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

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KEYWORDS:
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Oxidative stress
TGF
Mitochondria
Caspases

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-inflammatoty 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-lysosomal 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].
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 pharmacologic 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 anti-oxidative 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 C4d tnf-βRII transgenic mice or TGF mice generated by Dr Richard Flavell is obtained from red grapes with SUD (Cruviers-Lascours, France) as Exgrape® (Lalilac Inc, Durham, NC, USA); anti-β tubulin (Millipore, Billerica, MA, USA); nitrotyrosine (Metsciences); 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 and ImageJ plug-in measured and plotted.

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), IκB, caspase-9, caspase-3 (Santa Cruz Biotechnology, Dallas, TX, USA); antimonyscin heavy chain (MHC) fast, anti-α tubulin (Millipore, Billerica, MA, USA); nitrotyrosine (Metsciences); 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 CTIL 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 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).

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).
obtained from the NCBI Reference Sequences database. Primer sequences are in Table 1. For PCR amplification (30 cycles) the amount of each cDNA was adjusted for each primer pair to be in the linear range of amplification and to give the same quantity of amplified complementary DNA as with the eukaryotic translation elongation factor 1α (eEF1α) primers. After 1.2% agarose gels analysis PCR products were quantified with Eastman Kodak 1 D3.6 software (Rochester, NY).

Statistical analysis

All quantitative data are reported as means value ± SEM. One-way ANOVA and post hoc Bonferroni’s multiple comparison tests were used to determine statistical significance between groups using SigmaStat 3.5 (San Jose, CA, USA) software. Bars not sharing a common letter are significantly different at $P < 0.05$.

Results

Skeletal muscle of TGF mice develop inflammation and oxidative stress

TGF mice are characterized by high-grade inflammation [33,36]. However, as inflammation profile and oxidative stress...

Table 1

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sequences sens</th>
<th>Sequences antisens</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td>TACGAACCTCCGGGTTGATGTC</td>
<td>CACGCTTGTCCCTTGAAGAGAC</td>
</tr>
<tr>
<td>IFNγ</td>
<td>GAACGTGGAAAAAGATGGTGAT</td>
<td>CGGCTTCCACACACCAAG</td>
</tr>
<tr>
<td>OAS1</td>
<td>AGCTATAAATCAGGCAAATCT</td>
<td>GGTCTCCACACACCAAG</td>
</tr>
<tr>
<td>OAS2</td>
<td>CTGCTCCAACATCTGCTTCT</td>
<td>AGAAACTCCCTAAGGGGACTG</td>
</tr>
<tr>
<td>Cyto c</td>
<td>TCCATGCAGGTTCTCCTCT</td>
<td>GGAGACCAAGATGAGATGTG</td>
</tr>
<tr>
<td>PGC1α</td>
<td>CCGCTGATGAAAGCCGGATG</td>
<td>GTGGGCGGTCTGCTGCTGAT</td>
</tr>
<tr>
<td>TFAM</td>
<td>ATGCTCCGAGCTGTTCAC</td>
<td>CCAACACTGCTGCTGCTG</td>
</tr>
<tr>
<td>MURF1</td>
<td>GCAGACTGACTAACGACATT</td>
<td>CACAGCCGTCTCAGACTC</td>
</tr>
<tr>
<td>Atrogin-1</td>
<td>GAGAATCTGTTGACACCT</td>
<td>ATCAGCTCAAACAGCTTAC</td>
</tr>
</tbody>
</table>

TNFα, tumor necrosis factor alpha; IFNγ, interferon γ; OAS, oligoadenylate synthetase; Cyto c, cytochrome c; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; TFAM, mitochondrial transcription factor A; MURF1, muscle RING-finger protein-1.
have not been characterized in TGF mice skeletal muscle (TGFp SM), we first analyzed inflammation and oxidative stress in TGFp SM compared to control mice skeletal muscle (CTL SM).

Inflammatory cytokines TNFα, IFNγ, and two interferon (IFN)-stimulated genes oligoadenylate synthetase 1 and 2 (OAS1 and OAS2) mRNA levels were significantly increased in TGFp SM compared to CTL SM (Fig. 1A,B). Further, macrophage infiltration was increased in TGFp SM, whereas macrophages were only occasionally observed in CTL SM (Fig. 1C). In addition, as shown in Figure 1D, the amount of IkBα (inhibitor of NF-κB) protein was significantly lower in TGFp SM than in CTL SM. These results indicated of a high-grade inflammation state in TGFp SM.

TGFp SM was also characterized by a significant increase in nitrotyrosine proteins (Fig. 2A). 4 HNE-modified protein (4-hydroxy-2-nonenal) and carbonylated protein levels were also increased in TGFp SM, but without reaching significance (Fig. 2B,C). Moreover, no significant change in TGFp SM levels of antioxidative enzymes: super oxidative dismutases (CuZnSOD and MnSOD), catalase and glutathione reductase (GR) were observed (Fig. 2, panels D-G). These data suggest that oxidative stress could develop in TGFp SM consecutively to an over-production of ROS, as there is no change in antioxidative enzymes levels.

