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Phosphorylation of Bad by Protein Kinase-C Activator Can Attenuate Tongue Muscle-derived Stem Cell Death Caused by Oxidative Stress

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Abstract **Background:** Improving the survival of transplanted cells is a critical issue in the use of stem cell transplantation therapy for cardiovascular disease. Many transplanted cells are lost through apoptosis or necrosis triggered by hypoxia or superoxide. **Methods and Results:** Tongue muscle-derived Sca-1(+) cells were isolated from the mouse and cultured for 2 weeks and then exposed to hydrogen peroxide (H_2O_2; 0.2 mM) for 24 h. The extent of cell death by either apoptosis or necrosis was measured by fluorescence-activated cell sorting using annexin V and propidium iodide. The effects of the protein kinase C activator phorbol 12-myristate 13-acetate (10 μM) on H_2O_2-induced cell death were investigated. Phorbol 12-myristate 13-acetate significantly improved cell survival (49.2 % ± 8.1 % to 64.3 % ± 5.1 %, $P < 0.01$) while its inhibitor, chelerythrine (1μM), abrogated the effect. The phosphorylation of Bad at serine112 residue was augmented by phorbol 12-myristate 13-acetate; which was inhibited by chelerythrine. **Conclusions:** Protein kinase C activator is useful to prevent cell death of tongue muscle-derived Sca-1(+) cells through the activation of Bad at serine 112 residue.

Key words: stem cells, cell death, oxidative stress, protein kinase C, bad

Introduction

Clinical trials have shown the efficacy of bone marrow-derived stem cell transplantation therapy for the treatment of intractable heart failure after myocardial infarction.[1,2] However, most of the beneficial effects of cell transplantation therapy may be attributed to paracrine effects rather than generating new cardiomyocytes from transplanted cells.[3,4] Improvement of cardiac function has been reported by cardiac-derived stem cell transplantation,[5,6] but the number of cells that differentiate into cardiomyocytes remains low. Several studies have shown that transplanted cells can be lost from the site of injection, either by washout by the blood stream or due to cell death after transplantation.[7,8] Transplanted cells are placed in hypoxic and superoxide and inflammatory cytokines-rich environment, which can lead to cell death of the transplanted cells by either apoptosis or necrosis.[8,9] Recently, we have reported a new tissue-derived stem cells from tongue muscle (TDSC) which can differentiate into cardiomyocytes and improves cardiac function and survival rate after myocardial infarction in mice.[10] However, most of the TDSC were lost after transplantation. Thus we need to investigate a new strategy to improve the survival of TDSC after transplantation. Here, we studied a strategy to improve the TDSC survival

after exposure to superoxide.

Materials and Methods

Animals

C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan) and bred in the Animal Center of Yamaguchi University Graduate School of Medicine. All experiments were approved by the Institutional Animal Care and Use Committee of Yamaguchi University. This investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Isolation and culture of TDSCs

As described in our previous study,[10] TDSCs were isolated using a magnetic cell sorting system (MACS, Miltenyi Biotec K.K. Tokyo, Japan). In brief, 8- week old C57BL/6 mice were anesthetized with sodium pentobarbital (50 mg/kg, I.P.) and euthanized by neck translocation. Tongue muscle from 3 mice was minced and digested with 10 mL of type II collagenase (0.4%, Worthington Biochemical Corp, NJ, USA) for 30 min at 37℃ with agitation. Collagenase was neutralized by adding PBS containing 2% serum albumin. Anti-Sca-1 antibody conjugated with biotin (1:100 dilution, Miltenyi Biotec) was added to isolated cells along with anti-biotin microbeads (1:4 dilution, Miltenyi Biotec). The cells were then incubated for 10 min at 4℃ and sorted twice using magnetic cell separation columns (Miltenyi Biotec). The isolated cells were cultured for 2 weeks in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), antibiotics, and growth factors including platelet-derived growth factor, (PDGF-BB, 10 ng/mL, R&D systems), epidermal growth factor (EGF, 10 ng/mL, Sigma), and leukemia inhibitory factor (LIF, 2.5 ng/mL, Sigma) at 37℃ in 5% CO_2. The Sca-1 positivity after 2 weeks of culture yielded more than 90% verified by flow cytometry analysis.

