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Apoptosis of Mesenchymal Stem Cells Induced by Hydrogen Peroxide Concerns Both Endoplasmic Reticulum Stress and Mitochondrial Death Pathway Through Regulation of Caspases, p38 and JNK

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ABSTRACT

Poor survival of mesenchymal stem cells (MSCs) compromised the efficacy of stem cell therapy for myocardial infarction. The increase of exogenous reactive oxygen species (ROS) in infracted heart is one of the important factors that challenged the survival of donor MSCs. In the study we aimed to evaluate the effect of oxidative stress on the cell death of MSCs and investigate its mechanisms in order to help with the identification of new biological compounds to reduce donor cells damage. Apoptosis of MSCs were evaluated with Hoechst 33342 staining and flow cytometry analysis. The mitochondrial membrane potential of MSCs was analyzed with JC-1 staining. Signaling pathways involved in H_2O_2 induced apoptosis were analyzed with Western blot. H_2O_2 induced apoptosis of MSCs in a dose- and time-dependent manner. H_2O_2 induced apoptosis of MSCs via both endoplasmic reticulum (ER) and mitochondrial pathways rather than extrinsic apoptosis pathway. H_2O_2 caused transient rather than sustained activation of p38 and JNK with no effect on ERK1/2 pathway. P38 was involved in the regulation of early apoptosis of MSCs while JNK was involved in the late apoptosis. P38 directed both ER stress and mitochondria death pathway in the early apoptosis. In conclusion, exogenous ROS was a major factor to induce apoptosis of MSCs. Both ER stress and mitochondria death pathway were involved in the apoptosis of MSCs. H_2O_2 activated p38 that directed the above two pathways in the regulation of early apoptosis of MSCs while JNK was involved in the late apoptosis of MSCs. J. Cell. Biochem. 111:967–978, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: MESENCHYMAL STEM CELL; MYOCARDIAL INFARCTION; REACTIVE OXYGEN SPECIES; APOPTOSIS; SIGNALING PATHWAY

A large number of studies have revealed mesenchymal stem cells (MSCs) transplantation had great promise in the improvement of heart function and neovascularization after myocardial infarction [Wang et al., 2001; Miyahara et al., 2006]. However, the therapeutic efficacy is greatly limited by the poor survival of donor MSCs in the infarcted heart [Wang et al., 2001; Saito et al., 2003]. The viability of transplanted cells is the key to the success of cell therapy. Therefore, it is necessary to identify the factors in the infracted hearts that challenge the survival of donor cells and how they cause donor cell death.

After being transplanted into the infarcted heart, MSCs face a complex environment with numerous factors that cause cell loss and/or death such as mechanical loss, inflammatory reaction, hypoxia, serum and glucose deprivation and oxidative stress, etc. In our previous study hypoxia and serum deprivation (hypoxia/SD) have been identified as an important factor to induce apoptosis [Zhu et al., 2006]. In addition to hypoxia/SD, oxidative stress in the micro-environment is another important factor. Evidences revealed a burst of excessive production of reactive oxygen species (ROS) was generated not only during reperfusion of ischemic cardiomyocytes [Ambrosio et al., 1993; Vanden Hoek et al., 2000], but in ischemic period before reperfusion [Becker et al., 1999; Lu et al., 2004]. ROS responsible for these oxidative stresses include hydrogen peroxide (H_2O_2), free radical superoxide anion (O^{2-}), and hydroxyl radical (OH^-). The balance

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between oxidase and antioxidase was disrupted after myocardial infarction and ROS increased both in the infarcted and uninfarcted areas of the heart where oxidative stress retained [Kinugawa et al., 2000; Sun, 2007]. Augmented ROS in the infarcted heart might be another important factor to induce apoptosis of transplanted MSCs. Understanding its mechanisms will contribute to the development of related bioactive compounds to alleviate the apoptosis of MSC.

ROS contributes to apoptosis through various distinct signaling pathways in various cells. However, the mechanisms of ROS effect on MSCs have not been fully understood to date. As an important signaling molecule, H_2O_2 is used as classical and common model of exogenous oxidative stress because of its unique biochemical properties such as relatively long half-life and solubility in both lipids and aqueous media [Droge, 2002; Ryter et al., 2007] In this study, H_2O_2 was used to treat MSCs to establish an oxidative stress model, and the effect of exogenous ROS on cell viability was observed. Moreover, possible pathways involved in apoptosis of MSCs induced by H_2O_2 were investigated.

MATERIALS AND METHODS

MATERIALS

Iscove's modified Dulbecco's medium (IMDM) and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY). Hoechst 33342 and H_2O_2 were purchased from Sigma-Aldrich (St. Louis), and Annexin V-FITC Apoptosis Detection Kit was from Oncogene (San Diego). The following inhibitors were used: Z-ATAD-FMK (BioVision, Mountain View, CA), Z-DEVD-FMK (R&D Systems. Minneapolis), SP600152 and SB202190 (Beyotime Inst. Biotech, Beijing, PR China), 4-phenylbutyrate (4-PBA) (Biomol, Enzo Life Sciences, Inc. NY). Mitochondrial Membrane Potential Detection Kit (JC-1) and the cytochrome *c* Releasing Apoptosis Assay Kit were from Beyotime Inst. Biotech. Anti-caspase-12, anti-caspase-8, anti-phospho-p38 MAPK (Tyr182), anti-β-actin antibody and the horseradish peroxidase-conjugated secondary antibody to rabbit, mouse or rat were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase-3, anti-phospho-JNK (Thr183/Tyr185), anti-p38 MAPK, anti-JNK, and anti-m-calpain were from Cell Signaling Technology (Danvers, MA).

CELL CULTURE AND TREATMENT WITH H_2O_2 OR INHIBITORS

MSCs were isolated from Sprague–Dawley rats (Vital River Laboratory Animal, Inc., Beijing, http://www.vitalriver.com.cn) as previously described [Zhu et al., 2006]. Bone marrow was collected from tibia and femur of the rats, plated in IMDM with 10% inactivated FBS and incubated at 37°C in a humid atmosphere containing 5% CO_2. The medium was replaced after 24 h to discard nonadherent hematopoietic cells. Adherent MSCs were further grown in the medium replaced every 2 days. Passages 3 of the cells were used in the experiment. In order to mimic conditions in vivo, cells were switched to IMDM containing 2% FBS and incubated with different concentrations of H_2O_2 (0.06, 0.09, or 0.12 mM) for 6, 12, or 24 h. To evaluate the effect of inhibitors, cells were preincubated for 1 h with caspase-12 inhibitor Z-ATAD-FMK (10 μM), caspase-3 inhibitor Z-DEVD-FMK (50 μM), p38 MAPK inhibitor SB202190 (15 μM), JNK inhibitor SP600152 (5 μM), or the chemical chaperone 4-PBA (5 mM) before H_2O_2 treatment.

