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Induction of Ca²⁺ signal mediated apoptosis and alteration of IP3R1 and SERCA1 expression levels by stress hormone in differentiating C2C12 myoblasts

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ABSTRACT

Glucocorticoid (GC) are stress hormones, whose cytotoxicity has been shown in various cells. The imbalance of calcium homeostasis is believed to be associated with the dexamethasone (DEX, a synthetic GC)-induced apoptosis. Here we show that in C2C12 myoblasts, DEX markedly up-regulated the expression of inositol 1,4,5-triphosphate receptor 1 (IP3R1) and down-regulated the expression of SERCA1 (sarcoendoplasmic reticulum Ca²⁺-ATPase 1), leading to calcium overload. Furthermore, the imbalance of calcium homeostasis increased the level of BAX, decreased the level of Bcl-2, induced cytochrome c release and activated caspase-3, leading to intranucleosomal DNA fragmentation and plasma membrane damage, eventually resulting in cell apoptosis. Taken together, by using C2C12 myoblasts as a model system, we demonstrated a novel mechanism for stress hormone-induced apoptosis: it is dependent on the induction of intracellular calcium overload via the alterations of IP3R1 and SERCA1 expressions.

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1. Introduction

In the practice of animal husbandry, stress has usually been considered as a reflex reaction that occurs ineluctably when animals have the inability to cope with an adverse environmental conditions, and which have a range of many unfavorable outcomes from minor discomfort to death. Stress leads to an increase of blood glucocorticoid (GC) such as cortisol via the hypothalamic–pituitary–adrenal axis (HPA). This has been described in mammals, including goat (Kannan et al., 2003), horse (Werner and Gallo, 2008) and pig (Li et al., 2008). It has been long known that meat quality is strongly affected by behavioral and physiological status of the animals before slaughter and poor meat quality usually accompanies with an exaggerated release of GC (Beattie et al., 2000; Yoshioka et al., 2005). In addition, previous studies have shown that GC induces apoptosis in many cell lines such as Leydig cells (Gao et al., 2002), osteoblast cells (Yun et al., 2009) and T-cells (Tanaka et al., 2006). Calcium has been implicated as a mediator of GC-induced apoptosis. Two lines of evidences support a role of calcium in GC-induced apoptosis. First, inhibitors of calcium-activated neutral protease could inhibit GC-induced apoptosis (Squier and Cohen, 1997). Second, elevation of intracellular free

calcium levels have been detected in lymphoid cells undergoing GC-induced apoptosis (Khan et al., 1996). It is not known, however, whether tissue injury and the decline of meat quality caused by accumulation in circulating GC due to muscle cells apoptosis is initiated through calcium signaling pathway.

The main aim of this study was to explore hormonal mechanisms by which stress affects apoptosis in C2C12 myoblasts, a well-characterized model of skeletal muscle for studying the molecular mechanisms of meat quality (Lambert et al., 2001; Xiong et al., 2009) in vitro. We hypothesized that dexamethasone (DEX, a synthetic GC) would increase biomarkers of apoptosis such as cytotoxicity, membrane damage, DNA fragmentation and apoptotic proteins. In addition, we also sought to determine whether stress hormones cause an increase in intracellular calcium concentration that is associated with alterations in inositol 1,4,5-triphosphate receptor 1 (IP3R1) and sarcoendoplasmic reticulum Ca²⁺-ATPase 1 (SERCA1). We tested this hypothesis by employing a stress model treated with DEX in vitro to mimic the physiological levels of the hormone to determine the apoptotic mechanisms.

2. Materials and methods

2.1. Reagents and antibodies

Antibodies for IP3R1 and BAX were purchased from Santa Cruz (CA, USA). All other antibodies were purchased from Abcam

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(Cambridge, UK). The horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, anti-mouse IgG and rabbit anti-goat IgG were from Chemicon International (Chemicon, California, USA). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA, USA). Anti-GAPDH antibody and all other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell line and cell culture

Mouse-derived C2C12 myoblasts were maintained at subconfluent density at 37 °C in a 5% CO₂ humidified atmosphere in DMEM with 10% FBS (Invitrogen), 100 U/ml penicillin G and 100 µg/ml streptomycin. To induce differentiation, confluent cells were shifted 72 h after seeding to a differentiation medium containing DMEM supplemented with 2% heat-inactivated horse serum. Differentiation was followed by observation of cell morphology under a phase-contrast microscopy. Cells were serum-starved for 10 h before stimulation experiments.

2.3. MTT assay

C2C12 myoblasts were seeded on 96-well plates for 24 h, and then were exposed to final concentrations (0, 0.1, 1, 5, and 10 µM in the absence or presence of 1 or 10 µM RU486) of DEX and further incubated at 37 °C for 4, 10, 24, 48, and 72 h. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, 5 mg/ml in PBS) solution was then added and cells were cultured at 37 °C for 4 h. Formazan crystals formed were dissolved in 150 µl/well of DMSO. The absorbance of solubilized formazan was read at 570 nm using El-x 800 universal microplate reader (BioTek Instruments Inc., Winooski, VT, USA).

2.4. Lactate dehydrogenase (LDH) assay

After C2C12 myoblasts were exposed to the indicated stimuli (0, 0.1, 1, 5, and 10 µM DEX in the absence or presence of 1 or 10 µM RU486) and further incubated at 37 °C for 4, 10, 24, 48, and 72 h, the culture medium was collected, the enzyme activity of LDH was assayed using a commercial kit (JianChen Co, Nanjing, China) according to the manufacturer's instruction.

2.5. DNA extraction and detection of DNA fragments

For the time-dependent experiments, C2C12 myoblasts were treated with 1 µM of DEX for 0.5, 1, 4, 10, 24, 48, and 96 h. For the dose-dependent experiments, myoblasts were treated with DEX in a final concentration of 0, 0.1, 1, 5, and 10 µM in the absence or presence of 10 µM RU486 for 24 h. Then, cells were harvested by scraping and total DNA was isolated according to the protocol of DNA Purification Kit (Beyotime, Beijing, China). DNA fragments were separated in a 0.8% agarose gel, stained with EB, and visualized under UV light.

