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Taiwan Cobra Phospholipase A_2-Elicited JNK Activation is Responsible for Autocrine Fas-Mediated Cell Death and Modulating Bcl-2 and Bax Protein Expression in Human Leukemia K562 Cells

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ABSTRACT

Phospholipase A_2 (PLA_2) from *Naja naja atra* venom induced apoptotic death of human leukemia K562 cells. Degradation of procaspases, production of tBid, loss of mitochondrial membrane potential, Bcl-2 degradation, mitochondrial translocation of Bax, and cytochrome *c* release were observed in PLA_2-treated cells. Moreover, PLA_2 treatment increased Fas and FasL protein expression. Upon exposure to PLA_2, activation of p38 MAPK (mitogen-activated protein kinase) and JNK (c-Jun NH_2-terminal kinase) was found in K562 cells. SB202190 (p38 MAPK inhibitor) pretreatment enhanced cytotoxic effect of PLA_2 and led to prolonged JNK activation, but failed to affect PLA_2-induced upregulation of Fas and FasL protein expression. Sustained JNK activation aggravated caspase8/mitochondria-dependent death pathway, downregulated Bcl-2 expression and increased mitochondrial translocation of Bax. SP600125 (JNK inhibitor) abolished the cytotoxic effect of PLA_2 and PLA_2-induced autocrine Fas death pathway. Transfection ASK1 siRNA and overexpression of dominant negative p38α MAPK proved that ASK1 pathway was responsible for PLA_2-induced p38 MAPK and JNK activation and p38α MAPK activation suppressed dynamically persistent JNK activation. Downregulation of FADD abolished PLA_2-induced procaspase-8 degradation and rescued viability of PLA_2-treated cells. Taken together, our results indicate that JNK-mediated autocrine Fas/FasL apoptotic mechanism and modulation of Bcl-2 family proteins are involved in PLA_2-induced death of K562 cells. J. Cell. Biochem. 109: 245–254, 2010.

KEY WORDS: PHOSPHOLIPASE A_2; JNK; FAS; FASL; BCL-2 FAMILY PROTEINS

Mitogen-activated protein kinases (MAPKs) transducer signals from the cell membrane to transcription sites in the nucleus in response to a variety of different stimuli and participate in various intracellular signaling pathways that control a wide spectrum of cellular processes, including growth, differentiation, and stress responses [Krishna and Narang, 2008]. The family of MAPKs includes extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38 MAPK. Although exception exists, ERK activation is generally associated with enhanced cell survival, whereas JNK and p38 MAPK are associated with cell death [Xia et al., 1995]. Numerous components of the signaling networks that control the proliferation and survival of hematopoietic cells including RAF/MEK/ERK and PI3K/AKT pathways are deregulated in myeloid leukemia [Van Etten, 2007; Scholl et al., 2008]. Downregulation of the survival signaling pathways including RAF/MEK/ERK and PI3K/AKT pathways has been suggested to be targeted therapy of myeloid leukemia [Wu et al., 2004; McCubrey et al., 2008; Scholl et al., 2008].

The enzyme phospholipase A_2 (PLA_2) hydrolyzes fatty acids at the *sn*-2 position of phospholipids, which generates free fatty acids and lysophospholipids are involved in several cell functions including the processing of pro-inflammatory mediators, cell proliferation, and apoptosis [Taketo and Sonoshita, 2002; Sun et al., 2004; Triggiani et al., 2006; Lambeau and Gelb, 2008]. Cytosolic PLA_2, Ca^{2+}-independent PLA_2, and secreted PLA_2 ($sPLA_2$) have been reported to be able to induce apoptotic process, and cell death

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induced by the $sPLA_2$ is usually attributed to ROS generation and increase in intracellular Ca^{2+} [Taketo and Sonoshita, 2002; Sun et al., 2004, 2007; Muralikrishna Adibhatla and Hatcher, 2006]. Recent study showed that *Naja naja atra* PLA_2 induced apoptotic death of acute myeloid leukemia U937 cells through p38 MAPK-mediated Fas/FasL upregulation and ERK inactivation [Liu et al., 2009]. Given that the primary cause of treatment failures in acute myeloid leukemia is usually the emergence of multidrug resistance arising from abnormalities in the apoptotic response [Schneider et al., 1995; Wang, 2003; Testa and Riccioni, 2007], restoring the sensitivity of leukemic cells to apoptotic stimuli by $sPLA_2$-elicited MAPKs signaling pathway may have the benefit of treating leukemia. Chronic myeloid leukemia is characterized by a mutant gene encoding the constitutively active Bcr/Abl kinase, which signals downstream to a variety of cytoprotective pathways including ERK, Akt, NF-κB, and Jak/STAT [Hochhaus et al., 2002; Donata et al., 2004; Van Etten, 2007]. Thus, the goal of the present study is to explore whether $sPLA_2$ treatment could abolish cytoprotective pathways and induced MAPK-mediated Fas/FasL protein expression in chronic myeloid leukemia cells. In the present study, treatment with human chronic myeloid leukemia K562 cells with *N. naja atra* PLA_2 led to activation of p38 MAPK and JNK but inactivation of ERK. Moreover, it was found that JNK-mediated Fas/FasL upregulation and modulation of Bcl-2/Bax protein expression was related to PLA_2-induced cell death.

MATERIALS AND METHODS

PLA_2 from the venom of *N. naja atra* (Taiwan cobra) was isolated as previously described [Chang et al., 1998]. Digitonin, MTT, MG-132, propidium iodide (PI), SB202190, and SP600125 were obtained from Sigma–Aldrich Inc., and rhodamine-123 was the product of Molecular Probes. Annexin V-FITC/PI flow cytometry assay kit was purchased from Invitrogen. Anti-Fas (N-18) and anti-ASK1 antibodies were obtained from Santa Cruz Biotechnology, Inc.

