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Inhibition of Apoptosis by Progesterone in Cardiomyocytes

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Summary

While gender-based differences in heart disease have raised the possibility that estrogen (ES) or progesterone (PG) may have cardioprotective effects, recent controversy regarding hormone replacement therapy has questioned the cardiac effects of these steroids. Using cardiomyocytes, we tested whether ES or PG has protective effects at the cellular level. We found that PG but not ES protects cardiomyocytes from apoptotic cell death induced by doxorubicin (Dox). PG inhibited apoptosis in a dose dependent manner, by 12 ± 4.0% at 1 μM and 60 ± 1.0 % at 10 μM. The anti-apoptotic effect of PG was also time dependent, causing 18 ± 5% or 62 + 2% decrease in caspase-3 activity within 1 or 72 hours of pretreatment. While PG causes nuclear translocation of its receptor within 20 mins, the cytoprotective effect of PG was cancelled by mifepristone (MF), a PG receptor antagonist. Analyses using Affymetrix high-density oligonucleotide array and RT-PCR found that PG induced Bcl-xL, metallothionine, NADPH quinone oxidoreductase 1, glutathione peroxidase-3, and 4 isoforms of glutathione S-transferase. Western blot analyses revealed that PG indeed induced an elevation of Bcl-xL protein in a dose and time dependent manner. Nuclear run-on assay indicated that PG induced Bcl-xL gene transcription. Inhibiting the expression of Bcl-xL using siRNA reduced the cytoprotective effect of PG. Our data suggests that PG induces a cytoprotective effect in cardiomyocytes in association with induction of Bcl-xL gene.

Keywords

Cytoprotection; nuclear receptor; gene expression; antioxidant; oxidative stress; Bcl-xL

Introduction

Gender-based differences in heart disease have raised the possibility that estrogen (ES) or progesterone (PG) have cardioprotective effects. Women tend to develop heart disease later than men (Low *et al.* 2002). Also, the duration between the onset of symptoms and heart failure is longer for women than men. Many studies including the Framingham Heart Study have shown an increased risk of heart disease in postmenopausal women relative to their age matched cycling counterparts (Murabito 1995; Wexler 1999). While these studies have lead to the assumption that supplementation of ES and PG in postmenopausal women may be beneficial for heart health, recent controversies regarding hormone replacement therapy in

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postmenopausal women have raised questions about whether ES or PG is indeed cardiac protective. While the effect of ES on the heart and cardiovascular system has been studied extensively, little is known about the cardiovascular effect of PG.

ES or PG binds to its receptor in the cytosol, causing nuclear translocation of the receptor. The PG receptor (PR) is present in cardiac muscle of a variety of mammalian species including the human (Lin *et al.* 1982; Ingegno *et al.* 1988; Grohe *et al.* 1997; Pasanen *et al.* 1997; Knowlton & Sun 2001; Goldstein *et al.* 2004). Administration of PG has been shown to induce a number of physiologic effects including increasing cardiac output (Kuebler *et al.* 2003), decreasing arterial pressure (Pecins-Thompson & Keller-Wood 1997; Roesch & Keller-Wood 1997), dilating coronary arteries (Minshall *et al.* 2002), induction of angiotensin I (Nickenig *et al.* 2000), and cardiac muscle protein synthesis (Goldstein *et al.* 2004). During pregnancy, physiological levels of ES and PG increase significantly along with a concomitant increase in cardiac output in addition to increases in blood volume. Although the PR is expressed in cardiac muscle, the molecular action of PG or PR remains largely undefined in cardiomyocytes.

The PR is encoded by a single gene that produces two isoforms, A and B, from alternative promoters (Graham & Clarke 1997; Conneely *et al.* 2002). The A isoform is essentially a truncated version of the B isoform and lacks the amino-terminal 128 amino acids of PR-B (Weigel *et al.* 1995). Both isoforms can bind to PG and change conformation upon ligand binding. The receptors then dimerize (A-A, B-B or A-B) and bind to hormone response elements (HRE) in the promoter regions of target genes. In addition, PRs also interact with co-activators, co-repressors and transcription factors (Weigel *et al.* 1995; Graham & Clarke 1997; Conneely *et al.* 2002). The ratio of PR-A versus PR-B differs by tissues and contributes to the difference in downstream genes regulated by PG (Richer *et al.* 2002; Smid-Koopman *et al.* 2003).

Many cardiac protective agents act to prevent death of cardiomyocytes from damaging agents. Doxorubicin (Dox) is an anthracycline chemotherapeutic agent currently used for the treatment of many neoplastic conditions such as lymphoma, leukemia, breast, ovarian, gastric, thyroid and bronchogenic carcinomas. The effectiveness of this drug is limited due to its dose dependent cardiac toxicity (Keizer *et al.* 1990; Singal & Iliskovic 1998; Singal *et al.* 2000). The administration protocol has been revised to reduce acute cardiac toxicity. However, chronic cardiac toxicity usually develops 2–10 years after drug administration as patients start to show signs of dilated cardiomyopathy. This toxicity is associated with Dox induced oxidative radicals, since Dox can accept electrons from oxoreductive enzymes in the mitochondria to form semiquinone free radicals, which can initiate a chain of redox reactions (Keizer *et al.* 1990; Singal & Iliskovic 1998; Singal *et al.* 2000). Dox has been shown to produce superoxide and H_2O_2 when incubated with the mitochondrial fraction of cardiac tissue extracts (Doroshow & Davies 1986). In addition to inducing oxidative stress, Dox at high concentrations can interact with DNA topoisomerase and cause DNA strand breaks (Gewirtz 1999). At the cellular level, Dox has often been used as a model compound for inducing apoptosis in a number of experimental systems including cardiomyocytes (Kumar *et al.* 1999; Arola *et al.* 2000; Kalyanaraman *et al.* 2002; Ueno *et al.* 2006; Bruynzeel *et al.* 2007).

Apoptosis is known to be an important pathological event in cardiac injury and heart failure. Recent studies have shown the presence of apoptosis in biopsy samples of failing human hearts and in the myocardium of experimental animals (Narula *et al.* 1996; Anversa *et al.* 1997; Bialik *et al.* 1997; Condorelli *et al.* 1999; Webster *et al.* 1999). There is evidence that inhibiting apoptosis may alleviate certain clinical manifestation of heart failure in experimental animals (Feuerstein *et al.* 1998; Ma *et al.* 1999; Kotamraju *et al.* 2000). Therefore agents protect cells from apoptosis may exhibit therapeutic potential to prevent cardiac injury and heart failure.

Results

Progesterone Inhibits Apoptosis Induced by Dox

Cardiomyocytes are adherent in culture but round up, detach and show Annexin V binding when undergo apoptosis. To test the effect of PG, cardiomyocytes were pretreated with PG for 24 hours before being placed in fresh medium for Dox treatment. With PG pretreatment, inhibition of apoptosis was observed through morphology and annexin V binding (Fig. 1A&B). Under normal culture conditions, cardiomyocytes beat synchronally in clusters at a frequency of 27.2 ± 1.7 beats per minute. PG treatment did not affect the beating frequency. By 24 hrs of Dox treatment, a fraction of cells undergo apoptosis while remaining cells survived but could no longer beat. With PG pretreatment, surviving cells beat at a frequency of 17.6 ± 2.9 beats per minute.

To quantify the inhibitory effect of PG on Dox induced apoptosis, we measured caspase-3 activity. Dox causes a dose dependent increase of caspase-3 activity in primary cardiomyocytes (Chen *et al.* 2005). When pretreated with PG at various doses before 0.8 μM Dox treatment, PG at 1 or 10 μM reduced caspase activity by 12 ± 4% ($p<0.05$) or 60 ± 1% ($p<0.005$) respectively (Fig. 2A). The inhibitory effect was dependent on how long cells being pretreated with PG. A decrease in caspase-3 activity was evident with 1 hr (18 ± 5% inhibition, $p<0.05$) pretreatment with 10 μM PG and was maximal with 72 hours of PG pretreatment (62 ± 2% inhibition, $p<0.005$, Fig. 2B). In addition to primary cultured cardiomyocytes, PG showed a protective effect in H9C2 rat cardiomyocyte cell line (data not shown).

A number of studies suggest that ES can be cytoprotective. We determined whether ES can prevent Dox from inducing apoptosis of cardiomyocytes. Cardiomyocytes were pretreated for 24 hours with doses of 17β–estradiol varying from 1 to 50 μM before treatment with Dox. No protective effect was observed with any of the dose of 17β-estrodial tested (data not shown). Male sex hormone testosterone (TS) did not show significant protective effect (Fig 2E). To determine if there was a synergistic effect of ES or TS with PG, we pretreated cells with ES or TS with PG. The results show that ES or TS did not potentiate the inhibitory effect of PG (Fig. 2D, E).

