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Overexpression of Cytochrome P450 CYP2J2 Protects against Hypoxia-Reoxygenation Injury in Cultured Bovine Aortic Endothelial Cells

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ABSTRACT

CYP2J2 is abundant in human heart and its arachidonic acid metabolites, the epoxyeicosatrienoic acids (EETs), have potent vasodilatory, antiinflammatory and cardioprotective properties. This study was designed to examine the role of CYP2J2 in hypoxia-reoxygenation-induced injury in cultured bovine aortic endothelial cells (BAECs). Early passage BAECs were exposed to 24-h hypoxia followed by 4-h reoxygenation (HR). HR resulted in cell injury, as indicated by significant increases in lactate dehydrogenase (LDH) release and trypan blue stained cells ($p < 0.01$) and was associated with a decrease in CYP2J2 protein expression. Transfection of BAECs with the CYP2J2 cDNA resulted in increased CYP2J2 expression and arachidonic acid epoxygenase activity, compared with cells transfected with an irrelevant green fluorescent protein (GFP) cDNA. HR induced significant injury in GFP-transfected BAECs, as indicated by increases in LDH release and trypan blue-stained cells ($p < 0.01$); however, the HR-induced injury was markedly attenuated in CYP2J2-transfected cells ($p < 0.01$). HR increased cellular 8-iso-prostaglandin $F_{2\alpha}$ ($p < 0.05$), and decreased eNOS expression, L-arginine uptake and conversion, and nitrite production ($p < 0.01$) in GFP-transfected BAECs. CYP2J2 transfection attenuated the HR-induced increase in 8-iso-prostaglandin $F_{2\alpha}$ ($p < 0.05$) and decreased the amount of extracellular superoxide detected by cytochrome *c* reduction under normoxic conditions ($p < 0.05$) but did not significantly affect HR-induced decreases in eNOS expression, L-arginine uptake and conversion, and nitrite production. Treatment of BAECs with synthetic EETs and/or epoxide hydrolase inhibitors also showed protective effects against HR injury ($p < 0.05$). These observations suggest: (1) HR results in endothelial injury and decreased CYP2J2 expression; (2) transfection with the CYP2J2 cDNA protects against HR injury; and (3) the cytoprotective effects of CYP2J2 may be mediated, at least in part, by antioxidant effects.

The role of cytochromes P450 in the NADPH-dependent epoxidation of arachidonic acid is well documented (Capdevila et al., 1981; Morrison and Pascoe, 1981; Oliw et al., 1981). The primary products formed are four regioisomeric *cis*-epoxyeicosatrienoic acids (5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET), which are hydrated to the corresponding *vicinal* dihydroxyeicosatrienoic acids (DHETs) by epoxide hydrolases (Capdevila et al., 1981; Morrison and Pascoe, 1981; Oliw et al., 1981). The EETs have been demonstrated to hyperpolarize and relax vascular smooth muscle cells by activating calcium-sensitive potassium channels (Gebremedhin et al., 1998; Campbell and Harder, 1999), increase coronary artery blood flow (Oltman et al., 1998), and protect the myocardium against ischemia-reperfusion injury (Wu et al., 1997). EETs have also been shown to inhibit cytokine-induced endothelial cell adhesion molecule expression by inhibiting nuclear factor κB (NF-κB) and increase endothelial capacitative calcium entry (Graier et al., 1995; Node et al., 1999). Importantly, their role in the response of endothelial cells to hypoxia-reoxygenation (HR) injury is unknown. The cytochrome P450 CYP2J2 has been shown to be highly expressed in human heart and vascular tissue and active in the biosynthesis of EETs (Wu et al., 1996; Node et al., 1999). We have shown that this P450 is particularly abundant in endo-

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ABBREVIATIONS: EET, epoxyeicosatrienoic acid; DHET, dihydroxyeicosatrienoic acid; NF-κB, nuclear factor κB; HR, hypoxia-reoxygenation; P450, cytochrome P450; NOS, nitric-oxide synthase; PG, prostaglandin; BAEC, bovine aortic endothelial cell; eNOS, endothelial nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; AT1, angiotensin II type 1; HPLC, high-performance liquid chromatography; HETE, hydroxyeicosatetraenoic acid; GC/MS, gas chromatography/mass spectrometry; SOD, superoxide dismutase; GFP, green fluorescent protein; LDH, lactate dehydrogenase.

thelial cells lining large and small coronary arteries, and in endothelium of the pulmonary and intestinal vasculature (Zeldin et al., 1996, 1997; Node et al., 1999). Other P450 isoforms are also expressed in endothelial cells including members of the CYP1A and CYP2C subfamilies (Lin et al., 1996; Fisslthaler et al., 1999); however, the relative contribution of these P450 enzymes to endothelial EET biosynthesis remains enigmatic.

Vascular endothelial cells play a central role in cardiovascular physiology and pathobiology. Endothelial cell dysfunction is an important event in virtually all forms of ischemia-reperfusion injury (Dzau and Gibbons, 1991; Lefer and Lefer, 1993; Liao, 1998; Biegelsen and Loscalzo, 1999; Tan et al., 1999). The dysfunction seems to be triggered within 2.5 min of endothelial generation of oxygen free radicals such as superoxide anion (Lefer and Lefer, 1993). One of the most important functions of endothelial cells is to produce nitric oxide (NO) via an L-arginine-NO synthase (NOS) pathway (Moncada, 1990). NO stimulates the enzyme guanylate cyclase, which results in increased cyclic guanosine monophosphate and consequent vasodilation. Like the EETs, NO also inhibits the NF-κB pathway and has anti-inflammatory properties (DeCaterina et al., 1995). Endothelial dysfunction is associated with decreased NO availability, probably caused by destruction of NO via a free radical-dependent mechanism (Lefer and Lefer, 1993). Maintenance and/or improvement of endothelial function has been shown to improve overall cardiac function after myocardial ischemia-reperfusion injury (Murohara et al., 1995; Shin et al., 1998). Furthermore, the nitrates, a related group of vasodilator drugs, supply exogenous NO to the vascular wall and are routinely used for management of ischemic heart disease (Abrams, 1996).

The relevance of reactive oxygen species generation and resultant lipid peroxidation in the pathogenesis of ischemia-reperfusion injury has been extensively documented (Morrow and Roberts, 1990; Mathews et al., 1994; Reilly et al., 1997). Cellular F_2-isoprostane levels have been shown to be highly sensitive and specific markers for lipid peroxidation and also affect vascular tone (Morrow and Roberts, 1990; Mathews et al., 1994). These eicosanoids are formed in vivo in humans via a nonenzymatic mechanism involving free radical-catalyzed peroxidation of arachidonic acid and have been proposed to participate as pathophysiological mediators in oxidant injury (Morrow and Roberts, 1990). Mathews et al. (1994) reported that a significant 60 to 250% increase in plasma F_2-isoprostane levels was observed during hepatic reperfusion after ischemia. Reilly et al. (1997) also provided evidence for increased oxidant stress during coronary artery reperfusion in humans (i.e., increased formation of the isoprostanes $IPF_{2\alpha}$-I and 8-iso-$PGF_{2\alpha}$ in acute coronary angioplasty).

Because CYP2J is abundant in heart tissue and localized to vascular endothelium, endothelial function is important in determining the degree of cellular injury after ischemia-reperfusion, and because CYP2J products (the EETs) are cardioprotective, we hypothesized that reduced endothelial CYP2J expression contributes to cellular dysfunction and is at least partly responsible for some of the pathophysiologic manifestations that follow ischemia-reperfusion. Furthermore, we postulated that the cellular injury could be abrogated either by maintaining the levels of CYP2J protein or by direct application of CYP2J-derived eicosanoids. Thus, the present study was designed to determine whether maintenance of CYP2J2 levels in cultured bovine aortic endothelial cells (BAECs) affects HR-induced cell injury and, if so, to begin to understand the mechanisms involved.

