Effect of Quercetin and DMSO on Skeletal Myogenesis from C2C12 Skeletal Muscle Cells with Special Reference to PKB/*Akt* Activity, Myogenin and Bcl-2 Expression

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Abstract

Conflicting data regarding the effect of antioxidants on skeletal myogenesis prompted us to study the action of superoxide anion and hydroxyl radical scavengers on differentiating murine C2C12 myoblasts. The onset of myotube formation was delayed by quercetin and DMSO while DNA synthesis was stimulated in response to elevated doses of both factors. Cell viability measured by MTT assay was inhibited either by 100 μM quercetin or 1% or 2% of DMSO whereas elevated number of apoptotic cells was detected at the same time. Muscle cell differentiation retarded by quercetin or DMSO was reflected by delay in myogenin expression and lowered distribution of myotubes with low (< 10) number of cell myonuclei. For large myotubes (> 10) low scores for DMSO, and high scores for quercetin were observed. Based on phosphorylation status, both antioxidants delayed PKB activation and PKB-dependent differentiation, as well as antiapoptotic effect of PKB. Bcl-2 antiapoptotic protein level was elevated earlier for control than for experimental treatment. Muscle creatine kinase activity reflected the reduced rate of myogenesis. In conclusion, promitogenic activity of quercetin and DMSO disturbs differentiation programme of myoblasts and might explain why more apoptotic cells was found after high doses of both factors. In contrast to DMSO, quercetin-induced delay in myogenesis may result in larger muscle mass. Results of this study support the idea that muscle differentiation can be regulated by scavengers of superoxide anion and hydroxyl radical.

Key words: antioxidants, apoptosis, Bcl-2, muscle differentiation, myogenin, PKB.

Basic Appl Myol 11 (1): 31-44, 2001

Muscle growth results from hypertrophying myofibers, which increase their myonuclear number by recruitment of satellite cells. Determined myoblasts, under conditions that favour differentiation, align and fuse to form multinucleated myotubes [44]. The mechanisms underlying the recruitment of satellite cells to hyperplastic or hypertrophic growth have not been established. It is widely accepted, that high proliferation rate delays the myogenesis and results in higher muscle mass [8, 13, 37]. The higher the rate of cell multiplication the higher the mass of growing muscle. Some growth factors and oncogenes as well as agents present intracellularly or in the extracellular matrix (ECM) also can repress the activity of myogenin and subsequently inhibit myogenesis [5, 31, 51]. However, there is also evidence that accelerated mitogenesis in the circumstances the promote enhanced muscle differentiation impairs the survival of muscle precursor cells [53, 54]. With regard to transduction of signals carrying orders to survive, proliferate or differentiate attention is put on protein kinase B a product of *Akt* gene (PKB/*Akt*). PKB protects against cell death either through downstream phosphorylation of Bad protein, subsequent release of BclXL or Bcl-2 from heterodimers with Bad, sequestration of Bax forming heterodimers with BclXL or Bcl-2, and consequent inhibition of pore formation in the mitochondrial membrane which unable the release of cytochrome C (cyt C) and apoptosis inducing factor (AIF)[36] or by phosphorylation-dependent inactivation of caspase 9 [15]. Two potent antagonists of reactive oxygen species (ROS) action in muscle cells, namely, quercetin and DMSO were evaluated in order to find the importance of these antioxidants to myogenesis. Quercetin biological activities are pleiotropic, opposite to

ROS, and include cell specific inhibition of ubiquitous enzymes (xanthine oxidase, tyrosine kinases, ornithine decarboxylase (ODC), PI-3K, MAPK-s, calmodulin (CaM), lipooxygenase (LOX) and cyclooxygenase (COX) [10, 16, 17, 56]. The role of antioxidants in cell transformation is uncertain, although several groups of authors have reported that, for instance, once activated by specific stimuli (inflammatory cytokines, ROS) hepatic stellate cells differentiate into myofibroblast-like cells, while antioxidants including quercetin inactivate this process [22, 23]. DMSO was well recognized for its ability to modulate the expression of various genes negatively [7, 32] or positively inducing muscle cell differentiation [2, 42]. However, up to date no data are available to elucidate quercetin biological activity in relation to muscle gene expression. According to studies on digestion and absorption, quercetin is the main dietary flavonoid consumed by Caucasians, so its relevance for muscle development would be of great value [11]. In rats fed diet enriched with 0.5% w/w blood plasma quercetin may rise up to 100 μM [29], but in human beings who consume approximately 50-80 mg of quercetin per day, the plasma level generally does not exceed 1 μM [20].

Another equally important issue is the understanding of abrogated myogenesis brought about by DMSO. Munoz-Canoves et al. [34] showed that DMSO inhibited myogenesis in cultured C2C12 myoblasts by repressing urokinase type plasminogen activator (uPA) gene expression. Unfortunately, studies of Munoz-Canoves et al. [34] were not verified by the examination of cell survival. DMSO is a skin permeable popular vehicle and compound used by body builders for topical treatment of the muscle pain and stiffness. It is also present in the anti-inflammatory and anti-rheumatoid medicaments. The issue whether and how DMSO influences muscle cell differentiation is a therefore a merit of considerable importance.

Moreover, with regard to muscle cell differentiation it should be pointed out that delayed myogenesis with extended period of cell multiplication but not apoptosis might be a characteristic feature of increased muscle mass. The aim of this paper was to provide a description of the relation between the cell growth, formation of myotubes, cell viability and apoptosis of cultured murine C2C12 muscle cells. Concomitantly, we scrutinized the expression of myogenin, Bcl-2 protein, PKB and MCK activity during differentiation. In this study we demonstrated that antioxidants quercetin and DMSO delay the expression of molecular and metabolic markers of differentiation. The results of our study suggest that superoxide anion (quercetin) and hydroxyl radical (DMSO) scavengers retard the onset of muscle cell fusion but, at least, in the case of quercetin this effect was transient and once initiated myogenesis was significantly accelerated by quercetin (larger myotubes). Both agents caused marked delay in PKB activation which was hardly followed by reduced rate of apoptosis; furthermore antiapoptotic properties of quercetin and DMSO diminished with the increasing dose of each factor. Every chosen biochemical index of muscle cell differentiation (myogenin, MCK) confirmed pictures obtained from microscopic observations (myotubes formed).

A deeper understanding of the role of phytochemicals and thiols in the mechanism of muscle growth, development and regeneration may have important implications for the prevention and treatment of neuromuscular disorders. Further studies are needed to address the above-mentioned effects and ascertain whether the physicochemical properties other then antioxidant cause delayed myogenesis.