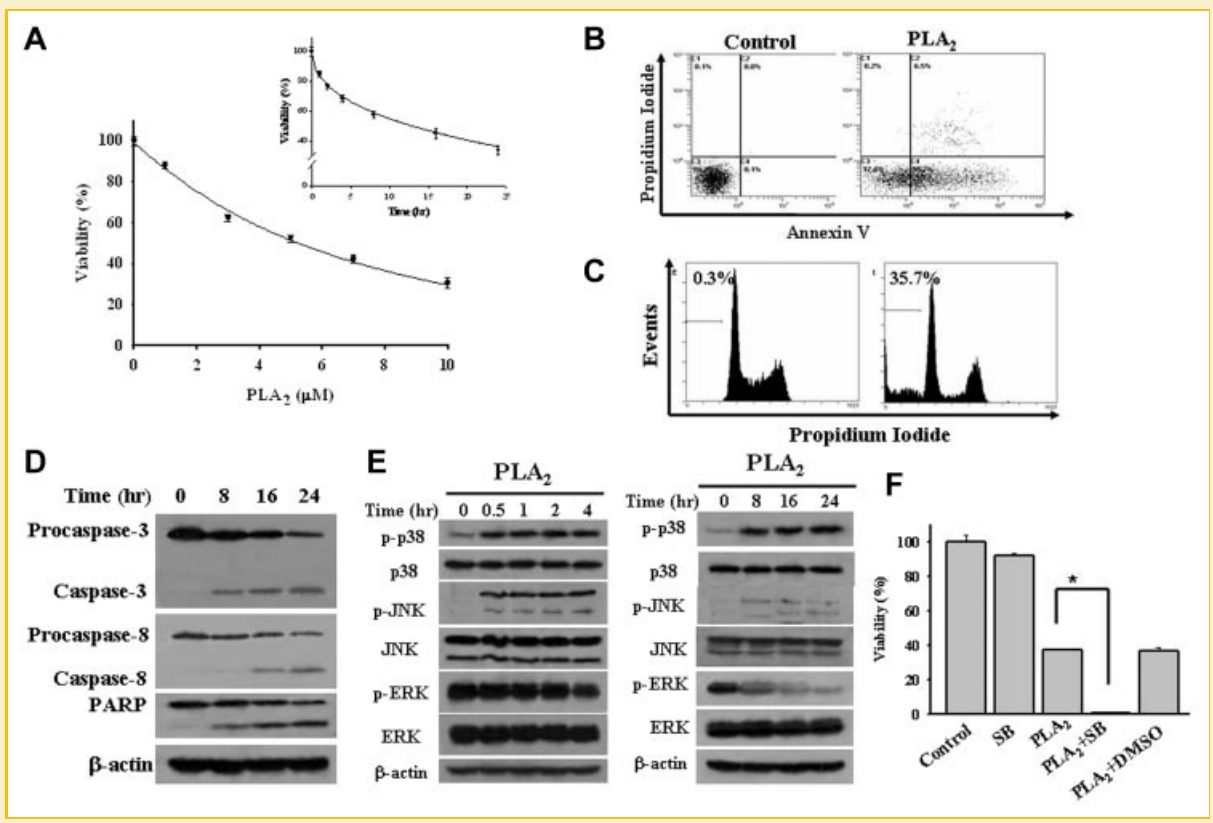

Fig. 1. PLA_2-induced apoptotic death of K562 cells. A: PLA_2-induced cell death in a concentration-dependent manner. K562 cells were incubated with varying concentrations of PLA_2 for 24 h. (Inset) K562 cells were incubated with 10 μM PLA_2 for indicated time periods. Cell viability was determined by MTT assay. Results are expressed as the percentage of cell proliferation relative to the control. Each value is the mean ± SD of six determinations. B: Cell deaths were analyzed by dual-parameter flow cytometry utilizing annexin V-FITC and PI. K562 cells were incubated with 10 μM PLA_2 for 24 h. On the flow cytometric scatter graphs, the left lower quadrant represents remaining live cells. The right lower quadrant represents the population of early apoptotic cells. The right upper quadrant represents the accumulation of late apoptotic cells. C: Cell cycle analysis of K562 cells treated with PLA_2. Flow cytometry analyses showed an increase in the sub-G_1 DNA content of K562 cells after treatment with 10 μM PLA_2 for 24 h. D: Degradation of procaspase-3, procaspase-8, and PARP in PLA_2-treated K562 cells. Cells were treated with 10 μM PLA_2 for indicated time periods. E: Western blot analyses of phosphorylated MAPKs in PLA_2-treated cells. K562 cells were treated with 10 μM PLA_2 for indicated time periods. F: SB202190 (p38 MAPK inhibitor) pretreatment potentiated PLA_2-induced death of K562 cells. K562 cells were pretreated with 10 μM SB202190 for 1 h and then incubated with 10 μM PLA_2 for 24 h. Cell viability was analyzed by MTT assay, and the data are the mean ± SD of six experiments (*$P < 0.05$).

Anti-FasL, anti-p38 MAPK, anti-p38α MAPK, anti-p38β MAPK, anti-phospho-p38 MAPK, anti-ERK, anti-phospho-ERK, anti-JNK, anti-phospho-JNK, anti-phospho-ASK1 (Thr845), anti-caspase-9, anti-c-$FLIP_L$, anti-Bax, anti-Bcl-2, and anti-Bid antibodies were products of Cell Signaling Technology. Z-IETD-FMK (caspase-8 inhibitor), JNK inhibitor 1 (JNKI-1), anti-caspase-3 antibody, and anti-caspase-8 antibody were purchased from Calbiochem, and anti-β-actin antibody were obtained from Chemicon. Anti-cytochrome *c*, anti-Bcl-xL, and anti-Bak antibodies were purchased from BD Pharmingen, and horseradish peroxidase-conjugated secondary antibodies were obtained from Pierce. Cell culture supplies were purchased from GIBCO/Life Technologies Inc. Unless otherwise specified, all other reagents were of analytical grade.

CELL VIABILITY ASSAY

Human chronic myeloid leukemic cell line K562 was obtained from ATCC (Rockville, MD) and cultured in RPMI 1640 supplemented with 10% fetal calf serum, 1% sodium pyruvate, 2 mM glutamine and penicillin (100 units/ml)/streptomycin (100 μg/ml) in an incubator humidified with 95% air and 5% CO_2. Exponentially growing cells (1×10^5) were plated in 96-well plates and treated with PLA_2 in serum-free medium for indicated time periods. At suitable time intervals, MTT solution was added to each well at a final concentration of 0.5 mg/ml and incubated for 4 h. Formazan crystals resulting from MTT reduction were dissolved by addition of 100 μl DMSO per well. The absorbance was detected at 595 nm using a plate reader.

DETECTION OF APOPTOTIC CELLS

Annexin V/PI staining was carried out according to the manufacturer's protocol (annexin V-FITC kit from Molecular Probes). After specific treatment, K562 cells were incubated with annexin V-FITC and PI. Then the cells were analyzed by a Beckman Coulter Epics XL flow cytometer.

SUB-G_1 ANALYSIS

Sub-G_1 distribution was determined by staining DNA with PI. Briefly, 1×10^6 cells were incubated with PLA_2 for 24 h. Cells were