2.6. AO/EB assay

Morphological analysis of apoptosis was performed 48 h after treatment of the cells with DEX in the absence or presence of RU486 by AO/EB staining. Monolayer cell cultures in 96-well plates were used for these studies. After removal of the incubation medium, cells were rinsed and treated with 4 µl of AO-EB working solution (AO: 100 µg/ml; EB: 100 µg/ml). The cells were then visualized immediately under a fluorescence microscope.

2.7. Measurement of cytosolic free calcium levels

Free cytosolic calcium levels were measured using the cell-permeable calcium-sensitive fluorescent dye Fluo-3/AM. DEX (0, 0.1, 1, 5, and 10 µM in the absence or presence of 10 µM RU486) was added to the culture medium for 48 h. The medium then was removed from the tissue culture plates and replaced with 5 µM Fluo-3/AM diluted in D-hanks buffer for 30 min. The dishes were then washed once with 5 ml D-hanks to remove residual dye. The resulting fluorescence as the indicator of calcium concentration was observed under the fluorescent microscopy (Nikon, Chiyodaku, Tokyo, Japan) at 488 nm excitation wavelength.

2.8. RT-PCR

Total RNA was extracted using Trizol (Invitrogen) and cDNAs were synthesized from 1 µg of RNA using SuperScript II reverse transcriptase (Invitrogen). The PCR primer sequences used are as follows: *GRα*, 5'-GCTTTGCTCTGATCTGATT-3' (forward) and 5'-TGGCTCTTACAGACCTTCCTT-3' (reverse); *SERCA1*, 5'-GCGAGGTGGTC TGTATCTTCTTG-3' (forward) and 5'-TGTCAGGTGAGGTGGCTTG-3' (reverse); *IP3R1*, 5'-CGAAGAAACGGAACAGGATAAG-3' (forward) and 5'-CAGCAGCATGGAGCACAGATAG-3' (reverse); *Bcl-2*, 5'-TGAGT ACCTGAACCGGCATCT-3' (forward) and 5'-TTCCCTTTGGCAGT AAAT AGC-3' (reverse); *BAX*, 5'-CGGCGAATTGGAGATGAACAG-3' (forward) and 5'-GGTCACTGTCTGCCATGTGGG-3' (reverse); *cytochrome c*, 5'-CAAATCTCCAGGTCTGTTCG-3' (forward) and 5'-CTGCCCTTTC TCCCTTCTTCT-3' (reverse); *caspase-3*, 5'-CTGACTGG AAAGCCGAA ACTC-3' (forward) and 5'-AGGGACTGGATGAACCACGAC-3' (reverse); *β-Actin*, 5'-CTGGCTGGCCGGGACCTGAC-3' (forward) and 5'-CCGCTCGTTGCCAATAGTGATGAC-3' (reverse).

2.9. Western blotting

Cells were lysed in 50 mM Tris-HCl (pH 7.6) buffer containing 0.15 M NaCl, 1 mM EDTA, protease inhibitor cocktail, and 1% Triton X-100. Proteins in cell lysates were separated by SDS-PAGE using 6–12% gel and transferred to PVDF membranes. Membranes were blocked in 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS)-Tween 20 (0.05%) and incubated overnight at 4 °C with specific primary antibodies at 1:200–1000 dilutions followed by HRP-conjugated secondary antibodies at 1:5000 dilutions. Detection was performed using enhanced chemiluminescence system.

2.10. Measurement of caspase-3 activity

Cells were lysed in 50 mM Tris-HCl (pH 7.6) buffer containing 0.15 M NaCl, 1 mM EDTA, protease inhibitor cocktail, and 1% Triton X-100. Then, these lysate cells were centrifuged at 10,000g for 10 min, and supernatant was used for caspase-3 activity assay according to manufacturer's instructions (BD, CA, USA). Briefly, 50 µg of total protein was incubated with 30 µl of caspase assay buffer and 2 µl of caspase-3 colorimetric substrate (Ac-DEVD-pNA) at 37 °C for 4 h. Absorbance was measured using spectrophotofluorometer (Rochester, USA) at 405 nm.

2.11. Construction of IP3R1 and SERCA1-promoter plasmid and Luciferase reporter assay

The 2.5 kb DNA fragment of pig IP3R1 and SERCA1 upstream of the transcription start sites were obtained using a Universal Genome-Walker™ kit (BD Biosciences), following the manufacturer's instruction. The desired promoter regions of chromosomal DNA were amplified by PCR with the following primers: IP3R1, 5'-CGGGGTACCTGATTTTCTTCTCTAAG-3' (forward) and 5'-CCGCTC GAGTCCACATGGTTACTG-3' (reverse); SERCA1, 5'-CGGGGTAC

CACCTTTCTCTC-3' (forward) and 5'-CCGCTCGAGGCTTATCTTCCCTC-3' (reverse). All amplified DNA sequences were confirmed by nucleotide sequence analysis and then cloned into the pGL3 basic vector (Promega, WI, USA). Cells were transiently transfected with 1 μ g pGL3-IP3R1-Luc or pGL3-SERCA1-Luc plasmids or PRL-TK-TK-Renilla-luc plasmids as an internal control mixed with 2.5 μ L Lipofectamine 2000 reagent in 24-well plates before addition of DEX or in combination with RU486. The luciferase activities were determined using the dual luciferase assay system (Promega, WI, USA).

2.12. Statistical analysis

Data are presented as means \pm SE of at least three independent experiments. Statistical analysis was performed by Duncan's test.

3. Results

3.1. Cell viability and cytotoxicity

To examine the cytotoxic effect of stress hormone on C2C12 myoblasts, cells were cultured with DEX and quantified using both the LDH and MTT assays with cells cultured in DEX-free media as control. As shown in Fig. 1A and B, cell viability declined drastically and plasma membrane integrity was damaged significantly after incubation with DEX at indicated times. LDH activities were elevated and formazan formation was reduced in dose- and time-dependent manners. When the cells were treated with DEX together with 1 or 10 μ M RU486, these changes were reversed ($P < 0.01$). Taken together, these results suggest that DEX exerts a cytotoxic effect on C2C12 myoblasts and RU486 (a specific inhibitor of GR) has a protective effect against DEX-induced membrane damage (Fig. 1A and B).