Materials and Methods

Reagents

All reagents were cell culture tested, of high purity, and unless otherwise stated they were purchased from Sigma-Aldrich Chemical Co., plastics were from Corning-Costar, while sera, media and antibiotics were from Gibco Life Technologies. Phosphate buffered saline (PBS) and ultra pure agarose were obtained from Gibco BRL. Primary rabbit polyclonal anti-Myogenin IgG antibody (M-225, sc-576), rabbit anti-Bcl-2 IgG antibody (N-19; sc-492), and goat anti-Akt-1 IgG antibody (C-20; sc-1618) as well as fluorescein conjugated secondary goat anti-rabbit IgG antibody for cytoimmunofluorescent studies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Sodium dodecyl sulphate (SDS) 10% (w/v), Sequi-Blot PVDF Membrane 0.2 μm and all reagents for immunoblotting were obtained from Bio-Rad Laboratories (CA, USA). Tris, EDTA, NaCl, proteinase K, RNAse A from bovine pancreas and Lambda DNA EcoR I *Hin*dIII Digest were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Other reagents were purchased as stated in the description of the respective methods (see below). Protein content was assayed both by Lowry method (Sigma Chemical Co. St. Louis, MO, USA) and the bicinchoninic acid method (BCA, Pierce Chemical Co., Rockford, IL, USA).

Cell culture

Murine C2C12 myoblastic cell line (satellite cells from thigh muscle) purchased from European Collection of Animal Cell Cultures (ECACC) was maintained in exponential phase of growth (20% (v/v) FCS/DMEM with Glutamax) designed as GM (growth medium) supplied with antibiotic-antimycotic mixture (Penicillin G sodium salt 50 IU/mL, Streptomycin sulphate 50 μg/mL; Gentamycin sulphate 20 μg/ml; Anti PPLO agent - Tylocine base 6 μg/mL, Fungizone - Amphotericin B 1 μg/ml), in a controlled humidified air atmos-

phere supplied with 5% CO_2, at 37°C in a multiwell or tissue culture Petri dishes (Corning-Costar Inc. U.S.A.).

Experimental procedure

Every other day the cells were washed twice with phosphate buffered saline (PBS) and medium was changed until they reached 100% confluence. Confluent cells (myoblasts of the same cell density fully covering surface dish) were then guided to post mitotic status, and differentiation and fusion were initiated by replacing GM with 2% (v/v) horse serum HS/DMEM designed as DM (differentiating medium). In the above mentioned conditions C2C12 myoblasts easily and fully differentiate into myotubes, therefore we could follow up modifications of differentiation process during 5 subsequent days. During the study freshly prepared media without or with the experimental factors were changed every 24 hours. Quercetin dissolved in DMSO (1, 10, 100 μM) or DMSO (0.1, 1, 2% v/v) were added to the medium. With regard to quercetin, the lowest DMSO concentration present (0.1% v/v) played the role of the control system, while for DMSO treatments fresh DM medium became the control system additionally shown on figures.

Cell viability

Assessment of cell viability based on mitochondrial function was assayed by the ability of cells to convert soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) into an insoluble purple formazan reaction product with minor modifications to protocol described by Jacobson et al. [21]. The GM medium was replaced with DM medium with or without experimental factors (changed every 24 h), and for this assay during the last 4 h of incubation time of each day these media were replaced with MTT solution (0.5 mg/mL in DMEM without phenol red, sterilised by filtration). MTT solution was then aspirated and formazan was dissolved by addition of 100 μL dimethylsulfoxide (DMSO). The absorbance (570-630 nm) was measured with ELISA Reader type ELx808, BIO-TEK Instruments (U.S.A) and % survival was defined as ((experimental-blank)/(control-blank)) x 100, where the blank was the value obtained from wells containing DMSO without cells. In all cases, the cells were examined under phase-contrast microscopy before application of MTT to visually assess the degree of cell death. Percent viability (MTT conversion into purple formazan in comparison with control value of 2% HS/DMEM for DMSO or 0.1% DMSO in 2% HS/DMEM for quercetin) indicates cell viability (mitochondrial respiration or activity of mitochondrial dehydrogenases).

Apoptosis

Cytotoxicity with resultant cell death was monitored by microscopic observations (Olympus BX-20). Apoptosis was evaluated by *in situ* uptake of bisbenzimide (HO 33342) and propidium iodide (PI) as described by Abu-Shakra et al. [1]. Percentage of apoptotis-apoptotic index (the number of apoptotic nuclei expressed per total number of nuclei) was calculated. Multiwell (6) dishes were used to grow myoblasts on cover slips coated with a 10% gelatine film. Experimental media were added, and at the end of each following 24 hours of study fluorochromes (HO 33342 or PI) were added. Firstly, for the last 30 min. bisbenzimide HO 33342 (stock solution of 25 mg/mL in H_2O) was introduced to give final concentration of 0.3 mg/mL, then for the final 5 min. propidium iodide (stock solution of 10 mg/mL in PBS) was added to give working solution of 5 μg/mL. Media were aspirated, cells gently washed with ice cold PBS, and mounted on slides using mounting medium anti-fading solution (DAKO, Denmark). Since PI does not enter cells that are alive, dead cells (late apoptotic and necrotic) were stained with this fluorochrome. On the other hand, HO 33342 penetrates every cell and also stains nuclear DNA. Fluorescent microscope BX-60 Olympus equipped with a PM20 automatic photomicrograph system was used for photographic recording. In the ultraviolet light at least one thousand nuclei in total were counted in ten (or more if necessary) randomly chosen visual fields per each slide and cells were qualified as follows: regular oval shaped blue nuclei (alive cells); condensed white-blue nuclei (apoptotic). In the same visual field the excitation of propidium iodide light led to the appearance of red nuclei of dead cells, mainly apoptotic.

Proliferation and protein assays

Simultaneous labelling with (^{3}H)-methyl-thymidine and (^{14}C)-leucine (Amersham PLC, 1 μCi/mL, 5 Ci/mmol) was used every day during 5 days (24 h of treatment) in order to determine changes in both the proliferation assay (mitogenicity) and protein synthesis (anabolism). When cells became fully confluent, cultures were washed twice with PBS and this day was set as day "0". Control and experimental media with quercetin and DMSO were poured into multiwell (24) plates. A volume of 10 μL of mixture of 30 μCi/mL of each label, to give final concentration of 1 μCi/mL of the radioisotope mixture was added to the one of the plates and the plates were transferred to the incubator as described for cell culture. Total label time was 24 hours. Every day cells were washed twice with PBS and immersed with fresh media, whereas one plate was supplemented with a radioactive label. After 24 h incubation with the label the cells were subject to fixation. The wells were poured with 0.5 mL of TCA (10% w/v) to precipitate protein and DNA. After overnight incubation at 4°C, TCA solution was aspirated and the cells were washed for 5 min with 70% v/v methanol followed by a 5 min wash with 90% v/v methanol. The cells were dissolved in 0.25 mL of 0.5 M NaOH supplied with 0.2% v/v Triton X-100, kept in the incuba-

tor for 24-48 h (37°C, 95% humidity). The completion whether the cells were dissolved was controlled microscopically. Soluble cell solution was neutralised with 5 M formic acid, mixed with scintillation cocktail (Aquasol, New England Nuclear), vigorously agitated, and counted in Packard TRI-CARB 1600 CA β-counter. The results obtained in d.p.m. were expressed in arbitrary units as % values (experimental values at the following days divided by the control value at day 1. of experiment multiplied by 100).