Materials and Methods

Cell Culture. Endothelial cells were isolated from bovine aorta as described (DeCaterina et al., 1995; Node et al., 1999). Early passage (third or fourth) bovine aortic endothelial cells (BAECs) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine and 10% fetal bovine serum (Hyclone Laboratory, Logan, UT) under 95% air/5% carbon dioxide at 37°C.

Plasmid Preparation and Transfection of Endothelial Cells. The CYP2J2 cDNA (1.876 kilobase pairs; GenBank accession number U37143) or GFP cDNA (0.75 kilobase pairs) were subcloned into the plasmid pcDNA3.0 (Invitrogen, Carlsbad, CA) at the *Eco*RI/*Xho*I and *Eco*RI/*Xba*I sites, respectively. Restriction enzyme digestion and sequence analysis using the dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and an ABI model 377 Automated DNA Sequencer (Applied Biosystems) confirmed the identity of the resulting pcDNA3.0/GFP and pcDNA3.0/CYP2J2 plasmids. Plasmids were purified using a Plasmid Purification Kit (QIAGEN, Inc., Chatsworth, CA) according to the manufacturer's instructions.

BAECs grown to ~50% confluence were transfected with either the pcDNA3.0 empty vector, pcDNA3.0/GFP, or pcDNA3.0/CYP2J2 (0.1 μg DNA/cm^2) using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's transfection protocol (DNA/FuGENE 6 = 1 μg/3 μl). Forty-eight hours after transfection, BAECs were examined for CYP2J2 expression and arachidonic acid metabolism (Wu et al., 1996, 1997; Node et al., 1999). The pcDNA3.0/GFP transfected cells were also examined for GFP expression using a Zeiss Model LSM-410 inverted confocal laser scanning microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a Fluar 10×/0.5 objective lens to determine transfection efficiency.

Treatment of Endothelial Cells with Eicosanoids and Epoxide Hydrolase Inhibitors. EETs were prepared by total chemical synthesis as described previously (Corey et al., 1980; Falck and Manna, 1982). DHETs were prepared by chemical hydration of EETs as described previously (Capdevila et al., 1990). Synthetic EETs and DHETs were purified by reverse-phase HPLC before use (Capdevila et al., 1990). BAECs, grown to ~85% confluence, were treated with either synthetic 11,12-EET, 11,12-DHET, or 14,15-EET in ethanol vehicle (1 μM each, final concentration) for 10 min before exposure to HR (see below). In parallel experiments, the specific soluble epoxide hydrolase inhibitor dicyclohexylurea (K_i = 30 nM, 10 μM final concentration) (Morisseau et al., 1999) and the specific microsomal epoxide hydrolase inhibitor elaidamide (10 μM final concentration) in dimethylformamide vehicle were added together with 11,12-EET. Cells treated with vehicle alone served as control cells.

Exposure of BAECs to Hypoxia-Reoxygenation and Cell Injury Determination. BAECs, either untransfected, at 48 h after transfection with pcDNA3.0/GFP or pcDNA3.0/CYP2J2, or 10 min after treatment with eicosanoids, epoxide hydrolase inhibitors, or vehicle, were exposed to hypoxia (95% nitrogen/5% carbon dioxide) for 24 h, followed by reoxygenation (95% air/5% carbon dioxide) for 4 h using an environmental chamber within a 37°C incubator. Cells maintained under continuous (28 h) normoxic conditions served as control cells. The hypoxia resulted in a significant decrease in oxygen tension in the culture medium to 30 mm Hg (Yang et al., 1999). After HR, culture medium was assayed for lactate dehydrogenase (LDH) release using the CytoTox 96 NonRadioactive Cytotoxicity Assay Kit (Promega, Madison, WI) and nitrite levels using the Griess reagent system (Promega) (Green et al., 1982). Total cell number and the

number of trypan blue stained cells were determined using a hemocytometer. The expression of CYP2J2, eNOS, iNOS, and angiotensin type 1 (AT1) receptor protein was determined by immunoblotting (Wu et al., 1996, 1997; Li et al., 1999; Node et al., 1999). NOS activity was also monitored by measuring the conversion of [L-^{3}H]arginine to [L-^{3}H]citrulline (Hiki et al., 1991).

Immunoblotting for CYP2J2 Protein. For determination of CYP2J2 expression after transfection, BAECs were trypsinized and used to prepare microsomal and mitochondrial subcellular fractions by differential centrifugation at 4°C as described previously (Isaya et al., 1988). For determination of CYP2J2 levels after HR, BAECs were lysed in 1% SDS, 0.1% Triton X-100, 10 mM Tris-HCl, pH 7.4. Proteins were separated on SDS-12% (w/v) Tris-Glycine precast gels (Invitrogen, Carlsbad, CA) and the resolved proteins were transferred electrophoretically onto nitrocellulose membranes. The membranes were then immunoblotted with affinity-purified rabbit polyclonal anti-human CYP2J2 IgG (1:2000 dilution), goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA), and the enhanced chemiluminescence Western blotting detection system (ECL; Amersham Pharmacia Biotech, Arlington Heights, IL) as described (Wu et al., 1996, 1997; Node et al., 1999). Previous work has shown that the anti-CYP2J2 IgG cross-reacts with known CYP2J subfamily P450s in human, rabbit, rat, and mouse but does not recognize other P450 isoforms, including members of the CYP1A, CYP2A, CYP2B, CYP2C, CYP2D, CYP2E, and CYP4A subfamilies (Wu et al., 1996, 1997; Node et al., 1999).

Endothelial Cell Arachidonic Acid Metabolism. Forty-eight hours after transfection with either pcDNA3.0/GFP or pcDNA3.0/CYP2J2, BAECs were incubated with freshly purified [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (185 Ci/mmol, 4–5 μCi/175-mm^2 flask) in serum-free culture medium at 37°C for 30 to 120 min. In some experiments, the P450 inhibitor SKF-525A (100 μM, final concentration) was added before the addition of arachidonic acid. The BAECs and culture medium were then collected and the reaction products extracted into ethyl ether, dried under a nitrogen stream, analyzed by reverse-phase HPLC, and quantified by on-line liquid scintillation using a Radiomatic Flo-One β detector (Radiomatic Instruments, Tampa, FL) as described previously (Wu et al., 1996; Node et al., 1999). Products were identified by comparing their reverse-phase HPLC properties with those of authentic EET, DHET, hydroxyeicosatetraenoic acid (HETE) and prostaglandin standards, and by gas chromatography/mass spectrometry.

Immunoblotting for AT1 receptor, eNOS, and iNOS. BAECs collected after HR were lysed in 1% SDS, 0.1% Triton X-100, 10 mM Tris-HCl, pH 7.4, and the total cellular lysate was clarified by centrifugation at 10,000 rpm for 2 min. Proteins were separated on 12% Tris-Glycine gels (Invitrogen), transferred to nitrocellulose, and then immunoblotted with rabbit polyclonal anti-AT1 receptor IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse monoclonal anti-eNOS IgG (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA), or rabbit polyclonal anti-iNOS IgG (BIOMOL Research Laboratories, Inc.) according to the manufacturer's instructions. Corresponding anti-rabbit IgG or anti-mouse IgG were used as the secondary antibody and the blots were visualized with ECL Western Blotting Detection System (Amersham) (Li et al., 1999). Densitometry was performed on autoradiographs using a ChemiImager 4000 Imaging System (Alpha Innotech Corp., San Leandro, CA).

