Pretreatment with insulin-like growth factor I protects skeletal muscle cells against oxidative damage via PI3K/Akt and ERK1/2 MAPK pathways

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Oxidative stress has an important role in the pathogenesis of many muscle diseases. The major contributors to oxidative stress in muscle tissue are reactive oxygen species such as oxygen ions, free radicals, and peroxides. Insulin-like growth factor I (IGF-I) has been shown to increase muscle mass and promote muscle cell proliferation, differentiation, and survival. We, therefore, hypothesized that IGF-I might also be cytoprotective for muscle cells during oxidative stress. Exogenous hydrogen peroxide (H₂O₂) was used to induce oxidative stress/damage in two types of skeletal muscle cells. Apoptotic pathways were assessed after the oxidative damage and the effects of IGF-I on oxidative stress in muscle cells were examined. Different IGF-I sub-pathways were analyzed with measurement of the expression of pro- and anti-apoptotic proteins. It was found that H₂O₂ diminishes muscle cell viability and induces a caspase-independent apoptotic cell death. Pretreatment with IGF-I protects muscle cells from H₂O₂-induced cell death and enhances muscle cells survival. This effect appears to result from the promotion of the anti-apoptotic protein, Bcl2. Further investigation shows that protection is via an IGF-I sub-pathway: PI3K/Akt and ERK1/2 MAPK pathways. Protecting muscle cells from oxidative damage presents a potential application in the treatment of the muscle wasting, which appears in many muscle pathologies including Duchenne muscle dystrophy and sarcopenia.

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Many pathological muscle conditions, such as Duchenne muscle dystrophy (DMD) and sarcopenia, have been found to be associated with an increase in oxidative stress.¹ DMD is a severe progressive X-linked muscle disease with the absence of dystrophin, a protein providing structural integrity on the muscle cell membrane. Owing to the fragility of the DMD muscle membrane, normal muscular contraction in DMD destabilizes the myocyte membrane, causing intracellular accumulation of calcium. This stimulates oxidative metabolism, which generates free radicals and triggers the key pathological processes.² Sarcopenia is a condition with degenerative loss of skeletal muscle mass and strength associated with ageing. Mitochondrial (Mt) production of reactive oxygen species (ROS) has been shown to increase in skeletal muscle over the course of ageing,³ which in turn reduces bioenergetic efficiency and leads to muscle fiber

atrophy/loss.⁴ Although DMD and sarcopenia have different pathological properties, they share a common syndrome, that is, muscle wasting. Abundant evidence implicates oxidative stress as a potential regulator of proteolytic pathways leading to muscle wasting.⁵

Oxidative stress results from the activities of the reactive compounds called ROS. ROS are natural by-products in the normal metabolism of oxygen and have important roles in cell signalling. ROS including oxygen ions, free radicals, and peroxides usually have highly reactive properties because of the presence of unpaired valence shell electrons. During physiological homeostasis overall oxidative balance is maintained by the removal of ROS via a variety of antioxidants to match the production of ROS from a variety of sources.⁶ Overproduction of ROS or lack of antioxidants leads to oxidative stress and cause oxidative damage such as

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deleterious cellular effects, cell death, and diverse pathological conditions. One major contributor to oxidative damage is hydrogen peroxide (H_2O_2), which is produced from superoxide that leaks from the mitochondria. Exogenous H_2O_2 has been used to induce myotube oxidative stress⁷ and atrophy⁸ in previous studies.

Preventing oxidative stress or protection against oxidative damage offers a potential strategy for cure or delaying muscle wasting.^{1,4} Insulin-like growth factor I (IGF-I) has been found to contribute to oxidative balance and has a protective effect against iron-induced-lipid oxidative stress.⁹ We, therefore, hypothesized that IGF-I might be able to protect muscle cells from oxidative stress. To test this hypothesis exogenous H_2O_2 was used to induce skeletal muscle cell oxidative stress/damage. The effects of IGF-I on oxidative stress in muscle cells was examined and different IGF-I pathways were analyzed. It was found that IGF-I has a protective effect on muscle cells after oxidative stress. Protecting muscle cells from oxidative damage by IGF-I offers a potential application in a variety of muscle pathologies.

MATERIALS AND METHODS

Reagents

Recombinant human IGF-I was purchased from Peprtech EC (UK). Caspase 3/7, 8, and 9 activity assay kits and CellTiter-Blue cell viability assay kits were purchased from Promega (Madison, USA). Yo Pro-1 iodide was purchased from Invitrogen (Paisley, UK). Polyclonal anti-Akt and Phospho-Akt (Ser473) antibodies were purchased from New England Bio-labs (Hitchin, UK). Polyclonal anti-human Bax and Bcl2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Anti-rabbit IgG antibody was purchased from GE Healthcare (Amersham, UK). LY294002, SB 203580, and MEK1/2 inhibitor were purchased from Merck (UK). TUNEL assay kit was purchased from Calbiochem (UK). Apoptotic DNA ladder detection kit was purchased from Millipore (USA). All cell culture media, serums, and antibiotics were purchased from GIBCO (UK).