Dependence on Receptor of PG Induced Cytoprotection

PG can diffuse through the plasma membrane and binds to its receptor in the cytosol, causing nuclear translocation. To verify that the PG receptor is indeed expressed in

cardiomyocytes, hearts from neonatal rats, adult male or female rats, and adult male or female mice were used for Western blot analyses. Rat uterus tissue was included as a positive control. Neonatal rat cardiomyocytes express both A and B isoforms of PR, while adult mice or rat hearts express mainly the A isoform (Fig. 3A). The H9C2 rat cardiomyocyte cell line contains the B isoform of PR (Fig. 3A). Since PG shows a protective effect in H9C2 cardiomyocytes, the B isoform likely plays a critical role in cytoprotection.

To determine if PG treatment causes activation of PR in cardiomyocytes, nuclear translocation was measured after cells were incubated with PG for various time points from 15 mins to 72 hours. The cells were harvested for isolation of cytoplasmic and nuclear fractions. The results show that PG indeed induces PR-B translocation in cardiomyocytes detectable 20 mins after PG exposure (Fig. 3B).

The cytoprotective effect of PG was eliminated by mifepristone (MF), a PR antagonist, as measured by caspase-3 activity (Fig. 4A). The ability of MF in canceling the cytoprotective effect of PG was confirmed with measurements of caspase-3 cleavage (Fig. 4B) and mitochondrial cytochrome c release (Fig. 4B).

Progesterone Induces Antioxidant and Anti-apoptosis Genes

To understand the mechanism of cytoprotection induced by PG, we utilized Affymetrix gene array to screen the expression of 20,000 genes. RNAs were harvested at 24 hrs after incubating cardiomyocytes with 10 μM PG or vehicle. The results show that PG caused 180 genes and 207 ESTs to be upregulated 1.5 fold or higher ($p<0.05$). Among the long list of genes are the anti-apoptosis gene Bcl-xL and 12 antioxidant/detoxification genes (Table 1 in Supplement). In contrast to these upregulated genes, 73 genes and 123 ESTs showed downregulation at 1.5 fold or lower. The downregulated genes include 8 DNA replication factors and enzymes, 5 cell cycle regulators, 12 cytoskeletal binding or morphogenesis proteins, and 7 cytokines/chemokines or their regulators (Table 2 in Supplement).

To verify the data from microarray, we have chosen 15 genes, 9 of which belong to the category of antioxidant, detoxification and anti-apoptosis genes. With every gene we picked, RT-PCR was able to verify the upregulation in PG treated cells (Fig. 5).

Bcl-xL Expression in PG Induced Cytoprotection

Bcl-xL gene is a prosurvival member of bcl-2 family and was identified by microarray as a PG inducible gene in cardiomyocytes. To characterize PG induced expression of Bcl-xL, cells were treated with PG for 4 hrs to 3 days. Bcl-xL elevation was first detectable at 4 hrs, reached the highest level at 24 hrs, and remained elevated for 3 days (Fig. 6A). In primary cultured cardiomyocytes, a dose dependent increase of Bcl-xL was observed (Fig. 6B). In H9C2 cardiomyocytes, the best dose for inducing Bcl-xL was 0.5 μM (data not shown). MF prevented PG from inducing Bcl-xL expression (Fig. 6C).

To address whether Bcl-xL elevation resulting from transcriptional activation of bcl-x gene, we measured the level of Bcl-xL transcription *in vitro* using nuclear run-on assay. PG treated cells showed a clearly elevated level of Bcl-xL transcription (Fig. 7A). Unlike corticosterone (CT), which induces transcription of COX-2 gene (Sun *et al.* 2008a; Sun *et*

al. 2008b), PG did not cause elevation of COX-2 per microarray analyses, allowing COX-2 to serve as a negative control for *in vitro* transcription assay (Fig. 7A). Measurements of Bcl-xL mRNA decay rate indicate that PG did not cause Bcl-xL mRNA stabilization (Fig. 7B). Our previous studies show that CT induces Bcl-xL and cytoprotection in a Glucocorticoid Receptor (GR) dependent manner (Chen *et al.* 2005). To differentiate PG effect from CT, we measured the GR transactivity using a luciferase reporter construct under the control of Mammary Tumor Virus (MTV) GR cis-element (GRE). While CT activated GRE, PG did not (data not shown). With CT induced Bcl-xL expression, 905 bp bcl-x promoter is activated (Fig. 8A). In contrast, PG did not activate this promoter fragment (Fig. 8A). With 3.2 kb bcl-x promoter, a minor but significant activation was observed with 10 μM PG treatment (Fig. 8B).

To determine whether Bcl-xL induction was necessary or sufficient for the cytoprotective effect of PG, we used siRNA to inhibit the expression of Bcl-xL mRNA. Cells were transfected with a combination of two siRNAs against Bcl-xL. Transfected cells were pretreated with PG for 24 hours before Dox treatment to induce apoptosis. Western blot analyses show that the expression of Bcl-xL was indeed decreased in the siRNA treated samples (Fig. 9A). Caspase-3 activity assay indicated that Bcl-xL siRNAs abolished PG-mediated cytoprotection (Fig. 9B).

Induction of Bcl-xL suggests that PG may inhibit apoptosis from other toxicants. We tested the effect of PG on toxicants such as hydrogen peroxide, rotenone, palmitate, 2-deoxyglucose and glutamate. PG pretreatment significantly decreased caspase-3 activity with any of these toxicants (Fig. 10A&B).

Discussion

This study found that PG decreases apoptotic-like cell death induced by Dox and several toxicants in primary cultures of rat cardiomyocytes. Affymetrix gene array analyses revealed that PG induces the expression of anti-apoptotic gene Bcl-xL. This finding was confirmed by measurements of Bcl-xL protein or mRNA using Western blot or RT-PCR. Blocking Bcl-xL induction by siRNAs results in a reduction in the protective effect of PG, indicating that this gene mediates the observed anti-apoptotic effect of PG. Overexpression of Bcl-xL has been shown to inhibit Dox induced apoptosis in cardiomyocytes (Kunisada *et al.* 2002).

While PG induces apoptosis in various tumor cells, PG has been shown to inhibit apoptosis in several tissue types, including the ovaries (Svensson *et al.* 2001; Okuda *et al.* 2004), uterus (Pecci *et al.* 1997; Kurita *et al.* 2001), breast (Feng *et al.* 1995; Moore *et al.* 2000; Berg *et al.* 2002), and anterior pituitary (Candolfi *et al.* 2005). In our study, the doses of PG necessary to inhibit Dox induced apoptosis, i.e. 1 – 10 μM, are comparable to that required to inhibit TNF-α induced apoptosis in rat lactotropes and somamotropes of the anterior pituitary (Candolfi *et al.* 2005). Cyclical release of PG in females is important for normal cyclical ovulatory function, which involves apoptosis during periods of the endometrial cycle with low PG concentrations (Martin *et al.* 1970; Terada *et al.* 1989; Sato *et al.* 1997). While it is not known why PG induces apoptosis in one cell type while inhibiting apoptosis

in others, induction of Bcl-xL correlates with anti-apoptotic effect of PG in endometrial cells (Pecci *et al.* 1997).

The mechanism of Bcl-xL induction by PG appears to involve transcriptional regulation. The Bcl-xL protein is encoded by bcl-x gene with a complex promoter structure and alternative splicing patterns. The Bcl-xL mRNA is the major transcript of bcl-x gene, which encodes 4 additional mRNA species, bcl-x_s, bcl-x_β, bcl-x_γ and bcl-$x_{\Delta TM}$, due to alternative splicing (Pecci *et al.* 2001). The mouse bcl-x gene has 5 promoters, P1 – P5, and is predicted to produce five mRNA species sharing the same translational start site with various lengths of 5′-untranslated region (5′UTR). P1 – P5 promoter is located from −151, −802, −1886, −2721 and −3412 bp from the translational start site respectively. In mouse mammary epithelial cells, glucocorticoids induce elevation of Bcl-xL mRNA from P4 promoter (Viegas *et al.* 2004). Two hormone response element (HRE)-like sequences have been identified at positions −3040 (TG*g*T*g*TGTCTGTTC*c*) and −3001 (aGcTCTCCAGcACA) upstream of P4 promoter. In addition to HRE binding sites, several cis-elements have been identified for binding of transcription factors such as Sp1, AP-1, Oct-1, Ets, Rel/NF-kB, GATA-1 and STATs within −3.2 kb promoter region of mouse bcl-x gene (Grillot *et al.* 1997). Within −1.2 kb of human bcl-x gene, binding of Rel/NF-kB, Ets, STATs or AP-1 transcription factors mediates transcriptional activation of bcl-x gene encoding Bcl-xL protein (Grad *et al.* 2000). The Sp1 transcription factor has been shown to mediate PG induced gene expression (Owen *et al.* 1998; Hewetson & Chilton 2003; Sriraman *et al.* 2003). PG also upregulates GATA-1 transcription factor and activates AP-1 promoter (Bamberger *et al.* 1996; da Silva Santos Duarte *et al.* 2002). While lack of activation of 905 bcl-x promoter excludes the possibility of some of these transcription factors, the discrepancy between a minor activation of 3.2 kb bcl-x promoter and a clearly elevated bcl-x gene transcription suggests the possibility of additional mechanisms such as sequence upstream of 3.2 kb in the promoter regulating PG induced Bcl-xL expression. Therefore it is possible that multiple pathways may mediate PG induced Bcl-xL gene expression in cardiomyocytes.

