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Shengmai-san Enhances Antioxidant Potential in C2C12 Myoblasts Through the Induction of Intracellular Glutathione Peroxidase

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Abstract. Cellular and tissue injury associated with reactive oxygen species (ROS) has been reported in many kinds of disorders. While the antioxidant enzymes play critical roles in inhibiting the ROS-mediated injury, glutathione peroxidase (GPx) is scavenging hydroperoxides including H_2O_2. We previously reported that *Shengmai-san* (SMS), a traditional Chinese medicine, prevented ischemia/reperfusion injury of the brain and other organs in rats. To clarify the effect of SMS on intracellular responses of muscle cells against oxidative stress, C2C12 myoblasts were subjected to H_2O_2 abuse. SMS pre-incubation prevented the decreasing cell viability after H_2O_2 treatment. The accumulations of cellular protein carbonyl associated with apoptotic cell death were also inhibited by the SMS pre-incubation prior to oxidative damage induction. At the same time, enhanced activity, protein, and mRNA expression levels of GPx were observed in cells pre-incubated with SMS prior to H_2O_2 abuse. Moreover, intracellular GSH was subsequently decreased after H_2O_2 treatment. These findings suggest that SMS improved the antioxidant capacity against acute oxidative stress through the constitutive enhancement of GPx expression in C2C12 myoblasts. Because of its antioxidative property, SMS might be useful not only for the oxidative damage associated diseases but also for the transplantation of myoblasts into muscular dystrophy patients.

Keywords: oxidative stress, *Shengmai-san*, glutathione peroxidase, skeletal muscle

Introduction

The cell injury associated with reactive oxygen species (ROS) has been implicated in a wide variety of muscle diseases and pathologic conditions (1). Duchenne muscular dystrophy (DMD), a severe progressive muscle disease due to a mutation of the dystrophin gene, and also the mdx mouse, an animal model for DMD, exhibits the absence of the membrane-associated protein dystrophin (2). Besides the functional defect of dystrophin, recent studies have shown that the muscular dystrophy is associated with an increase in oxidative stress (3 – 6). The redox status in the degrading muscles of patients is more oxidative compared to those of other organs. Muscular stem cells including exogenously reconstituted myoblasts with proteins such as dystrophin are expected to have clinical application to muscular dystrophy as an effective cell therapy (7, 8). It will be quite important to enhance the antioxidant potentials of myoblasts or stem cells against oxidative stress conditions, especially for the purpose of regeneration of muscle such as in the cell or gene therapies. Hence, the skeletal muscles, not only matured myofibrils and myotubes but also myoblasts and myogenic stem cells, are clinically awaited to have high capacity against oxidative stress conditions.

Skeletal muscle is susceptible to ROS injury even under physiological condition because there are rapid changes in energy supply and oxygen flux during the contraction (9). Moreover, extreme exercise leads to muscle injury by increased production of ROS (10, 11). Thus, the enhancement of antioxidative potential of skeletal muscles will have an advantage in preparing for the redox imbalance occurring under exercise conditions or in diseases.

Glutathione peroxidase (GPx) plays a critical role to

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inhibit tissue or cellular damages caused by oxidative stress in collaboration with other antioxidants and enzymes (12, 13). By either the Fenton or Harber-Weiss type reactions using GSH as a co-substrate, GPx scavenges hydroperoxides including H_2O_2, which can otherwise produce hydroxyl radicals, one of the most aggressive ROS (14). Therefore, the induction of GPx expression is critical to attenuate cellular antioxidant potential and thus constitutively enhanced GPx expression might be valuable in terms of prevention or management of disease associated with oxidative stress. In this sense, traditional medicine formula used for disease prevention is an interesting target of study because their basic function is to modulate the physiological resistance against exogenous stimuli including oxidative stress through manipulating cellular processes (15).

Recent reports demonstrated that *Shengmai-san* (SMS), a traditional Chinese medicine formula, significantly prevented the oxidative damage in heart, brain, and other tissues (4, 16). We also showed that SMS had high antioxidant potential to prevent cerebral oxidative damages induced by ischemia-reperfusion treatment in experimental animals (17). In the experiments, SMS pretreatment inhibited the GPx activity loss that occurred after the ischemia-reperfusion stress in rats as well as the prevention of protein carbonyl formation and TBARS in many organs.

Here, we demonstrate further mechanisms of the antioxidant effect of SMS that improved antioxidative potential against H_2O_2-induced oxidative stress through increases in the expression of intracellular GPx and its activity in C2C12 myoblasts.

Materials and Methods

Preparation of Shengmai-san (SMS)

The SMS decoction was prepared as described previously (18). The three composite herbs of SMS: *Panax ginseng* (48 g), *Ophiopogon japonicus* (48 g), and *Schisandra chinesis* (24 g) were kindly provided by Kotaro Kanpo Co., Ltd., Osaka; they were kept in a −80°C freezer until used. The specimen numbers of herbs (*Ginseng*: 06100501, *Ophiopogon japonicus*: 06190101, *Schisandra chinesis*: 06020301) for this study were recorded and stored for 10 years at Niigata University of Applied Life Sciences. The herbs were suspended in 1200 ml distilled water, soaked for 1 h, and then boiled for 1 h in each experiment. After the filtration, the extracts were used for the addition to the culture. The concentration of SMS was calculated and indicated as weight per volume (w/v) of dry herb into medium. The quality control of SMS is secured by HPLC analysis with standard solutions of active ingredients (18). To estimate the pharmacological effect of SMS, α-tocopherol (α-Toc), and *N*-acetyl cysteine (NAC) were compared as antioxidant references (positive control) in the present study (Fig. 1C).

