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Phosphorylation of proteins and apoptosis induced by c-Jun N-terminal kinase1 activation in rat cardiomyocytes by H_2O_2 stimulation

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Abstract

Cytokines and various cellular stresses are known to activate c-Jun N-terminal kinase-1 (JNK1), which is involved in physiological function. Here, we investigate the activation of JNK1 by oxidative stress in H9c2 cells derived from rat cardiomyocytes. H_2O_2 (100 μM) significantly induces the tyrosine phosphorylation of JNK1 with a peak 25 min after the stimulation. The amount of JNK1 protein remains almost constant during stimulation. Immunocytochemical observation shows that JNK1 staining in the nucleus is enhanced after H_2O_2 stimulation. To clarify the physiological role of JNK1 activation under these conditions, we transfected antisense JNK1 DNA into H9c2 cells. The antisense DNA (2 μM) inhibits JNK1 expression by 80% as compared with expression in the presence of the sense DNA, and significantly blocks H_2O_2-induced cell death. Consistent with the decrease in cell number, we detected condensation of the nuclei, a hallmark of apoptosis, 3 h after H_2O_2 stimulation in the presence of the sense DNA for JNK1. The antisense DNA of JNK1 inhibits the condensation of nuclei by H_2O_2. Under these conditions, the H_2O_2-induced phosphorylation of proteins with molecular masses of 55, 72, and 78 kDa is blocked by treatment with the antisense DNA for JNK1 as compared with the sense DNA for JNK1. These findings suggest that JNK1 induces apoptotic cell death in response to H_2O_2, and that the cell death may be involved in the phosphorylations of 55, 72, and 78 kDa proteins induced by JNK1 activation. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: c-Jun N-terminal kinase; Hydrogen peroxide; Antisense; Apoptosis

1. Introduction

In response to ischemia and reperfusion, mammalian cells induce various pathways that lead to cell death and organ dysfunction [1,2]. The cellular injury is known to be caused mainly by oxidative stress, such as by H_2O_2 generated during ischemia and reperfusion. Oxidative stress can trigger the activation of multiple signaling pathways including phosphorylation cascades such as those involving mitogen-acti-

Abbreviations: JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium

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vated protein kinase (MAPK) superfamily members and protein kinase C isoforms, which might regulate cellular injury [3–13]. In particular, members of the MAPK superfamily are responsible for the phosphorylation of a variety of proteins, including downstream kinases and transcription factors related to physiological functions in response to oxidative stresses [14–18]. The MAPK superfamily is classified to three subfamilies, extracellular regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and $p38^{MAPK}$ [19]. Among members of the MAPK superfamily, JNK has been shown to be important in the signaling pathways to apoptosis. JNK is activated in response to a variety of cellular stresses, including DNA damaging agents, heat shock, proinflammatory cytokines, or hypoxia and reoxygenation via the phosphorylation of threonine and tyrosine residues by SEK1 or MKK4 [20–22]. These cellular stresses can produce hydrogen peroxide, a major oxidative stress, as well as ischemia and reperfusion, indicating that oxidative stresses may be involved in cellular injury through the JNK pathway. Therefore, increased interest has focused on the role of the JNK pathway in response to oxidative stress. Recently it was reported that JNK activated by H_2O_2 participates in the induction of apoptosis through CPP32, a caspase family member [16]. However, downstream factors in the JNK activation by H_2O_2 are poorly understood. We recently demonstrated that in the heart, JNK1 translocates to the nucleus from the cytoplasm during ischemia, and is activated in the nucleus during postischemic reperfusion [23]. The nuclear activation of JNK1 rapidly induces the expression of the *c-jun* gene, suggesting that JNK1 activation may play a role in ischemia and reperfusion [23]. However, the role of JNK1 activation during postischemic reperfusion in the heart is presently unknown. Neither a specific inhibitor of JNK1 nor mice in which the targeted gene for JNK1 is disrupted have not been produced, although inhibitors of ERK [24–26] and $p38^{MAPK}$ [27] have been developed.

