. Values shown are mean $\pm$ 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 (50  $\mu$ mol/L) for 20 minutes before ischemia. Values represent mean $\pm$ 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 (1  $\mu$ mol/L) for 20 minutes before ischemia. Values represent mean $\pm$ SE; n=4 per group; \* $P$ <0.05 vs vehicle.

presence of the sarc $K_{ATP}$  and mito $K_{ATP}$  inhibitor GLIB or the selective mito $K_{ATP}$  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  $K_{ATP}$  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  $K_{ATP}$  in the cardioprotective effect of CYP2J2 overexpression.

### Endogenous Flavoprotein Fluorescence

To further investigate the effect of CYP2J2 overexpression on mito $K_{ATP}$  activity, we measured endogenous flavoprotein fluorescence, an index of mitochondrial redox state,<sup>25,26</sup> in

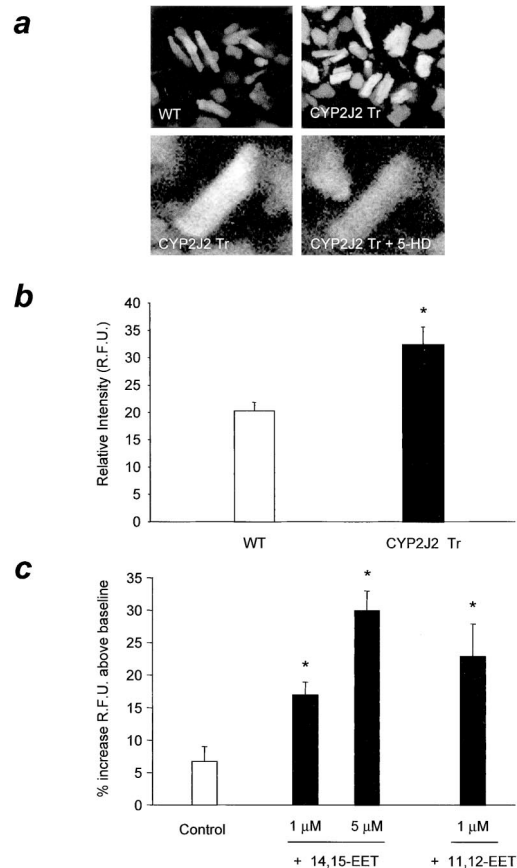


**Figure 4.** Effects of  $K_{ATP}$  effectors on postischemic LVDP recovery in CYP2J2 Tr and WT mice. Hearts were administered vehicle, GLIB (20  $\mu\text{mol/L}$ ), 5-HD (100  $\mu\text{mol/L}$ ), or PIN (100  $\mu\text{mol/L}$ ) for 20 minutes before ischemia. Values represent mean  $\pm$  SE,  $n=3$  to 11 per group; \* $P<0.05$  vs WT; ‡ $P<0.05$  vs vehicle control of same genotype.

WT and CYP2J2 Tr cardiomyocytes. 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 mito $K_{ATP}$  activity. We also examined the effect of CYP2J2-derived metabolites on flavoprotein fluorescence in WT cardiomyocytes. Application of 14,15-EET (1 to 5  $\mu\text{mol/L}$ ), the major CYP2J2 product, resulted in a dose-dependent increase in flavoprotein fluorescence (Figure 5c). A similar effect was observed after application of 1  $\mu\text{mol/L}$  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 mito $K_{ATP}$ . Together with the inhibitor studies, these data suggest that mito $K_{ATP}$  activation is one mechanism for improved postischemic functional recovery in CYP2J2 Tr mice.

