Role of Nitric-Oxide Synthase, Free Radicals, and Protein Kinase C δ in Opioid-Induced Cardioprotection

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ABSTRACT
Opioids generate free radicals that mediate protection in isolated cultured cardiomyocytes. We hypothesize that the nature of these radicals is nitric oxide, and that nitric oxide activates the protein kinase C (PKC) δ isoform. Through this signal transduction pathway, opiates protect cardiomyocytes during hypoxia and reoxygenation. Cell viability was quantified in chick embryonic ventricular myocytes with propidium iodide. Oxygen radicals were quantified using a molecular probe, 2′,7′-dichlorofluorescin diacetate (DCFH-DA). After a 10-min infusion of the opioid δ receptor agonist BW373U86 (BW; 2 or 20 pM) and a 10-min drug-free period, cells were subjected to hypoxia for 1 h followed by reoxygenation for 3 h. BW produced a concentration-dependent reduction in cardiomyocyte death (2 pM, 35.3 ± 3.9%, n = 5; 20 pM, 21.5 ± 4.0%, n = 8, p < 0.05 versus controls) and attenuated oxidant stress compared with controls (43.3 ± 4.2%, n = 8). The increase in DCFH-DA oxidation with BW before hypoxia was abolished by the specific nitric-oxide synthase inhibitors nitro-L-arginine methyl ester (L-NAME) or Nω-nitro-L-arginine (L-NMMA) (100 μM each). L-NAME or L-NMMA blocked the protective effects of BW. BW selectively increased the activity of PKC δ isoform in the particulate fraction, and its protection was abolished by the selective PKC δ inhibitor rottlerin (1 μM). Similar to BW, infusion with 5 μM of the nitric oxide donor S-nitroso-N-acetylpenicillamine (SNAP) reduced cardiomyocyte death (24.6 ± 3.7, n = 8), and this protection was blocked by chelerythrine or rottlerin. Chelerythrine and rottlerin had no effect on BW-generated oxygen radicals before hypoxia, but they abolished the protection of SNAP. The nature of DCFH oxidation produced by opioid δ receptor stimulation is nitric oxide. Nitric oxide mediates cardioprotection via activating PKC δ in isolated myocytes.

Opioids protect against ischemia-reperfusion injury in vivo (Schultz et al., 1998a) and in vitro (Liang and Gross, 1999; Huh et al., 2001). Intravascular administration of opioids affects the coronary endothelium, circulating blood elements, and activates a signal transduction cascade in cardiomyocytes. Which effect (on endothelium, blood cells, or cardiomyocytes) accounts for cardioprotection remains unclear.

Opioids increase nitric oxide synthesis from vascular endothelial cells and monocytes (Fimiani et al., 1999). In anesthetized rats, intravenous infusion of opioids reduces myocardial infarct size (Fryer et al., 2000). The role of nitric oxide in opioid-induced cardioprotection has not been defined. Several recent studies strongly suggest that nitric oxide from vascular endothelium mediates the cardioprotection of early and late preconditioning (Bolli et al., 1998, 2000). Since there are many confounding factors present in in vivo settings, we chose isolated cultured cardiomyocytes to determine whether nitric oxide, which originates from cardiomyocytes, mediates opioid-induced cardioprotection.

Stimulation of opioid δ receptors causes mitochondria to release oxygen radicals in cardiomyocytes, and this effect correlates with cardioprotection (McPherson and Yao, 2001a,b). These radicals are thought to activate protein kinase C (PKC) (Gopalakrishna and Anderson, 1989) and mediate cardioprotection (Simkhovich et al., 1998). The goal of this study is to determine the nature of these radicals produced by opioids (H₂O₂, nitric oxide, or both).

Translocation of activated PKC δ, e, and η from cytosol to membranes has been detected in preconditioned hearts (Ping et al., 1997; Kawamura et al., 1998). Ping and colleagues (1999) have shown that nitric oxide induces translocation of the PKC ε isoform and mediates the late phase of preconditioning in a conscious rabbit model of cardiac ischemia-reperfusion (Bolli, 2000). To determine whether this signaling pathway mediates the cardioprotection of opioids, we studied the effects of the selective opioid δ receptor agonist BW373U86 (Chang et al., 1993) on the enzyme activity of PKC δ, e, and η in isolated cultured cardiomyocytes.