In this study we prepared an antisense oligoDNA of JNK1 which can be applied in vivo, and investigated the role of JNK1 activation in response to H_2O_2, a major molecule in reperfusion injury, with special attention paid to the physiological substrates of JNK.

2. Materials and methods

2.1. Materials

Anti-JNK-1 antibody (C-17), anti-phospho-JNK-1 antibody (G-7), anti-Bax antibody, and anti-HSP27 antibody were bought from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p53 antibody was bought from PharMingen (San Diego, CA). Anti-α-actin antibody was from Sigma Chemical Co. (St. Louis, MO). An enhanced chemiluminescence reaction kit assay and anti-rabbit immunoglobulin G antibodies-coupled to peroxidase were obtained from Amersham., and the cell counting kit (modified MTT assay) was from Dojindo Chemicals (Kumamoto, Japan). Milli-Q water was used in all experiments (synthesis A10, Millipore, Bedford, MA, USA). All other chemicals were commercially available.

2.2. Cell culture and ischemic hypoxia/reoxygenation

The embryonic rat heart-derived cell line, H9c2 cells, were plated at a density of 5×10^4 cells per dish in 100-mm culture dishes. After incubation in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum for 72 h, the cells were cultured in serum-free DMEM for 60–72 h [5].

2.3. Electrophoresis and immunoblotting

The extracts and molecular mass standards were electrophoresed on 10% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) and transferred to nitrocellulose membranes (0.45 μm). The blots were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20, and incubated with antibody. After the blots were washed, the antigens were visualized by enhanced chemiluminescent detection reagents.

2.4. Immunofluorescent staining

H9c2 cells were seeded in a Chamber Slide at a density of 3×10^4 cells per well. The cells were subjected to H_2O_2 for 2 h, and then fixed with 1% glutaraldehyde for 30 min. Following fixation, the cells were incubated with 1 mM Hoechst 33258 in phos-

phate-buffered saline (PBS), and washed with PBS. The cells were viewed under a confocal fluorescence microscope (LSM 510, Carl Zeiss, Heidelberg, Germany). For JNK1 staining, the cells were fixed with acetone/methanol (50:50) for 3 min at −20°C. Following fixation, the cells were blocked with 10% fetal bovine serum in PBS for 1 h, then incubated for 24 h at 4°C with antibody against JNK1 at 1:100 dilution in PBS containing 3% bovine serum albumin, washed with PBS, and incubated for an additional hour at room temperature with Cy2-conjugated anti-rabbit IgG at 1:800 dilution in PBS containing 3% bovine serum albumin. The cells were viewed under a fluorescence microscope (Axioplan 2, Carl Zeiss).

2.5. Transfection of DNAs into H9c2 cells

Transfection of the oligonucleotide for JNK1 was determined as described previously [5,28]. Briefly, a phosphorothioate oligonucleotide with the sequence 5′-ACT TCT GCT CAT GAT GGC-3′ for JNK1 was synthesized as an antisense DNA. A control phosphorothioate oligonucleotide sequence 5′-GCC ATC ATG AGC AGA AGT-3′ for JNK1 (sense) was also synthesized. The cells (typically 80% confluent in 24-well dishes) were washed three times with PBS. Appropriate dilutions of oligonucleotides in 200 μl of serum-free DMEM including liposomes (Tfx-50, Promega Co., Madison, WI) were preincubated at room temperature for 15 min. The cells were incubated for 1 h at 37°C in the presence of 5% CO_2. At the end of the incubation period, 1 ml of medium containing 10% fetal bovine serum was added. After incubating for 12 h, the cells were incubated in serum-free medium for 60–72 h. The expression of JNK1 was confirmed by immunoblotting using anti-JNK1 antibody. After incubation under conditions of oxidative stress, the cells were used for biochemical assays. Densitometric measurements were performed with a plate analyzer (ETY3A, Toyo Sokki Co., Kanagawa, Japan).

