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PAF exerts a direct apoptotic effect on the rat H9c2 cardiomyocytes in Ca^{2+}-dependent manner

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Abstract

Background: Previous studies suggested that platelet-activating factor (PAF) plays an important role in ischemic diseases. Apoptosis has been implicated in myocardial infarction-related cell death. The present study was designed to determine whether PAF could induce apoptosis in cardiac myocytes and the underlying mechanisms by which PAF causes apoptosis.

Methods: H9c2 cardiac myocytes were used to investigate the effect of PAF on intracellular calcium concentration, cell viability and cell apoptosis. Signaling pathway of caspase-3, cytochrome *c* and MAPK (ERK, JNK, p38) was determined during the PAF induced apoptosis.

Results: First, our results showed that treatment of H9c2 cardiomyocytes with PAF (0.2 to 20 μM) caused apoptosis in these cells and the apoptotic process was suppressed by either BN52021 (an antagonist of PAF receptor) or BAPTA/AM (an intracellular Ca^{2+} chelator), suggesting an involvement of PAF and its receptor mediated calcium-dependent signaling. Second, we found that activity of p38-MAPK (mitogen-activated protein kinase) and caspase-3 was elevated in the cells treated with PAF, without altering activity of ERK and JNK, and that PAF-induced enhancement of caspase-3 activity was attenuated by application of either BAPTA/AM or SB203580 (p38 inhibitor). Furthermore, PAF-induced apoptosis and release of cytochrome c from mitochondria was blunted by SB203580, and PAF-induced enhancement of p38 activity was also attenuated by BAPTA/AM.

Conclusion: Our data implicate that a PAF and its receptor in triggering apoptosis occurs in cultured H9c2 cardiac myocytes via a calcium-dependent p38 MAPK activated cytochrome *c*/caspase-3 apoptosis signaling pathway.

Keywords: PAF; Ca^{2+}; p38; Apoptosis; Ischemia; Caspase-3

1. Introduction

Platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine, PAF) is an autacoid, which has been shown to have profound effects on many systems, including muscle, vascular tissue and brain [1,2]. As far as cardiovascular diseases are concerned, PAF as a major mediator seems to be involved in ischemia, infarction and sudden cardiac death [3]. Under normal physiological conditions, PAF is minimally expressed; however, under conditions of oxidative stress, as occurs in ischemia/reperfusion (I/R) injury, PAF is released by neutrophils and monocytes and is expressed on the outer leaflet or luminal side of endothelial cells [4]. Furthermore, evidence implicating PAF synthesis in the heart is provided by detection of PAF release from animal and human myocardial tissue under immunologic and non-immunologic (e.g. ischemia)

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stimuli [5]. Recently, Dr. Elizabeth and co-workers demonstrated PAF acetylhydrolase (PAF-AH, an isolated naturally occurring enzyme) hydrolyzes PAF, renders PAF inactive and prevents I/R injury [6]. These studies strongly suggested that PAF probably involved in process of I/R injury and directly exerted injury on cardiac myocytes.

Apoptosis is a specific type of cellular death observed in a variety of tissues, under physiological or pathological conditions [7]. In recent years it has been recognized that apoptosis is involved in cardiac myocyte death resulting from I/R injury [8,9]. The role of PAF and its receptor has been studied within the context of apoptosis. The contribution of PAF to hepatocyte apoptosis was tested in an ethanol/endotoxin model of alcoholic hepatitis [10]. In the central nervous system PAF and/or the PAF receptor appear to have dual effects [see below]. On the one hand, PAF is required for neuronal survival. However, additional evidence suggests that PAF induces neuronal apoptosis. For example, Bennett and co-workers reported that expression of PAF receptor mRNA is restricted to neurons undergoing apoptosis after excitotoxic injury *in vivo* [11]. Hostettler and coworkers demonstrated that PAF induces cell death in both astrocytes and oligodendrocytes and that the effects are prevented by antagonists of the receptor [12]. Apoptosis can be induced in cells by a variety of mechanisms. The ability of PAF to induce apoptosis has been reported to be mediated by caspase-3 in some cases [12]. Furthermore, Lu's group has also demonstrated PAF-induced apoptosis is blocked by Bcl-2 in rat intestinal epithelial cells [13]. It is well known that release of Ca^{2+} into the cytosol is required for apoptosis in many cases and overload of intracellular Ca^{2+} ($[Ca^{2+}]_i$) plays a key role in process of apoptosis [14]. Increase in intracellular Ca^{2+} induced by PAF has been consistently observed in the smooth muscle of the intestine, stomach and uterus [15–17].

As stated above, both PAF and apoptosis may have an etiological role in myocardial ischemia. Signaling through the PAF receptor can lead to the stimulation or inhibition of apoptotic responses, depending on the cell type studied. Unfortunately, the effect of PAF on apoptosis in cardiac myocytes has not been elucidated yet. We also recently found that PAF could induce $[Ca^{2+}]_i$ overload in the cardiac myocytes of guinea pig [18,19]. These observations promoted us to investigate whether PAF could induce apoptosis of cardiac myocytes through a Ca^{2+}-dependent mechanism.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and other cell culture reagents were obtained from Gibco (Grand Island, NY, USA). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) detection kit was purchased from Roche Company (Penzberg, Germany). Caspase assay system, fluorometric kit was obtained from Promega (WI, USA). Antibodies against total or phospho-ERKs, phospho-JNKs, phospho-p38 MAPK (rabbit anti-rat), cytochrome *c* (rabbit anti-rat) and HRP-conjugated goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). BAPTA/AM, fluo-3/AM, BN52021 and other reagents were purchased from Sigma (St. Louis, MO, USA). PAF stock solution (10 mM) was diluted in sterile saline (0.9%. w/v) containing 0.25% bovine serum albumin (BSA).