Determination of NOS Activity. Cellular NOS activity was determined by measuring the conversion of L-arginine to L-citrulline (Hiki et al., 1991). After 24 h of hypoxia and 4 h of reoxygenation, BAECs (10^7 cells/100 mm dish) were washed once with 25 mM HEPES buffer (25 mM HEPES, 140 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) and then incubated with 2 ml HEPES buffer containing [L-^{3}H]arginine (300,000–400,000 cpm/dish) at 37°C for 30 min. The incubation was terminated by addition of 4 ml of stop buffer (118 mM NaCl, 4.7 mM KCl, 1.8 mM K_2HPO_4, 24.5 mM $NaHCO_3$, 4 mM EDTA, and 5 mM *N*-arginine, pH 5.5). The cells were then washed once with stop buffer, disrupted by adding 1 ml 0.3 N $HClO_4$, and the solution was neutralized by addition of ~80 ml of 3 M K_2CO_3. Exactly 0.1 ml of the resulting solution was counted in a Beckman LS 6500 Scintillation Counter to measure total [L-^{3}H]arginine uptake; 0.25 ml of this solution was applied to Dowex A50W-X8 Na^+ columns, washed with 10 mM HEPES buffer, pH 5.5, and the eluent counted to determine the amount of [L-^{3}H]citrulline production (Hiki et al., 1991). The percentage conversion of L-arginine to L-citrulline was calculated using the formula % conversion = {[L-^{3}H]citrulline production × 4} / {total [L-^{3}H]arginine uptake × 10} × 100.

Measurement of Cellular 8-Iso-PGF$_{2\alpha}$. Total 8-iso-PGF$_{2\alpha}$ levels in BAECs were measured using a modification of the procedure described by Morrow and Roberts (1999). Briefly, cells were collected by treatment with trypsin and stored at −80°C until analyzed. Butylated hydroxytoluene was added to the freshly thawed samples to inhibit oxidation during sample processing. The samples (4×10^7 cells) were incubated with KOH for 30 min at 40°C to release phospholipid bound isoprostanes. The samples were then acidified to pH 2.0 to 3.0, 3 ng of a d_4 8-iso-PGF$_{2\alpha}$ internal standard was added, and the precipitated protein was removed by centrifugation at 375*g*. The F$_2$-isoprostanes in the supernatant were concentrated by passage over C_{18} Sep-pak columns (Millipore, Marlborough, MA), followed by Silica Sep-pak (Millipore) columns. The eluant, containing a mixture of the d_4 internal standard and endogenous total cellular F$_2$-isoprostanes, was dried under vacuum and derivatized to the pentafluorobenzyl ester using α-bromo-2,3,4,5-pentafluorotoluene according to the protocol of Schweer et al. (1997). The pentafluorobenzyl-derivatized isoprostanes were purified by thin-layer chromatography, and the bands were collected based on comparison of their R_f values to those of authentic standards. The derivatized isoprostanes were eluted from the silica gel with the thin-layer chromatography developing solvent and further derivatized with bis(trimethylsilyl)trifluoro-acetamide as described previously (Morrow and Roberts, 1999). Quantification was done by GC/negative chemical ionization/MS on a 25-m Supelco DB 5 column (Supelco, Bellefonte, PA) under experimental conditions that allowed separation of 8-iso-PGF$_{2\alpha}$ from other isoprostane peaks. The ratio of the (M-181) peak height of the d_0 8-iso-PGF$_{2\alpha}$ from the sample (m/z 569) was compared with that of the d_4 8-iso-PGF$_{2\alpha}$ internal standard (m/z 573). All injections were done in duplicate.

Determination of the Amount of Extracellular Superoxide in BAECs. Cytochrome *c* reduction was used to assess extracellular superoxide anion levels in BAECs (Arnal et al., 1996; Barbacanne et al., 2000). Forty-eight hours after transfection with either pcDNA3.0/GFP or pcDNA3.0/CYP2J2, BAECs (400,000 cells/well) were washed once with 50 mM sodium phosphate buffer, pH 7.4, and then incubated with 1 ml of a solution containing 5.55 mM glucose, 1.36 mM Ca_2Cl, 20 μM deferoxamine mesylate, 77.69 mM NaCl, 4.96 mM KCl, 10 μM calcium ionophore A23187, with/without 100 IU/ml superoxide dismutase (SOD) at 37°C (Barbacanne et al., 2000). Cytochrome *c* reduction was determined at time 0 (to obtain basal values) and again 15 min later. The absorbance of the medium was read spectrophotometrically at 550 nm against a distilled water blank. Values in the presence of SOD were subtracted from values in the absence of SOD to determine the amount of cytochrome *c* reduction attributable to superoxide (Barbacanne et al., 2000). Control studies demonstrated that addition of A23187 had no effect on the amount of cytochrome *c* reduction by BAECs.

Statistical Analysis. Data are expressed as a percentage of values obtained from control cells maintained under normoxic conditions. Data were compared by analysis of variance using SYSTAT software (SYSTAT Inc., Evanston, IL). When *F* values indicated that a significant difference was present, Fisher's least significant difference test for multiple comparisons was used. Values were considered significantly different for $P < 0.05$. All data were obtained from 3 to 24 separate experiments and expressed as mean ± S.E.M.

Results

Hypoxia-Reoxygenation Causes Cell Injury and Decreases Endothelial CYP2J Protein Expression. BAECs maintained under control (normoxic) conditions continued to grow and showed little evidence of cell injury. There were $1.56 \pm 0.12 \times 10^5$ cells/cm^2 surface area, LDH release into the culture medium was 11.8 ± 0.7 mU/10^5 cells, and $9.9 \pm 1.1\%$ of the cells stained with trypan blue. In contrast, BAECs exposed to 24 h of hypoxia followed by 4 h of reoxygenation exhibited a significant 40% decrease in cell number ($p < 0.01$), LDH release into the culture medium was increased by 120% ($p < 0.01$), and there was a 34% increase in the number of trypan blue-stained cells ($p < 0.01$) (Fig. 1A). These data demonstrate significant HR-induced endothelial cell injury and are in agreement with previous data on the effects of this stress in endothelial cells (Samarasinghe and Farrell, 1996; Blanc et al., 1999).

Protein immunoblotting using a polyclonal antibody to recombinant human CYP2J2 that cross-reacts with CYP2J isoforms in rabbit, rat, and mouse revealed the presence of an abundant 56-kDa protein band in control BAEC lysates (Fig. 1B). This protein, which has an electrophoretic mobility slightly lower than that of recombinant CYP2J2 (57 kDa) but similar to CYP2J2 present in human tissues, probably represents the bovine ortholog of human CYP2J2. Interestingly, the expression of this CYP2J2 immunoreactive protein was markedly reduced in BAECs that were exposed to HR (Fig. 1B). This decreased expression was relatively selective for CYP2J2 in that the expression of other proteins (e.g., angiotensin II type I receptor) remained unchanged after exposure of endothelial cells to HR. Moreover, the effect of HR on CYP2J2 expression is not limited to bovine aortic endothelial cells. In cultured human coronary artery endothelial cells, we observed a similar reduction in CYP2J2 expression with HR (data not shown). In contrast, the expression of other endothelial proteins (e.g., ICAM-1 and VCAM-1) was increased in the human coronary artery endothelial cells after HR.

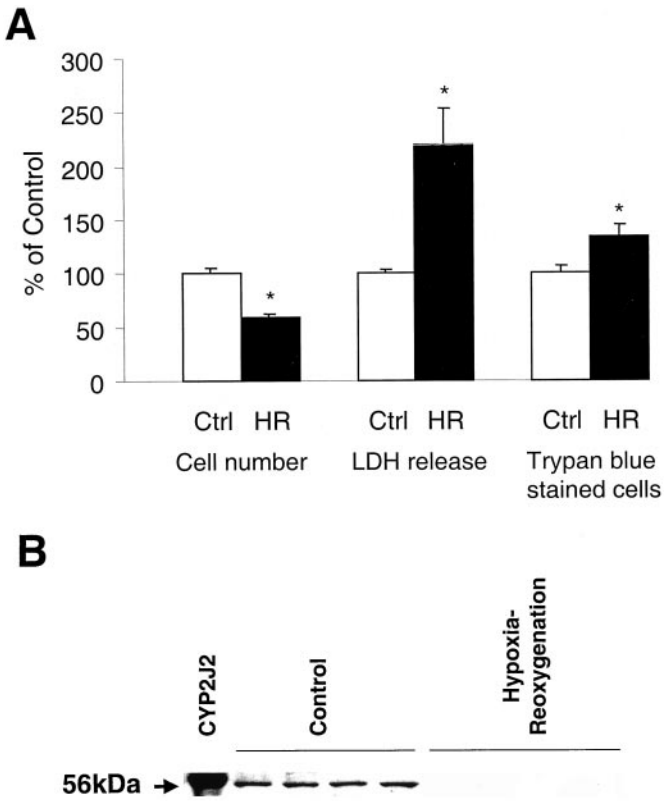

Fig. 1. Effect of HR on cell injury and CYP2J2 expression in cultured BAECs. BAECs were either maintained under normoxic conditions or exposed to HR. A, exposure of BAECs to HR results in decreased cell number, increased LDH release, and more trypan blue-stained cells compared with BAECs maintained under normoxic conditions. Ctrl, control. *$p < 0.01$ versus control; $n = 12$ in each group. B, immunoblot showing that exposure of BAECs to HR decreases CYP2J2 protein expression. Lane 1, recombinant CYP2J2; lanes 2 to 5, lysates from BAECs maintained under normoxic conditions; lanes 6 to 9, lysates from BAECs exposed to HR. Results are representative of four separate experiments.