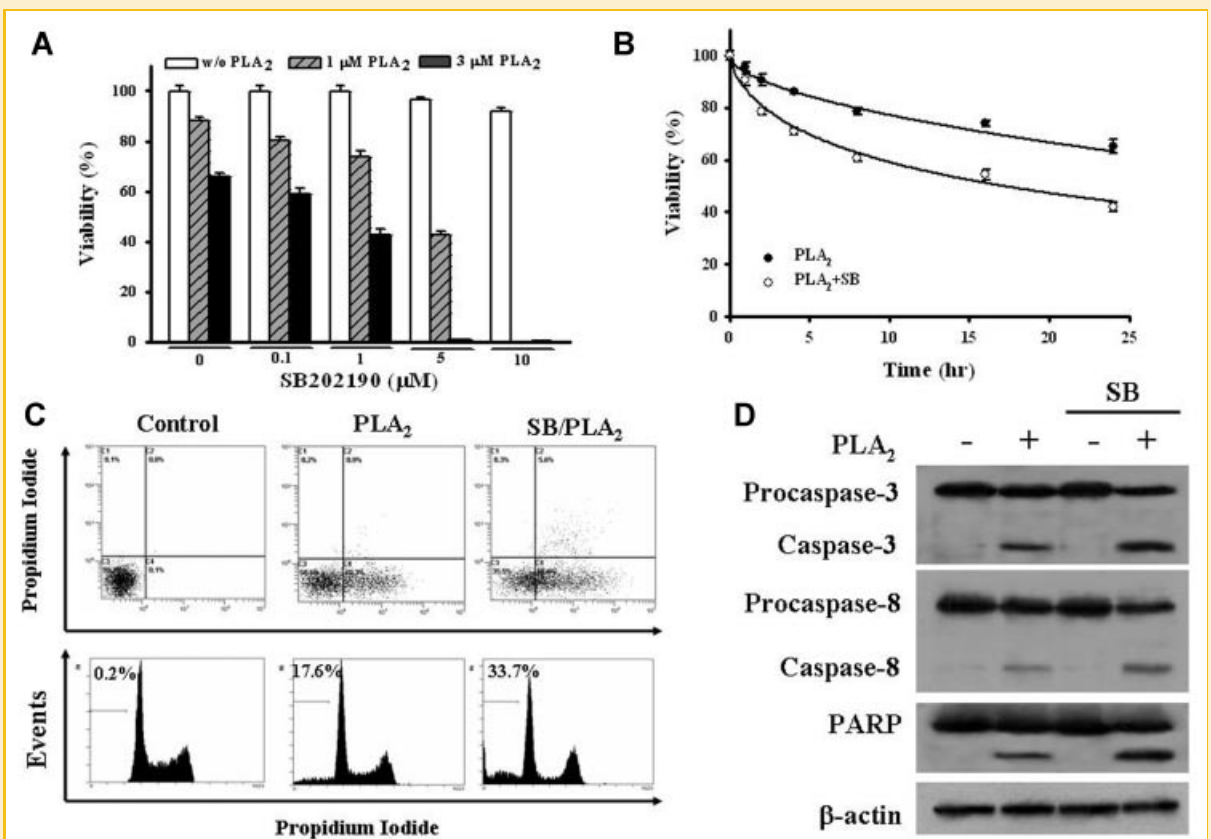

Fig. 2. SB202190 pretreatment promotes the cytotoxic effect of PLA_2 toward K562 cells. A: SB202190 pretreatment enhanced the cytotoxicity of PLA_2 in a concentration-dependent manner. K562 cells were treated with varying concentrations of SB202190 for 1 h, and then incubated with 1 or 3 μM PLA_2 for 24 h. Cell viability was analyzed by MTT assay, and the data are the mean ± SD of six experiments. B: SB202190 pretreatment enhanced the cytotoxicity of PLA_2 in a time-dependent manner. K562 cells were treated with 1 μM SB202190 for 1 h, and then incubated with 3 μM PLA_2 for indicated time periods. Alternatively, K562 cells were treated with 3 μM PLA_2 for indicated time periods. Cell viability was analyzed by MTT assay, and the data are the mean ± SD of six experiments. C: Flow cytometry analyses of annexin V-staining cells (top panel) and sub-G_1 DNA content (bottom panel) after PLA_2 treatment. K562 cells were treated with 1 μM SB202190 for 1 h, and then incubated with 3 μM PLA_2 for 24 h. D: SB202190 pretreatment led to an increase in the degradation of procaspase-3, procaspase-8, and PARP in PLA_2-treated K562 cells. K562 cells were treated with 1 μM SB202190 for 1 h, and then incubated with 3 μM PLA_2 for 24 h.

then washed in phosphate buffer saline (PBS) and fixed in 70% ethanol. Cells were again washed with PBS and then incubated with PI (10 μg) with simultaneous treatment of RNase at 37°C for 30 min. The percentages of cells having the sub-G_1 DNA content were measured with a Beckman Coulter Epics XL flow cytometer and analyzed using EXPO32 ADC software.

DETECTION OF MITOCHONDRIAL MEMBRANE POTENTIAL

PLA_2-treated cells were incubated with 20 nM rhodamine-123 for 20 min prior to harvesting, and then washed with PBS. Rhodamine-123 intensity was determined by flow cytometry.

SUBCELLULAR FRACTIONATION

Following specific treatment, cytosolic and pellet (mitochondrial) fractions were generated using a digitonin-based subcellular fractionation technique [Liu et al., 2009]. Cytochrome *c* and proteins of Bcl-2 family were detected by Western blot analysis.

DNA TRANSFECTION

Dominant negative p38α MAPK and p38β MAPK plasmids, pcDNA3-DN-p38α and pcDNA3-DN-p38β, were kindly provided by Dr. J. Han (Xiamen University, China). The plasmids were transfected into K562 cells using pipette-type Electroporator (MicroPorator-MP100, Digital Bio Tech. Co., Korea).

RNA INTERFERENCE

ASK1 siRNA expression plasmid (pKD-ASK1-V2) and control vector (pKD-Negcon-V1) were obtained from Upstate Biotechnology Inc. FADD siRNA (catalog number sc-35352) and negative control siRNA (catalog number sc-37007) were purchased from Santa Cruz Biotechnology, Inc. Transfections were performed with Lipofetamine™ 2000 (Invitrogen) according to the manufacturer's instructions.

WESTERN BLOT ANALYSIS

After specific treatments, proteins of cell lysate were resolved on SDS–PAGE and transferred onto a PVDF membrane. The transferred membranes were incubated with appropriate primary antibodies and horseradish peroxidase-conjugated secondary antibodies. The immune complexes were detected by SuperSignal West Pico Chemiluminescent substrate kit (Pierce).

