Oxidative Damage of Cardiomyocytes Is Limited by Extracellular Regulated Kinases 1/2-mediated Induction of Cyclooxygenase-2*

(Received for publication, September 11, 1998, and in revised form, December 1, 1998)

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Oxidative stress causes cardiac damage following ischemia/reperfusion and in response to anthracyclines. Extracellular signal-regulated kinases (ERK) 1/2 are activated by oxidative stress in cardiac myocytes and protect cardiac myocytes from apoptosis. Prostaglandins (PG) also protect cells from injury in a number of tissues, including the cardiomyocyte. Cyclooxygenase (COX) is the rate-limiting enzyme in PG biosynthesis has two isoforms, the constitutive COX-1 and an inducible COX-2. Here, we examined the effects of two oxidative stresses, hydrogen peroxide (H2O2) and the antioxidant doxorubicin on the activity of ERK1/2 and the expression of COX isoforms and PG formation in neonatal rat primary cardiomyocytes. These cells expressed COX-1 at rest and both COX isoforms on treatment with phorbol 12-myristate 13-acetate. Exposure to 50 μM H2O2 for 10 min or doxorubicin at 10 and 100 μM caused expression of COX-2 that was prevented by free radical scavengers. COX-2 induction was associated with activation of ERK1/2 and the specific ERK-inhibitor PD098059 abolished COX-2 expression. Treatment of cells with decay oligonucleotides corresponding to COX-2 promoter elements implicated the AP-1 and NF-kB but not the NF-kB1 in the transcription of COX-2. Induction of COX-2 mRNA and protein was accompanied by increased prostacyclin formation, which was abolished by the selective COX-2 inhibitor, NS-398, and PD098059. H2O2 and doxorubicin enhanced the release of lactate dehydrogenase and free radical scavengers prevented this. NS-398 enhanced the release of lactate dehydrogenase in response to H2O2 and doxorubicin, whereas the injury was prevented by iloprost, a stable prostacyclin analogue. In cardiomyocytes cell injury by H2O2 and doxorubicin is limited by an increase in prostacyclin formation that reflects induction of COX-2 mediated by ERK1/2 activation.

Reperfusion of ischemic myocardium results in an abrupt aggravation of cardiomyocyte injury, demonstrated experimentally by the re-introduction of oxygen into hypoxic myocardium (1). This injury is due in part to the generation of reactive oxygen species (2, 3), and the injury is limited by antioxidants and free radical scavengers (4). Potential sources of reactive oxygen species generation during ischemia/reperfusion of vascular tissue include superoxide (O2−) via neutrophil NADPH oxidase (5, 6) or from leakage of electrons from the electron transport chain in the mitochondria (7). The O2− produced is converted to hydrogen peroxide (H2O2) by superoxide dismutase, a weak free radical that reacts with O2− to generate the highly active hydroxyl radical (·OH). Anthracyclines, such as doxorubicin induce cardiac injury in a similar manner, in that they are metabolized to the corresponding semiquinone free radical by broadly distributed enzymes, flavin reductases (7, 8).

In addition to causing tissue injury, free radicals induce genes that are protective, such as hemoxygenase-1, which generates the antioxidant, biliverdin (9). Cyclooxygenase is likewise cytoprotective in several tissues (10) and plays a role in preventing apoptosis (11). Moreover, cyclooxygenase activity is known to be sensitive to free radicals (12). In this study, we explored the expression of cyclooxygenase in response to free radical-induced injury of primary cardiomyocytes using H2O2 and doxorubicin, both of which induce cardiac prostaglandin (PG) formation (13). Cyclooxygenase (COX), the rate-limiting enzyme in prostaglandin synthesis, exists as two isoforms (14, 15). COX-1 is present in most cells and is responsible for constitutive PG formation, whereas COX-2 is largely absent but is induced by cytokines, growth factors, and hormones (16). A recent study has reported the expression of COX-2 in ischemic human myocardium and in dilated cardiomyopathy, but not in normal cardiomyocytes (17). How COX-2 is induced in the heart and its function in these conditions is unknown. Cardiac fibrosis has been reported in mice where the COX-2 isoform has been disrupted (18), suggesting that COX-2 expression may be protective.

