Free Radical Research, September 2008; 42(9): 798–806

informa
healthcare

Phosphoinositide 3-kinase/Akt signalling is responsible for the differential susceptibility of myoblasts and myotubes to menadione-induced oxidative stress

JEONG A. LIM, JOO HONG WOO, & HYE SUN KIM

Department of Biological Science, College of Natural Sciences, Ajou University, Suwon, 443-749, Korea

Accepted by Dr J. Keller

(Received 20 May 2008; revised 21 August 2008)

Abstract
In this study, it was found that undifferentiated myoblasts were more vulnerable to menadione-induced oxidative stress than differentiated myotubes. Cell death occurred with a relatively low concentration of menadione in myoblasts compared to myotubes. With the same concentration of menadione, the Bcl-2/Bax ratio decreased and nuclei containing condensed chromatin were observed in myoblasts to a greater extent than in myotubes. However, myotubes became increasingly susceptible to menadione when phosphoinositide 3-kinase (PI3-K) was blocked by pre-incubation with LY294002, a PI3-K inhibitor. Actually, PI3-K activity was reduced by menadione in myoblasts but not in myotubes. In addition, the phosphorylation of Akt, a downstream effector of PI3-K, was inhibited in myoblasts by menadione but increased in myotubes. Both LY294002 and API-2, an Akt inhibitor, decreased the Bcl-2/Bax ratio in menadione-exposed myotubes. These results suggest that the differential activity of PI3-K/Akt signalling is responsible for the differential susceptibility of myoblasts and myotubes to menadione-induced oxidative stress.

Keywords: *PI3-K/Akt signalling, oxidative stress, menadione, L6 muscle cell lines, apoptosis, Bcl-2/Bax ratio*

Introduction

Myoblasts, the myogenic precursors of skeletal muscle, proliferate, differentiate into multinucleated myotubes and form new muscle fibres [1]. In the course of myoblast differentiation, various signalling pathways related to the regulation of myogenesis are activated, such as the p38 mitogen-activated protein kinase (p38 MAPK), nuclear factor-κB (NF-κB) and phosphoinositide 3-kinase (PI3-K)/Akt pathways [2,3]. In addition, the expression and accumulation of muscle-specific proteins increase with differentiation [4]. Thus, differentiated myotubes have many biochemical and morphological characteristics that differ from those of undifferentiated myoblasts.

With high concentrations of myoglobin and rapid changes in oxygen flux, skeletal muscles are extremely susceptible to reactive oxygen species (ROS)-induced oxidative stress [5,6]. Menadione (2-methyl-1,4-naphthoquinone, vitamin K3) is a quinone that generates free radicals intracellularly by undergoing redox cycles [7] and has previously been shown to induce oxidative stress in muscle cells by producing superoxide and hydrogen peroxide [8]. However, the intracellular signalling pathway(s) leading to menadione-induced death in muscle cells has not been identified. The regulation of oxidation within the cell plays a critical role in cell survival by modulating key signalling pathways [9]. Indeed, oxidative stress has been shown to stimulate cell survival signalling pathways in addition to causing cell death. The PI3-K/Akt pathway is one such pathway regulated by ROS through activation of this signalling cascade, which

Correspondence: Hye Sun Kim, Department of Biological Science, College of Natural Sciences, Ajou University, Suwon, 443-749, Korea. Tel: +82 31 219 2622. Fax: +82 31 219 1615. Email: hsunkim@ajou.ac.kr

ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760802429021

is critical for cell survival [10]. Akt, also known as protein kinase B, is a serine/threonine kinase that is activated in response to signals that are transmitted through PI3-K [11]. Akt is an important survival signal that suppresses cell death [12] and is activated in response to oxidant injury [13]. Akt is phosphorylated by phosphoinositide-dependent protein kinases (PDKs) on Thr308 or Ser473 [14].

Although activation of the PI3-K/Akt pathway by various stresses has been studied in a variety of cell types, its role against oxidative stress in skeletal muscle cells has not been well-studied. Specifically, the activity of PI3-K/Akt signalling under menadione-induced oxidative stress in skeletal muscle cells has not been reported. The present study examined the differential response of myoblasts and myotubes in the cultured rat L6 skeletal muscle cell lines to menadione-induced oxidative stress. It was found that undifferentiated myoblasts were more vulnerable to the stress than differentiated myotubes. This study demonstrates that the PI3-K/Akt signalling pathway is responsible for the differential susceptibility.

Materials and methods

Materials

Anti-Bcl-2, anti-Bax and anti-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p85 antibody was from Upstate Biotechnology (Lake Placid, NY). Anti-Akt, anti-phospho-Akt (Ser473) and anti-poly (ADP-ribose) polymerase (PARP) antibodies were from Cell Signaling Technology (Beverly, MA). SB203580, NF-κB activation inhibitor (6-amino-4-[4-phenoxyphenylethylamino] quinazoline) and Akt/PKB signalling inhibitor-2 (API-2) were from Calbiochem (La Jolla, CA). DAPI (4′,6-diamidino-2-phenylindole) was from Molecular Probes-Invitrogen (Carlsbad, CA). The silica gel 60 thin-layer chromatography (TLC) plates were from Merck (Darmstadt, Germany). [γ-^{32}P]ATP was from DuPont NEN (Boston, MA). Other materials were obtained from Sigma (St. Louis, MO).

Cell culture and oxidative stress

Undifferentiated myoblasts and differentiated myotubes of L6 cells were prepared as previously described [15]. Briefly, cells cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal bovine serum for 3 days were termed myoblasts. Confluent myoblasts were induced to differentiate by transferring them into medium containing 5% horse serum and culturing them for 7 days. The fused multinucleated cells were called myotubes. To induce oxidative stress, myoblasts and myotubes were pre-incubated in serum-free DMEM for 1 h and then exposed to either menadione or 0.05% DMSO as a vehicle.

