

# Involvement of NF- $\kappa$ B and Muscle Specific E3 Ubiquitin Ligase MuRF1 in Cigarette Smoke-Induced Catabolism in C2 Myotubes

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## Abstract

Cigarette smoking has been identified as a risk factor for muscular damage and sarcopenia, the age-related loss of muscle mass and strength in old age. Cigarette smoke (CS)-induced oxidative stress and p38 MAPK activation have been shown to be the main cellular mechanisms leading to skeletal muscle catabolism. In order to investigate the involvement of NF- $\kappa$ B as another possible cellular mechanism by which CS promotes muscle catabolism, C2 myotubes, from an in vitro skeletal muscle cell line, were exposed to different time periods of whole vapor phase CS in the presence or absence of NF- $\kappa$ B inhibitor, IMD-0354. The CS-induced reduction in diameter of myotubes and time-dependent degradation of the main contractile protein myosin heavy chain were abolished by NF- $\kappa$ B inhibition. Also, C2 exposure to CS resulted in I $\kappa$ B- $\alpha$  degradation and NF- $\kappa$ B activation, which led to upregulation of the muscle specific E3 ubiquitin ligase MuRF1, but not MAFbx/atrogen-1. In conclusion, our results demonstrate that vapor phase CS exposure to skeletal myotubes triggers NF- $\kappa$ B activation leading to skeletal muscle cell damage and breakdown of muscle proteins mediated by muscle specific E3 ubiquitin ligase MuRF1. Our findings provide another possible molecular mechanism for the catabolic effects of CS in skeletal muscle.

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**Keywords**

Cigarette smoke • E3 ubiquitin ligases • NF- $\kappa$ B • Muscle atrophy • Protein degradation • p38 MAPK

## 1 Introduction

Approximately 20 % of the population smoke tobacco worldwide (Basu et al. 2011). Cigarette smoke (CS) is composed of several semi-liquid particles within a mixture of combustion gases (Green and Rodgman 1996). Vapor phase CS consists of aldehydes, nitrogen oxides (NO) and over 1,015 free radicals per puff (Swan and Lessov-Schlaggar 2007) including various reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Smith and Fischer 2011). Smoking is associated with an increased incidence of cardiovascular, cerebrovascular and vascular diseases. Additionally, it is the primary cause of chronic obstructive pulmonary disease (COPD) (Swan and Lessov-Schlaggar 2007). It has been shown that inhalation of both the particulate and vapor phase components of cigarette mainstream smoke lead to pulmonary inflammation in smokers (Rennard and Daughton 1993).

Cigarette smoking has previously been identified as a risk factor for sarcopenia, the loss of skeletal muscle mass and strength in old age (Castillo et al. 2003; Lee et al. 2007; Szulc et al. 2004). Vapor phase CS causes atrophy and degradation of muscle proteins in cultured skeletal myotubes. These catabolic effects are mediated by CS associated activation of p38 mitogen-activated protein kinase (MAPK) and up-regulation of the muscle specific E3 ubiquitin ligases: muscle atrophy F-box protein (MAFbx/atrogen-1) and muscle ring finger-1 protein (MuRF1) which are responsible for determining the exact proteins targeted for proteasomal degradation in skeletal muscle.

Similarly to the p38 MAPK pathway, another major pathway that is activated by CS is the nuclear factor  $\kappa$ B (NF- $\kappa$ B) that modulates many

vital cellular activities, such as immune, inflammation, survival, and proliferation. Reactive oxygen species (ROS) and TNF- $\alpha$  both activate NF- $\kappa$ B which regulates myogenic activity. Inhibition of the NF- $\kappa$ B pathway in atrophy models prevents muscle degeneration and myofiber death (Meng and Yu 2010). CS has been identified as one of the environmental stimuli that lead to NF- $\kappa$ B activation (Ahn and Aggarwal 2005). Previous work has shown that NF- $\kappa$ B is activated in human lymphocytes exposed to vapor phase CS (Hasnis et al. 2007). In addition, also cigarette smoke condensate (CSC) exposure causes NF- $\kappa$ B activation in various cell lines (Anto et al. 2002).