3.2. The analysis of DNA fragmentation

In order to quantify the induction of apoptosis by DEX, we measured DNA fragmentation using DNA electrophoresis and fluorescent staining following the time- and dose-dependent experiments. DNA ladders, considered as one of the hallmarks of apoptosis, were present in all the tested times (1, 4, 10, 24, 48, and 72 h) except the earliest time point (0.5 h) after treatment with 5 μ M DEX (Fig. 2A and B). As shown in Fig. 2C and D, following 24 h exposure, all concentrations of DEX used (0.1, 1, 5, and 10 μ M) significantly increased the apoptotic DNA fragmentation in the extracted DNA. DEX (10 μ M)-induced apoptosis was attenuated by the addition of 10 μ M RU486. Thus, these results demonstrate that DEX induces fragmentation of chromosomal DNA leading to apoptosis.

3.3. Morphological observation by inverted microscope (AO/EB staining)

To further characterize the effect of stress hormone on apoptosis, a fluorescence microscopy analysis of cell death was made using AO/EB staining after cells was exposed to various concentrations of DEX. Because of the cell membrane integrity, bright green nuclei with normal structure were seen in most of control cells. The cells treated with DEX for 48 h exhibited significant morphological apoptotic changes, with yellow/orange nuclei, nuclear condensation and formation of apoptotic bodies. More than 40% of cells were found to be undergoing apoptosis in a dose-dependent manner with 0.1, 1, 5, and 10 μ M of DEX treatment, while percentage of apoptotic cells was less than 20% when incubated with 10 μ M DEX in the presence of RU486 (10 μ M) (Fig. 3). Therefore,

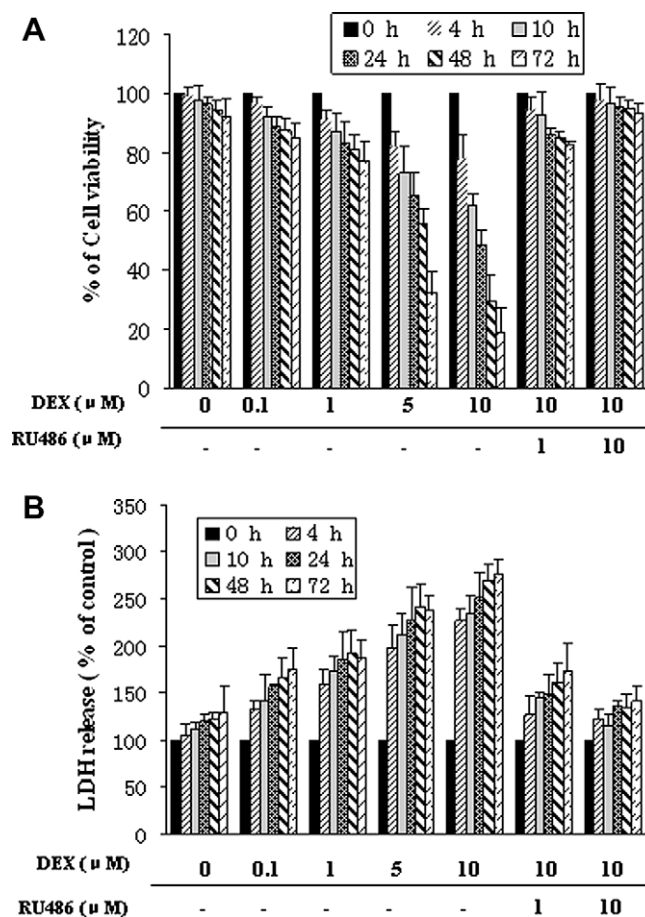


Fig. 1. Effects of DEX on cell viability of C2C12 myoblasts. Cells were treated with indicated concentrations of DEX or/and RU486 for 4, 10, 24, 48, and 72 h, and Percentage of cell death was assessed by MTT test (A) and LDH release assay (B). The viability of control cells at time 0 was set at 100%, and viability relative to the control was expressed.

the results suggest that DEX is able to induce noticeable apoptotic morphology in C2C12 myoblasts.

3.4. Effect of DEX on $[Ca^{2+}]_i$

To examine whether stress hormone-induced apoptosis is associated with an increase in calcium, we evaluated the intensity of intracellular fluo-3 fluorescence, an indicator of calcium, in C2C12 myoblasts treated with or without RU486. Following treatment with 0.1, 1, 5, and 10 μ M of DEX for 48 h, intracellular calcium was found to be showing significantly ($P < 0.05$ or < 0.01) higher than that of the control. Addition of equimolar concentration of RU486 significantly attenuated the effects of DEX (10 μ M) on intracellular calcium (Fig. 4). These results indicate that an increase in intracellular calcium by DEX might induce apoptosis.

3.5. Effect of DEX on $GR\alpha$ expression levels

To explore whether expression of the $GR\alpha$ was modulated by DEX and whether this modulation occurs at transcriptional or post-transcriptional level in C2C12 myoblasts, RT-PCR and Western blotting analyses of $GR\alpha$ mRNA and protein were performed. The $GR\alpha$ mRNA expression was down-regulated by stimulation with increasing concentrations of DEX (Fig. 5A and B). DEX treatment also reduced $GR\alpha$ protein levels in a dose-dependent manner (Fig. 5C and D). These effects could be prevented by RU486. The results indicate that DEX might exert its biological effects via

