Journal of Cellular Physiology

Peritoneal Dialysate Effluent During Peritonitis Induces Human Cardiomyocyte Apoptosis by Regulating the Expression of GATA-4 and Bcl-2 Families

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Cardiovascular event and infection are leading causes of death from peritoneal dialysis (PD). This study examined in vitro cellular mechanism for cardiotoxicity induced by PD-related peritonitis. Cultured human cardiomyocytes were treated with PD effluent (PDE) during peritonitis (PPDE), and effects of PPDE on cultured cardiomyocytes in terms of apoptosis, with expression its related genes assessed. Results showed PPDE treatment of cardiomyocyte leading to onset of apoptosis, as confirmed by phosphatidylserine exposure plus DNA fragmentation and damage. This apoptosis is mediated by reduced Bcl-2/Bax and Bcl-x_L/Bax ratios, as well as reduced expression of GATA-4, an important cardiomyocyte survival factor, at the level of transcription. These changes activated pro-apoptotic pathways. PPDE treatment also inhibited ERK signals, contributing to cardiotoxicity. Our findings revealed that PPDE contains potent pro-apoptotic factors that regulate expression of GATA-4 and Bcl-2 families, inducing cultured cardiomyocyte apoptosis. This pinpoints a key role of apoptosis in PD-associated cardiovascular events, along with a potential therapeutic target.

J. Cell. Physiol. 226: 94–102, 2010. © 2010 Wiley-Liss, Inc.

Cardiovascular event and infection are the first and second leading causes of death in the peritoneal dialysis (PD) populations (Parfrey and Foley, 1999; Go et al., 2004; Schiffrin et al., 2007; USRDS, 2008); both events are closely related. PD-related peritonitis is the crucial infection in PD patients (Aslam et al., 2006; Bender et al., 2006). Peritoneal toxin should be absorbed to the systemic circulation and might induce cardiotoxicity. After an episode of severe infection in dialysis patients, risk of death from cardiovascular events is increased sevenfold for 6 months and continues to rise for up to 48 months (Ishani et al., 2005; Bender et al., 2006). It has been considered to play a significant role in up to one sixth of patient deaths occurring during the course of PD therapy (Fried et al., 1996). In 41.5% of patients with peritonitis-related mortality, immediate cause of death was a cardiovascular event (Pérez Fontan et al., 2005). Clinical findings indicate that a peritonitis episode may culminate in cardiovascular event (Fried et al., 1996; Bender et al., 2006): high incidence of peritonitis is accompanied by greater risk of death (Maiorca et al., 1993; Fried et al., 1996; Piraino, 1998), and cardiovascular events contribute to risk of peritonitis-related death in patients undergoing PD (Digenis et al., 1990; Firanek et al., 1991; Lupo et al., 1994). However, the possible mechanisms connecting PD-related peritonitis and cardiac mortality have not been addressed.

Growing evidence implicates cardiomyocyte apoptosis as a mechanism contributing to various types of heart disease (Olivetti et al., 1997; Haunstetter and Izumo, 1998; Narula et al., 1999). Cardiomyocyte apoptosis could result in a loss of contractile tissue, compensatory hypertrophy of myocardial cells, reparative fibrosis, and heart failure. In animal models, endotoxin (Natanson et al., 1989; Ramana et al., 2006), exotoxin (Natanson et al., 1989; Sibelius et al., 2000), and inflammatory mediator (Mann, 1999) play important roles in cardiomyocyte apoptosis. In PD patients with infectious peritonitis, expression of inflammatory mediators and cytokines increase in PD effluent (PDE) and correlate with treatment outcome (Lai et al., 2000; Wang and Lin, 2005). Yet there are no data on effects of peritonitis PD effluent (PPDE) on cardiomyocytes viability and apoptosis.

Hsin-Hui Wang and Ping-Chun Li contributed equally to this work.

Contract grant sponsor: China Medical University;
Contract grant number: CMU97-212.

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Received 14 May 2010; Accepted 24 June 2010

Published online in Wiley Online Library (wileyonlinelibrary.com.), 12 July 2010.
DOI: 10.1002/jcp.22309

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Bcl-2 protein family members are the best characterized proteins that are directly involved in the regulation of apoptosis (Cory and Adams, 2002). Bcl-2 and its closest homologues, Bcl-x_L and Bcl-w, potently inhibit apoptosis in response to many cytotoxic insults. Bax and Bak are well known proapoptotic members of the Bcl-2 protein family. Regulation of apoptosis is highly dependent on the ratio of anti-apoptotic to pro-apoptotic proteins. Conditions that induce myocardial stress cause complex alterations in levels of Bcl-2 family proteins (Bishopric et al., 2001).

Cardiac Bcl-2 gene expression has been shown to be regulated by GATA-4 both in vitro and in vivo (Kobayashi et al., 2006). GATA-4 is a transcription factor enriched in cardiac tissue that is essential for various cardiomyocyte physiological and adaptive responses. An early event in the cardiotoxicity induced by the antitumor drug doxorubicin is GATA-4 depletion, which in turn causes cardiomyocyte apoptosis (Aries et al., 2004; Suzuki and Evans, 2004). GATA-4 has also been shown to upregulate transcription of the anti-apoptotic genes Bcl-2 (Kobayashi et al., 2006) and Bcl-x_L (Aries et al., 2004; Suzuki and Evans, 2004) in cardiomyocytes, and to play a central role in regulating the survival or apoptosis of cardiomyocytes. Although previous studies have suggested the importance of apoptosis regulation and GATA-4 expression in various heart diseases, their role in PD peritonitis-related cardiotoxicity has not been elucidated.

To clarify the relationship between PD-related peritonitis and high cardiac mortality, we examined the contributions of PD-related peritonitis to cardiotoxicity. We postulated that during PD-related peritonitis, proapoptotic pathways are activated in cardiomyocytes. To test this hypothesis, human cardiomyocytes were cultured and treated with PPDE. The possible underlying signaling pathways of cardiotoxicity induced by PPDE were examined.

Subjects and Methods

Human cardiomyocytes culture

This research was approved by the China Medical Hospital Institutional Review Board. Written informed consent was obtained from each individual. Human cardiomyocytes obtained from the myocardial ventricular resection specimens of patients undergoing cardiac surgery were isolated as previously described (Ancey et al., 2002). Cells were cultured for a period of 8 days, and culture medium was completely replaced every 3 days. Cultured medium was Dulbecco's modified Eagle medium (Hyclone, Logan, UT) containing equal Volume of F12, 1% L-glutamine, 25% fetal bovain serum, 1% non-essential amimo acid, 1% pyruvate, 250 μl hydrocortisone, 250 μl interferrin, and insulin 250 μl in each 500 ml. For keeping its cardiomyocytes property, the cells were passaged before 90% confluent about 2–3 days.