Cell culture

C2C12 myoblasts were maintained in Dulbecco's modified Eagle medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma). The cells were seeded in ϕ60-mm dishes (Nalge Nunc Int., Rochester, NY, USA) at a density of 1×10^4 cells/cm^2. All cultures were kept in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Initially, the cells were cultured for 1 day in 3 ml of DMEM, which was supplemented with 100 U/ml penicillin (Invitrogen, Carlsbad, CA, USA), 100 U/ml streptomycin (Invitrogen), and 10% FBS. In the first experiment, C2C12 myoblasts were cultivated for 24 h with or without SMS (Kotaro Kanpo Co., Ltd.). The cultures were washed with 3 ml serum-free DMEM; and subsequently, the culture mediums were replaced with 0.1, 1, 10, and 100 mM of H_2O_2 (Wako Pure Chemical Co., Osaka). Cultured myoblasts dissociated by 1 h after H_2O_2 addition. The control was cultured with 0 mM of H_2O_2 in DMEM. In the second experiment, 1 mM H_2O_2 was used.

Cell viability

Cell viability was determined by the MTT [3,(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide] method as described previously (19). The myoblasts were cultured with or without SMS for 24 h after the pre-incubation in GM for 24 h in 96-well multi-titer plates. Then H_2O_2 was added to the culture and incubated for 30 min. After replacing the medium for 0.05% MTT with GM, the myoblasts were incubated for 4 h at 37°C. The cells were lysed in the lysing buffer [20% SDS, 50% *N*,*N*-dimethyl formamide (DMF), pH 4.7], and the absorbance at 570 nm was measured by a microplate reader.

In situ cell death detection

C2C12 myoblasts were seeded onto a Lab-tech chamber (Nalge Nunc). Cells were fixed for 5 min with cold methanol and then rehydrated with PBS. Slides were rinsed with PBS and incubated in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice. Apoptotic cells were detected by the TUNEL method using the In Situ Cell Death Detection Kit (Roche Molecular Biochemicals, Mannheim, Germany). The cells were examined and photographed under a confocal laser microscope (Bio-Rad, Hercules, CA, USA) after nuclei were counterstained with 1 mM

Propidium iodide (PI, Sigma). At least 500 cells in 6 randomly selected fields were counted for each treatment.

Protein carbonyl

Protein carbonyls were determined in C2C12 myoblasts by measuring the reactivity of carbonyl derivatives with 2,4-dinitrophenylhydrazine (DNPH) as previously described (20). In brief, cells were harvested with ice cold PBS, and the reaction with 10 mM DNPH in 2 N HCl was performed for 1 h at 37°C in a dark place. The aliquoted proteins were washed with ethanol – ethyl acetate (1:1). The precipitated proteins were dissolved in 6 M guanidine at pH 2.5. The carbonyl content was determined by the absorbance at 360 nm with a UV-spectrophotometer.

GPX activity

GPX activity was determined according to the method of Albercht (21). Briefly, an aliquot of cell homogenate in 0.05 M phosphate buffer containing 1.15% (w/v) KCl was mixed in a cuvette with 935 μl of the coupling solution (2.0 mM EDTA-2Na, 1.0 mM NaN_3, 1 mM GSH, 0.2 mM NADPH, and 100 U/ml GSH reductase in 50 mM Tris-HCl, pH 7.6). Kinetic decay of NADPH absorbance at 340 nm was measured after the addition of 25 μl of 1.0 mM H_2O_2 as substrate using a spectrophotometer.

Immunocytochemistry

For immunofluorescence analysis, C2C12 myocblasts were seeded onto a Lab-tech chamber. After the incubation, the cells were fixed for 5 min with cold methanol, rehydrated with PBS, and then incubated in PBS containing 0.05% saponin. Chamber slides were blocked for 15 min in normal goat serum (in PBS) and incubated for 1 h with FITC conjugated antibody (anti GPx-1 Ig; Biogenesis, Inc., NH, USA) for immunofluorescent labeling. For the GPx-1 staining, antibody was diluted 1:200. After extensive washes, nuclei were counterstained with PI. The cells were examined and photographed under a confocal laser microscope (Bio-Rad).

Immunoblotting

Cells were harvested by scraping into ice cold phosphate-buffered saline (PBS), and extracts were prepared by lysis buffer containing 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 0.5% NP-40, 1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride (PMSF), 2 μg/ml pepstatin, 2 μg/ml aprotinin, and *p,p'*-dichlorodiphenyltrichloroethane (DTT). Protein concentrations of extracts were determined by a protein assay kit (Bio-Rad). Cell lysates were applied to 10% SDS polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane. For immunoblotting, the membrane was blocked with 5% skim milk in Tris-buffered saline containing 0.1% Triton X-100 (TBS-T) for 1 h at room temperature. As the primary antibody, anti-GPx-1 rabbit anti serum (N-20; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and anti β-tubulin monoclonal antibody (T4026, Sigma), for even loading, were used. After the incubation with primary antibodies for 16 h at 4°C, the membrane was washed 3 times with TBS-T followed by the incubation with second antibody (Zymed) for 1 h at room temperature. Target proteins were visualized by an ECL reaction kit (Amersham, Piscataway, NJ, USA) and chemiluminescence film (Amersham).