Myotube formation

Cell cultures were also analysed morphologically after fixing and staining with Giemsa reagent. Mononuclear myocytes (spindle shaped) and multinuclear (3-10 myonuclei per myotube) as well as (10 > myonuclei per myotube) myotubes were identified with a contrast-phase microscope (Olympus BX-20) in 10 randomly chosen microscopic fields and photographed. Since the number of cells does not increase in confluent cultures myotube indices (3 > 10 myonuclei per myotube) and (10 myonuclei > per myotube) were calculated by scoring the number of myotubes containing certain number of myonuclei present per microscopic field.

Creatine kinase activity

Muscle creatine kinase (MCK) activity in cell lysates was determined using CK assay kit (Sigma Diagnostics, St. Lois, MO, USA) with N-acetyl cysteine included as the reagent to stimulate MCK activity.

Immunoblotting

Immunoblotting was performed as follows: an aliquot of ice-cold extraction buffer containing: 50 mM Tris-acetate, 50 mM NaF, 2.5 mM EDTA, 1 mM EGTA, 5 mM sodium pyrophosphate, 5 mM β-glycerophosphate, 1 mM sodium orthovanadate (Na_3VO_4), 2 mM dithioerytreitol (DTT), 1 mM benzamidine, 4 μg leupeptin, and 1% (v/v) SDS, pH 7.2) was added to a tube and the cellular suspension was dissolved by repetitive triturating with a pipette tip and left to stay for 10 min at room temperature. Viscous solution was then transferred to a fresh tube, frozen in liquid nitrogen and stored at -70°C until used. Western blot analysis was carried out using equal amounts of protein (100 μg) subjected to SDS-PAGE under reducing conditions. Electrotransfer of proteins to PVDF membranes (0.2 μm) was performed for 1 h at 100 V and followed by blocking in TBS buffer (20 mM Tris, 500 mM NaCl, pH 7.5) supplemented with 5% non-fat powdered milk. The membranes were then probed with primary antibody - rabbit polyclonal anti-Myogenin (1 μg/ml), rabbit polyclonal anti-Bcl-2 (1 μg/ml,) and goat polyclonal anti-Akt-1 (1 μg/ml) for 16 h at 4°C, washed three times in TBS containing 0.05% Tween-20 and were incubated with goat anti-rabbit or anti-goat antibody conjugated with horseradish peroxidase. The blots were developed using the enhanced chemiluminescence (ECL) detection system (Amersham) according to the manufacturer's protocol. The membranes were scanned and analysed using a JX-330 Sharp scanner and densitometry of bands was performed using Diversity One™ version 1.3 software (*pdi*, New York, NY, USA).

Cytoimmunofluorescence studies

The presence and intracellular location of certain regulatory proteins was based on the immunocytochemical detection performed by two-step reaction. In 6 well (35 mm diameter) tissue culture dishes cells grown on cover slips coated with 10% gelatine were fixed with methanol free 1% (w/v) formaldehyde in PBS for 15 min (37°C, 100% humidity), washed twice with PBS and transferred into 70% ethanol (stored up to 48 h at -20°C). Subsequently, the cells were washed twice with PBS supplemented with 1% (w/v) bovine serum albumin (PBS-BSA). The cells were than immersed under 100 μl of sterile primary rabbit anti-Myogenin or anti-Bcl-2 IgG antibody solution (10 μg/ml w/v, Santa Cruz, CA, USA) in 1% PBS-BSA and incubated for 1.5 h (37°C, 100% humidity). Rabbit anti-IgG antibody (Santa Cruz, CA, USA) served as isotype negative control. The cells were washed twice with PBS, immersed under 100 μl of secondary FITC-conjugated goat-anti rabbit IgG diluted 1:100 v/v (Santa Cruz, CA, USA) and incubated again for 1.5 h (37°C, 100% humidity). After a subsequent double wash with PBS cover slips were drained and mounted upside down on microscopic slides covered with a drop of mounting medium (DAKO Corp. Denmark). Sometimes the myonuclei were labelled with Actinomycin D (5 μg/ml H_2O_2, 10 min, 4°C) before microscopic evaluation. The cells were observed under a fluorescent microscope and photographed (fluorescent microscope BX-60 Olympus equipped with the PM20 automatic photomicrograph system).

Oligonucleosomal fragmentation of DNA

The method for agarose-gel electrophoresis of whole cells described by Wolfe et al. [55] was adopted with minor modifications. Briefly, approximately 1 x 10^6 of cells grown on tissue culture Petri dishes (35 mm diameter) was aseptically harvested with rubber policeman in PBS-D, monolayer and medium transferred into sterile Eppendorf tubes and centrifuged (800 g, 10 min, 4°C), the supernatant was aspired, and the cell pellet after wash with PBS and concomitant centrifugation was held in sterile Eppendorf tubes until assay (4°C). The cells were resuspended by triturating in 15 μL of sterile deionized water, vortexed for 1 sec. Approximately 6 μL of RNAse A solution (10 mg/mL in 10 mM Tris, 15 mM sodium acetate, pH 7.5, pre-

heated for 15 min in 95°C) was added to each tube, the samples were mixed by spin and incubated at 56°C for 1 h. Afterwards, 9 μL of loading buffer (12% Ficoll 400 in TAE, 0.25% bromophenol blue and 0.25% xylene cyan blue) was added and the samples were spun again. While running digestion, the gels were prepared by dissolving 1.8 g agarose in TAE buffer 1.8% (w/v) (10 mM Tris, 10 mM sodium acetate, 1 mM EDTA) and melted it in a microwave oven. 1 ml of 10% SDS (w/v) was added to liquid gel to give the final content of 2% followed by addition of 20 μL of proteinase K (20 mg/ml) after cooling to 55°C. The solution was then immediately poured into the gap above the wells. Each sample was transferred into each well. 1 μL of λ DNA EcoR I HindIII Digest (125-21,226 bp) mixed with 10 μL TAE and 5 μL of loading buffer was applied to each gel to provide a size marker. Electrophoresis of DNA was initially performed with 2V/cm (20 Volts) for 1 h, followed by 3 h with 8V/cm (80 Volts) with TAE as running buffer. After electrophoresis the gels were stained for 20 min with ethidium bromide (1 μg/mL) followed by 5 min washing with deionized water. The gels were then UV illuminated and photographed by Biometra BioDoc II video imaging system (U.S.A.).

Statistical analysis

Value on day 1. in the control group for each series of treatments was set as 100%. All remaining data (unless otherwise stated) were expressed as % of this value (% of control). Since quercetin was dissolved in DMSO (present as 0.1% v/v of experimental medium), 0.1% of DMSO is to be chosen as a control group for quercetin. The statistical analysis was performed by one-way analysis of variance. Determination of significance between the differences of the means was carried out with Tukey's multiple range test. In order to compare the treatment means at the same time points the results were analysed by two-way analysis of variance. The results were expressed as the means and SEM with a value of $P < 0.05$ taken as significant, $P < 0.01$ as highly significant and $P < 0.001$ as very highly significant.

Results

Proliferation assay and protein synthesis

Simultaneous determination of both DNA synthesis and protein synthesis provide indirect information about the rate of cell growth occurring during tissue development [38]. In the control system (DM) the slope of curve representing time-course for multiplicity (proliferation assay - DNA synthesis) was falling (by 74% in extreme; $P < 0.001$, Fig. 1a), whereas the slope of curve illustrating time-course of protein synthesis was elevating (by 70% in extreme; $P < 0.001$; Fig. 1b).