Cell Lines and Culture Conditions

C_2C_{12} (mouse) and L_6E_9 (rat) skeletal muscle cell lines were purchased from European Collection of Cell Cultures (ECACC). Cells were cultured and maintained at 37 °C in 5% CO_2 /95% air using DMEM medium containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (PenStrep). All cells were used at passages 10–30 after their receipt from the supplier.

Cell Treatments

Pretreatment with IGF-I

C_2C_{12} or L_6E_9 cells were seeded in 96-well plates at a density of 2×10^4 cells/well in DMEM medium containing 10% FBS, 1% PenStrep, and maintained for 24 h. After seeding, cells were pretreated with different concentrations (0, 50, and 100 ng/ml) of IGF-I (Peprtech EC, UK) in DMEM medium

containing 10% FBS and 1% PenStrep at 37 °C in 5% CO_2 /95% air for 24 h. After pretreatments, cells were challenged with different concentrations of H_2O_2 .

H_2O_2 induce cell oxidative stress

After seeding or pretreatment with IGF-I, cells were challenged by the addition of H_2O_2 solution at the desired final concentrations (0.25, 0.5 and 1 mM) in DMEM medium containing 10% FBS and 1% PenStrep at 37 °C in 5% CO_2 /95% air for 2 h. After treatments and oxidative challenge, cells were subjected to cell viability or cell death assays.

Inhibition of PI3K/Akt, p38, and ERK1/2 MAPK pathways

To determine which pathway IGF-I executes its cell protection from oxidative injury, a cell permeable phosphatidylinositol 3 kinase (PI3K) inhibitor (LY294002), p38 mitogen-activation protein kinase (MAPK) inhibitor (SB 203580), or MEK1/2 inhibitor (cat no. 444939, Merck) were used to inhibit PI3K/Akt, p38MAPK, or MEK1/2 MAPK pathways. Briefly, C_2C_{12} and L_6E_9 cells were seeded in 96-well plates at a density of 1×10^4 cells/well in DMEM medium containing 10% FBS and 1% PenStrep and maintained for 24 h. After seeding, cells were treated with IGF-I at a concentration of 100 ng/ml plus LY294002 (20 μ M), SB203580 (20 μ M), or MEK1/2 inhibitor (20 μ M) in DMEM medium containing 10% FBS and 1% PenStrep at 37 °C in 5% CO_2 /95% air for 24 h before they were challenged with different concentrations of H_2O_2 . After treatments and oxidative challenge, cells were subjected to cell viability or cell death assays.

Cell Viability Assay

After 24 h of pretreatment with different concentration of IGF-I and 2 h of oxidative challenge, cell viability was assessed using a previously reported method.^{10,11} Briefly, after treatment with IGF-I and oxidative stress, 20 μ l of CellTiter-Blue reagent (Promega) was added to each well without the removal of the medium. The plate was rocked on an orbital shaker gently for 2 min to help mixing of the CellTiter-Blue reagent with culture medium. Cells were then further incubated at 37 °C in 5% CO_2 /95% air for 5 h. Fluorescence was measured with excitation at 530 and emission at 620 nm using a fluorescent plate reader (Fluoroskan Ascent FL; Labsystems, Helsinki, Finland). The experiments were performed in triplicate and repeated on two separately initiated cultures.

Cell Death Assay

After pretreatment by IGF-I and oxidative stress by H_2O_2 , dead cells were assessed using a previously reported method.^{11,12} Briefly, cells were seeded in 96-well plates with DMEM containing 10% FBS and 1% PenStrep at a density of 2×10^4 cells/well. After overnight culture, cells were pretreated with multiple IGF-1 concentrations for 24 h and oxidatively stressed with different concentrations of H_2O_2 . After treatment, YOPRO-iodide (Invitrogen) was added to

each well at a final concentration of 4.0 $\mu\text{M/l}$ and mixed for 2 min on an orbital shaker. Cells were incubated at 37 °C in 5% $\text{CO}_2/95\%$ air for 5 h. Fluorescence was measured with excitation at 485 nm and emission at 510 nm using a fluorescent plate reader (Fluoroskan Ascent FL; Labsystems). For comparison purposes results were expressed as the percentage of dead cells to viable cells, which was calculated using the following formula:

$$\text{Dead cells (\%)} = \text{FD}/\text{FV} \times 100$$

where FD represents fluorescence reading for dead cells, FV represent fluorescence reading for viable cells determined in the cell viability assay. The experiments were performed in triplicate and repeated on two separately initiated cultures.

