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Over-expression of JunB inhibits mitochondrial stress and cytotoxicity in human lymphoma cells exposed to chronic oxidative stress

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Activator protein-1 can induce either cell survival or death, which is controlled by opposing effects of different Jun members. It is generally accepted that c-Jun is pro-apoptotic, but that JunD is anti-apoptotic in stress-exposed cells. Additionally, although there are reports suggesting that JunB plays a protective role, its role in stress-induced apoptosis remains unclear. Here, we investigated the role of JunB in H_2O_2-induced cell death using cells that over-expressed the protein or were transfected with si-JunB. Inhibition of JunB expression accelerated H_2O_2-mediated loss of mitochondrial membrane potential (MMP) and cytotoxicity. Conversely, over-expression of JunB protein led to significant inhibition of the MMP loss and cell death. The increase in JunB expression also attenuated nuclear relocation of apoptosis-inducing factor and mitochondrial Bcl-2 reduction that occurred following H_2O_2 exposure. These results suggest that JunB can signal survival against oxidant-mediated cell death by suppressing mitochondrial stress. [BMB reports 2010; 43(1): 57-61]

INTRODUCTION

Activator protein-1 (AP-1) regulates a broad range of cellular events in response to extracellular signals. This transcription factor can induce either cell survival or death under stress conditions, and this induction is controlled by opposing effects of different Jun members, including c-Jun, JunB, and JunD (1-3). It is generally accepted that c-Jun is pro-apoptotic, but JunD is anti-apoptotic in stress-exposed cells, whereas c-Jun N-terminal kinase (JNK) signals both cell survival and apoptosis by mediating JNK/JunD and JNK/c-Jun signaling, respectively (2-4). This dual role depends on the patterns of JNK activation, specifically, whether there is a transient or prolonged activation in response to stresses (5). Additionally, we recently found that the JNK/c-Jun pathway could signal cell death under chronic oxidative stress.

There have been several reports of the protective role of JunB against stress-mediated apoptosis. Additionally, it was reported that JunB inhibits endoplasmic reticulum stress and apoptosis in pancreatic beta cells (6). The extracellular signal-regulated kinase (ERK)-mediated pathway is believed to be related to the expression of JunB (7); however, the exact role of this protein in cellular signaling in response to stress is still unclear. Moreover, the dual role of the Jun family in regulation of pro- and anti-apoptotic signals varies from cell to cell and under different stress conditions (8). In this study, we investigated the role of JunB in cell death caused by continuous exposure to H_2O_2. To accomplish this, we compared the response of JunB over-expressing or si-JunB transfected human lymphoma cells to H_2O_2 generated by glucose oxidase. We found that JunB mediates survival response in cells exposed to H_2O_2 by reducing mitochondrial stress.

RESULTS

Inhibition of JunB expression by siRNA transfection promotes MMP loss and cell death in H_2O_2-exposed cells

Previously, we found that the continuous presence of H_2O_2 induces mitochondrial stress and subsequent cell death in a time- and dose-dependent manner (9). To determine the role of JunB in H_2O_2-induced cell death, we transfected cells with JunB-specific siRNA. siRNA targeting of the JunB gene almost completely attenuated the cellular level of the protein at 24 h after transfection (Fig. 1A). Additionally, siRNA transfection significantly augmented the H_2O_2-induced loss of mitochondrial membrane potential (MMP) and cytotoxicity in both BJAB and Jurkat cells (Figs. 1B, C).

Over-expression of JunB signals cells to protect against H_2O_2-mediated cytotoxicity by inhibiting mitochondrial stress

To better understand the role of JunB in H_2O_2-exposed cells, we

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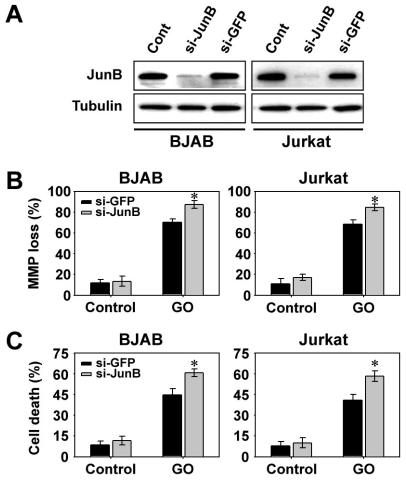

Fig. 1. Transfection of cells with JunB-specific siRNA accelerates MMP loss and cell death caused by H_2O_2 exposure. (A) Western blot analysis of the cellular JunB level 24 h after siRNA transfection. si-GFP or si-JunB transfected cells (2 x 10^6 cells/ml) were exposed to 10 mU/ml glucose oxidase at 24 h after transfection. Twelve hours after exposure, the MMP level (B) and cytotoxicity (C) were evaluated. *P < 0.05 vs. si-GFP transfected cells.