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ABBREVIATIONS: PKC, protein kinase C; DCFH, 2′,7′-dichlorofluorescin; DCFH-DA, DCFH diacetate; DCF, oxidized DCFH; BW, BW373U86; SNAP, S-nitroso-N-acetylpenicillamine; BSS, balanced salt solution; PI, propidium iodide; PBS, phosphate-buffered saline; L-NMMA, Nω-nitro-L-arginine; L-NAME, nitro-L-arginine methyl ester; BNTX, benzylidenenaltrexone; Chel, chelerythrine.
total PKC, and the ε and δ isoforms, in cytosol and particulate fractions.

**Materials and Methods**

**Cardiomyocyte Isolation and Culture.** Ventricular myocytes from 10-day-old chick embryos were prepared according to a method described previously (McPherson and Yao, 2001a,b). Briefly, hearts were harvested and placed in Hanks’ balanced salt solution lacking magnesium and calcium (Invitrogen, Carlsbad, CA). Ventricles were minced, and myocytes were dissociated by use of four to six repeated exposures to trypsin degradation (0.025%; Invitrogen) at 37°C with gentle agitation. Then, isolated cells were transferred to a solution with a trypsin inhibitor for 8 min, filtered through a 100-µm mesh filter, centrifuged for 5 min at 1200 rpm at 4°C, and finally resuspended in a nutrient medium described previously (McPherson and Yao, 2001b). Resuspended cells were placed in a petri dish in a humidified incubator (5% CO2, 95% air at 37°C) for 45 min to promote early adherence of fibroblasts. Nonadherent cells were counted with a hemocytometer, and viability was measured with trypan blue (0.4%). Approximately 1 × 10⁶ cells in a nutritive medium were pipetted onto coverslips (25-mm) and incubated for 3 to 4 days, after which synchronous contractions of the monolayer were noted. Experiments were performed on spontaneously contracting cells at day 3 or 4 after isolation.

The myocyte culture system was checked for nonmuscle cell contamination by staining with anti-myosin heavy chain monoclonal antibodies (CCM-52) labeled with horseradish peroxidase. More than 95% of plated cells stained for myosin. The remaining cells, less than 5% of total cells, consisted mainly of fibroblasts. This experiment was reported in 1996 (Vanden Hoek et al., 1996) and routinely performed to ensure purity of cardiomyocyte isolation and culture. Endothelial cell contamination was minimal.

**Hypoxia System.** Glass coverslips containing spontaneously beating chick myocytes were placed in a stainless steel, 1-ml, flow-through chamber (Penn Century Co., Philadelphia, PA). The chamber was sealed with Kynar film (McMaster-Carr, Elmhurst, IL) placed between the coverslip and the metal hypoxic chamber to minimize oxygen exchange between the chamber wall and the perfusate and then mounted on a temperature-controlled platform (37°C) on an inverted microscope. A water-jacketed glass equilibration column mounted above the microscope stage was used to equilibrate the perfusate to known oxygen tensions (PO2). The standard perfusion medium was equilibrated for 1 h before the experiment by bubbling with a gas mixture of 21% oxygen, 5% carbon dioxide, and 74% nitrogen. A hypoxic solution, composed of balanced salt solution (BSS) containing no glucose with 2-deoxyglucose (20 mM) added to inhibit glycolysis, was bubbled with a gas mixture of 2% carbon dioxide and 80% nitrogen for 1 h before the experiments. The pH of the perfusion solution was routinely verified (normoxic BSS, 7.4; hypoxic BSS, 6.8). Stainless steel or polymer tubing with low oxygen solubility connected the equilibration column to the flow-through chamber to minimize ambient oxygen transfer into the perfusate. PO2 in our hypoxic chamber was routinely monitored by Oxyspot (Medical Systems Inc., Greenvale, NY) under conditions identical to those of experiments using an optical phosphorescence quenching method (Wilson et al., 1988; Lo et al., 1996). PO2 in the chamber was 5.33 ± 0.71 mm Hg (n = 6) during hypoxia and 136 ± 3.65 mm Hg (n = 6) during normoxia perfusion.