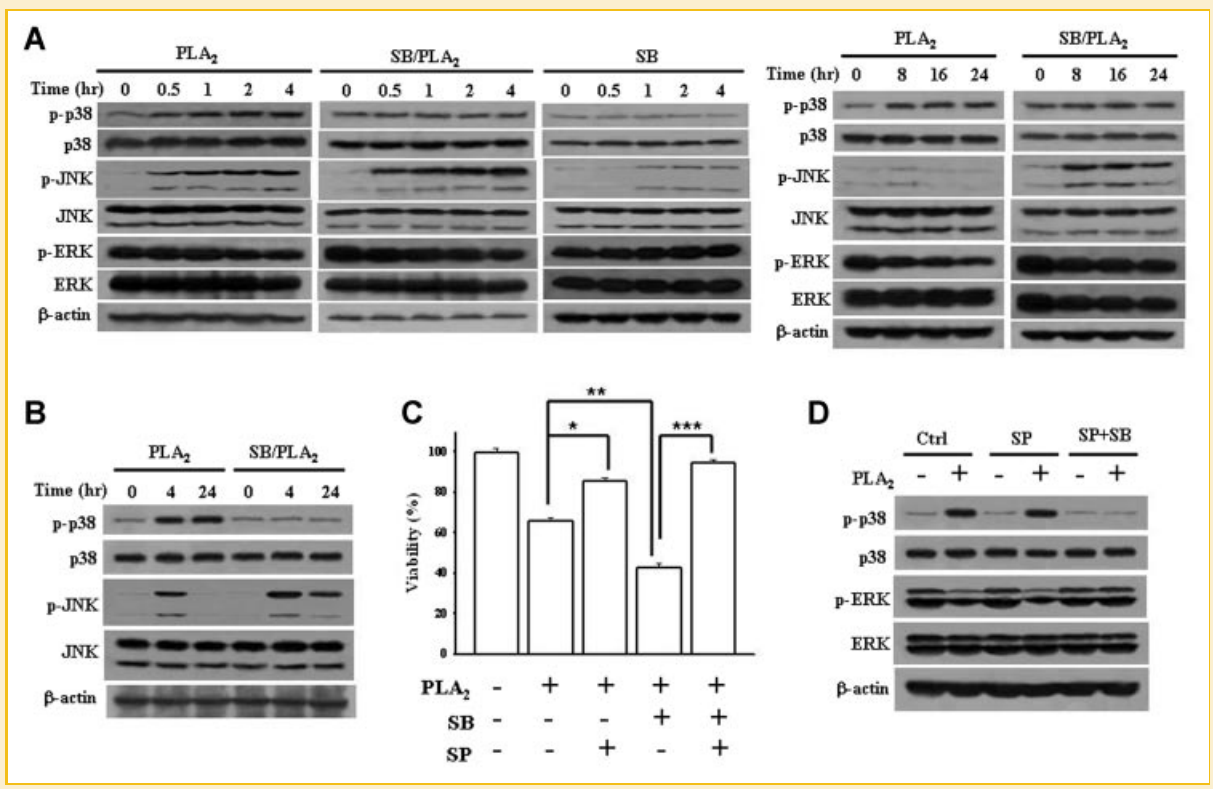

Fig. 3. SB202190 pretreatment led to prolonged JNK activation. A: Persistent phosphorylation of JNK in PLA_2-treated K562 cells after pretreatment with SB202190. K562 cells were treated with 1 μM SB202190 for 1 h, and then incubated with 3 μM PLA_2 for indicated time periods. B: Suppression of p38 MAPK led to dynamically persistent JNK activation in PLA_2-treated cells. K562 cells were treated with 1 μM SB202190 for 1 h, and then incubated with 3 μM PLA_2 for 4 or 24 h. C: Suppression of JNK activation restored viability of PLA_2-treated cells. K562 cells were treated with 10 μM SP600125, 1 μM SB202190, or a combination of 10 μM SP600125 and 1 μM SB202190 for 1 h, and then incubated with 3 μM PLA_2 for 24 h. Cell viability was analyzed by MTT assay, and the data are the mean ± SD of six experiments (*****$P < 0.05$). D: PLA_2-induced ERK inactivation was abolished by pretreatment with SP600125 and SB202190. K562 cells were treated with 10 μM SP600125 or a combination of 10 μM SP600125 and 1 μM SB202190 for 1 h, and then incubated with 3 μM PLA_2 for 24 h.

STATISTICAL ANALYSIS

All data are presented as mean ± SD. Significant differences among the groups were determined using the unpaired Student's *t*-test. A value of $P < 0.05$ was taken as an indication of statistical significance. All the figures shown in this article were obtained from at least three independent experiments with similar results.

RESULTS AND DISCUSSION

Upon exposure to PLA_2, K562 cells showed a concentration- and time-dependent decrease in cell viability (Fig. 1A). A reduction in approximately 70% of cell viability was observed after treatment with 10 μM PLA_2 for 24 h. Figure 1B shows that PLA_2 treatment caused an increase in annexin V staining, while no evidence of only positive PI staining (upper left quadrants) was detected in PLA_2-treated K562 cells. As shown in Figure 1C, flow cytometric analyses of hypodiploid cell populations revealed that PLA_2 induced an increased accumulation of cells in the sub-G_1 phase (35.7%) as compared with untreated control cells (0.3%). Immunoblotting analyses revealed decrease in the levels of procaspase-3 and procaspase-8 after PLA_2 treatment (Fig. 1D). Moreover, the production of active caspases and degradation of PARP (a caspase-3 substrate) were also noted. These supported the notion that PLA_2 induced apoptotic death of K562 cells.

Figure 1E shows that PLA_2 treatment led to a notable increase in phosphorylation of JNK and p38 MAPK and inactivation of ERK in K562 cells. Persistent phosphorylation of p38 MAPK was noted after PLA_2 treatment up to 24 h, while PLA_2-elicited JNK activation was diminished after post-PLA_2 treatment for 8 h. Given that p38 MAPK was critical for PLA_2-induced apoptotic death of U937 cells [Liu et al., 2009], the role of activated p38 MAPK in PLA_2-induced apoptosis of K562 cells was examined. Astonishingly, SB202190 pretreatment was found to further reduce viability of PLA_2-treated K562 cells (Fig. 1F).

Figure 2A shows that SB202190 enhanced cytotoxicity of PLA_2 in a dose-dependent manner. Because the dose required for approximately half-maximum inhibition of viability was achieved when cells were treated with a combination of 1 μM SB202190 and 3 μM PLA_2 for 24 h, this single treatment was used for further assessment of cytotoxicity. As shown in Figure 2B, co-incubation with SB202190 and PLA_2 showed a time-dependent reduction in viability