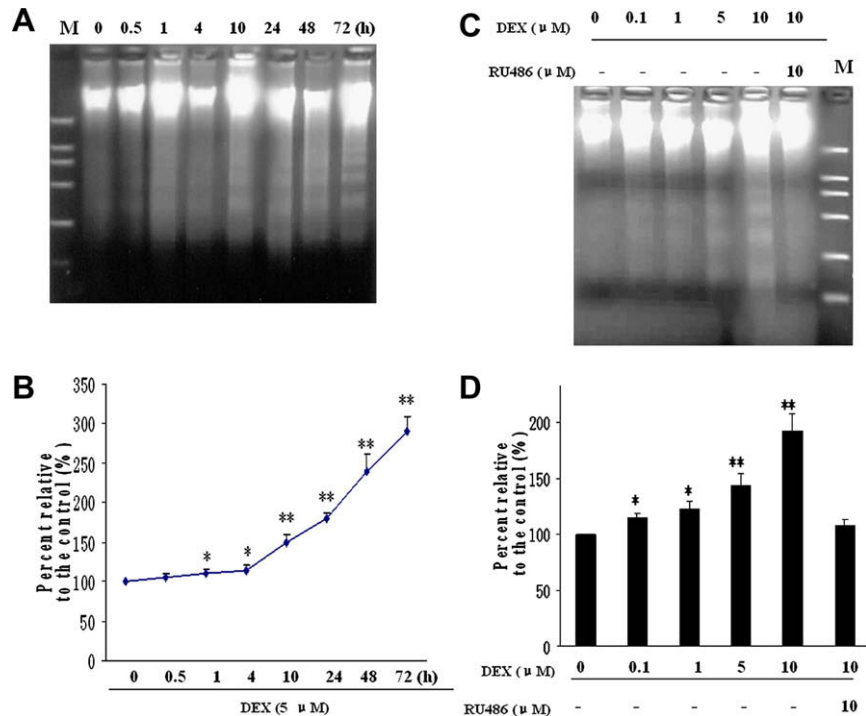


Fig. 2. DNA fragmentation in DEX-treated C2C12 myoblasts. C2C12 myoblasts were treated with 5 μM DEX at indicated periods of time (A) and indicated concentrations (B) of DEX or/and 10 μM RU486 for 24 h. Results shown in the curve chart (B) and diagram (D) are the densities of the bands DNA fragmentation in (A) and (C), respectively. M: DL 2000 ladder.

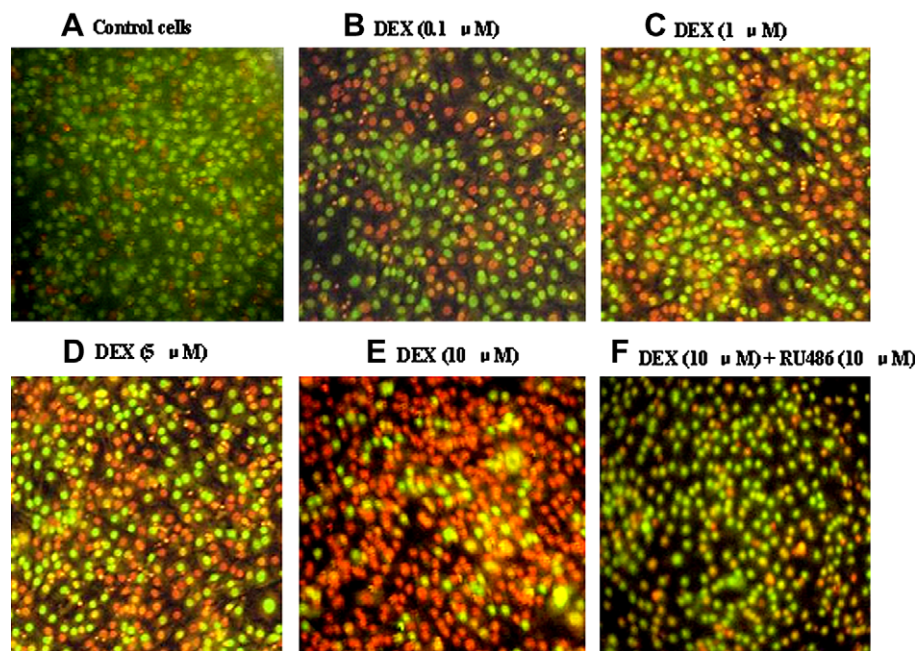


Fig. 3. Fluorescence microscope detection of normal and apoptotic cells with AO/EB double staining. C2C12 myoblasts were treated with indicated concentrations of DEX or/and RU486 for 48 h and harvested, washed with PBS (4 °C), and stained with AO/EB (100 mg/l). (A) Control cells. (B) Cells treated with 0.1 μM DEX. (C) Cells treated with 1 μM DEX. (D) Cells treated with 5 μM DEX. (E) Cells treated with 10 μM DEX. (F) Cells treated with 10 μM DEX in the presence of 10 μM RU486.

down-regulation of GR expression. This down-regulation is at the transcriptional level.

3.6. Effect of DEX on the expression levels of Ca^{2+} channel release related genes/proteins (SERCA1 and IP3R1)

To explore the role of Ca^{2+} channel in the mechanism of stress hormone-induced apoptosis, we examined the effects of DEX on

SERCA1 and IP3R1 mediated calcium signaling pathways in C2C12 myoblasts. RT-PCR analysis showed that there was an increased mRNA level of *IP3R1* and decreased mRNA level of *SERCA1* in DEX-treated C2C12 myoblasts in a dose-dependent manner (Fig. 6A and B). Data from Western blotting showed a similar increase of IP3R1 and decrease of SERCA1 expression at the protein level when cells were treated with 5 and 10 μM DEX for 24 h (Fig. 6C and D). A combination of 10 μM DEX with 10 μM RU486

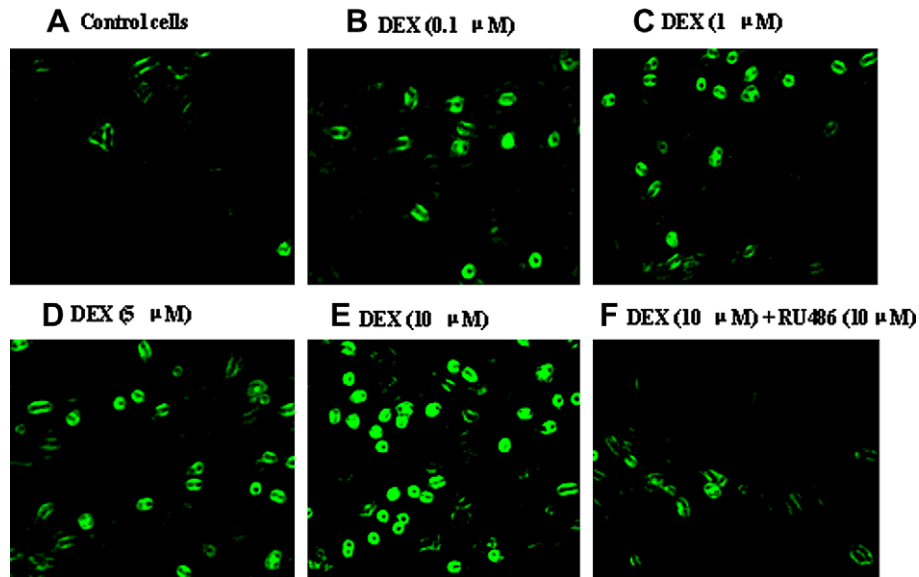


Fig. 4. Intracellular calcium level in C2C12 myoblasts revealed by using laser scanning confocal microscope with Fluo-3/AM as an intracellular free Ca^{2+} fluorescent probe. After being treated with indicated concentrations of DEX or/and RU486 for 48 h, C2C12 myoblasts were harvested, washed with D-hanks (4°C), and incubated with Fluo-3/AM ($5\ \mu\text{M}$) for 30 min, and then prepared for microscopic examination. (A) Control cells. (B) Cells treated with $0.1\ \mu\text{M}$ DEX. (C) Cells treated with $1\ \mu\text{M}$ DEX. (D) Cells treated with $5\ \mu\text{M}$ DEX. (E) Cells treated with $10\ \mu\text{M}$ DEX. (F) Cells treated with $10\ \mu\text{M}$ DEX and $10\ \mu\text{M}$ RU486.