Quercetin and DMSO, regardless of the dose used, were apparently mitogenic ($P < 0.001$). However, DNA synthesis rate was not in direct proportion to the concentration of DMSO or quercetin (Fig. 1a). Mitogenicity stimulated by quercetin, in extremes, amounted to 50%, 58%, and 12% for 1, 10 and 100 μM, respectively, whereas for DMSO reached 20%, 0, 40% for 0.1%, 1% and 2%, respectively. Also protein synthesis did not directly respond to the dose of experimental factors ($P < 0.001$). For quercetin, protein formation was inhibited by 12% after 1 μM ($P < 0.05$) and by 60% after 100 μM ($P < 0.001$), except stimulation by 80% after 10 μM of quercetin (Fig 1b; $P < 0.01$). This relation was especially evident for DMSO, where protein synthesis rate did not differ from control after 0.1% DMSO, whereas it was inhibited after 1% and 2% of DMSO, respectively (Fig. 1b; $P < 0.001$).

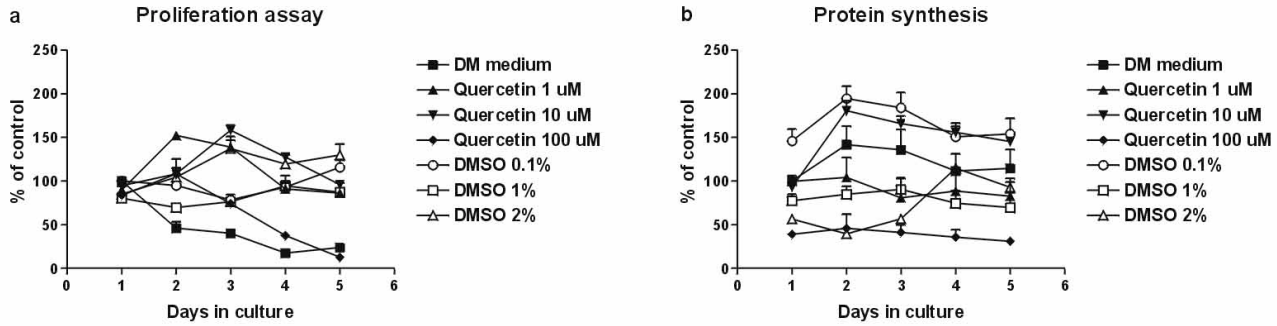

Figure 1. Effects of various concentrations of quercetin or DMSO on DNA synthesis (proliferation assay) (a) or protein synthesis (b). Confluent murine C2C12 myoblasts grown on multiwell (24) plates were incubated in DMEM supplemented with 2% HS (DM) in the absence or presence of quercetin (1, 10, 100 μM) or DMSO (0.1, 1, 2% v/v) for 5 days. At each time point (day 1., 2., 3., 4., 5.), cells from three wells of multiwell plate per each treatment taken and radioactivity (d.p.m.) of TCA precipitable fractions of (^{3}H)-thymidine labeled DNA and (^{12}C)-leucine labeled protein were assessed as indicated. The experiment was repeated at least twice with similar results. Values are means with SEM.

Effect of quercetin and DMSO

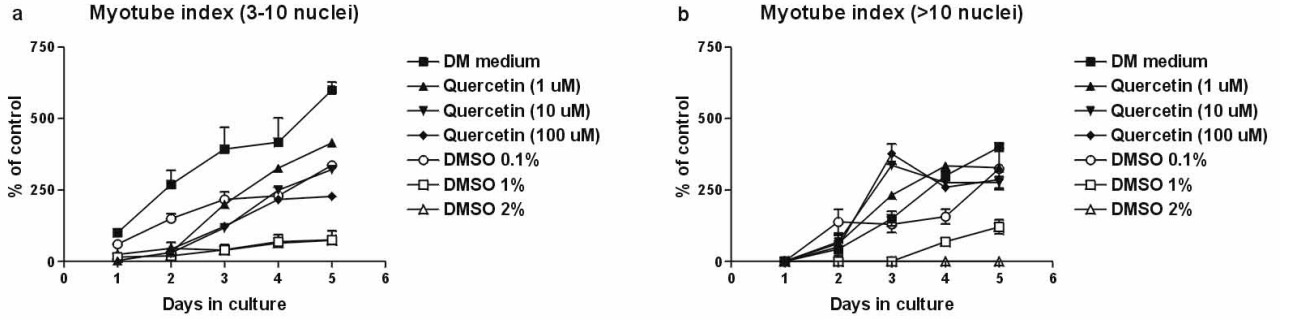

Figure 2. Effects of various concentrations of quercetin or DMSO on myotube indices (3-10 myonuclei per myotube) (a) or (10 > myonuclei per myotube) (b). Confluent murine C2C12 myoblasts were incubated in DMEM supplemented with 2% HS (DM) in the absence or presence quercetin (1, 10, 100 μM) or DMSO (0.1, 1, 2% v/v) for 5 days. At each time point (day 1., 2., 3., 4., 5.), cells from three wells of multiwell plate per each treatment were fixed and stained with Giemsa and myotubes counted as indicated. The experiment was repeated at least twice with similar results. Values are means with SEM.

Myotube formation

Since the intensity of myotube formation may differ, we decided to study the effect of each of the experimental agent on the presence of either myotubes with 3-10 myonuclei (initial step of fusion) or myotubes with 10 or more myonuclei (late step of fusion), separately.

Both quercetin and DMSO were observed to delay the onset of myotube formation (< 10 myonuclei) in comparison to controls (Fig. 2a, $P < 0.001$). Also the number of myotubes formed after the replacement of GM with DM was higher in controls than in the experimental cultures ($P < 0.001$). While quercetin was shown to accelerate the formation of already existing myotubes (10 > myonuclei) manifested by more numerous large myotubes (Fig. 2b; increase by 250%; $P < 0.001$), DMSO was found to maintain inhibitory effect until the very end of experiment (Fig. 2b; decrease by 250%; $P < 0.001$). Furthermore, during 2% DMSO treatment no myotube with more than 10 myonuclei was found all over the experimental period. Both effects were time-dependent (Fig. 2).

Myogenin expression

Activation of *myogenin* gene was monitored in the aim to find out whether morphological alterations in the size and nuclei content of myotubes are mirrored by the transcriptional activity of the important molecular inductor of E-box dependent muscle specific genes. Myogenin expression was examined either by cytoimmunofluorescence or by immunobloting. After quercetin or DMSO, we observed microscopically a marked gap (24 h) of myogenin expression, which reflects the absence of myotubes at the same time (data not shown). For the first time myogenin protein had been observed by immunoblotting at the onset of myotube formation - after 24 h for controls, and after 48 hours for the experimental treatments. During subsequent days, the expression of myogenin was significantly accentuated in growing myotubes both in controls and after quercetin and DMSO ($P < 0.001$). In great part the results of immunobloting were consistent with cytoimmunofluorescent studies. The blots representing the myogenin protein concentration showed a time-dependent increase in density (Fig. 3). The blots illustrating myogenin proved the inhibitory effect of both antioxidants on the expression of *myogenin* gene.

