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Peroxynitrite Induces Degradation of Myosin Heavy Chain via p38 MAPK and Muscle-Specific E3 Ubiquitin Ligases in C2 Skeletal Myotubes

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Abstract

Oxidative stress and inflammation play an important role in the catabolism of skeletal muscles. Recently, cigarette smoke (CS) was shown to stimulate muscle catabolism by activation of p38 MAPK and up-regulation of the muscle-specific E3 ubiquitin ligases (E3s) atrogin-1 and MuRF1 which are over-expressed during muscle atrophy. Peroxynitrite ($ONOO^-$), an oxidative ingredient of CS, also produced during oxidative stress and inflammation, was previously shown to induce ubiquitination and degradation of muscle proteins. To investigate the involvement of p38 MAPK and the muscle-specific E3s in $ONOO^-$-induced muscle catabolism, C2 myotubes, differentiated from a myoblast cell line, were exposed to $ONOO^-$ (25 μM) in a time-dependent manner. Following exposure, degradation of myosin heavy chain (MyHC) and actin, activation of p38 MAPK, and levels of atrogin-1 and MuRF1 were studied by Western blotting. Peak phosphorylation of p38 MAPK was observed at 1 h of $ONOO^-$ exposure. $ONOO^-$ caused a significant increase in the levels of atrogin-1 and MuRF1. In accordance, a significant decrease in MyHC levels was observed in a time-dependent manner. These findings support previous studies in which the catabolic effects of $ONOO^-$ were shown. In addition, $ONOO^-$ was demonstrated to induce degradation of muscle proteins by activation of p38 MAPK and up-regulation of the muscle-specific E3s atrogin-1 and MuRF1.

Keywords

Atrogin-1 • Cigarette smoke • Inflammation • Muscle catabolism • MuRF1 • Oxidative stress • p38 MAPK • Reactive nitrogen species

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1 Introduction

The effects of reactive oxygen species (ROS) and reactive nitrogen species (RNS) on atrophy of skeletal muscle have been studied extensively. Oxidative and nitrative stress, in which high levels of ROS and RNS are produced, were suggested to promote muscle atrophy by regulation of muscle proteolysis (Rom et al. 2012a; Sukhanov et al. 2011; Meng and Yu 2010; Supinski and Callahan 2007). Degradation of skeletal muscle proteins is mainly regulated by the ubiquitin-proteasome system (UPS). The E3 ubiquitin-ligating enzymes (E3s) of the UPS are responsible for determining which proteins are targeted for degradation by the proteasome (Rom et al. 2012b; Meng and Yu 2010). Muscle atrophy F-box protein (atrogin-1) and muscle RING finger-1 protein (MuRF1) are muscle-specific E3s that are over-expressed in various conditions of muscle atrophy. These E3s target specific muscle proteins for ubiquitination and subsequent degradation by the proteasome during skeletal muscle atrophy (Foletta et al. 2011).

ROS and RNS can induce cellular damage through oxidation and nitration of biomolecules, including DNA and proteins which can affect signal transduction pathways and cellular processes (Yeo et al. 2008; Bar-Shai and Reznick 2006a, b). ROS were previously shown to promote muscle catabolism by affecting the UPS. Li et al. (2003) found that exposure of skeletal myotubes to hydrogen peroxide (H_2O_2) stimulated ubiquitin conjugation to muscle proteins and up-regulation of the muscle-specific E3s atrogin-1 and MuRF1. In accordance, increased catabolism of muscle proteins and decreased expression of myosin heavy chain (MyHC) were found in C2C12 myotubes treated with H_2O_2 (Gomes-Marcondes and Tisdale 2002).

Peroxynitrite ($ONOO^-$) is a potent oxidizing and nitrating RNS that promotes the development of various pathologies (Bar-Shai and Reznick 2006a, b). Increased production of $ONOO^-$ occurs during conditions of inflammation in which excess nitric oxide (NO) is generated by neutrophils and phagocytes. Excess NO reacts with superoxide and forms various RNS, including $ONOO^-$ (Yeo et al. 2008; Hasnis et al. 2007). The role of $ONOO^-$ in muscle proteolysis was previously studied by Bar-Shai and Reznick (2006a, b). It was found that exposure of L6 skeletal myotubes to $ONOO^-$ caused degradation of muscle-specific proteins that was mediated by ubiquitination of muscle proteins. In addition, $ONOO^-$ exposure resulted in a non-transient activation of nuclear factor-κB (NF-κB) in skeletal muscle cells.