3. Results

We used cells incubated in serum-free DMEM for 60–72 h since JNK activity was detected for up to 48 h in untreated cells after the removal of serum. We first examined the time course of JNK1 activation after H_2O_2 stimulation by immunoblotting using an anti-phospho-JNK1 antibody that recognizes the tyrosine phosphorylation of JNK1 (Tyr-185) necessary for activation. JNK1 phosphorylation increased significantly by approximately 4-fold by 25 min after H_2O_2 stimulation (Fig. 1A), and the phosphorylation of JNK2 with a molecular mass of 54 kDa was also detected by the antibody. The amount of JNK1 remained almost constant up to 30 min after stimulation (Fig. 1B). JNK1 phosphorylation correlated with the JNK activity measured by immunoprecipitation-kinase assay using c-Jun as a substrate (data not shown). Immunocytochemical observation using the anti-JNK1 antibody showed that JNK1 was localized mainly in the cytosol under control conditions (Fig. 2A), and nuclear staining was enhanced after H_2O_2 stimulation for 25 min (Fig. 2B). To investigate the role of JNK1 in H_2O_2-induced cell death, we transfected an antisense oligonucleotide against JNK1 into H9c2 cells, and found JNK1 expression to be inhibited by 85% at a concentration of

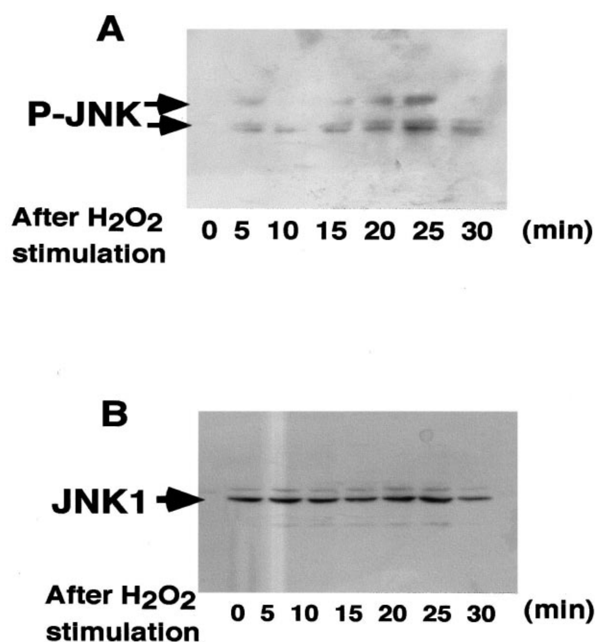

Fig. 1. Time course of tyrosine phosphorylation of JNK1 (Tyr-185) by H_2O_2 stimulation. Cell extracts (40 μg protein) were prepared from H9c2 cells exposed to H_2O_2 (100 μM) for the indicated times, and subjected to immunoblotting with anti-phospho-JNK1 antibody (A) or anti-JNK antibody (B). The figure shows representative immunoblots obtained from four independent experiments as described in Section 2.

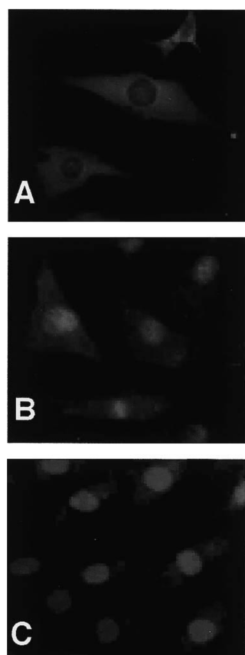

Fig. 2. Localization of JNK1 after H_2O_2 stimulation. H9c2 cells were incubated for 0 min (A,C), for 25 min after the addition of H_2O_2 (B), fixed in cold acetone/methanol (50:50), and stained with anti-JNK1 antibody (A,B), or with anti-histone antibody (C) as described in Section 2. The figure shows representative photographs obtained from four independent experiments. Final magnification ×400.