2.2. Cell culture

H9c2 cells, a clonal cell line derived from fetal rat heart, is a gift from Dr. Wang Zhiguo at the Montreal Heart Institute, Canada. H9c2 cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Cells were maintained in 95% air and 5% CO_2 at 37 °C. Cells were passaged regularly and subcultured to ~90% confluence before use. For measurement of cell viability and apoptosis experiments, cells were serum-deprived for 24 h.

2.3. Cell viability assay

Cell viabilities were determined by mitochondrial dehydrogenase activity, using MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide, Sigma] assay. Briefly, cells were cultured using 96 wells plate and incubated with MTT (0.5 mg/ml) medium for 2 h at 37 °C. Subsequently, MTT medium was discarded and 0.04 N HCl containing acidic isopropanol was added to incubate the cells. Thirty min after incubation, absorbance rate was measured at 570 nm using a microplate reader (BIO-RAD, Hercules, CA). Cell viability expressed as relative viability (%) and calculated as follow: $A \times 100\%$, where A represents the value of absorbance rate obtained under different experimental conditions.

2.4. TUNEL assay

The H9c2 cells were cultured on 2×2 cm slides. TUNEL detection was carried out according to the instruction provided by the manufacture. After counterstaining with hematoxylin, the slides were mounted and analyzed under a confocal microscope (Fluoview-300, Olympus, Japan). For quantitative analysis, TUNEL-positive cells in five different visual fields with approximately 200 cells/field were counted.

2.5. Assessment of electron microscopy

To perform electron microscopy analysis, H9c2 cells were grown in 25-cm^2 culture flasks. After induction of apoptosis, these cells were fixed in 2.5% glutaraldehyde in PBS for 2 h at 4 °C and then postfixed in 1% osmium tetroxide. After dehydration in a series of graded ethanol baths (30–100%) and in propylene oxide, cells were embedded in Epon. Cell sections (80–200 nm) were obtained using a Reichert Ultracut E microtome and stained with uranyl acetate. Grids were examined with a Jeol 1200 EXII electron microscope (Tokyo, Japan).

2.6. Assessment of intracellular Ca^{2+}

After the cultured H9c2 cardiac myocytes were adhered to the cover-slips of the chamber, cells were rinsed once with the standard Tyrode's solution (in mM: 126 NaCl, 5.4 KCl, 10 HEPES, 0.33

$NaH_2PO_4 \cdot 2H_2O$, 1.0 $MgCl_2 \cdot 6H_2O$, 1.8 $CaCl_2$ and 10 Glucose; pH was adjusted to 7.40±0.05 with NaOH) and then incubated with a working solution containing fluo-3/AM (20 mM) and Pluronic F-127 (0.03%) at 37 °C for 45 min. After loading, cells were washed with the standard Tyrode's solution to remove the extracellular fluo-3/AM. Concentration of $[Ca^{2+}]_i$ was measured using Ca^{2+} indicator fluo-3/AM as probe. The images were captured using a 20× objective (488 nm excitation, 530 nm emission). Scanning time lasts for 30 min. Reagents were added between the 3rd and 4th scans (10 s apart) and the images were stored in hard disks. Fluorescent intensities before (FI_0) and after (FI) the drug administration were recorded. Fold change in $[Ca^{2+}]_i$ was represented by the ratio of FI/FI_0.

2.7. Caspase-3 activity assay

To assess the activity of caspase-3, the H9c2 cells were scraped from the plates in ice-cold PBS 30 min after PAF treatment. The cells from each 75-cm^2 plate were then lysed in 160 μl of ice-cold cell lysis buffer for 30 min. The lysate was centrifuged at 13,000 *g* for 30 min at 4 °C. The supernatant was used for subsequent assay. The fluorogenic substrates for caspase-3 were labeled with the fluorochrome 7-amino-methyl coumarin (AMC). AMC was released from these substrates upon cleavage by caspase-3. The enzyme activity was determined by monitoring the fluorescence produced by free AMC using a spectrofluorophotometer (SHIMADZU Corporation, RF-5301PC, KYOTO, Japan) at 360/460 nm. Caspase-3 activity was expressed in picomoles AMC liberated as per minute per microgram of protein.