Although Bcl-xL plays a critical role in cell survival response, other genes identified from the microarray study may also play a role in PG induced cytoprotection. Several genes induced by PG have been shown to have cytoprotective effects, such as metallothionein I, a metal binding protein, which can function as an antioxidant and protect cardiomyocytes from Dox toxicity (Kang 1999). NADPH Quinone-Oxidoreductase I (NQO1) is able to reduce quinones similar to Dox (Lind *et al.* 1982; Floreani *et al.* 2002). Since Dox causes oxidative stress, induction of GPx3 may also contribute to the cytoprotective effect of PG. In addition to cytoprotective genes, we observed induction of several muscle contractile genes by PG, consistent with a recent report (Goldstein *et al.* 2004). Therefore not only PG can prevent apoptosis, it likely enhances the synthesis of muscle proteins and the contractile function of cardiomyocytes.

Rat cardiomyocytes express A and B-isoforms of PR proteins. As a member of the conserved nuclear receptor family, PR contains an N-terminal region with two transcription activator domains (AF1, AF3), a Zinc finger-containing central DNA binding domain (DBD) and a C-terminal ligand binding domain. Both PR isoforms are able to bind to PG

and undergo conformational change upon ligand binding. Subsequently, the receptors dimerize (AA, AB or BB), can become phosphorylated, bind to the HRE in the promoter regions of target genes, or interact with co-factors or transcription factors (Weigel *et al.* 1995; Graham & Clarke 1997; Conneely *et al.* 2002). PR-A and PR-B may turn on the expression of different sets of genes (Richer *et al.* 2002). The Bcl-xL has been found to be upregulated by PR-A in human breast cancer cells (Richer *et al.* 2002). Since H9C2 cells express PR-B and respond to PG by increasing Bcl-xL expression, PR-B may be a key player in Bcl-xL expression in cardiomyocytes. While the myocardium of adult rats or mice express predominant PR-A, it is not known whether the human expresses both isoforms in the myocardium and whether the ratio of the isoforms changes during growth and development. This type of information is important since PG may provide therapeutic option for treatment against Dox induced cardiomyopathy if PR-B is expressed in the human myocardium.

Experimental Procedures

Cell Culture and Treatment of Drugs

Cardiomyocytes were prepared from 1 to 2 days old neonatal Sprague-Dawley rats (Harland, Indianapolis, IN) as previously described (Chen *et al.* 2000). The myocytes were seeded at a density of 2×10^6 cells per 100 mm dish, 0.3×10^6 cells per well of 6-well plates or 7.5×10^4 cells per well of 24-well plates. Cells were cultured in low glucose DMEM with 1 mM pyruvate, 10% fetal bovine serum, 100 units/ml penicillin and 100 units/ml streptomycin for 3–4 days before experiments. Using this protocol, over 90% of the cells are myocytes at the time of experiments as judged by sarcomeric myosin content. At 3–4 days after plating, cardiomyocytes were cultured in DMEM containing 0.5% FBS for treatment of PG at 10 μM or indicated doses.

Caspase Activity Assay

Detached cells were collected by centrifugation and were combined with adherent cells harvested from the same well in 6-well plates. The combined cells were dissolved in 250 μl of lysis buffer (0.5% Nonidet P-40, 0.5 mM EDTA, 150 mM NaCl, and 50 mM Tris pH 7.5). Cell lysates (50 μl) were incubated 1 hour at 37°C with 40 μM of N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC, Alexis Biochemicals, San Diego, CA) in 100 μl reaction buffer (10 mM HEPES, pH 7.5, 0.05 M NaCl and 2.5 mM DTT). The released AMC was measured using a 96-well fluorescence plate reader (Cambridge Bioresearch Model 7620) with an excitation wavelength of 365 nm and an emission wavelength of 450 nm.

Annexin V Binding Assay

Cells were seeded onto coverglasses in 24-well plates. Detached cells in the supernatant were collected by 5 min centrifugation at 1000 rpm. After washing the detached cells and adherent cells with PBS, detached cells were combined with its corresponding group of cells remained adherent to the coverglass. Annexin V-FLUOS (Roche Applied Science, Indianapolis, IN) was diluted 1: 50 with incubation buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$) and cells were incubated with 25 μl/well labeling solution. The

cells were examined under a Nikon E800M fluorescent microscope. The images were acquired using a Hamamatsu C5180 digital camera with the Adobe Photoshop software.

Western Blot

Cells in 100 mm dishes were lysed by scraping in EB buffer (1% Triton X-100, 10 mM Tris pH 7.4, 5 mM EDTA pH 8.0, 50 mM NaCl, 50 mM NaF, 2 mM Na_3VO_3) for PR or Laemini buffer [0.5 M Tris, pH 6.8, 2.4% (w/v) SDS, 50% (v/v) glycerol] for Bcl-xl measurements. Both types of lysate buffer contain freshly added protease inhibitors, 10 μg/ml aprotinin and 1 mM phenylmethanesulfonyl fluoride. Protein concentration was measured by the Bradford method (Bio-Rad, Richmond, CA), the bicichoninic acid (BCA) method (Pierce, Rockford, IL) or the Warburg-Christian method (Layne 1957). Proteins were separated by SDS polyacrylamide gel electrophoresis using a mini-Protean II electrophoresis apparatus (Bio-Rad, Richmond, CA) and transferred to immobilon-P membranes (Millipore, Bedford, MA) by electrophoresis. The membrane was incubated with primary antibodies and then secondary antibodies conjugated with horseradish peroxidase. The bound antibodies were detected via an enhanced chemiluminescent reaction.

Cytosolic and Mitochondrial Fractionation

The mitochondria and cytosolic fractions were prepared using a fractionation kit as instructed by the manufacturer (BioVision, catalog# K256-100). Briefly, 1×10^7 cells were scraped and washed in PBS, and suspended in 500 μl of cytosol extraction buffer containing 1 mM DTT and 1 tablet of protease inhibitors. After 10 mins incubation on ice, the cells were lysed using an ice cold Dounce homogenizer. The homogenates were examined under a microscope for complete breakage of cells. After 4°C centrifugation at 700 g for 10 mins to remove the nuclei, the supernatant was transferred to a new tube. The cytosolic fraction was collected as the supernatant by centrifugation at 10,000 g for 30 mins at 4°C. The pellet was resuspended in 50 μl of mitochondrial extraction buffer containing DTT and protease inhibitors as mitochondrial fraction.

Microarray

Cardiomyocytes were harvested by Trizol reagent (Sigma, St. Louis, MO) for extraction of total RNA. RNA was cleaned with an RNeasy mini kit (Qiagen). The quality of RNA was examined by agarose gel electrophoresis and the Agilent 2100 bioanalyzer (Agilent Technologies) to ensure the purity and integrity of RNA suitable for microarray. The RNA was converted to cDNA by reverse transcription using a SuperScript Choice kit from Invitrogen with a T7-(dT)24 primer incorporating a T7 RNA polymerase promoter. The cRNA was prepared and labeled with biotin via *in vitro* transcription using the BioArray High Yield RNA Transcript labeling kit (Enzo Biochemical). Labeled cRNA was fragmented by incubation at 94°C for 35 mins. For hybridization, 15 μg of fragmented cRNA was incubated for 16 hrs at 45°C with a Rat Expression 230A Gene chip. After hybridization, the gene chips were automatically washed and stained with streptavidin-phycoerythrin using a fluidics station (Affymetrix). The probed arrays were scanned at 3 μm resolution using the Genechip System confocal scanner made for Affymetrix by Agilent. Affymetrix Microarray Suite 5.0 was used to scan and analyze the relative abundance of

each gene from the average difference of intensities. Analysis parameters used by the software was set to values corresponding to 1.5 fold increase or decrease (Purdom-Dickinson *et al.* 2007).

RT-PCR

Total RNA extracted using Trizol was used as a template for RT-PCR. Superscript II was used for reverse transcription at 37–50°C for 1 hr. For PCR, the denaturation and primer extension were carried out at 94°C and 72°C. The annealing temperature was calculated based on GC content of the primers as suggested by the oligonucleotide synthesis company (Biosynthesis, Lewisville, TX). The products were detected by agarose gel electrophoresis with ethidium bromide staining or during real-time PCR (BioRad CFX96) using SYBR Green dye.

Nuclear Run-on Assay

A non-radioactive method was used as described (Sun *et al.* 2008b). Briefly, the nuclei from 5×10^7 trypsinized cells were collected by centrifugation following lysis in a buffer (10 mM Tris, pH 7.4, 3 mM $MgCl_2$, 10 mM NaCl, 150 mM sucrose, and 0.5% NP-40). The nuclei were incubated in 2× transcription buffer (200 mM KCl; 20 mM Tris-HCl, pH 8.0; 5 mM $MgCl_2$; 5 mM dithiothreitol; 4 mM each of ATP, GTP, and CTP; 200 mM sucrose; and 20% glycerol). The reaction was initiated by addition of 4 mM biotin-16-UTP and was carried out with 2 hrs incubation at 30°C. The reaction was terminated by addition of Trizol for extraction of RNA. Streptavidin agarose was used to isolate biotin-labeled newly synthesized RNA. The RNA was reextracted with Trizol for reverse transcription and subsequent real-time PCR (BioRad CFX96) using SYBR Green dye.