Activation of NF- $\kappa$ B in muscle-specific transgenic expression of activated IKK (MIKK) mice have been demonstrated to induce significant atrophy through expression of the muscle specific E3 ubiquitin ligase MuRF1, but not MAFbx/atrogen-1 (Cai et al. 2004). A signaling pathway of IKK/NF- $\kappa$ B/MuRF1 was proposed, in which atrophic stimuli activates NF- $\kappa$ B, which in turn results in upregulation of MuRF1 (Rom et al. 2012). Other findings have also demonstrated that MAFbx/atrogen-1 upregulation is not required for NF- $\kappa$ B-induced muscle loss (Cai et al. 2004).

The aim of this study was to investigate NF- $\kappa$ B signaling involvement in CS-induced muscle atrophy and degradation of muscle proteins. This was done by exposing C2 myotubes from an in vitro skeletal muscle cell type culture to different levels of whole vapor phase CS in the absence and presence of an NF- $\kappa$ B inhibitor. We assumed that inhibition of NF- $\kappa$ B prior to exposure of cultured myotubes to vapor phase CS will prevent the atrophy of myotubes and protein breakdown through reversed upregulation of the muscle-specific E3 MuRF1, but not MAFbx/atrogen-1.

## 2 Methods

### 2.1 Cell Culture

The C2 mouse skeletal myoblast cell line was a generous gift from Prof. E. Bengal (Faculty of Medicine, Technion, Israel). C2 myoblasts were grown in 24 wells, 35 and 100 mm plates in growth medium (GM) consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS), 1 % (v/v) penicillin/streptomycin and 1 % (v/v) L-glutamine at 37 °C in humidified 95 % air-5 % CO<sub>2</sub> atmosphere. For the differentiation of myotubes, myoblasts were plated in 0.1 % gelatin-coated plates and were grown to 90 % confluence. At this point, GM was replaced by differentiation medium (DM) consisting of DMEM supplemented with 2 % (v/v) heat-inactivated horse serum, 1 % (v/v) penicillin/streptomycin and 1 % (v/v) L-glutamine. During differentiation DM was replaced every 48 h for 6 days until cell fusion and multi-nucleated myotubes formation was achieved. Successful cell differentiation was determined by expression of the main contractile protein myosin heavy chain (MyHC) as measured by immunoblotting.

### 2.2 CS Exposure Experiments

Experiments were held on day 7 of differentiation when the cells have completed their differentiation into elongated multi-nucleated myotubes. Exposure to CS was performed by a system consisting of a chamber attached to a vacuum pump and a negative pressure gauge (up to 600 mmHg) at one end and a cigarette at the other end. Myotube plates were placed inside the chamber. Then, the vacuum pump was activated, valve B was closed and valve A was opened until a desired level of negative pressure was created inside the chamber. By using the vacuum pump the pressure inside the chamber was reduced relatively to the atmospheric pressure outside. Subsequently, a TIME commercial cigarette containing 14 mg of tar and 0.9 mg of

nicotine and filter (Dubek Ltd., Tel Aviv, Israel) was lit, valve A was closed and valve B between the burning cigarette and chamber was opened for 10 s allowing CS to enter the chamber. Creating reduced pressure inside the chamber allowed the drawing of CS from the burning cigarette into the chamber. Thus, the level on negative pressure inside the chamber equated the dose of CS entering the chamber. Smoke passing through the cigarette filter was considered as vapor phase CS. After exposure to CS, the chamber with the myotube plates was sealed and transferred for different incubation times at 37 °C. Sham-air exposed myotube plates were used as control. In the experiments that examined the effects of CS in increasing incubation periods, control plates were subjected to the most prolonged incubation time with exposure to air instead of CS.

### 2.3 Measurement of Myotube Diameters

Myotube plates were photographed after CS exposure experiments using a digital camera (Olympus UC30) mounted on a phase contrast microscope (Olympus CK40-SLP) at  $\times 20$  magnification. Following experiments, nine fields of view were chosen randomly and ten largest myotubes in each field were measured in a blinded fashion without knowledge of treatment using Image J software. Mean values constituted a measure of 90 myotubes for each experiment. Results were expressed as percent of myotube diameters of the sham-air exposed control plates.