2.8. Immunoblotting

Western blotting was used to determine activity of extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), p38 mitogen-activated protein kinases (p38 MAPK) and cytochrome *c* in the presence and in the absence of PAF. Mitochondria and cytosol were separated by differential ultracentrifugation [20]. Proteins (20 μg) were first separated by SDS-PAGE on 10% polyacrylamide gels and then transferred electrophoretically onto nitrocellulose membranes. Nonspecific binding sites were blocked with 5% nonfat milk powder in TBS-T (20 mM Tris–HCl pH 7.5, 137 mM NaCl, 0.1% Tween 20) for 30 min at room temperature. Subsequently, the membranes were incubated overnight with the appropriate primary antibody at 4 °C. After washing in TBS-T (3×5 min), blots were incubated with the respective horseradish peroxidase-conjugated secondary antibody (diluted at 1:5000) in TBS-T containing 1% nonfat milk powder for 60 min. After washing in TBS-T (3×5 min), bands were detected using enhanced chemiluminescence (ECL) (Amersham Biosciences, Uppsala, Sweden) and quantified by scanning densitometry (Gel Analyzer v. 1.0), the intensities of interesting band were adjusted by the β-actin band intensity.

2.9. Statistical analysis

Data were presented as Mean±SD. Statistical comparison was performed by the Student *t-test* and with an analysis of variance (ANOVA), with a value of $p<0.05$ considered significant.

3. Results

3.1. The effect of PAF on increase in $[Ca^{2+}]_i$ and cell viability

To investigate whether PAF can induce an increase in $[Ca^{2+}]_i$, H9c2 cardiac myocytes were loaded fluo-3/AM in a standard Tyrode's solution and these cells were subsequently treated with the same solution containing no PAF, 0.2 μM, 2 μM, and 20 μM PAF. As indicated by Fig. 1, we observed an elevated FI/FI_0 upon application of additional 0.2 μM PAF, reflecting an increase in $[Ca^{2+}]_i$. Fig. 1A represents the images of cells under various conditions (590 s, 0 s defined as the time point of taking the first image prior to adding PAF). As shown in the Fig. 1B, FI/FI_0 increased dramatically upon application of 2 μM and 20 μM PAF suggesting $[Ca^{2+}]_i$ overload. The results shown in first four bars from the left of Fig. 1B suggest that PAF induces $[Ca^{2+}]_i$ overload in cultured H9c2 cells in a dose-dependent manner. On average, 0.2 μM, 2 μM, and 20 μM PAF evoked a 1.56±0.17 ($n=15$), 3.55±0.58 ($n=27$) and 4.78±0.78 ($n=32$) fold increase in $[Ca^{2+}]_i$, respectively ($p<0.05$, compared to that of time point of 0 s, *i.e.* control). We reasoned that PAF-induced $[Ca^{2+}]_i$ overload could be mediated by PAF receptor on the membrane of H9c2 cells. Therefore, we assessed measurements of FI/FI_0 in H9c2 cardiac myocytes in the presence of

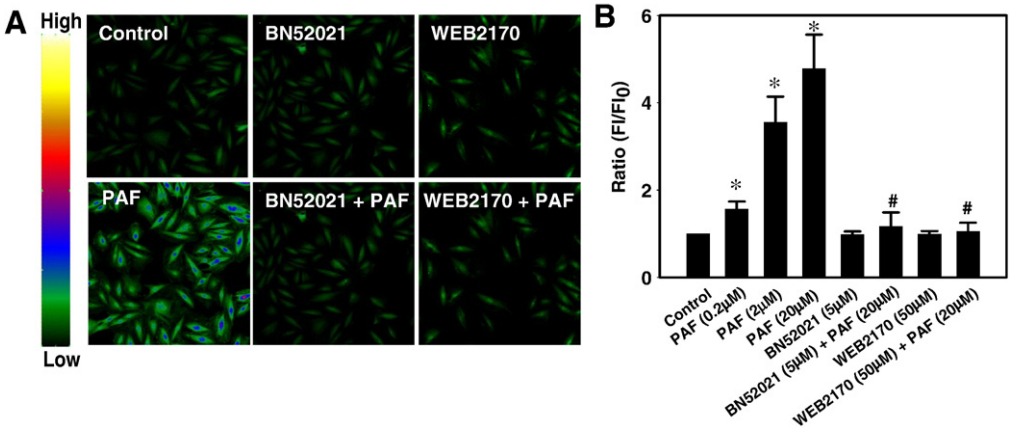

Fig. 1. PAF induced an increase in $[Ca^{2+}]_i$ in H9c2 cells. A: Representative confocal images were taken under different experimental conditions as indicated in the figures (magnification: ×20). B: Summarized results of FI/FI_0 obtained from experiments as shown in A. Data were obtained from six experiments ($n=6$) for each condition. * indicates $p<0.05$ *vs.* control; # represents $p<0.05$ *vs.* 20 μM PAF treatment.

BN52021 or WEB2170, antagonists of PAF receptor, to determine whether the effect of PAF on $[Ca^{2+}]_i$ is a consequence of PAF binding to its receptors. The H9c2 cells were treated with 5 μM BN52021, or 50 μM WEB2170 2 h prior to application of 20 μM PAF. The effect of PAF on $[Ca^{2+}]_i$ was nearly abolished by BN52021 or WEB2170. In addition, 5 μM BN52021 or WEB2170 alone did not alter FI/FI_0. These results suggest that PAF induced $[Ca^{2+}]_i$ overload in H9c2 cells is mediated by its receptor, albeit we don't have direct evidence.