Cell viability assay

The cells were grown in 96-well plates under the indicated culture conditions and the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-di-phenyltetrazolium bromide) reagent was added to each well and incubated for 4 h at 37°C. The dark blue formazan crystals were solubilized in DMSO and the absorbance was measured at a wavelength of 570 nm with background subtraction at 655 nm on the ELISA plate reader.

Immunoblotting

Cells were disrupted by ultra-sonication in lysis buffer (50 mM Tris-HCl, pH 7.5 containing 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM EDTA, 10% glycerol, 1 mM PMSF, 1 mM Na_3VO_4, 50 mM NaF and 0.1% protease inhibitor cocktail). Cell extracts were subsequently centrifuged at 15 000 $\times g$ to remove debris. The proteins were separated by SDS-PAGE and subjected to immunoblotting as previously described [15]. The density of the immunoreactive bands was quantified using Scion Image.

PI3-K activity assay

PI3-K activity was determined as previously described [15]. In brief, p85 was immunoprecipitated with anti-p85 antibody and the precipitated enzymes were incubated with 2.5 μg of phosphatidylinositol (PI) and 5 μCi of [γ-^{32}P] ATP for 10 min at room temperature. The phospholipids were separated by TLC and the radiolabelled PI3P products were identified as previously described [16].

DAPI staining

Cells were grown on coverslips and treated with either menadione or DMSO. The cells were fixed with 3.7% formaldehyde for 10 min and then stained with 5 μg/ml DAPI. The cells were then observed by fluorescence microscopy.

Statistical analysis

All averaged data are expressed as mean $\pm$ SEM. The significance of the difference from the respective control for each experiment was assayed using Student's t-test. A p-value of < 0.005 was considered significant.

Results

Undifferentiated myoblasts are more vulnerable to menadione-induced oxidative stress than differentiated myotubes

During the differentiation of skeletal muscle cells, proliferating myoblasts withdraw from the cell cycle and form multinucleated myotubes by spontaneous membrane fusion. Oxidative stress is one of the most

dangerous stresses to skeletal muscle cells. Because many biochemical changes occur during the morphological differentiation of myoblasts, it was hypothesized that the cellular response to oxidative stress may change during the course of myogenesis. To test this hypothesis, undifferentiated myoblasts and differentiated myotubes of rat L6 skeletal muscle cells were prepared and the response of cells under menadione-induced oxidative stress was examined. At relatively low concentrations of menadione (lower than 5 μM), no cytotoxic effects were evident and there were no differences in the responses of myoblasts and myotubes to menadione (Figure 1A). However, myoblasts were greatly damaged when the concentration of menadione increased to ~10 μM. Thereafter, the relative viability sharply decreased with further increases in menadione concentration. In contrast, multinucleated myotubes were more resistant to the same concentration of menadione than myoblasts. Myotubes were also damaged at higher doses, but the relative viability declined gently compared to that of myoblasts. Several experiments revealed that prominent differences in viability between myoblasts and myotubes occurred between 10–20 μM menadione and 2–3 h of exposure; these concentrations and exposure periods were used in subsequent experiments. Differential susceptibility in myoblasts and myotubes was also observed according to incubation times. At 10 μM menadione, the relative viability of myoblasts was less than 50% following 1 h incubation, whereas there were no significant changes in myotubes during the 24 h incubation (Figure 1B). The damaged myoblasts became rounded and detached from the substrate, which resulted in increased vacant areas in the culture dish (Figure 1C). In contrast, the morphology of myotubes did not change with the same concentration of menadione.

We next examined whether the menadione-induced death of myoblasts occurred via an apoptotic pathway by assaying of the expression of Bcl-2 and Bax, DAPI staining and cleavage of PARP. Because menadione-exposed myoblasts detached from the dish bottom, all the cells were harvested in the culture dishes including detached cells. Bcl-2, an anti-apoptotic protein, neutralizes pro-apoptotic effects by forming heterodimers with Bax, a pro-apoptotic protein. Thus, any agent that decreases the Bcl-2/Bax ratio may promote apoptosis [17]. As shown in Figure 2A, expression of Bcl-2 was greatly decreased (0.44) and that of Bax was slightly increased (1.14) by 20 μM menadione in myoblasts. The ratio of Bcl-2 to Bax (0.39) indicated that menadione induced apoptosis in myoblasts. In contrast, neither Bcl-2 nor Bax expression was significantly changed by menadione in myotubes. The ratio of Bcl-2/Bax (1.03) indicated that 20 μM menadione did not induce apoptosis in myotubes. The immunoblotting data are representatives for three independent experiments and the values of the