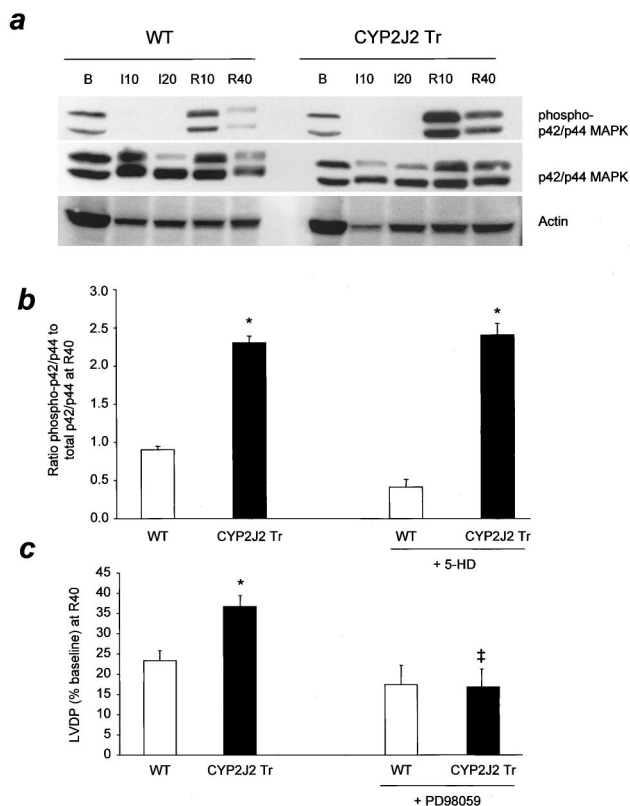
### Role of p42/p44: MAPK Activation in Postischemic Functional Recovery

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 signifi-



**Figure 5.** Endogenous flavoprotein fluorescence in cardiomyocytes from CYP2J2 Tr and WT mice. a, Representative confocal images showing flavoprotein fluorescence in WT and CYP2J2 Tr cardiomyocytes at baseline. Lower panel shows images of a CYP2J2 Tr cardiomyocyte at baseline and after treatment with 5-HD (100  $\mu\text{mol/L}$ ). b, Flavoprotein fluorescence (arbitrary units) in WT and CYP2J2 Tr cardiomyocytes at baseline. Data represent measurements from 55 to 60 cells from each of 4 animals per genotype and are expressed as mean  $\pm$  SE; \* $P<0.05$  vs WT. c, Flavoprotein fluorescence in WT cardiomyocytes after treatment with either vehicle (control), 14,15-EET (1  $\mu\text{mol/L}$ , 5  $\mu\text{mol/L}$ ), or 11,12-EET (1  $\mu\text{mol/L}$ ). Data represent measurements from 15 to 20 cells from each of 4 to 7 animals per group and are expressed as mean  $\pm$  SE; \* $P<0.05$  vs vehicle control.

cantly 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 mito $K_{ATP}$  opening<sup>27</sup>; hence, we examined whether inhibition of mito $K_{ATP}$  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



**Figure 6.** Role of MAPK pathway in improved LVDP recovery in CYP2J2 Tr hearts. **a**, Representative immunoblots showing expression of total p42/p44 MAPK, phospho-p42/p44 MAPK, and actin C-11 in hearts at baseline (B), after 10 minutes (I10) or 20 minutes ischemia (I20), and 10 minutes (R10) or 40 minutes reperfusion (R40). **b**, Ratio of phospho-p42/p44 MAPK to total p42/p44 MAPK expression after 40 minutes reperfusion after treatment with vehicle or 5-HD (100  $\mu$ mol/L). Values represent mean  $\pm$  SE; n=4 animals per group; \* $P$ <0.05 vs WT. **c**, Effect of MEK inhibitor on LVDP recovery at 40 minutes reperfusion in CYP2J2 Tr and WT mice. Hearts were administered vehicle or PD98059 (10  $\mu$ mol/L) during reperfusion. Values represent mean  $\pm$  SE, n=8 to 10 per group; \* $P$ <0.05 vs WT; ‡ $P$ <0.05 vs vehicle control of same genotype.

MAPK during reperfusion is another mechanism for improved postischemic functional recovery in CYP2J2 Tr mice.

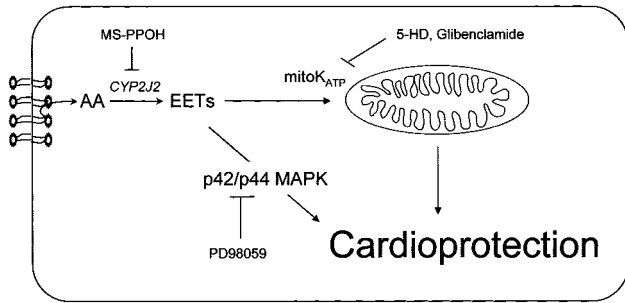
## Discussion

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.<sup>2</sup> The discovery of CYP2J2 as a primarily cardiac P450 active in the epoxidation of AA to EETs,<sup>4</sup> together with recent studies documenting that these P450-derived eicosanoids can affect cardiomyocyte function in vitro,<sup>3,13–17,28</sup> has led to the hypothesis that this enzyme may also have important endogenous 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 positive<sup>16,17</sup> and negative<sup>28</sup> inotropic effects in the heart under basal conditions. After ischemia/reperfusion, EETs are reported to have both cardioprotective<sup>3</sup> and cardiodepressant effects.<sup>17</sup> In light of these controversies, we developed a transgenic model to study the effects of CYP2J2 overexpression 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 biosynthesis is enhanced in stenosed coronary arteries<sup>8</sup> and during cardiac ischemia/reperfusion injury,<sup>7</sup> these findings may also have important therapeutic implications.