TUNEL Assay

To examine whether H_2O_2 induces apoptotic cell death, C_2C_{12} and L_6E_9 cells were seeded in chambers mounted on a glass slide with DMEM media containing 10% FBS and 1% PenStrep at a density of 2×10^5 cells/slide. After overnight incubation, cells were treated by H_2O_2 (C_2C_{12} and L_6E_9 cells at concentrations of 1 mM and 0.25 mM, respectively) for 1 and 2 h. One group of cells was treated by camptothecin (20 μM) for 2 h as a positive control. After treatment, cells were fixed with formalin (10%), and apoptotic cells were detected using a terminal deoxynucleotidyl transferase mediated dUTP nick-end-labeling (TUNEL) kit (Calbiochem).

Detection of DNA Fragmentation

DNA fragmentation, the hallmark of apoptosis, was examined using DNA ladder detection kit (Millipore). Briefly, C_2C_{12} and L_6E_9 cells were seeded in a 5-ml flask at a density of 2×10^6 cells/flask with DMEM media containing 10% FBS and 1% PenStrep. After overnight incubation, cells were treated with H_2O_2 (C_2C_{12} and L_6E_9 cells at concentrations of 1 and 0.25 mM, respectively) for 2 h. After the treatment, cells were washed three times with PBS and pelleted by centrifugation. The DNA fragments were isolated and purified according to the manufactory instruction and visualized by electrophoresis.

Caspase Activity Assay

After oxidative stress, cells were subjected to caspase 3/7, 8, and 9 activity measurements with Caspase-Glo assay kit (Promega). Briefly, the plates containing cells were removed from the incubator and allowed to equilibrate to room temperature for 30 min. A volume of 100 μl of Caspase-Glo reagent was added to each well, the contents of wells were gently mixed with a plate shaker at 300–500 r.p.m. for 30 s. The plate was then incubated at room temperature for 2 h. The luminescence of each sample was measured in a plate-reading luminometer (Thermo Labsystems) with parameters of 1-min lag time and 0.5 s/well read time. The experiments were performed in triplicate and repeated on two separately

initiated cultures. To assess whether the Caspase-Glo assay kit is suitable for measurement of caspase activity in muscle cells, both muscle cell lines were treated by cycloheximide at 0.1 mM for 24 h (cycloheximide is a protein translation inhibitor, which has been proven to cause both muscle cell apoptosis), caspase 3/7, 8, and 9 activity was measured with Caspase-Glo assay kit using the above protocol.

Protein Extraction and Western Blotting

C_2C_{12} and L_6E_9 cells were seeded in a six-well plate at a density of 18×10^5 cells/well with DMEM medium containing 10% FBS and 1% PenStrep and incubated at 37 °C in 5% $\text{CO}_2/95\%$ air for 24 h. After seeding, cells were washed with serum-free medium (99% DMEM, 1% PenStrep) three times and then cultured in serum-free medium for 24 h. The volume of medium in each well was 3 ml. After serum deprivation, the cells were divided into four groups with each group containing two wells. For the first group, cells were incubated with serum-free medium, the second group were incubated with serum-free medium containing IGF-I (100 ng/ml), the third group were incubated with serum-free medium containing IGF-I (100 ng/ml) and LY294002 (20 μM), and the fourth group were incubated with serum-free medium containing IGF-I (100 ng/ml) and SB203580 (20 μM). All treatments lasted 24 h. After treatment, cells were washed three times with PBS, lysed by the addition of RIPA buffer containing protease inhibitor cocktail (Roche), and harvested with a cell scraper. To assist cell lysis, cell suspensions were repeatedly frozen and thawed. Total protein concentration in the samples was determined using a modified Lowry protein assay kit (Pierce Biotechnology, USA). In total, 60 μg of total protein was mixed with the same volume of the laemmli sample buffer containing 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M tris HCl, pH approximately 6.8 (Sigma, UK). The samples were then denatured by heating at 95 °C for 5 min. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins, which were then electro-blotted on to polyvinylidene fluoride (PVDF) membrane (Bio-Rad, USA). The membrane was blocked with 5% milk in PBS (Marvel semi-skimmed milk powder) and incubated with primary antibodies at 4 °C overnight followed by incubation with anti-rabbit IgG antibody conjugated with horse radish peroxidase (Dako, UK) at 1/2000 dilution. The primary antibody dilution for Bax, Bcl2 and Akt was 1/200, for Phospho-Akt was 1/1000. After secondary antibody incubation, the membrane was washed three times with PBS and incubated with Super Signal West Dura extended duration Substrate (Pierce, USA) for 5 min and the protein bands were illuminated with a chemi-doc system (Bio-Rad). The density of protein bands was measured using densitometry software (Molecular Analyst, windows software for Bio-Rad image analysis system version 1.5, USA).

Statistical Analysis

All data have been examined and shown a normal distribution. All results were expressed as mean $\pm$ s.e.m. One-way ANOVA (Prism version 4 2004 edition, USA) with multiple comparison tests were used. Statistical analysis was performed on $n=6$ samples and Bonferroni's Multiple Comparison Test was used after ANOVA. $P<0.05$ was considered as significant and indicated as $*P<0.01$ was considered as higher significance and indicated as $**P>0.05$ was considered as not significant and marked as NS.