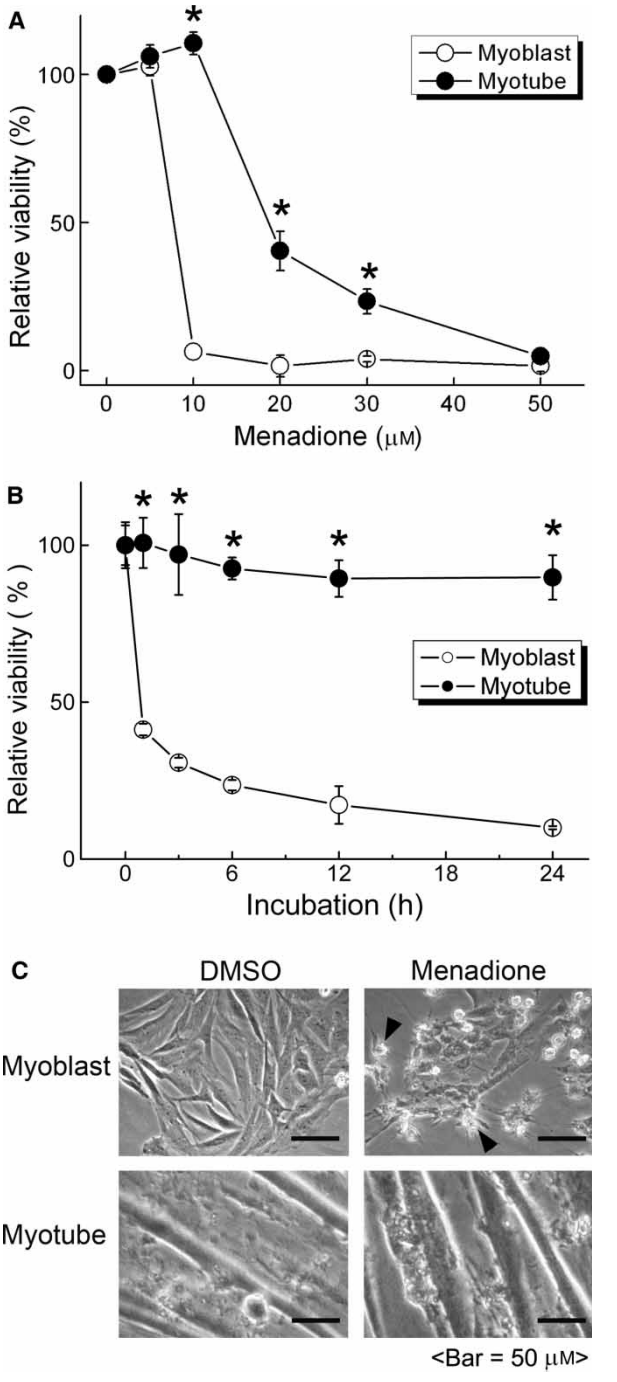

Figure 1. Effect of menadione on the cell survival of L6 myoblasts and myotubes. (A) Myoblasts and myotubes were prepared as described in Materials and methods. They were exposed to increasing doses of menadione and cultured for 24 h. (B) Myoblasts and myotubes were incubated with 10 μM menadione and cultured for the indicated times. Cell viability was assayed by MTT method. Relative viability was expressed as a ratio of viability of each treatment to that of DMSO treatment, a vehicle. Values are mean ± SEM of three separate experiments. The asterisks indicate significant differences from myoblasts (*$p < 0.005$). (C) Both myoblasts and myotubes were exposed to 20 μM menadione for 3 h and observed under a phase-contrast microscope. Arrow heads in the menadione-exposed myoblasts indicate damaged and undetached myoblasts.

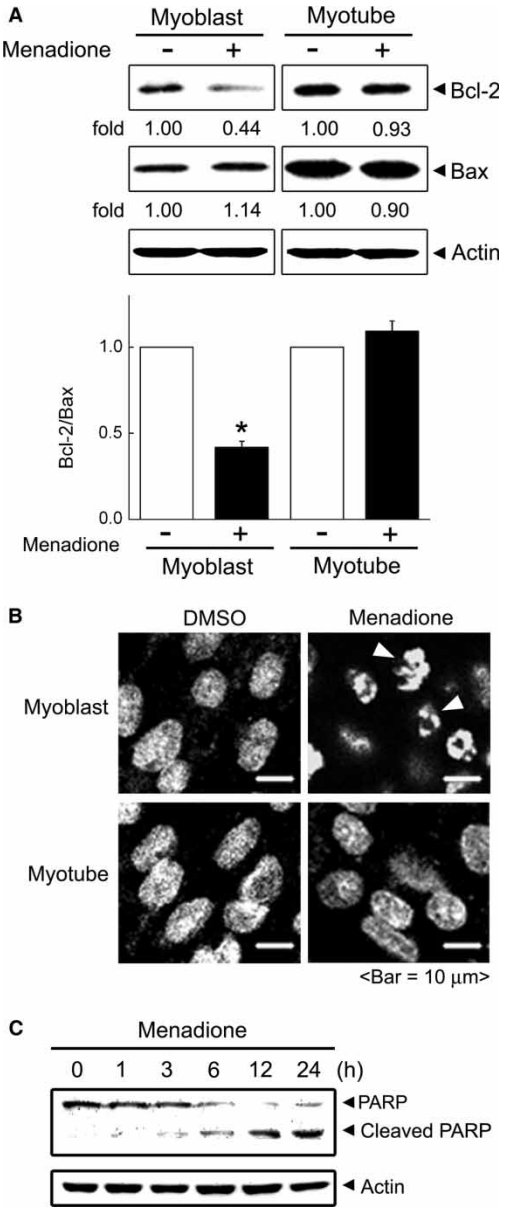

Figure 2. Activation of apoptotic pathway under menadione-induced oxidative stress. (A) Both myoblasts and myotubes were exposed to DMSO (−) or 20 μM menadione (+) for 3 h. The expression levels of Bcl-2 and Bax were determined by immunoblotting with the corresponding antibodies. The band density of DMSO-treated myoblasts and myotubes was defined as 1.00 and the relative intensities of the bands were expressed as folds. Actin was used as a loading control. Relative ratio of Bcl-2/Bax expression of three independent experiments was calculated and shown by graph. Values represent mean±SEM. The asterisk indicates significant difference from DMSO-treated myoblasts (*$p < 0.005$). (B) Both myoblasts and myotubes were exposed to DMSO or 20 μM menadione for 2 h. Cells were stained with DAPI and observed under a fluorescent microscope. Arrow heads indicate condensed nuclei of apoptotic cells in the menadione-exposed myoblasts. (C) Myoblasts were exposed to 20 μM menadione and cultured for the indicated periods. Cleavage of PARP was assayed by immunoblotting with the anti-PARP antibody. Actin was used as a loading control.

graph show the mean of Bcl-2/Bax ratio of the experiments (Figure 2A). On the other hand, the expressions of both Bax and Bcl-2 increased in myotubes even without stress. The level of Bcl-2 indicated 1.55 and that of Bax 2.73 in myotubes compared to myoblasts; therefore, the Bcl-2/Bax ratio in myotubes decreased. These results are consistent with the previous report that apoptosis occurs in the progress of myogenesis and that the expression of both Bcl-2 and Bax increases during differentiation [18]. This study next observed the nuclei of the treated cells by DAPI staining. Chromatin condensation in the nuclei was observed in menadione-treated myoblasts, but not in myotubes (Figure 2B). It has been reported that activation of caspase-3 is required for menadione-induced apoptosis in C2C12 muscle cells [19]. To confirm apoptosis by menadione, the cleavage of PARP, known as endogenous substrate for caspase-3 and a marker for apoptosis [20], was assayed. Cleaved PARP was detected in the menadione-exposed myoblasts after 1 h exposure (Figure 2C). These results clearly show that myoblasts are more vulnerable to menadione-induced oxidative stress than myotubes and that the cell death of myoblasts is related to an activated pro-apoptotic pathway.