How does CYP2J2 overexpression produce beneficial cardiac 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 cardiomyocytes released more DHETs into culture media than did WT cardiomyocytes. In contrast, there were no significant differences 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 contractile 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. Importantly, MS-PPOH completely abolished the improved postischemic LVDP recovery in CYP2J2 Tr mice, further suggesting that the cardioprotective effects of CYP2J2 overexpression 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  $K_{ATP}$ . Two pharmacologically distinct  $K_{ATP}$  types have been identified in cardiomyocytes, sarc $K_{ATP}$  and mito $K_{ATP}$ .<sup>29</sup> Sarc $K_{ATP}$  is activated during cardiac ischemia when cytoplasmic ATP is depleted and affects membrane excitability. Activation leads to shortening of the cardiac action potential and reduced intracellular calcium overload.<sup>30,31</sup> Several sarc $K_{ATP}$  openers produce beneficial effects on the myocardium in animal models of ischemia, and several sarc $K_{ATP}$  inhibitors block ischemic preconditioning.<sup>30,31</sup> Structurally, cardiac sarc $K_{ATP}$  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 sarc $K_{ATP}$  by reducing channel sensitivity to ATP; however, the exact site on the channel that interacts with EETs remains enigmatic.<sup>13,14</sup> Although the precise molecular composition of mito $K_{ATP}$  remains unknown, preliminary studies suggest the presence of a multi-protein complex containing succinate dehydrogenase.<sup>32</sup> Importantly, recent pharmacological data indicate that selective activation of mito $K_{ATP}$  confers cardioprotection after ischemia<sup>25,26,30,31</sup>; however, a role for P450 epoxygenase metabolites in this process has not been investigated. In this regard,



**Figure 7.** Schematic of proposed mechanisms of cardioprotection in CYP2J2 Tr mice. CYP2J2-derived eicosanoids activate  $\text{mitoK}_{\text{ATP}}$  and the p42/p44-MAPK pathway leading to cardioprotection.

we observed that the beneficial effects of CYP2J2 overexpression are abolished by 5-HD, which is selective for  $\text{mitoK}_{\text{ATP}}$ .<sup>29</sup> Interestingly, we also demonstrated increased flavoprotein fluorescence, a marker of mitochondrial redox status,<sup>25</sup> in CYP2J2 Tr cardiomyocytes consistent with enhanced  $\text{mitoK}_{\text{ATP}}$  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 is activation of  $\text{mitoK}_{\text{ATP}}$ . Although the precise pathways by which  $\text{mitoK}_{\text{ATP}}$  activation confers cardioprotection remain unknown, potentially beneficial consequences of opening  $\text{mitoK}_{\text{ATP}}$  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.<sup>26,30,31</sup>

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.<sup>33,34</sup> Recent studies demonstrate that EETs activate the MAPK pathway in endothelial and vascular smooth muscle cells.<sup>11,12</sup> 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<sup>35</sup> showing that activation of p42/p44 MAPK at reperfusion is essential for preconditioning-induced protection. In contrast,  $\text{mitoK}_{\text{ATP}}$  activation was enhanced before ischemia in CYP2J2 Tr hearts. Gross and coworkers<sup>27</sup> recently proposed that activation of p42/p44 MAPK occurs downstream of  $\text{mitoK}_{\text{ATP}}$  opening. However, inhibition of  $\text{mitoK}_{\text{ATP}}$  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  $\text{mitoK}_{\text{ATP}}$  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  $\alpha\text{MHC}$  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  $\text{mitoK}_{\text{ATP}}$  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 ES02710 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 Demianemko, and Page Myers for assistance with various aspects of this work.

