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Dietary resveratrol confers apoptotic resistance to oxidative stress in myoblasts

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Abstract

High levels of reactive oxygen species (ROS) contribute to muscle cell death in aging and disuse. We have previously found that resveratrol can reduce oxidative stress in response to aging and hindlimb unloading in rodents *in vivo*, but it was not known if resveratrol would protect muscle stem cells during repair or regeneration when oxidative stress is high. To test the protective role of resveratrol on muscle stem cells directly, we treated the C2C12 mouse myoblast cell line with moderate (100 μM) or very high (1 mM) levels of H_2O_2 in the presence or absence of resveratrol. The p21 promoter activity declined in myoblasts in response to high ROS, and this was accompanied a greater nuclear to cytoplasmic translocation of p21 in a dose-dependent matter in myoblasts as compared to myotubes. Apoptosis, as indicated by TdT-mediated dUTP nick-end labeling, was greater in C2C12 myoblasts as compared to myotubes ($P<.05$) after treatment with H_2O_2. Caspase-9, -8 and -3 activities were elevated significantly ($P<.05$) in myoblasts treated with H_2O_2. Myoblasts were more susceptible to ROS-induced oxidative stress than myotubes. We treated C2C12 myoblasts with 50 μM of resveratrol for periods up to 48 h to determine if myoblasts could be rescued from high-ROS-induced apoptosis by resveratrol. Resveratrol reduced the apoptotic index and significantly reduced the ROS-induced caspase-9, -8 and -3 activity in myoblasts. Furthermore, Bcl-2 and the Bax/Bcl-2 ratio were partially rescued in myoblasts by resveratrol treatment. Similarly, muscle stem cells isolated from mouse skeletal muscles showed reduced Sirt1 protein abundance with H_2O_2 treatment, but this could be reversed by resveratrol. Reduced apoptotic susceptibility in myoblasts as compared to myotubes to ROS is regulated, at least in part, by enhanced p21 promoter activity and nuclear p21 location in myotubes. Resveratrol confers further protection against ROS by improving Sirt1 levels and increasing antioxidant production, which reduces mitochondrial associated apoptotic signaling, and cell death in myoblasts.

Keywords: Myogenesis; Oxidative stress; Myoblasts; Myotubes; Apoptosis; Reactive oxygen species

1. Introduction

Satellite cells are muscle stem cells that are critical for repair from muscle injury. When skeletal muscle damage occurs, satellite cells are activated and proliferate to become myogenic precursor cells or myoblasts, which migrate to the injury site, then differentiate to form new muscle. These events are similar to processes that regulate myogenesis, and they can be studied in culture model systems. Reactive oxygen species (ROS) regulate cell signal transduction pathways and many cellular functions [1,2] including myogenesis [3]. However, excessive ROS levels both initiate and mediate the dysfunction in a variety of cells including muscle cells. This includes disruption of cell signaling, metabolism, transcription and apoptosis, and therefore, ROS may impair myogenesis [4–7].

Aging is associated with high levels of muscle ROS *in vivo*, which may contribute to increased apoptosis and cell death and reduced myoblast differentiation leading to poor muscle repair [8]. High levels of ROS are associated with metabolic diseases like diabetes [8–10], which may contribute to the loss of myoblast function, increase myoblast cell death [11] and further exacerbate muscle repair in aging. Fulle and colleagues [12] have demonstrated that a high percentage of the myogenic precursor cells from elderly muscles undergo apoptosis triggered by mitochondrial-associated caspase-9, and this appears to be closely linked to the high ROS levels that are found in aged muscles [8]. Thus, we predict that strategies to attenuate high ROS levels should reduce apoptosis in myoblasts and improve muscle differentiation/repair in aging and in other diseases that have elevated ROS levels.

In vitro studies have shown that resveratrol (3,5,4′-trihydroxystilbene) increases antioxidants to reduce the impact of ROS, increases protein synthesis [13], inhibits protein degradation and attenuates atrophy of skeletal muscle fibers [14–17]. A high dose of resveratrol that was fed to rodents (400 mg/kg/day) was shown to reduce muscle fiber atrophy after hindlimb unloading [18]. We also have found that resveratrol that was given at 12.5 mg/kg/day [19] tended ($P=.06$) to blunt atrophy in fast-contracting muscles in response to the high-ROS environment associated with hindlimb unloading in aged rodents, but it did not improve muscle stem cell (satellite cell) activation/proliferation in old animals after reloading [20]. The

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blunting of satellite cell activity in response to muscle reloading in old animals by resveratrol [20] could have been due to the direct inhibitory effects of excessive ROS levels in muscle and systemically, and potentially, the apoptotic events that occur in muscle in aged hosts. In this paper, we tested the hypothesis that resveratrol would directly improve myoblast survival by reducing mitochondrial-associated apoptotic signaling in myoblasts and myotubes in response to a high-ROS environment.

