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MAPK signaling in H9c2 cardiomyoblasts exposed to cholesterol secoaldehyde – Role of hydrogen peroxide

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ABSTRACT

3β-Hydroxy-5,6-secocholestan-6-al (cholesterol secoaldehyde or ChSeco), an oxysterol known to be formed in ozone- and singlet oxygen-mediated oxidations of cholesterol, has been detected in the atherosclerotic plaque and in the brain of patients suffering from Alzheimer's disease and Lewy body dementia. Previously, we have shown that, in H9c2 cardiomyoblasts, ChSeco induces oxidative stress followed by apoptosis involving both intrinsic and extrinsic signaling pathways. In the present study, we investigated the nature of reactive oxygen species (ROS) and its associated redox signaling in H9c2 cells upon treatment with ChSeco. Both catalase and deferoxamine, which lowered intracellular ROS, were found to alleviate the ChSeco-induced cytotoxicity. ChSeco-treated H9c2 cells showed a significant decrease in the intracellular catalase activity, suggesting the involvement of H_2O_2 in the associated cytotoxicity. Additionally, in ChSeco-exposed cells, there was a marked increase in lipid peroxidation and pre-treatment with SB 203580 (p38 MAPK inhibitor) and MEK1/2 inhibitor (ERK1/2 and JNK inhibitor) rendered protection against the cytotoxicity. An early increase in the expression of p-SAPK/JNK or delayed p38 MAPK did not alter ATF-2 but decreased c-Jun expression in these cells. Overall, these findings are consistent with MAPK signaling resulting from increased cellular H_2O_2 in ChSeco-induced cytotoxicity in cardiomyoblasts.

1. Introduction

Oxidized cholesterol (oxysterols), a major class of biologically important molecules, is involved in numerous cell signaling pathways [1]. Oxysterol-induced physiological changes are implicated in many pathological conditions such as aging, cancer and cardiovascular and cerebrovascular diseases [2–5]. Studies conducted on cultured cellular and animal models have shown the involvement of extracellular signal-related kinases (ERK) in survival signals and stress-activated protein kinases (SAPK) and p38 mitogen-activated protein kinases (MAPK) in non-survival signals under the influence of oxysterol-mediated oxidative stress [6].

3β-Hydroxy-5,6-secocholestan-6-al (cholesterol secoaldehyde or ChSeco), a product of cholesterol oxidation, has been implicated in the pathogenesis of atherosclerosis and Alzheimer's disease [7–9]. Recent studies indicate that ChSeco could be formed at the inflammatory sites through the singlet-oxygen mediated oxidation of cholesterol followed by Hock cleavage of the product, cholesterol 5α-hydroperoxide [10–12]. The detection of ChSeco in arterial plaques [7] and brain samples of Alzheimer's patients [9] suggest the association of the oxysterol in those pathological states. Earlier, we have shown that ChSeco induces apoptosis in H9c2 cardiomyoblasts [13,14], GT1-7 hypothalamic neurons [15], and primary cortical neuronal cells [16]. In these cell lines, intracellular generation of reactive oxygen species (ROS) and subsequent depletion of GSH have been observed predisposing the cell death through mitochondrial and the death receptor pathways. Despite these observations, the participation of specific ROS and the associated cell signaling mechanism(s) have not yet been established to date and therefore, in the current study, we investigated the involvement of ROS and stress-induced signaling pathways in ChSeco-induced cytotoxicity in H9c2 cardiomyoblasts.

2. Materials and methods

2.1. Chemicals, cell culture supplies, and H9c2 cardiomyoblasts

Bovine serum albumin (BSA), mouse monoclonal anti-β-actin antibody, catalase, cholesterol, deferoxamine (DFO), dimethyl sulfoxide (DMSO), Dulbecco's modified Eagle's medium (DMEM), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), H_2O_2, 2-mercaptoethanol, N-acetyl-L-cysteine (NAC), penicillin–streptomycin (stabilized solution containing 10,000 units/mL penicillin and 10 mg/mL streptomycin), phosphate buffered saline (PBS),