**Necrosis Assay.** Fluorescent cell images were obtained with an X10 objective lens (Nikon Fluor; Nikon, Tokyo, Japan). Data were acquired and analyzed with Metamorph software (Universal Imaging Corp., Downingtown, PA). There were approximately 600 cardiomyocytes under the selected field for each experiment. Multiple fields were examined and compared before each study; the field with normal synchronous contraction was chosen and monitored throughout experiments. Cell viability was quantified with the nuclear stain propidium iodide (PI; 5 µM) (Molecular Probes, Eugene, OR), an exclusion fluorescent dye that binds to chromatin upon loss of membrane integrity (Altman et al., 1993). PI is not toxic to cells over a course of 8 h, permitting its addition to the perfusate throughout the experiments. At the completion of each experiment, digitonin (300 µM) was added to the perfusate for 1 h. Digitonin disrupts the membrane integrity of all cells allowing PI to enter. Percent loss of viability (cell death) was expressed relative to the maximum value after 1 h of digitonin exposure (100%).

**Quantification of Oxygen Radicals.** Oxygen radicals generated in cells were assessed with the probe 2',7'-dichlorofluorescin (DCFH). The membrane-permeable diacetate form of DCFH (DCFH-DA) was added to the perfusate at a final concentration of 5 µM. Within the cell, esterases cleave the acetate groups on DCFH-DA, thus trapping DCFH intracellularly (Sawada et al., 1996). Oxygen radicals in the cells lead to oxidation of DCFH, yielding the fluorescent product DCF (Vanden Hoek et al., 1996). DCFH in cardiomyocytes is readily oxidized by H2O2 or hydroxyl radical but is relatively insensitive to superoxide (Vanden Hoek et al., 1996). Fluorescence was measured with an excitation wavelength of 480 nm, dichroic 505-nm long pass and emitter bandpass of 535 nm (Chroma Technology, Brattleboro, VT) with neutral density filters to attenuate the excitation light intensity. Fluorescence intensity was assessed in clusters of several cells identified as regions of interest. The background was identified as an area without cells or with minimal cellular fluorescence. Intensity was reported as the percentage of initial value after subtraction of the background value.

**Permeabilization of Cardiac Myocytes.** We used a technique described by Gray et al. (1997) to permeabilize cardiomyocytes to allow a peptide (εV1-3) in this study to enter cardiomyocytes before experiments. The temperature of isolated and cultured myocytes was slowly reduced by two sequential 2-min incubations, each with 2 ml (for 35-mm culture dishes) of fresh phosphate buffer solution. The first incubation with phosphate buffer solution was carried out at room temperature; the second, with chilled PBS in an ice bath. The PBS was discarded, and the cells were incubated with 1 ml of freshly prepared permeabilization buffer [20 mM HEPES, pH 7.4, 10 mM EGTA, 140 mM KCl, 50 µg/ml saponin (Sigma-Aldrich, St. Louis, MO), 5 mM NaN3, and 5 mM oxalic acid di potassium salt] containing the desired peptides for 10 min in an ice bath. ATP was added just before adding the permeabilization buffer to cells (i.e., 30 µl of 200 mM ATP, pH 7.4, per milliliter of permeabilization buffer). The cells were then gently washed four times on ice with 2 ml of chilled PBS. Then, an additional 2 ml of chilled PBS was added to the cells for a 20-min recovery on ice. After the chilled PBS was removed, 2 ml of room temperature PBS was added, and the cells were placed at room temperature for 2 min. This step was repeated with PBS at 37°C, after which the original cell media were added back to the cells at 37°C. The cells were further incubated for 30 min at 37°C before interventions.