### 2.4 Cell Lysates Preparation and Western Blot Analysis

Following the CS exposure experiments, cells were washed twice by PBS and lysed for cytosolic proteins using 400  $\mu$ l/plate lysis buffer consisting of 50 mM Tris HCl pH 7.4, 300 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 200 mM EDTA and 0.1 % Triton  $\times 100$ . Protease inhibitor, diluted 40 $\times$ , and phosphatase inhibitor cocktails (Sigma-Aldrich,

St. Louis, MO) were added to lysis buffer just prior to use. Cells were scraped and transferred to micro-centrifuge tubes for incubation on ice for 10 min followed by centrifugation at 4 °C and 14,000 RPM for 10 min. Supernatants containing cytosolic proteins were collected and kept at –80 °C. Total protein concentrations were measured by Bradford assay (Bio-Rad) using bovine serum albumin as standard. A total protein of 20 µg/lane was loaded and separated by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following SDS-PAGE, proteins were transferred to nitrocellulose membranes. Membranes were blocked with 5 % non-fat milk powder in TBS-T (0.125 % Tween) for 1 h and exposed overnight to primary antibody at 4 °C. Primary antibodies against the following proteins were used: MyHC (1:1,000), MAFbx/atrogen-1 (1:1,000), MuRF1 (1:1,000) (Santa Cruz Biotechnology, Santa Cruz, CA), actin (1:4,000) (Chemicon International; Temecula, CA), p38 mitogen-activated protein kinase (MAPK) (1:1,000), phospho-p38 MAPK (1:1,000) (R&D Systems; Minneapolis, MN). The next day, membranes were washed with TBS-T followed by 1 h incubation at ambient temperature with appropriate secondary antibodies conjugated to horse-radish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Detection was performed by enzyme-linked chemiluminescence (ECL) using ImageQuant LAS 4,000 digital imager system (GE Healthcare Life Sciences; Rehovot, Israel). Protein quantities were determined by densitometry and analyzed using Total Lab Software.

## 2.5 Protein Loading Control – The Ponceau S Staining Technique

Since one of our objectives was to examine the effects of CS on actin protein, this protein could not be used as an internal control for protein loading because it was degraded by our treatments. Therefore, we used a quantitation of total proteins by the Ponceau staining before antibody probing as an alternative to housekeeping

protein blotting. Romero-Calvo et al. (2010) have shown that reversible Ponceau S staining can be used advantageously over actin detection for equal loading control in Western blotting. Ponceau S is a non-specific protein dye; all proteins in the membrane are colored. After transfer of proteins to nitrocellulose membranes, the membranes were rinsed in Ponceau S solution (Bio-Rad) for 10 min, followed by a brief rinse in double-distilled water (DDW), so that the lanes and bands were clearly visible. Membranes were then inserted in-between transparency sheets and scanned using a standard scanner. Total protein quantity in each lane was determined by densitometry of the scanned membrane using Total Lab Software and used for normalization. At each lane, ECL detected proteins were quantified relatively to total protein quantification found by densitometry of Ponceau S staining. Subsequently, membranes were rinsed once more in DDW until the staining was completely eliminated. From that point on, the blocking and antibody incubation steps were continued as usual.

## 2.6 RNA Purification, Reverse Transcription, and Quantitative Real Time PCR (qPCR)

Purification of total RNA from myotubes was performed by High Pure RNA Isolation Kit (Roche) according to the manufacturer's instruction. RNA concentrations were quantified at 260 nm by a nano-drop spectrophotometer. Samples were diluted to equal concentrations containing 1 µg of RNA. Samples were used to synthesize cDNA with High Capacity cDNA Revers Transcription Kit (Applied Biosystems; Foster City, CA) using MultiScribe Reverse Transcriptase, RT buffer, 100 mM dNTP mix, RT random primers, RNase inhibitor, and nuclease free H<sub>2</sub>O for a final volume of 20 µl.