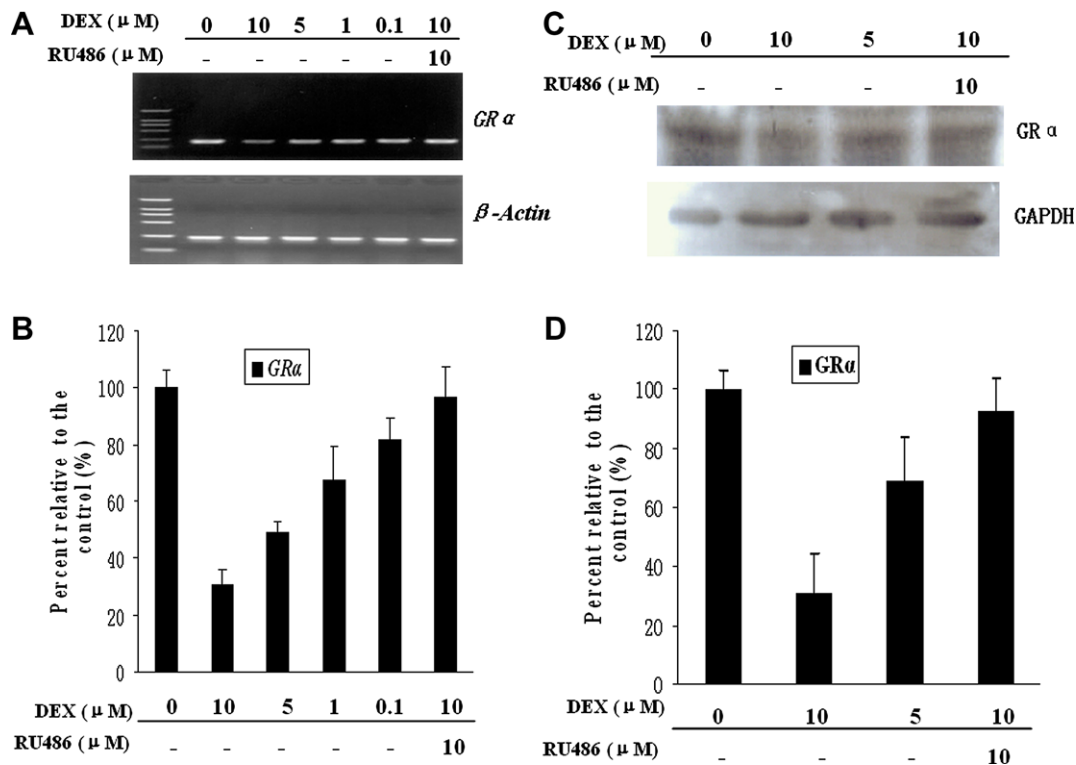


Fig. 5. Detection of endogenous gene and protein expression of $\text{GR}\alpha$ by semi quantitative RT-PCR (A) and Western blotting (C) from total RNA or protein extracted from C2C12 myoblasts, respectively. Cells were serum-starved for 10 h before indicated concentrations of DEX or/and $10\ \mu\text{M}$ RU486 was added for 24 h, and then lysed for total RNA extraction and total protein collection. The bottom panels depict quantification of $\text{GR}\alpha$ gene (B) and protein (D). β -Actin levels that were set as 100% served as the internal gene controls and GAPDH as the protein controls. The left lane in (A): DL2000 ladder.

for 24 h blocked DEX-induced alteration of IP3R1 and SERCA1 at both mRNA and protein expression levels. These results suggested that the regulatory effect of DEX on the expression of IP3R1 and SERCA1 was mediated by the GR.

In order to determine whether stress hormones regulate IP3R1 and SERCA1 gene expression by GR-dependent transcriptional acti-

vation or stabilization, C2C12 myoblasts were transfected with the IP3R1 and SERCA1 luciferase reporter plasmid along with indicated DEX or RU486, respectively. DEX induced a gradual increase in the promoter activity of IP3R1 in a dose-dependent manner (Fig. 8A). The luciferase activity of cells treated with $10\ \mu\text{M}$ DEX was 2.3-fold of that of control cells, and was completely attenuated by addition