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protease inhibitor cocktail (4-(2-aminoethyl)-benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin), sodium dodecyl sulfate (SDS), thiobarbituric acid (TBA), Tris base (Trizma), Triton X-100, trypsin–EDTA (2.5 g porcine trypsin and 0.2 g EDTA-Na_4 per liter of HBSS), and 1,1,3,3-tetraethoxypropane were purchased from Sigma (St. Louis, MO). Other chemicals and reagents were obtained from the following sources: SB203580, MEK1/2 inhibitor, and caspase-3/7 assay kit from Calbiochem (LaJolla, CA); 5-(and-6)-chloromethyl 2′,7′dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) from Invitrogen (Carlsbad, CA); acrylamide/bis-acrylamide (30% w/v in water) and polyvinylidene-difluoride (PVDF) membranes from Bio-Rad (Hercules, CA); fetal bovine serum (FBS) from Atlanta Biologicals (Lawrenceville, GA); bicinchoninic acid (BCA) protein assay kit and SuperSignal West Femto chemiluminescent substrate from Pierce (Rockford, IL); CellTiter 96® AQueous One solution from Promega (Madison, WI); and primary antibodies to ATF-2, c-Jun, p38 and p-SAPK/JNK (Thr183/Thr185) from Cell Signaling (Danvers, MA). Rat embryonic H9c2 cardiomyoblasts were purchased from ATCC (Rockville, MD). Stock solutions of DFO, CM-H_2DCFDA and other additives were prepared in DMSO, ethanol, or PBS.

2.2. Synthesis of cholesterol secoaldehyde

ChSeco was synthesized by ozonation of cholesterol in methanol/dichloromethane (1/1 v/v) followed by reduction of the product mixture with Zn/acetic acid [13,17]. The purity of the final product was determined by RP-HPLC [17]. Stock solutions of ChSeco (20 mM, each) were prepared in DMSO and stored in small aliquots at −80 °C until use.

2.3. Cell culture and treatments

H9c2 cardiomyoblasts were sub-cultured to pre-confluence and harvested by trypsinization once every 2–3 days as described in our previous publications [13,14]. For experimental use, cells were seeded in either 24-well plates (cell density: *ca.* 5×10^4/well; volume: 0.5 mL) or 6-well plates (cell density: *ca.* 1.5×10^6/well; volume: 2 mL). At 70% confluence, cells were treated with 0–15 μM ChSeco in DMEM containing 2% (v/v) FBS and antibiotics and incubated for designated time periods. In certain assays, H9c2 cells were pre-treated with catalase, DFO, SB203580, and MEK1/2 inhibitor for 1 h and then exposed to ChSeco.

2.4. Cell viability

H9c2 cardiomyocytes in 24-well plates (control as well as cells previously treated with ChSeco and other additives) were washed twice with PBS. Thereafter, the cells were incubated for 1–4 h with 500 μL of 1:25-diluted CellTiter 96® AQueous One solution containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS). The product of MTS reduction (MTS formazan) formed during the incubation was measured at 490 nm using a BioTek EL 800 micro plate reader (Winooski, VT) [14].

2.5. Measurement of intracellular peroxides

H9c2 cardiomyoblasts in 24-well plates were pre-incubated for 30 min with 10 μM CM-H_2DCFDA. Thereafter, the cells were washed free of exogenous CM-H_2DCFDA and exposed to designated concentrations of ChSeco in Krebs–Ringer-Hepes (KRH) buffer. In certain experiments, H9c2 cells, 1 h prior to exposure to ChSeco, were incubated with catalase (100 and 500 units/mL) or DFO (25 and 50 μM). The appearance of fluorescence (excitation/emission: 485/530 nm), which marks the intracellular formation of CM-DCF, was monitored continuously for periods up to 12 h using a SpectraMax Gemini EM spectofluorometer (Sunnyvale, CA) [14].

2.6. Assay of catalase activity

Following treatment with ChSeco for desired time periods, H9c2 cardiomyoblasts (*ca.* 5×10^6) were washed with ice-cold PBS and then lysed in 1 mL of 0.1 M Tris–HCl buffer, pH 6.80 that also contained 1% (w/v) SDS and 1% (v/v) protease inhibitor cocktail. The supernatant of cell lysate, obtained after centrifugation 300*g*, was used as the source of catalase. Protein content in the supernatant was determined using a BCA kit. Catalase activity was measured based on the initial rates of breakdown of H_2O_2 [18]. Typically, the assays were performed in a final volume of 3 mL of 0.1 M phosphate buffer, pH 7.5 containing 10–12 mM H_2O_2 (initial absorbance at 240 nm: 0.45–0.50) and an aliquot cell-free supernatant (protein content: 100 μg). The decrease in absorbance was monitored over a period of 3 min using a Genesys 10 UV spectrophotometer (Thermo Spectronic; Madison, WI) at the ambient temperature.