Another major source of exposure to $ONOO^-$ is cigarette smoke (CS). CS contains numerous of ROS and RNS, including H_2O_2, superoxide, and NO. These ROS and RNS can enter the bloodstream and cause macromolecular damage (Rom et al. 2012a; Csiszar et al. 2009). CS is considered the main source of human exposure to NO. Superoxide and NO from CS can react and produce $ONOO^-$ (Hasnis et al. 2007). Recently, we have shown that exposure of C2 skeletal myotubes to CS stimulated muscle catabolism by increased oxidative stress, phosphorylation of p38 mitogen-activated protein kinase (MAPK), activation of the NF-κB pathway and up-regulation of the muscle-specific E3s atrogin-1 and MuRF1 (Kaisari et al. 2013; Rom et al. 2013a). These findings led us to propose a cellular model of CS-induced catabolism of skeletal muscle. According to this model, components of CS enter the bloodstream and reach skeletal muscle of smokers. In skeletal muscle, CS components lead to increased oxidative stress that induces the activation of intracellular signaling pathways including the p38 MAPK and the NF-κB pathways. Activation of these signaling pathways results in up-regulation of muscle-specific E3s leading to increased breakdown of muscle proteins (Rom et al. 2012b).

Various component of CS such as aldehydes, ROS, and RNS have the potential to promote catabolism of skeletal muscle (Rom et al. 2012a). For instance, it was recently found that exposure of C2 myotubes to acrolein, a toxic unsaturated aldehyde present in high levels in CS, stimulated muscle catabolism by activation

of the p38 MAPK pathway and up-regulation of muscle-specific E3s (Rom et al. 2013b). H_2O_2, also present in CS, was shown to induce breakdown of muscle proteins by activation of the UPS and over-expression of atrogin-1 and MuRF1 (Li et al. 2003; Gomes-Marcondes and Tisdale 2002). The RNS $ONOO^-$ is also present in CS and was previously shown to stimulate degradation of muscle proteins (Bar-Shai and Reznick 2006a, b). However, the effects of $ONOO^-$ on the p38 MAPK pathway and muscle-specific E3s during muscle catabolism have yet to be studied. Therefore, the present study aims to investigate the effects of $ONOO^-$ on muscle catabolism as mediated by p38 MAPK and the muscle-specific E3s atrogin-1 and MuRF1.

2 Methods

The study was approved by an institutional Ethics Committee.

2.1 Cell Culture

The C2 cell line of mouse myoblasts was a generous gift from Prof. Eyal Bengal (Rappaport Faculty of Medicine, Technion, Israel). C2 myoblasts were grown in 24 wells and 100 mm plates at 37 °C in humidified 95 % air and 5 % CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % heat-inactivated fetal bovine serum, 1 % penicillin/streptomycin and 1 % L-glutamine (Biological Industries, Bet HaEmek, Israel). For differentiation into myotubes, myoblasts were plated in 0.1 % gelatin-coated plates and were grown to 90 % confluence. Then, medium was replaced by DMEM, supplemented with 2 % heat-inactivated horse serum, 1 % penicillin/streptomycin, and 1 % L-glutamine (Biological Industries, Bet HaEmek, Israel), which was replaced every 48 h for 6 days until cell fusion and formation of multi-nucleated myotubes was achieved. A successful cell differentiation was determined by expression of MyHC as measured by Western blotting.

2.2 Cell Treatments

Experiments were held on Day 7 of differentiation when the cells had completed their differentiation into myotubes. The culture medium was replaced by fresh medium at the beginning of each experiment. Stock solutions of $ONOO^-$ (Cayman Chemical Company, Ann Arbor, MI) were prepared in 0.3 M NaOH, due to its stability in alkaline pH. $ONOO^-$ was added to the culture medium at a final concentration of 25 μM followed by incubation for increasing periods at 37 °C. Control myotubes were treated with fresh medium without $ONOO^-$.

2.3 Cell Viability

To assess the effects of $ONOO^-$ on the viability of myotubes, 2×10^5 cells were seeded in 24-well plates and grown to 90 % confluence for differentiation into myotubes. On Day 7 of differentiation, myotubes were treated with 25 μM $ONOO^-$ and incubated for increasing periods. Following incubation with $ONOO^-$, viability of myotubes was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma-Aldrich, St. Louis, MO, USA) as previously described (Rom et al. 2013a, b). For each experiment, OD was measured in triplicate at 570 nm using ELISA reader (Biochrom Anthos Zenyth, Cambridge, UK). The viability was expressed as a percentage of the values of control myotubes (corresponding to 100 %).