PI3-K/Akt signalling is involved in the differential susceptibility of myoblasts and myotubes to menadione-induced oxidative stress

It was hypothesized that the differential susceptibility of myoblasts and myotubes to menadione is related to the differential activity of signalling pathway(s) during the stress. Therefore, this study examined the activity of several signalling pathways that are known to be related to differentiation of myoblasts and to anti-oxidation activity, such as PI3-K, NF-κB and p38 MAPK pathways [2,21,22]. This study did not investigate the activity of C-Jun N-terminal kinase (JNK), one of the stress signalling molecules, because JNK is not activated in the differentiation of L6 myoblasts [2] and the influence of JNK activation on cell survival following oxidative stress is complex and still highly controversial [10]. For these assays, the signalling pathways in myoblasts and myotubes were blocked by pre-incubation with specific inhibitors and then cells were exposed to menadione. The inhibitors used were LY294002, a PI3-K inhibitor, NF-κB activation inhibitor [23,24] and SB203580, a p38 MAPK inhibitor. The effective concentrations of the inhibitors were tested by modification of the manufacturer's recommendation. The inhibitor tests were performed with 10 μM menadione, when myoblasts were damaged but myotubes were not significantly damaged (black bars in Figure 3A and B). When myoblasts were pre-incubated with these inhibitors, there were no significant changes in cell viability between inhibitor-treated cells and non-treated cells

under menadione-induced oxidative stress. In myotubes, however, a prominent change in cell survival was observed in LY294002-treated cells. The relative viability of myotubes pre-incubated with LY294002 was greatly reduced with menadione compared to inhibitor-untreated myotubes. Neither NF-κB activation inhibitor nor SB203580 produced differential effects on cell survival at the tested doses (Figure 3B); menadione-induced damage occurred in myoblasts, but to a lesser extent than in myotubes, regardless of the inhibitors.

To further examine the role of PI3-K in menadione-induced oxidative stress, it was determined whether menadione affected PI3-K activity. Myoblasts and myotubes were exposed to 10 μM menadione and were assayed for phosphorylation of exogenous PI. As shown in Figure 4A, the activity of PI3-K increased ~2-fold in differentiated myotubes compared to that in undifferentiated myoblasts

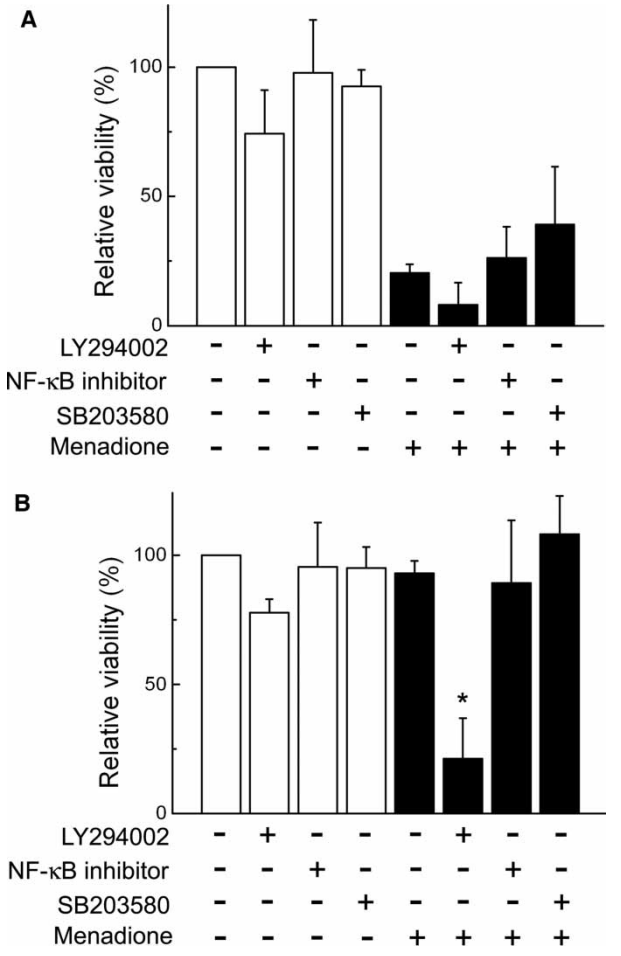

Figure 3. Effect of signalling pathway inhibitors on the cell survival under menadione-induced oxidative stress. Myoblasts (A) and myotubes (B) were pre-incubated with 50 μM LY294002, 50 nM NF-κB activation inhibitor or 20 μM SB203580 for 1 h. Cells were then exposed to 10 μM menadione or not and cultured for the next 24 h. Cell survival was assayed by MTT method. Results represent the average value of three independent experiments. The asterisk indicates a significant decrease from LY294002-non-treated myotubes ($^{\star}p < 0.005$).

at the almost same concentration of p85, the regulatory subunit of PI3-K. Of interest was the finding that its activity was greatly inhibited by menadione in myoblasts (0.26). Under the same stress, however, PI3-K in myotubes remained active to phosphorylate exogenous PI. This study next examined the phosphorylation of Akt, a downstream effector of PI3-K. The phosphorylation of Akt on Ser473 was detected both in myoblasts and myotubes even without menadione (0 h). However, it decreased significantly from 1 h after menadione exposure in myoblasts. In contrast, the phosphorylation in myotubes increased from the early time after menadione exposure (30 min). The protein level of Akt did not significantly change both in myoblasts and myotubes regardless of menadione. Therefore, the ratio of phospho-Akt/Akt decreased with increasing exposure periods in myoblasts, whereas it increased in myotubes (Figure 4B). These results show that the PI3-K/Akt pathway is inhibited in myoblasts by menadione-induced oxidative stress, whereas it is rather activated in myotubes.