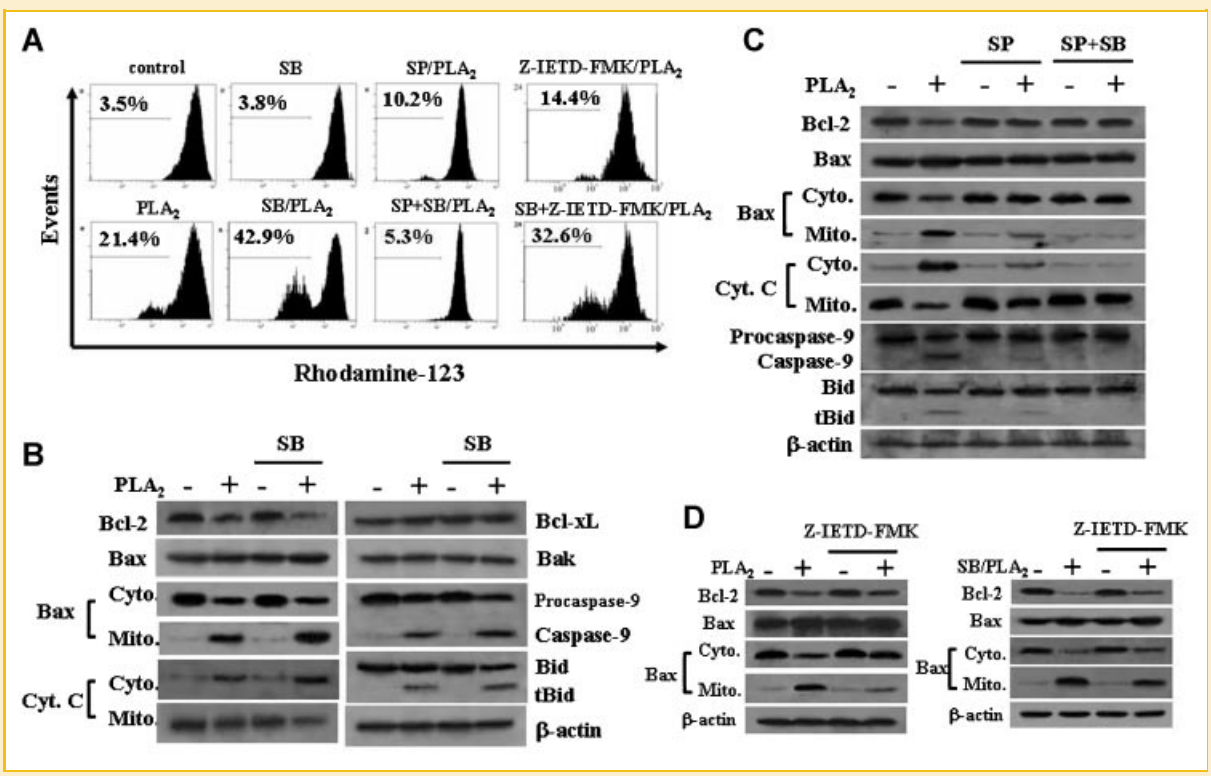

Fig. 4. PLA_2 induced the loss of mitochondrial membrane potential (ΔΨm) and modulated expression of Bcl-2 family proteins. **A:** Analyses of ΔΨm by flow cytometry. K562 cells were treated with 10 μM SP600125, 1 μM SB202190, a combination of 10 μM SP600125 and 1 μM SB202190, 100 μM Z-IETD-FMK, or a combination of 1 μM SB202190 and 100 μM Z-IETD-FMK for 1 h, and then incubated with 3 μM PLA_2 for 24 h. **B:** Western blot analyses of proteins of Bcl-2 family, cytochrome *c* release, and caspase-9 activation in PLA_2- or SB202190/PLA_2-treated cells. K562 cells were treated with 1 μM SB202190 for 1 h, and then incubated with 3 μM PLA_2 for 24 h. **C:** Abolition of JNK activation suppressed PLA_2-induced Bcl-2 degradation, mitochondrial translocation of Bax, cleavage of t-Bid, cytochrome *c* release, and caspase-9 activation. K562 cells were treated with 10 μM SP600125 or the combination of 10 μM SP600125 and 1 μM SB202190 for 1 h, and then incubated with 3 μM PLA_2 for 24 h. **D:** Effect of Z-IETD-FMK on protein expression of Bcl-2 and Bax in K562 cells treated with PLA_2 or a combination of SB202190 and PLA_2. K562 cells were treated with 100 μM Z-IETD-FMK or a combination of 1 μM SB202190 and 100 μM Z-IETD-FMK for 1 h, and then incubated with 3 μM PLA_2 for 24 h.

of K562 cells. Flow cytometric analyses revealed that SB202190 increased the population of annexin V-staining cells and an accumulation of cells in the sub-G_1 phase after PLA_2 treatment (Fig. 2C). Immunoblotting analyses showed that SB202190 pretreatment increased notably the activation of caspase-3 and -8, and degradation of PARP in PLA_2-treated cells (Fig. 2D).

Figure 3A shows that phospho-JNK was no longer observed after treatment with 3 μM PLA_2 for 8 h. Compared with cells treated with PLA_2 alone, JNK phosphorylation was dynamically persistent in cells co-incubated with SB202190 and PLA_2 (Fig. 3A,B). This suggested a causal relationship between p38 MAPK inactivation and prolonged JNK activation. Figure 3C shows that SP600125 rescued markedly viability of K562 cells treated with PLA_2 or a combination of SB202190 and PLA_2. Noticeably, PLA_2-elicited ERK inactivation was restored by pretreatment with a combination of SB202190 and SP600125 (Fig. 3D). This was in line with the suggestion that ERK activation is negatively regulated by p38 MAPK/JNK pathway [Junttila et al., 2008].

Increasing evidence suggests that altered mitochondrial function is linked to apoptosis and a decreasing mitochondrial transmembrane potential is associated with mitochondrial dysfunction [Green and Reed, 1998]. As shown in Figure 4A, flow cytometry analysis showed that, in untreated control K562 cells, more than 96% of cells were functionally active with high rhodamine-123 signals. Increasing population of K562 cells exhibited the loss of mitochondrial membrane potential ($\Delta\Psi m$) after PLA_2 treatment (Fig. 4A). SB202190 pretreatment enhanced the loss of $\Delta\Psi m$ in PLA_2-treated cells, and SP600125 markedly attenuated the dissipation of $\Delta\Psi m$ in K562 cells induced by a combination of SB202190 and PLA_2. During mitochondrion-mediated apoptosis, cytochrome *c* is released from the mitochondria into the cytosol, where it promotes caspase activation [Green and Reed, 1998]. As shown in Figure 4B, PLA_2 induced the release of cytochrome *c* into cytosol and caspase-9 activation. Moreover, cleavage of Bid to produce activated Bid (tBid) was observed with PLA_2-treated cells. tBid has been reported to be associated with the mitochondrial outer membrane and cause disruption to $\Delta\Psi m$, thus resulting in cytochrome *c* release [Hengartner, 2000]. These results reflected that the cytotoxicity of PLA_2 was mediated through mitochondrial membrane alteration.

Figure 4B shows that SB202190 pretreatment slightly increased cytochrome *c* release, downregulated Bcl-2 expression, slightly upregulated Bax expression, increased mitochondrial translocation of Bax, slightly increased caspase-9 activation, and the production of t-Bid in PLA_2-treated cells. SP600125 pretreatment largely reversed these molecular events associated with mitochondria-mediated death pathway (Fig. 4C). This suggested that SB202190-elicited prolonged JNK activation augmented caspase-8/mitochindria-dependent death pathway in PLA_2-treated cells. It is known that the antiapoptotic Bcl-2 proteins protect cells against apoptosis