Doxorubicin (Sigma, St. Louis, MO) (DOXO) was prepared as 50 μM stock solution in DMSO and then diluted to 0.5 and 1 μM in 1× PBS for working concentration.

We also measured electrophysiological character of cultured human cardiomyocytes, including action potential duration (APD) and peak L-Type calcium current (IcaL) (Chang et al., 2008).

Immunostaining of human cardiomyocytes and confocal microscopic imaging

To characterize cardiomyocytes in culture, cells were incubated with a desmin monoclonal antibody (mAb) (Vision BioSystems, Mount Waverley, Victoria, Australia), α-sarcomeric actinin mAb (Sigma), or a rabbit polyclonal anti-CAPON antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by a FITC-labeled IgG (Santa Cruz Biotechnology, Inc.) secondary antibody. Cells were double labeled with WGA (Invitrogen, San Francisco, CA). Staining was assessed and photomicrographs were obtained using a Leica TCS SP2 Confocal Spectral Microscope.

Patient population and peritonitis

Turbid PPDE prior to antibiotic treatment were collected in eight culture positive peritonitis episodes from PD patients treated in our hospital. Microorganisms were gram-positive bacteria in five episodes and gram-negative bacteria in three episodes. All patients had a Tenckhoff peritoneal catheter inserted and were treated with the standard double-bag system (Baxter Healthcare Corp., Deerfield, IL). These five men and three women had a mean age of 47.8 years. Causes of renal failure included chronic glomerulonephritis, reflux nephropathy, obstructive nephropathy, and renal hypoplasia. Time on PD ranged from 10 to 56 months. Peritonitis was defined as presence of two of the following criteria: microorganisms on gram staining, subsequent positive culture of PD fluid; cloudy fluid (leukocyte count $>$100 cells/ml with $>$50% polymorphonuclear cells) and/or peritoneal inflammation symptoms. Exclusion criteria included: (a) tunnel-tract or exit site infections, (b) completion of antibiotic therapy for peritonitis within 28 days of study enrollment, (c) the presence of peritonitis attributed to fungus or mycobacterial infection or negative culture, (d) drug sensitivity showing resistance to initial antibiotic therapy, (e) previous immunosuppressive therapy, (f) anemia from a disorder other than chronic renal failure, and (g) insulin-dependent diabetes mellitus.

Overnight dwell bags of PDE samples (SPDE) were collected also from five stable PD without peritonitis patients (two men, three women, mean age $= 50.23 \pm 11.67$ years) treated in our hospital. No patient was diabetic, and no peritonitis episode was noted over the past year. Causes of renal failure included chronic glomerulonephritis, obstructive nephropathy, and polycystic kidney disease. Patients signed an informed consent form for these studies.

Collection of PDE

The PDE were collected as previously described (Wang et al., 2008): PDE was centrifuged at 400*g*, 4°C for 10 min. After centrifugation, supernatants were collected, refrigerated, then lyophilized, and dried to powder using Freeze Dryer (Eyela, Tokyo, Japan). Powder was stored at −70°C until analysis. The lyophilized PPDE samples were reconstituted as a 625 mg/ml stock solution with 1 × PBS, and filtrated by 0.45 μM filter. PPDE stock solutions were storage at −20°C and diluted with 1 × PBS to working concentration before use.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay

Cardiomyocyte cell viability was determined by a MTT assay. In a 96-well microplate, 2.5×10^4 cells/well were incubated in 100 μl of culture media and exposed to different concentrations of DOXO or PPDE for varying time periods. 12.5 μM MTT dye (stock; 5 mg/ml) were added into each well and the cells were further incubated at 37°C for 4 h. Viable cells could convert MTT dye dark blue product, the cells were lysed with dimethyl sulfoxide (DMSO) (Sigma) and cell lysates were collected. Optical density (OD) values of the absorbance 540 nm were obtained using a microplate reader (mode FL 331, Bio-Tek Instruments, Winooski, VT).

Apoptosis assays by flow cytometry

Apoptosis was quantitatively gauged by detecting phosphatidylserine exposure on cell membrane with Annexin V staining, as described above (Chen et al., 2001a). Cells were simultaneously stained with Annexin V-FITC (25 ng/ml; green

fluorescence, R&D Systems, Minneapolis, MN) and dye exclusion (propidium iodide, 20 μg/ml, red fluorescence). Data were obtained by flow cytometry analysis with FACS-SCAN (Becton-Dickinson, Heidelberg, Germany) FACS Canto in cell populations from which debris was gated out and analyzed.

Evaluation of apoptosis by TUNEL and DAPI staining

Cultured human cardiomyocytes were plated at approximately 2×10^5 cells/well in 12-well plates with DOXO or PPDE and incubated at 37°C for 24 h. Cell nuclei were stained with 4, 6-Diamidino-2-phenyindole (DAPI, Sigma) and DNA fragments labeled with AlexaFluor 488 dye-labeled anti-BrdU antibody (Apo-Brd U^{TM} TUNEL assay kit. Invitrogen). Cells were observed and photomicrographs were obtained using a Leica TCS SP2 Confocal Spectral Microscope. An individual blinded to the experimental conditions counted at least 300 cells in six different high power fields for each experiment.

Comet assay

Approximately 5×10^3 cells/ml of cardiomyocytes were incubated with doxorubicin or PDE during peritonitis for 24 h at 37°C, isolated, then examined for DNA damage by Comet assay previously described (Kim et al., 2003). Treated cells were embedded in situ in 1% agarose, then placed in lysis solution for 30 min. Cell nuclei were subsequently electrophoresed for 20 min at 1 V/cm, followed by staining with PI and visualization with a fluorescence microscope.

RNA isolation and reverse transcription

RNA was extracted from cells using RNAzol B (TEL-TEST, Inc., Friendswood, TX). Resulting RNA suspension was forthwith converted to cDNA by reverse transcription, cDNA samples stored at −70°C for analysis.