Inhibition of PI3-K/Akt signalling results in decrease of Bcl-2/Bax ratio in menadione-exposed myotubes

To further assess the involvement of PI3-K/Akt signalling in menadione-induced oxidative stress, it was examined whether LY294002 and API-2, an Akt inhibitor, influence the expression of Bcl-2 and/or Bax in myotubes. Myotubes were incubated with LY294002 or API-2 for 1 h before menadione exposure. The cells were then exposed to 20 μM menadione and were assayed for Bcl-2 and Bax expression. As shown in Figure 5A, the expression of Bcl-2 greatly decreased and that of Bax increased when myotubes were pre-incubated with LY294002 and then exposed to menadione. Therefore, the ratio of Bcl-2/Bax (0.36) was significantly decreased by menadione in LY294002-pretreated myotubes. Similarly, the expression of Bcl-2 was significantly decreased by API-2 and the ratio of Bcl-2/Bax (0.37) was also decreased (Figure 5B). The immunoblotting data are representatives for four independent experiments and the values of graphs show the mean of Bcl-2/Bax ratio of the experiments. These results show that inhibition of PI3-K/Akt signalling in myotubes results in activation of the apoptotic pathway during menadione-induced oxidative stress.

Discussion

This study investigated the differential susceptibility of cultured undifferentiated myoblasts and differentiated myotubes to menadione-induced oxidative stress. Undifferentiated myoblasts were more vulnerable than differentiated myotubes. Under the stress, the ratio of Bcl-2/Bax was significantly down-regulated in myoblasts, but not in myotubes, which

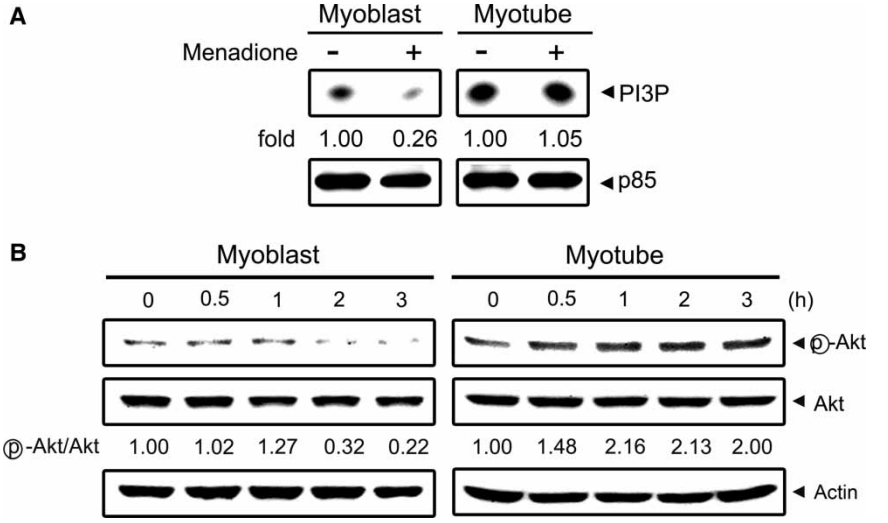

Figure 4. Activity of PI3-K/Akt signalling under menadione-induced oxidative stress. (A) Myoblasts and myotubes were exposed to DMSO (−) or 10 μM menadione (+) for 3 h. Cells were then assayed for PI3-K activity as described in Materials and methods. The intensity of radio-labelled PI3P in DMSO-treated myoblasts and myotubes was defined as 1.00 and the relative intensities of the PI3P were expressed as folds. (B) Myoblasts and myotubes were exposed to 20 μM menadione for the indicated times. Expression and phosphorylation of Akt were assayed by immunoblotting with anti-Akt and anti-phospho-Akt antibodies (Ser473), respectively. Relative intensities of phospho-Akt/Akt ratio were calculated and expressed as folds. Actin was used as a loading control.

indicates that apoptosis occurred in myoblasts. PI3-K/Akt signalling was stimulated in myotubes, but inhibited in myoblasts under the same stress. Here it is demonstrated that the differential activity of PI3-K/Akt signalling is responsible for the differential susceptibility of myoblasts and myotubes to menadione-induced oxidative stress by providing evidence. First, myotubes showed increased susceptibility to menadione when PI3-K was blocked by LY294002 (see Figure 3B). Second, PI3-K activity in myoblasts, but not in myotubes, was significantly blocked by menadione when myoblasts were damaged but myotubes were not (see Figure 4A). Third, with the same concentration of menadione, phosphorylation of Akt decreased shortly after exposure in myoblasts, while it increased in myotubes (see Figure 4B). Finally, LY294002 and API-2 significantly reduced the ratio of Bcl-2/Bax in menadione-exposed myotubes (see Figure 5).

Due to various properties of skeletal muscle cells, these cells are continuously exposed to ROS and they have the capacity to withstand oxidative stress [25]. Several endogenous enzymatic (e.g. superoxide dismutase (SOD), catalase and glutathione peroxidase) and non-enzymatic (e.g. glutathione, vitamins A, C and E and flavonoids) antioxidants constitute well-defined defense systems in muscle cells [10]. The antioxidant abilities of muscle cells appear to vary with cell types, differentiation stages, pro-oxidant molecules and activation level of antioxidant systems. Transcription and activity levels of several antioxidant enzymes, such as catalase and glutathione peroxidase, decreased with differentiation of C2C12 cell lines, which resulted in increased susceptibility to paraquat-induced oxidative stress [25]. In contrast, L6C5 myotubes acquired an apoptosis-resistant phenotype with progress of differentiation, accompanied by drastic increases in NF-κB and activating protein 1 (AP-1) [26]. In the same report, C2C12 cells were found to be more resistant than L6C5 rat skeletal muscle cells to H_2O_2-induced oxidative stress. This study also examined whether menadione influenced the expression of the antioxidant enzymes such as catalase, MnSOD and CuZnSOD in L6 cells. However, the results showed that the expressions of those enzymes were not altered by the treatment of menadione both in myoblasts and myotubes (data not shown).