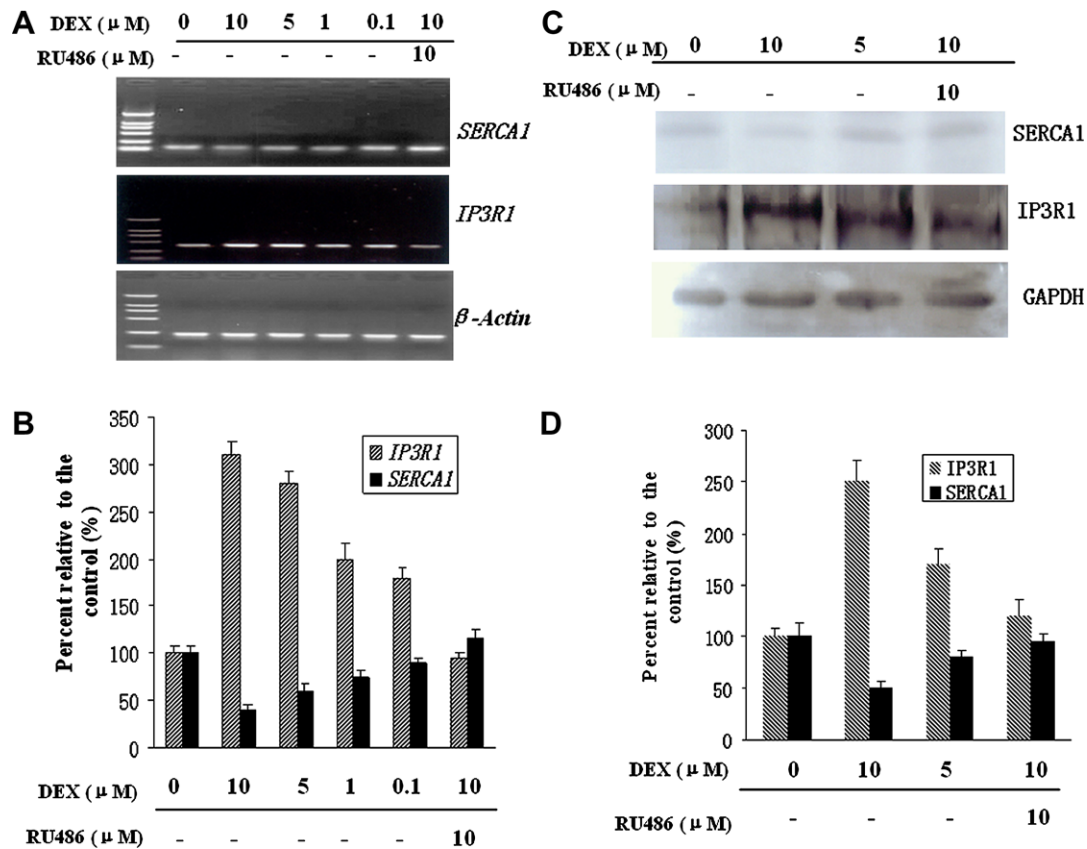


Fig. 6. The detection of endogenous Ca^{2+} channel release related genes and protein expression (SERCA1 and IP3R1) by semi quantitative RT-PCR (A) and Western blotting (C) from total RNA or protein extracted from C2C12 myoblasts, respectively. Cells were serum-starved for 10 h before indicated concentrations of DEX or/and 10 μM RU486 was added for 24 h, and then lysed for total RNA extraction and total protein collection. The bottom panels depict quantification of SERCA1 and IP3R1 gene (B) and protein (D). β -Actin levels that were set as 100% served as the internal gene controls and GAPDH as the protein controls. The left lane in (A): DL2000 ladder.

of 10 μM RU486. In contrast, DEX had no effect on SERCA1-promoter activity (Fig. 8B). These results suggested that the expression of IP3R1 is directly mediated by the GR whereas the change in transcription of SERCA1 by DEX might be through other mechanism(s).

3.7. Effect of DEX on the expression levels of apoptosis related genes (*Bcl-2*, *BAX*, *cytochrome c* and *caspase-3*)

To identify molecular mechanisms mediating DEX-induced apoptosis of C2C12 myoblasts, we tested for an alteration of *Bcl-2*, *BAX*, *cytochrome c* and *caspase-3*, which have been shown to play a central role in almost any form of apoptotic cell death. As shown in Fig. 5A and B, after 24 h incubation with 0.1, 1, 5, and 10 μM of DEX treatment, the mRNA level of *Bcl-2* was down-regulated while that of *BAX* was up-regulated as compared with that of the control, leading to increased ratio of pro-apoptotic *BAX/Bcl-2*. RT-PCR analysis also revealed a marked increase in the *cytochrome c* release and elevation of *caspase-3* in a dose-dependent manner (Fig. 7A and B). Next, we tested for an alteration of apoptotic proteins. The results of Western blotting (Fig. 7C and D) were consistent with RT-PCR. The effect of DEX on *caspase-3* activation, an important mediator of apoptosis, was also examined. As can be seen in (Fig. 7E), DEX activates *caspase-3* in a dose-dependent manner. This alteration induced by 10 μM DEX was almost completely prevented by co-incubation with 10 μM RU486. Together, these results indicate that apoptosis induced by the stress hormone in C2C12 myoblasts might be due to changes in the mitochondrial membrane permeability.

4. Discussion

Calcium is one of the most important intracellular messengers that translate extracellular stimuli into intracellular pathways regulating cell survival, development, gene expression and differentiation (Berridge et al., 2000). Therefore, proper intracellular calcium signaling is essential for normal cell functions and needs to be precisely regulated. Calcium overload has been suggested to be the final common pathway of most types of cell death (Liu et al., 1998; Demareux and Distelhorst, 2003). In this study, we showed that DEX-induced apoptosis through the increase in intracellular calcium (Fig. 4). Fluorescent microscopy analysis revealed that DEX markedly induced calcium increase in C2C12 myoblasts. This result was similar with previous reports that myotoxin II and Cartap treatment caused an increase of calcium in C2C12 myoblasts to induce apoptosis (Liao et al., 2006; Villalobos et al., 2007). The imbalance of calcium homeostasis in sarcoplasmic reticulum (SR) has been suggested to result in structural damage of the SR and an increase in cytoplasmic calcium, leading to further activation of proteases and lipases that finally destroyed the structure of cell membrane and the ensuing apoptosis of the cell (Pretorius and Bornman, 2005). Under our experimental conditions, treatment with DEX significantly increased LDH leakage and decreased cell viability, both of which are indicators of plasma membrane integrity (Fig. 1B). And the AO/EB staining assay further demonstrated that DEX increased the numbers of apoptotic cells at 48 h after DEX treatment in C2C12 myoblasts (Fig. 3).

Endonuclease activation is another important biochemical event in apoptosis. In normal cells the inactive endonuclease has

characteristic silencing and was shown to be a $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent activity and whose activation occurred as an early event leading to DNA damage and cell death (Collins et al., 1996; Hagar et al., 1996). Our results showed that intracellular free calcium concentration and DNA fragmentation exhibited similar patterns in response to the indicated concentrations of DEX (Fig. 2), suggesting that calcium may play a major role during DEX-induced DNA damage. But, it still remains to determine whether the increased intracellular calcium might activate endonuclease directly, or through a calcium-dependent protease like calpain, leading to DNA fragmen-

tation. Further studies are required to elucidate the precise mechanism of DEX-induced DNA damage.