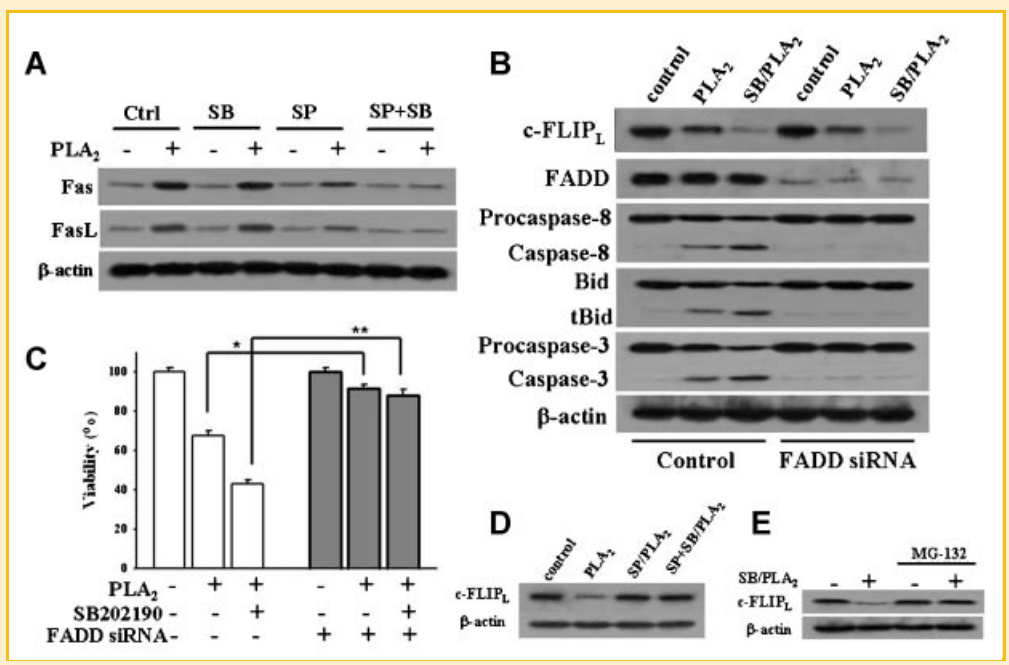

Fig. 5. PLA_2 induced Fas-mediated death pathway in K562 cells. A: Western blotting analyses of Fas and FasL protein expression. K562 cells were treated with 10 μM SP600125, 1 μM SB202190, or a combination of 10 μM SP600125 and 1 μM SB202190 for 1 h, and then incubated with 3 μM PLA_2 for 24 h. B: Effect of downregulation of FADD on PLA_2-induced degradation of procaspase-8, cleavage of Bid, and c-$FLIP_L$ downregulation. K562 cells were transfected with 100 nM control siRNA or FADD siRNA, respectively. After 24 h post-transfection, FADD protein expression, c-$FLIP_L$ protein expression, degradation of procaspase-8, and cleavage of Bid were analyzed. C: Viability of control siRNA- and FADD siRNA-transfected cells after treatment with 3 μM PLA_2 for 24 h (*,**$P < 0.05$). D: Effect of SP600125 on c-$FLIP_L$ protein expression in PLA_2- or SB202190/PLA_2-treated cells. K562 cells were treated with 10 μM SP600125 or a combination of 10 μM SP600125 and 1 μM SB202190 for 1 h, and then incubated with 3 μM PLA_2 for 24 h. E: MG-132 abrogated c-$FLIP_L$ downregulation in SB202190/PLA_2-treated cells. K562 cells were treated with 1 μM SB202190 or a combination of 10 μM MG-132 and 1 μM SB202190 for 1 h, and then incubated with 3 μM PLA_2 for 24 h.

via interaction with proapoptotic proteins of the Bcl-2 family such as Bax. An excess of Bax proteins results in their insertion into the outer mitochondrial membrane where they oligomerize and form protein-permeable channels which promote cell death by releasing cytochrome *c* and other lethal factors from the mitochondria [Reed, 2006]. Thus, $\Delta\Psi$m loss noted with SB202190/PLA_2-treated cells should be mildly associated with JNK-modulated Bcl-2/Bax protein expression. Alternatively, Z-IETD-FMK (caspase-8 inhibitor) pretreatment partly rescued the loss of $\Delta\Psi$m in K562 cells treated with PLA_2 or a combination of SB202190 and PLA_2 (Fig. 4A). Moreover, Z-IETD-FMK did not completely abrogate Bcl-2 downregulation and mitochondrial translocation of Bax (Fig. 4D). This reflected that enhancement of caspase-8 activation by sustained JNK activation was not exclusively responsible for mitochondrial alteration, and suggested that JNK played a role in directly modulating protein expression of Bcl-2 and Bax.

Death receptors of the tumor necrosis factor (TNF) family such as Fas and TNFR1 are the best understood death pathways and recruit FADD and procaspase-8 to the receptor. Recruitment of procaspase-8 through FADD leads to its auto-cleavage and activation, and in turn activates effector caspases such as caspase-3 [Thorburn, 2004]. Immunoblotting analyses displayed an increase in Fas and FasL protein expression after PLA_2 treatment (Fig. 5A). Pretreatment with SB202190 did not alter protein expression of Fas and FasL, while SP600125 decreased the effect of PLA_2 on Fas and FasL expression in PLA_2-treated cells or SB202190/PLA_2-treated cells. Downregulation of FADD expression with 100 nM siRNA targeted to a unique sequence, which reduced FADD protein level by more than 95%, abolished markedly PLA_2-induced activation of procaspases and cleavage of Bid (Fig. 5B). Compared with control siRNA, FADD siRNA rescued viability of cells treated with PLA_2 or a combination of SB202190 and PLA_2 (Fig. 5C). These findings suggest that