2.7. Thiobarbituric acid-reactive substances (TBARs)

Quantitative determination of TBARs as an index of lipid peroxidation in H9c2 cardiomyoblasts following treatment with ChSeco was performed spectrophotometrically [19]. H9c2 cells at sub-confluence were prepared and exposed to ChSeco as described earlier. Approximately 5×10^6 cells were lysed in 150 μL of cold 0.1 M Tris–HCl buffer, pH 6.80 containing 1% (w/v) SDS and 1% (v/v) protease inhibitor cocktail. The lysate was clarified by centrifugation for 15 min at 15,000*g* at 4 °C. The clear lysate (125 μL) was mixed with 50 μL of 10% (w/v) SDS, 250 μL of 20% (v/v) acetic acid (pH 3.0), and 750 μL of 0.67% (w/v) TBA in a test tube. The reaction mixture was incubated at 100 °C (water bath) for 1 h, allowed to cool, and 500 μL of water was added to each test tube and centrifuged. The absorbance of the resultant pink color (an indicator of TBARs including MDA) was measured at 532 nm using a Genesys 10 UV spectrophotometer.

2.8. Western blot analysis

H9c2 cardiomyoblasts in 6-well plates were exposed to ChSeco for 0–24 h. At various time points, the medium was removed and the cells were washed twice with PBS. Thereafter, the cells were lysed in 150 μL of ice-cold 62.5 mM Tris–HCl buffer, pH 6.80 that also contained 1% (w/v) SDS and 1% (v/v) protease inhibitor cocktail. To facilitate cell lysis, each sample was sonicated for 2×10 s using a probe sonicator set at 2 Hz. The cell lysates were centrifuged at 10,000*g* for 15 min at 4 °C. The amount of protein in the supernatant was determined using the BCA protein kit. Approximately 50 μg of cellular protein was resolved by 10% SDS–PAGE, after which the resolved proteins were transferred onto PVDF membranes, which were blocked for 1 h with 5% (w/v) skim milk powder in Tris–HCl buffer saline (pH 7.5) containing 0.1% (v/v) Tween 20 (TBST). Following an overnight incubation with mouse primary monoclonal antibodies against p38, p-SAPK/JNK (Thr183/Thr185) ATF-2 and c-Jun (diluted 1:1000) at 4 °C, the PVDF membranes were washed and incubated with specific secondary antibody-HRP conjugate for 1 h at room temperature. To remove the unbound antibody-HRP conjugate, the membranes were washed with TBST and incubated with SuperSignal West Femto substrate for 5 min. Thereafter, the chemiluminescence signal was detected using an Alpha imager as described earlier [14].

3. Results and discussion

Earlier, we have demonstrated that intracellular accumulation of peroxides or peroxide-like molecules (collectively referred to as ROS) are responsible for cytotoxicity in ChSeco-exposed H9c2 cardiomyoblasts [14]. In the present study, we show that H_2O_2 is one of the major peroxides formed in ChSeco exposures and that the observed cytotoxicity is mediated through stress-activated signaling pathways. There are three different lines of evidence in favor of the formation of H_2O_2. First, exogenous addition of catalase (100 units/mL) inhibited the increase in CM-DCF fluorescence in H9c2 cells exposed to ChSeco (Fig. 1A). Second, addition of catalase (100 and 500 units/mL) partially reversed the cytotoxicity induced by ChSeco (Fig. 1B). Third, there was little or no attenuation of ChSeco-induced cytotoxicity by HI catalase (Fig. 1B), meaning that catalase in the active state only was able to protect the cells against oxidative stress and the associated cytotoxicity.