RESULTS

H₂O₂-Induced Muscle Cell Oxidative Stress

Two mammalian skeletal muscle cell lines (C2C12 and L6E9) were used in the experiment. After 2 h of exposure to different concentrations of H₂O₂, survival of both C₂C₁₂ and L₆E₉ cells decreased in a dose-dependent manner (Figures 1a and b); the higher the concentration of H₂O₂, the less the survival of cells. When the ratios of dead cells to survival cells was examined, it revealed that exposure to H₂O₂ also increased the number of dead cells in a dose-dependent manner (Figures 1c and d). This indicates exogenous H₂O₂-induced muscle cell oxidative stress, which not only reduced cell viability, but also caused cell death. It is interesting to note that L₆E₉ cells are more susceptible to H₂O₂ than C₂C₁₂ cells as it is observed that all three concentrations (0.25, 0.5, and 1 mM) of H₂O₂ decrease L₆E₉ cell viability significantly compared with the untreated control cells (Figure 1b), while only the highest concentration (1 mM) of H₂O₂ reduces the C₂C₁₂ cell viability significantly (Figure 1a). Similar to cell survival, the ratio of dead cells is also significantly higher in L₆E₉ (Figure 1d) cells than C₂C₁₂ cells (Figure 1c).

To analyze whether H₂O₂-induced cell death is a type of apoptosis, TUNEL assay was used to examine dead cells after H₂O₂ exposure. The results showed that H₂O₂-induced apoptosis in both C₂C₁₂ and L₆E₉ cells after 1- and 2-h exposure (Figure 1e). It seems that 2-h exposure caused more apoptosis than 1-h exposure. To validate the method, camptothecin that has been proven to be able to induce muscle cell apoptosis previously,¹³ was included in the experiment as a positive control. It was found that exposure to camptothecin for 2 h also caused apoptosis in both cell lines.

To further prove H₂O₂-induced cell death is a type of apoptosis, DNA fragmentation was examined. The results showed that H₂O₂-induced DNA fragmentation in both cell lines after 2-h exposure (Figure 1f). It seems that H₂O₂ caused more severe DNA destruction in C₂C₁₂ cells than in L₆E₉ cells; this may be due to the different concentration of H₂O₂ used in the experiment, for C₂C₁₂ cells 1 mM of H₂O₂ was used, while for L₆E₉ cell 0.25 mM of H₂O₂ was used.

H₂O₂-Induced Muscle Cell Death is a Type of Caspase-Independent Apoptosis

To analyze by which apoptotic pathway H₂O₂-induced muscle cell death occurs, caspase activity was assessed after

cells had been exposed to H₂O₂ at 1 mM for 2 h. It was found that H₂O₂ significantly induced cell apoptotic death (Figures 1b and d–f), however, it does not affect caspase 3/7, 8, and 9 activity (Figures 2a and b) in either cell lines. It was also shown that cycloheximide treatments (which has been proven to be able to cause both cell lines apoptotic death) can significantly increase the activity of caspase 3/7 and 9 in both cell lines and caspase 8 in C₂C₁₂ but not in L₆E₉ cells (Figures 2c and d) measured by Caspase-Glo assay kit indicating the kit used in experiments is suitable for the measurement of caspase activity in both muscle cell lines. All together these data suggest that H₂O₂-induced muscle cell death is a type of caspase-independent apoptosis.

IGF-I Increase Muscle Cell Survival After H₂O₂-Induced Oxidative Stress

To determine whether IGF-I has a protective effect on muscle cells from H₂O₂-induced oxidative injury, muscle cells were pretreated with different concentrations of IGF-I for 24 h before they were exposed to a variety concentrations of H₂O₂. It was found that the viability of both muscle cell lines was higher in the IGF-I pretreated groups than the untreated control group (Figures 3a and b). The increase in the viability of the pretreated cells was in a dose-dependent manner in both cell lines indicating that IGF-I can increase muscle cell survival after oxidative stress. It is interesting to note that the response to oxidative challenge and IGF-I treatment were different in the two cell lines. The greater viable cell loss in L₆E₉ cells (Figure 3b) compared with C₂C₁₂ cells (Figure 3a), after H₂O₂ treatment, indicates that L₆E₉ cells are more sensitive to oxidative challenge than the C₂C₁₂ cells. Although IGF-I has a protective effect in both cell lines in a dose-dependent manner, the extent of protection is different. In C₂C₁₂ cells, the highest concentration of IGF-I (100 ng/ml) always brings the cell survival level to the level of the untreated normal cells no matter what concentration of H₂O₂ is used in oxidative stress (Figure 3a). In the L₆E₉ cells, the highest concentration of IGF-I (100 ng/ml) only brings the cell survival level to the level of the untreated normal cells in the lowest level of H₂O₂ treatment (0.25 mM), while in the higher levels of H₂O₂ treatments (0.5 and 1 mM), the highest concentration of IGF-I only brings cell survival to the level of 65 and 45% of the untreated normal cells, respectively (Figure 3b). The difference may be due to the fact that L₆E₉ cells are more vulnerable to the higher level of H₂O₂ treatments (0.5 and 1 mM), which decrease cell viability to the 45 and 25% of the untreated normal cells, respectively (Figure 3b) and IGF-I protection may not be influential enough to recover these severely damaged cells.