Real-time PCR with SYBR green assay

Five μl of cDNA (1–10 ng) was mixed with SYBR green PCR core reagent or master mix reagent (Applied Biosystems Inc., Foster City, CA). The thermal cycling conditions were determined according to rules of "Thermal cycling parameters for primer optimization." Each RNA samples was also analyzed with β-actine which serve as internal control for correcting relative specific gene expression levels. Primers were designed using Primer Express Primer Design software, as follows:

Bcl-2	Sense primer: ATGTGTGTGGAGAGCGTCAA
	Antisense primer: ATCACCAAGTGCACCTACCC
Bcl-x_L	Sense primer: ACAGCAGCAGTTTGGATGC
	Antisense primer: TGGGATGTCAGGTCACTGAA
Bax	Sense primer: TTGGGTGAGACTCCTCAAGC
	Antisense primer: CACTGTGACCTGCTCCAGAA
GATA-4	Sense primer: AGCTCCTTCAGGCAGTGAGA
	Antisense primer: CTGTGCCCGTAGTGAGATGA
β-actine	Sense primer: CAGGTATGCACCCAGAGTGA
	Antisense: GATATGGAGAAGATTTGGCA

According to amplification plot, cycle number over the threshold means Ct value. The Ct value of non-template control is 45. The relative expression ratio among untreated RNA and different RNA samples could be calculated with 2-Ct.

Western blotting

Ten to fifty micrograms protein extracts were separated by 10–12% SDS–PAGE and transferred to nitrocellulose (PVDF) membranes blocked overnight with $1 \times$ TBS buffer containing 5% skim milk. Membranes were incubated with optimal concentrations of primary antibodies: anti-GATA-4 mAb (Abcam, Abgent, San Diego, CA), anti-extracellular signal-regulated kinase (ERK) mAb (Cell Signaling Technology, Beverly, MA), and anti-β actin mAb (Sigma) in $1 \times$ TBS buffer containing 5% skim milk. Membranes were washed and then incubated with appropriate secondary antibody (goat anti-mouse mAb conjugated with HRP), and visualized by enhanced chemiluminescence ECL detection kit (Perkin Elmer, Wellesley, MA). The antibody for GATA-4 (1:100), Bcl-X_L (1:100), Bax (1:100) were purchased from Santa Cruz Biotechnology, Inc. and Bcl-2 (1:1,000) was purchase from Cell Signaling (Santa Cruz, CA).

Statistics

All data are presented as mean ± standard deviation (SD). Differences between groups were analyzed by Mann–Whitney *U* test or ANOVA, values of $P < 0.05$ considered statistically significant.

Results

Characterization of human cardiomyocytes in primary culture

To characterize cardiomyocytes, muscle markers desmin and myocyte-specific protein α-sarcomeric actinin were detected (Fig. 1). CAPON, recently documented as endogenous protein expressed in guinea pig cardiomyocytes, interacts with nitric oxide synthase to accelerate cardiac repolarization by inhibition of L-type calcium channels. Expression of endogenous CAPON

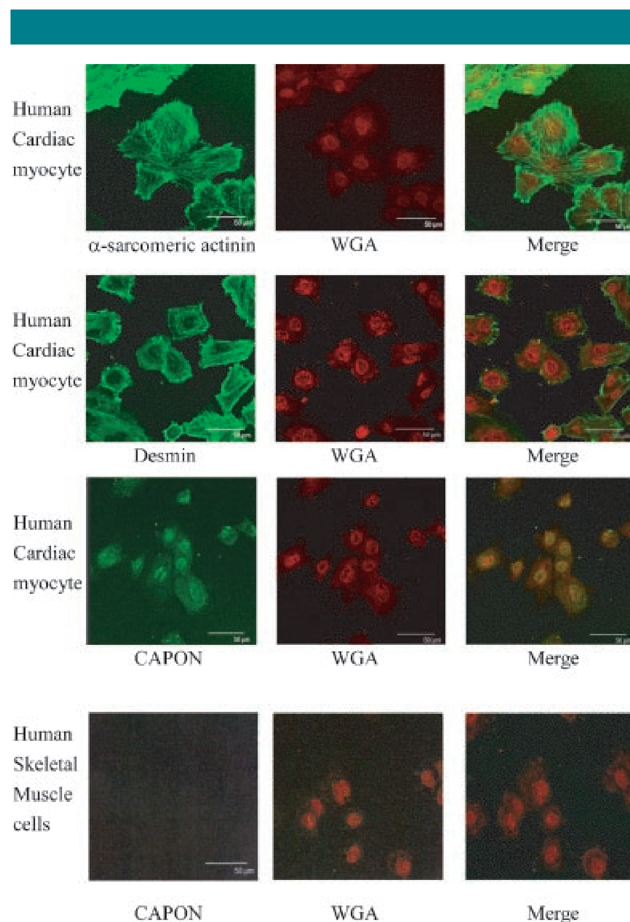

Fig. 1. Characterization of human cardiomyocytes by immunostaining of cardiomyocyte markers. Double labeling of cardiomyocytes with WGA (red) and α-sarcomeric actinin (green) (first line) or desmin (green) (second line) or CAPON (green) (third line). Negative control using cultured human skeletal muscle cells was stained with CAPON (last line).