Muscle creatine kinase activity

The activity of MCK rose with the time of incubation ($P < 0.001$), but in relation to the time of collection (1., 3., 5. day) the highest values of the mean were observed for controls, lower for quercetin and the lowest for DMSO (Fig. 4, $P < 0.01$). Elevated activity of MCK corresponded to the accelerated respiration of myotubes determined by MTT test.

PKB activity

Once activated, protein kinase B has phosphorylated form, therefore the additional upper band of PKB of higher molecular weight is found. The density of dot blots is directly proportional to the extent of phosphorylation. During 5 days of induced myogenesis, separated and established identity of PKB was found to be both free form and phosphorylated form, with the remark that after DM phosphorylation of PKB reached maximum on day 2. (Fig. 5). Then the activation gradually lessen. Quercetin and DMSO delayed PKB phosphorylation, so the blots of phospho-PKB were observed during subsequent days with the maximum on day 4 (Fig. 5).

Apoptosis

Differentiation and fusion of myoblasts might be attenuated by other mechanisms than influence on signal transduction and gene expression. A good example is illustrated by the fact that cells, which undergo the cell cycle exit or those, which are on the way to fusion process in certain circumstances can be deleted (pre-

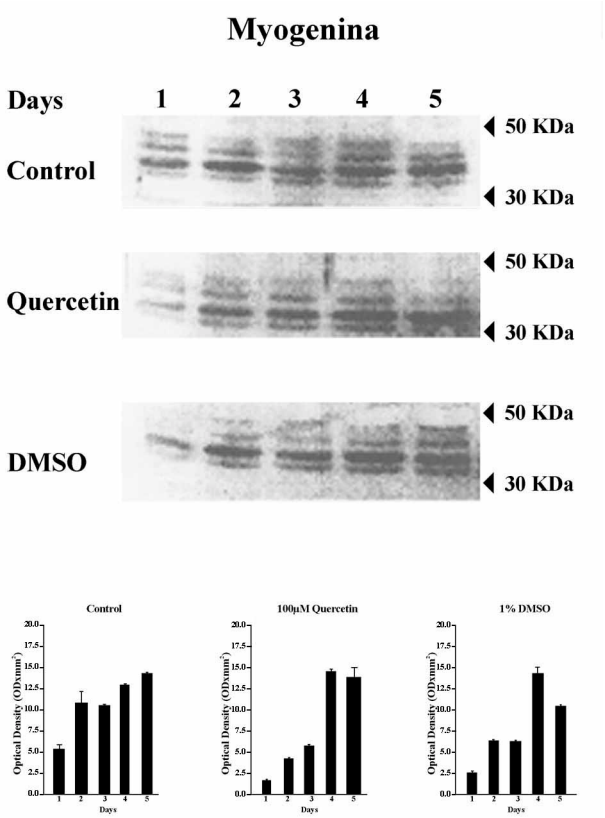

Figure 3. The results of densitometric analysis (optical density, OD x mm^2) of myogenin (filled bars) expression during differentiation of C2C12 myoblasts. Confluent murine C2C12 myoblasts grown on Petri dishes (60 mm diameter) were incubated in DMEM supplemented with 2% HS (DM) in the absence (Control); or the presence quercetin (100 μM Quercetin) or DMSO (1% DMSO). At each time point (day 1., 2., 3., 4., 5.), plate of cells from each day was taken and proteins were analyzed by SDS-PAGE followed by Western blot. The results are representative of the experiments that were repeated three times with similar results.

mature mitosis or mitotic catastrophe). During apoptosis cell membrane remains intact, chromatin is condensed and finally cell blabbing occurs with the cell fragmentation to apoptotic bodies. Regardless of the route of cell elimination, the essential question is whether dying cells are those, which are currently forming myotubes, or others, which are not involved in the fusion process. Observations performed on cells simultaneously stained *in situ* with HO33342 and PI enable identification of floating cells, mononuclear cells and myonuclei marked with dense chromatin, a symptom of apoptotic cell death. The apoptotic index (AI) was calculated, and a photographic record performed to visualize the necrobiology of the muscle cells. Assuming the lack of injury, the cells might disappear by the mechanism of cell death during differentiation well documented as cited below. Consistently with the previous reports [1, 15, 45, 48, 53, 54] apoptosis of mononuclear myogenic cells was exaggerated with the time of differentiation reaching the maximum on day 4. (increase from 1% on day 1. to 8% on day 5., or from 2% on day 1. to 9% on day 5.; $P < 0.001$, Fig. 6a). In comparison, both antioxidants at low level were less apoptotic, so AI at last was equal to controls, but elevated i.e. for 2% DMSO. Therefore, the less apoptogenic activity of quercetin and DMSO was adversely proportional to the dose of each agent. Quercetin at 1 μM inhibited apoptotic cell death (0-2% at maximum; $P < 0.001$), whereas a further increase in concentration caused elevation to 7% at maximum ($P > 0.05$) (Fig. 6a). After 1 day of the experiment very similar representation of condensed nuclei was observed for DMSO treatments, approximately 3% vs. 1% of control ($P < 0.05$). At the maximum, AI amounted to 8% for control and almost to 9% for 2% of DMSO ($P < 0.05$). The concentration of 1% and to a higher degree 0.1% of DMSO led to a very highly significant reduction in AI value. Since the AI reached maximum on day 4. of experiment ($P < 0.001$), we decided to check whether fragmented DNA was present in cell cultures treated with either of the antioxidants. Both quercetin and DMSO caused the presence of small molecular weight DNA fragments but the formation of oligonucleosomes was not observed, although smears of lysed DNA were found for 1% and 2% of DMSO (Fig. 7). It seems, that both antioxidants at high doses directly or indirectly inhibited viability of the cells.

Viability of the cells

In order to examine whether quercetin and DMSO disturb vital processes in the cells, we carried out a series of viability tests (MTT assay). The reduction of MTT into insoluble formazan occurs exclusively at the expense of reducing equivalents, the essential condition of life supported by energy demands. In control systems the viability rose with time up to 40% above the reference value ($P < 0.001$). Quercetin at 1 and 10 μM did affect cell viability by stimulating cellular respiration (15% above control, $P < 0.01$), but at 100 μM the process of a progressive increase in MTT reduction was inhibited (by 10% on day 2., $P < 0.05$) and remained unchanged until the end of the study (Fig. 6b). On the other hand, for DMSO we found dose- and time-dependent loss of viability, slight for 0.1% (reduction by 14%; $P < 0.05$), but higher for 1% (85%; $P < 0.001$), and 2% (100%; $P < 0.001$) at the end of the examination period. Actually, restricted respiration was concomitant with the elevated rate of apoptosis, and for quercetin the cells that were alive respired with higher intensity than controls.