ASSESSMENT OF MORPHOLOGICAL CHANGES

The chromatin dye Hoechst 33342 was used to assess morphological change of cells. MSCs were fixed for 30 min in phosphate-buffered saline (PBS) containing 1% glutaraldehyde at room temperature, then washed twice with PBS and exposed to 5 μg/ml Hoechst 33342 for 30 min at room temperature. The cells were observed with a fluorescence microscope. Apoptotic cells were characterized by morphological alterations such as condensed nuclei and cell shrinkage.

FLOW CYTOMETRY ANALYSIS OF CELL APOPTOSIS AND MITOCHONDRIAL MEMBRANE POTENTIAL

The Annexin V-FITC/PI Apoptosis Detection Kit was used to evaluate apoptosis of cells. After being rinsed with ice cold PBS, the cells were resuspended in 200 μl of binding buffer. Ten microliters of Annexin V stock solutions was added to the cells and incubated for 30 min at 4°C. The cells were then further incubated with 5 μl propidium iodide (PI) and immediately analyzed with a FACScan. Ten thousands events were acquired on a FACSC-LSR (Becton-Dickinson, San Jose, CA) and analyzed with CellQuest (Becton-Dickinson) software.

Mitochondrial Membrane Potential Detection Kit (JC-1) was used to analyze mitochondrial membrane potential of cells. Cells were resuspended in 1 ml IMDM supplemented with 10% FBS after being rinsed with PBS, then stained with 2.5 g/ml JC-1 for 20 min at 37°C. After being rinsed, the cells were resuspended in 0.5 ml IMDM with 10% FBS and analyzed with FACScan.

PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

After being treated with H_2O_2, the cells were harvested and centrifuged at 500*g* for 10 min at 4°C. The pellets were resuspended in lysis buffer (1% Triton X-100, 20 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM β-glycerol-phosphate, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml each of leupeptin, aprotinin, and pepstatin) for 30 min, then centrifuged at 14,000*g* for 10 min at 4°C. The supernatants were collected and quantified with Bradford Protein assay.

To analyze protein levels, supernatants with equal amounts of proteins were mixed with 5× SDS sample buffer and boiled for 5 min then separated through 8–15% SDS–PAGE gels. The proteins were transferred to nitrocellulose membranes by a semi-dry blotting system after electrophoresis. The membranes were incubated with 5% skim milk to block nonspecific binding at 4°C followed with primary antibody in 5% skim milk overnight at 4°C. After being washed in TBST, the membranes were incubated for 2 h with horseradish peroxidase-conjugated secondary antibody. Bands were visualized by enhanced chemiluminescence and exposed to radiography film after the membranes were washed with TBST.

To analyze cytochrome *c* and Bax in different subcellular fractions, cytosolic and mitochondrial fractions were obtained, respectively, with a cytochrome *c* releasing apoptosis assay kit. Bax and cytochrome *c* in the cytosol and mitochondria were determined with Western blotting.

STATISTICAL ANALYSIS

Data are expressed as mean ± standard deviation. Differences among groups were tested by one-way ANOVA. Comparisons between two groups were evaluated using Student's *t*-test. A value of $P \leq 0.05$ was considered as significantly different.

RESULTS

H_2O_2 INDUCED APOPTOSIS OF MSCS IS DOSE AND TIME DEPENDENT

In order to investigate the effect of H_2O_2 on MSCs, the cells were treated with different concentration of H_2O_2 (0.06, 0.09, and 0.12 mM) for 6–24 h. Characterization of cell death was analyzed by Hoechst 33342 staining and flow cytometry facilitated Annexin V-FITC/PI staining. Hoechst 33342 staining showed control cells had the normal elongated MSCs morphology with large regular nuclei while H_2O_2 treated cells appeared apoptotic characterized with condensed, fractioned nuclei, and cell loss (Fig. 1A,C).

Annexin V-FITC/PI staining revealed 3.73 ± 1.51% of MSCs appeared apoptotic after treatment with 0.06 mM H_2O_2 for 24 h and the percentage of apoptotic cells increased obviously to 13.55 ± 4.56% and 21.85 ± 5.92% when H_2O_2 concentration was raised to 0.09 and 0.12 mM, respectively. The percentage of late apoptotic and necrotic cells demonstrated the same dependency on the concentration of H_2O_2. To determine the time course of H_2O_2 induced apoptosis MSCs were exposed to 0.12 mM H_2O_2 for different period of them (6, 12, and 24 h, respectively). MSCs (5.30 ± 3.06%) appeared apoptotic after 6 h H_2O_2 treatment. Apoptotic cells increased to 16.08 ± 5.23% after 12 h and 21.79 ± 6.58% after 24 h of treatment. Longer treatment of H_2O_2 resulted in a greater population of both early and late apoptotic cells (Fig. 1C,D). Taken together, H_2O_2 induced apoptosis of MSCs was dependent on the dose and duration of the treatment.

H_2O_2 INDUCED MITOCHONDRIAL DYSFUNCTION OF MSCS

Mitochondrion is one of the primary sources of ROS and the key regulator of apoptosis. On the other hand, mitochondrial functions could also be altered by ROS [Olson and Kornbluth, 2001; Tsutsui et al., 2006]. To investigate the change of mitochondrial functions during apoptosis of MSCs induced by H_2O_2 mitochondrial transmembrane potential was measured with JC-1 staining. JC-1 is a mitochondrial $\Delta\Psi$m-sensitive dye. When mitochondrial $\Delta\Psi$m is high, JC-1 accumulates in the matrix of mitochondria by forming J-aggregates with red fluorescence. However, when mitochondrial $\Delta\Psi$m is low JC-1 becomes monomer with green fluorescence. In flow cytometry analysis the lateral and longitudinal axes of coordinates represent distribution of green and red fluorescence, respectively. The ratio of green and red fluorescence indicates depolarization percentage of mitochondria. The results revealed loss in mitochondrial $\Delta\Psi$m after 6 h H_2O_2 treatment despite the fact that only a small percentage of MSCs appeared apoptotic. Therefore, H_2O_2 caused changes of mitochondrial $\Delta\Psi$m earlier than the occurrence of apoptosis. In addition, reduction of mitochondrial $\Delta\Psi$m correlated with the duration of H_2O_2 treatment (Fig. 2A,B).

Cytochrome *c* release from mitochondria into cytosol is another mitochondrial event during apoptosis. In order to investigate cytochrome *c* release during H_2O_2 induced apoptosis of MSCs, cellular fractionation was performed and cell lysates from cytosolic and mitochondrial fractions were subjected to Western blot. The data demonstrated that upon H_2O_2 treatment there was an increase of cytochrome *c* in the cytosolic fraction accompanied with a decrease of that in the mitochondrial fraction (Fig. 2C–F). Bax could oligomerize and translocate to the mitochondrial membrane and contribute to the formation of pores that alter mitochondrial membrane permeability, which in turn facilitate the release of cytochrome *c* from the mitochondria [Saito et al., 2000]. The effect of H_2O_2 on the translocation of Bax was also investigated. The result showed H_2O_2 treatment increased Bax in the mitochondrial fraction accompanied by a decrease of Bax in the cytosolic fraction (Fig. 2G–J). Our data demonstrated that H_2O_2 treatment compromised the mitochondrial potential, caused mitochondrial cytochrome *c* release and Bax mitochondrial translocation in MSCs.