2.4 Western Blot Analysis

Following $ONOO^-$ exposure, myotubes were lysed for cytosolic proteins as previously described (Kaisari et al. 2013; Rom et al. 2013a, b). Then, 20 μg of cytosolic proteins were loaded in each lane, separated by SDS-PAGE and later transferred to nitrocellulose membranes. Membranes were blocked with 5 % non-fat milk powder in TBS-T (0.125 % Tween) (Sigma-Aldrich,

St. Louis, MO, USA) for 1 h and exposed to primary antibodies overnight at 4 °C. Primary antibodies were MyHC (1:1,000), MAFbx/atrogin-1 (1:1,000), MuRF1 (1:1,000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), actin (1:4,000) (Millipore, Billerica, MA, USA), p38 MAPK (1:1,000), and phosphorylated p38 MAPK (1:1,000) (R&D Systems, Minneapolis, MN, USA). The next day, membranes were washed with TBS-T followed by 1 h incubation at ambient temperature with the appropriate secondary antibodies (Jackson Immuno-Research, West Grove, PA, USA). Detection was performed by enzyme-linked chemiluminescence (ECL) (Biological Industries, Bet HaEmek, Israel) using Image-Quant LAS 4000 digital imager system (GE Healthcare, Chalfont St. Giles, Bucks, UK). Protein quantities were determined by densitometry and analyzed using Total Lab Software V2006C (Nonlinear Dynamics, Newcastle, UK).

2.5 Protein Loading Control

Since the effects of $ONOO^-$ on the main contractile muscle proteins, MyHC and actin, were investigated in the present study, actin could not be used as a housekeeping protein for loading control. Alternatively, quantification of total proteins by reversible Ponceau staining was used for protein loading control as previously described (Kaisari et al. 2013; Rom et al. 2013a, b; Romero-Calvo et al. 2010). Briefly, membranes were rinsed in Ponceau S solution (Bio-Rad, Hercules, CA, USA) for 10 min before antibody probing, followed by a brief rinse in DDW until bands were clearly visible. In each lane, total protein quantities were determined by densitometry and used for normalization of ECL detected proteins. This method was validated as an alternative to actin blotting (Romero-Calvo et al. 2010).

2.6 Statistical Analysis

Results are expressed as means ± SE of 3 independent experiments. A *t*-test and one-way ANOVA followed by Tukey's or Dunnett tests were used for statistical analysis. $p < 0.05$ was considered statistically significant. Statistical analysis was performed by SPSS 17 software (SPSS Inc., Chicago, USA).

3 Results

3.1 Effects of $ONOO^-$ on Viability of Myotubes

Differentiated myotubes were exposed to 25 μM $ONOO^-$ and incubated for 1, 2, and 6 h. Following incubation, viability of myotubes was assessed by the MTT assay as described earlier in the Methods section. Exposure of myotubes to 25 μM $ONOO^-$ caused a time-dependent decrease in cell viability. However, compared with control, viability of myotubes remained higher than 80 % and the change in viability was not significant in all time points examined (Fig. 1). Therefore, experiments were held with $ONOO^-$ at the concentration of 25 μM for up to 6 h, the time in which $ONOO^-$ was found to be non-cytotoxic.

3.2 $ONOO^-$ Activates p38 MAPK

p38 MAPK is a stress-activated kinase that responds to various stimuli including oxidative stress. In addition, p38 MAPK is a key mediator of catabolic signaling in skeletal muscles and was previously shown to mediate the

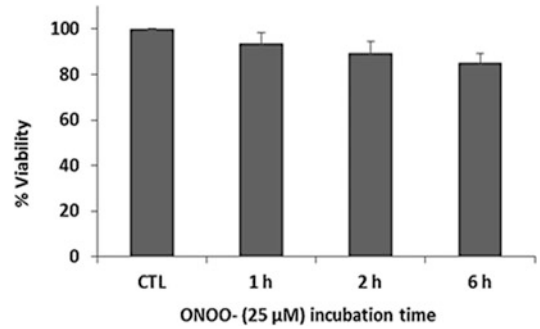

Fig. 1 Effects of $ONOO^-$ on viability of myotubes. Myotubes were exposed to 25 μM $ONOO^-$ for 1, 2, and 6 h. Untreated myotubes served as control (CTL). Following incubation, viability of myotubes was assessed by the MTT assay. Results are means ± SE of 3 independent experiments.