It is well known that the expressions of many apoptotic gene products, such as BAX, bcl-2, cytochrome c and caspase-3, are influenced by cytoplasmic calcium overload (Bezprozvanny and Hayden, 2004). The results in the present study by RT-PCR assay (Fig. 7A) and Western blotting analysis (Fig. 7C) showed that C2C12 myoblasts exposed to DEX had an increase in BAX and a decrease in Bcl-2, thus increased the BAX/Bcl-2 ratio, indicating that the apoptosis induced by DEX might be related to the regulated

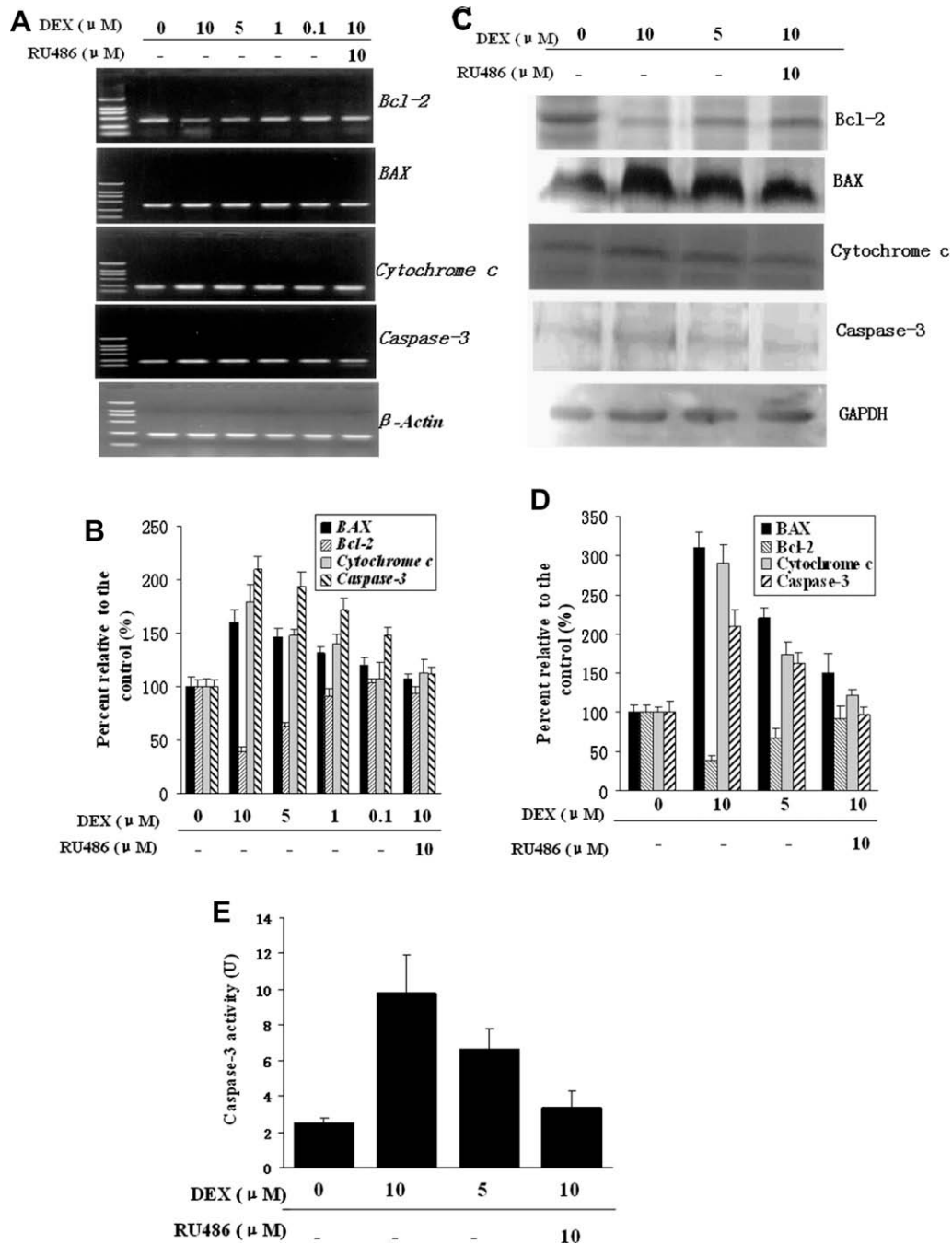


Fig. 7. The detection of endogenous apoptosis related genes and protein expression (Bcl-2, BAX, cytochrome c and caspase-3) (A) and Western blotting (C) from total RNA or protein, and the activity of caspase-3 (E) from total protein extracted from C2C12 myoblasts, respectively. Cells were serum-starved for 10 h before indicated concentrations of DEX or/and 10 μM RU486 was added for 24 h, and then lysed for total RNA extraction and total protein collection. The bottom panels depict quantification of Bcl-2, BAX, cytochrome c and caspase-3 gene (B) and protein (D). β -Actin levels that were set as 100% served as the internal gene controls and GAPDH as the protein controls. The left lane in (A): DL2000 ladder.

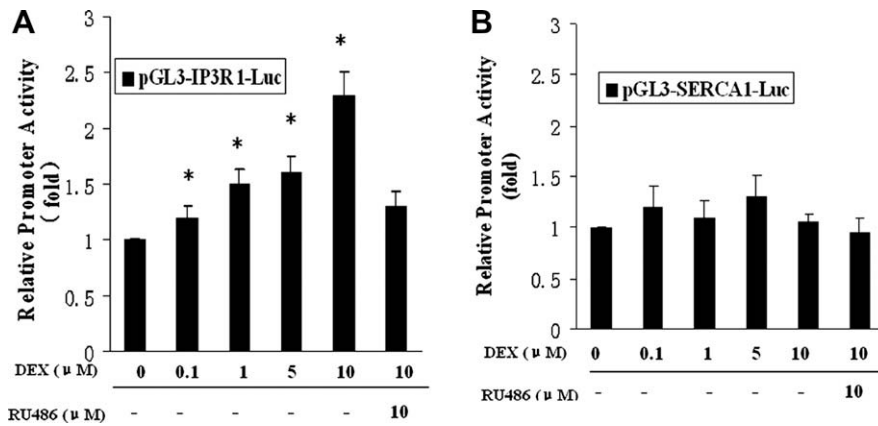


Fig. 8. The detection of *IP3R1* and *SERCA1*-promoter activity. C2C12 myoblasts were transiently cotransfected with 1 μ g of pGL3-IP3R1-Luc (A) or pGL3-SERCA1-Luc (B) plasmids and 2.5 μ l Lipofectamine 2000 reagent, and then incubated with indicated concentrations of DEX or/and RU486 for 48 h, followed by cell lysis and analysis of luciferase activity. $P < 0.05$, statistically significant difference between a treated group and the control.