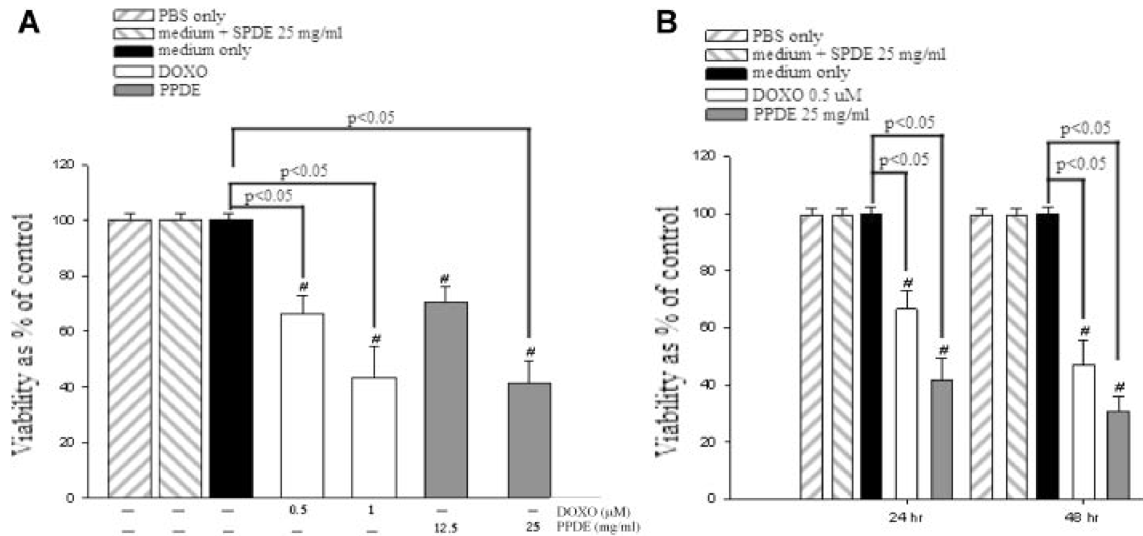

Fig. 2. Cell viability reduced after treatment with PPDE. Human cardiomyocytes were treated with PPDE during peritonitis. Cell viability was determined by MTT assay. A: Cells were incubated with various concentration of peritoneal dialysate effluent during peritonitis (PPDE) (12.5, 25 mg/ml) (n = 8) or doxorubicin (0.5, 1 μM; DOXO) as a positive control for 24 h. B: Cell were treated with PPDE (25 mg/ml) for 24 or 48 h (n = 8) and controls data are expressed as mean ± SD of eight different PPDE. #*P* < 0.05 versus medium only control. (▨: treated with PBS only; ▧: treated with medium and stable peritoneal dialysate effluent (SPDE) without peritonitis; ■: treated with medium only; □: Doxo pretreatment; ■: treated with medium and peritonitis peritoneal dialysate effluent (PPDE)).

protein in cultured cardiomyocytes was detected by immunofluorescent staining and confocal microscopy (Fig. 1). Both action potential duration (APD) and peak L-Type calcium current (IcaL) were APD_{10}, APD_{50}, APD_{75} and APD_{90}: 95.4 ± 10.6, 289.2 ± 15.6, 308.2 ± 15.4, and 318.4 ± 16.4 msec, respectively, with peak IcaL density of −10.2 ± 0.9 pA/pE at + 10 mV (n = 6).

PPDE induces cell death in human cardiomyocytes

Cardiac cell death is believed to play a major contributory role in development and progression of myocardial dysfunction (Haunstetter and Izumo, 1998). To assess whether PPDE treatment induced cardiac cell death, cell viability were evaluated by MTT assay. Doxorubicin-induced cardiotoxicity, which has been well described (Shan et al., 1996), was used as a positive control. MTT assay showed PDE during peritonitis- and doxorubicin-induced human cardiomyocyte cell death as both dose- (Fig. 2A) and time-dependent (Fig. 2B). When cardiomyocytes were pre-exposed to 12.5, 18, or 25 mg/ml PDE during peritonitis for 24 h, cell viabilities were 70.6 ± 5.7%, 58.7 ± 9.7%, and 41.6 ± 7.8%, respectively, all significantly lower than in cardiomyocytes without pre-treatment ($P < 0.05$) (Fig. 2A). This change was even more profound in the 48 h treatment group (Fig. 2B). When cardiomyocytes were pre-exposed to 25 mg/ml PDE from stable PD patients for 24 and 48 h, cell viabilities were similar with cardiomyocytes without pre-treatment (data not shown).

PPDE induces apoptosis in human cardiomyocytes

The above lend substantial evidence of apoptosis playing a critical role in cardiomyocyte cell death associated with several cardiac diseases (Olivetti et al., 1997; Haunstetter and Izumo, 1998). To explore whether PPDE during peritonitis challenge induces human cardiomyocyte apoptosis, we assessed apoptotic cell death by flow cytometry. TUNEL staining and Comet assays were performed for determination of DNA damage. Doxorubicin, which can induce cardiomyocyte

TABLE 1. Dose dependent manner of PPDE induced cell apoptosis in cultured human cardiomyocytes

Treatment	% of apoptosis		
	0	12.5 mg/ml	25 mg/ml
Medium only	1.0 ± 2.7	10.4 ± 2.8	11.8 ± 3.2
PBS only	2.2 ± 3.1	10.2 ± 3.2	10.8 ± 3.6
Medium + DOXO	9.7 ± 2.4	28.5 ± 4.1[a]	41.2 ± 4.5[a]
Medium + SPDE	0.7 ± 2.5	10.2 ± 2.7	12.4 ± 3.5
Medium + PPDE	9.7 ± 2.6	32.4 ± 3.8[a]	48.6 ± 4.8[a]

Cultured human cardiomyocytes were exposed to medium only, PBS only, medium plus DOXO (0.5 μM), medium plus SPDE (24 h) and medium + PPDE (24 h). Annexin V-FITC apoptosis analysis were determined by FACS. Each value is the mean from eight independent peritonitis episodes.
[a]Significant effect of the agonist compared with medium only control, $^*P < 0.05$.

TABLE 2. Time dependent manner of PPDE induced cell apoptosis in cultured human cardiomyocytes

Treatment	% of apoptosis		
	0	24 h	48 h
Medium only	1.0 ± 2.7	11.5 ± 2.7	12.1 ± 3.2
PBS only	2.2 ± 3.1	10.4 ± 2.6	11.5 ± 3.4
Medium + DOXO	9.7 ± 2.4	34.8 ± 3.6[a]	42.2 ± 4.2[a]
Medium + SPDE	0.7 ± 2.5	12.2 ± 3.2	13.6 ± 3.2
Medium + PPDE	9.7 ± 2.6	48.2 ± 4.7[a]	57.6 ± 4.4[a]

Cultured human cardiomyocytes were exposed to medium only, phosphate buffer solution (PBS) only, medium plus DOXO (0.5 μM), medium + SPDE (stable peritoneal dialysate effluent) (25 mg/ml) and medium + PPDE (peritonitis peritoneal dialysate effluent) (25 mg/ml). Annexin V-FITC apoptosis analysis were determined by FACS. Each value was the mean from eight independent peritonitis episodes.
[a]Significant effect of the agonist compared with medium only control, $^*P < 0.05$.