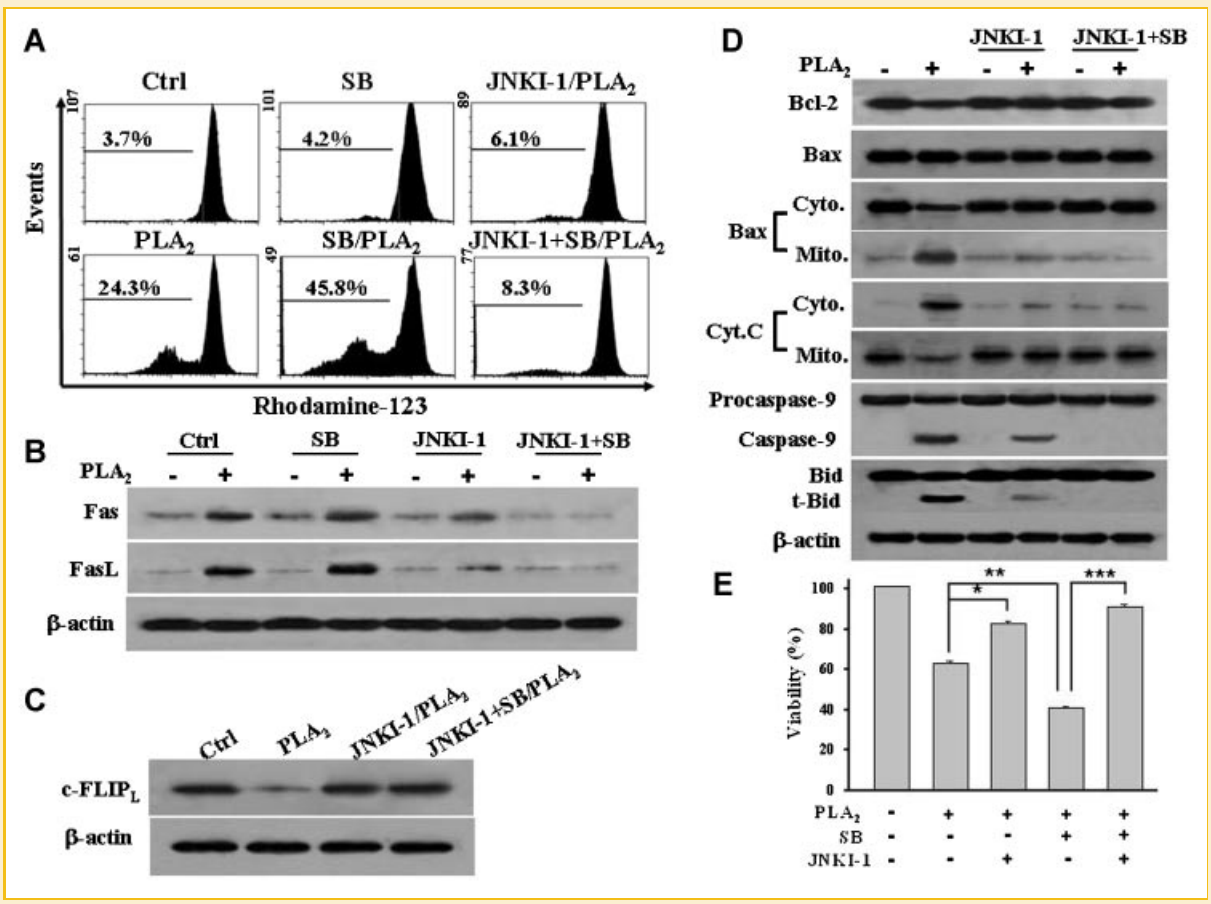

Fig. 6. Effect of JNK inhibitor I on mitochondrial membrane potential ($\Delta\Psi$m), expression of Bcl-2 family proteins, and Fas-mediated death mechanism in PLA_2-treated K562 cells. A: Analyses of $\Delta\Psi$m by flow cytometry. K562 cells were treated with 5 μM JNK inhibitor 1 (JNKI-1), 1 μM SB202190, a combination of 5 μM JNKI-1 and 1 μM SB202190 for 1 h, and then incubated with 3 μM PLA_2 for 24 h. B: Western blotting analyses of Fas and FasL protein expression. K562 cells were treated with 5 μM JNKI-1, 1 μM SB202190, or a combination of 5 μM JNKI-1 and 1 μM SB202190 for 1 h, and then incubated with 3 μM PLA_2 for 24 h. C: Effect of JNKI-1 on c-$FLIP_L$ protein expression in PLA_2- or SB202190/PLA_2-treated cells. K562 cells were treated with 5 μM JNKI-1 or a combination of 5 μM JNKI-1 and 1 μM SB202190 for 1 h, and then incubated with 3 μM PLA_2 for 24 h. D: Pretreatment with JNKI-1 suppressed PLA_2-induced Bcl-2 degradation, mitochondrial translocation of Bax, cleavage of t-Bid, cytochrome *c* release, and caspase-9 activation. K562 cells were treated with 5 μM JNKI-1 or a combination of 5 μM JNKI-1 and 1 μM SB202190 for 1 h, and then incubated with 3 μM PLA_2 for 24 h. E: Pretreatment with JNKI-1 restored viability of PLA_2-treated cells. K562 cells were treated with 5 μM JNKI-1, 1 μM SB202190, or a combination of 5 μM JNKI-1 and 1 μM SB202190 for 1 h, and then incubated with 3 μM PLA_2 for 24 h. Cell viability was analyzed by MTT assay, and the data are the mean ± SD of six experiments (*,**,***$P < 0.05$).

PLA_2-induced cell death is related to activation of Fas-mediated death pathway and PLA_2-elicited JNK activation is responsible for Fas and FasL upregulation. Noticeably, Chang et al. [2006] reported that JNK-mediated phosphorylation and activation of the E3 ubiquitin ligase ITCH induces proteasome-dependent degradation of c-FlLP_L, an inhibitor of caspase-8. Compared with that of PLA_2-treated cells, the level of c-$FLIP_L$ was notably downregulated in cells treated with SB202190 and PLA_2 (Fig. 5B). Figure 5D shows that SP600125 rescued c-$FLIP_L$ downregulation in PLA_2- or SB202190/PLA_2-treated cells. Moreover, MG-132 (proteasome inhibitor) abrogated c-$FLIP_L$ downregulation elicited by treatment with SB202190 and PLA_2 (Fig. 5E). This indicated that sustained JNK activation led to increase in caspase-8 activation and cleavage of Bid through increased degradation of c-$FLIP_L$.

In order to rule out nonspecific action of SP600125 on abolishing the cytotoxic effects of PLA_2, the effect of JNKI-1 on PLA_2-induced mitochondrial depolarization, Fas/FasL upregulation, and c-$FLIP_L$ downregulation was investigated. As shown in Figure 6A, JNKI-1 markedly attenuated the dissipation of $\Delta\Psi m$ in K562 cells induced by a combination of SB202190 and PLA_2. Moreover, JNKI-1 attenuated PLA_2-elicited Fas/FasL upregulation and rescued c-$FLIP_L$ downregulation in PLA_2-treated cells or SB202190/PLA_2-treated cells (Fig. 6B,C). In the meantime, JNKI-1 pretreatment largely reversed the molecular events associated with mitochondria-mediated death pathway including cytochrome *c* release, downregulated Bcl-2 expression, increased mitochondrial translocation of Bax, increased caspase-9 activation, and the production of t-Bid in PLA_2-treated cells (Fig. 6D). Figure 6E shows that JNKI-1 rescued markedly viability of K562 cells treated with PLA_2 or a combination of SB202190 and PLA_2. These results again emphasized a crucial role of JNK activation in the cytotoxicity of PLA_2.

Given that ASK1 is critical for p38 MAPK and JNK activation, the effect of PLA_2 on ASK1 activation was examined [Fujii et al., 2004]. Figure 7A shows that PLA_2 induced phosphorylation of ASK1. Transfection of pKD-ASK1 markedly reduced PLA_2-evoked p38 MAPK and JNK activation (Fig. 7B), indicating that ASK1 was an upstream event responsible for activation of p38 MAPK and JNK in PLA_2-treated cells. p38 MAPK has four isomers including p38α, p38β, p38γ, and p38δ [Krishna and Narang, 2008]. Previous studies reveal that SB202190 inhibits mainly p38α MAPK and p38β MAPK, but it has no effect on p38γ and δ [Kumar et al., 1997]. Given that SB202190 pretreatment led to prolonged JNK activation in PLA_2-treated cells, p38α MAPK and/or p38β MAPK were then be suggested to be involved in negatively regulating JNK activation. In contrast to that of dominant negative mutant of p38β MAPK, dominant negative mutant expression of p38α MAPK resulted in notable increase in the level of phospho-JNK in K562 cells after PLA_2 treatment (Fig. 7C). This suggested that PLA_2-evoked p38α