### References

- Zeldin DC. Epoxygenase pathways of arachidonic acid metabolism. *J Biol Chem.* 2001;276:36059–36062.
- Thum T, Borlak J. Gene expression in distinct regions of the heart. *Lancet.* 2000;355:979–983.
- Wu S, Chen W, Murphy E, Gabel S, Tomer KB, Foley J, Steenbergen C, Falck JR, Moomaw CR, Zeldin DC. Molecular cloning, expression, and functional significance of a cytochrome P450 highly expressed in rat heart myocytes. *J Biol Chem.* 1997;272:12551–12559.
- Wu S, Moomaw CR, Tomer KB, Falck JR, Zeldin DC. Molecular cloning and expression of CYP2J2, a human cytochrome P450 arachidonic acid epoxygenase highly expressed in heart. *J Biol Chem.* 1996;271:3460–3468.
- Spiecker M, Darius H, Hankeln T, Soufi M, Sattler AM, Schaefer JR, Node K, Borge J, Muge A, Lindpaintner K, Huesing A, Maisch B, Zeldin DC, Liao JK. Risk of coronary artery disease associated with polymorphism of a cytochrome P450 epoxygenase, CYP2J2. *Circulation.* In Press.
- King LM, Ma J, Srettabunjong S, Graves J, Bradbury JA, Li L, Spiecker M, Liao JK, Mohrenweiser H, Zeldin DC. Cloning of the CYP2J2 gene and identification of functional polymorphisms. *Mol Pharmacol.* 2002; 61:840–852.
- Nithipatikom K, DiCamelli RF, Kohler S, Gumina RJ, Falck JR, Campbell WB, Gross GJ. Determination of cytochrome P450 metabolites of arachidonic acid in coronary venous plasma during ischemia and reperfusion in dogs. *Anal Biochem.* 2001;292:115–124.
- Rosolowsky M, Falck JR, Willerson JT, Campbell WB. Synthesis of lipoxygenase and epoxygenase products of arachidonic acid by normal and stenosed canine coronary arteries. *Circ Res.* 1990;66:608–621.
- Hecker M, Bara AT, Bauersachs J, Busse R. Characterization of endothelium-derived hyperpolarizing factor as a cytochrome P450-derived arachidonic acid metabolite in mammals. *J Physiol.* 1994;481:407–414.
- Campbell WB, Gebremedhin D, Pratt PF, Harder DR. Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ Res.* 1996;78:415–423.