Transfection

Primary cultured cardiomyocytes were transfected with 0.5 μg of DNA from pGL3 firefly luciferase under the control of MTV GRE, −905 bp human bcl-x promoter or −3.2 kbp mouse bcl-x promoter using FuGene-6 liposomes on the 3rd day of plating. A TK-Renilla luciferase plasmid (0.05 μg) was cotransfected as a reference for transfection efficiency. For siRNA, the cells in 6 well plates were incubated 6 hrs with 100 nM each 5′-GGCUGGCGAUGAGUUUGAAtt-3′ (abbreviated as SI, Ambion) and 5′-GGUAGUGAAUGAACUCUUUtt-3′ (abbreviated as SII, Ambion) or a silencer negative control siRNA of 19 bases oligonucleotide derived from a scrambled sequence (Ambion) in 1 ml mixture of Opti-MEM (Invitrogen) and DMEM containing 3 μl Oligofectamine (Invitrogen). At 48 hours after transfection, cells were placed in fresh media and treated with 10 μM PG for 24 hours for measurements of luciferases or for treatment with Dox to measure for apoptosis.

Statistics

Student's t-test was used to compare the means of two groups of samples. Significant differences ($p<0.05$) were indicated with asterisks (*). ANOVA one-way analysis of variance ($p<0.05$) followed by Bonferroni correction was used to verify the significant difference using Prism 4.0 software. Means that are not significant different from each other

are labeled with a common letter. Therefore, means in the “a” group are significant different from means labeled with “b”, “bc” or “c” and so on. Means labeled with “bc” are not significant different from those with “b” or “c”, although means labeled with “b” is significant different from that labeled “c”.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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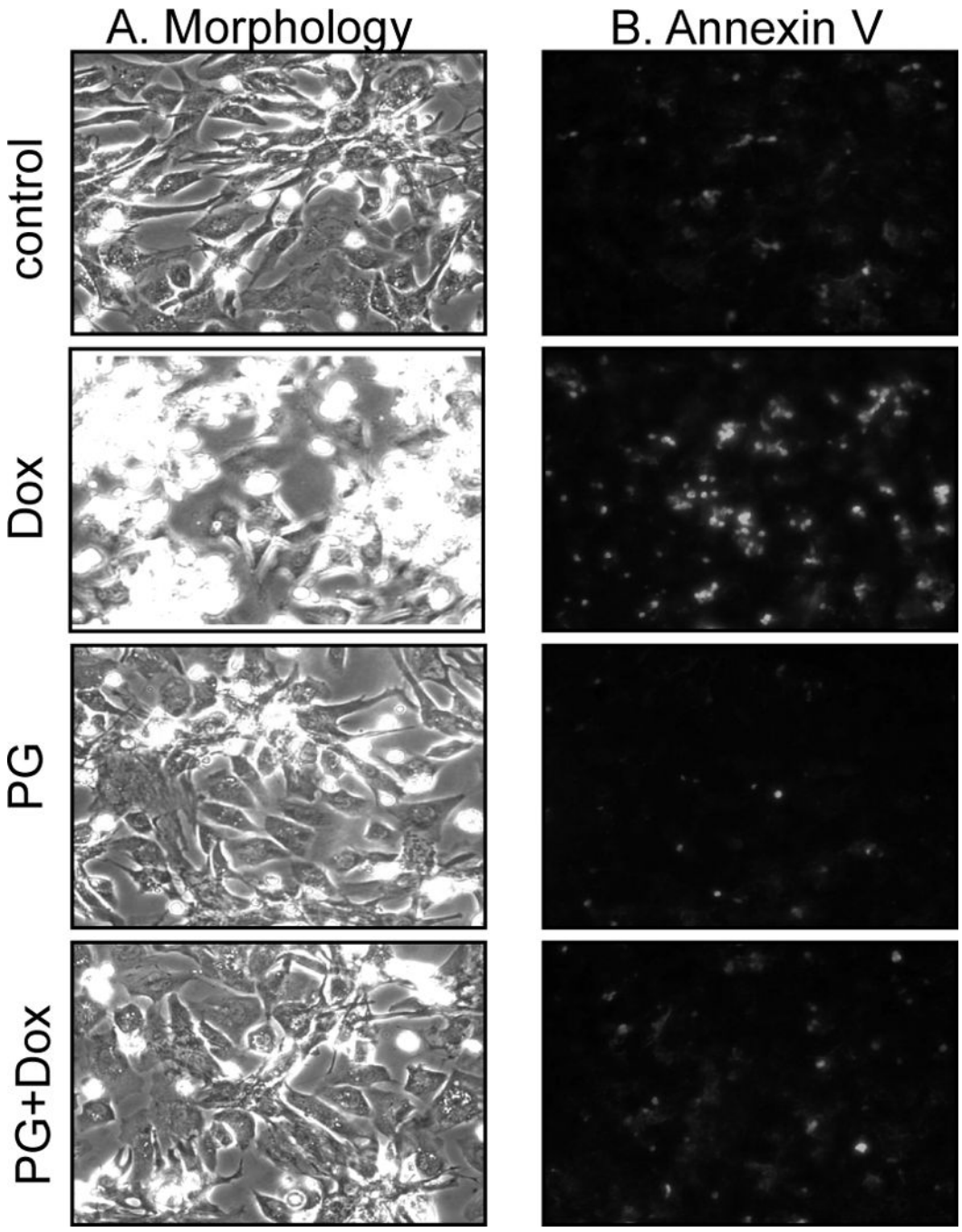

Figure 1. Morphological Evidence of PG Inhibiting Dox Induced Apoptosis

Primary cultured cardiomyocytes were pretreated with 10 μM PG for 24 hrs and then treated with 0.8 μM Dox in the absence or presence of 10 μM PG. At 24 hrs after Dox treatment, the morphology was recorded under a phase contrast microscope. Detached cells were combined with adherent cells for Annexin V-Fluo staining for recording under a fluorescent microscope.