ROLES OF CASPASES IN H_2O_2 INDUCED APOPTOSIS OF MSCS

We next sought to investigate the caspases that are involved in H_2O_2 induced apoptosis of MSCs. We speculated both extrinsic and intrinsic apoptotic pathways are involved. Once the extrinsic apoptotic pathway is initiated, the interaction between a death ligand and its corresponding cell surface receptor leads to activation of caspase-8 and subsequent cleavage of caspase-3 [Muppidi et al., 2004]. In this study H_2O_2 treatment failed to cause appreciable change in the amount of cleaved caspase-8 (Fig. 3A,B). Thus, caspase-8 was not involved in H_2O_2 induced apoptosis in MSCs. This also suggested extrinsic apoptotic pathway did not play an important role in H_2O_2 induced apoptosis of MSCs. Since both extrinsic and intrinsic apoptotic pathway contribute to the activation of caspase-3, we next examined whether caspase-3 was activated by H_2O_2 treatment. Western blot was performed using an anti-caspase-3 antibody. Significant cleaved caspase-3 was observed after H_2O_2 treatment (Fig. 3C,D). Moreover, pretreatment of MSCs before H_2O_2 with Z-DEVD-FMK, a specific inhibitor of caspase-3 reduced the percentage of apoptosis of MSCs (Fig. 3I,J). These results indicated H_2O_2 induced apoptosis of MSCs via the activation of caspase-3.

Previous reports demonstrated that in addition to mitochondria, other organelles such as endoplasmic reticulum (ER) or nucleus could trigger apoptosis of cells [Brenner, 2000; Nakagawa et al., 2000]. ER stress induced apoptosis involves several pathways, among which caspase-12 is believed to be one of the regulators [Nakagawa et al., 2000; Jimbo et al., 2003; Kalai et al., 2003]. In the present study H_2O_2 obviously increased the cleavage of caspase-12 (Fig. 3E,F). The involvement of caspase-12 was further investigated by treating MSCs with H_2O_2 plus Z-ATAD-FMK, a caspase-12 specific inhibitor. Flow cytometric analysis revealed Z-ATAD-FMK reduced the amount of both early and late apoptotic cells (Fig. 3I,J). These results indicated H_2O_2 induced apoptosis of MSCs via the activation of caspase-12. To further investigate the role of ER in H_2O_2 induced-apoptosis, MSCs was treated H_2O_2 plus with 4-PBA, which is known to attenuate ER stress as a chemical chaperone [Ozcan et al., 2006; Sawada et al., 2008; Ozcan et al., 2009]. Flow cytometric analysis indicated that 4-PBA significantly reduced the percentage of early and late apoptotic cells (Fig. 3I,J). In addition,

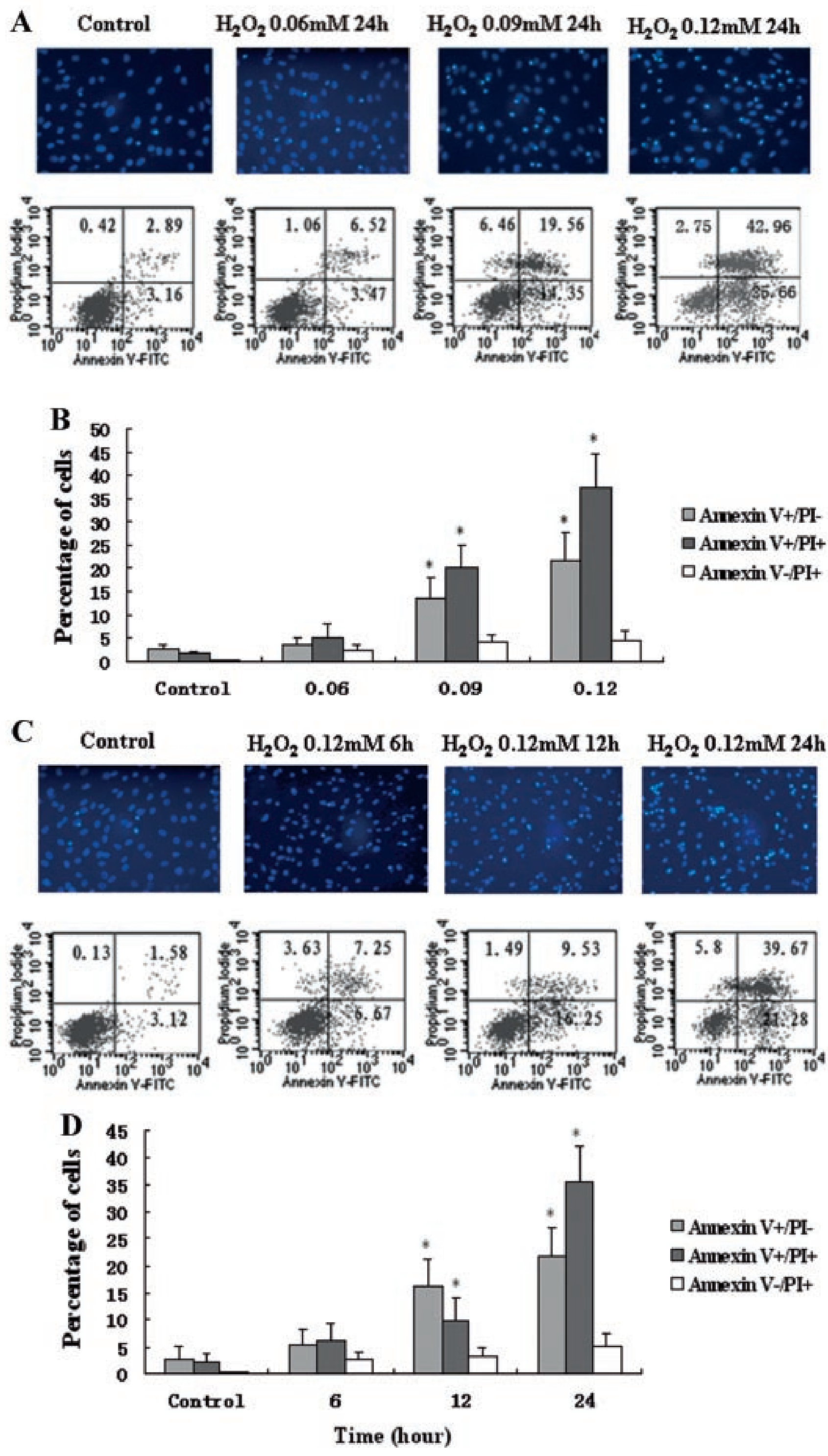

Fig. 1. MSCs were treated with different concentration of H_2O_2 for 24 h and cell death were evaluated with Hoechst 33342 staining (A, top panel) and flow cytometry analysis (A, bottom panel and B). MSCs were treated with 0.12 mM H_2O_2 for different period of time, and cell death was evaluated with Hoechst 33342 staining (C, top panel) and flow cytometry analysis (C, bottom panel and D). Annexin V−/PI−: viable cells; Annexin V+/PI−: early apoptotic cells; Annexin V+/PI+: late apoptotic or necrotic cells; Annexin V−/PI+: necrotic cells. The results represent the mean ± standard of three independent experiments performed in triplicate. $^*P < 0.05$, versus the control cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

