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Basic nutritional investigation

Grape polyphenols supplementation reduces muscle atrophy in a mouse model of chronic inflammation

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ABSTRACT

Objectives: Polyphenols (PP) have demonstrated beneficial effects on low-grade inflammation and oxidative stress; however, little is known about their effect on highly inflamed muscle. The purposes of this study were (i) to evaluate muscle alteration induced by high-grade inflammation, and (ii) to test the effects of red grape PP supplementation on these alterations.
Methods: We used a transgenic mice model (transforming growth factor [TGF] mice) to develop a high T cell–dependent inflammation and C57 BL/6 control (CTL) mice model. Skeletal muscles of TGF and CTL mice were investigated for inflammation, atrophy and oxidative stress markers. Isolated mitochondria from hindlimb muscles were used for respiration with pyruvate as substrate and oxidative damages were measured by Western blot. TGF mice were supplemented with a mixture of red grape polyphenols (50 mg/kg/d) for 4 wk. Data were analyzed by one-way analysis of variance (ANOVA) and post hoc Bonferroni's multiple comparison tests.
Results: TGF mice presented skeletal muscle inflammation, oxidative stress, mitochondrial alteration and muscle atrophy. Atrophy was associated with two distinct pathways: (i) one linked to inflammation, NF-κB activation and increased ubiquitin ligase expression, and (ii) one dependent on reactive oxygen species (ROS) production leading to damaged mitochondria accumulation and activation of caspase-9 and 3. Supplementation of TGF mice with a mixture of red grape polyphenols (50 mg/kg/d) for 4 wk improved mitochondrial function and highly decreased caspases activation, which allowed muscle atrophy mitigation.
Conclusions: These observations suggest that nutritional dosages of red grape polyphenols might be beneficial for reducing skeletal muscle atrophy, even in a high-grade inflammation environment.

Introduction

Muscle atrophy is frequently associated with a high-inflammatory state such as cancer cachexia, rheumatoid arthritis, infection or chronic obstructive pulmonary disease [1,2]. Moreover, muscle atrophy is an independent predictor of increased mortality in such disorders [3,4].

Mechanisms implicated in muscle atrophy involved two proteolysis pathways: the ubiquitin-proteasome system (UPS) and the autophagy-lysosome pathway. The UPS is an essential regulator of sarcomeric protein degradation via their attachment to multiple ubiquitin molecules and then their degradation by the 26 S proteasome. In muscle, the ubiquitination of proteins is regulated by the muscle specific ubiquitin ligase E3: muscle atrophy F-box (MAFbx)/Atrogin-1 and muscle RING-finger protein-1 (MuRF1) [5,6]. The autophagy-lysosomal pathway triggers the removal of specific organelles such as mitochondria or protein aggregates ensuring cell metabolism and cell component turnover [7,8]. This pathway begins with the capture of substrates into an autophagosome that fuses with lysosome to form an autophagolysosome. The sequestered material is subsequently degraded by lysosomal hydrolases [7]. However, during muscle proteolysis, myofilaments are first released via proteolytic action of calpain and/or caspase-3 before their degradation by proteasome, as this latter can degrade only monomeric proteins [9].

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Activation of inflammation and oxidative stress are both involved in muscle atrophy [10,11]. Nuclear factor κB (NF-κB) pathway activation leads to inflammatory cytokine production as tumor necrosis factor α (TNFα), interleukin 6 (IL6) and interferon γ (IFNγ), which plays an essential role in muscle atrophy and could activate NF-κB [11].

Oxidative stress also contributes to muscular atrophy, through activation of the ubiquitin-proteasome pathway [12,13] and of caspase-3 via caspase-9 [14]. Caspase-3 seems to be the initial step triggering muscle proteolysis [15]. Inhibiting caspase-3 activation results in muscle atrophy attenuation [16].

Currently, there is no pharmacologic treatment effective in restoring or maintaining skeletal muscle (SM) mass during chronic inflammatory disease. Therefore, non pharmacological alternative care strategies need to be developed to allow improving quality of life and life expectancy in inflammatory disease as previously demonstrated with physical activity [17].

Recently, several studies determined that supplementation with polyphenols (PPs) could modulate inflammation and oxidative stress [18–20]. PPs are phytochemical compounds abundantly present in fruits and vegetables [21]. They possess aromatic ring(s) with one or more hydroxyl moieties allowing ROS scavenging [22]. Moreover, in vivo, their antioxidative capacity could also be due to their ability to regulate antioxidative enzyme expression at posttranscriptional mRNA level via ARE- and Nrf2-mediated mRNA decay [23]. PPs have also antiinflammatory capacity in that they inhibit NF-κB signaling [24] and expression of inflammatory genes [25]. Because of this, PP supplementation has been extensively tested in pathologies characterized by low-grade inflammation and oxidative stress, such as insulin resistance [26,27], type 2 diabetes [28] and cardiovascular diseases [29]. There is little data concerning PP supplementation effects on muscle atrophy in pathologies characterized by high-grade inflammation [30,31].

Therefore, the purposes of this study were first to evaluate muscle alteration induced by high-grade inflammation in a transgenic mice model (transforming growth factor [TGF] mice), and second, to test the effects of PP supplementation on these alterations.

Materials and methods

Ethical approval

All procedures conformed to the Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes (agreement number: A34-172-38).

Animal experiments

The C57 BL/6 strain of mice used during our study was bred in our facility. The CD4 dnTGFβRII transgenic mice or TGF mice generated by Dr Richard Flavell is deposited at Jackson Laboratory (B6.Cg-Tg (Cd4-TGFBR2)16 Flv/J, stock no 005551). This mouse model express a dominant-negative form of the TGF-β receptor type II under the control of the murine CD4 promoter leading to the loss of T cell activation inhibition by TGF-β. These mice develop a high T cell–dependent inflammation [32,33], inducing a large systemic, liver, kidney, colon and lung inflammation [32,33].