Cell viability was determined by MTT assays as described in Materials and methods section. As illustrated in Fig. 2A, under control condition the relative viability of H9c2 cardiac myocytes was ~100%. In the cells treated with 10^{-10}–10^{-7} M PAF, it appears that viability of the cells were similar to that of control. However, as the dose of PAF increased (10^{-6}–10^{-4} M), the cell viability was reduced in a dose-dependent manner. To further confirm that the effect of PAF on cell viability is mediated by its receptor, we pretreated the cells with a variety of concentration of BN52021 2 h prior to application of PAF. Fig. 2B shows that BN52051 reduced the effect of PAF on cell viability in a dose-dependent manner, suggesting PAF affect cell viability via its receptor. Furthermore, as shown in Fig. 2C, the effect of PAF on cell viability was completely abolished by pretreatment of H9c2 cardiac myocytes with BN52021, WEB2170 or BAPTA/AM 2 h prior to PAF treatment. These results also suggest that PAF-induced $[Ca^{2+}]_i$ overload contributes to reduction of cell viability in the cultured H9c2 cardiac myocytes.

3.2. PAF-induced apoptosis and activity of caspase-3 is prevented by chelating $[Ca^{2+}]_i$ with BAPTA/AM

We hypothesized that PAF-induced $[Ca^{2+}]_i$ overload could cause cardiac myocytes apoptosis and reduction of viability of these cells. Therefore, we performed TUNEL assays to determine whether PAF could induce cardiac myocytes apoptosis. Under control condition, it is hard to detect nuclear staining in H9c2 cardiac myocytes Fig. 3A (a). In contrast, as shown in Fig. 3A (b) and A (c) in the cells treated with 0.2 μM, 2 μM PAF we observed a gradually increased amount of stained nuclei as indicating apoptosis occurred in H9c2 cardiac myocytes. As shown in Fig. 3A (c), A (d) and A (g), it appears that PAF-induced amount of stained nuclei reached plateau at the concentration of 2 μM PAF. To determine if PAF-induced apoptosis in these cells is due to $[Ca^{2+}]_i$ overload, we assessed similar experiments as described above in the presence of BN52021 and BAPTA/AM (an intracellular calcium chelator). As shown in Fig. 3A (e), A (f) and A (g), the percentage of apoptotic cells was decreased dramatically upon treatment with BN52021 and BAPTA/AM 2 h before PAF application.

We also examined the micro-morphological changes induced by PAF using electron microscope at an original magnification of ×8000, as an alternative indication of apoptosis. As shown in Fig. 3B (a), the micro-structure of the cell under control condition appears to be normal. However, in the cells treated with PAF there were considerable amount of cells that exhibited a robust change in micro-structure including chromosomal DNA condensation, segmentation of the nucleus, sunken nucleus membrane, mitochondrial swelling and destruction of cristae as shown in Fig. 3B (b) and B (c). In contrast, changes in cellular micro-structure induced by PAF was, at least in part, prevented by pretreatment of cells with BN52021 [Fig. 3B (d)] or BAPTA/AM [Fig. 3B (e)]. The results obtained from TUNEL assays and electron microscopy examinations are consistent with the notion that PAF indeed induces apoptosis and these results suggest that intracellular calcium plays an important role in PAF-induced apoptosis in cultured H9c2 cardiac myocytes.

In addition, as shown in Fig. 3C (black bars), caspase-3 activity was significantly increased after treatment of H9c2 cells with 2 μM PAF for 2 h. To elucidate if an increase in caspase-3 activity induced by PAF was due to an intracellular calcium overload, we performed experiments with respect to caspase-3 activity in H9c2 cells that were pretreated with either BN52021 or BAPTA/AM 2 h prior to PAF treatment. As shown in Fig. 3C, caspase-3 activity was not altered by pretreatment of these cells with BN52021 or BAPTA/AM alone. Interestingly, PAF-induced caspase-3 activity was dramatically attenuated by pretreatment with either BN52021 or BAPTA/AM. These results suggest that an enhanced activity of caspase-3