Enzyme-linked immunosorbent assay (ELISA)

GPx expression level was compared by using ELISA. An aliquot of cell lysates was applied to each well of a microtiter plate and incubated at 4°C for 16 h. The plate was washed 5 times with PBS and incubated with TBS (20 mM-Tris, 500 mM-NaCl) solution containing 1% bovine serum albumin for 1 h. Polyclonal antibody against GPx-1 (sheep IgG, Biogenesis) was applied to each well and incubated for 3 h. After washing again, the plate was further incubated for 1 h with goat anti-sheep IgG (Sigma) conjugated with alkaline phosphatase. After washings with PBS, substrate kit (Bio-Rad) solution was added to each well of the plate and the absorbance at 405 nm was determined after the mixtures were allowed to stand for 30 min at 37°C

Reverse transcription polymerase chain reaction (RT-PCR)

To examine the effect of SMS on GPx-1 gene expression, quantitative RT-PCR was performed with the total RNA from the cultured C2C12 myoblasts. Briefly, the total RNA was prepared by the method of acid guadinium-phenol-chloroform (AGPC) (22). For the synthesis of cDNA, the total RNA (1 μg) was reverse-transcribed for 1 h at 42°C in the reaction mixture (20 μl) containing 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 0.5 mM each of dNTPs, 1.25 μg of oligo(dT)$^{12\text{-}18}$, and 500 Units of Superscript II^{TM} (Invitrogen). PCR was performed under the following conditions: 33 cycles amplification with the following sequential process (denatured at 94°C, 30 s; annealed at 55°C, 30 s; extended at 72°C, 1 min) using the primer pairs for GPx-1 (forward: 5'-TCGGTTTC CCGTGCAATCAGTTC-3', reverse: 5'-GAGTGCAGC CAGTAATCACCAAG-3'). The extent of GPx gene expression was normalized against the expression level of a house keeping gene, glyceraldehyde-2-phosphate

dehydrogenase (GAPDH). The PCR amplification of GAPDH mRNA was examined by the RT-PCR kit (Toyobo Co., Ltd., Osaka) with the following primers: forward: 5'-TCCACCACCCTGTTGCTGTA-3', reverse: 5'-ACCACAGTCCATGCCATCAC-3'. The PCR products were separated by agarose gel electrophoresis (1% agarose; Nacalai Tesque, Inc., Kyoto) to confirm the target product of PCR for each reaction. For quantitative evaluation of the gene expression levels, the samples were analyzed by real-time RT-PCR before the products became saturated, and a relative frequency for the expression of GPx gene against GAPDH gene was calculated for standardization.

Cellular GSH levels

GSH level in C2C12 myoblasts was determined by the fluorescence method using monochlorobimane (MBCL) as described (23). C2C12 cells were incubated in a 96 well microtiter plate at the density of 2×10^5 cells per well with or without SMS for 24 h; then the cells were treated with H_2O_2 in serum-free DMEM for 1 h. After the extensive washing with KRB buffer (1.3 mM $CaCl_2$, 1.3 mM $MgSO_4$, 0.3 mM KH_2PO_4, 131 mM NaCl, 5 mM KCl, and 20 mM HEPES, pH 7.4), C2C12 myoblasts were lysed by 0.1% Triton-X100 in KRB buffer for 10 min at room temperature. The formation of the fluorescent MBCL-GSH complex was monitored after the addition of reaction buffer (0.04 U/ml glutathione *S*-transferase and 50 μM MBCL in KRB buffer) at an excitation wavelength of 395 nm and the emission wavelength of 470 nm using a microplate spectrofluorometer (Fluoroskan Ascent, Labsystems, MA, USA). The results were expressed as the percent of MBCL fluorescence relative to the control.

Statistics

Statistical analysis was performed by the General Linear Model procedure of SAS (1995) in conjugation with the Duncan's multiple range tests. The level of statistical significance was taken as $P<0.05$.

Results

Cell viability

The viability of C2C12 myoblasts after oxidative abuse by H_2O_2 was determined by MTT assay. H_2O_2-induced cell death occurred in a dose-dependent manner for the control cultures without SMS pre-incubation (Fig. 1A). In contrast, the cell death by H_2O_2 was alleviated by the pre-treatment of cells with 0.2% (w/v) SMS. Although the cell viability was not significantly different at 0.25 and 4 mM H_2O_2, the cell death at H_2O_2 concentrations of 0.5 – 2 mM was significantly inhibited