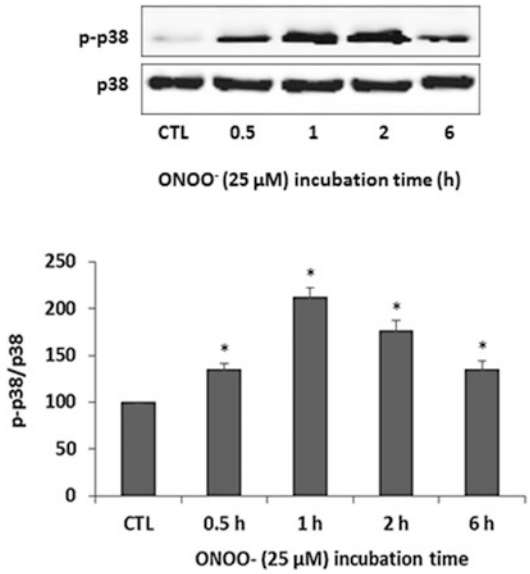

Fig. 2 ONOO⁻ activates p38 MAPK. Myotubes were exposed to 25 μM ONOO⁻ for 0.5, 1, 2, and 6 h. Untreated myotubes served as control (CTL). Following incubation, cell lysates were subjected to Western blot analysis with antibodies against p38 MAPK (p38) and phosphorylated p38 MAPK (p-p38). Protein levels of p-p38 and p38 were quantified by densitometry and the values of p-p38 were normalized to p38 and compared with CTL. Results are means ± SE of 3 independent experiments; *p < 0.05 *vs*. CTL

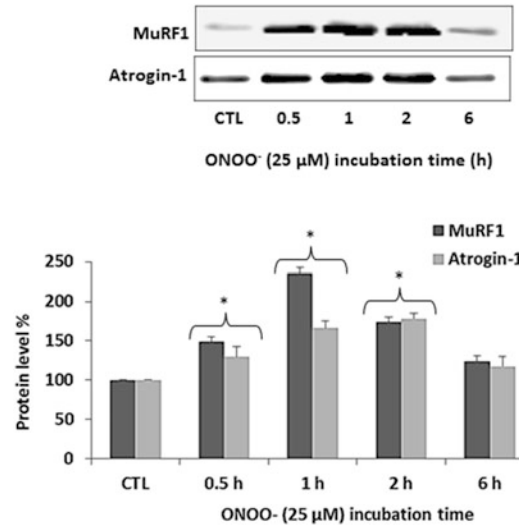

Fig. 3 ONOO⁻ up-regulates MuRF1 and atrogin-1. Myotubes were exposed to 25 μM ONOO⁻ for 0.5, 1, 2 and 6 h. Untreated myotubes served as control (CTL). Following incubation, cell lysates were subjected to Western blot analysis with antibodies against MuRF1 and atrogin-1. Protein levels were normalized by total protein densitometry detected by Ponceau S staining and expressed relative to the corresponding value of CTL. Results are expressed as mean ± SE of 3 independent experiments. *p < 0.05 *vs*. CTL

up-regulation of the muscle-specific E3s (Rom et al. 2012b, 2013a, b; Li et al. 2005). Therefore, it was of interest to study the effects of ONOO⁻ on p38 MAPK in skeletal myotubes. Myotubes were exposed to 25 μM ONOO⁻ for increasing periods and activation of p38 MAPK was studied by examining the levels of phosphorylated p38 (p-p38) relative to non-phosphorylated p38. Significant phosphorylation of p38 was observed from 0.5 h until 6 h of ONOO⁻ exposure and peak phosphorylation was evident at 1 h of exposure (Fig. 2).