**PKC Enzyme Assay.** Enzyme activity of total PKC and its ε and δ isoforms was measured by a method described previously (Ping et al., 1997, 1999). For each experiment, 5 million cells were collected in sample buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM each EGTA and benzamidine, 50 µg/ml phenylmethylsulfonyl fluoride, 10 µg/ml each of aprotonin, leupeptin, and pepstatin A, and 0.3% β-mercaptoethanol) (Sigma-Aldrich). The collection of cells was centrifuged at 45,000g for 30 min and separated into cytosol and particulate fractions. The particulate pellet was dissolved ultrasonically in sample buffer. Enzyme protein concentration was determined according to the Bradford method (Bradford, 1976). Each fraction, 50 to 100 µg, was assayed for activity of total PKC and its isoforms (assay kit; Amersham Biosciences, Piscataway, NJ). The activity of total PKC in the pellet (particulate) and the supernatant (cytosolic) was assayed separately. For PKC ε and δ assays, proteins were immunoprecipitated overnight by PKC ε and δ monoclonal antibody (BD Biosciences Pharmingen, San Diego, CA) in immunoprecipitation buffer (pH 7.4) (150 mM NaCl, 50 mM Tris, 1 mM EGTA, 1 mM EDTA, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride).
fluoride, 16 μg/ml benzamidine-HCl, and 10 μg/ml each for phenan- 
throline, aprotinin, leupeptin, and pepstatin A) (Sigma-Aldrich) with 
protein A/G beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). 
PKC ε-specific substrate (ERMRPRKRQGSVRRRV) (BIOMOL Re- 
search Laboratories, Plymouth Meeting, PA) was used for the 
phosphorylation reactions with [32P]ATP (Amersham Biosciences). Since 
there is no specific substrate available for PKC δ, the same substrate 
for total PKC was used for the phosphorylation reaction with 
[32P]ATP with the proteins that were immunoprecipitated overnight 
by PKC δ monoclonal antibody (BD Biosciences PharMingen). Ad- 
ditionally, we used rottlerin (1 μM) to block PKC δ and eV1 (100 μM) 
to block PKC ε phosphorylation to ensure the specificity of the 
increased enzyme activity.

Chemicals. BW373U86, 8-nitroso-N-acetylpenicillamine (SNAP), 
N6-monomethyl-L-arginine (L-NMMA), and nitro-L-arginine methyl 
ester (L-NAME) were purchased from Sigma-Aldrich. Chelerythrine 
was purchased from Calbiochem-Novabiochem Corp. (San Diego, 
CA). BW373U86, L-NMMA, L-NAME, or chelerythrine was dissolved 
in BSS buffer before administration. Rottlerin and BNTX were pur- 
blished from BIOMOL Research Laboratories and dissolved in a 1:5 
cocktail of ethanol/saline. PI and DCFH-DA were purchased from 
Molecular Probes.

Experimental Protocol. Figure 1 shows the experimental pro- 
tocol. Fifteen groups of cardiomyocytes [control, BW (2 pM), BW (20 
pM), BNTX (0.1 μM), BNTX + BW, L-NMMA (100 μM), L-NMMA + 
BW, L-NAME (100 μM), L-NAME + BW, rottlerin (1 μM), rottlerin + 
SNAP (5 μM), rottlerin + SNAP, chelerythrine (4 μM), and SNAP + 
Chel] were studied. Cardiocytes were subjected to 1 h of 
hypoxia followed by 3 h of reoxygenation. Ethanol/saline (1:5) (con- 
trol series) or BW (2 or 20 pM) was added to the perfusate for 10 min 
followed by 10 min of a drug-free period before the cells were sub- 
jected to hypoxia and reoxygenation. For the corresponding series, 
rottlerin (1 μM), L-NMMA (100 μM), or L-NAME (100 μM) was added 
to the perfusate during baseline (1 h) before 60 min of hypoxia. Nine 
additional series of experiments were used to determine the effects of 
the above interventions on production of oxygen radicals before and 
during hypoxia and reoxygenation.

An additional six groups of cardiomyocytes were used to examine 
whether the nitric oxide donor SNAP (5 μM) activates PKC δ and 
mediates cardioprotection [control, SNAP, chelerythrine (4 μM), 
SNAP + Chel, rottlerin (1 μM), and SNAP + rottlerin].

For the PKC enzyme activity assay, BW (20 pM) was adminis- 
tered for 10 min followed by a 10-min drug-free period, then cardiocytes 
were collected for the assay. In the control group, vehicle (ethanol/ 
saline 1:5) was given for 10 min instead of BW administration.

Statistical Analysis. Data are expressed as mean ± S.E.M. Dif- 
ferences between groups for cell death and oxygen radical production 
were compared by a two-factor analysis of variance with repeated 
measures and Fisher's least significant difference test. Differences 
between groups were considered significant at values of P < 0.05.