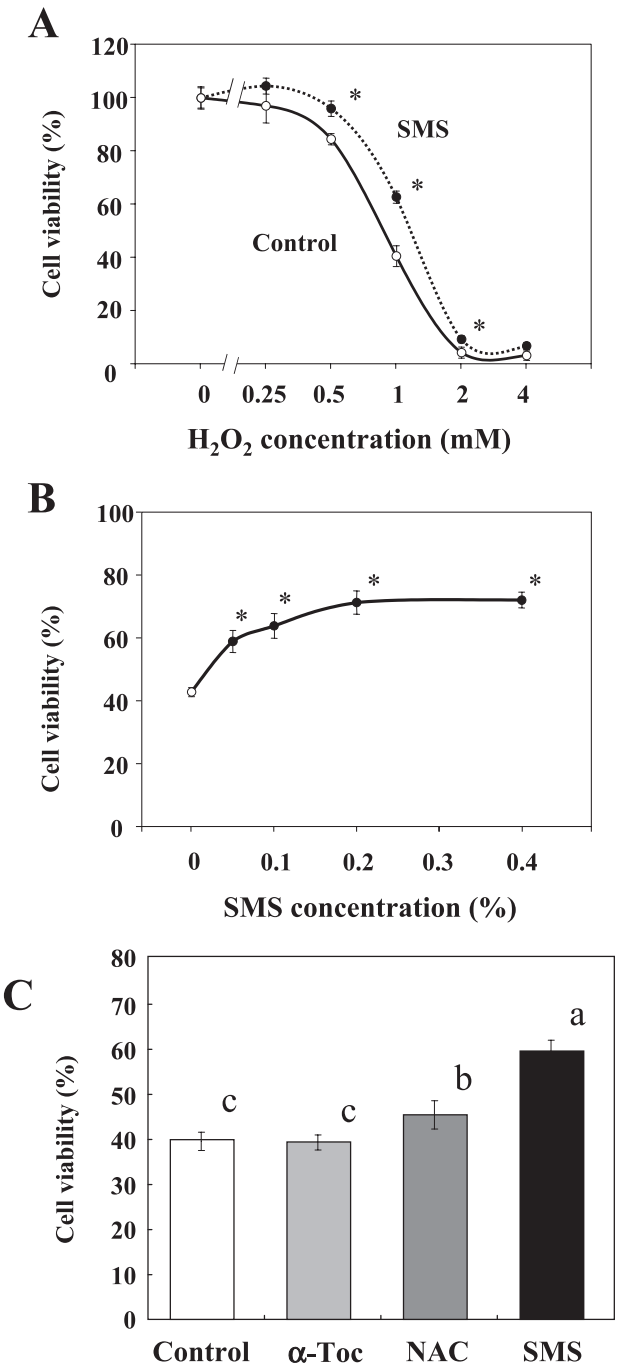

Fig. 1. Cell viability of C2C12 myoblasts. A: Dose-dependent effect of H_2O_2 on cell viability of C2C12 myoblasts. After the pre-incubation with or without SMS (0.2%) for 24 h, myoblasts cultures were treated with different concentrations of H_2O_2 as indicated for 1 h. B: Effect of SMS concentration on cell viability of myoblasts. After the pre-incubation with SMS, at the indicated concentration, myoblasts cultures were treated with 1 mM H_2O_2 for 1 h. Each value represents the mean ± S.D. obtained from quadruplicate at least (*$P<0.05$). C: The efficacy of SMS on cell viability was compared to α-Tocopherol (α-Toc)- and *N*-acetyl cysteine (NAC)-treated cells. After the pre-incubation with SMS (0.2%), α-Toc (10 μM), or NAC (5 mM) for 24 h, cells were treated with H_2O_2 (1 mM) for 24 h. Each value represents the mean ± S.D. obtained from at least quadruplicate determinations ($^{abc}P<0.05$).

by SMS pre-treatment. At 1.0 mM H_2O_2, the viability difference was almost 20%. The preventive effect of SMS on H_2O_2-induced cell death was examined at various concentrations of SMS at fixed H_2O_2 concentration (1.0 mM) (Fig. 1B). Cell viability was recovered significantly with increasing concentrations of SMS from 0.05% – 0.4% and leveled off at 75% of viability at SMS concentrations higher than 0.2% SMS. These results indicate that the antioxidant potential of C2C12 myoblasts was definitely modulated by SMS pre-treatment, and thus the cells were rescued from the death induced by H_2O_2 abuse. In addition, the preventive effect of SMS on H_2O_2 cytotoxicity was compared with α-Toc and NAC, as antioxidant positive controls (Fig. 1C). Although the treatment of NAC significantly attenuated the cell death induced by H_2O_2, the protective potential of SMS was superior to that of NAC, while α-Toc did not have any preventive effect on H_2O_2-induced cytotoxicity in C2C12 cells. Since the recovering effect of SMS on H_2O_2-induced cell death was observed not only in C2C12 myoblasts but also in PC-12 pheochromocytoma, Jurkat lymphoma, and A549 lung carcinoma cells (data not shown), it is suggested that the protective effect of SMS against H_2O_2 stress is a general phenomenon in different cell types.

Apoptotic cell death

It is well known that H_2O_2 derived oxidative stress induces apoptotic cell death in a variety of cell lines and also at tissue level. Here, we examined primary