Microscopic observation

Cultured cells were observed by a microscope (Keyence, Japan) at x100 magnification and imaged at each stage.

Annexin V-FITC assay using flow cytometry

Apoptosis, necrosis, and apo-necrosis were assayed using the annexin V- fluorescein isothiocyanate (FITC) apoptosis detection kit (Sigma), according to the manufacturer's protocol.[11] In brief, cells were trypsinized and washed with serum-containing medium. Cells (1×10^5) were resuspended in 500 μL of annexin V binding buffer. Five μL of annexin V-FITC and equal volume of PI were added, and incubated for 5 min at room temperature in the dark. Annexin V-FITC binding was analyzed by flow cytometry (FC500; Beckman Coulter, USA) with excitation at 488 nm and emission at 530 nm using an FITC signal detector. PI staining was analyzed by the phycoerythrin emission signal detector. Upon apoptosis, phosphatidylserine translocates from the cytoplasmic side of the plasma membrane to the cell surface. Annexin V has a strong Ca^{2+}-dependent binding affinity for phosphatidylserine and is used as a probe to detect apoptosis.[10] Cells bound to annexin V-FITC with an intact plasma membrane (without PI staining) were determined to be apoptotic.[12] Cells that had lost membrane integrity resulting in PI staining were defined as necrotic.[12] Cells stained by both annexin V-FITC and PI were defined as apo-necrotic.[12]

Western blotting

Cultured TDSCs were lysed with RIPA buffer containing a protease inhibitor cocktail (Complete, Roche, IN, USA) in a glass homogenizer on ice. Homogenates were mixed with an equal volume of lysis buffer and centrifuged at 1,000 $\times g$ for 20 min. The supernatant was used to measure protein concentration by the Bradford method. An equal amount of protein from each group (50 μg) was resolved by electrophoresis on a 10% polyacrylamide gel (SDS-PAGE) and transferred to a nitrocellulose membrane. Non-specific binding on the membrane was blocked by using 5% non-fat milk diluted in Tris-buffered saline for 1h, and then blots were incubated with primary antibodies against Bad, phospho-Bad (Ser112 , Ser136), Akt, and phospho-Akt (Ser473) and β-Tubulin as a loading control overnight at 4℃. Blots were

incubated with secondary antibody for 2h at room temperature. Bands were visualized by enhanced chemiluminescence (ECL) and quantified using a Lumino image analyzer (LAS-1000; Fuji Film, Japan). Band density was normalized by loading control *β*-Tubulin and expressed as the ratio to control group.

DNA Microarray

TDSCs were cultured for 2 weeks, and 1×10^6 cells were used for gene chip analysis (Affymetrix). The expressed genes were analyzed using Gene Spring Viewer (Agilent Technologies).

Protocols

Microscopic observation and FACS analysis

TDSCs cultured for 2 weeks were trypsinized and resuspended in 6-well culture plate (Corning 3516, USA) at a density of $2x\ 10^5$ cells per well in DMEM supplemented with 0.5% FBS. Four groups were examined: in the control group, cells were cultured without H_2O_2 for 24h; in H_2O_2 group, cells were cultured for 24h in 0.2 mM H_2O_2; in H_2O_2 with phorbol 12-myristate 13-acetate (PMA) pretreatment group, PMA (1, 5, and 10 μM) was added for 30 min and then washed by centrifugation (1000g, 5 min), then cultured again with 0.2 mM H_2O_2 for 24h; in PMA with chelerythrine pretreatment group, PMA (10 μM) and chelerythrine (1 μM) were added for 30 min and washed, then cultured again for 24h with 0.2 mM H_2O_2. The cells of each group were isolated by trypsinization after 24h culture and used for FACS analysis. The number of experiment was 6 for each group.