Results

Effects of the Selective Opioid δ Receptor Agonist 
BW373U86 on Cell Death, Contraction, and Oxidant 
Stress. BW373U86 (2 and 20 pM) reduced cell death in a 
concentration-dependent manner. The pattern and extent of 
cell death were similar to that previously reported (McPherson 
and Yao, 2001b). After 3 h of reoxygenation, cardiomyo- 

e death was 43.25 ± 4.17% in controls (n = 8), 35.28 ± 3.87% 
in BW373U86 (2 pM)-treated cells (n = 5), and 21.51 ± 3.96% 
in BW373U86 (20 pM)-treated cardiocytes (n = 8). Sponta- 
neous contractile activity was noticed in 12 of 16 BW373U86-
treated cells (20 pM, 75%) and 1 of 15 hypoxic controls (7%). 
BW373U86 decreased oxidant stress in a concentration-re-

e manner (Fig. 2a) and conferred protection from cell 

d and oxygen radical production during hypoxia/reoxygen-

ation. The protection afforded by BW373U86 (20 pM) was 
lost in the presence of 0.1 μM BNTX (cell death: 40.21 ± 
4.72%, n = 6) compared with controls (cell death: 43.25 ± 
4.17%, n = 8). BNTX had no effect on cardiomyocyte death 
(46.12 ± 6.80%, n = 4) when compared with that observed in 
hypoxic controls (Fig. 3b) or on oxidant stress. The data 

d from BW373U86 (20 pM)-treated cells and control 
cells for DCF fluorescence and percent cell death were re- 
peatedly used in Figs. 2 through 5 for convenience of com-

parison.

Role of Nitric-Oxide Synthase. The protection afforded 
by BW373U86 (20 pM) was lost in the presence of 100 μM 
L-NAME or L-NMMA (cell death: 52.03 ± 3.5%, n = 6 and 
47.36 ± 5.42%, n = 6, respectively) compared with controls 
(cell death: 43.25 ± 4.17%, n = 8). The concentration of the 
nitric-oxide synthase inhibitors had no effect on cardiomyo-

1. Control
2. BW373U86 (BW) 2 pmol/L
3. BW 20 pmol/L
4. BNTX 0.1 umol/L
5. BNTX + BW 20 pmol/L
6. L-NMMA 100 umol/L
7. L-NMMA + BW 20 pmol/L
8. L-NAME 100 umol/L
9. L-NAME + BW 20 pmol/L
10. Rottlerin 1 umol/L
11. Rottlerin + BW 20 pmol/L
12. SNAP 5 umol/L
13. Rottlerin + SNAP
14. Chelerythrine 4 umol/L
15. Chelerythrine + SNAP

Fig. 1. Schematic diagram of experimental protocol.
cyte death (40.92 ± 3.28%, n = 4 and 38.54 ± 4.21%, n = 4) when compared with that observed in hypoxic controls (Figs. 4b and 5b) or on oxidant stress (data not shown).

Infusion of BW373U86 led to a moderate increase of DCFH oxidation prior to hypoxia but subsequently attenuated the free radical burst during reoxygenation (Fig. 2a). This moderate increase of DCFH oxidation before hypoxia was abolished by L-NAME or L-NMMA (Figs. 4a and 5a).

Role of PKC δ Isoform. The protection produced by BW373U86 was blocked by the selective PKC δ inhibitor rottlerin 5 μM (cell death: 41.65 ± 3.25%, n = 9) compared with controls (cell death: 43.25 ± 4.17%, n = 8). Rottlerin alone did not affect either cell death (Fig. 6) nor the increased DCFH oxidation produced by BW373U86 administered before hypoxia (Fig. 6a). These results indicate that PKC δ activation is a downstream signal of the oxygen radicals that mediate BW373U86-induced protection.

BW373U86 markedly increased the enzyme activity of PKC δ in the particulate fraction but had no effect on total PKC activity and the ε isoform compared with controls. The increased PKC δ activity in particulate fraction with BW373U86 was not affected by 100 μM V1–2 but was abolished when 1 μM rottlerin was added before the phosphorylation experiments (virgin cardiomyocytes, 21.1 ± 6.25, n = 3; BW-treated, 48.71 ± 6.39, n = 3; BW + rottlerin, 20.10 ± 3.44, n = 3; and BW + eV1–2, 59.22 ± 5.87, n = 3; picomoles per minute per gram of protein). In the cytosolic fraction, no difference was observed in the enzyme activity of total PKC or the δ or ε isoforms.