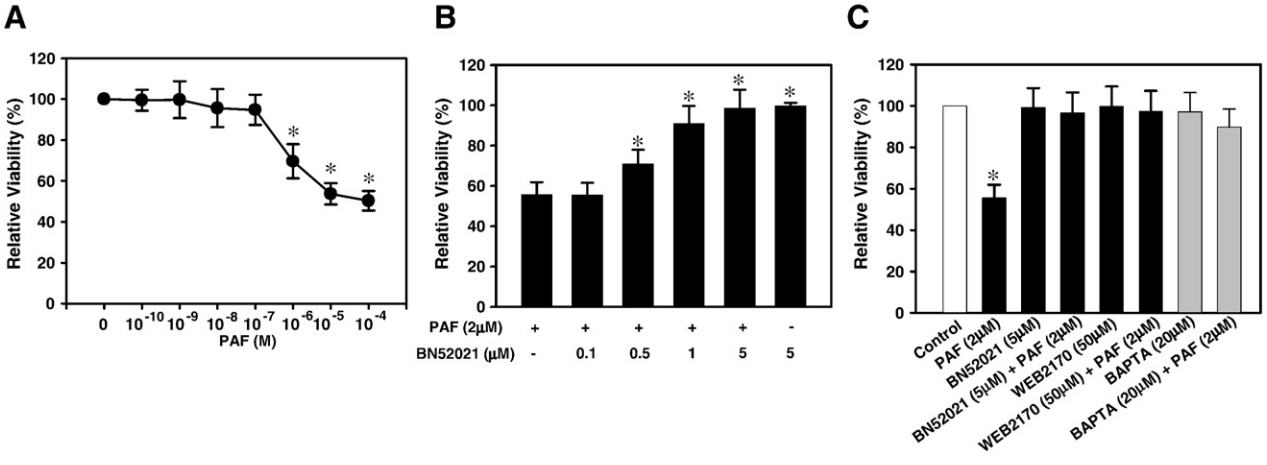

Fig. 2. Effect of PAF on cell viability in H9c2 cells. A: Viability of cultured H9c2 cells was decreased significantly upon treatment of cells with 10^{-6}–10^{-4} M PAF for 2 h *indicates $p<0.05$ *vs.* 0 M PAF group ($n=3$). B: H9c2 cells were preincubated with BN52021 (0.1, 0.5, 1, 5 μM) for 2 h and followed by 2 μM PAF for 2 h. Data were obtained from six experiments for each group ($n=6$). *indicates $p<0.05$ *vs.* 2 μM PAF group. C: PAF-induced reduction of cell viability was reversed by two hours' pretreatment of cells with 5 μM BN52021, 50 μM WEB2170 and 20 μM BAPTA/AM. Data were obtained from five experiments for each group ($n=5$). *indicates $p<0.05$ *vs.* control.

mediates PAF-induced apoptosis via $[Ca^{2+}]_i$ overload in cultured H9c2 cells.

3.3. Effect of PAF on activation of MAPKs in cultured H9c2 cardiac myocytes

MAPKs, including ERK, JNK and p38, are a family of central signaling molecules that respond to numerous stimuli and are known to involve in cell survival and death decisions. We hypothesized that PAF-induced apoptosis in cultured H9c2 cardiac myocytes might be due to an enhanced activity of these kinases. To this end, we performed Western blot analysis with respect to the expression level of these kinases in the absence and in the presence of PAF. As illustrated in Fig. 4, in the presence of 2 μM PAF, the activity of total and phosphorylated p38 MAPK was increased gradually in a time-dependent manner (Fig. 4C), but activity of ERK and JNK was not changed (Fig. 4A and B). These results suggest that p38 MAPK-dependent signaling may play a role in PAF-induced apoptosis in cultured H9c2 cells probably through activation of cytochrome *c*/caspase-3 pathways.

3.4. PAF-induced apoptosis through a p38 MAPK-dependent signaling is attenuated by BAPTA/AM

To identify whether p38 MAPK-dependent signaling is involved in the apoptosis process induced by PAF, we first performed experiments using a specific inhibitor of p38 MAPK to examine relative viability and apoptosis in H9c2 cells. Fig. 5A and B represents that SB203580, a specific inhibitor of p38 MAPK, partly attenuated reduction of cell viability brought about by PAF. These results suggest that enhanced p38 MAPK activity was most likely involved in PAF-induced reduction of viability and apoptosis in cultured H9c2 cardiac myocytes (Fig. 5A and B). We reasoned that enhanced caspase-3 activity in H9c2 cardiac myocytes treated with PAF might be a downstream consequence of p38 MAPK-dependent cytochrome *c* signaling. As shown in Fig. 5C and D, PAF induced cytochrome *c* release from mitochondria and increase of caspase-3 activity were attenuated by SB203580. The release of cytochrome *c* from the mitochondria to cytoplasm is an important feature of caspase 3-mediated apoptosis. Our findings strongly suggest that there is a link between p38 MAPK, cytochrome *c* and caspase-3 signaling; where PAF induces an increase in caspase-3 activity, at least in part, via p38 MAPK-dependent cytochrome *c* release signaling and thereby causes apoptosis and reduction of viability in H9c2 cardiac myocytes.

As illustrated in Fig. 1, there is an $[Ca^{2+}]_i$ overload upon treatment of these cells with PAF; therefore, we hypothesized that elevated p38 MAPK activity might be triggered by PAF-induced $[Ca^{2+}]_i$ overload. As shown in Fig. 5E, the activity of both p38 MAPK and phosphorylated p38 MAPK in the absence or in the presence of BAPTA/AM was identical under control condition. The activity of both p38 MAPK and phosphorylated p38 MAPK was increased ~3-fold ($n=3$) in PAF treated H9c2 cardiac myocytes. As expected, PAF-induced enhancement of p38 MAPK and