Transfection of BAECs with pcDNA3.0/CYP2J2 and EET Biosynthesis. Forty-eight hours after transfection of BAECs with the pcDNA3.0/GFP construct, ~20% of the cells exhibited strong green fluorescence, indicating significant GFP expression (Fig. 2A). Immunoblotting of microsomes prepared from these GFP-transfected cells with the anti-CYP2J2 IgG revealed expression of the constitutive 56-kDa bovine CYP2J2 ortholog (Fig. 2B), the abundance of which was unchanged compared with untransfected cells or with cells transfected with the empty pcDNA3.0 vector. These control GFP-transfected cells metabolized radiolabeled arachidonic acid to epoxygenase metabolites (EETs and DHETs) at a rate of ~8 pmol/min/10^7 cells (Fig. 2C). Transfection of endothelial cells with the pcDNA3.0 expression vector containing the CYP2J2 cDNA resulted in abundant expression of a 57-kDa CYP2J2 immunoreactive protein (Fig. 2B). This recombinant CYP2J2 protein was present in whole-cell lysates and in both microsomal and mitochondrial subcellular fractions. The increase in CYP2J2 expression was accompanied by a significant increase in endothelial arachidonic acid epoxygenase activity ($p < 0.05$) (Fig. 2C). The increased epoxygenase activity was inhibited in the presence of the P450 inhibitor SKF-525A. Based on this data, we conclude that: (1) CYP2J2 is constitutively expressed in GFP-transfected BAECs; (2) these cells biosynthesize EETs from arachidonic acid; and (3) increased CYP2J2 expression is accompanied by increased epoxygenase activity.

Protective Effect of CYP2J2 Transfection on Hypoxia-Reoxygenation-Induced Injury in BAECs. In preliminary experiments, we determined the effects of transfection alone on BAECs under normoxic conditions. Transfection of BAECs with either pcDNA3.0 empty vector (data not shown), pcDNA3.0/GFP, or pcDNA3.0/CYP2J2 resulted in 28 to 38% fewer cells, 20 to 33% increase in the number of trypan blue stained cells, 250 to 350% increase in cellular LDH release, and 40 to 50% lower cellular eNOS expression, compared with untreated cells (data not shown) or cells treated with FuGENE 6 reagent alone (all $p < 0.05$) (Fig. 3, A–D). There were no significant differences between GFP- and CYP2J2-transfected BAECs in any of these parameters under normoxic conditions (Fig. 3, A–D).

Compared with either GFP-transfected cells (data not shown) or CYP2J2-transfected cells maintained under normoxic conditions, GFP-transfected BAECs exposed to 24 h of hypoxia followed by 4 h of reoxygenation exhibited a 36% reduction in cell number, LDH release into the culture medium was increased by 230%, and there was a 86% increase in the number of trypan blue-stained cells (all $p < 0.01$) (Fig. 4, A–C). These data indicate significant HR-induced endothelial cell injury in GFP-transfected cells. Importantly, the

HR-induced cell injury was significantly attenuated in CYP2J2-transfected BAECs. Thus, cell number was slightly higher ($p = 0.154$), significantly less LDH was released into the culture medium ($p < 0.01$), and there were significantly fewer trypan blue-stained cells ($p < 0.01$) in CYP2J2-transfected BAECs than in GFP-transfected BAECs exposed to HR (Fig. 4, A–C). Immunoblots confirmed that CYP2J2-transfected cells had higher levels of immunoreactive CYP2J2 protein than GFP-transfected cells after exposure to HR (Fig. 4D). Together, these data demonstrate that the presence of CYP2J2 protein is associated with reduced HR-induced cellular injury. CYP2J2 and/or its products are protective in endothelial cells.

Effects of P450-Derived Arachidonic Acid Metabolites on Hypoxia-Reoxygenation-Induced Injury in BAECs. The beneficial effects of CYP2J2 transfection seem to be mediated, at least in part, by arachidonic acid metabolites. Thus, addition of 1 μM 11,12-EET to the culture medium 10 min before hypoxia significantly attenuates HR-induced cell death as measured by the number of trypan blue-stained cells ($p < 0.01$) (Fig. 5). Other epoxygenase products, such as 14,15-EET and the 11,12-DHET, were also

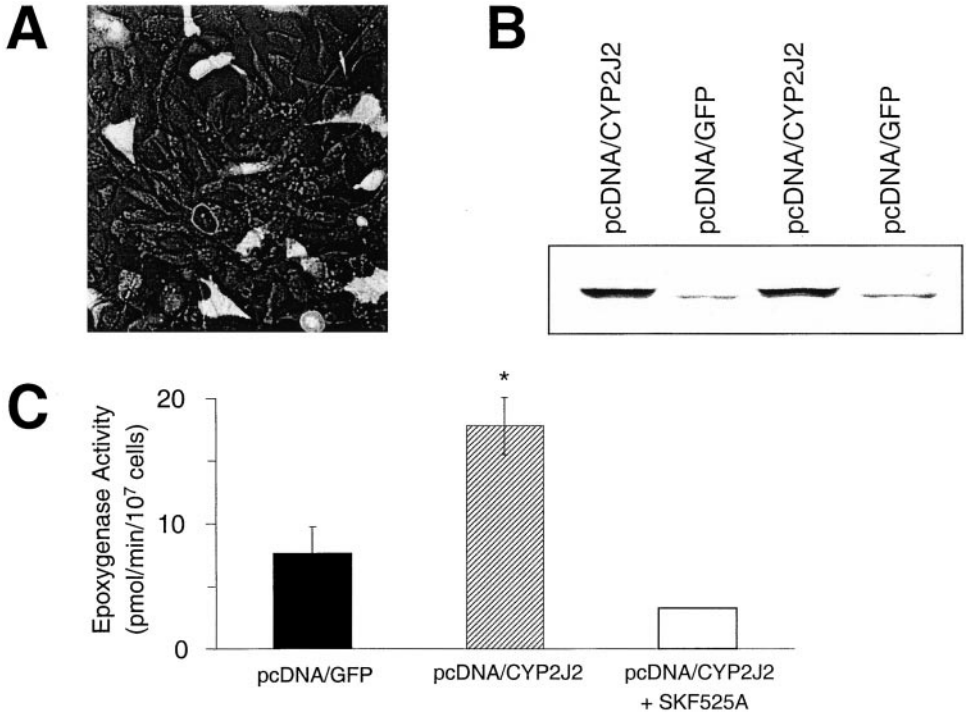

Fig. 2. CYP2J2 expression and arachidonic acid epoxygenase activity in transfected BAECs. Cells were transfected with either pcDNA3.0/GFP or pcDNA3.0/CYP2J2. A, 48 h after transfection of BAECs with pcDNA3.0/GFP, ~20% of cells exhibit strong green fluorescence. B, immunoblotting shows that CYP2J2 protein expression is increased in BAECs 48 h after transfection with pcDNA3.0/CYP2J2. Lanes 1 and 2, microsomes prepared from transfected BAECs; lanes 3 and 4, mitochondria prepared from transfected BAECs. C, 48 h after transfection, arachidonic acid epoxygenase activity is increased in CYP2J2-transfected BAECs ($n = 4$) compared with GFP-transfected cells ($n = 4$) and CYP2J2-transfected cells treated with SKF-525A ($n = 3$). *$p < 0.05$ versus GFP-transfected cells.