Nitric Oxide/Oxygen Radicals Activate PKC δ Isoform. Infusion of the nitric oxide donor SNAP (2 μM) for 10 min and followed by 10 min of a drug-free period before hypoxia reduced cell death similar to that of BW373U86 (Fig. 7a). This protection was blocked by the nonselective PKC inhibitor chelerythrine (4 μM) (44.00 ± 3.42%, n = 8) or the selective PKC δ inhibitor rottlerin 1 μM (47.12 ± 3.60%, n = 6) compared with controls (43.25 ± 4.17%, n = 8). Chelerythrine or rottlerin alone had no effect on cell death (40.58 ± 5.68%, n = 4 and 39.78 ± 5.04%, n = 4, respectively) (Fig.
7a). In contrast, the selective PKC inhibitor \( \text{H}9280 \text{V1-2}, 100 \mu M \) had no effects on the protection produced by either BW373U86 or SNAP (Fig. 7b). The dose of \( \text{H}9280 \text{V1-2} \) (100 \( \mu M \)) was chosen based on a preliminary study in which this dose completely blocked the ischemic preconditioning-increased PKC activity. These results indicate that nitric oxide activates PKC, which mediates BW373U86-induced protection.

**Discussion**

We have recently demonstrated that BW373U86, a selective \( \delta \) opioid receptor agonist (Chang et al., 1993), generates oxygen radicals from cardiomyocyte mitochondria (McPherson and Yao, 2001a,b). In the present study, BW373U86-generated oxygen radicals were abolished when the opioid \( \delta_1 \) receptor or nitric-oxide synthase was antagonized. Panel b, the reduction in cell death was blocked by l-NAME. l-NAME alone did not affect either cell death or oxygen radical production before hypoxia. *, \( P < 0.05 \) compared with the corresponding point in controls.

![Fig. 4](image-url) Panel a, treatment with the specific nitric oxide synthase inhibitor \( \text{l-NAME} \) abolished the increase in oxygen radicals before hypoxia and restored BW373U86-reduced oxidant stress to levels observed in controls during reoxygenation. Oxidant stress is referenced to free radical bursts during prolonged hypoxia and reoxygenation. Panel b, the reduction in cell death was blocked by l-NAME. l-NAME alone did not affect either cell death or oxygen radical production before hypoxia. *, \( P < 0.05 \) compared with the corresponding point in controls.

![Fig. 5](image-url) Panel a, treatment with the specific nitric oxide synthase inhibitor \( \text{l-NMMA} \) blocked the increase in oxygen radicals before hypoxia and restored BW373U86-reduced oxidant stress to levels observed in controls during reoxygenation. Panel b, the reduction in cell death was blocked by \( \text{l-NMMA} \). \( \text{l-NMMA} \) alone did not affect either cell death or oxygen radical production before hypoxia. *, \( P < 0.05 \) versus the corresponding point in controls.

Initially, we found that the selective opioid \( \delta \) receptor agonist BW373U86 reduced cardiomyocyte death during hypoxia and reoxygenation in a concentration-dependent manner. These results are consistent with previous reports in which BW373U86 attenuated hypoxia/reoxygenation injury in isolated cultured cardiomyocytes (McPherson and Yao, 2001a,b). Bofetiado et al. (1996) showed that BW373U86 increased mouse survival during acute lethal hypoxia. The protection of BW373U86 was abolished with pretreatment of a selective opioid \( \delta_1 \) receptor antagonist, BNTX (Sofuoglu et
Stimulation of opioid receptors also mimicked ischemic preconditioning to reduce myocardial infarction in anesthetized rats (Schultz et al., 1998a; Schultz and Gross, 2001).

Hypoxia/reoxygenation generates a large number of free radicals in our system. Such oxidant stress contributes to hypoxia/reoxygenation injury in vivo (Zweier et al., 1987; Lucchesi et al., 1989) and in vitro (McPherson and Yao, 2001a). Transient administration of BW373U86 markedly attenuated oxidant stress. Previously, we and others found that monophosphoryl lipid A limited infarct size by decreasing free radical production from neutrophils (Yao et al., 1993) and by activating inducible nitric-oxide synthase (Xi et al., 1999). The reduction in cardiomyocyte death with BW373U86 correlated with its effect on oxidant stress during hypoxia and reoxygenation. Because temperature, pH, perfusion rate, and partial pressure of oxygen and carbon dioxide were controlled throughout the experiment, BW373U86 exerted its salutary effects via an intracellular signaling mechanism.