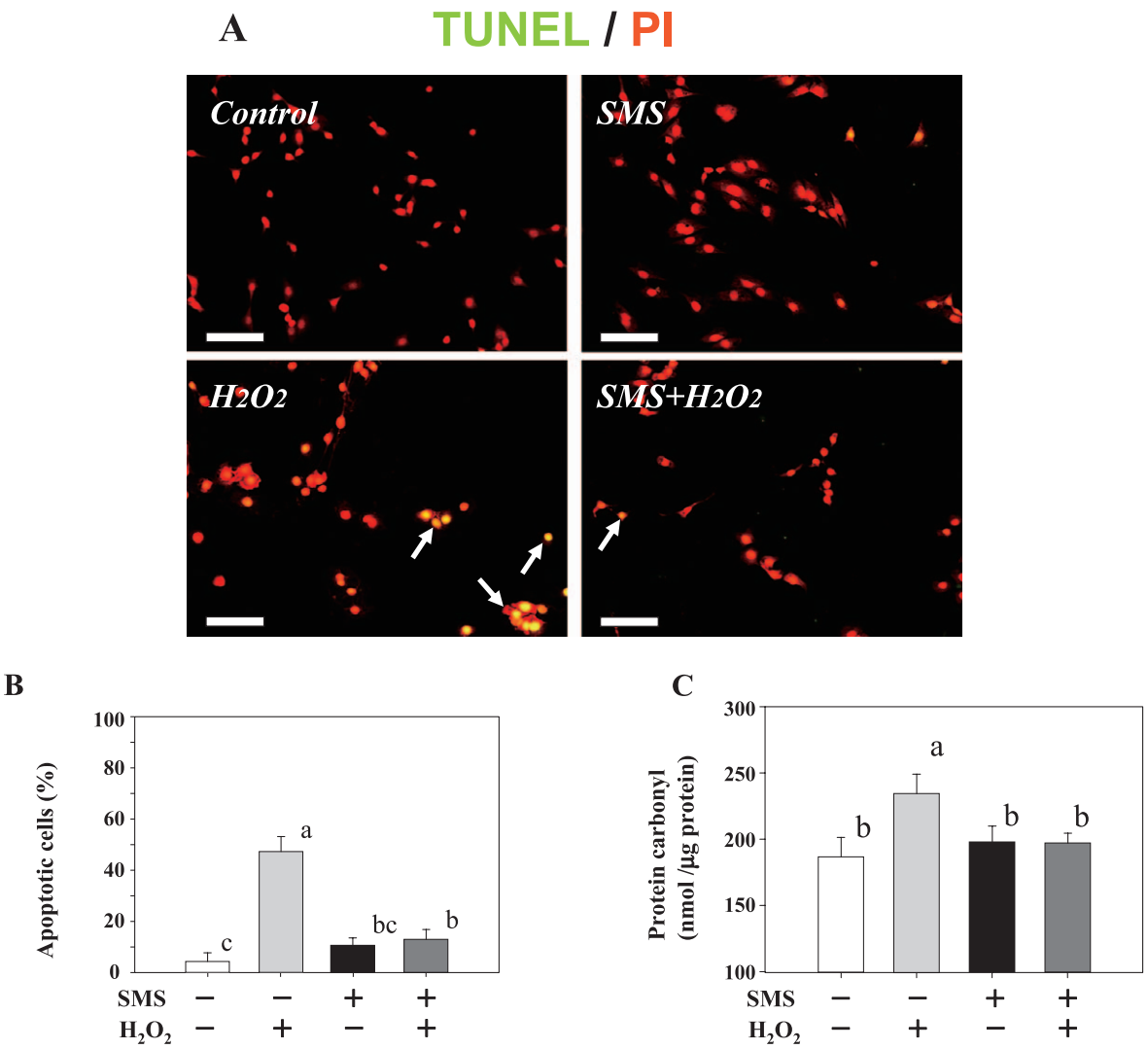

Fig. 2. Apoptotic cell death in C2C12 myoblasts. After the pre-incubation with or without SMS for 24 h, myoblasts cultures in Lab-Tec chamber slides were treated with or without 1 mM H_2O_2. Apoptotic cells were stained by the TUNEL method; subsequently, the cells were observed by a confocal laser microscope (A), and at least 500 cells in randomly selected 6 fields were counted (B). The amount of protein carbonyl was equalized by input of total protein and was determined (C). Each value represents the mean ± S.D. obtained from at least quadruplicate determinations ($^{abc}P<0.05$).

apoptotic cell death by the TUNEL method (Fig. 2A). A few apoptotic cells (5%) were observed even in the control culture, but the percentage of TUNEL-positive myoblasts was markedly increased to 47% after 1-h treatment of H_2O_2 (Fig. 2B). SMS itself slightly increased the apoptotic cell rate (11%) compared to the control culture, but the H_2O_2-induced cell death was significantly suppressed in the SMS–pre-treated myoblasts with the rate decreasing to as low as that of the 24-h SMS (0.2%)–pretreated cells without H_2O_2 abuse. The protective effect of SMS was also observed in the DNA breakage of C2C12 myoblasts after H_2O_2 stress when determined by both the flow cytometric analysis of PI-stained sub-G1 cells and Agarose DNA fragmentation test (data not shown). These results indicated that SMS pre-incubation modified cellular potential against apoptotic cell death induced by H_2O_2.

Protein carbonyl

In order to estimate the extent of protein damage in C2C12 myoblasts after oxidative abuse by H_2O_2, the content of protein carbonyl was measured (Fig. 2C). SMS itself did not alter the protein carbonyl level after the incubation for 24 h compared to the control culture. H_2O_2 abuse for 1 h, however, significantly increased the protein carbonyl contents. H_2O_2-induced protein carbonyl formation was completely suppressed in SMS pre-treated cells. It was thus suggested that pre-incubation with SMS for 24 h modulated cellular antioxidant potential of C2C12 myoblasts so as to resist to oxidative protein damage induced by H_2O_2.

GPx activity

GPx plays a major role in preventing free radical and ROS damages in numerous cells and is known to be expressed under oxidative stress (24, 25). The GPx activity was thus measured in cultures treated with SMS or H_2O_2 or both (Fig. 3). As expected, GPx activity was significantly enhanced after H_2O_2 abuse in C2C12 myoblasts to approximately 135% of the control. The pre-incubation with SMS for 24 h, on the other hand, significantly activated GPx in C2C12 myoblasts to a level 28% higher than that of the control culture. However, no further enhancement of GPx activity occurred in the SMS pre-treated cells even after H_2O_2 treatment for 1 h and the activity level was maintained as low as 26%. It was clearly indicated that the acquired cellular resistance of C2C12 myoblasts against H_2O_2 abuse resulted from the constitutively enhanced GPx activity during the pre-incubation with SMS for 24 h.

Protein expression of GPx

GPx expression at the protein level was further examined by immunohistochemical observation, immunoblot analysis, and ELISA using anti-GPx antibody (Fig. 4: A – D). A constitutive expression of GPx was observed in the control C2C12 myoblasts culture shown by immunohistochemical (Fig. 4A) and immunoblot analysis (Fig. 4: B and C). After the pre-incubation with 0.2% SMS for 24 h, the intracellular GPx protein in C2C12 myoblasts was increased (Fig 4: B and C) and widely distributed to the entire cell body as shown by the increased green fluorescence compared to the control culture (Fig. 4A). ELISA assay showed that the GPx protein was increased gradually with increasing time of pre-incubation with SMS and became saturated after 24 – 48 h of incubation (Fig. 4D). Indeed, the induction of GPx protein by SMS was statistically significant at 24 and 48 h. These data indicate that the enhanced resistance of C2C12 myoblasts against oxidative stress was due to the GPx protein expression manipulated by SMS pre-incubation.