Although extensive studies on oxidative stress in muscle cells have been completed, the signalling mechanism that leads to activation of antioxidant systems are not fully established and the activities of some signalling pathways remain controversial. It was hypothesized that the differential response of myoblasts and myotubes to the oxidative stress is related to the differential activity of signalling pathway(s) in the course of myoblast differentiation. Previous studies on PI3-K signalling in muscle cells have largely focused on its function in the regulation of myoblast differentiation; several findings have shown that PI3-K/Akt signalling plays an important role in myogenesis. Interfering with PI3-K activity with inhibitors such as LY294002 or wortmannin or using a dominant-negative mutant of p85 blocks myoblast fusion and the expression of muscle-specific proteins [27,28]. In addition, it has been reported that various environmental stresses including oxidative stress are capable of inducing apoptosis leading to the down-regulation of PI3-K/Akt signalling [29,30]. ROS elicit a wide spectrum of responses depending on the cell

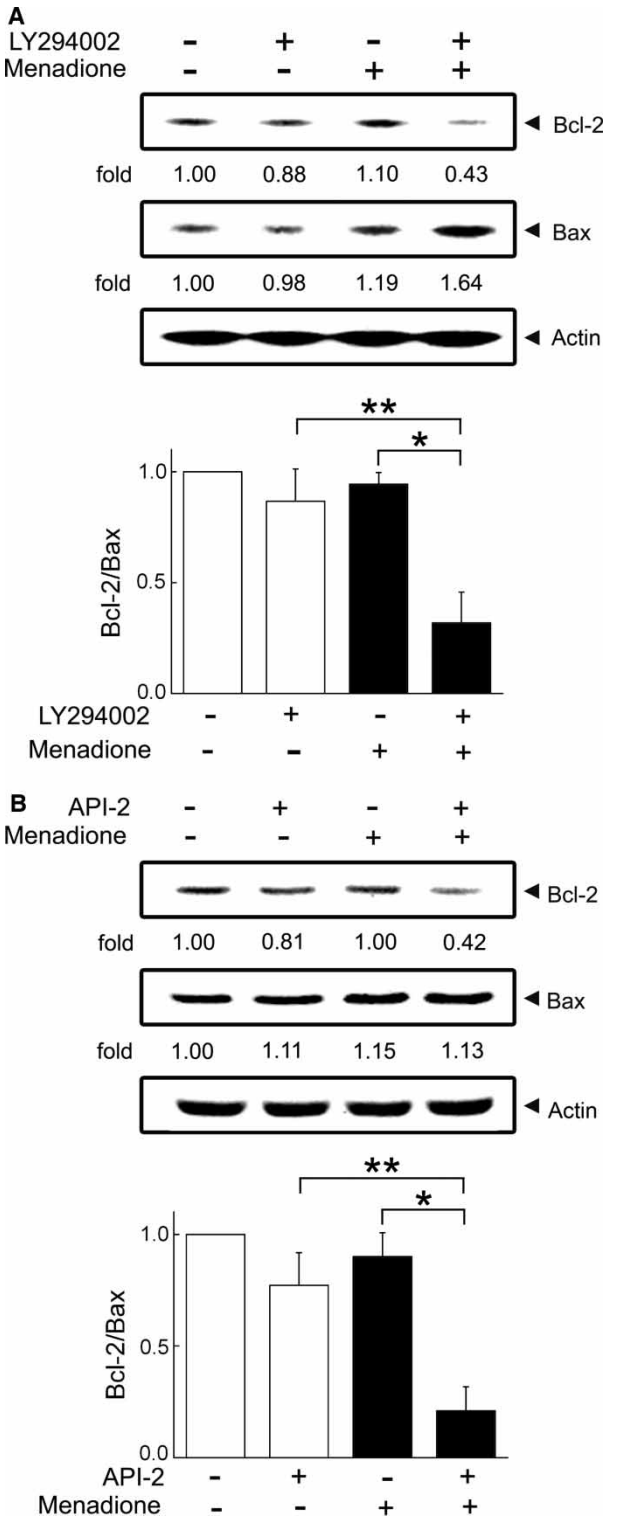

Figure 5. Effect of PI3-K/Akt signalling inhibitors on the expression of Bcl-2 and Bax in the menadione-exposed myotubes. Myotubes were pre-incubated with 50 μM LY294002 (A) or 1 μM API-2 (B) for 1 h and then exposed to 20 μM menadione or not for 3 h. The expression levels of Bcl-2 and Bax were determined by immunoblotting with the corresponding antibodies. Relative ratio of Bcl-2/Bax expression of four independent experiments was calculated and shown by graph. Values represent mean ± SEM. The asterisks indicate significant differences ($^{*}p < 0.005$, $^{**}p < 0.005$).

type, the magnitude of the dose and duration of the exposure via activation of numerous signalling pathways [10]. For instance, NF-κB and p38 MAPK pathways are activated by H_2O_2 in C2 myoblasts at specific doses and exposure periods [22]. However, involvement of those pathways in differentiated myotubes was not assayed. Because this study focused on the differential response of myoblasts and myotubes to the menadione-induced oxidative stress, the effect of menadione was assayed at the doses in which myoblasts were damaged but myotubes were not. Under the conditions, differential activity of the PI3-K/Akt signalling pathway is involved in the differential susceptibility between myoblasts and myotubes. Therefore, one cannot fully exclude the involvement of other pathways under the oxidative stress; menadione may modulate activities of those pathways under certain conditions.