The attenuated oxidant stress by BW373U86 during the hypoxic period could slow down depletion of endogenous antioxidants of the cardiomyocytes, which would preserve the ability of the cells to reduce oxidant stress at reoxygenation and increase cell survival. Such a mechanism may be as important as reduced oxidant stress at reoxygenation to explain the cardioprotection of BW373U86. Free radicals generated during hypoxia and reoxygenation contribute to the pathogenesis of cell damage (McPherson and Yao, 2001a).

BW373U86 increased oxygen radicals before the start of hypoxia, and this effect correlated with reduced cardiomyocyte death via the activating PKC δ isoform. Cell death was assessed by propidium iodide uptake. Treatment with chelerythrine (4 μM) (SNAP + Chel, n = 6) or rottlerin (1 μM) (SNAP + rottlerin, n = 8) abolished the protection produced by SNAP (panel a). ϵV1–2 (100 μM), a selective inhibiting peptide for the PKC ε isoform, had no effect on the protection produced by SNAP and BW373U86 (panel b). *

*, P < 0.05 versus the corresponding point in controls.
scavenger, N-2-mercaptopropionylglycine, and was only partially antagonized by BNTX. This suggests that BW373U86-mediated delayed cardioprotection in rats occurs via a free radical mechanism, which is only partially dependent on activation of opioid receptors. Our results only implicate a mechanism that is responsible for the acute phase of cardioprotection produced by BW373U86 in isolated chick embryonic cardiomyocytes. Nevertheless, the present finding and those of Patel et al. (2001) indicate an important role of oxygen radicals in signaling both acute and late phases of preconditioning.

The production of these radicals was abolished by two inhibitors of nitric-oxide synthase, l-NAME or l-NMMA (Yao and Gross, 1993), which alone had no effect on DCFH oxidation. Although DCFH is more sensitive to hydroxyl/hydrogen radicals, it is also oxidized by free radicals of other sources including OH\(^-\), \(H_2O_2\), nitric oxide, and peroxynitrate. These results suggest that nitric-oxide synthase plays an important role in generation of these radicals and that the nature of these radicals may be nitric oxide. Inhibition of the mitochondrial electron transport chain or blockade of mitochondrial K\(_{\text{ATP}}\) channels abolishes the increased DCFH oxidation with BW373U86 (McPherson and Yao, 2001a). Thus, mitochondria seem to be a significant source of nitric oxide, and mitochondrial K\(_{\text{ATP}}\) channels appear to be involved in the production.

The cardioprotection provided by BW373U86 was also abolished by specific inhibition of nitric-oxide synthase with l-NAME or l-NMMA. Nitric oxide may protect against hypoxia/reoxygenation-reperfusion injury in the myocardium (Vegh et al., 1992). In addition, the protection of opioid \(\delta\) receptor stimulation was abolished with 5-hydroxydecanoate, a selective mitochondrial K\(_{\text{ATP}}\) channel antagonist in vivo (Schultz et al., 1998b) and in vitro (McPherson and Yao, 2001a). Nitric oxide and mitochondrial K\(_{\text{ATP}}\) channel opening are important in BW373U86-induced cardioprotection.