Signal transduction of Bad and Akt

TDSCs cultured for 2 weeks were resuspended in 6 well-culture dishes containing $7x\ 10^5$ cells in each well. In Bad phosphorylation study, in control group PMA was not added; in PMA group PMA (10 μM) was added for 30 min and then cells were harvested for Western blot. In chelerythrine + PMA group, PMA (10 μM) with chelerythrine (1 μM) were added for 30 min and then cells were harvested for analysis. The number of experiment was 4 for each group.

In Akt phosphorylation study, in control group PMA was not added. In PMA group, PMA (10 μM) was added for 30 min and used for Western blot. In chelerythrine + PMA group, PMA (10 μM) and chelerythrine (1 μM) were added for 30 min and used for the analysis. The number of experiment was 4 for each group.

Statistical analysis

Values are presented as means ± standard error of the mean value (SEM). Cell survival rate, the rate of cell death mode, and the extent of phosphorylation of Bad and Akt were tested by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test (StatMate, ATMS Co. Ltd., Tokyo, Japan). $P < 0.05$ was considered significant.

Results

FACS analysis of freshly isolated TDSCs showed 92 ± 2% of Sca-1 (+). Representative data from FACS analysis of annexin V (horizontal axis) and PI (vertical axis) for each group are shown in Fig.1A. The representative microscopic observation for each group was shown in Fig. 1B.

Cell survival rate estimated by FACS

In the control group, the cell survival rate was 83.8 % ± 11.7% (Fig. 1C). In the H_2O_2 group, the cell survival rate was 49.2 % ± 6.1% ($P<0.01$ vs. control). In the H_2O_2 with PMA (10 μM) pretreatment group, the cell survival rate increased to 64.3 % ± 5.1% ($P<0.01$ vs. H_2O_2 group). The effect of PMA was not dose-dependent in the examined range (1-10 μM). In PMA and chelerythrine pretreatment group, the pro-survival effect of PMA was abrogated (N.S. vs. H_2O_2 group).

Different mode of cell death

Fig. 1D-F shows the rate of apo-necrotic, apoptotic, and necrotic cell rate depending on the criteria using the PI and Annexin V expression[11-13] in each group. Compared to the control group, apo-necrotic and apoptotic cells increased significantly by H_2O_2 (both $P<0.01$ vs. control), but necrotic cells were not changed (N.S. vs. control). Apoptotic cells decreased significantly by PMA pre-treatment in all doses examined, ($P<0.01$ vs. H_2O_2