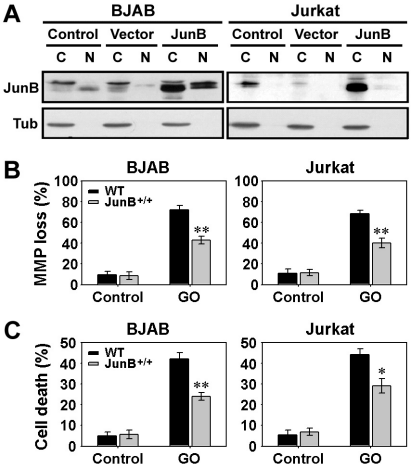

Fig. 2. Over-expression of JunB protein attenuates H_2O_2-mediated MMP loss and cytotoxicity. (A) BJAB or Jurkat cells were transfected with JunB expression vector and the cytosolic and nuclear JunB levels were determined by immunoblotting after 18 h of transfection. C and N represent the cytosol and nucleus, respectively. Control and JunB over-expressing cells were exposed to 10 mU/ml GO for 12 h, after which they were analyzed for the MMP level (B) and cytotoxicity (C). *P < 0.05 and **P < 0.01 vs. glucose oxidase-exposed control cells.

constructed a stable BJAB and Jurkat cell line that expressed JunB. Transfecting cells with JunB expression vector led to significant accumulation of the protein when compared to the control and vector-transfected cells (Fig. 2A). Additionally, over- expression of JunB significantly inhibited the loss of MMP (Fig. 2B) and cell

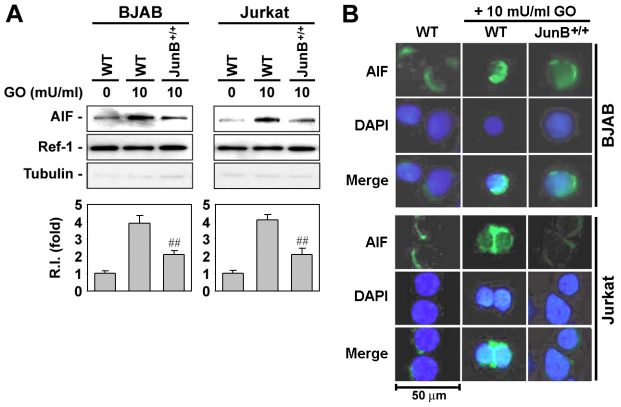

Fig. 3. The increase in JunB expression diminishes the nuclear AIF translocation and nuclear condensation in H_2O_2-exposed cells. (A) Nuclear proteins were prepared from control and JunB over-expressing cells incubated in the presence or absence of 10 mU/ml glucose oxidase for 12 h, after which western blot analysis was performed using AIF specific antibody. ##P < 0.01 vs. glucose oxidase-exposed control cells. (B) The cells were also immunostained with FITC-AIF (green) after 12 h of H_2O_2 exposure and then counterstained with DAPI (blue) prior to confocal microscopy analysis. A representative result from triplicate experiments is shown.

death that had increased after exposure to H_2O_2 (Fig. 2C).

Western blot analysis and confocal microscopy also showed that an increase in JunB expression efficiently blocked the nuclear translocation of apoptosis-inducing factor (AIF) and nuclear condensation that occurred in response to continuous exposure to H_2O_2 (Figs. 3A, B).

Over-expression of JunB inhibits the H_2O_2-mediated decrease of Bcl-2 in mitochondria

The loss of MMP is a key event in the apoptotic process that is strongly influenced by the mitochondrial status of Bcl-2 family proteins (10). We previously reported that expression of the Bcl-2 protein was apparently diminished in H_2O_2-exposed cells (11). Thus, we evaluated the effects of JunB protein on the mitochondrial status of Bcl-2 protein in H_2O_2-exposed cells. Exposure of the control BJAB cells to 10 mU/ml glucose oxidase for 6 h led to a significant decrease in Bcl-2 in the mitochondrial fraction, whereas there was no decrease in the transfected cells (Fig. 4). Additionally, transfection itself had no effect on the basal level of Bcl-2 protein in mitochondria. These findings were similar to that the results obtained when control or JunB transfected-Jurkat cells were exposed to the same conditions (data not shown).