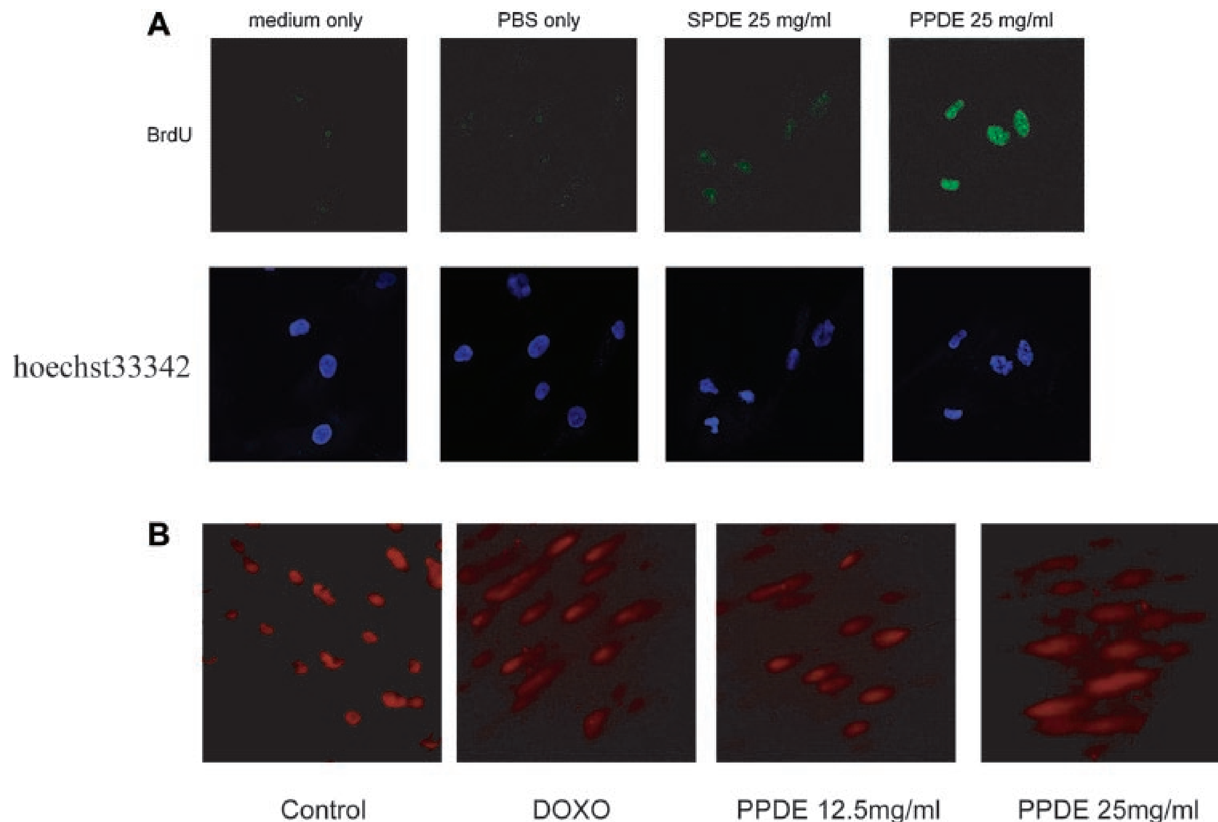

Fig. 3. PPDE treatment induces apoptosis in cultured human cardiomyocytes. A: APO-Brd U™ TUNEL assay. Cardiomyocytes were treated with PPDE, SPDE (25 mg/ml) or DOXO (0.5 μM) as a positive control for 24 h, then stained by the TUNEL method. Nuclei were counter stained with AlexaFluor 488dye. Confocal microscopy of cardiomyocytes showed all nuclei (blue), including apoptotic nuclei (vacuoles). B: Cardiomyocyte DNA damage was determined by the Comet assay. Cardiomyocytes were treated with PPDE (12.5 or 25 mg/ml) or DOXO (0.5 μM) as a positive control for 24 h, and then the Comet assay was performed.

apopotosis (Kim et al., 2003), was used as a positive control. After cell incubation with 25 mg/ml PPDE peritonitis for 24 h, apoptosis was detected by flow cytometry (Tables 1 and 2). TUNEL staining and confocal microscopy confirmed cardiomyocyte apoptosis (Fig. 3A). Analyses indicated little cardiomyocyte apoptosis with non-exposed condition (control group) and exposure with 25 mg/ml SPDE from stable PD patients (Tables 1 and 2). By contrast, doxorubicin and PPDE induced apoptosis in 34.8–48.6% of human cardiomyocytes after treated for 24 h. Finally, PPDE induced DNA damage was determined by Comet assay (Fig. 3B): higher concentrations of PPDE resulted in greater numbers of damaged cells.

PPDE induced Bax increase and suppression of GATA-4 expression in human cardiomyocytes

The Bcl-2 family of proteins are key regulators of the stress-induced apoptotic pathway (Bishopric et al., 2001); to determine their role in regulation of PPDE induced cardiomyocyte apoptosis, mRNA concentrations of prosurvival proteins Bcl-2 and Bcl-x_L and proapoptotic protein Bax were measured in human cardiomyocytes by quantitative real-time RT-PCR (Fig. 4A). Compared to the no-exposure control group, Bcl-2/Bax and Bcl-x_L/Bax ratios dropped significantly following 4 h of PPDE treatment (Fig. 4B; $P < 0.05$ vs. control). Western blotting analysis for Bcl-2, Bcl-xl, and Bax protein expression in the same experimental conditions obtained similar results (Fig. 6A). These data indicated that PPDE treatment decreased Bcl-2/Bax and Bcl-x_L/Bax ratios, resulting in increase Bax expression in human cardiomyocytes.

Transcription factor GATA-4 has been identified as a specific myocardial survival factor which induces transcription and expression of Bcl-2 and which is associated with cell survival (Ancey et al., 2002; Kitta et al., 2003; Aries et al., 2004; Suzuki and Evans, 2004). To characterize mechanisms underlying PPDE activity in human cardiomyocytes, mRNA and protein expression of GATA-4 were measured. For cardiomyocytes exposed to PPDE, GATA-4 mRNA expression decreased fivefold relative to no-exposure control cells by quantitative real-time RT-PCR ($P < 0.05$) (Fig. 5A). Western blots of nuclear GATA-4 protein expression in PPDE exposed human cardiomyocytes also showed lower levels than the control group (Fig. 5B,C), suggesting that PDE during peritonitis treatment decreases levels of GATA-4 gene expression in human cardiomyocytes.

PPDE does not contain inflammatory mediators

To evaluate whether PPDE was enriched in pro-apoptotic mediators, TRAIL, FasL, TNFα, IL-6, and IL-1 were rated by enzyme-linked immunoassay by commercial ELISA kit. Cultured supernatant from peripheral blood mononuclear cells stimulated with lippolysaccharide was used as positive control; TRAIL, FasL, TNFα, and IL-1 were undetectable in 25 mg/ml PPDE (data not shown). The lower limit of sensitivity was 0.70 pg/ml.