Alternatively, resistance to cell death in myoblasts and myotubes in a high-ROS environment may occur through cell signaling that is independent from buffering ROS. Several studies suggest that ROS may alter p21 levels and protein localization in a variety of cell lines [21–23]. Specifically, phosphorylation of Ser^{153} in p21 is thought to induce its translocation from the nucleus to the cytosol, thereby blocking the cell cycle inhibitory activity in C2C12 myoblasts [24]. Other data [23] show that oxidative stress induces p21 cytoplasmic localization and ubiquitination associated degradation. Thus, a secondary purpose of this study was to determine the effect of ROS on the p21 promoter activity in myoblasts and myotubes, and to investigate whether p21 promoter activity and protein abundance are associated with apoptotic resistance. In this study, we report that myotubes are more resistant to ROS-induced apoptosis than myoblasts, and the reductions of p21 promoter activity and nuclear loss of p21 colocalization are associated with apoptotic resistance within myoblasts. ROS treatment reduced silent mating type information regulation 2 homolog (Sirt1) in myoblasts, which is a putative target for resveratrol. Furthermore, resveratrol provided protection against high-ROS-induced apoptosis and apoptotic signaling proteins in ROS-sensitive myoblasts, potentially through a p21- and/or Sirt1-mediated antioxidant mechanism.

2. Materials and methods

2.1. Cell culture

Murine-derived C2C12 myoblasts were obtained from the American Type Cell Culture Collection (ATCC, Manassas, VA, USA). The myoblasts were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA) and antibiotics (100 U/ml penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B; Mediatech. Inc., Herndon, VA, USA). The cells were incubated at 37°C in a water-saturated atmosphere of 95% ambient air and 5% CO_2. To induce myotube formation, C2C12 myoblasts were plated at an initial density 1×10^5 cells/well in six-well culture dishes. After reaching 70%–80% confluency, the growth medium was replaced with DMEM supplemented with 2% heat-inactivated horse serum and antibiotics (differentiation medium) to induce myotubes formation. The medium was replaced with fresh medium each day. Myotubes were used for experiments after 6 days of incubation in differentiation medium.

Myoblasts and myotubes were treated with 0 μM, 0.1 mM or 1 mM H_2O_2 for 6, 12, 24 or 48 h and then were harvested in ice-cold lysis buffer [25].

2.2. Resveratrol treatment

Myoblasts or myotubes were transferred to fresh media containing 0, 10, 25 or 50 μM of resveratrol. After 24 h of resveratrol treatment, H_2O_2 was added to the medium to make a final concentration of 0 mM, 0.1 mM or 1 mM H_2O_2. Myoblasts or myotubes were transferred to fresh media containing 0–50 μM of resveratrol and 0–1 mM H_2O_2 each day. The cells were then harvested 6, 12, 24 or 48 h. after treatment with H_2O_2.

2.3. Detection of apoptotic cell death

DNA cleavage, which characteristically occurs in apoptotic cells, was measured by TdT-mediated dUTP nick-end labeling (TUNEL) (Roche Applied Science, Indianapolis, IN, USA). The C2C12 cells were grown on glass cover slips, fixed in 4% paraformaldehyde in PBS (pH 7.4) and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. The cells were incubated with TdT and fluorescein-dUTP at 37°C for 1 h. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Vectashield mounting medium, Vector Laboratories, Burlingame, CA, USA). The cells were visualized with an ECLIPSE E800 fluorescence microscope (Nikon Instruments, Melville, NY, USA). The apoptotic index was defined as the percentage of TUNEL-positive cells to DAPI-positive cells.

2.4. Fluorometric caspase activity assay

Caspase activity was measured as previously reported [26]. Briefly, the cytoplasmic protein fraction without a protease inhibitor was incubated in assay buffer (50 mM PIPES, 0.1 mM EDTA, 10% glycerol and 10 mM DTT, pH 7.2) with 1 mM fluorogenic 7-amino-4-trifluoromethyl coumarin (AFC)-conjugated substrate (Ac-DEVD-AFC for caspase-3, Ac-LEHD-AFC for caspase-9, Ac-IETD-AMC for caspase-8; Alexis, San Diego, CA, USA) at 37°C for 2 h. The change in fluorescence was measured on a spectrofluorometric (CytoFluor; Applied Biosystems, Foster City, CA, USA) with an excitation wavelength of 390/20 nm and an emission wavelength of 530/25 nm for caspase-3 and -9 and 460/40 nm for caspase-8 before and after the 2-h incubation. Caspase activity was estimated as the change in arbitrary fluorescence units normalized to micrograms of protein used in the assay.