Catalase is a crucial member of the cellular antioxidant defense as it converts H_2O_2 into water and O_2 without the expense of reduction equivalents. Catalase is not expected to be permeable through the plasma membrane of H9c2 cells. And H_2O_2, which has almost the same size, dielectric properties, and capacity to form hydrogen bonds as that of water, can be transported in and out of biological membranes through aquaporins [20]. Therefore, in the present context, it is possible that H_2O_2 formed in H9c2 cells, either through diffusion or aquaporin-mediated transport, is accessible to the exogenously added catalase (Fig. 1A and B). It is pertinent to mention that CM-H_2DCFDA, the probe used here for the determination of ROS (Fig. 1A), although membrane-permeable, is specific for intracellular peroxides [21]. This is because CM-H_2DCFDA, upon entering the cells, is hydrolyzed by cellular esterases and the resulting product 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein (CM-H_2DCF) is not permeable through biological membranes and therefore becomes locked inside the cells [22,23]. Finally, the fluorescence monitored in this assay results from oxidation of CM-H_2DCF (non-fluorescent) to CM-DCF (fluorescent) formed in peroxide-dependent reactions in the cellular milieu [21,23].

To further confirm the role of H_2O_2 in observed cytotoxicity, H9c2 cardiomyoblasts were exposed to low concentrations of ChSeco (1–5 μM) and the activity of total cellular catalase was assessed. As can be seen, the activity of catalase increased as the concentration of ChSeco increased (Fig. 2A). In a time-dependent study, an initial increase in catalase activity was observed following the exposure of cells to 15 μM ChSeco and over an extended time period (e.g., 12 h), the activity significantly declined (Fig. 2B). This early increase in catalase activity might have offered a protective effect to cells against the ChSeco-induced toxicity; however, subsequent loss of catalase activity possibly contributed to increased accumulation of H_2O_2, initially in the cellular milieu and subsequently in the extracellular environment due to diffusion. It is possible that the H_2O_2 accumulated (over the experimental period) could be a source for oxygen-derived free radicals (ODFRs) initiating peroxidative stress.

Hydrogen peroxide formed inside the cells can be metabolized into more deleterious hydroxyl radicals (·OH) through iron-catalyzed Fenton reactions [24,25]. Therefore, we studied the cytotoxicity of ChSeco in the presence of DFO. Unlike, catalase DFO is cell-permeable and the iron-chelates of DFO are catalytically inactive. It was found that DFO (25 and 50 μM) when added simultaneously with ChSeco (10 μM) inhibited intracellular peroxide (i.e., H_2O_2) formation (Fig. 3A) and also reversed cytotoxicity (Fig. 3B). These observations suggest that H_2O_2 accumulation (probably due to lowered cellular catalase activity) in the intracellular milieu might be converted by Fe(II) to ·OH. This was manifested by a 7-fold increase in the level of TBARs in H9c2 cells exposed to 15 μM ChSeco over a period of 8 h as compared to control cells (Fig. 3C). Intracellular H_2O_2 can induce membrane peroxidation generating more peroxides and carbonyl compounds. The enhanced levels of TBARs also indicate the extent of oxidative insult induced by ChSeco in H9c2 cells.

Apart from ODFRs and lipid peroxidation discussed above, elevated levels of H_2O_2 in H9c2 cells can mediate cytotoxic responses through mitochondrial cytochrome *c*. We have shown previously that, upon exposure of H9c2 cells to ChSeco, there has been a substantial loss of cytochrome *c* in the mitochondrial fraction with a concomitant increase in the cytosolic fraction [14]. The release of cytochrome *c* could be a consequence of enhanced oxidation of cardiolipin mediated through H_2O_2-dependent pseudoperoxidase activity of cytochrome *c* [26]. Once released into the cytosol, cytochrome *c* along with Apaf-1 protein can result in the formation of apoptosome which causes activation of procaspase-9 to caspase-9 and subsequent activation of caspase-3/7 [14]. Thus, H_2O_2 can promote apoptosis through the intrinsic pathway as proposed in our previous study [14].