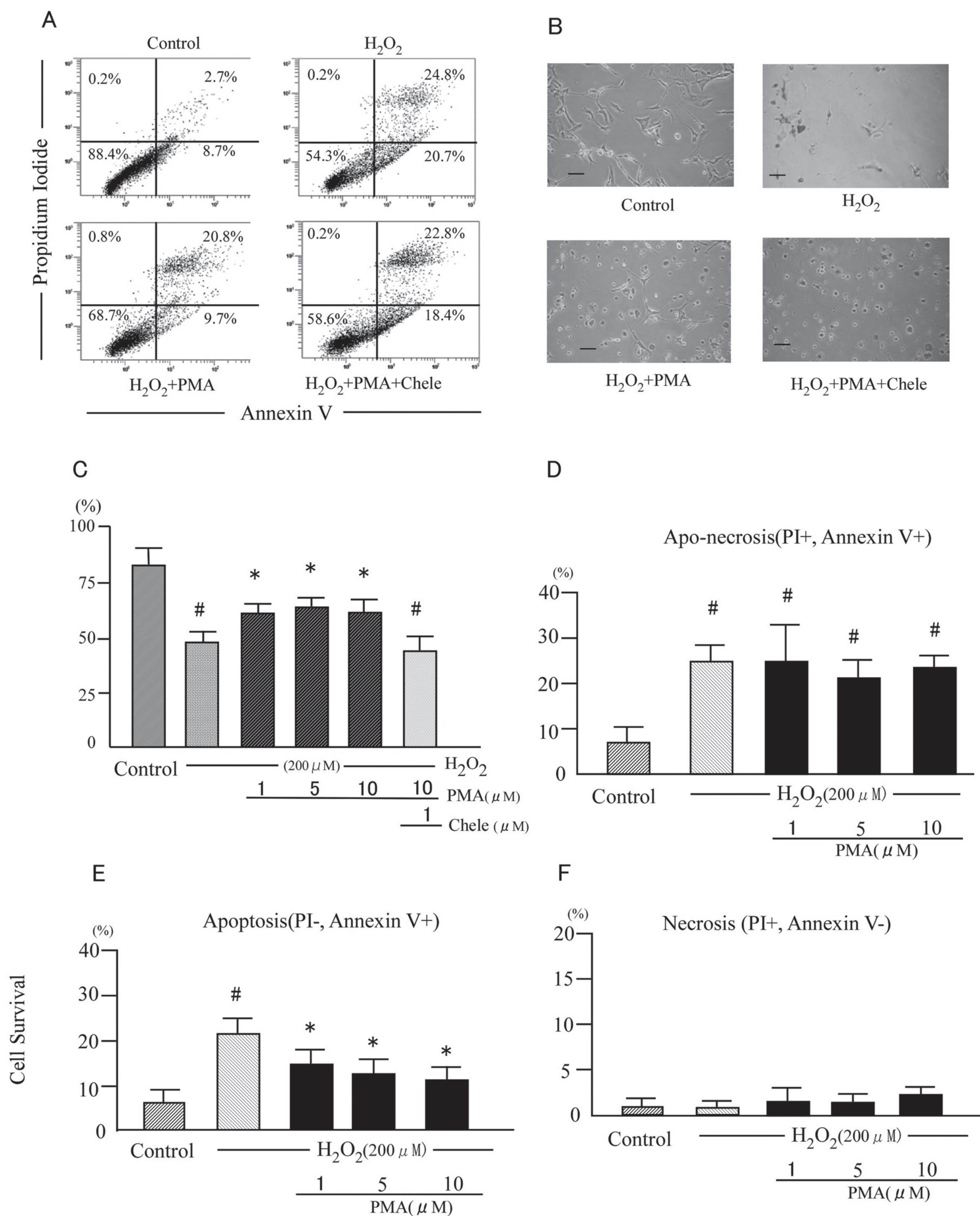

Fig. 1 A-F

Effect of PMA on 24h H_2O_2 exposure to TDSC. Representative data for FACS analysis of annexin V and propidium iodide, and photomicroscopic observation (x 200, bar indicates 20μm) (A,B) and the result of cell survival analyzed by FACS (C), and the cell death mode (D-F). PMA pretreatment increased the cell survival and its effect was abrogated by chelerythrine. Apo-necrosis (PI+, annexin V+) and apoptosis (PI-, annexin V+) was significantly increased by H_2O_2. PMA significantly suppressed apoptosis ($P<0.01$) dose-dependently, but not apo-necrosis or necrosis. # $P<0.01$ vs. control , * $P<0.01$ vs. H_2O_2. N=6 for each group. PMA: phorbol 12-myristate 13-acetate.

group), but apo-necrotic and necrotic cells were not changed (both N.S. vs. H_2O_2 group).

Bad phosphorylation by PMA

Bad phosphorylation (Ser136 and Ser112) was determined by Western blotting (Fig. 2A). Bad (Ser112) phosphorylation was significantly increased by PMA (P<0.01 vs. control), which was inhibited by chelerythrine (Fig. 2B). Conversely, Bad (Ser136) phosphorylation was not influenced by PMA (Fig. 2C).

Akt phosphorylation

Akt phosphorylation was determined by Western blotting (Fig. 3A-C). Akt (Ser473) phosphorylation was not influenced by PMA (Fig. 3C).