The mitogen-activated protein kinases (MAPKs) are serine/threonine protein kinases and 4 subfamilies have been described. One of the subfamilies, extracellular regulated kinases (ERKs)1/2, is activated by a variety of growth factors, cytokines, and phorbol esters, and regulates cell proliferation and differentiation (19, 20). In cardiac myocytes activation of ERK1/2 regulates gene expression and is implicated in the development of cellular hypertrophy (21, 22). Oxygen-derived free radicals induce activation of ERK1/2 and a second MAP kinase, p38 in cardiac myocytes and other cells (23, 24). H2O2-induced activation of ERK1/2 is mediated through the Ras/Raf-1/Mek pathway (23–25). PD098059, a selective inhibitor of ERK1/2 activation, aggravates H2O2-induced apoptosis of cardiomyocytes (23). The mechanism of the protective role played by ERK1/2 activation is unknown. We investigated a possible link between ERK1/2 activation and COX-2 expression in car-

* This work was supported by the Health Research Board of Ireland and the Irish Heart Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Printed in U.S.A.

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domyocytes injured by oxygen-free radicals and used a transcription factor decay approach to decipher the role of the promoter elements NF-κB and AP-1 in COX-2 induction.

EXPERIMENTAL PROCEDURES

Reagents—Culture medium (Dulbecco’s modified Eagle’s medium/ Ham’s F-12), pancreatic, newborn calf serum (NBS), LDH assay kit LD-L10, phorbol 12-myristate 13-acetate (PMA), hydroperoxide (H₂O₂), doxorubicin (DX), polyethylene glycol catalase (PEG-CAT), polyethylene glycol superoxide dismutase (PEG-superoxide dismutase), Immobilon-P polyvinylidene fluoride membranes, 3,3′-diaminobenzidine tetrahydrochloride, Harris Hematoxylin solution modified, permutant and common laboratory chemicals were from Sigma. Type II collagen was from Worthington Biochemical Corp., Cambridge, MA. Deuterated eicosanoid standards, 9,39-fluroprenisol (PGE₉α analog), and arachidonic acid were obtained from Cayman Chemical Co., Ann Arbor, MI. Goat polyclonal anti-COX-1 antibody was obtained from Oxford Biomedical Research Inc., MI. Mouse monoclonal anti-COX-2 antibody (R6), which cross-reacts with the rat COX-2, was a kind gift from Dr. Isakson, Monsanto, St. Louis, MO. Mouse monoclonals anti-ERK1/2 were obtained from Transduction Laboratories, Lexington, KY. Secondary anti-goat and anti-mouse biotinylated antibodies, chemiluminescence reagents for assaying Western blot detection, and p44/p42 MAP kinase assay kit were from New England Biolabs (UK) Ltd., Hertfordshire, UK. PCR prep kits were obtained from Promega, Southampton, UK. Primers for PCR and oligonucleotides for transcription factor decay experiments were purchased from Genosys Biotech Inc., Cambridge, UK. Iloprost was from Schering AG, Berlin, Germany. ERK1/2 inhibitor PD098059 and p38 MAP kinase inhibitor SB203580 were obtained from Calbiochem, La Jolla, CA.

Cell Culture—Primary cultures of neonatal rat cardiomyocytes were prepared by a modification of the method originally described by Simpson et al. (26). Briefly, the hearts from 1 to 3-day-old Wistar rats were minced and dissociated with approximately 80 units/ml type II collagenase and 0.06% pancreatin. Dispersed cells were incubated in 25-cm² flasks for 30 min at 37°C in a CO₂ incubator. Nonattached viable cells were washed with PBS and medium containing COX inhibitors were then added to cells/ml. To obtain a near-pure cardiomyocyte preparation, cells were incubated in Dulbecco’s modified Eagle’s medium with Ham’s F-12 supplemented with 1.2 g/liter sodium hydrocarbon carbonate, 10% (v/v) NBS containing 1 μM pyruvic acid, 1 μM glucose, 10 μg/ml insulin, 10 ng/ml sodium selenite, 250 μg/ml vitamin C, 100 μg/ml 5-bromo-2-deoxyuridine, 100 units/ml penicillin G, and 100 μg/ml streptomycin. This medium was replaced with medium containing NBS at 48 h. The cells were incubated for 24 h before addition of the test compounds.

Treatment of Cells with PMA—On day 4 following the isolation, cardiomyocytes grown in 25-cm² flasks were rinsed twice with PBS. PMA was added to medium without NBS and incubated with cells for 6 h. For negative controls, cardiomyocytes were incubated with medium alone. Following treatment the medium was removed and cells were washed with PBS and medium containing COX inhibitors were then added: 200 μM aspirin for 30 min or 1 μM NS-398 for 45 min. All cells were then washed twice with PBS and 2 ml of HBSS was added containing 50 μM arachidonic acid (peroxide free) for 10 min to measure the maximum amount of prostaglandin formed. The supernatants were stored at −70°C for PG analysis.