3.3 ONOO⁻ Upregulates the Muscle-Specific E3s MuRF1 and Atrogin-1

ONOO⁻ was previously shown to cause degradation of muscle proteins which was mediated by protein ubiquitination (Bar-Shai and Reznick 2006a). These findings suggest that ONOO⁻ can activate the UPS. The muscle-specific E3s MuRF1 and atrogin-1 play a major role in the UPS by determining which muscle proteins are targeted for degradation by the proteasome (Foletta et al. 2011; Meng and Yu 2010). To study the effects of ONOO⁻ on the expression of the above muscle-specific E3s, myotubes were exposed to 25 μM ONOO⁻ for increasing periods and protein levels of MuRF1 and atrogin-1 were examined by Western blotting as described earlier in the Methods section. Starting from 0.5 h of exposure, ONOO⁻ caused a significant increase in protein levels of both MuRF1 and atrogin-1. By 6 h of ONOO⁻ exposure, protein levels of MuRF1 and atrogin-1 decreased and were not significantly different from the control (Fig. 3).

3.4 ONOO⁻ Induces a Time-Dependent Degradation of MyHC

To study the effects of ONOO⁻ on the main contractile muscle proteins, myotubes were

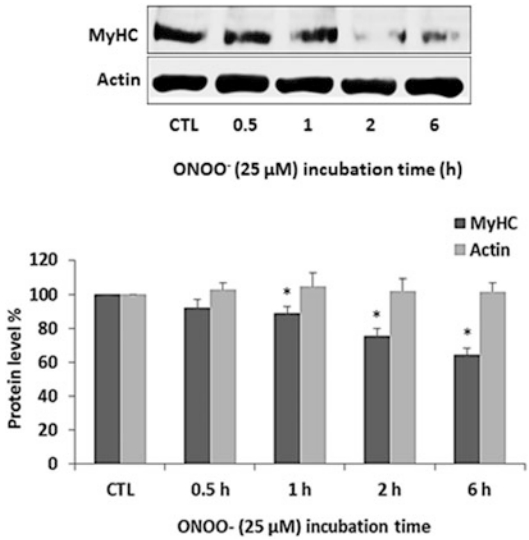

Fig. 4 $ONOO^-$ induces degradation of MyHC. Myotubes were exposed to 25 μM $ONOO^-$ for 0.5, 1, 2, and 6 h. Untreated myotubes served as control (CTL). Following incubation, cell lysates were subjected to Western blot analysis with antibodies against MyHC and actin. Protein levels were normalized to total protein and expressed relative to the corresponding value of CTL. Results are means ± SE of 3 independent experiments. *$p < 0.05$ *vs.* CTL

exposed to 25 μM $ONOO^-$ for increasing periods and protein levels of MyHC and actin were examined by Western blotting as described earlier in the Methods section. A significant decrease in protein level of MyHC was evident from 1 h of $ONOO^-$ exposure. The level of MyHC decreased as the time of exposure to $ONOO^-$ increased (Fig. 4). No significant change in the level of actin was found.

4 Discussion

The present study investigated the effects of $ONOO^-$ on muscle catabolism as mediated by p38 MAPK and the muscle-specific E3s atrogin-1 and MuRF1. It was found that exposure of C2 skeletal myotubes to $ONOO^-$ at the concentration of 25 μM stimulated a time-dependent degradation of MyHC that was mediated by phosphorylation of p38 MAPK and up-regulation of the muscle-specific E3s atrogin-1 and MuRF1.

It was previously shown that exposure of skeletal muscle cells to $ONOO^-$ resulted in a loss of the muscle-specific proteins MyHC and telethonin, which was mediated by a constitutive activation of NF-κB and ubiquitination of muscle proteins (Bar-Shai and Reznick 2006a, b). Moreover, exposure of rat muscle fibers to $ONOO^-$ resulted in a reduced maximum force of slow-twitch fibers and cross-linking of MyHC1 appearing as larger protein complexes (Dutka et al. 2011). Also, exposure of skeletal muscle S1-myosin ATPase (S1) to SIN-1 (3-morpholinosydnonimine), which mimics the effects of chronic exposure to $ONOO^-$, caused inhibition, oxidation, and a partial unfolding of S1 (Tiago et al. 2006). In addition, treatment of various proteins with $ONOO^-$ resulted in enhanced proteolytic susceptibility toward degradation by the proteasome, suggesting that $ONOO^-$ can react with proteins and lead to their recognition and degradation by proteasome (Grune et al. 1998). The findings from the present study suggest that additional signaling pathways of muscle catabolism are activated in skeletal muscle in response to $ONOO^-$ exposure.