Protein kinase C isoform expression in TDSCs

TDSCs expressed protein kinase C (PKC) isoforms α, β1, γ, δ, ε, ν, μ, ι, and η, but did not express isoforms θ and ζ shown by the DNA microarray analysis.

Discussion

Cell therapy for myocardial regeneration is hindered by a low efficiency of cell engraftment associated with this treatment. Terrovitis et al.[8] indicated that only 3.8% of stem cells injected into an infarcted rat heart retained after 24h. One of the reasons for the acute loss of transplanted cells may be caused by washed away by the blood stream. However, in the long term loss of stem cells could be caused by cell death, either through necrosis or apoptosis, when cells are exposed

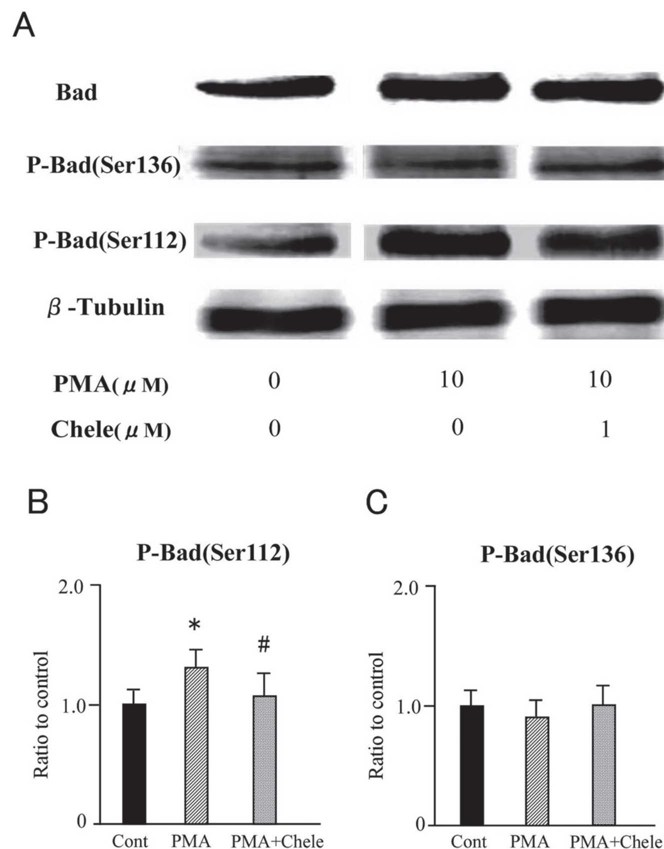

Fig. 2 A-C

Effect of PMA on the phosphorylation of Bad. Western blotting of Bad, P-Bad (Ser136) and P-Bad (Ser112) are shown (A) and the quantitative result of P-Bad (Ser112) and P-Bad (Ser136) are shown (B,C). P-Bad (Ser112) was increased significantly by PMA (P<0.01) and inhibited by chelerythrine, but P-Bad (Ser136) was not affected by PMA. β-tubulin is a loading control. * P<0.01 vs. control, # P<0.05 vs. PMA. (N=4).