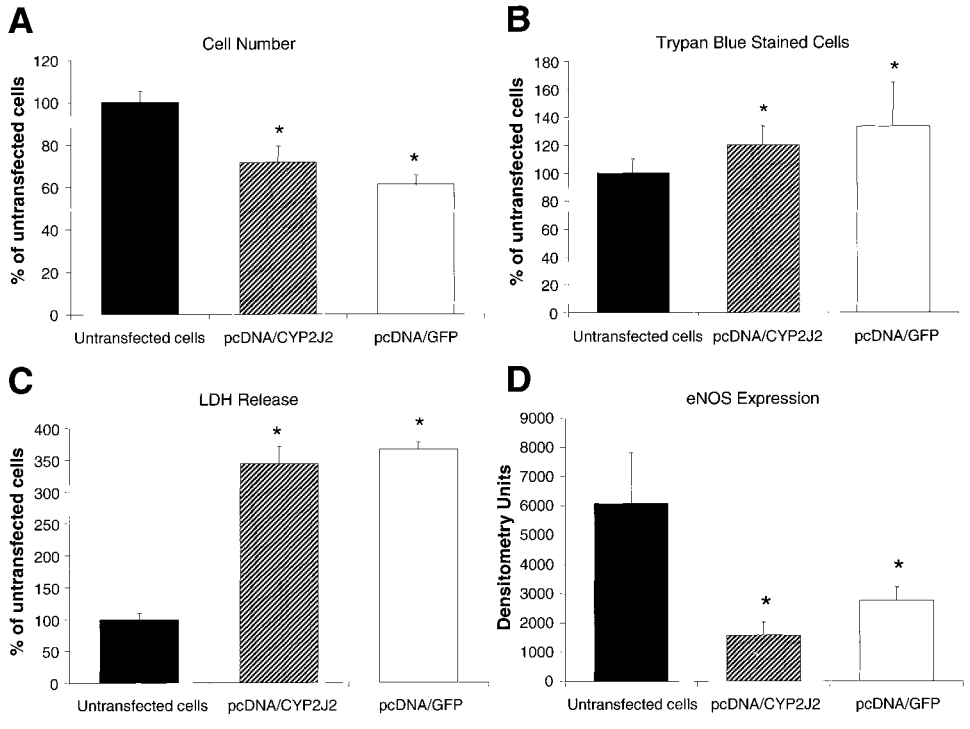

Fig. 3. Effect of transfection on cell injury parameters and eNOS expression in BAECs under normoxic conditions. Cell injury parameters and eNOS expression were evaluated under normoxic conditions in untransfected BAECs and in BAECs transfected with either pcDNA3.0/CYP2J2 or pcDNA3.0/GFP. Transfection results in significantly lower total cell number (A), more trypan blue-stained cells (B), higher LDH release (C), and lower eNOS expression (D) in both CYP2J2- and GFP-transfected BAECs. There were no significant differences between GFP- and CYP2J2 transfected cells in any of these parameters. *$p < 0.01$ versus untransfected cells, n = 5 to 6 in each group.

active in attenuating the effects of HR, albeit to a much lesser extent than 11,12-EET (data not shown). Similarly, addition of the soluble epoxide hydrolase inhibitor dicyclohexylurea and the microsomal epoxide hydrolase inhibitor elaidamide (which decrease EET hydrolysis and prolong the half-life of endogenous EETs) also limits HR-induced endothelial cell injury ($p < 0.05$) (Fig. 5). The combination of 11,12-EET and epoxide hydrolase inhibitors produced a larger effect than either 11,12-EET alone or epoxide hydrolase inhibitors alone ($p < 0.01$) (Fig. 5).

Superoxide Anion Generation, Lipid Peroxidation, and CYP2J2-mediated Protection in BAECs. To examine whether some of the beneficial effects of CYP2J2 overexpression were mediated by influences on oxygen free radical generation, antioxidant defenses and/or lipid peroxidation, we used GC/MS methods to quantify 8-iso-$PGF_{2\alpha}$ in GFP- and CYP2J2-transfected BAECs exposed to HR (Fig. 6A). We also used a cytochrome c reduction assay to assess the amount of extracellular superoxide in GFP- and CYP2J2-transfected BAECs maintained under normoxic conditions. Compared with CYP2J2-transfected cells maintained under normoxic conditions, GFP-transfected BAECs exposed to 24 h of hypoxia followed by 4 h of reoxygenation had 20 to 25% higher levels of cellular 8-iso-$PGF_{2\alpha}$ (2.45 ± 0.19 and 2.99 ± 0.32 pg/10^5 cells, respectively, $p < 0.05$). Interestingly, transfection with the CYP2J2 containing vector significantly attenuated this HR-induced 8-iso-$PGF_{2\alpha}$ increase (2.54 ± 0.26 pg/10^5 cells; $p < 0.05$ versus GFP-transfected cells). Furthermore, the amount of extracellular superoxide anion was significantly lower in CYP2J2-transfected cells compared with untransfected or GFP-transfected cells ($p < 0.05$) (Fig. 6B). Together, these observations suggest that the protective effects of CYP2J2 in endothelial cells may also involve an antioxidant pathway.

Nitric Oxide Pathway, AT1 Receptor, and CYP2J2-Mediated Protection of BAECs. To determine whether the nitric oxide pathway was involved in the protective effects of CYP2J2 transfection in BAECs, we examined NOS expression and activity. Exposure of either CYP2J2- or GFP-transfected BAECs to HR resulted in significantly lower eNOS expression (Fig. 7A), nitrite production (Fig. 7B), L-arginine uptake (Fig. 7C), and L-citrulline production (Fig. 7D) compared with CYP2J2- or GFP-transfected cells maintained under normoxic conditions (all $p < 0.01$). There were no significant differences in any of these parameters between GFP- and CYP2J2-transfected cells. The percentage conversion of L-arginine to L-citrulline, indicating eNOS activity, was not affected by hypoxia-reoxygenation (34.8 ± 3.1% in CYP2J2-transfected BAECs; 37.6 ± 1.2% in GFP-transfected BAECs; 42.9 ± 2.2 in CYP2J2-transfected BAECs after HR; and 37.2 ± 4.2% in GFP-transfected BAECs after HR). The inducible form of NOS was not detectable in any of the experimental groups. The angiotensin II type 1 receptor (AT1), which has been implicated in the pathogenesis of ischemic heart disease (Yang et al., 1998), was abundantly expressed in BAECs; however, there were no significant differences in AT1 expression after HR or after transfection with the CYP2J2 containing plasmid (Fig. 8).