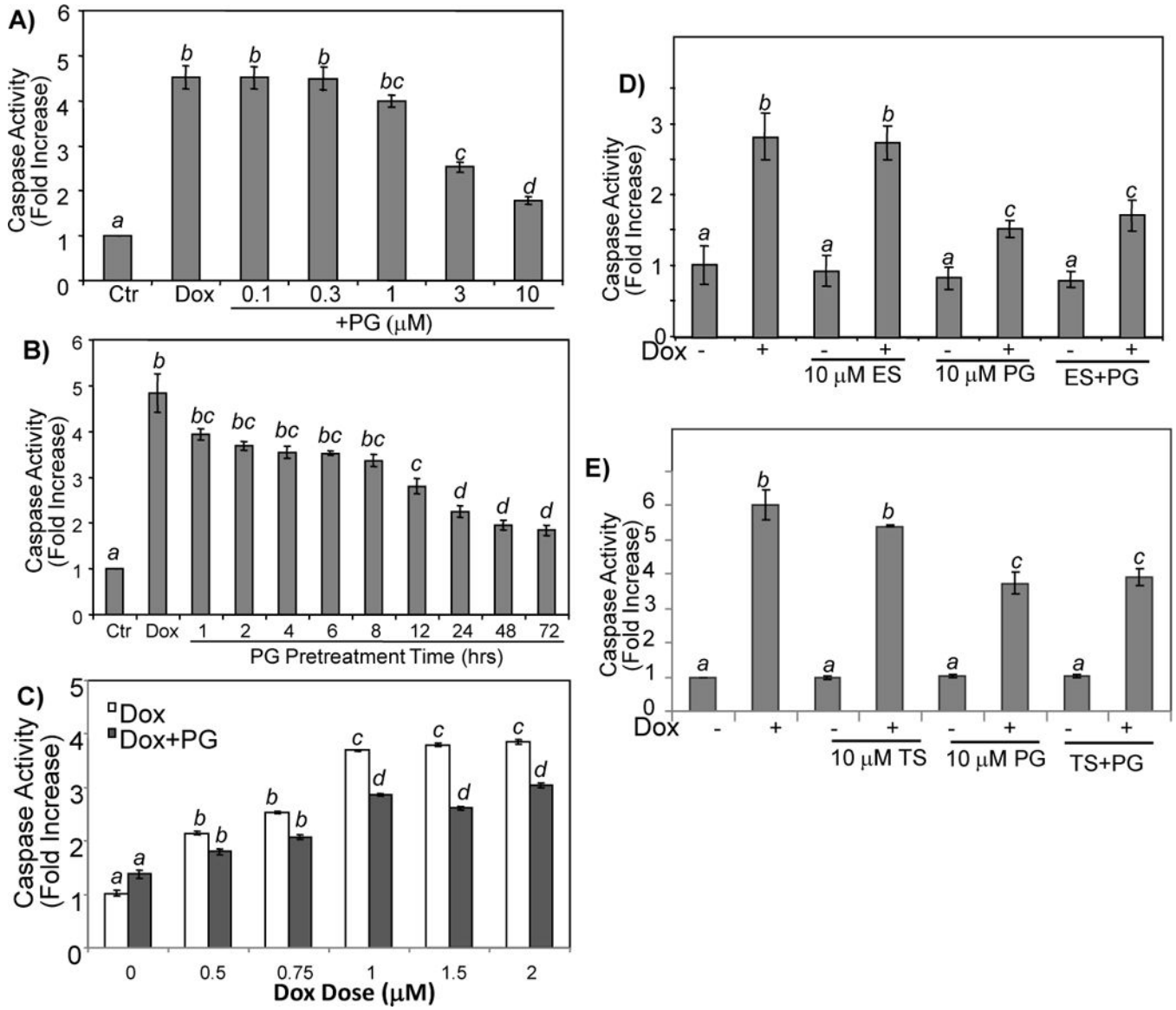

Figure 2. PG Inhibits Dox Induced Caspases

Primary cultured cardiomyocytes were treated with 0.8 μM Dox for 24 hrs following pretreatment of PG at indicated dose for 24 hrs (A) or at 10 μM over indicated time (B). For Dox dose response, cardiomyocytes were treated with 10 μM PG and Dox at indicated doses for 16 hrs (C). Following pretreatment with 10 μM PG in the absence or presence of 10 μM 17β-estradiol (ES) or 10 μM testosterone (TS), cells were treated with 0.8 μM Dox for 24 hrs before harvesting for measurements of caspase activity (D, E). The data are from one experiment representative of three with means ± standard deviations from three samples. A superscript letter indicates significant difference ($P<0.05$) from means labeled with a different letter as determined by ANOVA analysis.

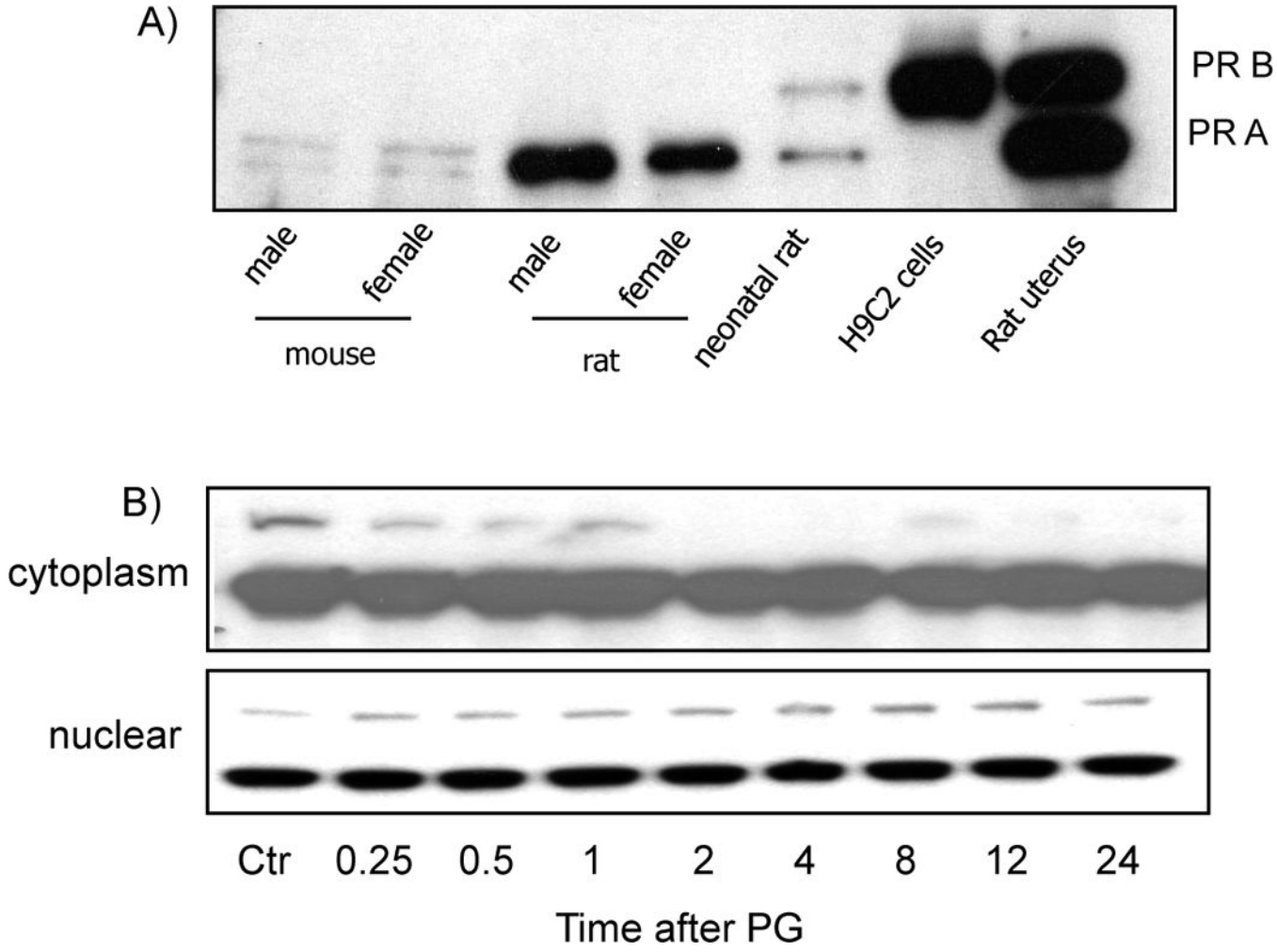

Figure 3. Expression of PR in Cardiac Tissue or Cardiomyocytes

Cardiac tissue was collected from 8 weeks old male or female C57BL6 mice or Sprague Dawley rats by quick frozen in liquid nitrogen. The uterus tissue from a pregnant rat was collected immediately after birth to serve as a positive control. Animal tissues were grinded in liquid nitrogen bath for dissolving in lysis buffer for Western blot analyses (A). Primary cultured cardiomyocytes were treated with 10 μM PG and harvested at indicated time for isolation of cytoplasmic or nuclear fractions (B). An equal amount of proteins (20 μg/lane) was loaded for Western blot analyses.