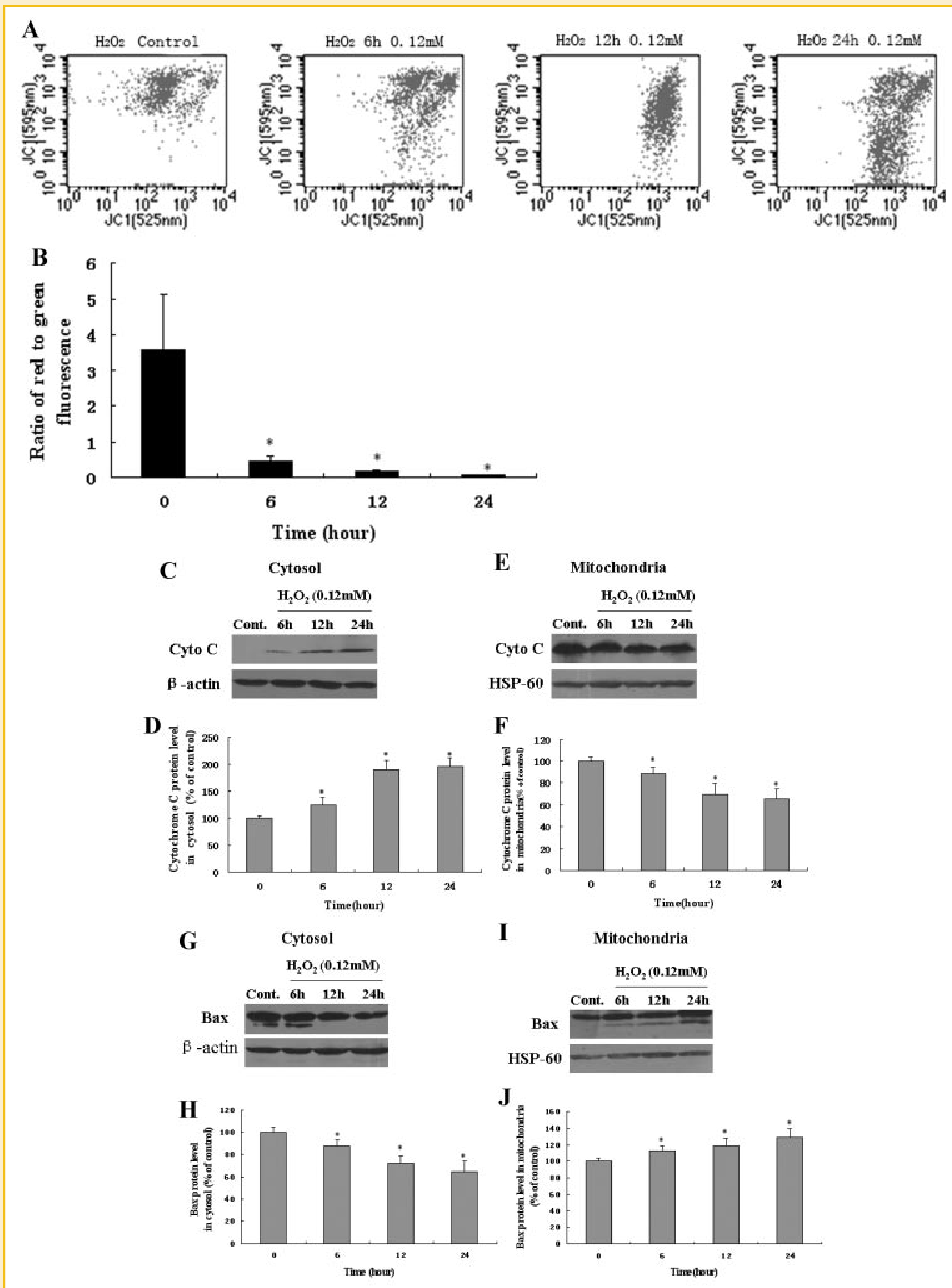

Fig. 2. MSCs were treated with 0.12 mM H_2O_2 for different period of time and mitochondria membrane potential was analyzed with JC-1 staining (A), the ratio of cells with red fluorescence to those with green fluorescence was analyzed in (B). MSCs were treated with H_2O_2 and control cells and treated cells were subjected to subcellular fractionation. Cell lysates from cytosol and mitochondria fraction were subjected to Western blot to detect cytochrome *c* (C–F) and Bax (G–J). Data shown are representative results from three independent experiments. $^{*}P < 0.05$, versus the control cells.

GRP78 protein in MSCs was also detected by Western blot after the cells were treated with H_2O_2. The result indicated that H_2O_2 increased the expression of GRP78 protein (Fig. 3G,H). The data suggested that ER stress was also involved in apoptosis of MSCs induced by H_2O_2.

Our data demonstrated both caspase-3 and caspases-12 were activated by H_2O_2 and they were both involved in H_2O_2 induced apoptosis of MSCs. How they work on each other is of interest. It was reported that caspase-12 activated caspase-3 independent of cytochrome *c* and Apaf-1 [Morishima et al., 2002; Rao et al., 2002]. In this study caspase-12 specific inhibitor Z-ATAD-FMK had no effect on the release of cytochrome *c* from mitochondria (Fig. 3K–N). Neither did it prevent the cleavage of caspase-3 (Fig. 3O,P). Our result suggested that caspase-3 was cleaved independent of the activation of caspase-12 in apoptosis of MSCs induced by H_2O_2, in