IGF-I Reduced Muscle Cell Death After H₂O₂-Induced Oxidative Stress

After pretreatment with IGF-I and challenge by H₂O₂, cell death was assessed by a previously reported method.^{11,12} The results show that a higher concentration of H₂O₂ increases

Figure 1 C₂C₁₂ (**a, c**) and L₆E₉ (**b, d**) skeletal muscle cells were challenged by H₂O₂ at concentrations of 0.25, 0.5, and 1 mM for 2 h. Cell viability was determined with CellTiter-Blue reagent (Promega), cell survival (%) (**a, b**) was expressed as percentage of viable cell in treated groups to the untreated control group (0 mM). Dead cell was assessed with YOPRO-iodide (Invitrogen); cell death/survival ratio (**c, d**) was calculated according to the method mentioned in the text. All data were represented as mean $\pm$ s.e.m. of six samples ($n = 6$). $P < 0.05$ was considered as significant and indicated as * $P < 0.01$ as higher significance and indicated as ** $P > 0.05$ as not significant and marked as NS. C₂C₁₂ and L₆E₉ cells were seeded in chambers mounted on glass slide for 24 h and treated by H₂O₂ (C₂C₁₂ and L₆E₉ cells at concentrations of 1 and 0.25 mM, respectively) for 1 and 2 h. One group of cells was treated by camptothecin (20 μ M) for 2 h as a positive control; one group was untreated as negative control. After treatment, apoptotic cells were detected by TUNEL assay. Dark blue stained cells are apoptotic cells (**e**). C₂C₁₂ and L₆E₉ cells were seeded in a 5-ml flask at a density of 2×10^6 cells/flask overnight and then treated with H₂O₂ (C₂C₁₂ and L₆E₉ cells at concentrations of 1 and 0.25 mM, respectively) for 2 h. Each cell line included one group that was untreated as negative control (N). The DNA fragments were detected by apoptotic DNA ladder detection kit (Millipore), and DNA fragmentation occurs in both cell line (**f**). It seems that H₂O₂ caused more severe DNA destruction in C₂C₁₂ cells than in L₆E₉ cells.

Figure 2 C₂C₁₂ (a) and L₆E₉ (b) skeletal muscle cells were challenged by H₂O₂ at concentrations of 1 mM for 2 h. Cellular caspase 3/7, 8, and 9 activity was determined by Caspase-Glo assay kit (Promega) and expressed as percentage of the treated cell to the untreated control cell. A positive control group (c, d) which received cycloheximide treatment (0.1 mM) was included in each cell line to assess whether the Caspase-Glo assay kit is suitable for the measurement of caspase activity in muscle cells. Caspase 3/7, 8, and 9 activity in control groups (c, d) was measured using the same method as (a, b) used. Caspase activity was represented as the percentage (%) to untreated control group, and data were represented as mean $\pm$ s.e.m. of six samples ($n = 6$). $P < 0.05$ was considered as significant and indicated as * $P < 0.01$ as higher significance and indicated as ** $P > 0.05$ as not significant and marked as NS.

Figure 3 C₂C₁₂ (a) and L₆E₉ (b) skeletal muscle cells were pretreated with different concentrations (0, 50, and 100 ng/ml) of IGF-I for 24 h and then challenged by H₂O₂ at concentrations of (0, 0.25, 0.5, and 1 mM) for 2 h. Cell viability was determined with CellTiter-Blue reagent (Promega); cell survival (%) was expressed as percentage of viable cell in treated groups to the untreated control group (0). All data were represented as mean $\pm$ s.e.m. of six samples ($n = 6$). $P < 0.05$ was considered as significant and indicated as * $P < 0.01$ as higher significance and indicated as ** $P > 0.05$ as not significant and marked as NS.

cells death in both cell lines (Figures 4a and b). In respect of cell death, the response of two cell lines to oxidative stress is different. In C₂C₁₂ cells, only the highest concentration (1 mM) of H₂O₂ causes significant cell death compared with the untreated cells (Figure 4a), while in L₆E₉ cells both concentrations of H₂O₂ (0.5 and 1 mM) cause significantly

more cell death compared with untreated cells (Figure 4b). This may also be due to the fact that L₆E₉ cells are more vulnerable to H₂O₂-induced oxidative stress. When cells were pretreated by IGF-I, the trends of cell death appear to be neutralized in both cell lines (Figures 4a and b) indicating IGF-I can prevent cells from death induced by oxidative stress.