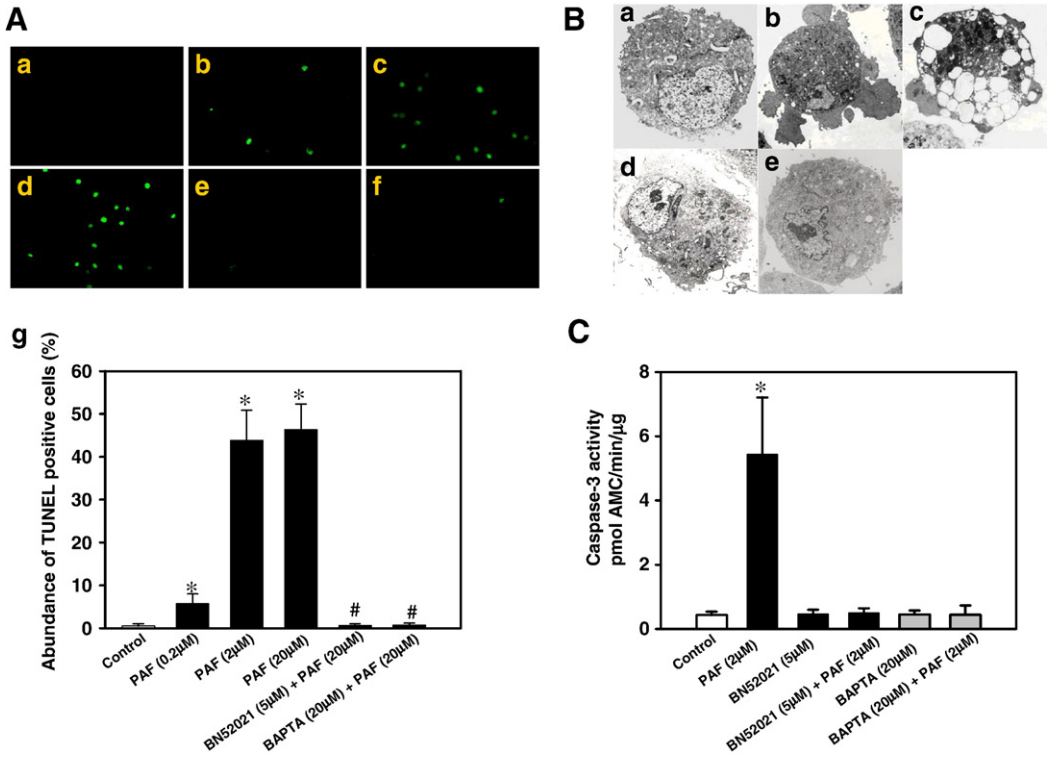

Fig. 3. PAF-induced apoptosis and activity of caspase-3 is prevented by chelating $[Ca^{2+}]_i$ with BAPTA/AM. A: TUNEL staining assays. (a) Control (b), (c) and (d) Green staining were observed in the cells pretreated with PAF (0.2, 2 and 20 μM) suggesting apoptosis occurred. (e) and (f) Cells were treated by BN52021 and BAPTA/AM 2 h prior to application of 2 μM PAF. (g) Percentage of TUNEL positive staining cell obtained from different experimental conditions. * indicates $p<0.05$ *vs*. control, $n=5$. B: Results from transmission electron microscope. The magnification was set at ×8000. (a) Control; (b) Cells were treated with 0.2 μM PAF and (c) 2 μM PAF, (d) 5 μM BN52021+2 μM PAF, (e) 20 μM BAPTA/AM+2 μM PAF. C: The activation of caspase-3 was detected using enzymatic assay. Data were obtained from five experiments for each group ($n=5$). * represents $p<0.05$ *vs*. control.

phosphorylated p38 MAPK expression level in H9c2 cardiac myocytes was attenuated by BAPTA/AM. These results suggest that PAF-induced $[Ca^{2+}]_i$ overload, in turn, activates p38 MAPK signaling and subsequently elevates cytochrome *c*/caspase-3 activity and finally causes apoptosis and reduction of viability in cultured H9c2 cardiac myocytes.

4. Discussion

Many efforts have been made to examine the effect of PAF on apoptosis and cell viability in different cell types and it has been shown that PAF induces a decrease in viability of a variety of cell types, including human lymphocytes, corneal epithelial cells, HaCaT Cells, and human colon carcinoma cells [21–24]. Interestingly, it has also been shown that PAF induces accelerated proliferation in some cell lines such as smooth muscle cells [25], and human bone marrow cells [26]. Moreover, even in the same cell type such as cultured human keratinocytes, contradictory results have shown by different groups [27,28]. It seems that whether PAF-induced decrease or increase in cell viability may depend upon the cell type and the concentration of PAF used. In this study, we used 0.2 μM, 2 μM, and 20 μM PAF, and 2 μM PAF was used in most experiments; the concentrations used were consistent with previous reported PAF concentrations that could cause apoptosis in cultured cells [10–13]. We observed that PAF at the concentration of 0.2 to 20 μM, indeed, can cause reduction of viability and apoptosis in cultured H9c2 cardiac myocytes with a dose-dependent manner. At the concentration of 2 μM, PAF-induced apoptosis and reduction of cell ability seemed to reach maximum. We also used low concentration of PAF (10^{-10} to 10^{-7} M) to examine its effect on cell viability in cultured H9c2 cardiac myocytes and we found that at these concentrations, PAF did not have notable effect on viability in these cells (Fig. 2A). A recent report suggests that PAF, at concentration of 2×10^{-11} M, appears to have a cardioprotective effect on isolated rat heart akin to ischemic preconditioning [29]. As discussed above, it appears that the effect of PAF on cardiac myocytes might depend on the concentration used. Although many groups reported that there is an increase in PAF level and PAF plays an important