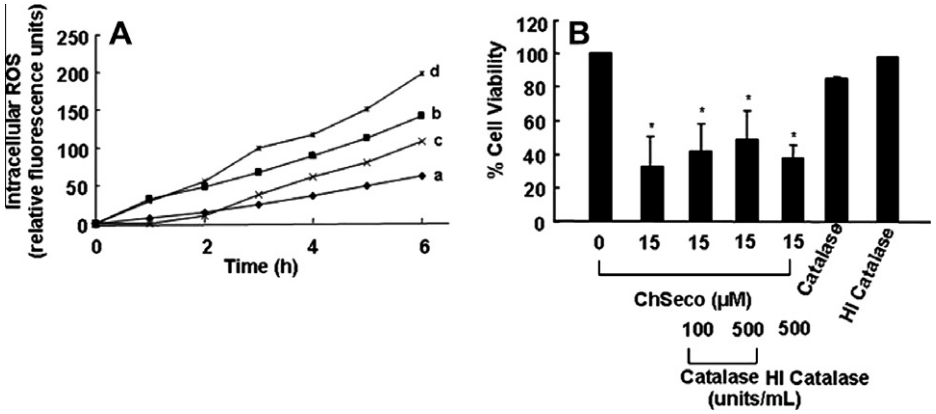

Fig. 1. (A) Intracellular formation of peroxide(s) in H9c2 cardiomyoblasts exposed to ChSeco and other additives: (a) none (control); (b) 15 μM ChSeco; (c) 15 μM ChSeco plus 100 units/mL catalase; and (d) 50 μM menadione (positive control). H9c2 cells (*ca.* 5×10^4) grown in 24-well plates were pre-incubated with10 μM CM-H_2DCFDA for 30 min at 37 °C. After washing the exogenous CM-H_2DCFDA, the cells were exposed to ChSeco, catalase, and menadione as indicated. The time-course of appearance of fluorescence due to the formation of CM-DCF was measured using a micro plate reader. Other details are as given in the Section 2. (B) Assessment of cytotoxicity in H9c2 cells exposed to ChSeco, catalase, and HI catalase. Cardiomyoblasts at *ca.* 70% confluence were exposed to 15 μM ChSeco along with catalase (100 and 500 units/mL; active or heat-inactivated). Cell viability was assessed 24 h later as described in Section 2. Values presented in A and B represent mean ± SD of three independent experiments (∗ indicates significance at $p < 0.05$ against the respective control).

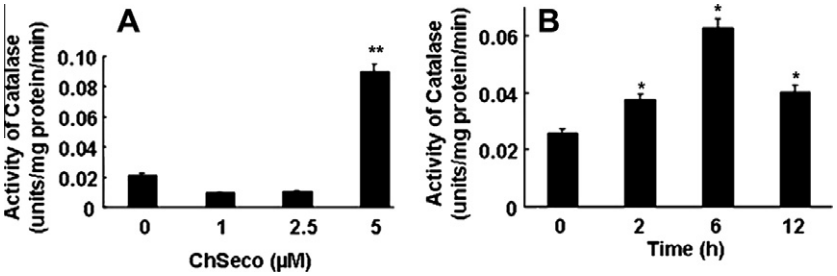

Fig. 2. (A) Effect of ChSeco concentration on the expression of catalase activity in H9c2 cardiomyoblasts. H9c2 cells (*ca.* 5×10^6) were exposed to 0–5 μM ChSeco for a period of 6 h. The activity of catalase in cell lysates (100 μg protein) was determined as described in Section 2. (B) Time-course of expression of catalase activity in H9c2 cells (*ca.* 5×10^6) exposed to 10 μM ChSeco for 0–12 h. At various periods of incubation, H9c2 cell lysates were analyzed for the activity of catalase (see above). In both A and B, data points represent mean ± SD of three independent experiments (∗ and ∗∗ indicate significance at $p < 0.05$ and $p < 0.01$ against the respective controls).