Treatment of Cells with Hydrogen Peroxide and Doxorubicin—On day 4 following isolation, the cardiomyocytes were rinsed twice with PBS. H₂O₂, 50 μM and 0.5 mM, or 100 μM/dX (172 μM) was added to medium without NBS and incubated with cells for 10 or 80 min, respectively. For negative controls cardiomyocytes were incubated with medium alone for equivalent amounts of time. Oxygen-free radical scavengers PEG-CAT (200 units/ml) and PEG-superoxide dismutase (200 units/ml) were added to cells 1 h before treating with DX. The medium was then removed, the cells washed with PBS and fresh medium added to allow cells recover for 10 min, 3 h, and 10 h. At these time points medium was removed and stored at −70°C for LDH analysis. The cells were then washed with PBS and medium containing COX inhibitors were added: 200 μM aspirin for 30 min or 1 μM NS-398 for 45 min. All cells were then washed twice with PBS and 2 ml of HBSS was added containing 50 μM arachidonic acid (peroxide free) for 10 min to measure the maximum amount of PG formed. This HBSS was stored at −70°C for analysis. The cells were then washed twice with PBS and either lysed in protein 1% Triton X-100 lysis buffer and stored at −20°C for Western blot analysis or scraped into Tri-Reagent and stored at −70°C for RNA isolation. In further experiments cells were treated with 1 μM NS-398 alone, or 10 μg/ml DX (17.2 μM) with or without 1 μM NS-398, or with 10 μg/ml DX in the presence of 200 units/ml PEG-CAT and 200 units/ml PEG-superoxide dismutase for 24 h. As above the medium was removed and stored at −70°C for LDH analysis, the cells washed with PBS and 2 ml of HBSS was added containing 50 μM arachidonic acid for 10 min. The HBSS was stored at −70°C for PG analysis. The cells were washed and scraped into Tri-Reagent and stored at −70°C for mRNA analysis.

Treatment of Cells with Iloprost, or Fluprostenol, followed by 0.5 mM H₂O₂ or 100 μg/ml Doxorubicin—Cells were treated with 30 nM iloprost alone, or with 1–100 nM iloprost, or medium alone (positive control) for 24 h, before treating with 0.5 mM H₂O₂ or 100 μg/ml DX, and LDH released into medium was measured. Cells were also treated both with the PGE₂α analog, 30 μM fluprostenol, or with 0.5 mM H₂O₂ or 100 μg/ml DX, and LDH released into medium was measured. The cells were subsequently scraped into Tri-Reagent for COX-2 mRNA analysis.

Treatment of Cells with H₂O₂ or Doxorubicin and the ERK Inhibitor PD098059 or the p38 MAP Kinase Inhibitor SB203580—Cells were pretreated with a denatured 50 μM PD098059 or 20 μM SB203580 for 1 h, before being exposed to 50 μM H₂O₂ or 100 μg/ml DX with PD098059 or SB203580 for 10 or 80 min, respectively. Following exposure cells were allowed to recover in serum-free medium with 50 μM PD098059 or 20 μM SB203580 for 3 h. As a positive control cardiomyocytes were incubated with 50 μM H₂O₂ or 100 μg/ml DX alone, for 10 or 80 min, respectively, followed by a 3-h incubation of cells in serum-free medium. As discussed above, all cells were washed with PBS and 2 ml of HBSS was added containing 50 μM arachidonic acid for 10 min. The HBSS was stored at −70°C for PG analysis. The cells were washed and either lysed in protein lysis buffer or scraped into Tri-Reagent for mRNA analysis.

ERK1/2 Activation by H₂O₂ and Doxorubicin—Cells were treated with 50 μM H₂O₂ or 100 μg/ml DX and 50 μM PD098059 or 20 μM SB203580 for 10 min, before being washed with PBS and ERK1/2 activity was measured using p44/p42 MAP kinase assay kit.