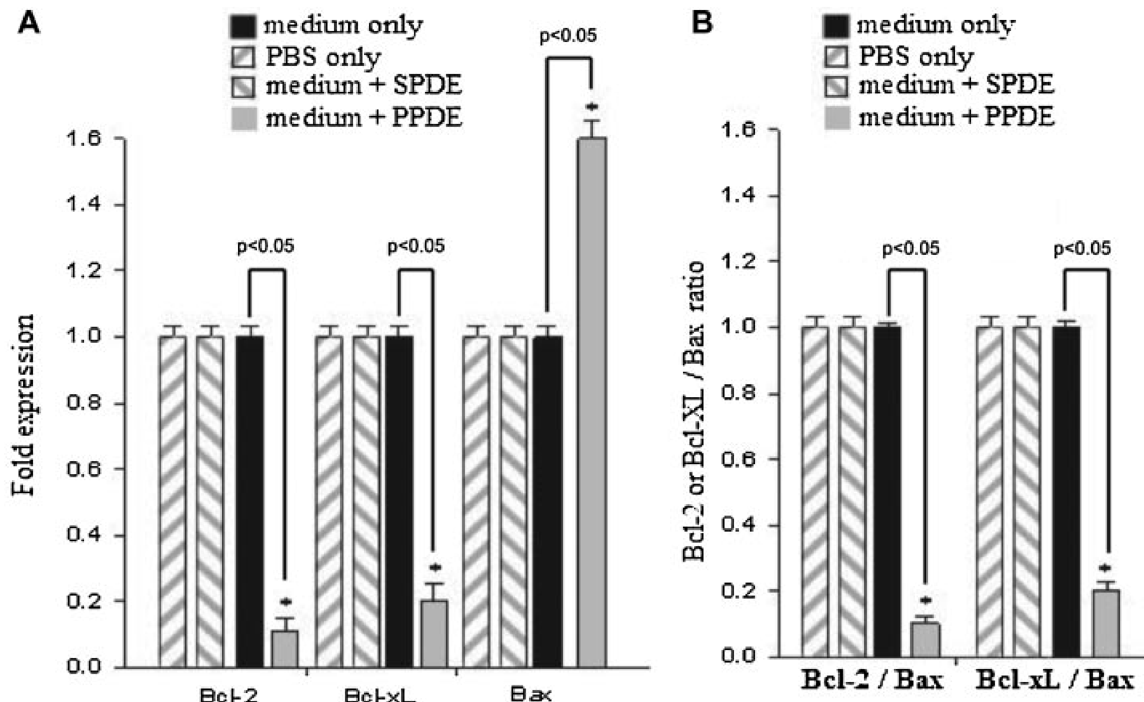

Fig. 4. A: PPDE treatment upregulates Bax gene expression in cultured human cardiomyocytes. Human cardiomyocytes were treated with or without PPDE (25 mg/ml), and then total RNA was prepared following 4 h of treatment. Bcl-2, Bcl-x_L, and Bax mRNA expression levels in cardiomyocytes were determined by quantitative real-time RT-PCR (n = 8). Data are expressed as the mean ± SD of eight different PPDE. *$P < 0.05$ versus control. B: Bcl-2/Bax and Bcl-xL/Bax ratio of experiment (A). Data are expressed as mean ± SD of eight different PPDE. *$P < 0.05$ versus control. (■) No treatment, medium only; (▨) no treatment PBS only; (▧) medium + stable PDE without peritonitis (SPDE) (■) treated with medium and peritonitis peritoneal dialysate effluent (PPDE).

Role of ERK pathway in PPDE induced cardiotoxicity

We next examined possible signaling mechanisms regulating PPDE-induced cardiomyocyte apoptosis. The GATA-4 molecule contains putative ERK phosphorylation sites, and recent studies have shown that some survival factors (Morimoto et al., 2000; Kitta et al., 2001, 2003) induce activity of GATA-4 via MEK/ERK-dependent phosphorylation. Therefore, we explored activity of MEK/ERK signaling pathways in PPDE treated cardiomyocytes. Figure 6 shows ERK phosphorylation significantly reduced in cells exposed to PPDE peritonitis, suggesting that PPDE inhibits the ERK signaling pathway, consistent with the idea that the ERK pathway is crucial for GATA-4 activity and cardiomyocyte survival.

Discussion

Our study demonstrates that PPDE contains potent pro-apoptotic factors and causes an imbalance between proapoptotic and prosurvival pathways, inducing apoptosis in human cardiomyocytes. This study revealed a possible mechanism of PD-related, peritonitis-induced cardiotoxicity. These novel findings constitute the first direct evidence linking PD peritonitis and cardiomyocyte apoptosis. Cardiovascular events are the major cause of death in PD patients with peritonitis. Our findings demonstrate the central role of apoptosis in PD peritonitis-associated cardiovascular events, and provide an explanation for the high incidents of cardiovascular events in PD-related peritonitis.

Cardiomyocyte death is important in the pathogenesis of cardiac disease in end stage renal disease (Parfrey and Foley, 1999). Cardiomyocyte death induces LV dilatation with compensatory LV hypertrophy, and eventually leading to systolic dysfunction. LV hypertrophy appears to be an important, independent, determinant of survival in patients with end stage renal diseases (Silberberg et al., 1989). Our study yields direct cellular evidence of PPDE from PD patients as cardiotoxic. In end-stage renal disease, cardiomyocyte death may be caused by continual LV overload, decreased large and small coronary vessel perfusion, hyperparathyroidism, and malnutrition (Parfrey and Foley, 1999). Our data provide another possible cause of cardiac cell death in patients undergoing PD.

Recent studies have proven various cardiac pathological states associating with cardiomyocyte apoptosis (Olivetti et al., 1997; Haunstetter and Izumo, 1998; Narula et al., 1999). Our study found that PPDE induced cardiomyocyte apoptosis and severity varied when diverse pathogens induced peritonitis (data not shown). Toxicity to cardiomyocytes was most profound with exposure to *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Streptococcus viridans*. Gram negative pathogens such as *Escherichia coli* and Proteus vulgaris were less cytotoxic. Prior animal study shows both endotoxin and exotoxin exposure inducing myocardial depression (Natanson et al., 1989; Sibelius et al., 2000; Ramana et al., 2006). Interestingly, pretreatment of animals or cells with lipopolysaccharide (LPS) induced LPS tolerance and largely reduced inflammation and dysfunction of the rat myocardium (Barroso-Aranda et al., 1991; Madorin et al., 2001). Further study should delineate effects of pathogen-related toxins on the PPDE-induced cardiotoxicity.