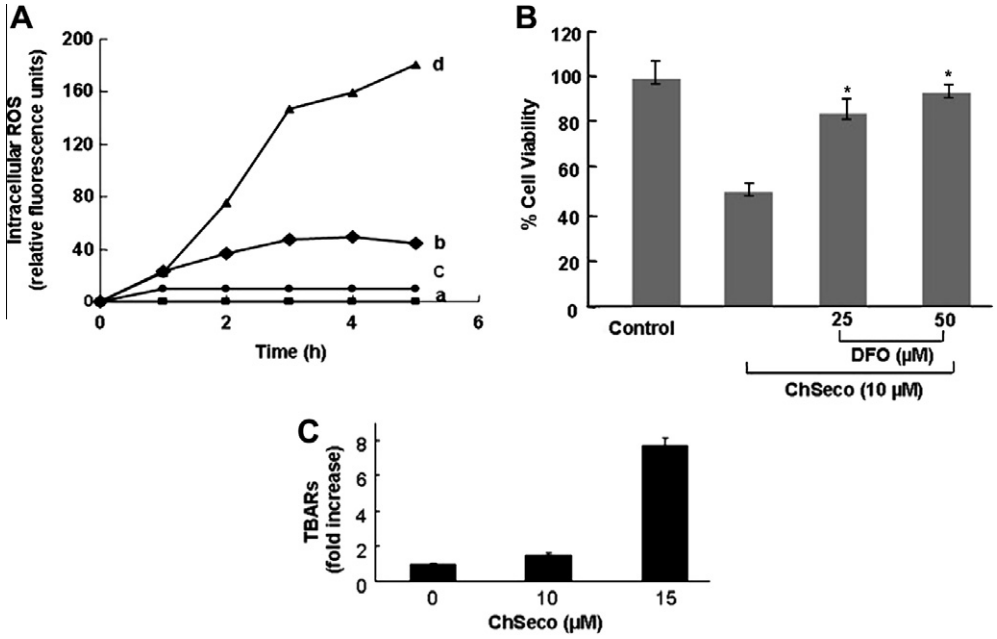

Fig. 3. (A) Intracellular formation of peroxide(s) in H9c2 cardiomyoblasts exposed to ChSeco and other additives: (a) none (control); (b) 10 μM ChSeco; (c) 10 μM ChSeco plus 50 μM DFO; and (d) 100 μM menadione (positive control). H9c2 cells (*ca.* 5×10^4) grown in 24-well plates were pre-incubated with10 μM CM-H_2DCFDA for 30 min at 37 °C. After washing the exogenous CM-H_2DCFDA, the cells were exposed to ChSeco, DFO, and menadione as indicated. The time-course of appearance of CM-DCF was measured using a micro plate reader as described in the text and legend to Fig. 1A. (B) Assessment of cytotoxicity in H9c2 cells exposed to ChSeco and DFO. At *ca.* 70% confluence, H9c2 cells were exposed to 10 μM ChSeco along with DFO (25 and 50 μM). Cell viability was assessed 24 h later as described in the text and legend to Fig. 1B. (C) Increased levels of TBARs in ChSeco-exposed H9c2 cells. Following exposure to 0, 10 or 15 μM ChSeco for 6 h, cells (*ca.* 5×10^6) were lysed in 150 μL of 0.1 M Tris–HCl buffer, pH 6.8 and the lysates were analyzed for the presence of TBARs as described in Section 2. MDA, which is representative of TBARs, was used for the construction of standard curve. Values presented in A–C represent mean ± SD of three independent experiments (∗ indicates significance at $p < 0.05$ against the untreated control cells).

It is generally believed that oxidative stress mediated through H_2O_2 involves activation of the MAP kinase (MAPK) family proteins [27]. Hence, we used SB203580, a specific chemical inhibitor of p38 MAPK to determine the involvement of MAPK pathway in ChSeco-mediated cytotoxicity in H9c2 cells. We observed that SB203580 offered significant protection against ChSeco-induced cytotoxicity in these cells (Fig. 4A). The protective effect of MEK1/2 inhibitor, which is believed to be exerted through inhibition of ERK kinase, was only marginal when compared to that of SB203580. This suggests that p38 MAPK plays a major role in ChSeco-induced cytotoxicity. The protein level of p-SAPK/JNK (a member of MAPK family) showed a dose-dependent increase on the concentration of ChSeco (Fig. 4Ba) and, in time course studies, a significant increase was noticed as early as 2 h and decreased thereafter (Fig. 4Bb). This ChSeco-induced increase in the levels of p-SAPK/JNK were found to be attenuated when H9c2 were treated with NAC (Fig. 4Ba). Further, from our Western blot analysis, we observed that p38 MAPK protein level increased over the time period of 12 and 24 h (Fig. 4Bc). MAPK p38 is reported to be induced by oxidative stress [28,29]. These results suggest that oxidative stress-induced activation of p-SAPK /JNK was consistent with the decrease in catalase activity and might be involved in the protective mechanisms of cells against ChSeco-induced cytotoxicity.