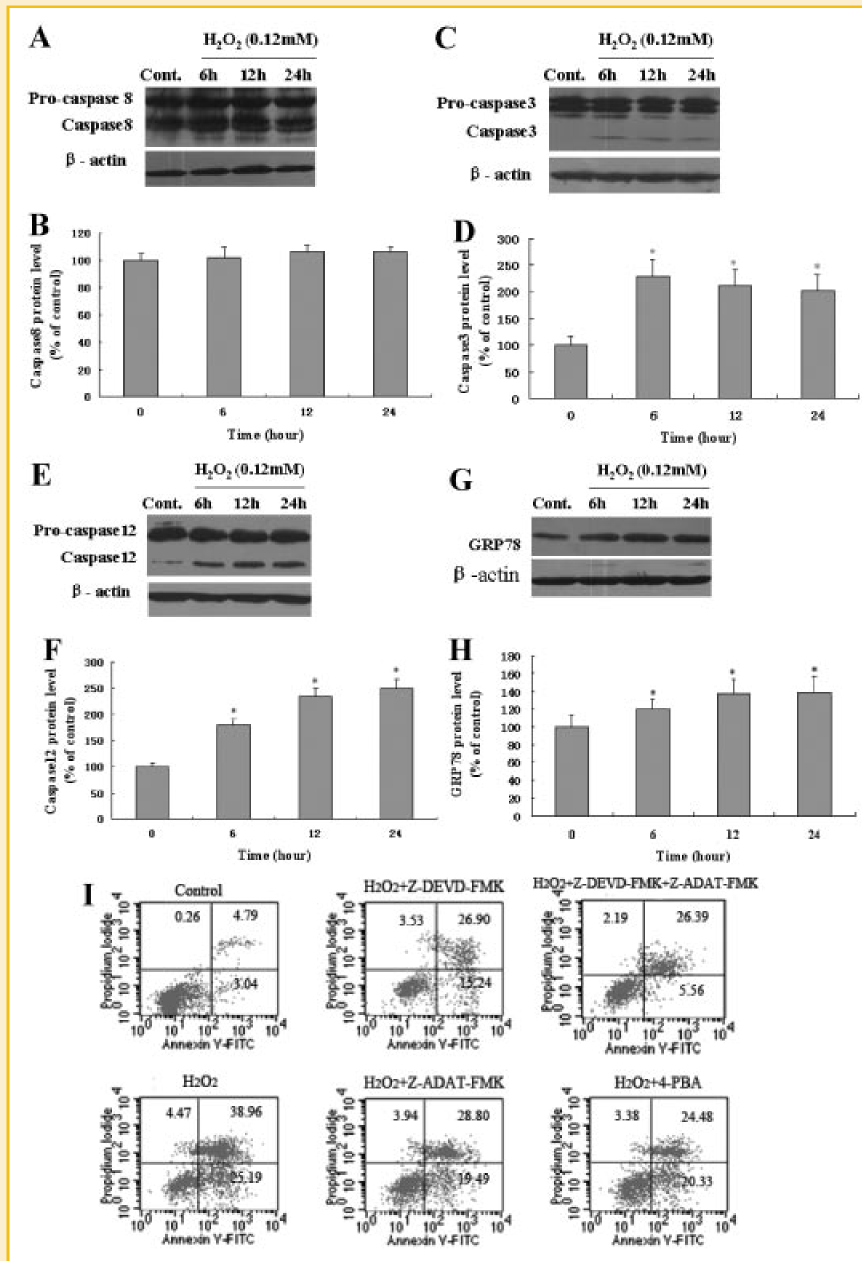

Fig. 3. MSCs were treated with 0.12 mM H_2O_2 for different period of time. Cell lysates were obtained and subjected to Western blot to detect caspase-3, caspase-8, and caspase-12, respectively, (A–F). MSCs were pretreated respectively with 10 μM Z-ATAD-FMK, 50 μM Z-DEVD-FMK or 5 mM 4-PBA for 1 h followed by 0.12 mM H_2O_2 treatment for 24 h. Apoptosis of the cells were evaluated with flow cytometry analysis (G,H). MSCs were pretreated with or without Z-ATAD-FMK for 1 h followed by 0.12 mM H_2O_2 treatment for 24 h. Subcellular fractionations were performed and cell lysates were subjected to Western blot to detect cytochrome *c* (I–L). The cells were preincubated with 10 μM Z-ATAD-FMK or 50 μM Z-DEVD-FMK, respectively, for 1 h followed by 0.12 mM H_2O_2 treatment for 24 h. Cell lysates were obtained and Western blot was performed to investigate the cleavage of caspase-3 (M,N). Data shown are representative results from at least three independent experiments. $^{*}P < 0.05$, versus the control cells. $^{\#}P < 0.05$, versus the cells treated by H_2O_2.

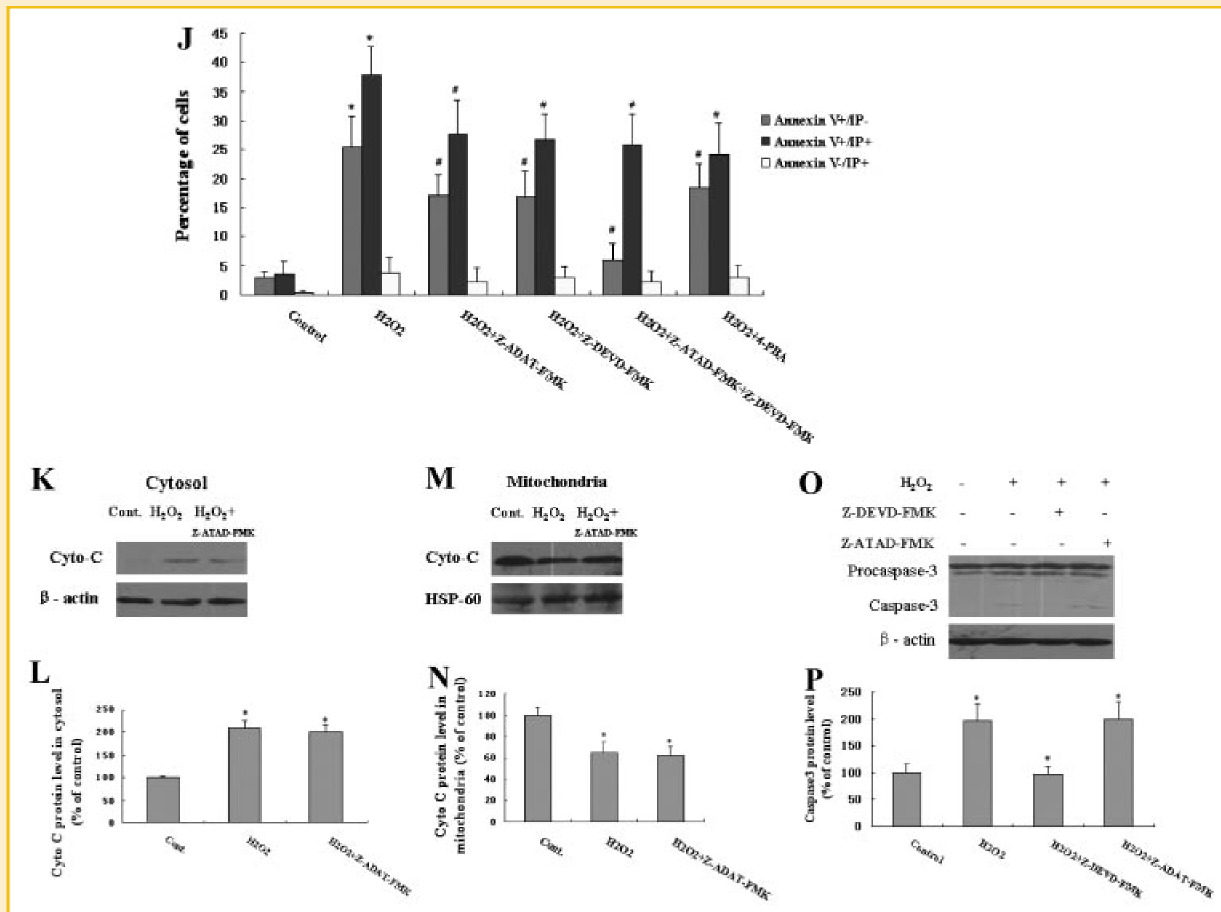

Fig. 3. (*Continued*)

addition, mitochondrial death pathway and ER stress were parallel in the regulation of MSCs apoptosis caused by H_2O_2.