Discussion

It has been known for more than 2 decades that cardiovascular tissues contain cytochromes P450 and associated monooxygenase activities (Guengerich and Mason, 1979; Stegeman et al., 1979); however, only recently have the identity and functional significance of these enzymes and their products been investigated. We described a human P450 (CYP2J2) that was highly expressed in the heart and vascular tissues, particularly

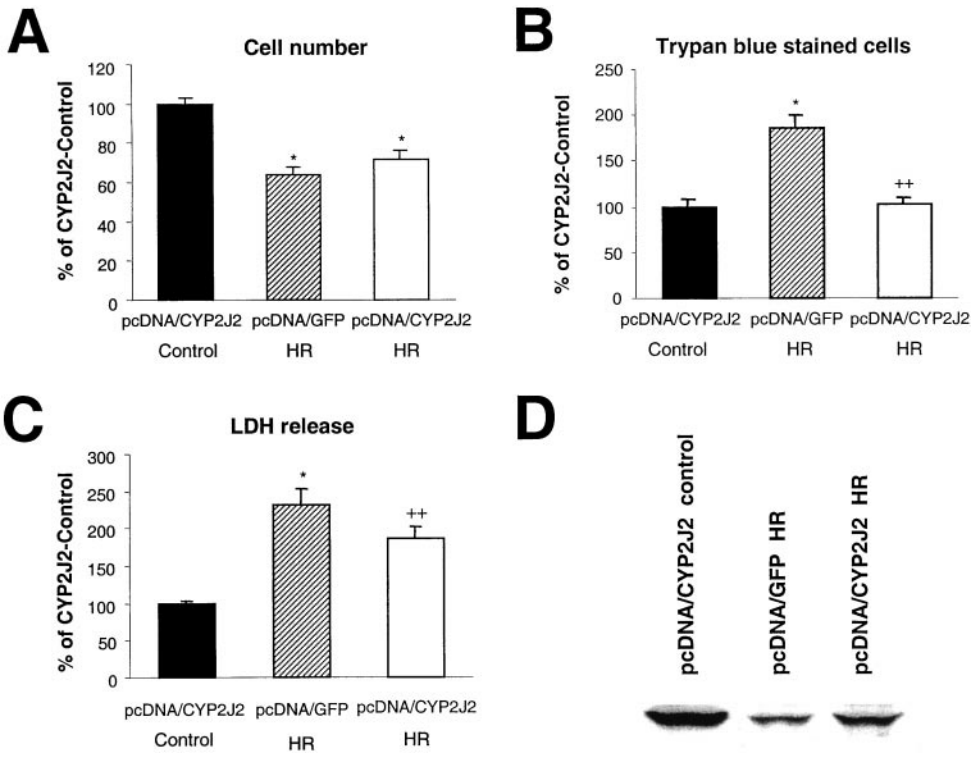

Fig. 4. Effect of CYP2J2 transfection on cell injury parameters and CYP2J2 protein expression in BAECs exposed to HR. Forty-eight hours after transfection of BAECs with either pcDNA3.0/GFP or pcDNA3.0/CYP2J2, cells were exposed to HR. A, cell number. B, trypan blue-stained cells. C, LDH release into the culture medium. HR results in significantly lower total cell number, more trypan blue-stained cells, and higher LDH release in GFP-transfected BAECs. The HR-induced changes in trypan blue stained cells and LDH release are significantly attenuated in CYP2J2-transfected BAECs. $^{*}p < 0.01$ versus CYP2J2-transfected cells maintained under normoxic conditions; $^{++}p < 0.01$ versus GFP-transfected cells exposed to HR; $n = 19$ to 24 in each group. D, CYP2J2 protein expression by immunoblotting. CYP2J2 protein levels were significantly higher in CYP2J2-transfected cells than in GFP-transfected cells under both normoxic and HR conditions. Results are representative of three separate experiments.

abundant in endothelial cells, and active in the epoxidation of arachidonic acid to EETs (Wu et al., 1996; Node et al., 1999). In the heart, the EETs were shown to improve postischemic recovery of cardiac contractile function, inhibit cardiac L-type Ca^{2+} channel activity, and increase coronary artery blood flow (Wu et al., 1997; Gebremedhin et al., 1998; Oltman et al., 1998; Chen et al., 1999). Indeed, EETs are leading candidates for endothelial-derived hyperpolarizing factor because they hyperpolarize and relax vascular smooth muscle by activating calcium-sensitive potassium channels (Gebremedhin et al., 1998; Campbell et al., 1999; Fisslthaler et al., 1999). More recently, we have shown that EETs have potent antiinflammatory actions; i.e., they decrease cytokine-induced endothelial cell adhesion molecule expression and inhibit leukocyte adhesion to the vascular wall by inhibiting NF-κB and IκB kinase (Node et al., 1999). In this report, we describe a new functional role for CYP2J2 and its eicosanoid products in limiting endothelial injury after exposure to HR. We demonstrate that CYP2J2 protein levels are markedly reduced after exposure of endothelial cells to HR and that transfection with the CYP2J2 cDNA attenuates the cellular injury. Given that endothelial injury is an important early event in the development of the atherosclerotic plaque and is associated with myocardial dysfunction in ischemic heart disease (Lefer and Lefer, 1993; Liao, 1998; Biegelsen and Loscalzo, 1999), we postulate that reduced CYP2J2 protein and/or activity may be involved in the pathogenesis of these cardiovascular disorders.

The reduction in endothelial CYP2J2 protein expression after exposure to HR seems to be relatively selective for this protein. We observed that endothelial expression of the AT1 receptor remains unchanged after this stimulus. We and others have observed an up-regulation of endothelial cell adhesion molecule expression (ICAM-1, ELAM-1, and E-selectin) after HR (Hess et al., 1994; B. Yang and D. C. Zeldin, unpublished observations). In addition, it is well documented that interleukin-1, interleukin-6, and vascular endothelial growth factor are also induced in endothelial cells after hypoxia (Ala et al., 1992; Marti et al., 2000). Thus, our observations cannot be explained simply on the basis of a generalized, nonspecific reduction in protein synthesis and/or increase in protein degradation in the hypoxic BAECs. It is interesting that eNOS expression, like that of CYP2J2, is also decreased after exposure of BAECs to HR. The percentage conversion of L-arginine to L-citrulline, indicating eNOS activity, was not affected by hypoxia-reoxygenation. Thus, decreased cellular uptake of L-arginine and reduced eNOS expression contribute to the lower nitrite levels observed in hypoxia-reoxygenated BAECs. One of the most important functions of the endothelium is nitric oxide production. Nitric oxide possesses potent vasodilatory, antiinflammatory, antithrombotic, and antiproliferative properties. Although some investigators have found that eNOS is up-regulated by ischemia-reperfusion (Felaco et al., 2000; Hangai et al., 1999), others have observed reduced eNOS expression and activity after ischemia-reperfusion injury (Liao et al., 1995; Giraldez et al., 1997). Although reasons for these discrepancies are not entirely clear, differences in experimental protocols, species and organ systems may be partially responsible. Impor-

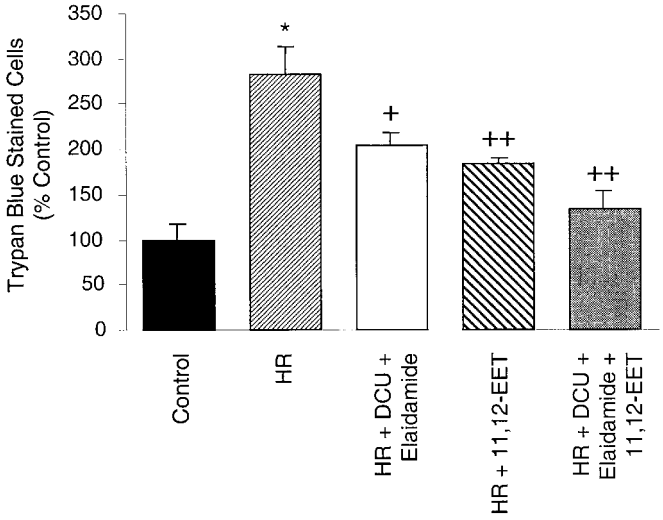

Fig. 5. Effect of synthetic arachidonic acid metabolites and epoxide hydrolase inhibitors on HR-induced cell injury in BAECs. Addition of epoxide hydrolase inhibitors dicyclohexylurea (10 μM) and elaidamide (10 μM) 30 min before hypoxia or addition of 1 μM 11,12-EET 10 min before hypoxia significantly attenuates HR-induced cell injury in BAECs. The effect of 11,12-EET and epoxide hydrolase inhibitors is larger than the effect of either 11,12-EET alone or epoxide hydrolase inhibitors alone. $^{*}p < 0.01$ versus control; $^{+}p < 0.05$ versus HR; $^{++}p < 0.01$ versus HR; $n = 5$ in each group.