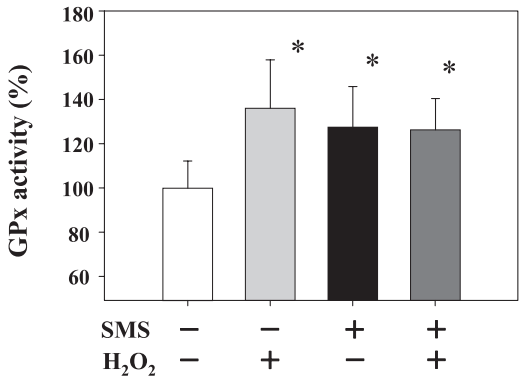

Fig. 3. GPx activity of C2C12 myoblasts. After the pre-incubation with or without SMS for 24 h, myoblasts cultures were treated with or without 1 mM H_2O_2. The percentage of GPX activity was compared to that in the control culture that was pre-incubated without SMS and treated without H_2O_2. Each value represents the mean ± S.D. obtained from at least quadruplicate determinations (*P<0.05).

mRNA expression of GPx

GPx expression in cultured C2C12 myoblasts was further examined at the gene transcriptional level. GPx mRNA expression was detected in all the cultures of C2C12 myoblasts as a PCR product of 0.43 kb by RT-PCR (Fig. 5A), and thus the expression levels were quantified with the band by real-time RT-PCR (Fig. 5B). When C2C12 myoblasts were treated with H_2O_2, GPx mRNA expression was significantly enhanced by 42% compared to the level in control culture. SMS pre-treatment itself also caused weak induction by 23% compared to the control. However, subsequent H_2O_2 abuse to the SMS pre-treated culture for 1 h did not

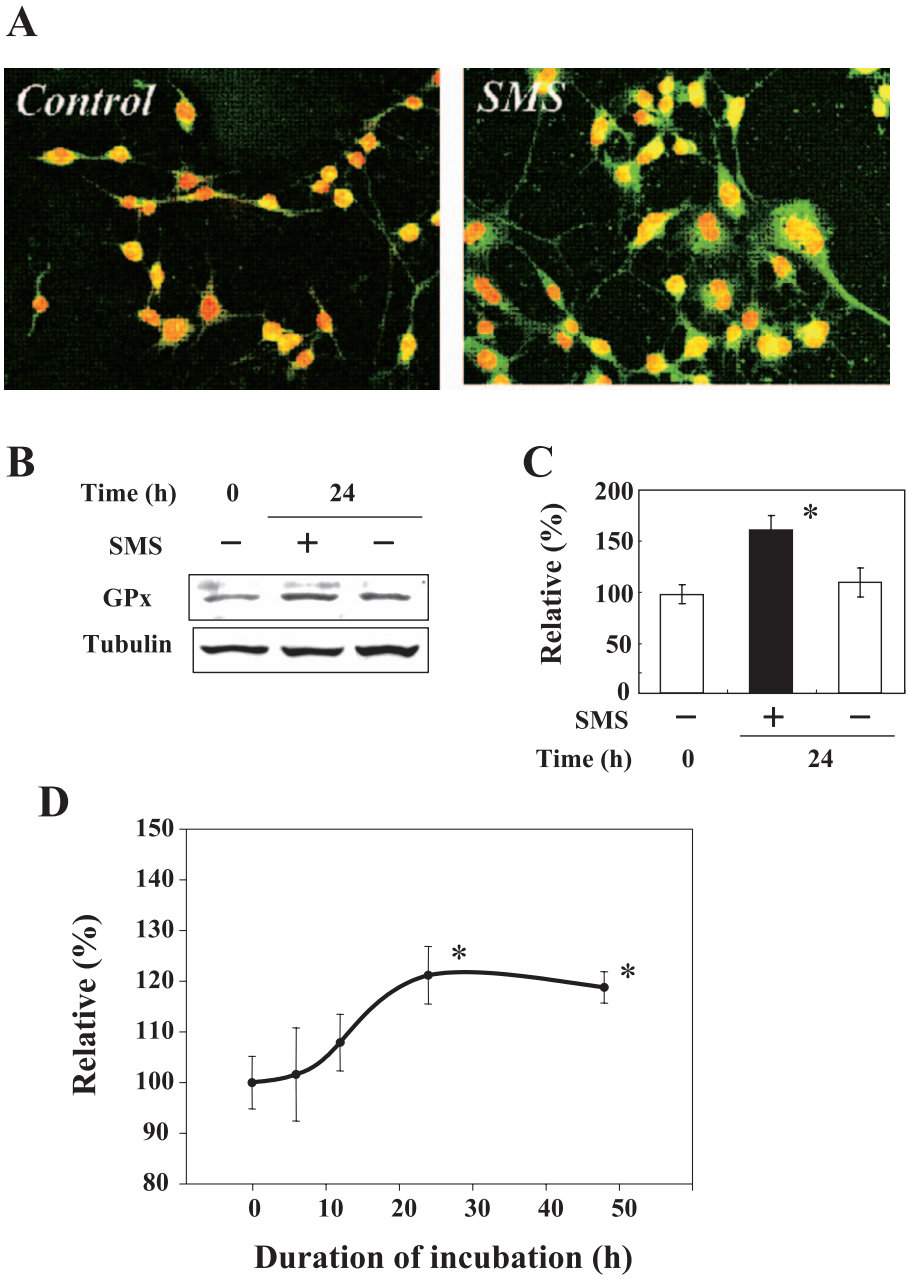

Fig. 4. GPx expression in C2C12 myoblasts. After the pre-incubation with or without SMS for 24 h, myoblasts cultures in Lab-Tec chamber slides were treated with or without 1 mM H_2O_2. Chambers were processed for indirect immunofluorescence assay with anti-GPx antibody and secondary antibody conjugated with FITC and subsequently counterstained with Propidium iodide (PI). Specimens were observed by a confocal laser microscope (A). Intracellular GPx in C2C12 myoblasts was estimated by immunobloting using anti-GPx antibody before and after incubation with SMS (B). The expression level of GPx was digitized by using NIH-image software and standardized by tubulin (C). Equal amount of protein, which was harvested at the indicated time, was applied and determined by the ELISA system using the same antibodies (D). Each value represents the mean ± S.D. obtained from at least quadruplicate determinations (*P<0.05).