qPCR was performed using Corbett Rotor-Gene 6,000 (Qiagen; Hilden, Germany) and qPCR SYBR Green ROX Mix (Thermo Fischer Scientific, Waltham MA). Before qPCR, the efficiency of amplification was determined for each primer set. All primer sets were tested for

efficiency >90 % as required for the  $\Delta\Delta C_t$  relative quantification algorithm. Three microliters of diluted cDNA was used as template; 2  $\mu$ l of forward and reverse primer mix (2  $\mu$ M) was added to 5  $\mu$ l of SYBR Green ROX Mix master. Reactions were performed in a 10  $\mu$ l reaction volume under the following conditions: Step 1–15 min at 95 °C; Step 2–5 s at 95 °C; Step 3–30 s at 60 °C, with 40 repeats of Steps 2 and 3. For each sample, a value of the threshold cycle ( $C_t$ ) was calculated using Rotor Gene 6,000 series software based on the time changes in mRNA expression level calculated subsequent to normalization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The abundance of target mRNA relative to GAPDH was determined by the  $\Delta\Delta C_t$  relative quantification method. Single products and specific melting temperatures were assessed by melting curve. The following primers (Sigma-Aldrich, St. Louis, MO) were designed by PrimerBank database and checked for specificity using BLAST: GAPDH forward: 5'-AGGTCG GTGTGAACGGATTG-3' and reverse: 5'-TGT AGACCATGTAGTTGAGGTCA-3'; MAFbx/atrogen-1 forward: 5'-CAGCTTCGTGAGCGAC CTC-3' and reverse: 5'-GCAGTCGAGAAGTCC AGTC-3'; MuRF1 forward: 5'-GTGTGAG GTGCCTACTTGCTC-3' and reverse: 5'-GCTC AGTCTTCTGTCCTTGA-3'.

## 2.7 NF- $\kappa$ B Signaling Inhibition

To investigate the involvement of NF- $\kappa$ B in CS induced catabolism of myotubes, cultures were pretreated with 10  $\mu$ M IMD-0354 (Sigma-Aldrich, St. Louis, MO), a selective I $\kappa$ B kinase (IKK) inhibitor (Tanaka et al. 2005), 24 h prior to CS exposure. Myotube diameters, MyHC levels, NF- $\kappa$ B activation, and muscle specific E3s expression were determined and compared with myotubes exposed to CS without IMD-0354 pretreatment.

## 2.8 p38 MAPK Inhibition

To investigate the involvement of p38 MAPK in CS induced catabolism of myotubes, cultures

were pretreated with 5  $\mu$ M SB203580 (Sigma-Aldrich, St. Louis, MO), a specific inhibitor of p38 MAPK (Li et al. 2005), 15 min prior to CS exposure. NF- $\kappa$ B activation was examined and compared with myotubes exposed to CS without SB203580 pretreatment.