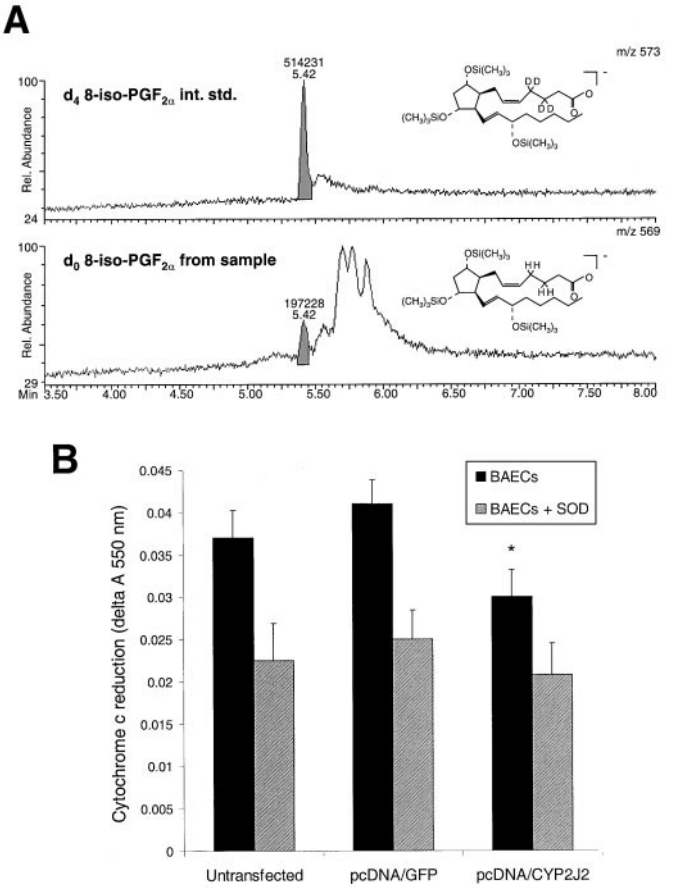

Fig. 6. Effect of CYP2J2 transfection on cellular F_2-isoprostanes and superoxide anion levels. A, GC/MS chromatogram showing quantitation of 8-iso-$PGF_{2\alpha}$ (shaded peak) in BAECs as described under *Materials and Methods*. The ratio of the (M-181) peak height of the d_0 8-iso-$PGF_{2\alpha}$ from the sample (m/z 569) was compared with that of the d_4 8-iso-$PGF_{2\alpha}$ internal standard (m/z 573). B, 48 h after BAEC transfection, the amount of extracellular superoxide anion (the SOD-inhibitable cytochrome c reduction) was significantly lower in CYP2J2-transfected BAECs than in GFP-transfected cells or untransfected cells. $^{*}p < 0.05$; $n = 24$ to 40 in each group.

tantly, CYP2J2 transfection significantly attenuated HR-induced injury in BAECs but had no significant effects on eNOS expression, L-arginine uptake, L-citrulline production, or nitrite production suggesting that the mechanism(s) by which CYP2J2 exerts its protective effects in endothelial cells are largely independent of the NOS-pathway.

We have previously shown that CYP2J2 is an active arachidonic acid epoxygenase, CYP2J2 products (the EETs) are present endogenously in cardiac tissues and, in addition to the cyclooxygenase and lipoxygenase pathways, the P450 monooxygenase pathway is an important member of the cardiac arachidonic acid metabolic cascade (Wu et al., 1996; Wu et al., 1997). The data presented herein demonstrate that BAECs metabolize radiolabeled arachidonic acid to EETs at appreciable rates, transfection of the endothelial cells with the CYP2J2 cDNA results in increased arachidonic acid epoxygenase activity, and this activity is reduced by the P450 inhibitor SKF-525A. Furthermore, recent gas chromatography/mass spectrometry analysis shows that EETs are endogenous constituents of BAECs and that CYP2J2 transfection increases endogenous EET levels by approximately 30% (Node et al., 2001). Together, these data establish a role for CYP2J2 in the biosynthesis of EETs from both exogenous and endogenous arachidonic acid pools in endothelial cells. However, other P450s may contribute to EET biosynthesis in endothelial cells. For example, Lin et al. (1996) have shown that the arachidonic acid epoxygenase present in human umbilical vein endothelial cells is a member of the CYP2C subfamily. Fisslthaler et al. (1999) showed that treatment of porcine coronary artery endothelial cells with β-naphthoflavone induces a CYP2C homolog and increases EET biosynthesis. In addition, the CYP1A enzymes are known to be induced in endothelial cells by aromatic hydrocarbons (Stegeman et al., 1989). Determining which of these enzymes is mainly responsible for basal EET production will not be an easy task given the complexity of the relevant P450 subfam-