Three-month-old male CD4 dnTGFβRII transgenic (TGF, n = 14) and C57 BL/B6 control (CTL; n = 10) mice were housed in plastic cages in a temperature-controlled environment with a 12-h light/dark cycle and free access to food and water. TGF mice were randomly divided into placebo (TGFp; n = 7) and PP supplementation (TGFpp; n = 7). PPs were administered in drinking water (50 mg/kg/d) for 4 wk. The polyphenolic extract administered was commercialized by GRAP'SUD (Cruviers-Lascours, France) as Exgrape® (Lalilab Inc, Durham, NC, USA) total extract, whom biochemical characteristics have been previously published [34]. It contains more than 92% of total PP as catechin equivalent, 15% procyanidines, 2% anthocyanes and 100 ppm of resveratrol. All these polyphenolic compounds were extracted and purified from red grapes with processes that preserved their characteristics. Preceding dissection, animals were euthanized by rapid cervical dislocation.

Morphometric analysis

Tibialis anterior muscles (TAs) were dissected, immediately frozen in liquid nitrogen after being immersed in a solution of cold isopentane, and stored at −80°C until further analysis. Ten μm TA transverse cryostat sections were stained with hematoxylin and eosin (H&E). Stained sections were then viewed under a Nikon (Tokyo, Japan) optiphot-2 microscope and images were captured with the Microvision Instruments' (Cedex, France) camera driven by Histolab (Gothenburg, Sweden) program version 5-13-1 software (Microvision Instruments). Morphometric analysis was respectively performed on three different cross-sections area (CSA) of each muscle studied using the Histolab program.

Immunohistochemistry

Ten μm TA cryostat sections were incubated with an anti-F4/80 antibody (AbDserotech, Kidlington, United Kingdom) for 1 h at room temperature. After being washed out with phosphate buffered saline solution, sections were incubated with an Alexa Fluor 488 conjugated antirat secondary antibody (Invitrogen) and DAPI. Fluorescence was viewed under a Leica Microscope (Leica DM6000, Wetzlar, Germany) using X63/1.40-0.60 HCX PL APO oil immersion objective. Images were captured as 16 TIFF files with the MicroMax 1300 CCD camera (RS-Princeton Instruments) driven by the MetaMorph (Nashville, TN, USA) (version 7; Universal Imaging, Bedford Hills, NY, USA) software.

Mitochondrial isolation and respiration

Mitochondria were isolated as previously described [35]. Rates of mitochondrial oxygen consumption were measured with a micro cathode oxygen electrode (Clark-type polarographic electrode) calibrated as previously described [35]. Fifty μL of isolated mitochondria were loaded into a respiratory chamber with respiration medium (RM) with pyruvate/malate as substrate and set at 37°C. Adenosine 5-diphosphate (ADP) at a final concentration of 500 μM was added to initiate state 3 respiration (ADP-stimulated). Respiratory control ratio (RCR) was obtained by dividing state 3 respiration rate by the recovery rate after ATP synthesis, i.e., state 4 respiration rate, defined as mitochondrial oxygen consumption after the depletion of exogenous ADP. ADP/O was expressed as number of nmol of ADP phosphorylated by nmol of O consumed. Each measurement was made in triplicate. Oxygen consumption was specified as the amount of oxygen disappearing from the respiration chamber over time per milligram of mitochondrial protein (nmol O/min/mg protein).

Total protein extract

TA muscles were thoroughly blended using a hand held homogenizer (Kinematica AG - POLYTRON PT 1300 D, Luzern, Switzerland) in lysate buffer complemented with protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged 10 min at 1000 g. Protein concentration of the supernatant was estimated with the BCA protein assay kit (Pierce).

Immunoblotting

Total or mitochondrial protein extracts were analyzed by western blot. Polyvinylidene fluoride (PVDF) membranes were incubated with the primary antibodies directed against: cytochrome C, 4-hydroxynonenal (4 HNE), IκBα, caspase-9, caspase-3 (Santa Cruz Biotechnology, Dallas, TX, USA); antimyosin heavy chain (MHC) fast, anti-α tubulin (Millipore, Billerica, MA, USA); nitrotyrosine (Mitosciences); oxidative phosphorylation complexes proteins (OXPHOS; Amersham, England, UK); MnSOD, CuZn SOD (assay designs, Farmingdale, NY, USA); glutathione reductase (GR), and catalase (AbFrontier, Seoul, Korea). After primary antibody incubation, PVDF membranes were incubated with secondary antibodies conjugated to peroxidase (Sigma-Aldrich, St. Louis, MO, USA) and protein expression levels were quantified with ImageJ software corrected with tubulin expression and normalized with CTL set at 100 %. Oxidized proteins were analyzed by western blot with the Oxyblot protein oxidation detection kit (Chemicon International, Temecula, CA, USA).

Quantitative RT-PCR amplification

Total RNAs were isolated from TA cells by blending muscle biopsies with a homogenizer (Kinematica AG - POLYTRON PT 1300 D) in the presence of the TRIzol reagent (Invitrogen, Omaha, NE, USA). To avoid genomic DNA contamination and amplification during PCR analysis, RNAs were treated with RNase-free DNase (Euromedex, Strasbourg, France) before their reverse transcription. Complementary DNAs (cDNAs) were generated by RT with oligo-(dT) primers and the Superscript II enzyme (Invitrogen). Gene sequences for primer design were