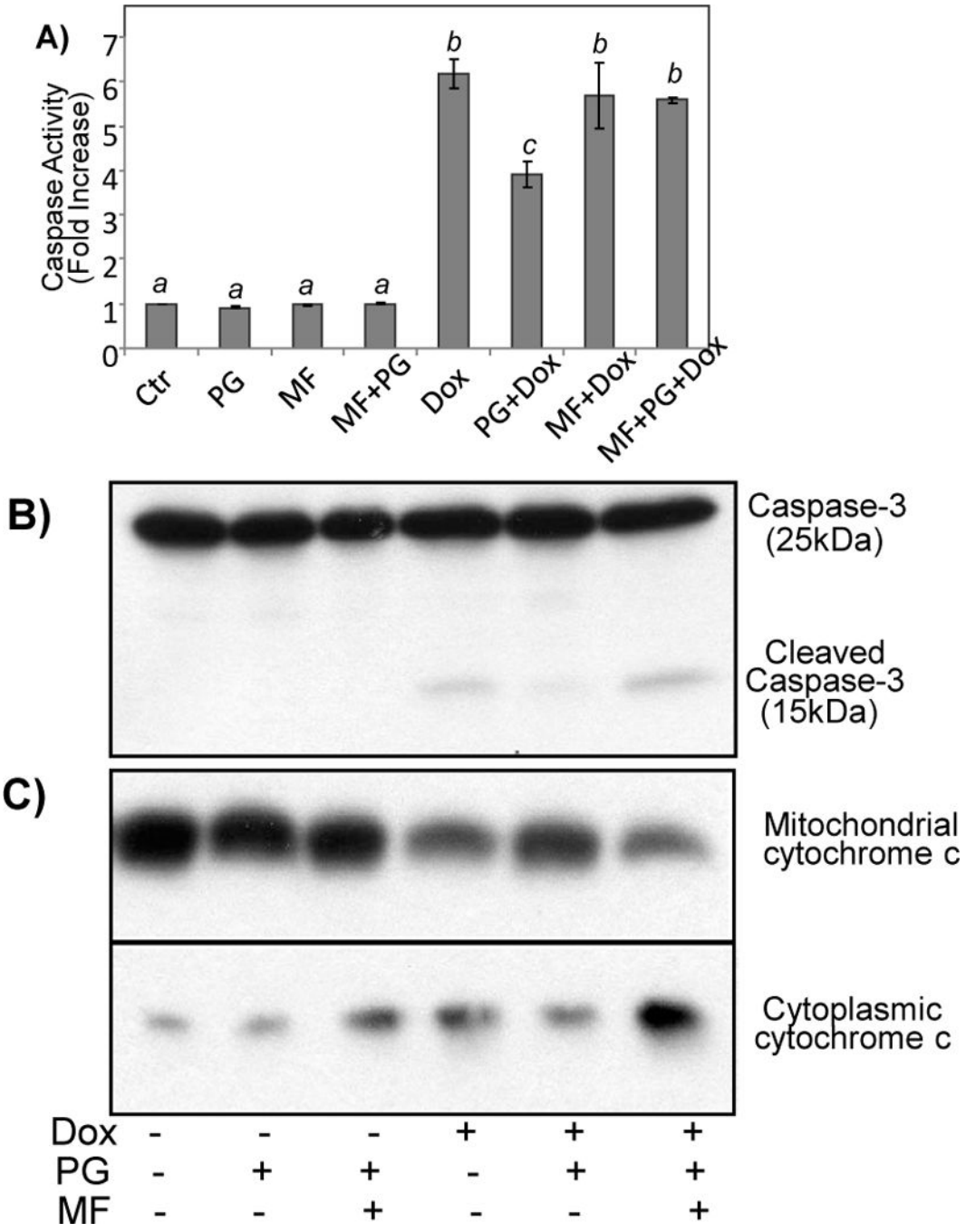

Figure 4. PR Receptor Antagonist MF Blocks Cytoprotective Effect of PG

Primary cultured cardiomyocytes were treated with 10 μM PG in the absence or presence of 1 μM MF for 24 hrs before addition of 0.8 μM Dox. The cells were harvested at 24 hrs later for measurements of caspase 3 using DEVD-AMC as a substrate (A), or for Western blot to measure caspase-3 cleavage or mitochondrial release of cytochrome c (B, C). A superscript letter indicates significant difference ($P<0.05$) from means labeled with a different letter as determined by ANOVA analysis.

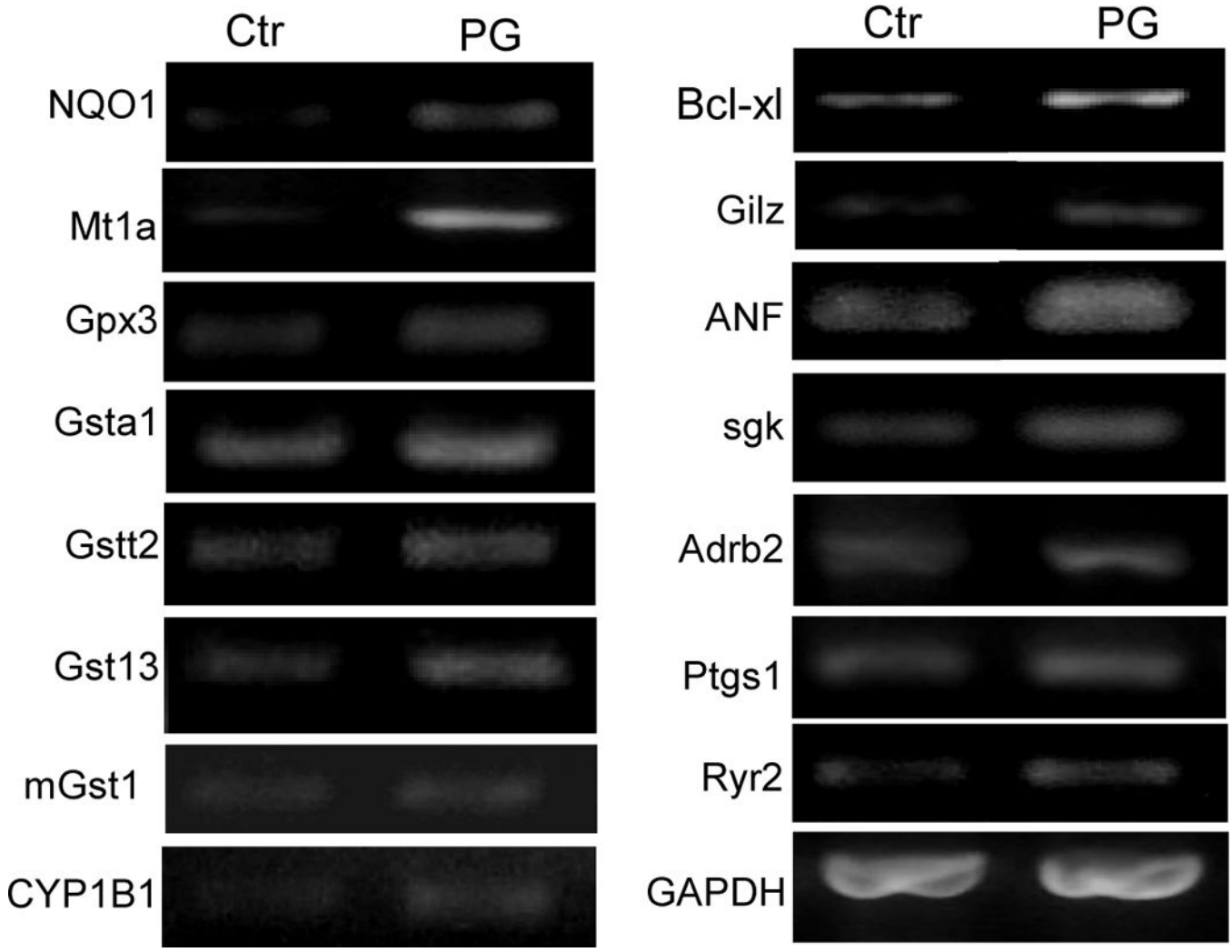

Figure 5. RT-PCR Verification of Microarray Detected Genes Induced by PG

Total RNA was harvested from primary cultured cardiomyocytes 24 hrs after 10 μM PG treatment for RT-PCR analyses as described in the Method. GAPDH was used as a loading control.

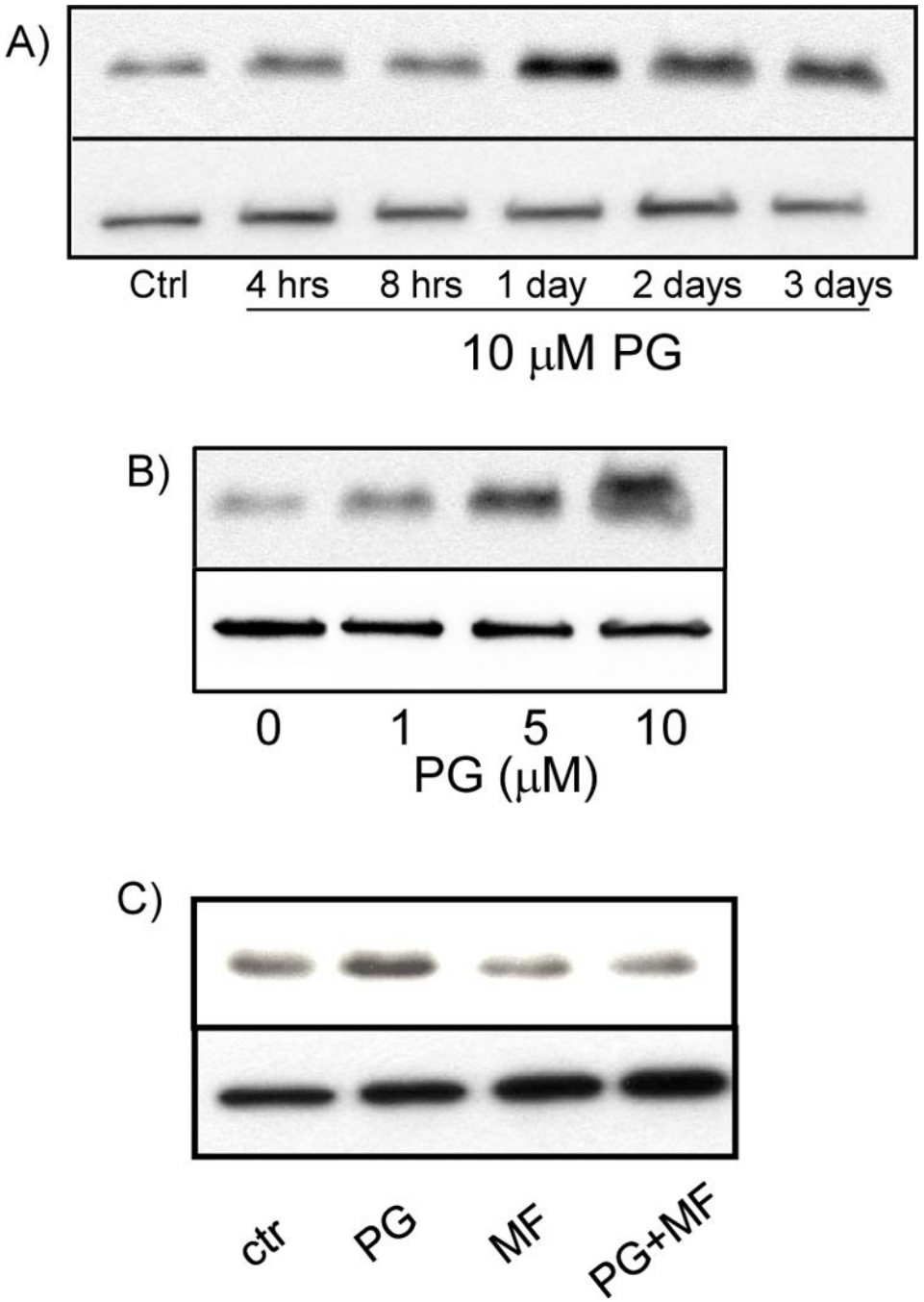

Figure 6. PG Induces Bcl-xL protein

Primary cultured cardiomyocytes were treated with 10 μM PG (A, C) or indicated dose (B) for the time points shown or 24 hrs (B, C). MF (1 μM) was added to cells 1 hr before PG (C). Cell lysates were used for Western blots to measure Bcl-xL protein (20 μg protein/lane, upper panels) with vinculin being used as a loading control (bottom panels). The Data shown is representative of 3 experiments.