Figure 4 C₂C₁₂ (a) and L₆E₉ (b) skeletal muscle cells were pretreated with different concentrations (0, 50, and 100 ng/ml) of IGF-I for 24 h and then challenged by H₂O₂ at concentrations of (0, 0.25, 0.5, and 1 mM) for 2 h. Dead cell was assessed with YOPRO-iodide (Invitrogen); cell death/survival ratio was calculated according to the method mentioned in the text. All data were represented as mean $\pm$ s.e.m. of six samples ($n=6$). $P<0.05$ was considered as significant and indicated as * $P<0.01$ as higher significance and indicated as ** $P>0.05$ as not significant and marked as NS.

IGF-I Execute Its Cell Protection from H₂O₂-Induced Oxidative Injury Likely via PI3K and ERK1/2 MAPK Pathways, but not via p38 MAPK Pathway

It has been established that the effects of IGF-I are mediated through the PI3K/Akt pathway or MAPK pathway. To determine by which pathway IGF-I executes its protective effect from H₂O₂-induced oxidative stress, a cell permeable, potent and specific PI3K inhibitor (LY294002) was used to examine the relation between the PI3K/Akt pathway and IGF-I protection in muscle cells undergoing oxidative stress. It was found that 1 mM H₂O₂ treatment reduced cell viability significantly and IGF-1 reversed the H₂O₂ effects. However, when cells were pretreated with both IGF-I and LY294002, the protection of IGF-I vanished in both cell lines (Figures 5a and c). Similar to cell survival, IGF-I preventing cells from death induced by H₂O₂ is also mediated by the PI3K/Akt pathway. It can be observed that IGF-I prevents cell from death caused by H₂O₂, while pretreatment by both IGF-I and LY294002 have neutralized IGF-I protection in both cell lines (Figures 5b and d). As previous studies have shown that the PI3K/Akt pathway has conflicting roles under different conditions, to make sure the PI3K/Akt pathway was activated by IGF-I in muscle cells and LY294002 inhibits PI3K activation at the same conditions used in the experiment, both cell lines were treated with IGF-I or IGF-I plus LY294002 for 24 h. The level of the total Akt and phosphor-Akt was determined by western blotting. The results show that the PI3K/Akt pathway can be activated by IGF-I and LY294002 can inhibit PI3K activation in both muscle cell lines under the same conditions in all experiments (Figures 6a–d).

The MAPK pathway is another major signal transduction cascade, which ultimately triggers multiple biological cell responses of IGF-I.¹⁴ To evaluate the role of the MAPK pathways in IGF-I protection cells from oxidative stress, two inhibitors of the MAPK pathway were used. One is SB203580, which specifically inhibits p38 MAPK and has been used to identify the physiological roles and targets of

p38MAPK;¹⁵ another one is the MEK1/2 inhibitor, which selectively inhibits MEK1 and MEK2. Interestingly, the MEK1/2 inhibitor not only markedly neutralized IGF-I protection in both cell lines (Figures 5e–h), but also significantly induced cell death (Figures 5f and h) and decreased cell survival (Figures 5e and g) in both cell lines even if cells have not been challenged by H₂O₂. This confirmed that MEK1 and 2 are major downstream signalling molecules of the IGF pathway and is involved with IGF-I protection for oxidative stress. In contrast to LY294002 and MEK1/2 inhibitors, the addition of SB203580 appears not to inhibit IGF-I protection cell from oxidative stress (Figures 5e–h). Together all these data suggest that IGF-I protection of muscle cells from oxidative damage appears to proceed via the PI3K/Akt and ERK1/2 MAPK pathways, but not proceed via p38 MAPK pathway.

IGF-I Affect Pro- and Pre- Apoptotic Proteins Expression in Muscle Cells

To test whether pretreatment with IGF-I has any effect on apoptotic regulation proteins, the expression level of Bax (a pro-apoptotic protein) and Bcl2 (an anti-apoptotic protein) were examined by western blotting after muscle cells were incubated either with IGF-I alone, or with IGF-I plus PI3K inhibitor or with the p38 MAPK inhibitor. It was found that the ratio of Bcl2 to Bax in IGF-I-treated cells was significantly higher compared with untreated cells (Figures 7a and b) indicating that pretreatment with IGF-I can reduce muscle cell apoptosis. When cells were pretreated with IGF-I and PI3K inhibitor (LY294002) the effect of IGF-I on the ratio of Bcl2 to Bax was neutralized (Figures 7a and b) confirming that IGF-I prevents cell from apoptosis via the PI3K/Akt pathway. If cells were pretreated with IGF-I plus a p38 MAPK inhibitor (SB203580), the effect of IGF-I on the ratio of Bcl2 to Bax was not significantly influenced compared with LY294002 suggesting that the p38 MAPK pathway seems not involved in the protective effect executed by IGF-I.