It is reported that age-related down-regulation of PI3-K/Akt is responsible for the increased susceptibility to menadione-induced oxidative stress in the rat kidney [31]. Then how does menadione influence the PI3-K/Akt pathway? This study suggests a possibility that ROS produced by menadione in muscle cells modulate the pathway. Menadione produces ROS including superoxide, H_2O_2 and others in muscle cells [8]. Activation of PI3-K/Akt pathway by superoxide and H_2O_2 is reported in C2C12 muscle cells [32] and in vascular smooth muscle cells [33]. Activation of Akt depends on its phosphorylation on Ser473 and Thr308 [11]. Akt activity is critical for cell survival. There are multiple downstream effectors of Akt in the survival effect. Akt has been shown to phosphorylate the pro-apoptotic protein Bad, thereby inhibiting its pro-apoptotic function [11,34,35]. Activation of Akt alters the expression of Bcl-2 and Bax, which results in inhibition of nitric oxide-induced apoptosis in the hippocampal neurons [36]. Even not examined in this study, the promoter region of Bcl-2 contains a cAMP-response element (CRE) site and the transcription factor CRE-binding protein (CREB) has been identified as a positive regulator of Bcl-2 expression [37,38]. Taken together, these results suggest that a survival signalling in myotubes under the menadione-induced oxidative is modulated via PI3-K-Akt-Bcl-2.

Menadione-induced death in several cancers is called autoschizis [39]. During this process, both apoptotic and necrotic morphologies are observed. The effect of menadione on C2C12 mouse skeletal muscle cells differs by dose: low concentrations induce apoptosis, while higher concentrations result in both apoptosis and necrosis [19]. Here, it is shown that relatively low doses of menadione induce apoptosis in L6 myoblasts. Menadione is used as an anticancer drug; the treatment of malignant cells in culture with menadione inhibits the cell cycle at the G_1/S and S/G_2 phases [40]. Both *in vivo* and *in vitro*

studies have shown a synergistic effect when menadione is combined with conventional chemotherapeutic agents [40]. Recently, non-selective PI3-K inhibitors, like wortmannin and LY294002, have also been proposed as anti-cancer drugs [41]. This study has shown that non-proliferating myotubes, as well as the actively proliferating myoblasts, can be damaged by menadione if the PI3-K/Akt pathway is also inhibited. These results suggest that the combined use of menadione and LY294002 could unexpectedly damage even non-malignant and non-proliferating cells.

In summary, these results of differential susceptibility in myoblasts and myotubes suggest a signalling mechanism for menadione-induced oxidative stress in cultured skeletal muscle cells. Differential activity of PI3-K/Akt against menadione-induced oxidative stress is responsible for the differential susceptibility in undifferentiated myoblasts and differentiated myotubes. The resistance of PI3-K/Akt activity to menadione sustained the expression ratio of Bcl-2/Bax, which resulted in increased survival in myotubes compared to myoblasts.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Jejurikar SS, Kuzon WM Jr. Satellite cell depletion in degenerative skeletal muscle. Apoptosis 2003;8:573–578.

[2] Sarker KP, Lee KY. L6 myoblast differentiation is modulated by Cdk5 via the PI3K-AKT-p70S6K signaling pathway. Oncogene 2004;23:6064–6070.

[3] Conejo R, Valverde AM, Benito M, Lorenzo M. Insulin produces myogenesis in C2C12 myoblasts by induction of NF-kappaB and downregulation of AP-1 activities. J Cell Physiol 2001;186:82–94.

[4] Pramanik SK, Bag J. Expression of muscle-specific proteins is necessary to regulate translation of the mRNA for a 40-kDa housekeeping polypeptide in rat L6 cells. Eur J Biochem 1989;182:687–698.

[5] Ostdal H, Skibsted LH, Andersen HJ. Formation of long-lived protein radicals in the reaction between H2O2-activated metmyoglobin and other proteins. Free Radic Biol Med 1997;23:754–761.

[6] Haycock JW, Jones P, Harris JB, Mantle D. Differential susceptibility of human skeletal muscle proteins to free radical induced oxidative damage: a histochemical, immunocytochemical and electron microscopical study *in vitro*. Acta Neuropathol 1996;92:331–340.

[7] Thor H, Smith MT, Hartzell P, Bellomo G, Jewell SA, Orrenius S. The metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes. A study of the implications of oxidative stress in intact cells. J Biol Chem 1982;257:12419–12425.

[8] Sun JS, Shieh KM, Chiang HC, Sheu SY, Hang YS, Lu FJ, Tsuang YH. Scavenging effect of benzophenones on the oxidative stress of skeletal muscle cells. Free Radic Biol Med 1999;26:1100–1107.

[9] Finkel T. Oxygen radicals and signaling. Curr Opin Cell Biol 1998;10:248–253.

[10] Martindale JL, Holbrook NJ. Cellular response to oxidative stress: signaling for suicide and survival. J Cell Physiol 2002;192:1–15.

[11] Kandel ES, Hay N. The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. Exp Cell Res 1999;253:210–229.

[12] Lee BH, Ruoslahti E. alpha5beta1 integrin stimulates Bcl-2 expression and cell survival through Akt, focal adhesion kinase, and Ca2+/calmodulin-dependent protein kinase IV. J Cell Biochem 2005;95:1214–1223.

[13] Huang C, Li J, Ding M, Leonard SS, Wang L, Castranova V, Vallyathan V, Shi X. UV Induces phosphorylation of protein kinase B (Akt) at Ser-473 and Thr-308 in mouse epidermal Cl 41 cells through hydrogen peroxide. J Biol Chem 2001;276:40234–40240.

[14] Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, Hemmings BA. Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J 1996;15:6541–6551.

[15] Woo JH, Kim JH, Inhee MJ, Kim HS. Lipid products of phosphoinositide 3-kinase abrogate genistein-induced fusion inhibition in myoblasts. Eur J Pharm 2006;529:84–94.

[16] Giorgino F, Pedrini MT, Matera L, Smith RJ. Specific increase in p85alpha expression in response to dexamethasone is associated with inhibition of insulin-like growth factor-I stimulated phosphatidylinositol 3-kinase activity in cultured muscle cells. J Biol Chem 1997;272:7455–7463.