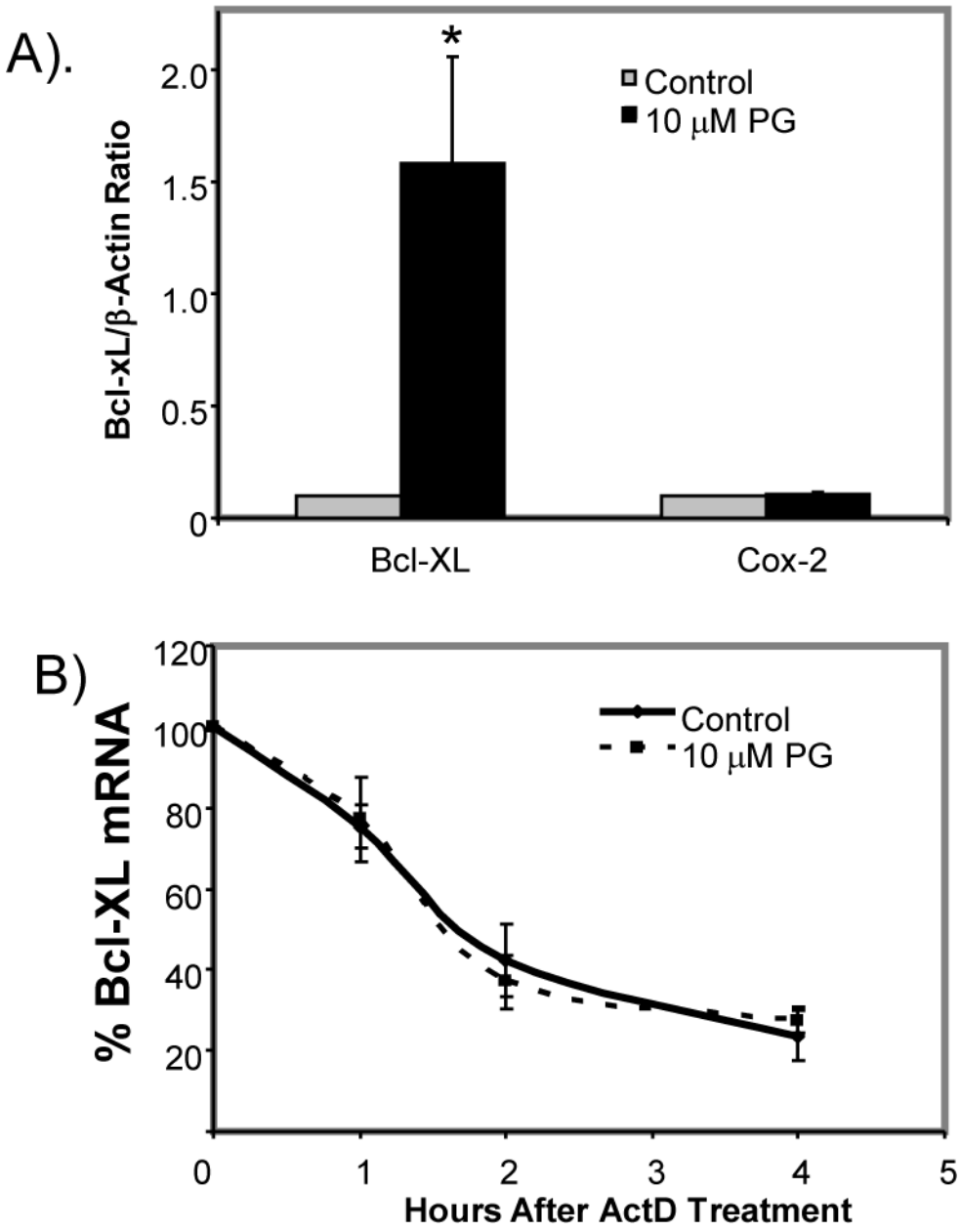

Figure 7. PG Causes An Increase in the Rate of Bcl-xL Transcription

Primary cultured cardiomyocytes were treated with 10 μM PG for 24 hrs before being harvested for nuclear run-on assay (A) or measurements of Bcl-xL mRNA stability by addition of 5 μg/ml actinomycin D (ActD) over indicated time (B). The relative Ct method was used to calculate mRNA levels with normalization to β-Actin (A). The level of Bcl-xL was set to 100 at 0 time point for determining Bcl-xL mRNA stability (B). The data represent average ± standard deviations of three independent experiments. An asterisk (*) indicates $p<0.05$ when the treated group was compared to control using Student's t test.

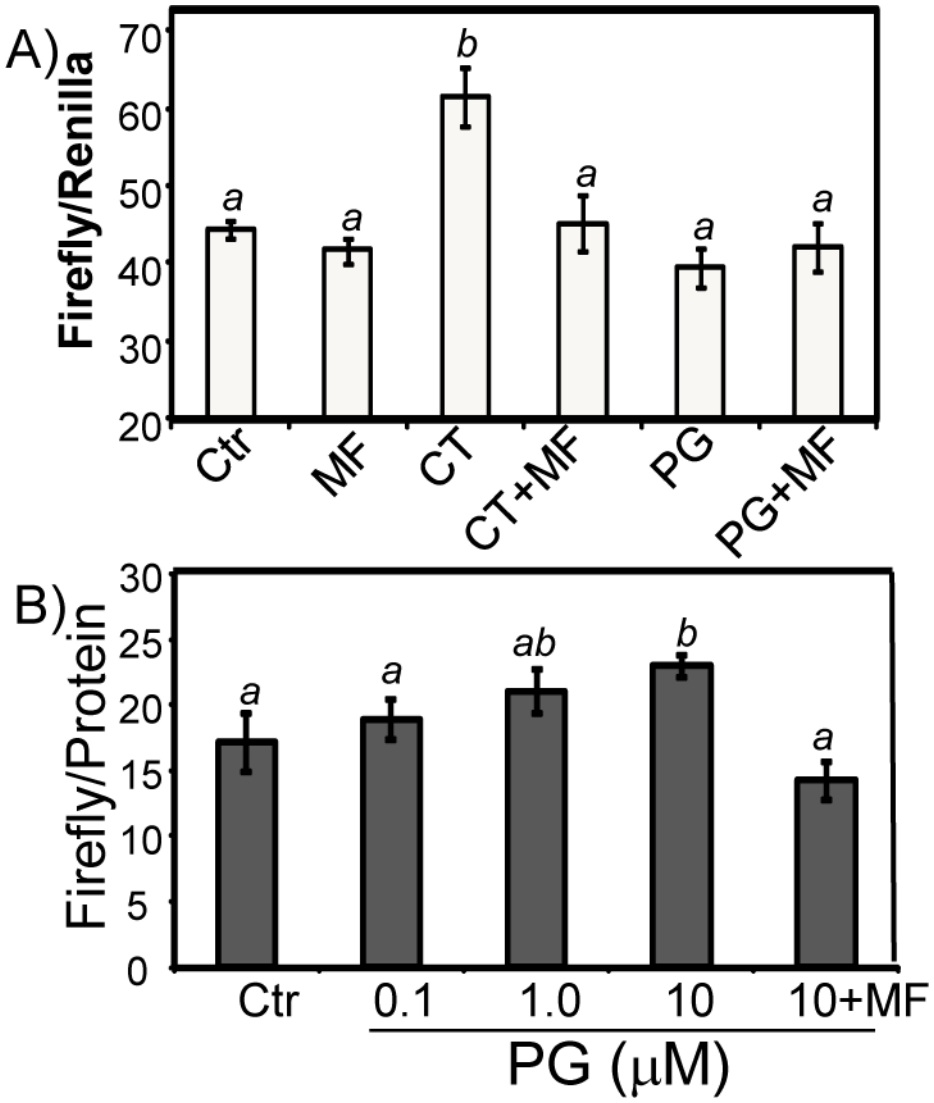

Figure 8. bcl-x Gene Promoter Activity by PG Treatment

Primary cultured cardiomyocytes were transfected with luciferase constructs under the control of −905 bp human bcl-x promoter (A) or −3.2 kb mouse bcl-x promoter (B) using Fugene6 liposomes. At 48 hrs after transfection, cells were placed in 0.5% FBS for 24 hrs before treatment with 1 μM corticosterone (CT, A), 10 μM PG (A) or PG at indicated doses (B) for 24 hrs before harvesting for measurement of luciferases. A superscript letter indicates significant difference ($P<0.05$) from means labeled with a different letter as determined by ANOVA analysis.