induce further increase in the GPx mRNA expression (26%). The increased GPx mRNA expression by H_2O_2 was completely inhibited in SMS treated cells. This result was well consistent with those of the enzyme activity and protein expression profile of GPx (Figs. 3 and 4). Notably, the mRNA expression level of PH-GPx (GPx-3), another subtype of GPx, was also determined but it was not altered under the same conditions (data not shown). This indicates that the improved modulation of the antioxidant potential of C2C12 myoblast by SMS

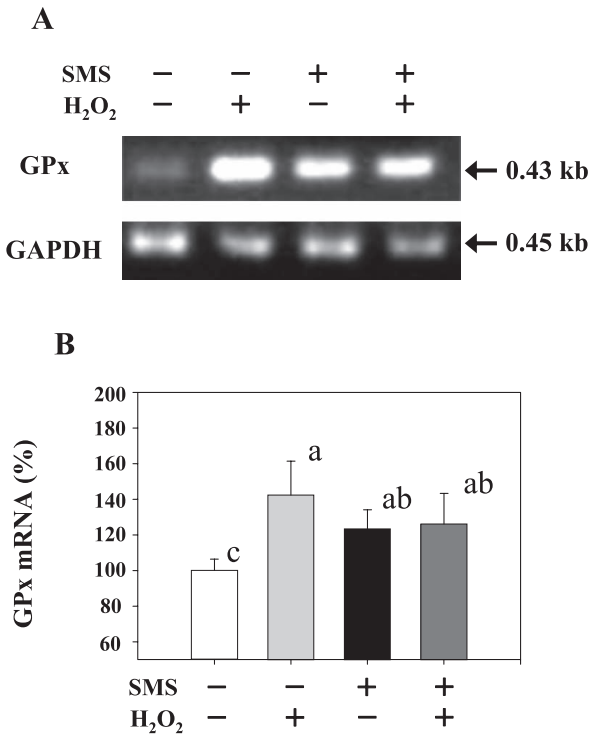

Fig. 5. mRNA expression of GPx in C2C12 myoblasts. GPx mRNA expression was studied by quantitative real-time RT-PCR using total cellular RNA from cultures that were incubated with or without SMS and treated with or without H_2O_2. To equalize input of total RNA, the level of RNA for the constitutively expressed GAPDH was determined (A) and digitized by Smart Cycler system software (B). PCR reaction was performed under conditions of linearity with respect to input RNA. PCR amplification was performed in quadruplicate. Each value represents the mean ± S.D. obtained from at least quadruplicate determinations ($^{abc}P<0.05$).

was due to an increase of cellular GPx at the transcriptional level.

GSH levels

The intracellular GSH level critically reflects cellular redox status. It is expected that GSH consumption is facilitated under the oxidative stress condition in which GPx uses GSH as the substrate to protect cells against free radicals such as OH^-, O_2^-, and $ONOO^-$ (12, 13). We, therefore, determined the intracellular GSH level by the use of MBCL as a fluorescent probe (Fig. 6). Although SMS pre-incubation did not alter intracellular GSH level, it was significantly decreased after treating the cells with 1.0 mM H_2O_2 as expected. GSH level in SMS pre-treated cells was decreased more significantly by additional treatment of cells with 1 mM H_2O_2. The decreased extent of GSH level was even greater than that observed in myoblasts treated with only H_2O_2. This result indicates that the enhanced GPx activity observed in the SMS pre-treated cells facilitated the intracellular

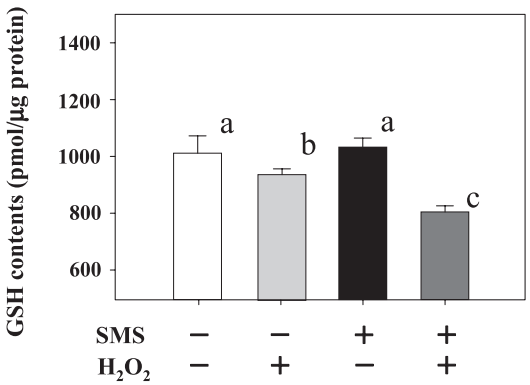

Fig. 6. GSH contents of C2C12 myoblasts. After the pre-incubation with or without SMS for 24 h, myoblast cultures were treated with or without 1 mM H_2O_2. To equalize input of total protein, the cellular protein was determined. Each value represents the mean ± S.D. obtained from at least quadruplicate determinations ($^{abc}P<0.05$).

GSH consumption after H_2O_2 abuse in order to protect the cells from oxidative stress.

Discussion

The muscle disorders such as muscular dystrophy are associated with an increase of oxidative stress (3 – 6). There are many clinical trials proposed for muscular dystrophy treatments such as gene therapy and transplantation of myoblasts (7, 8). On the other hand, skeletal muscle, either normal muscle or myoblast, is susceptible to oxidative stress that can be easily induced in normal muscles and myoblasts through the rapid changes in energy supply and oxygen flux during the contraction (9) or through extreme exercises (10, 11). Therefore, antioxidative protection of cells is one of the basic strategies to control the oxidative muscle injury and its related disease conditions. Here we demonstrated the way to modulate inherent cellular defensive potential against oxidative stress by SMS. SMS is a traditional herbal medicine formula that has been used for more than eight hundred years in China and is comprised of three herbs, *Panax ginseng, Fructus schisandrae*, and *Radix ophipogonis*. SMS is traditionally used for the treatment of excessive loss of essence Qi and body fluid and is currently prescribed for treating coronary heart diseases (26).