BW373U86-induced protection was abolished by rottlerin (1 \(\mu\)M). Rottlerin selectively inhibits the PKC \(\delta\) isoform with an IC\(_{50}\) value of 3 to 6 \(\mu\)M (Gschwendt et al., 1994). The IC\(_{50}\) for inhibition of other isoforms of PKC (\(\alpha\), \(\beta\), \(\gamma\), \(\epsilon\), and \(\zeta\)) is greater than 30 to 42 \(\mu\)M (Gschwendt et al., 1994). Fryer and colleagues (2001) also demonstrated that rottlerin, at a dose selective for PKC \(\delta\) inhibition, ablished the opioid-induced cardioprotection in anesthetized rats (Fryer et al., 2001). These authors also provided convincing evidence that translocation of PKC \(\delta\) to mitochondria was critical to cardioprotection afforded by \(\delta\) opioid receptor stimulation (Fryer et al., 2001). With a similar chick cardiomyocyte preparation, Liang showed that PKC activation protected cells against injury after hypoxia and reoxygenation (Huh et al., 2001). Others have also shown that PKC activation mediates cardioprotection in isolated hearts and in vivo models of hypoxia/reoxygenation (Brooks and Hearse, 1996; Ping et al., 1997). Furthermore, we found that the nitric oxide donor SNAP mimicked BW373U86-induced protection. The protection produced by BW and SNAP was abolished by either chelerythrine or rottlerin. Nakano et al. (2000) demonstrated that the exogenous nitric oxide donor SNAP activated PKC and triggered ischemic preconditioning in isolated rabbit hearts. Recently, Bolli (2000) suggested that preconditioning activates PKC via nitric oxide or nitric-oxide synthase. Because rottlerin did not affect the increased DCFH oxidation before hypoxia, PKC appears to be a downstream signal from nitric oxide in BW373U86-induced protection (Ping et al., 1999; Rakshit et al., 2000). Others have found, in isolated rabbit hearts, that nitric oxide activated PKC and mediated cardioprotection (Nakano et al., 2000; Rakshit et al., 2000).

Results from our laboratory showed that BW373U86 selectively increased the enzyme activity of the PKC \(\delta\) isoform in the particulate fraction without affecting total PKC activity and that of its \(\epsilon\) isoform (Liu et al., 2001b). The mechanisms by which nitric-oxide synthase and nitric oxide activate PKC, and which isoform(\(s\)) is activated, remain to be elucidated.

Ping and colleagues (1997) have shown that ischemic preconditioning translocates the PKC \(\epsilon\) isoform in conscious rabbit hearts and that such translocation correlates with cardioprotection. However, PKC \(\eta\) was also activated in their study. Our recent data with the same chick cardiomyocyte preparation showed that PKC \(\epsilon\) was activated following transient exposure to hypoxia or acetylcholine and these effects seemed to correlate with cardioprotection (Liu et al., 2001a). In contrast, a selective PKC \(\epsilon\)-inhibiting peptide (\(\epsilon\)V\(_1\)–\(\epsilon\)2, 100 \(\mu\)M) had no effect on BW- and SNAP-induced cardioprotection (Gray et al., 1997). Others have reported that PKC \(\alpha\) and \(\delta\) were translocated to the cell membrane during ischemic preconditioning and high-calcium preconditioning (Miyawaki et al., 1996; Miyawaki and Ashraf, 1997). These differences are likely due to the use of different experimental protocols to induce cardioprotection (opioid agonists versus transient ischemia or high calcium). Although the present study does not eliminate the importance of other PKC isoforms in limiting cardiac infarction, PKC \(\delta\) activation appears to be essential for opiates to confer cardioprotection.

The mechanism by which PKC \(\delta\) protects hypoxic/reperfused cardiomyocytes is not clear. A recent study by Wang and colleagues (1999) showed that ischemic preconditioning resulted in PKC \(\delta\) translocation to mitochondrial sites, where activation of PKC \(\delta\) might directly open mitochondrial K\(_{\text{ATP}}\) channels and result in phosphorylation of numerous rate-limiting enzymes or increase gene expression of heat shock proteins and nitric oxide synthase.

Taken together, BW373U86 transiently increased nitric oxide in virgin cardiomyocytes that activate protective signaling pathways and result in less cell damage. Either damaged cardiomyocytes generate fewer free radicals during subsequent hypoxia and reoxygenation, or the activated protective signal transduction pathway leads to an increased scavenging ability in cardiomyocytes. Both would lead to attenuated oxidant stress.

In conclusion, stimulation of \(\delta\) opioid receptors with BW373U86 activates nitric oxide synthase to produce nitric oxide. Nitric oxide activates the PKC \(\delta\) isoform directly or via oxygen radicals. The activated PKC \(\delta\) is likely to be redistributed to cardiomyocyte mitochondria. Through this signal transduction pathway, BW373U86 attenuates oxidant stress and reduces cardiomyocyte death during hypoxia and reoxygenation.

References


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