A second possible cause of cardiomyocyte apoptosis is inflammatory or pro-apoptotic mediators and cytokines in PPDE. Expression of inflammatory mediators and cytokines in

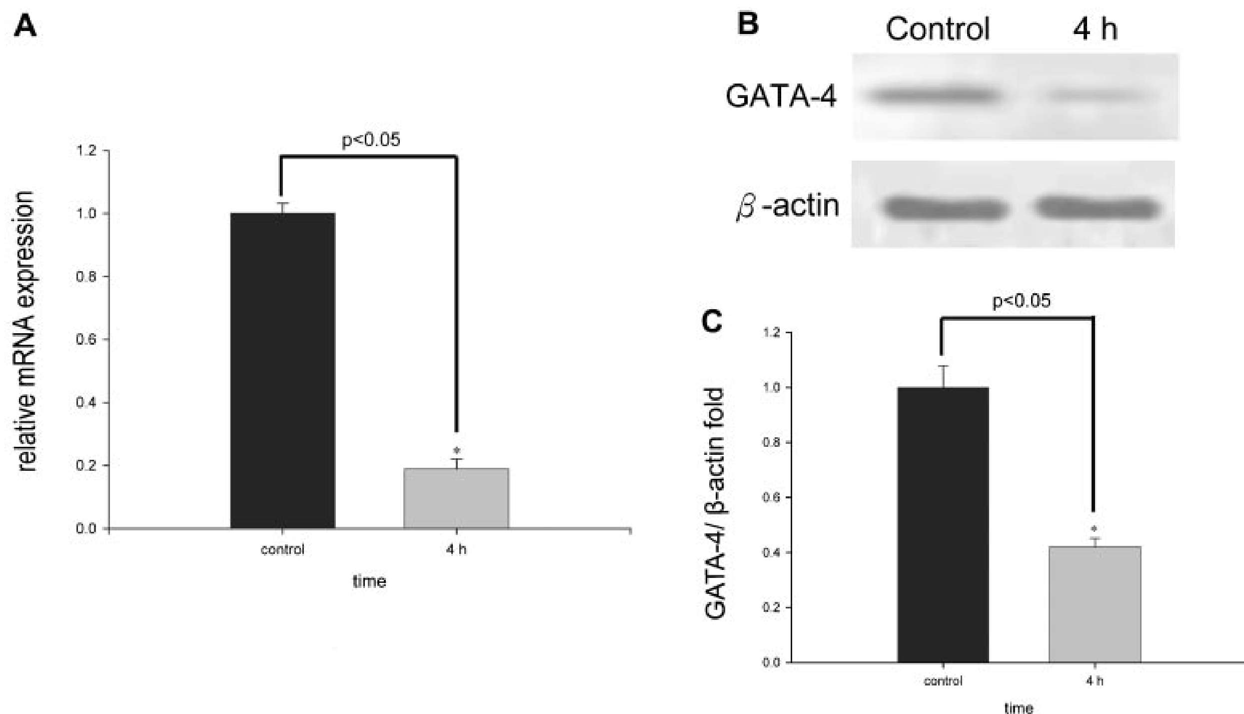

Fig. 5. Effects of PPDE on expression of cardiac GATA-4 mRNA and protein. Cultured human cardiomyocytes were treated with or without PPDE (25 mg/ml), and then protein extracts and total RNA were prepared following 4 h of treatment. A: GATA-4 mRNA expression levels in cultured human cardiomyocytes as determined by quantitative real-time RT-PCR. B: Western blot showing GATA-4 protein levels. C: GATA-4 protein levels in cultured human cardiomyocytes, ascertained by densitometry. Data are expressed as mean ± SD of eight different PPDE. *$P < 0.01$ versus control.

PPDE is reported to increase during acute peritonitis, then return to control levels (Lai et al., 2000; Cory and Adams, 2002; Wang and Lin, 2005). Inflammatory mediators and cytokines have been cited as playing a role in cardiomyocyte apoptosis and clinical cardiac dysfunction (Haudek et al., 2007). With TRAL, FasL, TNFα, or IL-1 undetectable in 25 mg/ml PPDE, inflammatory mediators and cytokines in PPDE may not contribute greatly to cardiomyocyte apoptosis.

GATA-4 is a survival factor in terminally differentiated cardiac myocytes (Aries et al., 2004; Suzuki and Evans, 2004) and may be an essential component of adaptive response of the adult heart (Suzuki and Evans, 2004). Experiments have shown 50% reduction in GATA-4 levels impairing drug-induced stress responses, yet not interfering with normal embryonic and postnatal mouse development (Suzuki and Evans, 2004). Our results indicate a mechanism by which PPDE inhibits transcription of the GATA-4 gene: lower GATA-4 levels in cardiomyocytes exposed to PPDE would impair their ability to respond to cardiac work or to stimuli that produce cardiac stress and then induce apoptosis. By our current findings, GATA-4 restoration or prevention of GATA-4 depletion may prove effective cardioprotection in PD patients with peritonitis.

The essential role of GATA-4 as a survival factor may be explained, in part, by its function as an upstream activator of the antiapoptotic Bcl-2 gene family. Previous research (Chen et al., 2001b) has shown that over expression of Bcl-2 attenuates myocardial apoptosis. In the present study, exposure of cardiomyocytes to PPDE caused decreases in the Bcl-2/Bax and Bcl-x_L/Bax ratios. Moreover, Bcl-2 is a major regulator of mitochondrial permeability and hence prevents activation of the "intrinsic" mitochondria-dependent apoptosis pathway (Foo et al., 2005). The intrinsic apoptotic pathway is activated in cardiac myocytes in response to various extracellular and intracellular stimuli (Foo et al., 2005). Our results provide evidence that activation of the intrinsic apoptotic pathway may be a possible mechanism for PPDE induced cardiomyocyte apoptosis.