2.0 μM DNA (Fig. 3). Incubation of cells with 2 μM of the sense or scrambled DNA for JNK1 had no significant effect on JNK1 expression for up to 4 days. MAPK expression remained almost unchanged in cells transfected with JNK1 DNA as compared with untreated cells (Fig. 3). We used the WST-1 assay to examine cell number in cells transfected with the antisense DNA for JNK1 after the addition of H_2O_2. Cells whose JNK1 protein was depleted by antisense DNA showed a complete inhibition of H_2O_2-induced cell death, while sense DNA-treated cells underwent cell death to the same extent

Fig. 3. Effect of JNK1 antisense DNA on JNK and MAPK expression in H9c2 cells. Cell extracts (40 μg protein) were prepared from H9c2 cells with 2 μM sense DNA or antisense DNA, and subjected to immunoblotting with anti-JNK1 antibody or anti-MAPK antibody. The figure shows representative immunoblots obtained from two independent experiments.

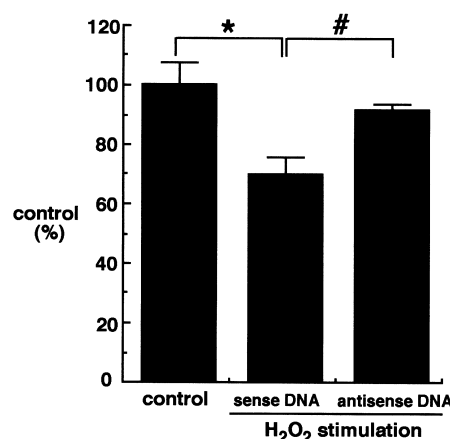

Fig. 4. Effect of JNK1 antisense DNA on cell death after H_2O_2 stimulation. H9c2 cells were pretreated with the sense or antisense DNA for JNK1 at the indicated concentrations in DMEM containing liposomes (Tfx-50), and exposed to H_2O_2 (100 μM) for 6 h. Cell numbers were determined by the WST-1 assay (mean ± S.E., $n = 5$; $^*P < 0.05$, $^\#P < 0.05$).

as untreated cells for up to 6 h under the same conditions (Fig. 4). Next, we evaluated cells subjected to H_2O_2 for nuclear condensation, a hallmark of apoptosis, using Hoechst 33258. One hour after H_2O_2 stimulation, no nuclear condensation was detectable under the conditions in this study (data not shown). Consistent with the decrease in cell number by stimulation with H_2O_2, shrinking of the cytoplasm, membrane blebbing, and nuclear condensation were observed in cells treated with the sense DNA for JNK1 after 3 h of H_2O_2 stimulation (Fig. 5). Treatment with the antisense DNA for JNK1 has no effect

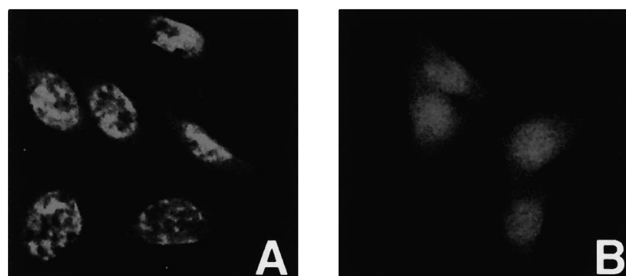

Fig. 5. Effect of JNK1 antisense DNA on apoptotic cell death after H_2O_2 stimulation. H9c2 cells were pretreated with the sense (A) or antisense (B) DNA for JNK1 at the indicated concentrations in DMEM containing liposomes (Tfx-50), and were exposed to H_2O_2 (100 μM) for 3 h. The cells were then fixed with glutaraldehyde solution (1% glutaraldehyde in PBS), and stained with 1 mM Hoechst 33256 as described in Section 2. The figure shows representative photographs from three independent experiments. Final magnification ×800.

on the loss of cell number or morphological changes in the nuclei after 3 h of H_2O_2 stimulation. These findings indicate that JNK1 participates in H_2O_2-induced apoptotic cell death. To investigate the physiological substrates of JNK1 involved in H_2O_2-induced cell death, we examined the phosphorylation of proteins after H_2O_2 stimulation in the presence of the antisense or sense DNA for JNK1. Stimulation by H_2O_2 induced the phosphorylation of proteins with molecular masses of 18, 55, 72 and 78 kDa in the presence of the sense DNA for JNK1. The phosphorylation of proteins with molecular masses of 55, 72, and 78 kDa was significantly inhibited in the presence of the antisense DNA for JNK1 (Fig. 6). These findings suggest that 55, 72, and 78 kDa proteins may be involved in H_2O_2-induced cell death through JNK1 activation.