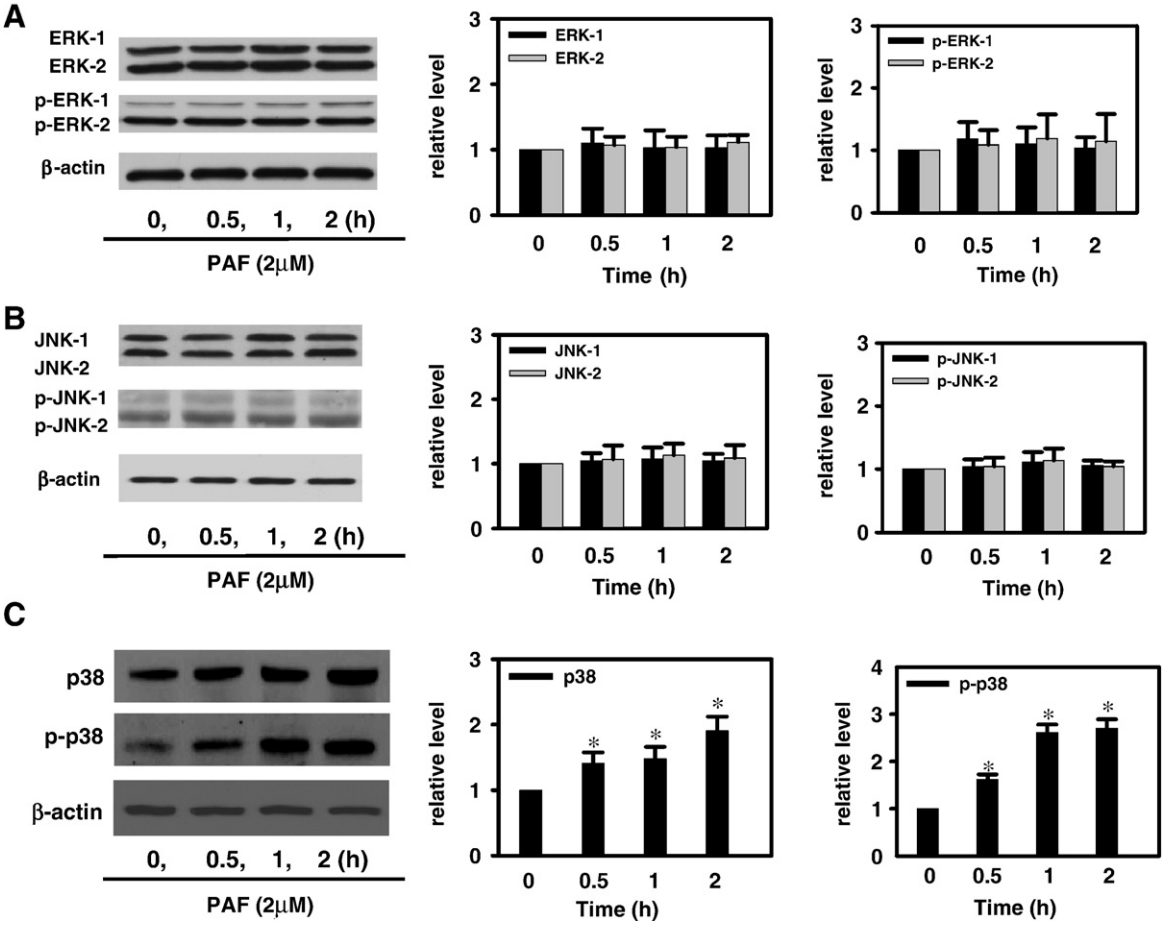

Fig. 4. Activation of MAPKs by PAF in H9c2 cardiac myocytes. Cells were treated with 2 μM PAF for indicated time periods. ERK and phospho-ERK (A), JNK and phospho-JNK (B) and p38 MAPK and phospho-p38 MAPK (C) were quantified by laser scanning densitometry. Results were normalized for β-actin and the data are presented as fold stimulations (bar graphs in middle and right column of A, B and C). Blots and results shown are representative of at least four independent experiments ($n=4$). ERK and JNK activity was not changed by PAF. The expression level of p38 MAPK and phosphor-p38 MAPK increased significantly upon treatment of these cells with PAF as indicated by bar graphs; * indicates $p<0.05$ *vs.* control.