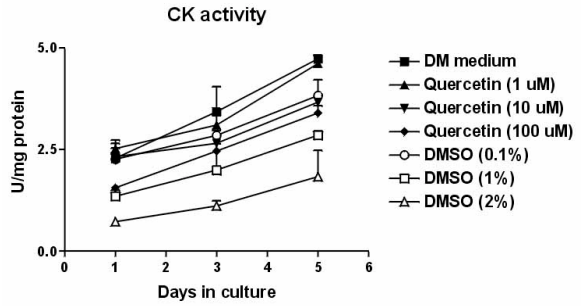

Figure 4. Effects of various concentrations of quercetin or DMSO on muscle cell creatine kinase (MCK) activity. Confluent murine C2C12 myoblasts grown on in Petri dishes (60 mm diameter) were incubated in DMEM supplemented with 2% HS (DM) in the absence or presence of quercetin (1, 10, 100 μM) or DMSO (0.1, 1, 2% v/v) for 5 days. At each time point (day 1., 3., 5.), three plates of cells from each treatment combination were taken and MCK activity was determined as indicated. The experiment was repeated twice with similar results. Values are means with SEM.

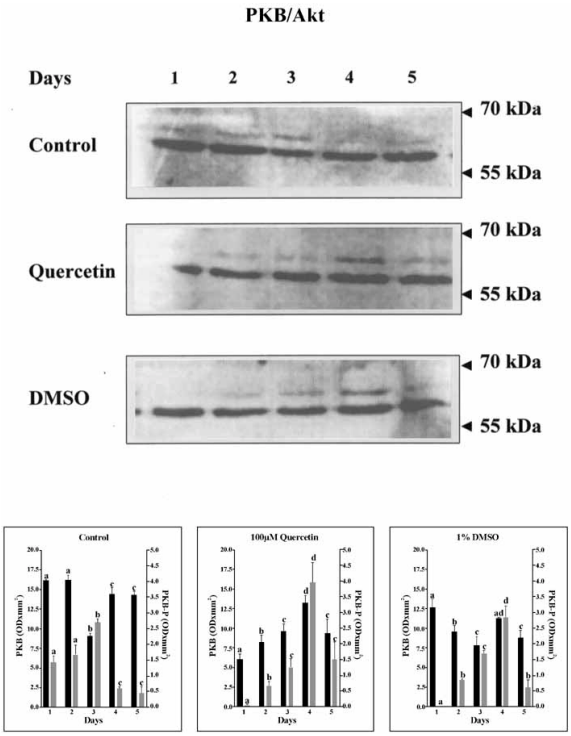

Figure 5. The results of densitometric analysis (optical density, OD x mm^2) of PKB (filled bars) and PKB-P (open bars) expression during differentiation of C2C12 myoblasts. Confluent murine C2C12 myoblasts grown on Petri dishes (60 mm diameter) were incubated in DMEM supplemented with 2% HS (DM) in the absence (Control); or the presence quercetin (100 μM quercetin) or DMSO (1% DMSO). At each time point (day 1., 2., 3., 4., 5.), plate of cells from each day was taken and proteins were analyzed by SDS-PAGE followed by Western blot. The results are representative of the experiments that were repeated three times with similar results.

Bcl-2 expression

Bcl-2 protooncogene product plays the key role in preventing apoptosis by sequestrating other apoptogenic proteins of the same family i.e. Bax, Bid [26]. Since the interplay between these proteins directly determines the occurrence of cyt C-dependent apoptosis either expression or intracellular translocation of Bcl-2 family proteins is regarded as an important determinant illustrating susceptibility of cells to apoptogenic signals. By immunobloting, reasonable similarities between the expression of Bcl-2 protein and PKB activation were observed. During myogenesis Bcl-2 protein peaked on day 2. whereas after quercetin or after DMSO treatment Bcl-2 protein level was elevated on day 4. ($P > 0.05$; Fig. 7). A similar picture was demonstrated on the basis of cytoimmunofluorescent studies.

Discussion

Up to date some factors that posses antioxidant activity or modulate intracellular redox status have been extensively studied and compared with regard to myogenic differentiation. Down-regulation of myogenin expression and myotube formation was described for aspirin in quail myoblasts [18]. Aspirin and/or salicylates are antioxidants, which inhibit oxidation initiated by superoxide/nitric oxide radicals [18], as well as inhibit experimentally induced lipid peroxidation and DNA damage [47]. When the hormone target is located at nuclear level (steroids or thyroid hormones) the direct transcriptional interplay between the nuclear receptors might also influence myogenesis [9]. For example, well characterised down-regulation by triiodothyronine (T3) of activating protein (AP-1) transcription complex via the suppression of redox sensitive 9-*cis*-retinoic acid receptor (RXR) mediated by cAMP pathway [30] and B-cell translocation gene 1 antiproliferative protein (BTG1) [41] led to enhanced muscle cell differentiation. In contrast studies performed with vitamin C derivative discovered its promoting action on muscle cell differentiation. After ascorbic acid 2 phosphate (Asc-2-P) the degree of fusion content in the culture was similar to control except the larger diameter of myotubes. However, the mechanism of increased differentiation after Asc-2-P was indirect, since no expression of molecular and biochemical markers of myogenesis were observed after implementation of specific collagen synthesis inhibitor [33]. On the other hand, retinoic acid and its derivatives either block both growth and fusion of L6 myoblasts [49], or stimulate myogenesis in C2C12 myoblasts by down-regulating the expression of syndecan-1, ECM

Effect of quercetin and DMSO

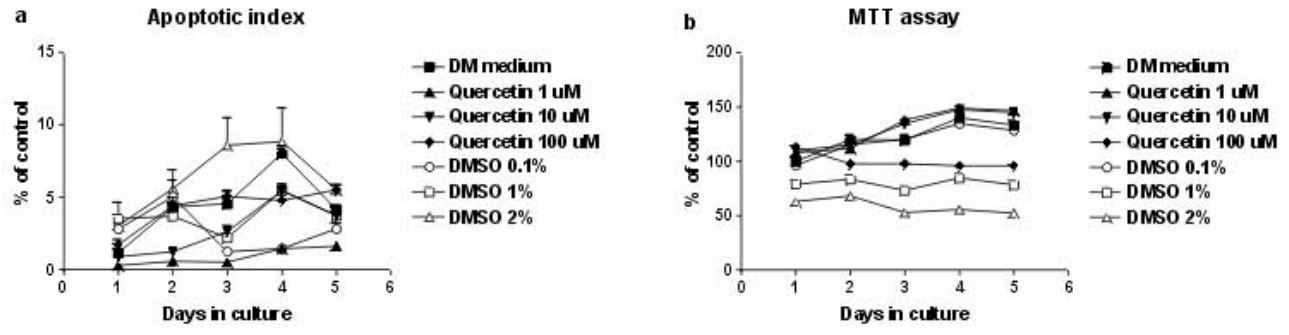

Figure 6. Effects of various concentrations of quercetin or DMSO on apoptotic index (AI) (a) or cell viability (MTT test) (b). Confluent murine C2C12 myoblasts were incubated in DMEM supplemented with 2% HS (DM) in the absence or presence quercetin (1, 10, 100 μM) or DMSO (0.1, 1, 2 % v/v) for 5 days. At each time point (day 1., 2., 3., 4., 5.), cells from three wells of multiwell plate per each treatment were fixed and all nuclei stained with HO 33342, as well as nuclei with condensed chromatin stained both with HO 33342 and PI were counted per microscopic field to assess the fraction of apoptotic nuclei or treated with MTT reagent. The experiment was repeated at least twice with similar results. Values are means with SEM.

proteoglycan involved in the antimyogenicity exerted by such mitogens as bFGF and TGF-β1 [28].