Since caspase-3 and caspase-12 inhibitors reduced the percentage of apoptosis of MSCs, respectively, the synergistic effects of the inhibitors were further investigated. The results indicated that pretreatment of MSCs with Z-DEVD-FMK and Z-ATAD-FMK reduced the amount of early apoptotic cells significantly, and had better cytoprotection than those with each of them alone (Fig. 3I,J).

ROLES OF MAPKS PATHWAYS IN H_2O_2 INDUCED APOPTOSIS OF MSCS

To analyze whether MAPKs pathways regulated H_2O_2 induced apoptosis of MSCs cell lysates were collected from MSCs treated with H_2O_2. Total and phosphorylated JNK, p38 and ERK were measured with Western blot, respectively. H_2O_2 caused a transient yet robust upregulation of phospho-JNK (Thr183/Tyr185) at 30 min and 1 h of H_2O_2 treatment. Significant activation of p38 MAPK (Thr180/Tyr182) was detected at 1 h of H_2O_2 treatment which was later of than that of JNK. Phospho-p38 MAPK peaked at 3 h of H_2O_2 treatment. After phosphor-JNK and phosphor-p38 MAPK peaked they declined rapidly to the level comparable to that of untreated MSCs. No significant change of phospho-ERK was observed from 30 min to 24 h of H_2O_2 treatment (Fig. 4A–F). The result indicated H_2O_2 differentially regulated the activities of the three subfamilies of MAPK.

To elucidate the roles of different MAPKs pathways in the apoptosis, MSCs were treated with JNK inhibitor SP600152 (5 μM), p38 inhibitor SB202190 (5 μM), or ERK1/2 inhibitor U0126 (20 μM), respectively. Then MSCs were subjected to H_2O_2 treatment and cell death were analyzed with Annexin V-FITC/PI staining. The results showed p38 MAPK inhibitor SB202190 significantly reduced the amount of early apoptotic cells, but had no obvious effect on the late apoptotic cells and necrotic cells. In contrast, JNK inhibitor SP600152 had no effect on early apoptotic cells but dramatically reduced the amount of the late apoptotic cells and necrotic cells (Fig. 4G,H). The results indicated p38 MAPK regulated the early apoptosis of MSCs induced by H_2O_2 while JNK was involved in the late apoptosis of the cells. ERK1/2 specific inhibitor U0126 had no effect on preventing cell death. Combining with the previous data that phospho-ERK was not upregulated by H_2O_2 treatment, our data suggested ERK1/2 was not involved in H_2O_2 induced cell death (Fig. 4G,H).

To further investigate the downstream target of JNK and p38 MAPK in the regulation of cell apoptosis, their effects on mitochondrial dysfunction were analyzed. Previous data showed