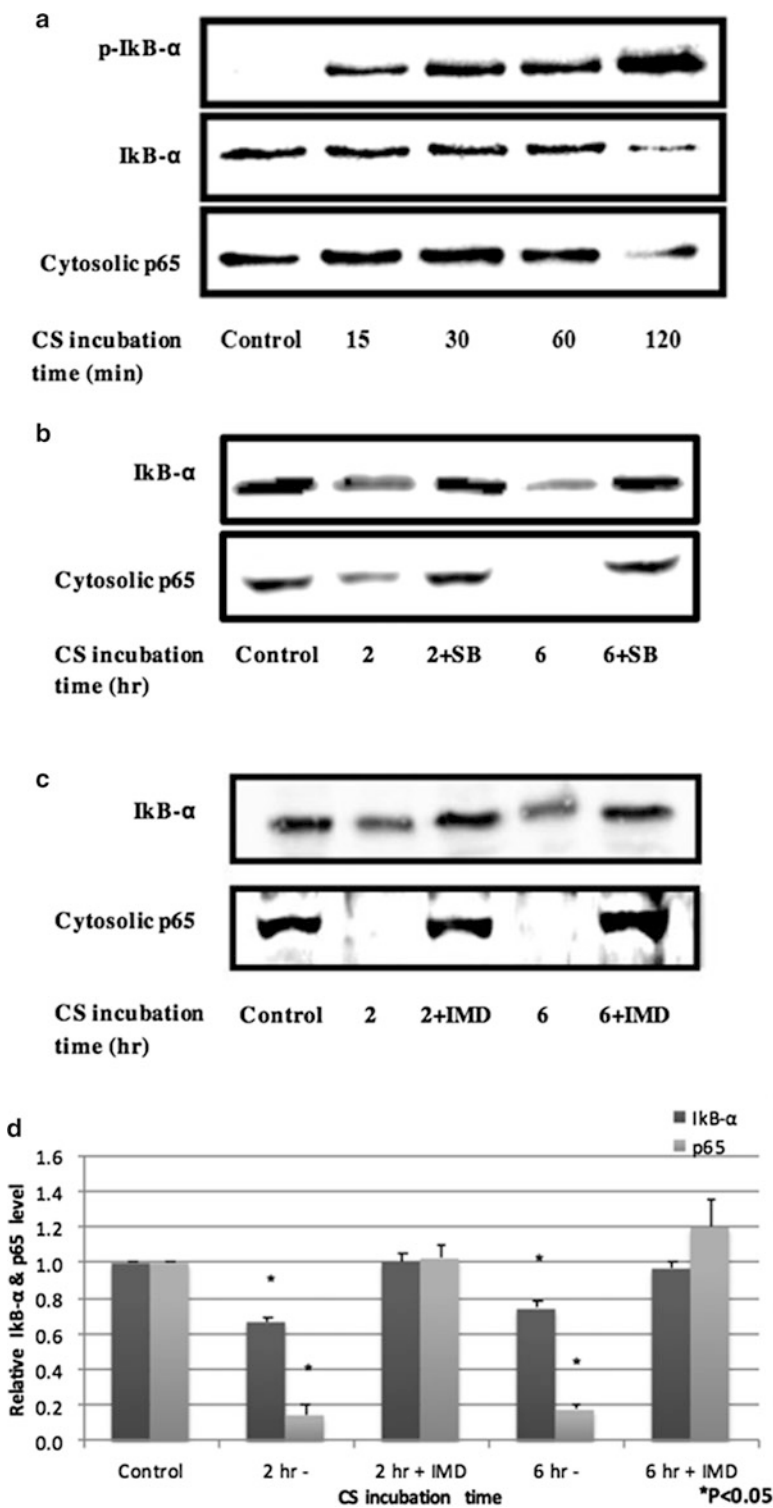
## 2.9 Statistical Analysis

Statistical analysis was performed with a *t*-test and one-way ANOVA followed by Tukey or Dunnett's tests using SPSS Statistics 16 software (SPSS, IBM, Chicago, IL).  $p < 0.05$  was considered statistically significant. Results were expressed as means  $\pm$  SE of three independent experiments.

## 3 Results

### 3.1 CS Stimulates NF- $\kappa$ B Signaling Activation

To explore the effects of CS on NF- $\kappa$ B activation, myotubes were exposed to CS at negative pressure level of 50 mmHg followed by incubation at increasing time periods up to 2 h. Afterwards, myotubes were lysed and subjected to Western blot analysis. Following 2 h of CS exposure, maximal I $\kappa$ B phosphorylation, I $\kappa$ B degradation, and a decrease in NF- $\kappa$ B p65 cytoplasmatic protein level were detected, which indicates NF- $\kappa$ B activation (Fig. 2.1a). Furthermore, it was essential to determine whether this activation was directly CS-induced or triggered indirectly by the known p38 MAPK pathway. For that, myotubes were exposed to CS at negative pressure of 50 mmHg in the presence and absence of p38 MAPK inhibitor, SB-203580. Following CS exposure, myotubes were incubated at increasing time periods up to 6 h, according to the delayed activation observed in Fig. 2.1a. Following incubation, myotubes were lysed and subjected to Western blot analysis to examine I $\kappa$ B- $\alpha$  and p65 protein levels. Control myotubes were exposed to air at the same level of negative pressure and incubated for 6 h. Pretreatment with 5  $\mu$ M, SB-203580 15 min prior to CS exposure prevented CS induced



**Fig. 2.1** CS exposure activates NF-κB activation via IκB-α degradation and p65 translocation from the cytoplasm into the nucleus in C2 myotubes. (a) Myotubes were exposed to CS at negative pressure of 50 mmHg and incubated for increasing time periods.