We carried out immunoblotting using anti-p53 antibody to examine whether a protein with molecular mass of 55 kDa is p53, apoptosis-related protein, since it was reported that JNK can phosphorylate directly p53 by H_2O_2 stimulation. In response to H_2O_2, the mobility shift of p53 protein, phosphorylation form of p53, was observed (Fig. 7A), and the mobility shift was inhibited by transfection of antisense DNA for JNK1. The findings suggest that 55 kDa protein phosphorylated by JNK activation may be p53. A transcriptional product of p53, Bax protein also increased by treatment with H_2O_2, the increase of which was blocked by antisense DNA for JNK1 (Fig. 7C). These findings suggest that JNK1 activates p53/Bax pathway in response to H_2O_2 stimulation. We performed the immunoblotting using anti-heat shock protein (HSP)27 antibody, since HSP27 forms a dimer through the phosphorylation in response to various stresses, and functions as a molecular chaperon. Anti-HSP27 antibody recognised only a protein with molecular mass of 27 kDa, a monomer form of HSP27, not 55 kDa pro-

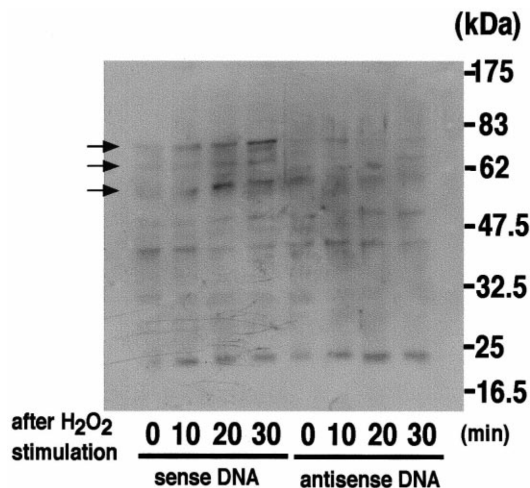

Fig. 6. Effect of JNK1 antisense DNA on the H_2O_2-induced phosphorylation of proteins. H9c2 cells were pretreated with the sense or antisense DNA for JNK1 at the indicated concentrations in DMEM containing liposomes (Tfx-50), and exposed to H_2O_2 (100 μM) for the indicated times. Cell extracts were prepared, and subjected to immunoblotting with anti-phospho-serine/phospho-threonine/phospho-tyrosine antibody. The figure shows representative immunoblots obtained from two independent experiments.