Once activated, the p38 MAPK and c-JNK pathways cause the activation of nuclear transcription factors such as c-Jun/ATF-2 leading to apoptosis in several cases [30–33]. In ChSeco exposed H9c2 cells, the level of ATF-2 however was unchanged but the c-Jun level decreases as the time of exposure was prolonged (Fig. 4Bc). Thus, it appears that ChSeco-induced apoptosis occurs irrespective of the activation of c-Jun/ATF-2. Rokutan et al. [34] have reported that elevated levels of GSH inhibit the oxidant-induced activation of c-Jun and ATF-2. In our previous studies, we have reported that GSH depletion and oxidative stress are early events in H9c2 apoptosis induced by ChSeco [13,14]. Loss of c-Jun level and unaltered level of ATF-2 as seen in ChSeco exposed H9c2 cells can now be better rationalized. The present study thus

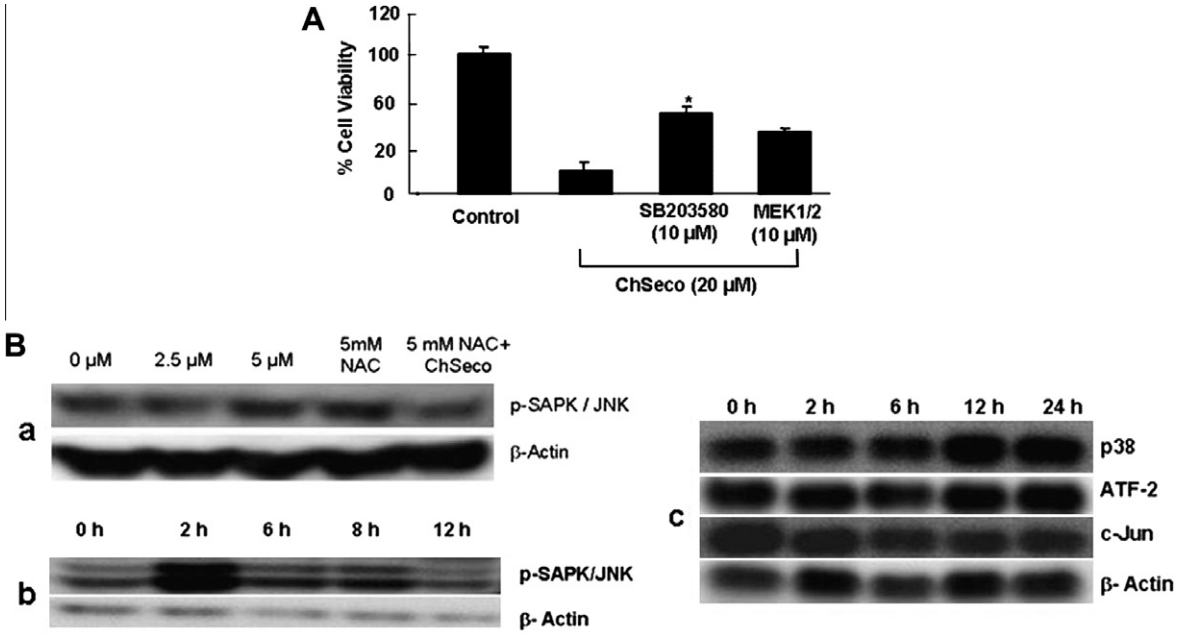

Fig. 4. Involvement of MAPK pathway in ChSeco-induced cell death in H9c2 cardiomyoblasts. (A) Inhibition of MAPK pathway offers protection against the ChSeco-induced cytotoxicity. H9c2 cells at *ca.* 70% confluence were pre-incubated for 1 h with 10 μM SB203580 (or MEK1/2 inhibitor) followed by exposure to 20 μM ChSeco. Cell viability was assessed 24 h later based on MTS reduction. Values presented are mean ± SD of three independent experiments (∗ indicates significance at $p < 0.05$ against control, untreated cells). (B) Western blot analysis of (a and b) p-SAPK/JNK and (c) p38, ATF-2 and c-Jun in H9c2 cells exposed to ChSeco: a refers to concentration-dependent changes whereas b and c refer to time-dependent changes in the expression of proteins. In assays where the concentration-dependence was examined (a), H9c2 cells were exposed to 0–5 μM ChSeco for 6 h in the presence or absence of 5 mM NAC. In assays where the time-dependence was studied (b and c), H9c2 cells were exposed to 10 μM ChSeco for periods up to 24 h as indicated. In all assays (a–c), for the purpose of normalization, the level of β-actin was monitored. Other details are as given in the text and legend to Fig. 1A. All experiments were replicated thrice and representative blots are presented.

offer a possibility that the generation of intracellular H_2O_2 initiates stress-activated kinase signaling that, in turn, induced apoptosis in H9c2 cells exposed to ChSeco.

Acknowledgments

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