2.5. Immunoblot analysis

C2C12 cells or myoblasts were harvested and fractionated by the method described by the Rothermel et al. [25]. Immunodetection was performed using established methods as described previously [27,28]. Protein concentrations of samples were determined by the Lowry method, and the purity of each fraction was confirmed as reported previously [29]. Tissue lysates were separated on 12% sodium dodecyl sulfate-polyacrylamide gels by SDS-PAGE, followed by electroblotting to a nitrocellulose membrane. The membranes were blocked with 5% milk in TBST and then incubated overnight (1:1000) in antibodies purchased from Cell Signaling Technology, Boston, MA, USA, including: Bcl-2 (#2876), Bax (#2772), cleaved caspase-3 (#9664), cleaved caspase-9 (#9509) AIF (#4642), p21 (#2946) and histone H3 (#5192) at 4°C. Finally, the membranes were incubated with the appropriate secondary antibody conjugated with HRP (1:50,000). The immunopositive bands were detected with ECL Advance (Amersham Biosciences, Piscataway, NJ, USA). The protein content of each band was quantified by densitometric analysis (Image J, NIH).

2.6. Immunohistochemistry analysis

Immunostaining for myosin heavy chain was visualized in myotubes by immunofluorescence as described previously [29]. In brief, cells were plated on glass cover slips in six-well dishes. After 24 or 48 h of H_2O_2 treatment, the cells were washed with PBS and fixed in 4% formaldehyde in PBS. MF20 (Developmental Hybridoma Bank, IA, USA) was used to detect the cellular location of this protein on myotubes, whereas desmin was used to identify myoblasts. The primary antibodies were followed by Alexa-546-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR, USA), and nuclei were stained with (DAPI). Samples were visualized using an Epi-fluorescence microscope (Nikon, Inc., Melville, NY, USA), and images were obtained using a SPORT RT camera (Diagnostic Instruments, Sterling Height, MI, USA).

2.7. Luciferase assay

A luciferase assay was used to determine the effect of H_2O_2 on p21 promoter activity. The myoblasts were maintained in DMEM supplemented with 10% fetal bovine serum at 37°C in 95% ambient air and 5% CO_2. Approximately 2×10^6 C2C12 myoblasts cells per sample were transfected with a luciferase reporter containing p21 promoter (a kind gift from Dr. Yonghua Yang, Department of Pathology and Cell Biology, University of South Florida College of Medicine) using Nucleofector Kit V (Amaxa, Gaithersburg, MD, USA) in a 0.2-cm gap cuvette (BioRad, Hercules, CA, USA), with four 200-V pulses each lasting 5 ms. After the electroporation, the cells were suspended in 500 μl of MEM (ATCC, Manassas, VA, USA) for 5 min at 37°C, plated into 10-cm plates and incubated in 5% CO_2 at 37°C for 24 h before any treatment. Twenty-four hours after electroporation, the cells were treated with 0 μM, 0.1 mM or 1 mM H_2O_2 and then harvested 24 h after treatment with H_2O_2. A pGL2 vector containing no promoter was used as a negative control for each experiment.

2.8. Isolation of mouse muscle stem cells

To follow up the studies from the C2C12 model, we tested the impact of ROS on skeletal muscle cells isolated from mice. Skeletal muscle satellite cells, which reside beneath the basal lamina of mature muscle fibers, function as muscle stem cells (C2C12 cells are a model for muscle stem cells), but they exhibit substantial phenotypic and functional heterogeneity. Although we have established the methods to isolate a population of muscle stem cells (MSCs) which are CD45−, Sca-1−Mac-1−, CXCR4+b1−, integrin+ [30,31], in our hands, this is a very small population (3% of all muscle stem cells), and it would take a great number of mice to obtain a usable sample. Instead, we have opted to use Fluorescence Activated Cell Sorting (FACS) to isolate and examine the properties of syndecan-4 (Syn4)-positive muscle stem cells [32], which, in our hands, represent~20% of MSCs. Muscle stem cells were isolated from six adult mouse (C57BL6) gastrocnemius muscles, aged 3 months, *via* Percoll gradient centrifugation and incubated with anti-Syn4 (7.5 μg/ml, based on optimization experiments) using published sorting methods [32]. The cells were sorted by FACS using gating strategies for side scatter, forward scatter and singlet gating.