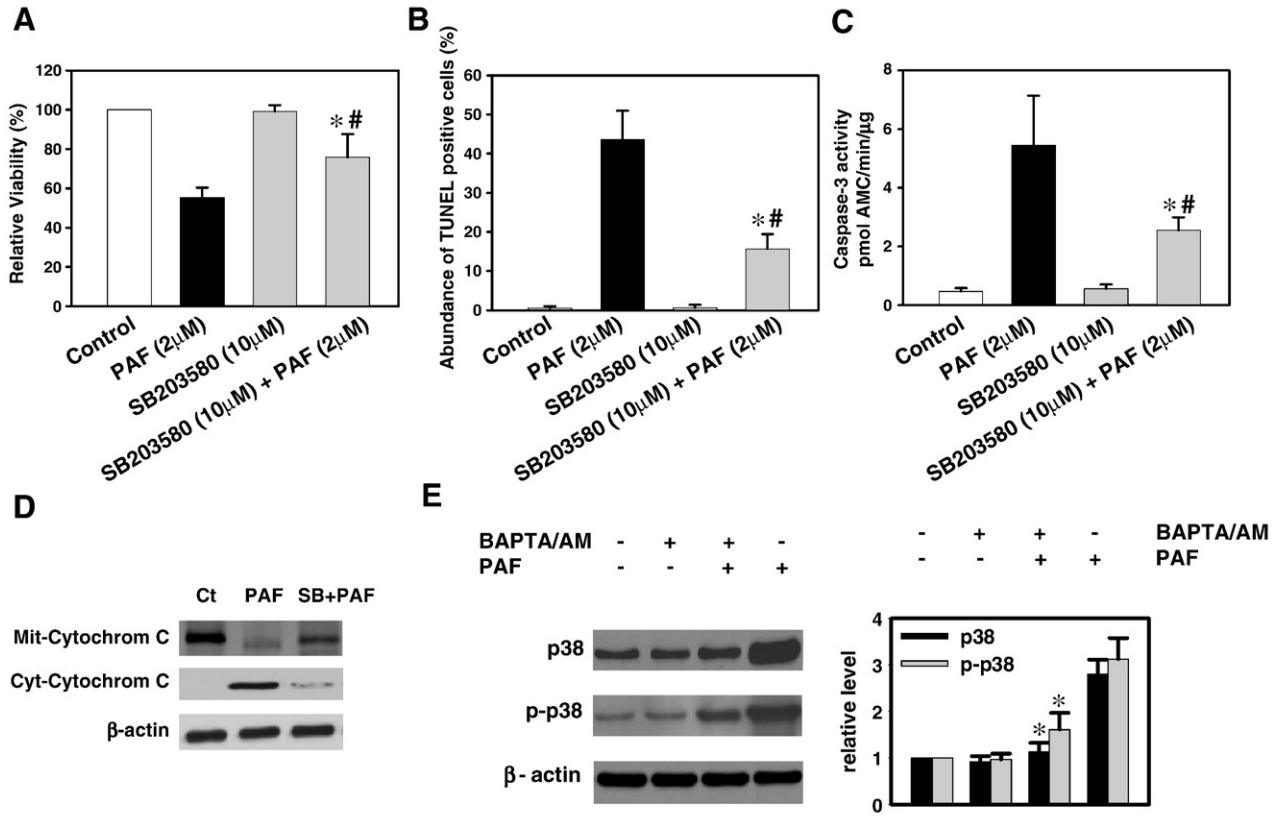

Fig. 5. Role of p38 MAPK in apoptosis signaling pathways. Cells were treated with 10 μM SB203850 (p38 MAPK inhibitor) 2 h prior to application of 2 μM PAF. A: Cell viability was significantly decreased by 2 μM PAF. SB203850, significantly attenuated PAF-induced reduction of cell viability. B: PAF-induced apoptosis in H9c2 cells was reduced dramatically by SB203850. C: PAF-induced enhancement of caspase-3 activity was significantly reduced by SB203850. * represents $p<0.05$ *vs*. control; # indicates $p<0.05$ *vs*. cells treated with PAF (2 μM) alone. D: SB203580 (10 μM) inhibited release of cytochrome *c* from mitochondria to cytoplasm by PAF. E: Inhibitory effect of BAPTA/AM on PAF-induced activation of p38 MAPK. Data were obtained from three experiments for each group ($n=3$). * indicates $p<0.05$ *vs*. data obtained from the cells treated with PAF (2 μM) alone.

risk factor during ischemia/reperfusion injury process; however, in those studies the authors did not measure the exact concentration of PAF or its metabolites in the plasma of those animals [5]. It is unfortunate that we do not know either the actual physiological concentration of PAF or its concentration under pathological conditions in the vicinity and/or tissues affected and in plasma because the detection of PAF level is extremely hard. Nevertheless, under pathological conditions such as ischemia/reperfusion injury, there is an elevated PAF level in the tissues and plasma [3]. Therefore, the current study may provide insight into the understanding of the pathophysiological role of PAF in cardiac diseases such as myocardial ischemia/reperfusion and cardiac failure.

PAF causes an increase in many known apoptotic agents and is known to activate MAPK signaling including p38 MAPK in a number of cell types [30–32]. Our data is congruous with these reports for PAF activated caspase-3 and p38 MAPK. Moreover, our results show that BAPTA/AM appears to play an important role in attenuation of PAF-induced apoptosis process. BAPTA/AM is an intracellular calcium chelator which prevented PAF-induced apoptosis and reduction of viability, and PAF-induced elevation of p38 MAPK and caspase-3 activity. These observations suggest that elevated p38 MAPK activity (phosphorylation) was dependent upon an increase in $[Ca^{2+}]_i$. The importance of changes in cytosolic Ca^{2+} for p38 MAPK activation was also demonstrated in human neutrophils [33].

As state above, our data demonstrate that in H9c2 cardiac myocytes, PAF induces an increase in $[Ca^{2+}]_i$ which subsequently triggers p38 MAPK phosphorylation. This phosphorylated p38 MAPK subsequently activates cytochrome *c*/caspase-3 signaling which leads to cell death via apoptosis. All experiments in current study were assessed using cultured H9c2 cardiac myocytes; however, it had been reported that high dose of PAF *in vivo* could also induce conduction arrhythmias and electrocardiogram signs of ischemia such as S–T segment elevation although it is not known whether PAF targets cardiac myocytes directly [34,35]. Thus, our study may shed a light on screening therapeutic agencies such as PAF receptor inhibitor to prevent apoptosis in the event of ischemia/reperfusion injury.

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The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology [36].

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