We previously showed that the preceding injection of SMS into duodenum to cerebral ischemia-reperfusion in rats prevented the loss of GPx activity, increase of protein carbonyl, and accumulation of lipid peroxide in brain and other tissues and thus finally alleviated the oxidative tissue injury caused by ischemia-reperfusion (17). The modulation and maintenance of tissue GPx activity by SMS treatment suggested that the preventive

effect of SMS was not only due to the radical scavenging activity but also due to modulation of antioxidative cellular response. The present study further revealed that C2C12 myoblasts, when pre-incubated with SMS, acquired the resistance to oxidative stress induced by H_2O_2 treatment; indeed, myoblasts that were preincubated with SMS and then subjected to H_2O_2 at the concentration of 0.5 – 1 mM showed significantly increased cell viability compared to the cells not pretreated with SMS (Fig. 1A). Since the extracellular SMS was washed out before H_2O_2 abuse, the protective effect of SMS on myoblasts against oxidative stress was not due to the direct scavenging of H_2O_2 by SMS in culture media, even though SMS has radical scavenging activity by itself in vitro (26). This is consistent with our previous observation in that DNA damage protection and repair in H_2O_2-treated PC-12 cells was attained by the preceding SMS treatment depending on the pre-incubation period (27). In the preliminary study, the improving effect of SMS was dependent on the length of the pre-incubation period and maximized after 24 h, and then it was constitutively maintained thereafter (data not shown). Thus, the following experiments were conducted with 24-h pre-incubation with SMS and the H_2O_2 concentration of 1 mM. H_2O_2-induced cell death of C2C12 myoblasts was significantly inhibited by SMS treatment, although the recovery of cell viability was saturated with SMS concentration higher than 0.2% (Fig. 1B). The inhibitory effect of SMS on H_2O_2-induced cytotoxicity was superior to that of NAC or α-Toc, known to have an antioxidant activity (28 – 30), treated cells. SMS, therefore, has more ability than the other antioxidants to modulate the antioxidative potential of C2C12 myoblasts against H_2O_2 abuse. The details of this mechanism in the C2C12 myoblasts were precisely examined under the experimental condition with 24-h pre-incubation with 0.2% SMS and 1 mM of H_2O_2 treatment.

It is commonly recognized that GPx plays a major role in the prevention of oxidative stress induced by free radical and ROS using GSH as the co-substrate (12 – 14), while the expression of intracellular GPx is induced in many cell types under the oxidative stress (24, 25). It was also observed in the present study that both the activity and mRNA expression of GPx were significantly enhanced in C2C12 myoblasts by H_2O_2 treatment for 1 h (Figs. 3 and 5), although it was no longer effective on the attenuation of the protein carbonyl, as an index of protein damage (Fig. 2C), and the number of the apoptotic cells (Fig. 2: A and B).

Increase of ROS level occurred under the oxidative stress condition and is sensed by redox-sensitive regulatory molecules such as thioredoxin in the cell, triggering homeostatic responses to prevent cellular injury (31). Franco et al. reported that mRNA expression of antioxidant enzymes was increased in muscle cells under oxidative stress, and the extent was correlated to the level of oxidative injury to cells (24). Chan et al. suggested that the levels and balance of antioxidant enzymes determine the susceptibility of muscle cells against oxidative injury (9). Indeed, it has been reported that the role of GPx is critical and the nerve cells overexpressing GPx showed higher resistance against H_2O_2 injury mediated by amyloid-beta (32, 33). In addition, we previously reported that SMS prevented the cell death induced by amyloid-beta in PC-12 with increasing intracellular GPx induction (18). In the present study, however, the induction of GPx activity by H_2O_2 treatment is not responsible for increasing the antioxidative potential of C2C12 myoblasts in view of the cell viability. It was shown, however, the induced GPx during the pre-incubation with SMS prior to H_2O_2 abuse inhibited oxidative injury and death of myoblasts in a dose-dependent manner (Fig. 1), that is, protein carbonyl accumulation (Fig. 2C) and increasing percentage of apoptotic cells (Fig. 2: A and B) were prevented. These observations were consistent with the results reported in other studies (17, 27) and suggest that SMS modulated the cellular redox status by enhancing the expression of GPx prior to H_2O_2 abuse. Thus, it was considered that the constitutive induction of the cellular GPx is necessary for the defense against the oxidative injury and its accumulation caused by ROS.

We ascertained that the intracellular GSH content was decreased in SMS pre-incubated culture more greatly than in the culture without SMS (Fig. 6). GSH plays a major role in cellular antioxidant defense, and thus the depletion of cellular GSH has been shown to increase the sensitivity of cells towards the toxic effects of ROS (34 – 36). It works not only as a strong free radical scavenger but also as a co-substrate of GPx (37, 38). It is thus indicated that the significant decrease of cellular GSH after H_2O_2 treatment observed in the SMS–pre-incubated myoblast cells resulted from the enhanced expression of GPx during the pre-incubation with SMS for 24 h. Since the *de novo* synthesis of GSH takes several hours (39), these results implicate that improved GPx level by SMS is more critical to suppress the oxidative tissue injury and following apoptotic cell death of C2C12 myoblasts. This also explains the observation that the decreased viability of cells was not effectively attenuated by SMS at the higher H_2O_2 concentration (>2 mM) (Fig. 1A), presumably because the availabilities of total GSH, as co-substrate for GPx and radical scavenger, were limited under the culture condition.

In conclusion, a constitutively enhanced GPx expression by SMS pre-incubation is valuable for protecting myoblasts against the oxidative cell damage. The present study defines the potential benefit of using herbal medicines as a multifunctional formula to treat oxidative stress in muscle and offers a new potent tool to optimize future cell therapy protocols such as treatments for the muscular dystrophies. Although we focused on GPx induction in myoblasts and showed the protective function in oxidative injury of cells in the present study, further study of SMS still remains and the antioxidative modulation in the various kinds of pathways involving other antioxidative enzymes and cellular components that have potentials such as free radical activity needs to be clarified because most of the antioxidative molecules have complex cross-talks in signal transduction pathways during the oxidative stress condition.

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