Furthermore, for instance DMSO has been long known [7, 32] to almost completely abrogate myogenesis. Recently it has been demonstrated that DMSO acts through the mechanism of transcriptional repression of uPA, that is the moderator of structural reorganisation of ECM that precedes the fusion of myoblasts [34]. Myogenin expression is also repressed by sodium butyrate, routinely used to block the checkpoint at the end of MyoD transcription family cascade [27]. Taken together, careful analyses of the results obtained from the studies with factors that express anti- or prooxidant behaviour have shown that myogenesis is often moderated by redox dependent mechanisms.

PKB activation accompanies discontinuing of cell divisions, hence protects differentiating myoblasts against death caused by the cessation of survival signals normally approaching the cells during proliferation but not if myogenin is to be activated. The perturbation in cell cycle activity or during cell cycle exit at the differentiation phase may lead to extensive death of myoblasts (see review by Sandri and Carraro [43]). Actually, from studies performed on cell lines and animals either deficient or with forced expression of certain genes, growing evidence proves that in fact muscle cell deletion is exacerbated after interrupted expression of protooncogenes [50, 57].

During our studies an increase in apoptosis by quercetin (100 μM) or DMSO (1,2%) was observed, although at low doses both antioxidants apparently inhibited apoptosis. Anyway, we could not demonstrate oligonucleosomal fragmentation of DNA after treatment with quercetin and DMSO despite the presence of apoptotic phenotype. Taken together, these findings and other from different laboratories indicate that, at least, a few alternative apoptotic pathways may exist [6, 24]. We guess that after reduced serum (DM) both antioxidants might act as survival factors when used at low concentrations. At the same time cell death machinery was markedly hampered, but with increased doses the outcomes of aberrant myogenesis led to the activation of apopoptotic cell death. The lack of DNA ladder could therefore be a characteristic sign of apoptosis induced by antioxidants or the result of cells washed off the DNA fragments in differentiating C2C12 myoblasts.

PKB/*Akt* is the main target of insulin signalling and regulation of cell survival [52]. Among the targets of PKB that delay cell death are Bad, caspase 9 and IKKs [15, 36]. As it was mentioned in the introduction, upon phosphorylation of Bad, antiapoptotic proteins Bcl-2 or BclXL are no longer bound to Bad and can effectively dimerize with Bax preventing the execution of apoptosis. Likewise, when PKB phosphorylate caspase-9, the enzyme is inactivated and the late phase of apoptosis is abrogated. New findings show that *Akt* is also involved in lowering the expression of Fas ligand, which by acting on Fas/Apo1 receptor (family of TNF-α receptors) induces cell death. The mechanism of such antiapoptotic action of PKB resulted from the phosphorylation of fork head related transcription factors (FKHR, FKHRL1), which translocate into cytosol, became sequestrated by 14-3-3 proteins and consequently are unable to target nuclear genes coding for Fas ligand [14]. Although in general a variety of apoptogenic signals are potentially blocked by PKB, overexpression of *Akt* does not fully protect mitotic C2C12 myoblasts from apoptosis during myogenesis [15]. This was also the case in our study, when PKB was at last activated either in the presence of quercetin or DMSO, the antiapoptotic activity diminished with elevated doses of each agent. It is most likely that other alternative antiapoptotic mechanism might explain the presence of apoptosis after activation of PKB.

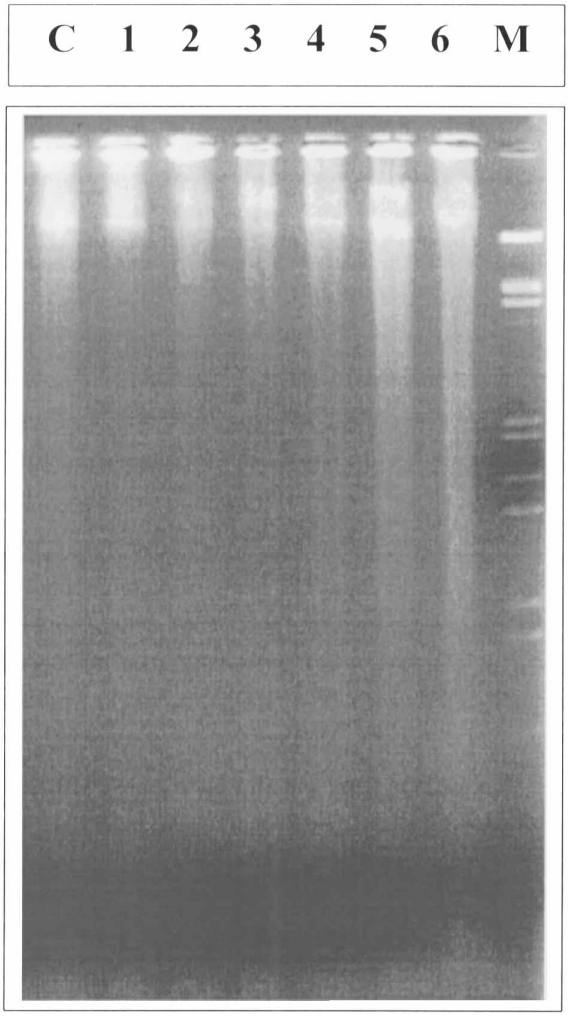

Figure 7. Effects of various concentrations of quercetin or DMSO on the presence of DNA fragmentation in cell lysates. Confluent murine C2C12 myoblasts grown on in Petri dishes (60 mm diameter) were incubated in DMEM supplemented with 2% HS (DM) in the absence or presence quercetin (1, 10, 100 μM) or DMSO (0.1, 1, 2% v/v). On day 4. formation of oligonucleosomal DNA in each well was assessed as indicated. The experiment was repeated at least twice with similar results. Approximately 1x10^6 cells from each treatment were taken for analyses. The cells were washed twice with PBS-D and subjected for further preparation as described. Lanes signed with subsequent numbers (1-7) represent: (C) control, (1, 2, 3) increasing quercetin doses (1, 10, 100 μM), (4, 5, 6) increasing doses of DMSO (0.1, 1, 2% v/v, respectively). Capital letter M stands for λ DNA EcoR I HindIII Digest (125-21,226 bp).