11. Fleming I, Fisslthaler B, Michaelis UR, Kiss L, Popp R, Busse R. The coronary endothelium-derived hyperpolarizing factor (EDHF) stimulates multiple signalling pathways and proliferation in vascular cells. *Pflugers Arch*. 2001;442:511–518.
12. Popp R, Brandes RP, Ott G, Busse R, Fleming I. Dynamic modulation of interendothelial gap junctional communication by 11,12-epoxyeicosatrienoic acid. *Circ Res*. 2002;90:800–806.
13. Lu T, VanRollins M, Lee HC. Stereospecific activation of cardiac ATP-sensitive K(+) channels by epoxyeicosatrienoic acids: a structural determinant study. *Mol Pharmacol*. 2002;62:1076–1083.
14. Lu T, Hoshi T, Weintraub NL, Spector AA, Lee HC. Activation of ATP-sensitive K(+) channels by epoxyeicosatrienoic acids in rat cardiac ventricular myocytes. *J Physiol*. 2001;537:811–827.
15. Lee HC, Lu T, Weintraub NL, VanRollins M, Spector AA, Shibata EF. Effects of epoxyeicosatrienoic acids on the cardiac sodium channels in isolated rat ventricular myocytes. *J Physiol*. 1999;519 Pt 1:153–168.
16. Xiao YF, Huang L, Morgan JP. Cytochrome P450: a novel system modulating Ca<sup>2+</sup> channels and contraction in mammalian heart cells. *J Physiol*. 1998;508:777–792.
17. Moffat MP, Ward CA, Bend JR, Mock T, Farhangkhoe P, Karmazyn M. Effects of epoxyeicosatrienoic acids on isolated hearts and ventricular myocytes. *Am J Physiol*. 1993;264:H1154–H1160.
18. Moran JH, Mitchell LA, Bradbury JA, Qu W, Zeldin DC, Schnellmann RG, Grant DF. Analysis of the cytotoxic properties of linoleic acid metabolites produced by renal and hepatic P450s. *Toxicol Appl Pharmacol*. 2000;168:268–279.
19. Fukushima A, Hayakawa M, Sugiyama S, Ajioka M, Ito T, Satake T, Ozawa T. Cardiovascular effects of leukotoxin (9, 10-epoxy-12-octadecenoate) and free fatty acids in dogs. *Cardiovasc Res*. 1988;22:213–218.
20. Gulick J, Subramaniam A, Neumann J, Robbins J. Isolation and characterization of the mouse cardiac myosin heavy chain genes. *J Biol Chem*. 1991;266:9180–9185.
21. Adams JW, Migita DS, Yu MK, Young R, Hellickson MS, Castro-Vargas FE, Domingo JD, Lee PH, Bui JS, Henderson SA. Prostaglandin F<sub>2</sub> alpha stimulates hypertrophic growth of cultured neonatal rat ventricular myocytes. *J Biol Chem*. 1996;271:1179–1186.
22. Newman JW, Watanabe T, Hammock BD. The simultaneous quantitation of cytochrome P450 dependent linoleate and arachidonate metabolites in urine by high-performance liquid chromatography-tandem mass spectroscopy. *J Lipid Res*. 2002;43:1563–1578.
23. Esposito G, Rapacciuolo A, Naga Prasad SV, Takaoka H, Thomas SA, Koch WJ, Rockman HA. Genetic alterations that inhibit in vivo pressure-overload hypertrophy prevent cardiac dysfunction despite increased wall stress. *Circulation*. 2002;105:85–92.
24. Gabel SA, London RE, Funk CD, Steenbergen C, Murphy E. Leukocyte-type 12-lipoxygenase-deficient mice show impaired ischemic preconditioning-induced cardioprotection. *Am J Physiol Heart Circ Physiol*. 2001;280:H1963–9.
25. Liu Y, Sato T, O'Rourke B, Marban E. Mitochondrial ATP-dependent potassium channels: novel effectors of cardioprotection? *Circulation*. 1998;97:2463–2469.
26. O'Rourke B. Evidence for mitochondrial K<sup>+</sup> channels and their role in cardioprotection. *Circ Res*. 2004;94:420–432.
27. Gross ER, Peart JN, Hsu AK, Grover GJ, Gross GJ. K(ATP) opener-induced delayed cardioprotection: involvement of sarcolemmal and mitochondrial K(ATP) channels, free radicals and MEK1/2. *J Mol Cell Cardiol*. 2003;35:985–992.
28. Rastaldo R, Paolucci N, Chiribiri A, Penna C, Gattullo D, Pagliaro P. Cytochrome P-450 metabolite of arachidonic acid mediates bradykinin-induced negative inotropic effect. *Am J Physiol Heart Circ Physiol*. 2001;280:H2823–H2832.
29. Hu H, Sato T, Seharaseyon J, Liu Y, Johns DC, O'Rourke B, Marban E. Pharmacological and histochemical distinctions between molecularly defined sarcolemmal KATP channels and native cardiac mitochondrial KATP channels. *Mol Pharmacol*. 1999;55:1000–1005.
30. Gross GJ, Peart JN. KATP channels and myocardial preconditioning: an update. *Am J Physiol Heart Circ Physiol*. 2003;285:H921–H9230.
31. Gross GJ, Fryer RM. Sarcolemmal versus mitochondrial ATP-sensitive K<sup>+</sup> channels and myocardial preconditioning. *Circ Res*. 1999;84:973–979.
32. Ardehali H, Chen Z, Ko Y, Mejia-Alvarez R, Marban E. Multiprotein complex containing succinate dehydrogenase confers mitochondrial ATP-sensitive K<sup>+</sup> channel activity. *Circulation*. 2003;108(suppl):1004.
33. Schulman D, Latchman DS, Yellon DM. Urocortin protects the heart from reperfusion injury via upregulation of p42/p44 MAPK signaling pathway. *Am J Physiol Heart Circ Physiol*. 2002;283:H1481–H1488.
34. Fryer RM, Pratt PF, Hsu AK, Gross GJ. Differential activation of extracellular signal regulated kinase isoforms in preconditioning and opioid-induced cardioprotection. *J Pharmacol Exp Ther*. 2001;296:642–649.
35. Hausenloy DJ, Mocanu MM, Yellon DM. Activation of the pro-survival kinases (PI3 kinase-Akt and Erk 1/2) at reperfusion is essential for preconditioning -induced protection. *Circulation*. 2003;108 (suppl IV):288.