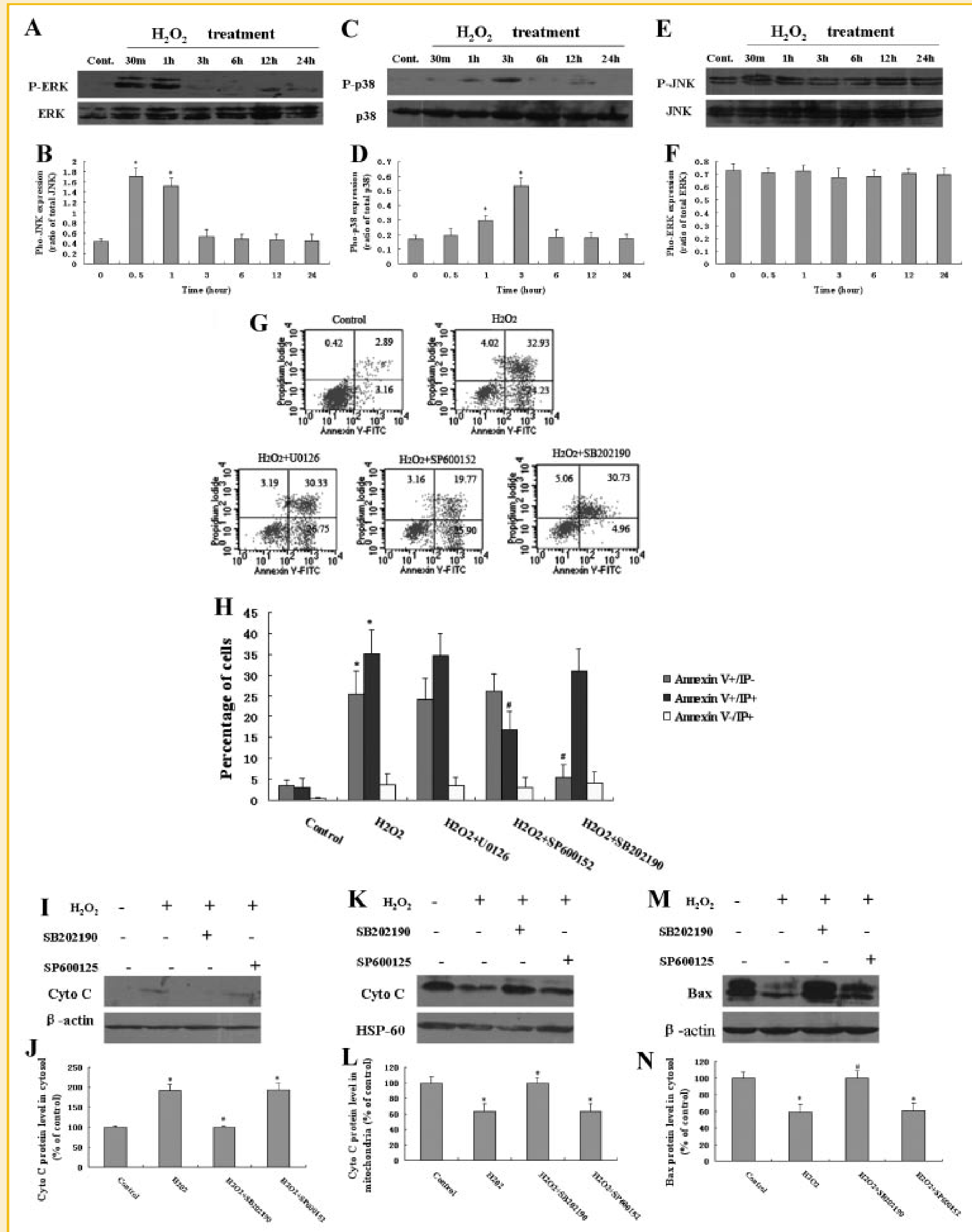

Fig. 4. MSCs were treated with 0.12 mM H_2O_2 for different period of time and cell lysates were obtained and subjected to Western blot to investigate activation of JNK, p38, and ERK, respectively (A–F). Moreover, MSCs were pretreated, respectively, with 15 μM SB202190, 20 μM U0126, or 5 μM SP600152 for 1 h followed by 0.12 mM H_2O_2 treatment for 24 h. Apoptosis of the cells were evaluated with flow cytometry analysis (G,H). To detect the effect of JNK/p38 specific inhibitors on mitochondria function, MSCs were preincubated with 15 μM SB202190 or 5 μM SP600152 for 1 h followed by incubation with 0.12 mM H_2O_2 for 24 h. The fractions of cytosol and mitochondria were isolated and immunoblotting was performed to detect the release of cytochrome *c* (I–L) and translocation of Bax (H–P). Cell lysates were obtained and Western blot was performed to investigate the cleavage of caspase-3 and caspase-12 (Q–T). Each data point represents mean ± standard deviation of three independent experiments. $^*P < 0.05$, versus the control cells. $^{\#}P < 0.05$, versus the cells treated by H_2O_2.

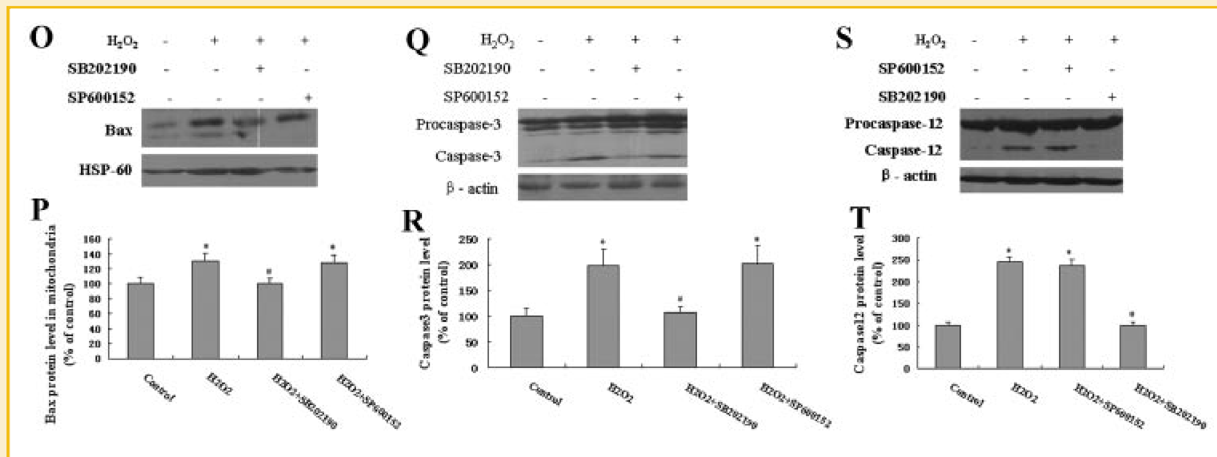

Fig. 4. (*Continued*)

upon H_2O_2 treatment cytochrome *c* was released from mitochondria into cytosol and Bax was translocated from cytosol onto mitochondria. Pretreatment of MSCs with p38 MAPK inhibitor SB202190 prevented translocation of Bax to mitochondria and release of cytochrome *c* from mitochondria into cytosol while JNK inhibitor SP600152 had no obvious effect on mitochondrial dysfunction of the cells (Fig. 4I–P). The roles of JNK and p38 MAPK on ER stress pathway were also investigated and the results indicated that SB202190 inhibited the cleavage of caspase-3 and caspase-12 respectively, while SP600152 had not such effects (Fig. 4Q–T). The previous data also proved that SB202190 also reduced significantly the amount of early apoptotic cells, therefore, p38 was involved in cell apoptosis by regulating both the mitochondria and ER pathway.

DISCUSSION

In this study we characterized H_2O_2 induced MSCs death and investigated the related signaling pathways. Our study indicated H_2O_2 induced MSCs death in dose- and time-dependent manners. H_2O_2 induced MSCs death involved both mitochondrial pathway and ER stress, but not death receptor pathway. p38 MAPK and JNK but not MAPK were involved in H_2O_2 induced MSCs death. Moreover, we are the first to demonstrate p38 MAPK and JNK played distinct roles in different phases of apoptosis of MSCs. p38 MAPK regulated early apoptosis while JNK regulated late apoptosis of MSCs. p38 MAPK targeted both mitochondria pathway and ER stress in H_2O_2 induced MSCs death (Fig. 5).

MSCs transplantation has emerged as one of the promising means to repair damaged myocardium. However, poor survival of transplanted MSCs in the infarcted area greatly limited its therapeutic efficacy. In our previous studies hypoxia/SD has been identified as one of the important factors to cause donor cell death [Zhu et al., 2006]. Evidently hypoxia/SD is not the only factor in the microenvironment of infarcted heart that led to the massive cell death. In the normal myocardium the balance between oxidase and antioxidase maintains a low level of ROS [Finkel, 1999]. When an infarction occurs, the decrease of antioxidase and the increase of oxidase shift the balance which results in elevated level of ROS in the infarcted heart [Hill and Singal, 1997; Fukui et al., 2001]. Oxidative stress in the microenvironment challenges the viability of the transplanted cells. Thus, it is necessary to explore how exogenous ROS causes the death of MSCs in order to improve the viability of the transplanted cells.

ROS act as second messengers in various signal transductions at physiological level [Park et al., 2005]. However, at elevated nonphysiological concentration ROS might cause progressive modification or degradation of cellular biochemicals including DNA, proteins, lipids, and carbohydrates which results in loss of cell function or even cell death [Halliwell and Gutteridge, 1999; Ryter et al., 2007]. In this study low level of ROS seemed to have little impact on the viability of MSCs. Apparent cell apoptosis occurred at high

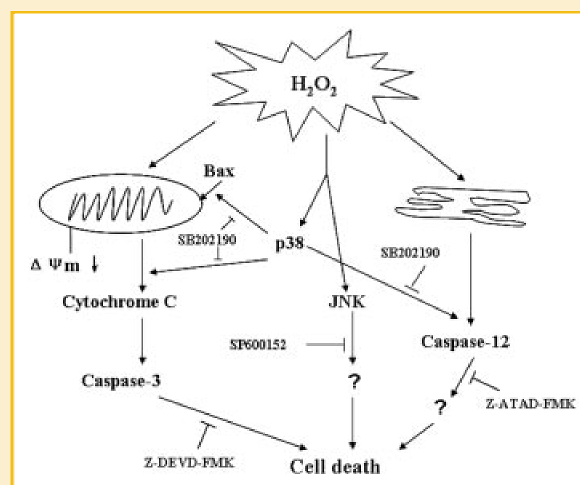

Fig. 5. Proposed signaling pathways in H_2O_2 induced apoptosis of MSCs.