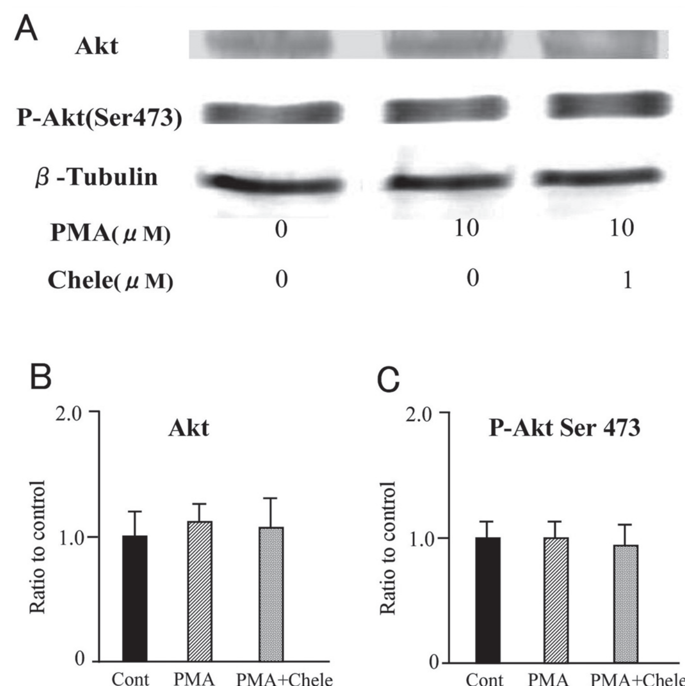

Fig. 3 A-C
Effect of PMA on the phosphorylation of Akt (Ser 473).Western blot of Akt and P-Akt (Ser473) were shown (A). The quantitative result of Akt and P-Akt (Ser473) was shown (B,C). P-Akt (Ser473)was not changed by PMA (10μM) pretretment, and chelerythrine (1μM) pretreatment did not affect on it. *β*-tubulin is a loading control. (N=4).

to hypoxia, superoxide[7-9] or inflammatory cytokines.[14] Thus, the prevention of cell death after stem cell transplantation is an important issue to investigate to facilitate the efficacy of stem cell transplantation. Ischemic preconditioning or pharmacological preconditioning has been shown to be effective in improving survival in skeletal myoblasts[4] and cardiac-derived stem cells.[15-17] However, these previously reported interventions are less effective on TDSC in our preliminary study (data not shown). Here, we demonstrate that PMA promoted cell survival by inhibiting apoptosis induced by H_2O_2 in TDSC, and this effect was abrogated by PKC inhibitor chelerythrine, indicating PKC activation plays an important role for TDSC survival.[18]

Survival of transplanted TDSCs

As shown in our previous study,[10] TDSCs can differentiate into beating cardiomyocyte-like cells. However, most of transplanted TDSCs died within 3 months. Thus, it is critical to improve cell survival of TDSCs after transplantation. Microarray analysis done in our study showed that TDSCs express several isoforms of PKC, thus we tested the effect of PKC activation on cell survival after exposure to superoxide.

Cell death caused by H_2O_2 and the effect of PMA

The survival of TDSCs decreased by 24h H_2O_2 exposure. Pretreatment with PMA improved survival of TDSCs after 24h H_2O_2 exposure. The effect of PMA was abrogated by a selective inhibitor of PKC, chelerythrine.[19]

The role of Bad in cell death

Bad is a member of the Bcl-2 family and promotes apoptosis by forming heterodimer with Bcl-2 and Bcl-xL.[20-22] Bad locates on the outer mitochondrial membrane and translocates to the cytoplasm upon activation. The phosphorylation of Bad plays a critical role in mediating cell death and survival.[21] Phosphoinositide 3-kinase (PI3K)/Akt has been shown to phosphorylate Bad (Ser136) and inhibit the apoptotic pathway of Bad.[22]

The role of PKC on cell death and survival

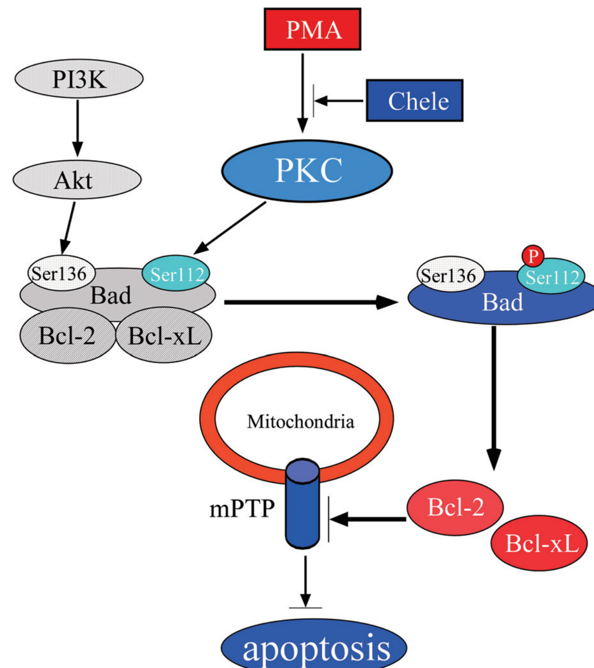

Fig. 4 Signaling cascade of PKC and PI3K in relation to Bad phosphorylation.