Transcription Factor Decays and COX-2 Expression—Transcription factor decay oligomers were synthesized by a method previously described by Schmedtje et al. (27) in vitro by annealing the complementary strands in a 1× annealing buffer (20 μM Tris-HCl, pH 7.5, 20 μM MgCl₂, and 50 μM NaCl). The mixture was heated to 80°C and allowed to cool to room temperature slowly over 3 h. The oligomers were designed from two NF-κB binding regions and one AP-1-like binding region on the rat COX-2 promoter. The NF-κB sequences used were, the distal site to the COX-2 promoter, (NF-κB-1) -413 5′-GGAG-GGCGGGGTTCCCTTAGTGGGAC-3′ – 386 and the proximal site to the COX-2 promoter, (NF-κB-2) -98 5′-GGGGGTTGGGAAACGGCAG-GGCGAAA-3′ – 72. The AP-1-like sequence was -173 5′-TACTTT-GGTGTGAATTCGCGTG-3′ – 149. A double-stranded DNA with a scrambled sequence (5′-CAGAGGATCTCTGCGATATCG-3′) was placed in the medium 30 min prior to co-exposure to the NF-κB and AP-1-like elements was used as a control in the decay experiments. Decay naked double-stranded DNA was placed in the medium at a concentration of 20 μM 1 h before exposing the cells to COX-2 promoter, (NF-κB-2) -98 5′-GGGGGTTGGGAAACGGCAG-GGCGAAA-3′ – 72. The double-stranded DNA and the scrambled DNA were added to medium at 20 μM, enabling sequestration of the transcription factor in the cytoplasm. As discussed under “Treatment of Cells with Hydrogen Peroxide and Doxorubicin,” all cells were washed with PBS and 2 ml of HBSS was added containing 50 μM arachidonic acid for 10 min. The HBSS was stored at −70°C for PG analysis. The cells were washed and...
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Reverse Transcription Polymerase Chain Reaction (PCR)—This procedure was used to compare the expression of COX-1 and COX-2 mRNAs between untreated and treated cells. Each pair of sense and antisense primers were designed to span at least one intron of the gene to exclude contaminating genomic DNA. Primers used were: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) 5' −AACCCATCACCAT-3', internal control.

For PCR, RNA samples of 1 μg were denatured at 65 °C for 10 min. 100 ng of random hexamers, 1 μM deoxyribonucleoside triphosphates, 0.5 μM of each sense and antisense primer, and 1 unit of Moloney murine leukemia virus reverse transcriptase were added and incubated at 37 °C overnight in a reaction volume of 20 μl. The reverse transcription reaction was stopped by heating to 95 °C for 5 min. A 1:10 volume of the generated cDNA reaction was used in the subsequent amplification reaction. PCR was performed in a 50-μl reaction with 1.5 μM magnesium chloride, 1 μM deoxynucleoside triphosphates, 0.5 μM of each sense and antisense primer, and 1 unit of Taq polymerase. The reaction cycles were denaturing at 94 °C for 1 min, annealing at 56 °C for GAPDH and COX-2 for 1 min, annealing at 52 °C for COX-1 for 1 min, and extension at 72 °C for 1 min. PCR cycle profiles for GAPDH, COX-1, and COX-2 were performed. Products were run on agarose gels (1.2%) with PGEM DNA markers. Polaroid instant photos were taken of the gels with a Polaroid DS34 direct screen instant camera. Expression of COX-1 and COX-2 were normalized with respect to GAPDH from parallel samples. In order to verify that the band produced at the correct size was rat COX-2 cDNA, this band was excised from gel, purified, cloned into PCR 2.1 vector, and sequenced.

Western Blotting—Lysates were prepared by treating cells with lysis buffer. Protein concentration was measured using a protein Bio-Rad assay, according to the manufacturer’s procedure. Lysate samples (20 μg of protein/lane) were applied to 10% SDS-polyacrylamide minigels, and 200 units of Moloney murine leukemia virus reverse transcriptase, and 200 units of Moloney murine leukemia virus reverse transcriptase, and 200 units of Moloney murine leukemia virus reverse transcriptase, were added and incubated at 37 °C overnight in a reaction volume of 20 μl. The reverse transcription reaction was stopped by heating to 95 °C for 5 min. A 1:10 volume of the generated cDNA reaction was used in the subsequent amplification reaction. PCR was performed in a 50-μl reaction with 1.5 μM magnesium chloride, 1 μM deoxynucleoside triphosphates, 0.5 μM of each sense and antisense primer, and 1 unit of Taq polymerase. The reaction cycles were denaturing at 94 °C for 1 min, annealing at 56 °C for GAPDH and COX-2 for 1 min, annealing at 52 °C for COX-1 for 1 min, and extension at 72 °C for 1 min. PCR cycle profiles for GAPDH, COX-1, and COX-2 were performed. Products were run on agarose gels (1.2%) with PGEM DNA markers. Polaroid instant photos were taken of the gels with a Polaroid DS34 direct screen instant camera. Expression of COX-1 and COX-2 were normalized with respect to GAPDH from parallel samples. In order to verify that the band produced at the correct size was rat COX-2 cDNA, this band was excised from gel, purified, cloned into PCR 2.1 vector, and sequenced (ABI Prism 310 Genetic Analyzer, Perkin-Elmer).