concentration of H_2O_2 ($\geq$0.09 mM) and with long treatment time ($\geq$12 h). H_2O_2 induced apoptosis of MSCs in dose- and time-dependent manners. This suggested exogenous ROS burst in the microenvironment of infracted heart was also a critical factor for apoptosis of MSCs. Therefore, various apoptotic factors including exogenous ROS and hypoxia/SD should be considered in order to improve the viability of transplanted MSCs in the infarcted heart, and the signaling pathway of exogenous ROS induced apoptosis should be investigated in order to provide targets for developing related bioactive molecules or drugs to inhibit apoptosis induced by oxidative stress in MSCs. Although preconditioning with diazoxide and antioxidant are proved to promote MSCs survival under oxidative stress [Afzal et al., 2010; Cui et al., 2010; Wang et al., 2010], the mechanism of ROS-induced MSCs apoptosis is not well known.

In order to elucidate the signaling pathways in H_2O_2 induced MSCs death we analyzed three major pathways involved in apoptosis: mitochondrial death pathway, death receptor pathway, and ER stress pathway. Mitochondrial-dependent pathway and death receptor-dependent pathway have been considered as the major signaling pathways in apoptosis of various cells induced by oxidative stress [Dumont et al., 1999; Maheshwari et al., 2009]. H_2O_2 treatment compromised mitochondrial function as evidenced by the release of cytochrome *c* from mitochondria into cytosol, Bax translocation from cytosol to mitochondria and the loss of mitochondrial $\Delta\Psi$m in MSCs. H_2O_2 treatment did not cause appreciable cleavage of caspase-8, which suggested extrinsic apoptosis pathway was not involved in the process of apoptosis, or act independently of caspase-8.

ER stress induces unfolded protein response (UPR) to protect cells from damage, but severe ER stress causes cell apoptosis by activating the proapoptotic transcription factor CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP), or JNK, or caspase-12 [Nakagawa et al., 2000; Urano et al., 2000; Marciniak et al., 2004]. Despite the reports presented the relationship between oxidative stress and ER stress during apoptosis, the mechanism has not been well understood, and particularly the crosstalk of ER stress and other apoptotic pathway in oxidative stress induced apoptosis. In our study, H_2O_2 activated caspase-12 and JNK, which specific inhibitors reduced H_2O_2 induced-apoptosis. In addition, 4-PBA reversed apoptosis of MSCs induced by H_2O_2, and the expression of GRP78 protein increased after H_2O_2 treatment. The data suggested H_2O_2 caused ER stress in MSCs. Death receptor-dependent pathway was not involved in H_2O_2 induced apoptosis of MSCs. Moreover, our data indicated for the first time both ER stress- and mitochondrial-dependent pathway contributed to the apoptosis of MSCs caused by H_2O_2.

Our data also suggested mitochondria apoptotic pathway led to the activation of caspase-3. Prevention of apoptosis by caspase-3 inhibitor Z-DEVD-FMK further proved caspase-3 played an important role in the apoptosis of MSCs induced by H_2O_2. Caspase-12 specific inhibitor Z-ATAD-FMK also reduced H_2O_2 induced apoptosis of MSCs but failed to prevent the release of cytochrome *c* and cleavage of caspase-3 despite the evidence that crosstalk exists between the activation of caspase-12 and cleavage of caspase-3 independent of cytochrome *c* [Morishima et al., 2002]. Pretreatment of MSCs with both Z-DEVD-FMK and Z-ATAD-FMK showed synergistic effects and better cytoprotection than those with each of them alone. Taken together, our results suggested mitochondrial death pathway and ER stress did not work on each other. Instead, they were parallel pathways in the regulation of apoptosis of MSCs induced by H_2O_2.

Previous reports showed activation of p38 and JNK were responsible for oxidative stress induced apoptosis but their respective roles in different phases of apoptosis were unknown. In this study p38 activated by H_2O_2 was involved in the early apoptosis of MSCs, and p38 is the common director for the activation of both mitochondria and ER stress induced apoptosis in MSCs, therefore, H_2O_2 activated p38 directed both ER stress pathway and mitochondria death pathway in the regulation of the early apoptosis of MSCs. In contrast with p38, JNK was involved in the late apoptosis of MSCs through independent of ER stress and mitochondria pathways. To our knowledge this is the first report to demonstrate the distinct roles of p38 and JNK in different apoptotic phases of cells induced by H_2O_2.

Transient activation of p38 and JNK in MSCs treated by H_2O_2 was another characteristic of the study. It has been shown oxidative stress led to sustained activation of JNK/p38 which caused apoptosis [Matsuzawa et al., 2002; Kamata et al., 2005]. For example, H_2O_2 results in sustained activation of ERK1/2, JNK, and p38 in HeLa cells and cardiomyocytes [Wang et al., 1998; Purdom and Chen, 2005]. Sustained activation of ERK1/2 was also associated with apoptosis of renal epithelial cells treated by H_2O_2 [Lee et al., 2006], and H_2O_2 leads to sustained activation of JNK in MLE12 and lung epithelial cells [Li et al., 2003; Pantano et al., 2003]. Transient activation of JNK/p38 was considered to induce cell growth and differentiation instead of apoptosis [Matsuzawa et al., 2002]. However, transient rather than sustained activation of p38 and JNK were induced by H_2O_2 in the apoptosis of MSCs in this study. And transient activation of JNK and p38 were also critical in the regulation of apoptosis in MSCs. The kinetics of the above two proteins in the study could be attributed to the specificity of MSCs. In MSCs treated with H_2O_2, JNK and p38 MAPK might switch on the pathways of apoptosis temporarily and transmit the signals, respectively, to their downstream targets which induced cell death. ERK activation has been shown to be responsible for apoptosis of some cells induced by H_2O_2 [Park et al., 2005; Lee et al., 2006], but it had no effect on apoptosis of MSCs in this study.

In conclusion, the study revealed exogenous ROS was one of the important factors to induce apoptosis of MSCs. Various apoptotic factors including hypoxia/SD and exogenous ROS should be put in the same context in order to appreciate their combined effects and improve the viability of transplanted MSCs. Apoptosis of MSCs induced by H_2O_2 led to the early and transient activation of p38 MAPK which directed both mitochondrial death pathway and ER stress that executed cell death via caspase-3 and caspase-12. Similarly activated JNK, on the other hand governed late apoptosis of MSCs. The in vivo relevance of the in vitro findings is awaiting further study. In spite of that, our study promoted the understanding of oxidative stress induced MSCs death and will help with the quest for new bioactive compounds to improve the viability of MSCs transplanted into infarcted hearts.

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