PMA activates PKC which phosphorylates Bad (Ser112), followed by the dissociation of Bcl-2 and Bcl-xL from Bad, which inhibits opening of mitochondrial permeability transition pore (mPTP) and subsequently attenuates apoptosis. PI3K activates Akt, which phosphorylates Bad (Ser136), but this pathway was not involved in the PMA-PKC pathway.

PI3K: phosphatidylinositol-3 kinase; PMA: phorbol 12-myristate 13-acetate.

The inactivation of PKC induces cell death in tumor cells and embryonic stem cells.[19,22] This has been shown to be related to PKC-μ and -ε. On the other hand, PKC inactivation by chelerythrine does not influence on the survival of mature cardiomyocytes.[23] In our study, chelerythrine (1μM) abolished the effect of PMA on reducing cell death of TDSCs upon exposure to H_2O_2. This indicates that PKC signaling is crucial for survival of TDSCs. Bad expressed in lung cancer cells and the phosphorylation of Bad (Ser112, Ser136) was induced by either PKC and PI3K/Akt pathways.[22] Villalba et al.[24] reported that PMA protected T cells from Fas-induced apoptosis through phosphorylation of Bad (Ser112) by PKC-θ. We showed that PMA phosphorylated Bad (Ser112) in TDSCs but not Bad (Ser 136). Our gene-chip analysis of TDSCs showed that PKC-θ is not expressed, indicating that other isoforms could phosphorylate Bad (Ser112) in TDSCs. Bertolotto et al.[25] showed that PKC-θ and -ε phosphorylate Bad (Ser112) in T cells. Taking these evidences together, PKC-ε may be a candidate isoform for the phosphorylation of Bad (Ser112) in TDSCs. Further studies on this signaling pathway are required. Figure 4 shows a schematic signaling cascade of PKC and PI3K/Akt pathways on Bad (Ser112) and Bad (Ser136) phosphorylation and the downstream cascade of apoptosis.[20-22] In our experiment, PI3K/Akt signaling was not involved in the effect of PMA on the protection of TDSCs from superoxide insult. A cross-talk between pro- and anti-apoptotic pathways at the level of PKC and Akt has been reported, but in our study, the phosphorylation of Akt (Ser 483) and Bad (Ser 136) was not observed, thus such cross talk is not working in the effect of PMA on cell survival of TDSCs.[26,27] The effect of PMA is effective when the stem cells are exposed to PMA in advance, then can transplanted to the tissues. However, superoxide dismutase (SOD) may be effective to diminish apoptosis induced by superoxide, this effect may not be effective by the pre-

medication as preconditioning. So the SOD should be administered for a long period after the transplantation of the stem cells. Thus, PMA as a strategy for preconditioning may be much easier to utilize.

Limitation

PMA can promote tumor cell progression.[28] This should be taken into account when using this drug for improving cell survival under superoxide-rich conditions. Using modalities other than PMA to activate PKC may be an alternative method to increase cell survival in TDSCs. The early loss of injected cells from the heart could be attributed to wash-out of injected cells from the beating heart. Thus, the effect of PMA pre-treatment to TDSC should be tested on in vivo model in the future study.

In conclusion, PMA pretreatment can improve survival of TDSC exposed to superoxide through activation of PKC. The phosphorylation of Bad (Ser 112) plays a key role to suppress apoptosis of TDSC upon exposure to superoxide.

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Conflict of Interest

The authors state no conflict of interest.

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