Western Blotting—Lysates were prepared by treating cells with lysis buffer. Protein concentration was measured using a protein Bio-Rad assay, according to the manufacturer’s procedure. Lysate samples (20 μg of protein/lane) were applied to 10% SDS-polyacrylamide minigels, electrophoresed, and transferred to nitrocellulose membranes overnight at 40 V constant voltage at 4 °C with use of a Western blot transfer apparatus. Immunostaining for COX expression was performed with a goat polyclonal anti-COX-1 antibody and a mouse monoclonal anti-COX-2 antibody, (R6), raised against the recombinant full-length mouse (m) COX-2 expressed by baculovirus. R6 antibody is...
significant rise, (*, measured as release of LDH. There was a injury in response to doxorubicin was enhanced significantly (\(p < 0.005\)), however, NS-398 en-

NS-398 alone over the 24-h period did not affect LDH release, however, NS-398 en-

hanced significantly (\(p < 0.005\)). C, cell injury in response to doxorubicin was measured as release of LDH. There was a significant rise, (\(p < 0.005\)) the re-

lease of LDH by doxorubicin.}

**PEG-CAT (**) enhance levels which was prevented by

a selective COX-2 inhibitor NS-398, aspirin, and PEG-superoxide dismutase and

PEG-CAT (\(p < 0.05\), \(p < 0.005\)). Treatment of cells with the selective COX-2 inhibitor

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pre-absorbed with COX-2 (21 \(\mu \text{M}\)) but not COX-1 (25 \(\mu \text{M}\)) (28). Immunostaining for ERK1/2 was performed with mouse monoclonal antibod-

eyes and developed with a horseradish peroxidase Western blot detection kit.

**LDH Assay**—Cytotoxicity was assessed by measuring LDH in the culture medium spectrophotometrically. The LDH release was standard-

ized with a cell injury index defined at \((A - B)/(C - B)\)

where \(A = \text{LDH activity in the test sample}\), \(B = \text{LDH activity measured in media with no cells (0% control)}\), and \(C = \text{LDH activity in samples from wells in which cells were lysed with Triton X-100 (100% control)}\).

**Determination of Eicosanoids by Gas Chromatography/Mass Spectrometry (GC/MS)**—GC/MS was used to examine the range of PG forma-

tion at 24 h (†, \(p < 0.05\) versus control) that was abolished by the selective COX-2 inhibitor NS-398, aspirin, and PEG-superoxide dismutase and PEG-CAT (\(p < 0.05\), \(p < 0.005\)).

Treatment of cells with the selective COX-2 inhibitor NS-398 alone over the 24-h period did not affect LDH release, however, NS-398 en-

hanced significantly (\(p < 0.05\)) the release of LDH by doxorubicin.

A

**expression and product formation in rat neonatal cardiomyocytes.** Morphological examination on the third day following isolation showed a synchronous and rhyth-

mically contracting monolayer of cardiomyocytes. GC/MS analysis showed that the primary prostaglandin product in car-

domyocytes is prostacyclin \(\rightarrow \text{PGF}_2\alpha > \text{PGF}_2\beta > \text{TXB}_2\) (Table 1). Similar levels of PG formation by cardiomyocytes have been reported (29). Formation of both 6-keto-PGF\(_{1\alpha}\) and PGE\(_2\) was significantly enhanced in response to PMA but neither PGF\(_{2\alpha}\) nor \(\text{TXB}_2\) were altered (Table 1). Selective inhibition of COX-2 activity by 1 \(\mu \text{M}\) NS-398 specifically abolished the PMA-in-

duced increase in 6-keto-PGF\(_{1\alpha}\) and PGE\(_2\), demonstrating that the increase in these PGs was COX-2-dependent. Inhibition of
COX activity by 200 μM aspirin prevented TXB₂ generation, showing that its generation was COX-1-dependent. PGF₂ₐ appears to be generated non-enzymatically.

H₂O₂-induced COX-2 Expression at 3 h—Cardiomyocytes were exposed to 50 μM H₂O₂ for 10 min which did not effect cell viability. RT-PCR analysis (Fig. 1A) showed that H₂O₂-induced COX-2 expression at 3 h did not affect mRNA levels of COX-1 or the housekeeping gene, GAPDH. There was a corresponding increase in COX-2 protein, whereas the expression of COX-1 did not change (Fig. 1B). H₂O₂ also induced 6-keto-PGF₁α production at 3 h, with levels returning to baseline by 10 h (Fig. 1C), as did the expression of COX-2 protein and mRNA. 1 μM NS-398 had no effect on product formation at baseline or at 10 min of recovery, but abolished the increase in 6-keto-PGF₁α at 3 h, demonstrating that this increase was COX-2-dependent. NS-398 did not effect the expression of COX-2 message and was therefore only inhibiting product activity (data not shown). Aspirin, a non-selective COX inhibitor, reduced the amount of 6-keto-PGF₁α formed at all time points.