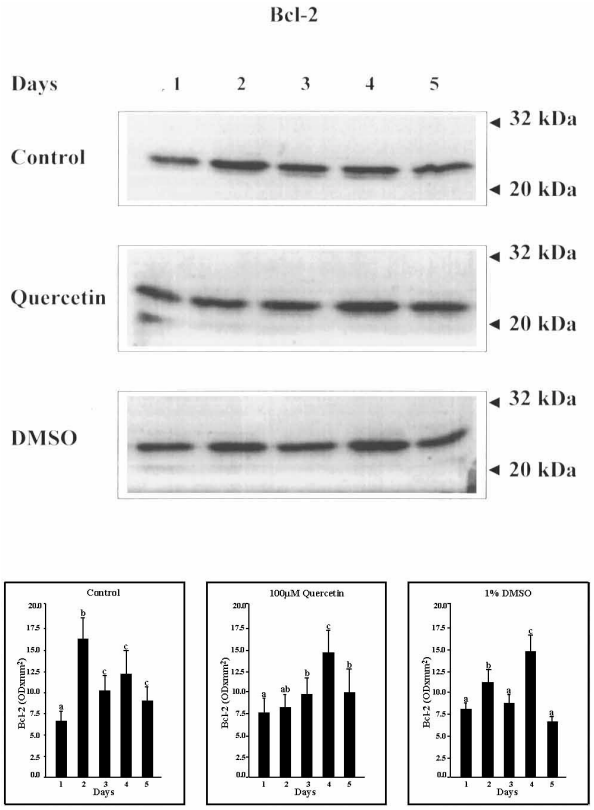

Figure 8. The results of densitometric analysis (optical density, OD x mm^2) of Bcl-2 (filled bars) expression during differentiation of C2C12 myoblasts. Confluent murine C2C12 myoblasts grown on Petri dishes (60 mm diameter) were incubated in DMEM supplemented with 2% HS (DM) in the absence (Control); or the presence quercetin (100 μM Quercetin) or DMSO (1% DMSO). At each time point (day 1., 2., 3., 4., 5.), plate of cells from each day was taken and proteins were analyzed by SDS-PAGE followed by Western blot. The results are representative of the experiments that were repeated three times with similar results.

The results obtained from our studies are consistent with the statement of time-dependent elevation in phosphorylation status of threonine/serine PKB during myogenesis. Actually, differentiation of C2C12 myoblasts is associated with the activation of PI-3K, an indirect upstream activator of *Akt* [42, 43], similarly to other myogenic cell types [19, 25]. However, stimulation of PKB/*Akt* activity by 100 μM of quercetin and 1% DMSO, observed in our experiment, was delayed and accompanied by symptoms of muscle differentiation. This may suggest that stimulatory signals probably originated from antioxidants and directed on PI-3K phosphorylation were retarded; therefore subsequent myogenesis was dependent on PKB downstream-regulated cascade. There are some striking parallels between the results presented

here and those previously reported by others [7, 32]. Our data indicate that cell elimination was a significant factor, which contributed markedly to determine abrogated myogenesis by DMSO. These observations are likely to have direct bearing on the role of factors affecting muscle cell death and restricted differentiation. In this scenario, DMSO would negatively regulate myogenesis and accelerates apoptosis of muscle cells. Quercetin activity on myogenesis differed from DMSO. A delay in muscle differentiation by quercetin was found at high dose whereas sustained DMSO-induced arrest of myogenesis was observed. Later on, the already formed myotubes grew faster after quercetin than in controls. Our results suggest that antioxidant properties of quercetin and DMSO should be responsible for similarities observed between affected muscle cells merely during initial 24 h of experiment.

We have demonstrated that apoptosis of C2C12 muscle satellite cells increased during myogenesis with the maximum on day 4. We also provided evidence that quercetin and DMSO were less apoptotic, but this property disappeared with elevated doses of both agents. In this regard, cellular respiration diminished with increasing rate of apoptotic cell death. However, when curves representing time-course of viability and apoptosis in control systems were plotted against each other, striking similarities appeared. No dissociation between the slopes of curves representing cell viability and apoptosis suggests that myotubes adapted to a higher energy demand by increasing activation of mitochondrial dehydrogenases to the extent that could overcome the loss of reducing equivalents from deleted cells. Such assumption is strongly supported by elevated MCK activity observed during myogenesis. However, this was not the case for DMSO indicating that aberrant myogenesis was linked to the failure of respiration of C2C12 myoblasts to execute a differentiation programme. With regard to quercetin merely modest inhibition of muscle differentiation was shown (day 1.). Taken together, these observations indicate the possibility that antioxidants with mitogenic activity at high doses might in some way trigger the apoptotic cell death of differentiating cells. Moreover, these cells cannot be rescued by concomitant activation of PKB/*Akt*. Additionally, it is conceivable that DMSO at most abrogated myogenesis partially due to the elimination of differentiating myoblasts that by DMSO had lost the resistance to apoptotis.

Apoptosis regulating proteins (Bcl-2 family) were studied in order to find their role in muscle development [12]. C2C12 myoblasts were Bcl-2 positive (15%), additionally it was thought that the expression of this protein correlates with early stages of myogenesis. In our experiment, we do provide the evidence that quercetin and DMSO modulated and changed *Bcl-2* gene expression during myogenesis. Therefore, we suppose that translocation among the members of Bcl-2 family or other apoptogenic/antiapoptotic proteins does not merely accounted for the C2C12 apoptosis. PKB activation was positively correlated with PKB phosphorylation and Bcl-2 expression.

Anyway, the requirement for satellite cell proliferation and differentiation to support muscle hypertrophy indicates that a transient delay in myogenesis after quercetin and possibly other mitogenic antioxidants might also play a putative role in the positive regulation of muscle growth. Just recently, some important observations were drawn by Puri et al. [39] who observed induction of differentiation of human rhabdomyosarcoma cells into fully developed myotubes by p38 MAP kinase which is well known to be activated by ROS. Similarly, activation of mitochondria due to the higher activity of complex I releases of H_2O_2 (Barja 2000) also led to differentiation of chick myoblasts [40]. The mechanisms underlying the recruitment of satellite cells for regenerative or hypertrophic processes have not been established, although the role of various growth factors until now was thought to be crucial [4, 35]. However, most mitogenic growth factors have been found to inhibit differentiation and fusion, and what we have found for the first time, also by antioxidants such as quercetin and DMSO. The course of changes in markers of myogenesis is sequential with the myogenin gene product being the ultimate determinant of molecular commitment of myoblasts to myogenesis. It would be advantageous to verify the working hypothesis if quercetin and DMSO directly or indirectly affect myogenin gene expression, and whether other kinases with possibly equal importance as PKB in Bad phosphorylation are PKA (which also inhibits myogenesis) and MAP kinases. Further work is required to understand the physiological relevance of the modified redox status within the muscle cells.

In summary, our findings suggest that superoxide anion (quercetin) and hydroxyl radical (DMSO) scavengers influence myogenesis. We observed suppressed myogenin expression, delayed activation of PKB/*Akt* and Bcl-2 expression, decreasing antiapoptotic effect of both agents with concomitant stimulation of mitogenicity and reduced anabolism. Less numerous but larger myotubes found at the end of the experimental period after quercetin could be considered as a sign of higher potency of myoblasts to fuse into existing myotubes- an indirect proof that quercetin might improve hypertrophying muscle. In contrast, DMSO inhibits myogenesis with high efficacity and therefore it should be avoided whenever accelerated muscle development, growth or regeneration is to be achieved.

Acknowledgements

This paper is dedicated to the memory of Professor Wies aw Barej who supported our efforts to obtain

funds necessary to complete this study. We thank Mrs. Justyna Olczak and Mrs. Teresa Hass for their contribution to microscopic evaluation of myotube formation and biochemical analyses. This work was supported by the grant No. 5 PO6K 029 15 from the Polish State Committee of Sciences.

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