Sham-air exposed myotubes served as control. Following incubation with CS, cell lysates were prepared and subjected to Western blot analysis using antibodies against: Phospho-IκB, IκB-α and NF-κB p65 proteins. (b) Myotubes were exposed to CS at negative pressure

significant I $\kappa$ B- $\alpha$  degradation and NF- $\kappa$ B p65 translocation from the cytoplasm (Fig. 2.1b). Subsequently, to investigate inhibition of CS-induced activation of NF- $\kappa$ B signaling activation, myotubes were exposed to CS at negative pressure of 50 mmHg in the presence or absence of IKK kinase inhibitor, IMD-0354. Following the described procedure, I $\kappa$ B- $\alpha$  and p65 protein levels were analyzed. A significant decrease in I $\kappa$ B- $\alpha$  and p65 protein were observed after 2 and 6 h of CS incubation. Pretreatment with 10  $\mu$ M of IMD-0354, 24 h prior to CS exposure prevented CS induced I $\kappa$ B- $\alpha$  degradation and NF- $\kappa$ B p65 translocation from the cytoplasm (Fig. 2.1c, d).

### 3.2 NF- $\kappa$ B Signaling Inhibition Prevents CS Induced Reduction in Myotubes Diameter Breakdown of MyHC

To evaluate NF- $\kappa$ B signaling involvement in CS induced catabolism of skeletal muscle, myotubes were exposed to CS at negative pressure level of 50 mmHg for 6 h in the absence or presence of 10  $\mu$ M of the NF- $\kappa$ B inhibitor IMD-0354, 24 h before exposure. Control myotubes were exposed to air at 50 mmHg for 6 h. Following incubation, myotubes were photographed and diameters were measured as described in the methods. The myotube diameter decreased depending on the CS incubation time. A significant reduction in myotube diameters was found following 6 h of incubation with CS in the absence of IMD. However, IMD prevented this decrease in myotube diameters following the same incubation protocols (Fig. 2.2a–c, e).

To examine the role of NF- $\kappa$ B signaling in CS-induced skeletal muscle catabolism, myotubes were exposed to CS at negative pressure of 50 mmHg followed by 6 h of incubation in the absence or presence of 10  $\mu$ M of IMD-0354, 24 h before exposure. Control myotubes were exposed to air at the same level of negative pressure and incubated for 6 h. Then, myotubes were lysed and subjected to Western blot analysis. MyHC levels decreased significantly following 6 h of incubation with CS in the absence of IMD. Nevertheless, MyHC levels did not change significantly in the presence of IMD. As expected, actin levels did not decrease significantly at any treatment (Fig. 2.2d, f).