Muscle mitochondria from TGF mice are characterized by increased oxidative stress and alteration of their respiratory chain function

Mitochondria are not only one of the major sources of ROS, but also the primary targets of oxidative-induced damage. Thus, we investigated oxidative stress and mitochondria activity on isolated mitochondria. Although there was no alteration in carbonylated mitochondrial protein level (Fig. 3A), the 4 HNE-modified protein level was significantly increased in isolated mitochondria of TGFp SM compared to CTL SM (Fig. 3B), suggesting mitochondria oxidative stress.

Isolated mitochondrial activity was first analyzed through oxygen consumption in the presence of pyruvate as substrate. Results are summarized in Table 2. Basal state 2 respiration and state 4, after ADP consumption, were not altered in mitochondria of TGFp SM compared to CTL SM. ADP-stimulated state 3 respiration was significantly decreased in mitochondria of TGFp SM compared to CTL SM. Consequently, the RCR was significantly decreased in mitochondria of TGFp SM.

In isolated mitochondria, there was no difference either in OXPHOS content (Fig. 3C) or in mitochondrial enzyme hydroxacylCoA dehydrogenase (HADH) activity (Fig. 3D) between TGFp and CTL mice. Furthermore, expression of several...
genes were involved in mitochondrial biogenesis: Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α), mitochondrial transcription factor A (TFAM) and cytochrome C were not significantly different between TGFp and CTL SM (Fig. 3E). Finally, when expressed per total amount of isolated mitochondrial OXPHOS protein content, the ADP/O ratio was not different in TGFp mice compared to CTL mice (Table 2). Interestingly, in muscle, OXPHOS protein content was significantly increased in TGFp SM extracts (Fig. 3F) suggesting an increase in mitochondria quantity. These results showed an increase in mitochondria oxidative damage associated with a higher OXPHOS content in TGFp SM extract with a decrease in mitochondrial respiration. These data suggest that SM mitochondria function was impaired in TGFp mice.

**TGF mice develop skeletal muscle atrophy**

SM weight (Fig. 4A) and mean values of SM fiber cross-sectional areas (CSA) (Fig. 4, panels B-C) were significantly decreased in TGFp mice. Further, the left shift of fiber CSA frequency distribution curve (Fig. 4D) illustrated an increase in the percentage of smaller fibers and a decrease in the percentage of larger fibers, demonstrating atrophy of SM in TGFp mice. Moreover, mRNAs of ubiquitin ligases MuRF1 and Atrogin-1 were significantly increased in TGFp mice (Fig. 4E). In the same way, activation of caspases-3 and 9 was increased in TGFp as attested by their higher level of cleaved fragment (Fig. 4F) and the myosin heavy chain (MHC) cleavage (Fig. 4, panels F-G). These data demonstrated that SM atrophy is associated with activation of caspases and UPS in TGFp SM.

**PP supplementation does not modulate skeletal muscle inflammation but reduces mitochondrial dysfunction**

To investigate the potential benefit of PP supplementation, TGF mice were supplemented with red grape polyphenol (TGFpp) or placebo (TGFp) for 4 wk. We analyzed TGFpp and TGFp SM for inflammation and oxidative stress. PP supplementation did not reduce inflammation in TGFpp SM, as attested by the absence of change in TNFα, IFNγ, OAS1, and OAS2 mRNA levels (Fig. 1A,B), macrophage infiltration (Fig. 1C), and IkB level (Fig. 1D). Moreover, in SM extract, nitrotyrosine, 4 HNE-modified...
proteins and carbonylated protein levels were not decreased after PP supplementation (Fig. 2, panels A-C). Also, antioxidative enzymes (Fig. 2, panels D-G) and mitochondrial HADH activity (Fig. 3D) were not modified, nor was mitochondrial biogenesis genes expression (Fig. 3E).

We next investigated oxidative stress markers in isolated mitochondria. We did not observe a decrease in 4 HNE-modified protein levels (Fig. 3B). However, surprisingly, carbonylated protein content was significantly reduced after PP supplementation (Fig. 3A), suggesting a reduction in mitochondrial oxidative stress on some specific ROS. Regarding mitochondrial respiration, a significant decrease in state 3 was noticed, although basal and state 4 were not modified after PP supplementation (Table 2). Thus, TGFpp mitochondria presented a significant decrease in RCR compared to TGFp (Table 2). However, PP supplementation had no effect on the expression of OXPHOS proteins in SM extract (Fig. 3F), although it decreased the total amount of OXPHOS proteins in isolated mitochondria.
Thus, mitochondrial efficiency measured with ADP/O was significantly increased after PP supplementation when normalized by the total amount of mitochondrial OXPHOS protein (Table 2) suggesting an improvement in mitochondrial function.