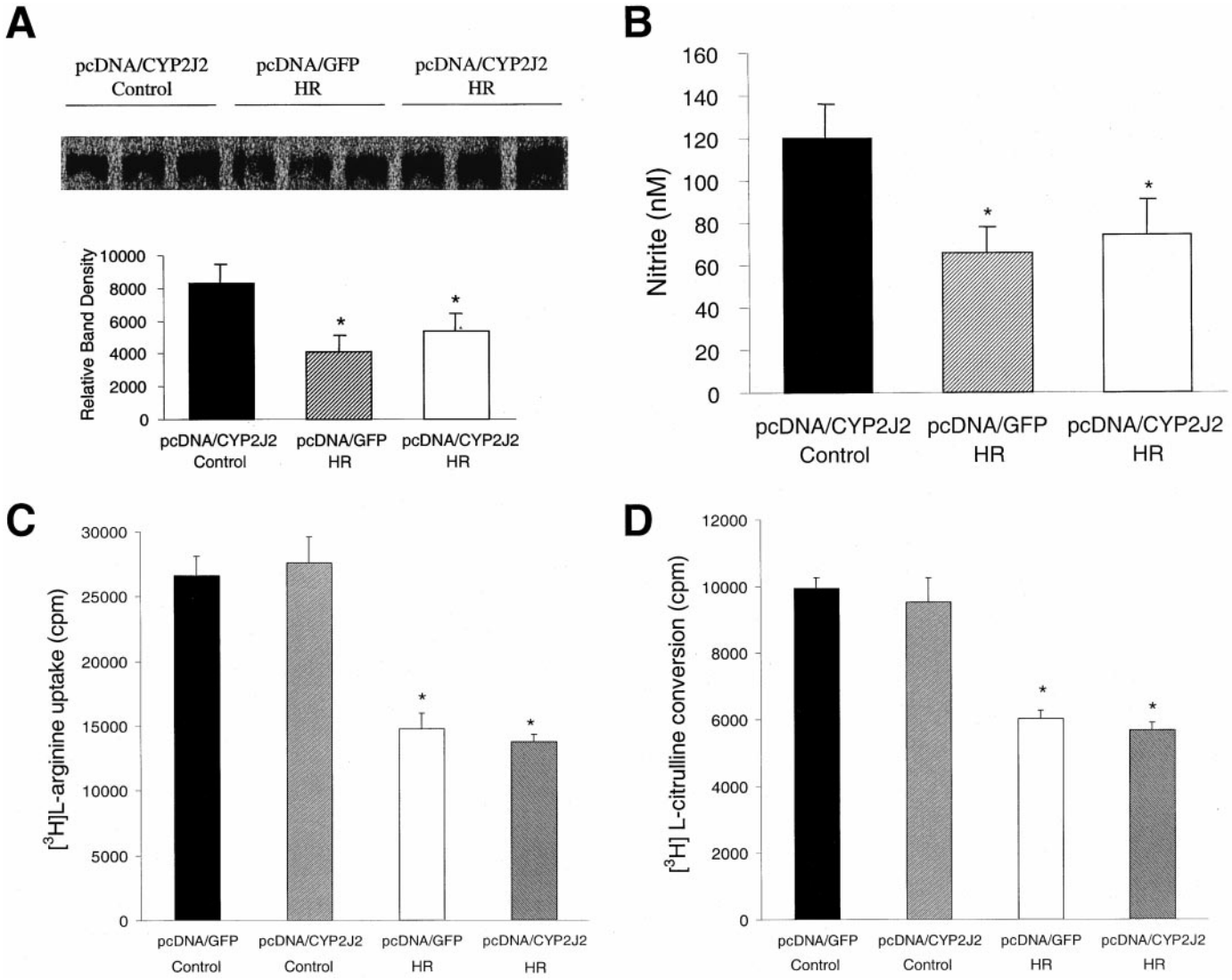

Fig. 7. Effect of HR and CYP2J2 transfection on eNOS expression and NOS activity. A, immunoblotting shows that HR decreased eNOS expression to a similar degree in both GFP- and CYP2J2-transfected BAECs. *$p < 0.01$ versus CYP2J2 transfected cells maintained under normoxic conditions; $n = 9$ in each group. B, nitrite production is lower in both GFP- and CYP2J2-transfected BAECs exposed to HR. *$p < 0.01$ versus CYP2J2 transfected cells maintained under normoxic conditions; $n = 9$ in each group. C, uptake of L-arginine is reduced in both GFP- and CYP2J2-transfected BAECs exposed to HR. *$p < 0.01$ versus GFP- and CYP2J2-transfected cells maintained under normoxic conditions; $n = 4$ in each group. D, production of L-citrulline is reduced in both GFP- and CYP2J2-transfected BAECs exposed to HR. *$p < 0.01$ versus GFP- and CYP2J2-transfected cells maintained under normoxic conditions; $n = 4$ to 8 in each group.

ilies and the absence of isoform specific inducers, inhibitors and inhibitory antibodies.

Our data demonstrate that EETs likely mediate some of the cytoprotective effects of CYP2J2 transfection after HR. Thus, micromolar concentrations of synthetic 11,12-EET significantly attenuate cell injury. We have previously demonstrated that micromolar concentrations of 11,12-EET (but not other P450-derived eicosanoids including 14,15-EET and 19-HETE) improve functional recovery after prolonged global ischemia-reperfusion in an isolated-perfused rat heart model (Wu et al., 1997). 11,12-EET was also the most active eicosanoid in inhibiting cytokine-induced adhesion molecule expression (Node et al., 1999) and tissue-plasminogen activator gene transcription (Node et al., 2001) in BAECs. For these reasons, we chose 11,12-EET rather than other eicosanoids for the experiments performed in Fig. 5. It has been shown that 14,15-EET, 11,12-EET, and 8,9-EET are present in roughly equal amounts in human heart (Wu et al., 1996), human plasma (Karara et al., 1992), rat heart (Wu et al., 1997), and bovine aortic endothelial cells (Node et al., 2001). The estimated concentrations of EETs in these studies are on the order of 0.1–0.3 μM. However, EETs are known to be taken up by endothelial cells and rapidly metabolized by epoxide hydrolases to DHETs (VanRollins et al., 1993). Because we chose to evaluate the effects of 11,12-EET at a slightly higher concentration than present endogenously, we cannot rule out that the concentrations we used were pharmacologic rather than physiologic. Importantly, our studies with exogenously added 11,12-EET were confirmed by showing that addition of epoxide hydrolase inhibitors, which prolong the half-life of endogenously formed EETs (Yu et al., 2000; Fang et al., 2001), also limit HR-induced endothelial cell injury. Indeed, the combination of 11,12-EET and epoxide hydrolase inhibitors produced a larger effect than either 11,12-EET alone or epoxide hydrolase inhibitors alone.

The magnitude of the effect of 11,12-EET was generally less pronounced than that of CYP2J2 transfection. One possible explanation for this observation is that the cytoprotective effects of CYP2J2 in endothelial cells may also involve EET-independent mechanisms. In this regard, we showed that CYP2J2 transfection significantly attenuates HR-induced increases in cellular 8-iso-$PGF_{2\alpha}$ and decreases extracellular superoxide levels in BAECs. The relevance of reactive oxygen species generation and lipid peroxidation in the pathogenesis of ischemia-reperfusion injury is well established (Morrow and Roberts, 1990; Mehta et al., 1993; Mathews et al., 1994; Reilly et al., 1997). Antioxidants are known to have beneficial cardiovascular functional effects after ischemia-reperfusion (Mehta et al., 1989; Meyer et al., 1996). Thus, our data suggest that the cytoprotective effects of CYP2J2 may be mediated, at least in part, by antioxidant effects.

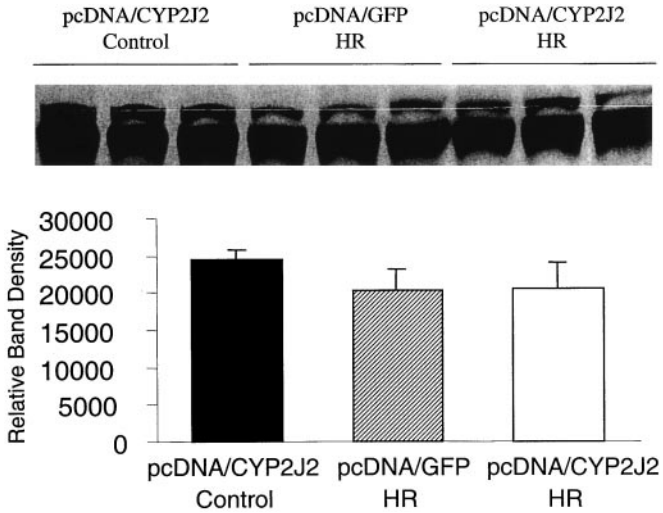

Fig. 8. Effect of HR and CYP2J2 transfection on AT1 receptor expression. Exposure of BAECs to HR does not change AT1 receptor expression either in GFP-transfected or CYP2J2-transfected cells. $n = 9$ in each group.

The role of cyclooxygenases in the formation of isoprostanes is controversial. Reports in the literature concerning the evidence for cyclooxygenase participation in the production of F_2-isoprostanes are conflicting. Several studies have found evidence for the production of F_2-isoprostanes via free radical catalyzed peroxidation of arachidonic acid, independent of the cyclooxygenase pathway (Morrow and Roberts, 1990; Morrow and Roberts, 1992; Wang et al., 1995). On the other hand, cyclooxygenase-dependent isoprostane formation has also been reported (Patrignani et al., 1996; Bachi et al., 1997; Klein et al., 1997). A recent multilaboratory, double-blind study designed to determine which of the biomarkers used for noninvasive measurement of oxidative stress are most specific, sensitive, and selective has reported that 8-iso-$PGF_{2\alpha}$ is formed predominately by nonenzymatic mechanisms and is thus a good indicator of oxidative stress (Graham et al., 2000).

Recently, we have shown that CYP2J2 can metabolize other polyunsaturated fatty acids such as linoleic acid to alcohols and monoepoxides (Moran et al., 2000). The *cis*-epoxyoctadecenoic acids are produced in vivo under certain pathologic conditions (e.g., severe burns, adult respiratory distress syndrome) and have been shown to be toxic to a variety of cell types at high concentrations (100–500 μM) (Kosaka et al., 1994; Moran et al., 2000). However, recent data suggest that at lower concentrations (500 nM-5 μM), these compounds can protect against cellular lysis (Moran et al., 2000). Thus, we cannot rule-out the possibility that some of the beneficial effects of CYP2J2 transfection in endothelial cells are caused, in part, by the production of linoleic acid metabolites.

In summary, we demonstrate that exposure of cultured BAECs to HR results in cell injury and reduced CYP2J2 protein expression. Transfection with the CYP2J2 cDNA, addition of synthetic 11,12-EET, or application of epoxide hydrolase inhibitors limits the HR-induced cellular injury. CYP2J2 transfection also attenuates the HR-induced increase in cellular 8-iso-$PGF_{2\alpha}$ and reduces the amount of extracellular superoxide anion in BAECs, but has no significant effect on eNOS expression or NOS activity. Together, these observations suggest that the cytoprotective effects of CYP2J2 in endothelial cells involve EET-dependent pathways and may be mediated, at least in part, by antioxidant effects.

Acknowledgments

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