[17] Fukamachi Y, Karasaki Y, Sugiura T, Itoh H, Abe T, Yamamura K, Higashi K. Zinc suppresses apoptosis of U937 cells induced by hydrogen peroxide through an increase of the Bcl-2/Bax ratio. Biochem Biophys Res Commun 1998;246:364–369.

[18] Dominov JA, Houlihan-Kawamoto CA, Swap CJ, Miller JB. Pro- and anti-apoptotic members of the Bcl-2 family in skeletal muscle: a distinct role for Bcl-2 in later stages of myogenesis. Dev Dyn 2001;220:18–26.

[19] Chiou TJ, Chu ST, Tzeng WF. Protection of cells from menadione-induced apoptosis by inhibition of lipid peroxidation. Toxicology 2003;191:77–88.

[20] Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS, Dixit VM. Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell 1995;81:801–809.

[21] Wang X, McCullough KD, Franke TF, Holbrook NJ. Epidermal growth factor receptor-dependent Akt activation by oxidative stress enhances cell survival. J Biol Chem 2000;275:14624–14631.

[22] Kefaloyianni E, Gaitanaki C, Beis I. ERK1/2 and p38-MAPK signalling pathways, through MSK1, are involved in NF-kappaB transactivation during oxidative stress in skeletal myoblasts. Cell Signal 2006;18:2238–2251.

[23] Tobe M, Isobe Y, Tomizawa H, Nagasaki T, Takahashi H, Fukazawa T, Hayashi H. Discovery of quinazolines as a novel structural class of potent inhibitors of NF-kappa B activation. Bioorg Med Chem 2003;11:383–391.

[24] Jack GD, Garst JF, Cabrera MC, DeSantis AM, Slaughter SM, Jervis J, Brooks AI, Potts M, Helm RF. Long term metabolic arrest and recovery of HEK293 spheroids involves NF-kappaB signaling and sustained JNK activation. J Cell Physiol 2006;206:526–536.

[25] Franco AA, Odom RS, Rando TA. Regulation of antioxidant enzyme gene expression in response to oxidative stress and during differentiation of mouse skeletal muscle. Free Radic Biol Med 1999;27:1122–1132.

[26] Catani MV, Savini I, Duranti G, Caporossi D, Ceci R, Sabatini S, Avigliano L. Nuclear factor kappaB and activating

protein 1 are involved in differentiation-related resistance to oxidative stress in skeletal muscle cells. Free Radic Biol Med 2004;37:1024–1036.
[27] Coolican SA, Samuel DS, Ewton DZ, McWade FJ, Florini JR. The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. J Biol Chem 1997;272:6653–6662.
[28] Jiang BH, Aoki M, Zheng JZ, Li J, Vogt PK. Myogenic signaling of phosphatidylinositol 3-kinase requires the serine-threonine kinase Akt/protein kinase B. Proc Natl Acad Sci USA 1999;96:2077–2081.
[29] Meier R, Thelen M, Hemmings BA. Inactivation and dephosphorylation of protein kinase Balpha (PKBalpha) promoted by hyperosmotic stress. EMBO J 1998;17:7294–7303.
[30] Zundel W, Giaccia A. Inhibition of the anti-apoptotic PI(3)K/Akt/Bad pathway by stress. Genes Dev 1998;12:1941–1946.
[31] Jin Q, Jhun BS, Lee SH, Lee J, Pi Y, Cho YH, Baik HH, Kang I. Differential regulation of phosphatidylinositol 3-kinase/Akt, mitogen-activated protein kinase, and AMP-activated protein kinase pathways during menadione-induced oxidative stress in the kidney of young and old rats. Biochem Biophys Res Commun 2004;315:555–561.
[32] Kosmidou I, Xagorari A, Roussos C, Papapetropoulos A. Reactive oxygen species stimulate VEGF production from C(2)C(12) skeletal myotubes through a PI3K/Akt pathway. Am J Physiol Lung Cell Mol Physiol 2001;280:L585–L592.
[33] Ushio-Fukai M, Alexander RW, Akers M, Yin Q, Fujio Y, Walsh K, Griendling KK. Reactive oxygen species mediate the activation of Akt/protein kinase B by angiotensin II in vascular smooth muscle cells. J Biol Chem 1999;274:22699–22704.

This paper was first published online on iFirst on 6 October 2008.

[34] Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 1997;91:231–241.
[35] del Peso L, Gonzalez-Garcia M, Page C, Herrera R, Nunez G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science 1997;278:687–689.
[36] Matsuzaki H, Tamatani M, Mitsuda N, Namikawa K, Kiyama H, Miyake S, Tohyama M. Activation of Akt kinase inhibits apoptosis and changes in Bcl-2 and Bax expression induced by nitric oxide in primary hippocampal neurons. J Neurochem 1999;73:2037–2046.
[37] Pugazhenthi S, Miller E, Sable C, Young P, Heidenreich KA, Boxer LM, Reusch JE. Insulin-like growth factor-I induces bcl-2 promoter through the transcription factor cAMP-response element-binding protein. J Biol Chem 1999;274:27529–27535.
[38] Wilson BE, Mochon E, Boxer LM. Induction of bcl-2 expression by phosphorylated CREB proteins during B-cell activation and rescue from apoptosis. Mol Cell Biol 1996;16:5546–5556.
[39] Gilloteaux J, Jamison JM, Neal DR, Summers JL. Cell death by autoschizis in TRAMP prostate carcinoma cells as a result of treatment by ascorbate: menadione combination. Ultrastruct Pathol 2005;29:221–235.
[40] Lamson DW, Plaza SM. The anticancer effects of vitamin K. Altern Med Rev 2003;8:303–318.
[41] Stein RC. Prospects for phosphoinositide 3-kinase inhibition as a cancer treatment. Endocrine-Related Cancer 2001;8:237–248.