Doxorubicin Induces COX-2 Expression Due to Free Radical Formation—Addition of 10 or 100 μg/ml doxorubicin to cells resulted in a marked and rapid reduction in their rate of contraction. Once doxorubicin was removed, however, and cells were allowed to recover for 20 min, a normal rate of contraction was restored. Addition of 100 μg/ml doxorubicin to cells for 80 min, followed by a 3-h recovery, did not cause cells to apoptose appreciably. However, 10 μg/ml doxorubicin over 24 h induced cell shrinkage, nuclear condensation, formation of apoptotic bodies, and blebs in the cell membrane in about 10% of cells. 100 μg/ml doxorubicin induced a time-dependent change in COX-2 mRNA levels (Fig. 2A), detectable after 10 min and peaking after 3 h recovery, without any change in COX-1 or GAPDH mRNA levels. 200 units/ml PEG-superoxide dismutase and PEG-CAT prevented COX-2 expression, demonstrating a role for free radicals in doxorubicin-mediated COX-2 induction. Doxorubicin (100 μg/ml) also caused an increase in 6-keto-PGF₁α formation after 3 h (Fig. 2B). This increase in 6-keto-PGF₁α was abolished by 1 μM NS-398 without preventing the expression of COX-2, demonstrating that the increase in product formation was COX-2-dependent. The increase in
6-keto-PGF$_{1x}$ was also abolished by pretreatment with 200 units/ml PEG-superoxide dismutase and PEG-CAT.

The release of LDH into cell medium was measured as a marker of cell injury (Fig. 2C). Following the initial 80-min exposure of cells to 100 μg/ml doxorubicin, there was no detectable rise in LDH at 10 min. After 3 h, there was a significant increase indicating that cell injury had occurred. There was no further increase in LDH released from 3 to 10 h. The increase in LDH release was suppressed by pretreatment with PEG-superoxide dismutase and PEG-CAT. PEG-superoxide dismutase alone increased the amount of LDH released, demonstrating the role of the hydroxyl radical in doxorubicin-mediated cell injury.

The lower concentration of 10 μg/ml doxorubicin also induced COX-2, but at a later time point. Doxorubicin (10 μg/ml) induced COX-2 message at 24 h without affecting mRNA levels of COX-1 or GAPDH. Doxorubicin (10 μg/ml) also caused an increase in COX-2-dependent 6-keto-PGF$_{1x}$ formation (Fig. 3, A and B). Again PEG-superoxide dismutase and PEG-CAT prevented COX-2 induction and product formation, suggesting a free radical-dependent mechanism. At this low drug concentration a continual accumulation of free radical metabolites of doxorubicin may be responsible for the COX-2 induction. It is also possible, however, that doxorubicin at this low concentration causes a steady increase in ceramide production by the cardiomyocyte (26) which is reported to induce apoptosis and mitochondrial H$_2$O$_2$ production (27).

10 μg/ml doxorubicin also caused the release of LDH which was prevented by PEG-superoxide dismutase and PEG-CAT (Fig. 3C). The COX-2 inhibitor NS-398 (1 μM) alone did not increase the release of LDH above that of controls. The release of LDH, however, was aggravated by 1 μM NS-398 (Fig. 3C), suggesting that a COX-2-dependent product protects the cells from injury.

Iloprost Protects Cardiomyocytes from Free Radical-induced Damage—Since COX-2-dependent PGs protect cardiomyocytes from injury, we examined the effects of iloprost (a stable analog of prostacyclin) on LDH release by cells exposed to high concentrations of H$_2$O$_2$ (as 50 μM H$_2$O$_2$ did not cause a substantial increase in LDH release following treatment). 30 μM iloprost reduced the amount of LDH released by H$_2$O$_2$ and doxorubicin-induced cell damage (Fig. 4, A and B). In contrast, an analog of PGF$_{2α}$, 30 μM fluoprostenol, did not prevent LDH release from cardiomyocytes, demonstrating that the reduction in LDH release was specific for iloprost.
Cyclooxygenase-2 has been identified recently in human cardiomyocytes in areas of myocardial infarction and from individuals with dilated cardiomyopathy, whereas no COX-2 was found in normal hearts (17). The mechanism for the induction of COX-2 in cardiomyocytes is unknown. In addition to growth factors and cytokines, shear stress, hypoxia, calcium ionophore, angiotensin II, bradykinin, thrombin, factors and cytokines, shear stress, hypoxia, calcium ionophore, angiotensin II, bradykinin, thrombin, 

Oxidant stress may also induce COX-2 (12). Feng et al. (12) showed that the inhibition of prostaglandin synthesis in rat mesangial cells by free radical scavengers was due to the suppression of COX-2 expression.