DISCUSSION

There is increasing evidence that AP-1 mediates both the positive and negative signals to cells in response to extracellular signals (4, 12). This mediation varies from cell to cell and depends on the combination of AP-1 sub-proteins (13, 14). When com-

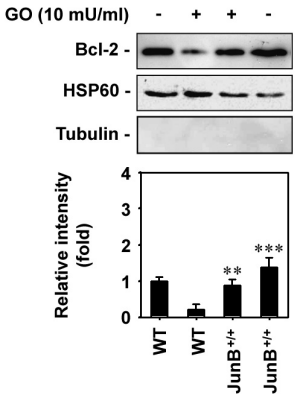

Fig. 4. Over-expression of JunB inhibits the H_2O_2-mediated decrease in the mitochondrial Bcl-2 level. The level of Bcl-2 in the mitochondria was determined by western blot analysis after 6 h of H_2O_2 exposure. The intensity of the bands was measured using a densitometer, and the results shown represent the means ± SD from triplicate experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. the glucose oxidase-exposed control cells.

pared to the abundant evidence that c-Jun and JunD proteins play important roles in determining the fate of cells, the role of JunB in H_2O_2-exposed cells has not been well established. Here, we showed that JunB mediates a protective role against chronic oxidative stress. Although the precise role of JunB in this mediation is unclear at present, it is insensitive to phosphorylation by JNK and a potent inhibitor of c-Jun transactivation that leads to cell death (15, 16). Inhibiting JNK activation increased the expression of JunB, whereas blockage of JunB by siRNA transfection facilitates cytokine-induced apoptosis (6, 17). Over-expression of JunB also protected pancreatic beta cells from cytokine-induced cell death (6). These findings combined with the results of the current study suggest that the predominant role of JunB is to protect cells from oxidative stress.

H_2O_2 leads to a change in the mitochondrial composition of Bcl-2 family proteins (18). In this study, we demonstrated that an increase in JunB expression attenuates H_2O_2-induced cell death by inhibiting MMP loss and nuclear AIF translocation. Over-expression of this protein also blocked the reduction of Bcl-2 protein in the mitochondria. These findings indicate that JunB leads to cell survival by attenuating mitochondrial stress through modulation of the mitochondrial Bcl-2 composition. It is a common concept that the mitochondrial pathway is involved in JNK-stimulated apoptosis (19). Furthermore, inhibition of JNK activation has been shown to increase the expression of JunB, but JunB can attenuate c-Jun expression (16, 20). Activation of JNK is also known to reduce Bcl-2 function (21, 22), while increased Bcl-2 expression has been shown to attenuate the activity of JNK and c-Jun (23). Taken together, these findings lead us to postulate that over-expression of JunB had a negative effect on JNK/c-Jun-mediated signaling and subsequently prevented mitochondrial stress by inhibiting degradation of the Bcl-2 protein. However, additional experiments are necessary to clarify how JunB interacts with JNK or c-Jun under oxidative stress.

Several reports have emphasized the controversial role of ERK in H_2O_2-induced apoptosis (24, 25). It has also recently been suggested that JunB expression is affected by ERK-mediated signaling. Additionally, an inhibitor of ERK was shown to suppress growth factor-induced expression of JunB, suggesting an interaction between ERK activation and JunB expression (7). Furthermore, it was reported that the ERK/JunB-mediated pathway plays a critical role in the induction of NOX1 expression in vascular smooth muscle cells (7). Our current findings also suggest that an inhibitor of ERK accelerates the cell death caused by H_2O_2 exposure, whereas inhibitor of JNK or p38 significantly diminishes the H_2O_2-mediated cytotoxicity (26). These findings indicate that the ERK-mediated pathway signals cell survival in H_2O_2-exposed cells, which can be mediated by JunB. More detailed mechanistic studies are currently being conducted in our laboratory to elucidate the relationship between ERK and JunB.

MATERIALS AND METHODS

Chemicals

Unless otherwise specified, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). All reagents were prepared immediately before use.