Figure 5 C₂C₁₂ (a, b, e, f) and L₆E₉ (c, d, g, h) muscle cells were pretreated with IGF-I (100 ng/ml) alone, IGF-I (100 ng/ml) plus LY294002 (20 μ M), IGF-I (100 ng/ml) plus SB203580 (20 μ M), IGF-I (100 ng/ml) plus MEK1/2 (20 μ M) for 24 h and challenged by H₂O₂ (1 mM) for 2 h. Cell viability was determined with CellTiter-Blue reagent (Promega), cell survival (%) (a, c, e, g) was expressed as percentage of viable cell in treated groups to the untreated control group. Dead cell was assessed with YOPRO-iodide (Invitrogen); cell death/survival ratio (b, d, f, h) was calculated according to the method mentioned in the text. All data were represented as mean $\pm$ s.e.m. of six samples ($n = 6$). $P < 0.05$ was considered as significant and indicated as * $P < 0.01$ as higher significance and indicated as ** $P > 0.05$ as not significant and marked as NS.

Figure 6 C₂C₁₂ (a, b) and L₆E₉ (c, d) cells were pretreated by IGF-I (100 ng/ml) alone, IGF-I (100 ng/ml) plus LY294002 (20 μ M) or IGF-I (100 ng/ml) plus SB203580 (20 μ M) for 24 h, cellular Akt and phosphor-Akt proteins were detected by western blotting (b, d) and quantized (a, c) by measuring the density of protein bands. All data were represented as mean $\pm$ s.e.m. of four samples ($n = 4$). The result was represented as the ratio of phosphorylated Akt/Akt (a, c).

DISCUSSION

Oxidative stress has a major role in the pathogenesis of many muscle diseases. The main contributors to oxidative stress in muscle tissue are ROS such as oxygen ions, free radicals, and peroxides. We used exogenous H₂O₂ to induce two types of muscle cell oxidative stress and found that exogenous H₂O₂ decreased muscle cell viability with an increase in caspase-independent apoptosis. IGF-I is able to prevent muscle cells from H₂O₂-induced cell death and recovers cell viability. After further investigation, it was found that it is likely IGF-I executes its cell protection via the PI3K/Akt and ERK1/2 MAPK, but not p38 MAPK pathways.

It has been known that caspases have a central role in most apoptotic cell death, however, in addition to the classic caspase-mediated apoptosis, mammalian cells can also undergo caspase-independent apoptosis,¹⁶ that is mediated by the

Figure 7 C₂C₁₂ cells were pretreated by IGF-I (100 ng/ml) alone, IGF-I (100 ng/ml) plus LY294002 (20 μ M) or IGF-I (100 ng/ml) plus SB203580 (20 μ M) for 24 h. Cellular Bcl2 and Bax proteins were detected by western blotting (b) and quantized (a) by measuring the protein band density. All data were represented as mean $\pm$ s.e.m. of two samples ($n = 2$). The result was represented as the ratio of Bcl2/Bax.

dissipation of the inner Mt membrane potential and the release of apoptosis factors. The caspase-independent apoptosis has been documented in various death models including exposure to some agents that affect protein redox status.¹⁷ Oxidation of redox-sensing inner membrane proteins was found to cause caspase-independent apoptosis in hybridoma T cells.¹⁸ Although H₂O₂ has been found to cause muscle myotube apoptosis,⁷ it is not clear that H₂O₂-induced muscle cell death is what type of apoptosis. As far as we know this is the first report that H₂O₂ induce muscle cell death is a type of caspase-independent apoptosis. It is interesting to note that two types of muscle cells used in the experiment have different sensitivity against H₂O₂ challenge and L₆E₉ cells are more susceptible to H₂O₂ than C₂C₁₂ cells. The reason for diverse responses to oxidative challenge in the two cell lines are not yet known. It is known that the level of IGF-I expression is very low or nonexistent in L₆E₉ cells but the IGF-I receptor is present, while in C₂C₁₂ cells both IGF-I and its receptor are highly expressed.¹⁹ The fact that L₆E₉ cells produce a very low level (if any) of IGF-I could be a reason why L₆E₉ cells are more vulnerable to H₂O₂-induced oxidative stress than C₂C₁₂ cells.

IGF-I is a single-chain peptide growth factor produced primarily by the liver and executes its action mainly through its receptor- insulin-like growth factor I receptor (IGF-IR). It can act as a systemic endocrine hormone or in a paracrine/autocrine manner in target tissues. A recent *in vivo* study has found that IGF-IR is a mediator of cardiac hypertrophy, which involved oxidative stress in cardiac muscle.²⁰ In skeletal muscle IGF-I can increase muscle mass, promote muscle cell proliferation, differentiation, and survival.²¹ Systemic administration of IGF-I or its analog has been shown to improve muscle function and reduce muscle susceptibility to

contraction-mediated damage in dystrophic *mdx* mice.^{22,23} The mechanism by which IGF-I and its analog confer protection from contraction-mediated damage in dystrophic skeletal muscle is not clear.²³ It was found that prolonged IGF-I treatment in *mdx* mice can induce muscle fibers from a fast-twitch phenotype toward the slow-twitch phenotype without a change in fiber cross areas.²² Compared with fast-twitch muscle, slow-twitch muscle fibers are rich in mitochondria,²⁴ have higher oxidative capacities,²⁵ and therefore use ATP in a more efficient way and produce less ROS, which would cause less or no damage to muscle cells. Our finding that IGF-I is able to prevent muscle cell from H₂O₂-induced cell death and recover cell viability may offer a rational explanation for IGF-I conferring protection on dystrophic muscle from contraction-induced damage. It is also interesting to note that this protective effect exists only when cells are pretreated by IGF-I for at least 24 h; if cells are treated with IGF-I and H₂O₂ simultaneously, there is no protection (unpublished data). This indicates that IGF-I executes its protection action through an indirect way.