As in the human cardiomyocyte, COX-1 was the only isoform expressed in resting rat cardiomyocytes while COX-2 was induced by treatment with a phorbol ester. COX-2 was also induced by two oxidants, doxorubicin at a therapeutically relevant concentration, and brief exposure to a relatively low concentration of H₂O₂. Doxorubicin is an anthracycline used as a chemotherapeutic agent and is well recognized to induce a chemotherapeutic agent and is well recognized to induce a
dilated cardiomyopathy (36). A hydroxyl radical originating from a superoxide radical has been implicated in l

The increase was abolished by the AP-1 double-stranded decoy oligomer and reduced by the proximalNF-κB-2 double-stranded decoy oligomer (*, p < 0.05).

DISCUSSION

Cyclooxygenase-2 has been identified recently in human cardiomyocytes in areas of myocardial infarction and from individuals with dilated cardiomyopathy, whereas no COX-2 was found in normal hearts (17). The mechanism for the induction of COX-2 in cardiomyocytes is unknown. In addition to growth factors and cytokines, shear stress, hypoxia, calcium ionophore, bradykinin, thrombin, and angiotensin II (30–35) induce COX-2 expression. Oxidant stress may also induce COX-2 (12). Feng et al. (12) showed that the inhibition of prostaglandin synthesis in rat mesangial cells by free radical scavengers was due to the suppression of COX-2 expression.

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dilated cardiomyopathy (36). A hydroxyl radical originating from a superoxide radical has been implicated in anthracycline-mediated cardiotoxicity (7, 8). Alternatively, toxicity may be due to accumulation of ceramide, a lipid formed when cells are exposed to anthracyclines and which may induce mitochondrial H₂O₂ production (37). Doxorubicin also induced toxicity in cultured cardiomyocytes detected as release of LDH. Both the expression of COX-2 and the release of LDH were abolished by the combination of superoxide dismutase and catalase confirming a free radical-dependent mechanism.

Oxygen-free radicals have indiscriminate effects on a variety of cellular targets including lipids, proteins, and oligonucleotides, resulting in cell death. At lower concentrations, however, oxygen-free radicals activate very specific pathways that in turn regulate the expression of genes influencing cell survival. Exogenous free radicals have been shown to activate p38 MAP kinase in several cell systems and less often to activate ERKs in these cells prevented H₂O₂-induced apoptosis (38) reported that free radical-induced activation of ERKs promotes cell survival, whereas activation of JNK and p38 MAPK induce apoptosis. Similarly, ceramide-induced activation of JNK is important for the induction of apoptosis (39). These results suggest that ERKs have a protective role, while activation of p38 MAPK/JNK leads to induction of apoptotic death on exposure to free radicals. The pattern of MAP kinase activation in response to oxidant stress varies between cells even in the same species. In rat vascular smooth muscle cells free radicals activate p38 MAP kinase but not ERK1/2 (40). In contrast, in rat neonatal cardiomyocytes H₂O₂ specifically activates ERKs through activation of the Src family of tyrosine kinases, Ras and RAF-1 (23). The activation of ERKs in these cells prevented H₂O₂-induced apoptosis al-
though the mechanism was not identified. Similarly in our studies ERK1/2 was activated by H₂O₂ and doxorubicin and was responsible for the activation of COX-2. Thus, PD0908059, which prevents MEK-dependent activation of ERK1/2, abolished the expression of COX-2 mRNA and protein and the increase in prostaglandin formation. In contrast, specific inhibition of p38 MAP kinase had no effect.