PP supplementation reduces muscle atrophy in TGF mice

Although increased muscle weight in TGFpp did not reach significance compared to TGFp (Fig. 4A), PP supplementation significantly increased mean CSA (Fig. 4, panels B-C) and a right-shift in fiber CSA frequency distribution curve was noticed (Fig. 4D). Thus, TGFpp SM had a decrease in smaller fibers percentage and an increase in larger fibers percentage. However, no change of the ubiquitin ligase MuRF1 and Atrogin-1 mRNAs levels was observed in TGFpp SM (Fig. 4E), while we observed a significant inhibition of activated caspases 3 and 9 expression (Fig. 4F). These results illustrated an inhibition of protein degradation pathways. As expected, TGFpp SM presented a significant reduction (88%) in cleaved MHC level compared to TGFp SM (Fig. 4G). Overall, TGFpp SM presented no more significant difference compared to CTL SM except for MuRF1 and Atrogin-1 mRNA expression levels.

Discussion

We demonstrated to our knowledge for the first time, that TGF mice, characterized by T cell–driven inflammation, developed (i) SM inflammation and (ii) SM atrophy. We further demonstrated that red grape PP supplementation mitigates muscular atrophy in TGF mice, by notably acting on mitochondrial function and caspase activation but not on Atrogin-1 and MuRF1 mRNAs levels.

TGF mice were previously characterized by a major systemic inflammation [32,33] with no data on muscle. Our results demonstrated that SM also developed an inflammatory state characterized by high expression of inflammatory gene (TNFα, IFNγ, and OAS1/OAS2) and NF-κB activation. Associated with this inflammation, TGF mice developed SM atrophy, described, to our knowledge for the first time, in this mice model.

We demonstrated that, despite an increased mitochondrial content in TGF SM, muscle atrophy was associated with mitochondrial dysfunction, increased mitochondrial oxidative stress, MuRF1 and Atrogin-1 genes expression and caspase-3 and 9 activation. Based on previous studies [11,13,14], we speculate that TGF SM mitochondria produced excessive amount of ROS, resulting in a deleterious effect on mitochondrial proteins leading to mitochondrial dysfunction. As previously described [37], our data highlight the role of mitochondria in the development of muscle atrophy. In fact, both disruption/impairment of mitochondrial function, network or mitophagy lead to the accumulation of damaged and dysfunctional mitochondria [38,39] that contribute to muscle atrophy [7,40].

We found that nutritional amount of grape PP was sufficient to mitigate muscle atrophy. As chronic inflammation in TGF mice cannot be negatively regulated [32], PP supplementation did not reduce muscular inflammation. Our data showed that PP supplementation in TGF mice acted mainly on ROS damage. PP supplementation reduced oxidized mitochondrial proteins, improved mitochondrial function and dramatically reduced caspases-9 and 3 activation. The TNFα/NF-κB/MuRF1-Atrogin-1/atrophy pathway was not affected by PP supplementation. Neither NF-κB activation nor the increased expression of TNFα and MuRF1-Atrogin-1 mRNA were decreased after PP supplementation. Although mRNA levels do not reflect exactly protein levels and activity, we speculate that MuRF1 and Atrogin-1 activity are increased in TGF mice secondary to their inflammatory status since these genes are known to be mainly regulated by inflammation [41]. Finally, this pathway appears to be independent of ROS regulation. In contrast, the caspase-9 and 3 activation were partially reverted in TGFpp (Fig. 5) indicating that their activation is dependent on ROS production. PP supplementation was not able to decrease the ROS-mediated formation of reactive aldehydes (i.e., 4 HNE) in both SM extract as in
isolated mitochondria, whereas there was a reduction in oxidized mitochondrial protein and decreased caspase-3 and 9 activation. These data suggest that PP supplementation did not have an impact on all sources of ROS.

Beneficial effects of PP supplementation on muscle atrophy appeared to be mediated through modulation of mitochondria function and caspase activation, as previously found in low-grade inflammation or muscle inactivity models [42,43].

Conclusion

Our results demonstrated to our knowledge for the first time, that in a murine model of chronic high-grade inflammation (TGF mice), characterized by SM inflammation, oxidative stress, mitochondrial dysfunction and muscle atrophy, a nutritional amount of red grape PP supplementation mitigates muscle atrophy, by principally acting at mitochondrial level. In fact, PP supplementation improves mitochondrial function and decreases caspases activation, even without acting on Atrogin-1 and MuRF1 expression.

Thus, supplementation with a nutritional amount of red grape PP might be beneficial for reducing SM atrophy and possibly improving quality of life and life expectancy in chronic high-grade inflammatory diseases. This beneficial effect needs to be validated in further human studies.

Acknowledgments

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