Several studies have suggested that in a subset of cell types, activation of ERK can protect against pro-apoptotic stimulants (Jarpe et al., 1998). However, inhibition of apoptosis by ERK depends upon cell type and stimuli. The present study found that the ERK pathway is involved in protection against PPDE-induced cultured cardiomyocyte apoptosis. Interestingly, GATA-4 contains putative ERK phosphorylation sites. Prior studies show HGF and endothelin-1 activating GATA-4 via MEK-ERK pathway-dependent phosphorylation, with dominant MEK mutant blocking HGF-induced Bcl-x_L expression in cardiac muscle cells (Ancey et al., 2002; Kitta et al., 2003). Studying phosphorylation of GATA-4 by ERK pathway will elucidate the role of MEK/ERK/GATA-4 pathway in PPDE-induced cardiomyocyte apoptosis. Apoptosis is not simple, linear chain reaction. Recent study found sustained inflammation activating multiple cell death pathways and spawns cardiomyocyte apoptosis (Haudek et al., 2007). Further research will focus on possible extrinsic apoptotic pathway involvement and relative importance of intrinsic and extrinsic pathways in PPDE-induced cardiomyocyte apoptosis. Unlike necrosis, viewed as basically irreversible, the step-by-step nature of apoptosis suggests existence of reversible checkpoints. Our results indicate therapeutic regimens that block intrinsic apoptosis signaling pathways presumably attenuate PPDE-induced cardiotoxicity.

In conclusion, this study demonstrates for the first time that PPDE contains potent pro-apoptotic factors that regulate

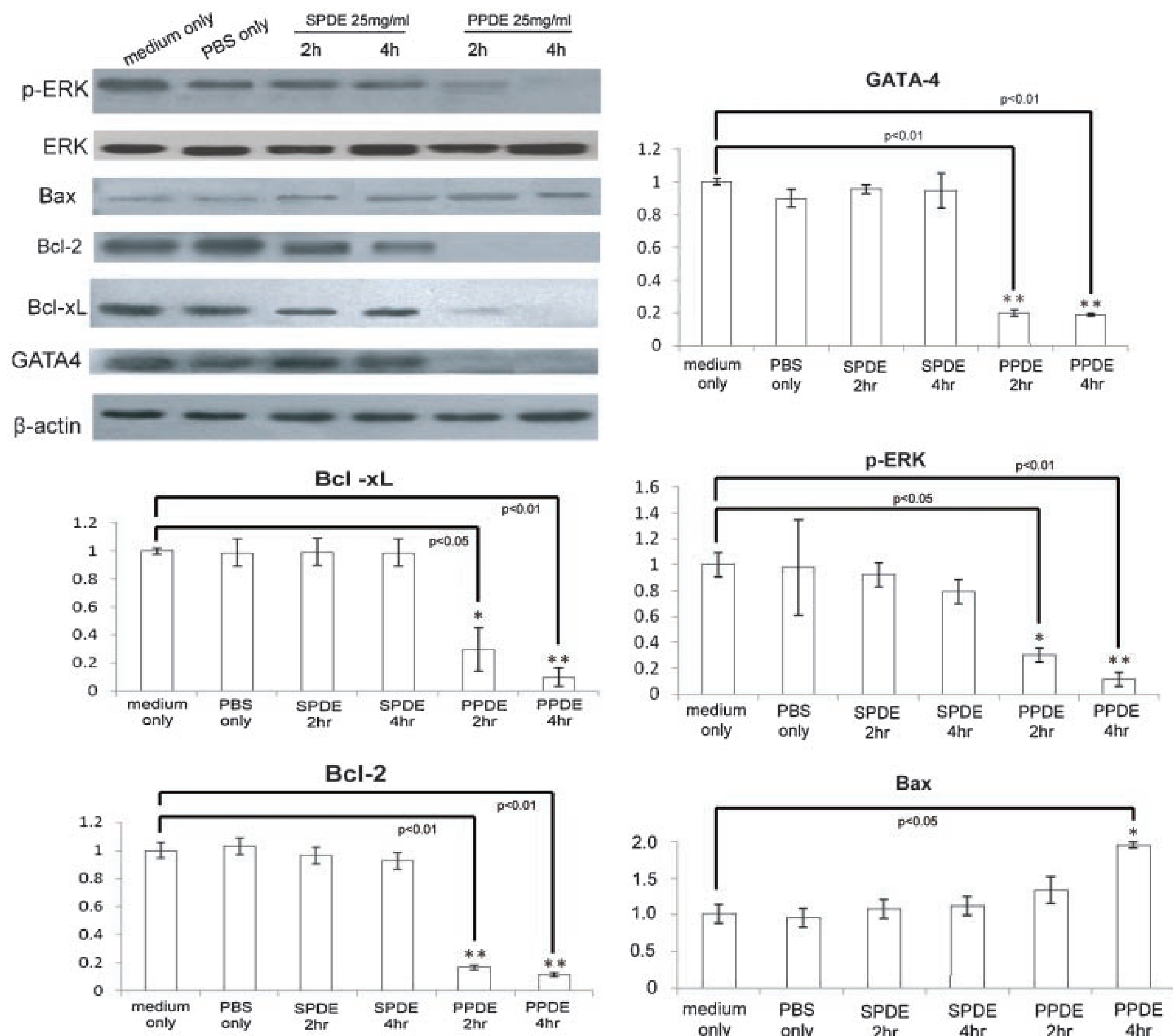

Fig. 6. PPDE treatment reduces ERK phosyhorylation, GATA4, Bcl-2, Bcl-xL expression and enhances Bax expression in cultured human cardiomyocytes. Cardiomyocytes were treated with medium only, PBS only, SPDE (25 mg/ml) for 2 and 4 h and PPDE (25 mg/ml) for 2 and 4 h. Cell lysates were separated by SDS–PAGE and specific monoclonal antibodies were used to detect phosphorylated and total ERK, and Bax, Bcl-2, Bcl-xL, GATA4 expression. Representative blots from eight separate experiments were shown. Quantitative densitometry expressed as phosphorylated protein relative to total protein. Data are expressed as the mean ± SD of eight different PPDE. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control. Medium only, PBS only and SPDE (25 mg/ml) in medium were used as negative controls.

expression of GATA-4 and Bcl-2 families, inducing cultured cardiomyocyte apoptosis. Findings illustrate a pivotal role of apoptosis in PD peritonitis-associated cardiovascular events, explain high cardiac mortality in PD-related peritonitis, and pinpoint apoptotic events as a marker and potential therapeutic target for PD peritonitis-induced cardiotoxicity.

Acknowledgments

This study was partially supported by the grant of China Medical University (CMU97-212).

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