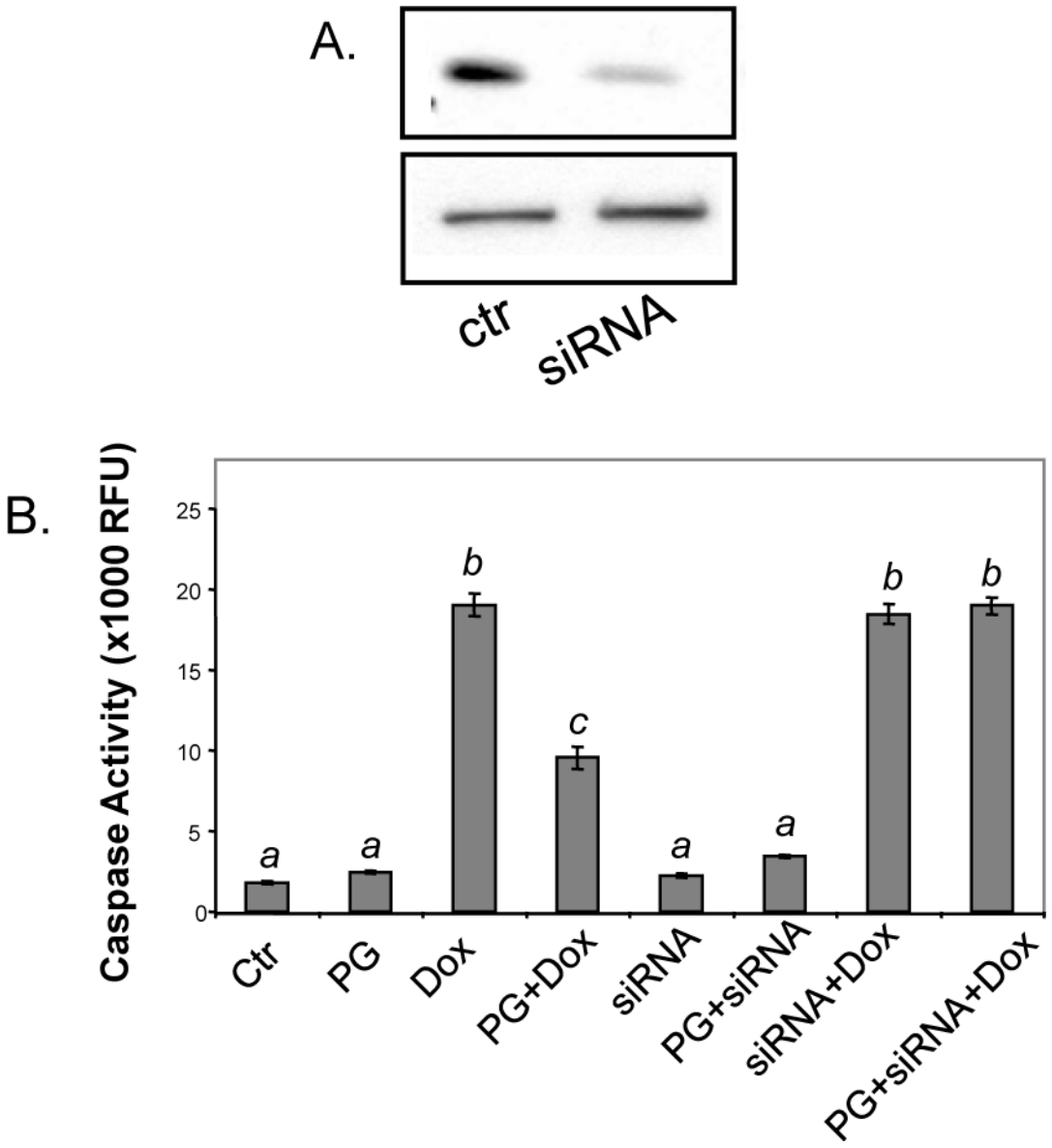

Figure 9. Cardioprotective Effect of PG Is Dependent on Bcl-xL

Primary cultured cardiomyocytes were transfected with Bcl-xL siRNA or negative control siRNA as described in the Methods. At 48 hrs after siRNA transfection, cells were collected to verify Bcl-xL down regulation (upper panel) by Western blot with vinculin (bottom panel) as a loading control (A) or were pretreated with 10 μM PG for 24 hrs prior to 16 hrs treatment with 1 μM Dox for measurement of caspase-3 activity (B). The data is from one experiment representative of three. A superscript letter indicates significant difference ($P<0.05$) from means labeled with a different letter as determined by ANOVA analysis.

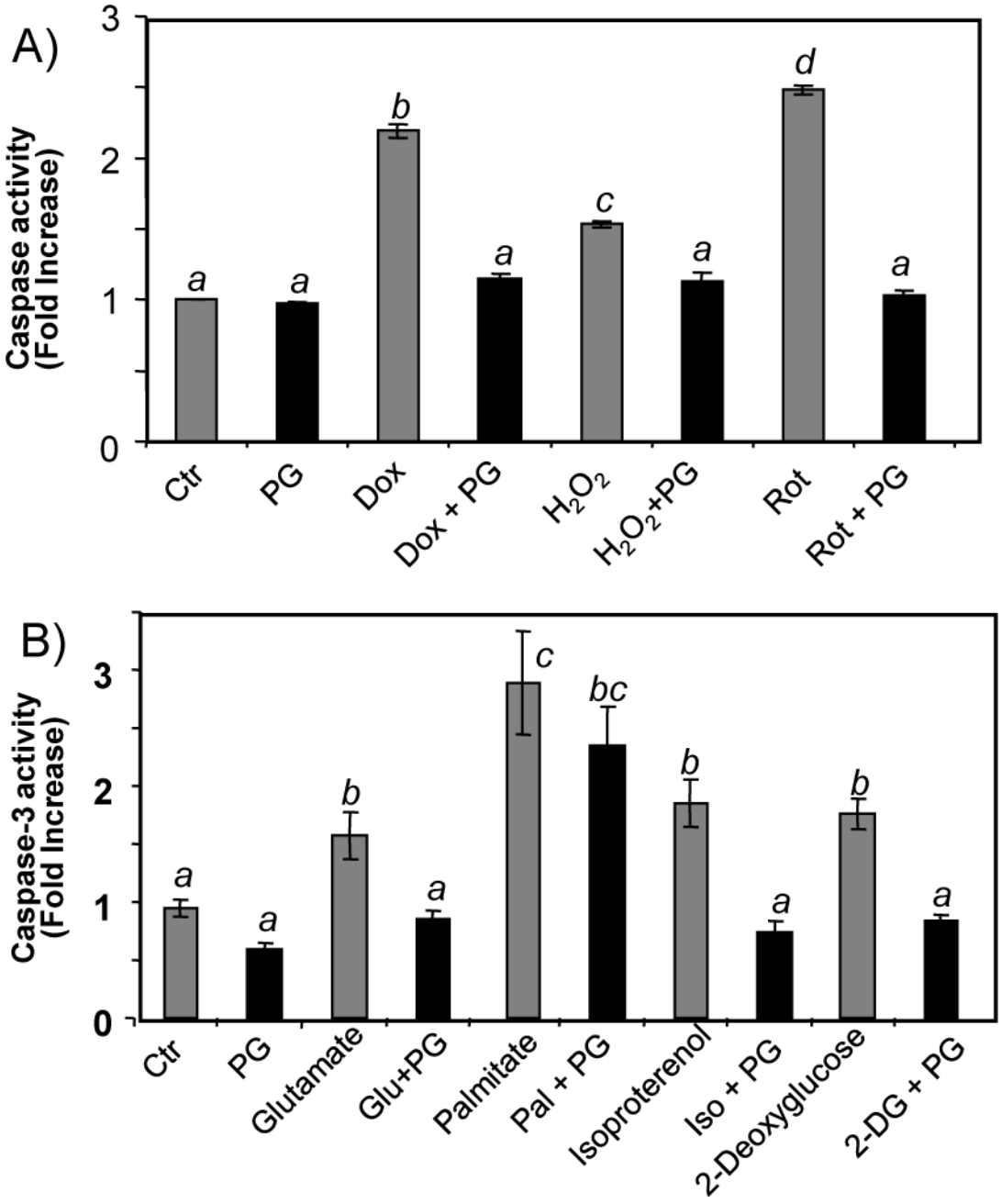

Figure 10. PG Inhibits Caspase-3 Activation by A Variety of Toxicants

Primary cultured cardiomyocytes were pretreated with 10 μM PG 24 hrs prior to treatment with 100 μM H_2O_2, 1 μM rotenone (Rot), 0.5 μM palmitate (Palm), 5 μM 2-deoxyglucose (2-DG) or 25 μM glutamate (Glu) in the absence or presence of 10 μM PG. Dox treatment (0.8 μM) was included for comparison. Cells were harvested at 24 hrs after for measurements of caspase-3 activity. The data represents mean ± SEM from triplicate samples of one experiment representative of three. A superscript letter indicates significant difference ($P<0.05$) from means labeled with a different letter as determined by ANOVA analysis.