The effects of IGF-I are mediated mainly through IGF-IR, which has tyrosine kinase activity and signals through the PI3K/Akt pathway and MAPK pathway. There have been a number of studies highlighting the contradictory roles of the PI3K/Akt pathway in cell survival and apoptosis. For instance, it has been previously found that the PI3K/Akt pathway is involved in cell survival and the prevention of apoptosis²⁶ while another study reported that PI3K/Akt pathway activation by IGF-I is not required for cell survival,²⁷ and not involved in the IGF-I anti-apoptotic function against IFN/TNF-induced apoptosis.^{28,29} These contradictory findings raise the possibility of conflicting roles of PI3K/Akt under different conditions. To make sure the PI3K/Akt pathway is activated by IGF-I in muscle cells under the same conditions used in the experiment, both cell lines were treated with IGF-I alone or IGF-I plus LY294002 for 24 h, the level of total Akt and phosphor-Akt was determined by western blotting. It was found that PI3K/Akt pathway was activated by IGF-I in muscle cells under the same conditions used in all experiments and LY294002 can inhibit PI3K activation under the same conditions. Further examination of cell viability and cell death after incubation with LY294002 revealed that IGF-I protects muscle cells from oxidative damage through the activation of PI3K/Akt pathway. Similarly, MAPK pathway is another major signal transduction cascade responding to the IGF-I stimulation. Using MEK1/2 inhibitor and SB203580 to block MAPK pathways, we confirmed MEK1 and 2 are major downstream signalling molecules of IGF pathway and involved with IGF-I protection for oxidative stress. P38 MAPK seems not to be involved in the muscle cell protection shown by IGF-I. It is interesting to note a different response between cell survival and cell death in IGF-I and p38 MAPK inhibitor (SB203580) treatment groups (Figures 5e and f). The difference may be due to the role that p38 has in the myoblast cell cycle. A previous report³⁰ has

shown that p38 has a critical role in the myoblast cell cycle. As p38 MAPK pathway is more implicated with the cell cycle, when it was inhibited, cell may stop proliferation and stay in the arrest state but not undergo apoptosis. As a result, the cell proliferation/survival will be severely disturbed, but cell death would not be affected.

It has been reported that caspase-independent apoptosis results from the protein damage caused by the unbalance of redox status¹⁶ and is linked with Mt membrane permeability.³¹ A recent study using IGF-I to treat old rats has found that IGF-I treatment has led to a reduction of free radical production in the liver and that this action is through a Mt protection.³² Bax and Bcl-2 are two main proteins to mediate Mt membrane permeability.³³ Bax is a pro-apoptotic protein, which forms a trans-membrane pore across the outer Mt membrane, leading to loss of membrane potential and enhances apoptosis. Bcl-2 is an anti-apoptotic protein, which prevents the pore formation and cell death. The ratio of Bcl2 to Bax can be a marker to indicate the level of apoptosis.³³ Our study shows that IGF-I reduces the muscle cell apoptotic level significantly and the PI3K inhibitor neutralise IGF-I action on apoptosis but p38 MAPK inhibitor has not affected apoptotic level. All these data suggest that IGF-I prevents cell from apoptosis through the PI3K/Akt and ERK1/2 MAPK pathways, but not via the p38 MAPK pathway.

Recombinant IGF-I has been produced on a large scale and has been in clinical trials for a variety of pathological conditions including growth failure,³⁴ type I³⁵ and type II diabetes.³⁶ Although there are many studies using IGF-I as a therapeutic agent to treat dystrophic muscle wasting in an animal model, the mechanism by which IGF-I protects cells from oxidative stress is still not clear. This study has offered a rational mechanism by which IGF-I prevents muscle from contract-mediated damage in a dystrophic animal model.

In summary, exogenous H₂O₂ can induce oxidative damage in skeletal muscle cells and cause muscle cell caspase-independent apoptotic death. Pretreatment by IGF-I can protect muscle cells from oxidative stress by increasing the ratio of the anti-apoptotic protein (Bcl 2) to the pro-apoptotic protein (Bax). The protection of IGF-1 seems to be via the PI3K/Akt and ERK1/2 MAPK pathways. Protecting muscle cells from oxidative damage presents a potential application in the treatment of muscle wasting, which occurs in many pathological muscle conditions including DMD and sarcopenia.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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