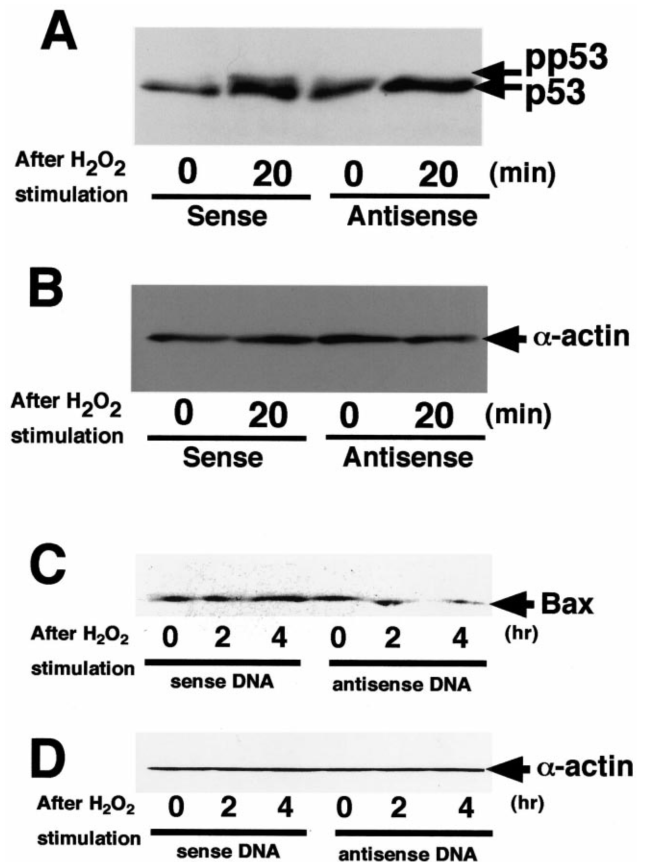

Fig. 7. Effect of JNK1 antisense DNA on the H_2O_2-induced p53 and Bax proteins. H9c2 cells were pretreated with the sense or antisense DNA for JNK1 at the indicated concentrations in DMEM containing liposomes (Tfx-50), and exposed to H_2O_2 (100 μM) for the indicated times. Cell extracts were prepared, and subjected to immunoblotting with anti-p53 antibody (A), anti-α-actin antibody (B,D), and anti-Bax antibody (C). The figure shows representative immunoblots obtained from three independent experiments.

tein in the SDS gel (data not shown). There might be mainly a monomer form of HSP27 in SDS gels as the interactions of protein–protein were broken by sample preparation using heating and SDS.

4. Discussion

In this report, we demonstrate that JNK1 is involved in H_2O_2-induced cell death, and that there are three proteins with molecular masses with 55, 72, and 78 kDa that are involved downstream of JNK1 in response to oxidative stress.

As shown in this study, the antisense DNA for JNK1 inhibits the cell death caused by oxidative stress, suggesting that there may be factor(s) involved in cell death downstream of JNK1. It appears that activated JNK directly phosphorylates proteins in response to H_2O_2, which may lead to apoptosis, since apoptotic cell death in H9c2 cells is rapidly induced after the addition of H_2O_2. In previous papers, several proteins related to apoptosis, such as p53 [29,30], bcl-2 [31,32], and Caspase3 [32] have been identified as substrates of JNK1. At least, we observed the phosphorylations of three proteins (p55, p72, and p78) as JNK substrates during oxidative stress. Among these proteins, a protein with a molecular mass of 55 kDa in SDS–polyacrylamide gel electrophoresis may be p53 as described by our data and Buschmann et al. [29]. As shown in this study, JNK1 translocates from the cytosol to the nucleus in response to H_2O_2. Transcription factor p53 is also known to localize in the nucleus, where it may be phosphorylated p53 by JNK1. The phosphorylation of p53 by JNK1 has been reported to play a role in H_2O_2-induced apoptosis, a finding consistent with our data. In response to oxygen species, HSP27 is phosphorylated, protecting cells against damages [33]. Within the cells, HSP27 can form dimeric complex of 54 kDa. A protein with molecular mass of 55 kDa present in downstream of JNK1 by H_2O_2 stimulation was not appeared to be HSP27, since molecular mass of 27 kDa, a monomer form of HSP27, was mainly detected by immunoblotting using anti-HSP27 antibody. It is possible that a protein with a molecular mass of approximately 70 kDa may be a HSP70 family member or a transcription factor related to heat shock proteins, since oxidative stresses, such as ischemia and hypoxia, induce HSP70 through HSF activation, and the induction of HSP 70 is reported to regulate JNK activity [34–36].

In cardiac myocytes that have lost their mitogenic activity, the preservation of cell viability by the inhibition of apoptotic cell death may be critical for the maintenance of normal cardiac function [1,37,38]. Since ischemia–reperfusion has been shown to induce apoptotic cell death in the heart, the activation of JNK1 might play a role in the signaling pathway of apoptosis in the heart exposed to oxidative stresses such as ischemia and hypoxia. It is possible that the antisense DNA for JNK1 developed in this study may contribute to a therapy for ischemic heart disease involving the inhibition of apoptosis during postischemic reperfusion.

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