Cell culture and treatment

Human lymphoma BJAB and Jurkat cells were cultured in RPMI-1640 medium supplemented with antibiotics (Gibco^{TM}, Grand Island, NY) and 10% fetal bovine serum (FBS; HyClone, Logan, UT). Cultures were placed into a fresh batch of RPMI-1640 medium supplemented with 0.5% FBS immediately before the cells were exposed to glucose oxidase. The addition of 10 mU/ml glucose oxidase into cultures generated 1 to 2.4 μM H_2O_2/min for up to 24 h (27).

Measurement of cytotoxicity and MMP

The cytotoxicity was determined by staining aliquots of cells with 0.4% trypan blue and then counting approximately 100 cells. Cell death was calculated as follows: % cytotoxicity = (total cells − viable cells)/total cells) × 100%. In addition, H_2O_2-exposed cells (2×10^6 cells/ml) were stained with 50 nM 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$; Molecular Probes, Eugene, OR) for 20 min at 37°C. The fluorescence related to MMP was measured using a FACS Calibur® system (Becton Dickinson, San Jose, CA). The level of MMP was determined using the WinMDI 2.9 program.

Western blot analysis

Whole cell lysates were made in a lysis buffer as described previously (9, 11), after which the protein content was quantified according to the Bradford method (28). Nuclear and mitochondrial fractions were prepared as described elsewhere

(9). An equal amount of protein (30 μg) per sample was separated by 12% SDS-PAGE and then blotted onto PVDF membranes before probing with primary and secondary antibodies. The blots were developed with enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK) before exposure to X-ray film. In this study, polyclonal antibodies specific to HSP60 (sc-1052), Bcl-2 (sc-783) and JunB (sc-73), and monoclonal antibodies specific to AIF (sc-13116) and Ref-1 (sc-17774) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody against α-tubulin was purchased from BD Bioscience Pharmingen (San Diego, CA).

Immunofluorescence assay

H_2O_2-exposed cells were fixed in 4% paraformaldehyde and then incubated with primary antibody specific to AIF in blocking solution for 2 h at room temperature prior to staining with FITC-conjugated secondary antibody. Cells were counterstained in blocking solution containing 1 μg/ml 4'-6-diamidino-2-phenylindole (DAPI) for 15 min. After washing three times with PBS, coverslips were mounted onto microscope slides using ProLong antifade mounting reagent (Molecular Probes, Eugene, OR).

Small interfering RNA transfection

siRNA-mediated silencing of the JunB gene was performed as described elsewhere, with slight modification (6, 9). Briefly, cells were transfected with 30 nM siRNA duplexes against JunB and GFP using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cellular level of JunB was checked by immunoblotting, and all assays were performed 24 h after transfection.

Vector construction and transfection

The JunB expression vector was constructed by cloning the coding sequences into pcDNA3.1 (Invitrogen, Burlington, Ontario, Canada). Briefly, the coding region for human JunB was generated by RT-PCR of mRNA from a laboring mouse myometrium using Taq polymerase (MBI Fermentas, Flamborough, Ontario). The following primers were used to amplify the full-length JunB coding region with the addition of a 5′ BamH1 and 3′ EcoRI site (please see Figure S1): JunB (cds)-U, 5′-AGG ATC CGA TGT GCA CTA AAA TGGC A-3′ and JunB (cds)-L, 5′-AGA ATT CTC AGA AGG CGT GTC CCT T-3′. The digested amplicon was then inserted into the pcDNA3.1 vector at the BamH1/EcoRI sites. The expression vector sequence was confirmed by nucleotide sequencing (sequences were provided in Figure S2).

For construction of the stable cell lines, BJAB and Jurkat cells were seeded at 100,000 cells/well in a 24-well plate to confirm that the cells were in the log phase of growth when transfected. The next day, the cells were transferred to serum free media immediately before transfection. The cells were transfected using 2 μl Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) in the presence of 0.8 μg of constructed vector. Briefly, the DNA-reagent complex was applied to the cells, after which they were transferred to fresh medium supplemented with 10% FBS and then incubated with the complex for 6 h at 37°C. After 48 h of incubation, over-expression of JunB was confirmed by Western blot analysis. For stable cell line selection, the transformed cells were treated with G418 during the culture period.

Statistical analysis

All data are expressed as the means ± standard deviation (SD). One-way analysis of variance (ANOVA) (SPSS version 16.0 software) followed by a Scheffe's test was used to determine significant differences between the groups. A value of $P < 0.05$ was considered significant.

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