The Src family of tyrosine kinases has been implicated in the induction of COX-2 in response to cytokines and growth factors, but not specifically the ERK kinase (41). The ERK1/2 kinase pathway, however, has been identified as the common signaling pathway of serotonin or phosphor ester-induced COX-2 expression in rat mesangial cells (42) and both ERK kinase and MAPK p38 have been shown to play a role in lysophosphatidic acid-mediated COX-2 expression by these cells (43). To identify how ERK1/2 induced COX-2 in cardiomyocytes we examined three putative sites on the promoter of the rat COX-2 gene. Two of the sites are homologous to the NF-κB element, which is also found in the promoters of human inflammatory genes, including the human COX-2 gene. The third, the AP-1-like site is similar to a promoter element common to early response genes. In high concentration, H₂O₂ induces translocation of NF-κB to the nucleus and subsequent gene expression in an endothelial cell line (27). This is by no means a ubiquitous event and in some cells there is gene induction in response to H₂O₂ without NF-κB translocation (27). Here, we show that AP-1 is the major promoter element involved, as a decoy promoter AP-1 sequence abolished COX-2 induction. ERK1/2 may induce c-Fos expression which complexes in a heterodimer with c-Jun forming the transcription factor that acts through AP-1. This is thought to explain interleukin-1β induction of nitric oxide in rat pancreatic islet cells, which is also ERK1/2 dependent (44). Moreover, increased activation of ERKs and of the AP-1 promoter has been shown in proliferating smooth muscle cells (45, 46). A double-stranded oligonucleotide decoy identical to the proximal NF-κB site showed that this site was also partially active, whereas the distal NF-κB site was inactive. These findings implicate both the proximal NF-κB and the AP-1 site, but suggest that the latter is the critical promoter element. It is worth noting that both AP-1 and NF-κB are activated by H₂O₂ in neonatal cardiomyocytes (47) and that intracellular oxidants may be the ultimate messengers common to all signals that activate AP-1 and NF-κB (48).

H₂O₂ and DX induced the release of LDH from cardiomyocytes, and the selective COX-2 inhibitor NS-398 potentiated this. In contrast, treating the cells with NS-398 alone did not affect the basal levels of LDH release. The injury response was prevented by iloprost, a stable analogue of the principle cyclooxygenase product of these cells, prostacyclin. The response to iloprost appeared to be specific as it was dose dependent and no protection was seen with fluprostenol, a stable analogue of prostacyclin. The response to iloprost was seen in cultured rat mesangial cells (42) and both ERK kinase and MAPK p38 have been shown to play a role in lysophosphatidic acid-mediated COX-2 expression by these cells (43). To identify how ERK1/2 induced COX-2 in cardiomyocytes we examined three putative sites on the promoter of the rat COX-2 gene. Two of the sites are homologous to the NF-κB element, which is also found in the promoters of human inflammatory genes, including the human COX-2 gene. The third, the AP-1-like site is similar to a promoter element common to early response genes. In high concentration, H₂O₂ induces translocation of NF-κB to the nucleus and subsequent gene expression in an endothelial cell line (27). This is by no means a ubiquitous event and in some cells there is gene induction in response to H₂O₂ without NF-κB translocation (27). Here, we show that AP-1 is the major promoter element involved, as a decoy promoter AP-1 sequence abolished COX-2 induction. ERK1/2 may induce c-Fos expression which complexes in a heterodimer with c-Jun forming the transcription factor that acts through AP-1. This is thought to explain interleukin-1β induction of nitric oxide in rat pancreatic islet cells, which is also ERK1/2 dependent (44). Moreover, increased activation of ERKs and of the AP-1 promoter has been shown in proliferating smooth muscle cells (45, 46). A double-stranded oligonucleotide decoy identical to the proximal NF-κB site showed that this site was also partially active, whereas the distal NF-κB site was inactive. These findings implicate both the proximal NF-κB and the AP-1 site, but suggest that the latter is the critical promoter element. It is worth noting that both AP-1 and NF-κB are activated by H₂O₂ in neonatal cardiomyocytes (47) and that intracellular oxidants may be the ultimate messengers common to all signals that activate AP-1 and NF-κB (48).

Adaptive responses of bacteria to the potential toxic effects of partially reduced oxygen metabolites (H₂O₂, •OH, and O₂⁻) include the transcriptional regulation of oxidative stress genes (57). Similar mechanisms exist in eukaryotic cells where reactive oxygen species cause expression of several enzymes involved in oxidant metabolism such as the human glutathione peroxidase gene (58, 59). The induction of COX-2 in cardiomyocytes may represent an additional example of an adaptive response that protects the cell from oxidant stress.

Acknowledgments—We thank Dr. Isakson for the generous gift of the mouse monoclonal anti-mouse COX-2 antibody (R6) and Louise Cullen, Mary-Rose Kenealy, Anne O’Neill, Theresa Keane, and Brendan Har-ken for technical assistance and advice.

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J. Biol. Chem. 1999, 274:5038-5046.
doi: 10.1074/jbc.274.8.5038

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