expression of BAX and Bcl-2 and the balance of these two proteins might lead to induction of cell apoptosis. The Bcl-2 protein plays a central role in controlling cell death, because of the formation of heterodimers with BAX, which is considered a promoter of cell apoptosis. In addition, this heterodimerization results in neutralization of the bound pro- and anti-apoptotic proteins. Therefore, the ratio of BAX to Bcl-2 rather than the absolute amount of either protein is critical for cell survival or apoptosis (Gross et al., 1999). Substantial evidence indicated that Bcl-2 protein prevented apoptosis by blocking the release of mitochondrial interspace proteins, including cytochrome *c* and the apoptosis inducing factor, while BAX stimulated release of cytochrome *c* from mitochondria, activated downstream of caspase in the cytochrome *c*-dependent apoptosis pathways resulting in apoptosis (Gross et al., 1999; Pinton and Rizzuto, 2006; Chai et al., 2008). Other studies suggested that the cytochrome *c* forms complex with Apaf-1/caspase-9 leading to the activation of caspase-3 and protease cascade (Chai et al., 2008). Therefore, the activation of caspase-3 caused by the decreased Bcl-2 and increased cytochrome *c* may be a possible mechanism of apoptosis in the DEX-inducing cell apoptosis.

For the disruption of cytosolic calcium homeostasis, two different channels, IP3R1 and SERCA1, have been characterized as releasing of calcium from internal stores and pumping calcium back into internal stores, respectively (Streb et al., 1983; Aubier and Viires, 1998; Lencesova et al., 2002). It was reported that stable knockdown of the IP3R1 in Jurkat T-lymphocytes can inhibit responsiveness to DEX-induced calcium elevation and apoptosis (Jayaraman and Marks, 1997) and siRNA for the IP3R1 also prevent calcium elevation and apoptosis in Jurkat lymphocytes (Wozniak et al., 2006). In contrast, in cerebellar granule neurons, a decrease in calcium resulting from inhibition of SERCA pumps by thapsigargin prevents the apoptotic death induced by mastoparan (Lin et al., 1997). In the present study, analyses of RT-PCR (Fig. 6A) and Western blotting data (Fig. 6C) showed increases in both mRNA and protein levels of IP3R1 in a dose-dependent manner with calcium elevation induced by DEX, whereas the levels of SERCA1 were decreased. Previous research showed that some genes were induced by GCs through transcriptional activation or repression by direct binding of GR to glucocorticoid responsive element (GRE) in the regulatory regions (Tseng et al., 2001; Waterman et al., 2006; Kim et al., 2008). A central problem addressed in this study is whether or not the changes of IP3R1 and SERCA1 induced by DEX cause calcium imbalance to stimulate further apoptotic events. Analysis of IP3R1-promoter activity (Fig. 8A) in C2C12 cells revealed an increase from 1.3- to 2.5-fold after DEX stimulation. This effect was promoter specific, and might depend on GRE se-

quences at 340 bp upstream of the transcription initiation site. This strongly suggests that the DEX-induced IP3R1 expression is at least in part a result of increased transcription of the IP3R1 gene, rather than altered stability of IP3R1 protein or the mRNA coding for it. However, no alteration of SERCA1-promoter activity (Fig. 8B) was detected in this study. It is thus tempting to speculate that there must be other molecular mechanism for altering calcium homeostasis by regulating SERCA1 expression in DEX-induced apoptosis. Several lines of evidence support this view. Over-expression of the anti-apoptotic proteins Bcl-2 affected the ER calcium store by up-regulating the SERCA1 expression (Kuo et al., 1998; Pinton and Rizzuto, 2006). However, the elucidation of the mechanisms by which DEX stimulated IP3R1 expression via GRE in the region of IP3R1-promoter and Bcl-2-mediated SERCA1 expression requires further experiments.

To further determine the effect of GR on DEX-induced apoptosis, the effects of RU486 on DEX-induced apoptosis were studied. Pretreatment with RU486 was shown to significantly block the DEX-induced biological effects. These results strongly suggest that DEX-induced effects are mediated by GR. The results of our studies can be summarized in a schematic presentation (Fig. 9). In conclusion, in this study, we demonstrated that C2C12 myoblasts is a suitable in vitro model for studying the skeletal muscle cell

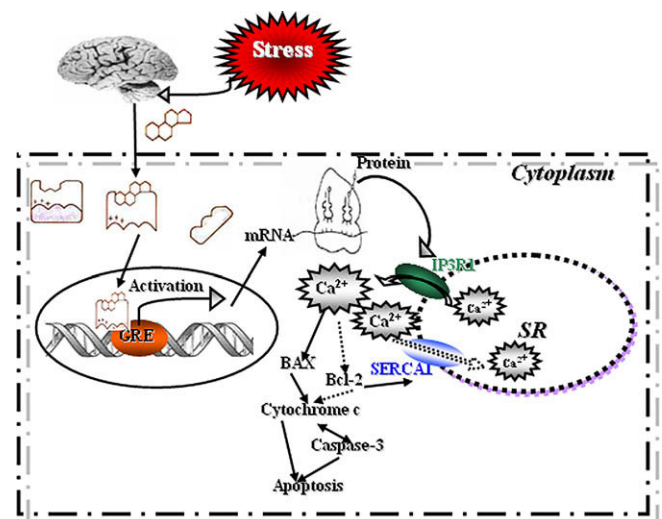


Fig. 9. Proposed model of the apoptotic pathway induced by stress hormone. SR, sarcoplasmic reticulum.

damage of stress hormone. DEX may exert its cytotoxicity, at least partially, to C2C12 cells by inducing calcium-dependent signal pathway. Calcium activates BAX and decreases Bcl-2, subsequently leading to activation of cytochrome *c* to stimulate further increase of caspase-3 via the up-regulation of IP3R1 level by direct binding of GR to GRE in its promoter. The decrease of Bcl-2 may in turn lead to a decline of SERCA1 expression to exacerbate the imbalance of calcium homeostasis. Then DNA fragmentation, membrane damage, LDH leakage and loss of cell viability follows and the cells ultimately undergo apoptosis.

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