p38 MAPK is a stress-activated protein kinase that is known to be phosphorylated in response to oxidative stress. In addition, activation of p38 MAPK was demonstrated in various pro-catabolic conditions such as limb immobilization, type 2 diabetes, aging, and exposure to CS (Kaisari et al. 2013; Rom et al. 2012b, 2013a; Li et al. 2003). Therefore, it was of great interest to study the effects of $ONOO^-$ on p38 MAPK in skeletal myotubes. Indeed, a significant phosphorylation of p38 MAPK was observed shortly after exposing myotubes to $ONOO^-$ (Fig. 2). It was previously shown that exposure of C2C12 myotubes to tumor necrosis factor-alpha (TNF-α) or H_2O_2 resulted in up-regulation of atrogin-1, which was blunted by SB203580, a specific inhibitor of p38 MAPK. Therefore, the muscle-specific E3 atrogin-1 was suggested to be a downstream target of p38 MAPK signaling (Li et al. 2003).

The main mechanism of protein degradation during conditions of muscle atrophy is the UPS. Addition of ubiquitin molecules to a protein

substrate requires the action of three enzymes: E1 ubiquitin activating enzyme, E2 ubiquitin-conjugating enzyme, and E3s which play the important role of determining which proteins will be targeted for proteasomal degradation. MuRF1 and atrogin-1 are two muscle-specific E3s that were shown to be up-regulated in various states of muscle catabolism including diabetes, cancer, renal failure, denervation, and exposure to CS and cytokine (Rom et al. 2013a; Foletta et al. 2011; Meng and Yu 2010). Due to the key role of these muscle-specific E3s in muscle catabolism, it was of great importance to study the effects of $ONOO^-$ on their expression. Interestingly, it was found that exposure of skeletal myotubes to $ONOO^-$ up-regulated both MuRF1 and atrogin-1, which was accompanied by degradation of MyHC, the main contractile muscle protein.

A major source of human exposure to $ONOO^-$ is CS (Rom et al. 2012a; Hasnis et al. 2007). $ONOO^-$ from CS can penetrate into the blood circulation and CS can stimulate increased generation of $ONOO^-$ within the cells (Csiszar et al. 2009). Yamaguchi et al. (2007) showed that sodium peroxynitrite and CS extract, which contains stable ROS and $ONOO^-$-like reactants, can penetrate into the blood through the lung alveolar wall and cause oxidative vascular injury. Also, exposure of rats to gas phase CS resulted in increased serum levels of 3-nitrotyrosine, which may indicate protein nitration by $ONOO^-$ (Yamaguchi et al. 2007; Bar-Shai and Reznick 2006a, b; Tiago et al. 2006). In addition, Barreiro et al. (2012) found that chronic exposure of mice to CS caused oxidative modifications to muscle proteins, including increased levels of 3-nitrotyrosine in the diaphragm and gastrocnemius muscles. The above studies suggest that $ONOO^-$ from CS is capable of reaching skeletal muscles and induce oxidative damage to proteins.

In a similar manner to $ONOO^-$ exposure, CS exposure to C2 skeletal myotubes was recently shown to stimulate muscle catabolism by activation of p38 MAPK and up-regulation of the muscle-specific E3s atrogin-1 and MuRF1 (Rom et al. 2013a). Various ROS and aldehydes which are present in CS can promote catabolism of skeletal muscle. For instance, H_2O_2, one of the ROS found in CS, was found to up-regulate atrogin-1 and MuRF1 and induce muscle breakdown in skeletal myotubes (Li et al. 2003; Gomes-Marcondes and Tisdale 2002). Also, we have recently shown that acrolein, a toxic unsaturated aldehyde present in CS, stimulated muscle catabolism by activation of the p38 MAPK pathway and up-regulation of the muscle-specific E3s in C2 myotubes (Rom et al. 2013b). Thus, our findings suggest that RNS from CS such as $ONOO^-$ may also have a catabolic effect on skeletal muscles.

In conclusion, $ONOO^-$ was found to induce a time-dependent degradation of MyHC that was mediated by activation of the p38 MAPK pathway and up-regulation of the muscle-specific E3s atrogin-1 and MuRF1 in skeletal myotubes. These findings are in line with previous studies that demonstrated the deleterious effects of $ONOO^-$ on skeletal muscle. This study provides additional cellular mechanisms that may explain the catabolic effects of $ONOO^-$ on skeletal muscle during condition of increased exposure to this RNS including inflammation, oxidative stress, and exposure to CS.

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Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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