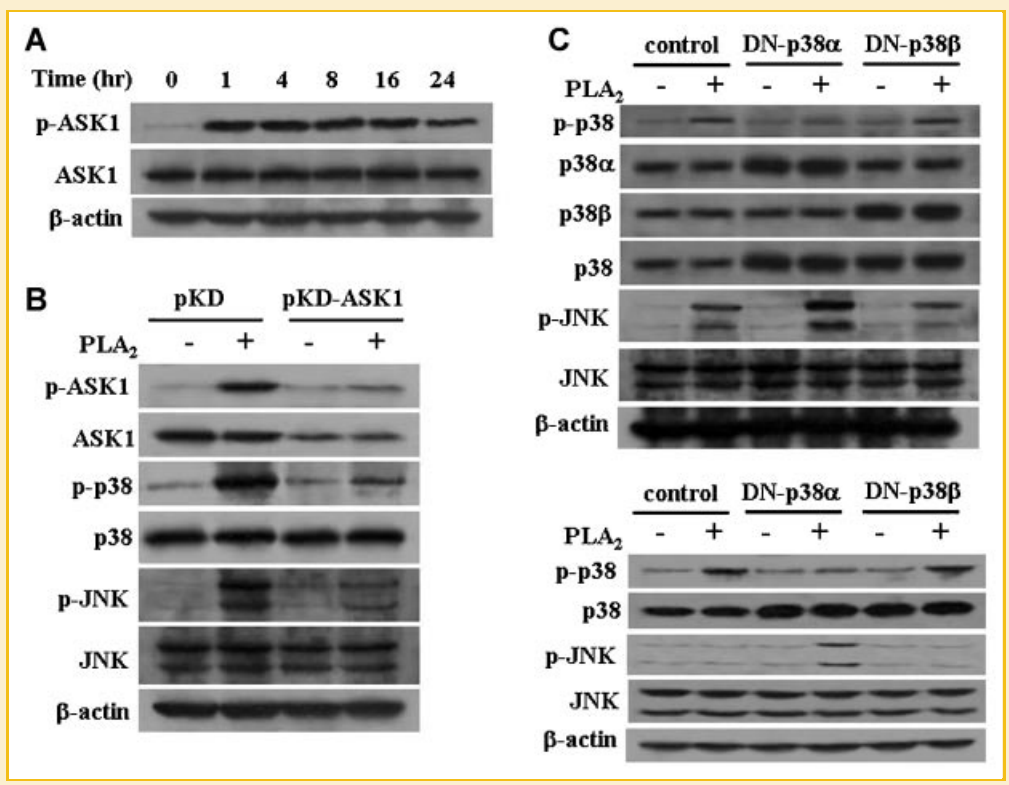

Fig. 7. p38α MAPK suppressed sustained JNK activation in PLA_2-treated K562 cells. A: PLA_2-induced activation of ASK1 in K562 cells. K562 cells were treated with 3 μM PLA_2 for indicated time periods. B: PLA_2-elicited p38 MAPK and JNK activation through ASK1. K562 cells were transfected with control vector (pKD) or pKD-ASK1, respectively. After 24 h post-transfection, cells were treated with 3 μM PLA_2 for 4 h. C: Transfection of dominant negative p38α MPAK led to a marked increase in the level of phospho-JNK. K562 cells were transfected with an empty expression vector, pcDNA-DN-p38α or pcDNA-DN-p38β, respectively. The cells were treated with 3 μM PLA_2 for 4 h (top panel) or 24 h (bottom panel) after 24 h post-transfection.

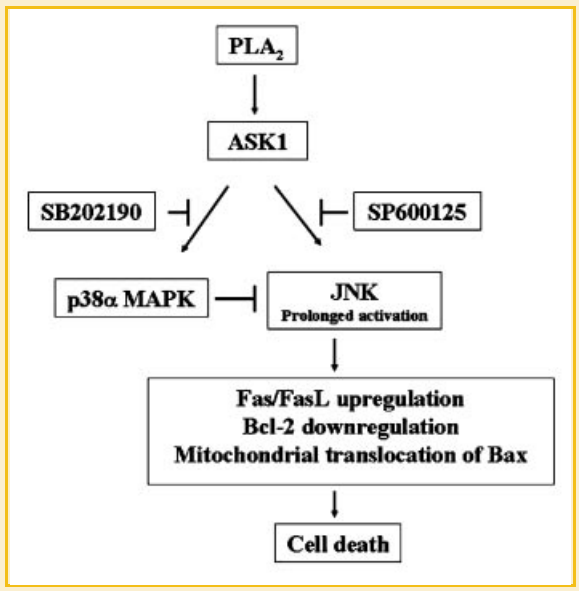

Fig. 8. Signaling pathway of PLA_2-induced apoptotic death of K562 cells. PLA_2 treatment led to p38α MAPK and JNK activation via ASK1 pathway. p38α MAPK activation inhibited persistent activation of JNK. JNK activation led to Fas/FasL protein expression and modulated expression of Bcl-2 family proteins. Finally, PLA_2 induced apoptotic death of K562 cells.

MAPK activation suppressed persistent phosphorylation of JNK. Previous studies revealed that JNK activation was negatively regulated by p38α MAPK in hepatocytes [Heinrichsdorff et al., 2008].

Our results show that PLA_2-induced death of K562 cells is partly associated with autocrine Fas-mediated death pathway. Suppression of JNK activation abolishes upregulation of Fas and FasL protein expression in cells treated with PLA_2 or a combination of SB202190 and PLA_2, suggesting that JNK regulates protein expression of Fas and FasL. This is in line with the findings that JNK is functionally involved in transcriptional regulation of Fas and FasL [Faris et al., 1998; Kasibhatla et al., 1998; Kuo et al., 2005]. Additionally, previous studies showed that JNK pathway regulates mitochondrial apoptotic cell death via modulation of Bcl-2 and Bax protein expression [Yamamoto et al., 1999; Schroeter et al., 2003; Asakura et al., 2008; Lee et al., 2008]. Consistent with these observations, our data reveal that sustained JNK activation further induces an increase in mitochondrial translocation of Bax and Bcl-2 downregulation. Thus, both Fas/caspase-8/mitocondrial pathway and modulation of Bax/Bcl-2 protein expression contribute to cell death elicited by treating with a combination of SB202190 and PLA_2.

Conclusively, PLA_2 treatment of K562 cells induces p38 MAPK and JNK activation through ASK1 pathway (Fig. 8). Suppression of p38α MAPK leads to prolonged JNK activation. PLA_2-induced JNK activation elicits autocrine Fas-mediated death pathway and modulates Bcl-2/Bak protein expression. Sustained JNK activation aggravates mitochondria alteration via increase in caspase-8 activation, Bcl-2 downregulation, and mitochondrial translocation of Bax. Thus, SB202190 pretreatment results in enhancing the cytotoxicity of PLA_2. In contrast to that of PLA_2-treated K562 cells, p38 MAPK activation is exclusively responsible for apoptotic death of PLA_2-treated U937 cells [Liu et al., 2009]. Moreover, JNK activation is insignificantly noted with PLA_2-treated U937 cells [Liu et al., 2009]. This likely reflected inherently different death and/or survival signaling pathways between acute myeloid leukemia cells and chronic myeloid leukemia cells. Understanding of PLA_2-induced cell death may suggest additional targets and/or rational strategies for leukemia therapy.

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