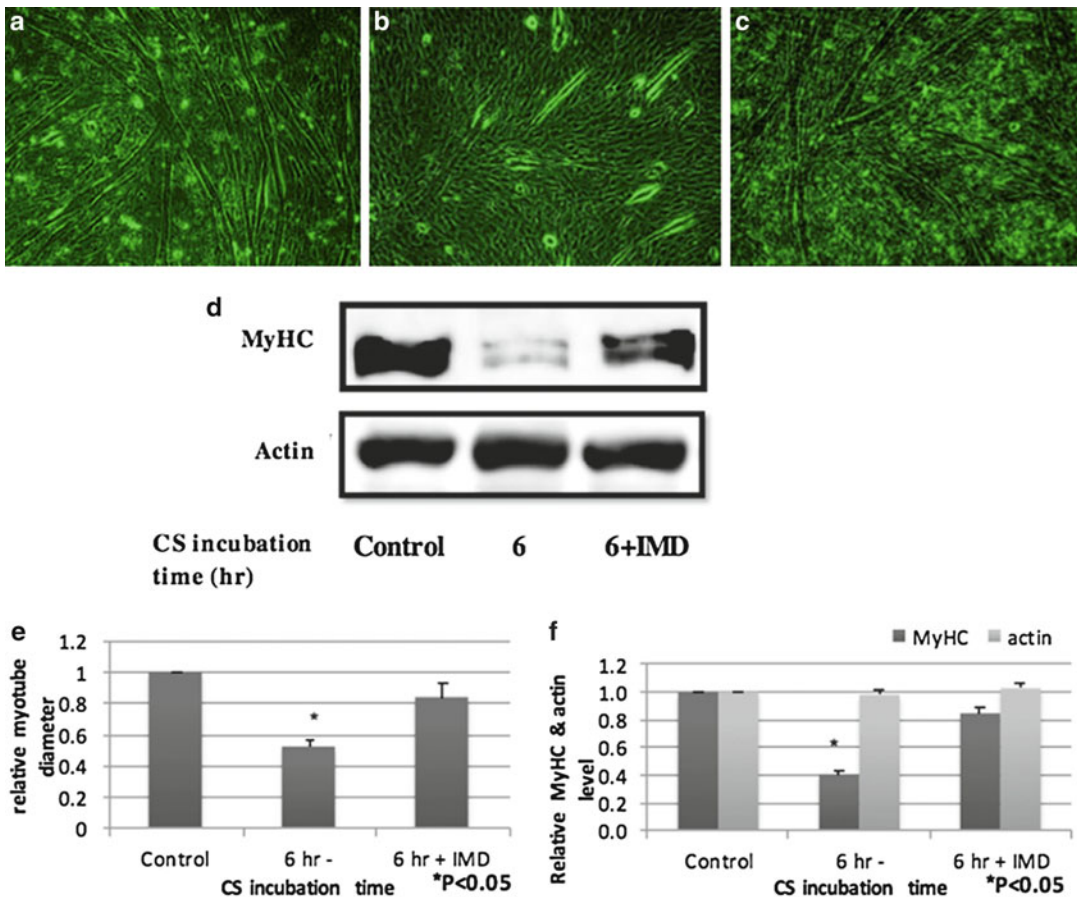
### 3.3 NF- $\kappa$ B Is Involved in CS-Induced Upregulation of MuRF1

To examine the involvement of NF- $\kappa$ B in CS-induced upregulation of E3s MAFbx/atrogen-1 and MuRF1 in skeletal myotubes, myotubes were exposed to CS at negative pressure of 50 mmHg and incubated for increasing time periods in the absence and presence of 10  $\mu$ M IMD-0354, 24 h before exposure. Control myotubes were exposed to air at the same level of negative pressure and incubated for 3 h. Following incubation, RNA purification, reverse transcription and qPCR were performed as described in the methods. A significant increase in MAFbx/atrogen-1 and MuRF1 mRNA levels was detected following 1 and 3 h of incubation with CS at 50 mmHg. However, no significant upregulation in MuRF1 mRNA was shown following pretreatment with IMD-0354 whereas in MAFbx/atrogen-1 the up-regulation persisted

**Fig. 2.1** (continued) of 50 mmHg and incubated for increasing time periods in the presence or absence of 5  $\mu$ M of p38 MAPK inhibitor SB-203580. Sham air exposed myotubes served as control. Following incubation with CS, cell lysates were prepared and subjected to Western blot analysis using antibodies against p65 and I $\kappa$ B- $\alpha$  proteins. (c) Myotubes were exposed to CS at negative pressure of 50 mmHg and incubated for increasing time periods in the presence or absence of 10  $\mu$ M of NF- $\kappa$ B inhibitor

IMD-0354. Sham air exposed myotubes served as control. Following incubation with CS, cell lysates were prepared and subjected to Western blot analysis using antibodies against p65 and I $\kappa$ B- $\alpha$  proteins. (d) p65 and I $\kappa$ B- $\alpha$  protein levels were normalized by total protein densitometry detected by Ponceau S staining and expressed relative to the corresponding value of sham-air exposed control myotubes. Results are expressed as means  $\pm$  SE of three different experiments. \* $p$  < 0.05 vs. control myotubes





**Fig. 2.2 NF- $\kappa$ B inhibition prevents CS-induced reduction in diameter and degradation of MyHC in C2 myotubes.** Myotubes were exposed to CS at negative pressure of 50 mmHg and incubated for 6 h in the presence or absence of 10  $\mu$ M of NF- $\kappa$ B inhibitor IMD-0354 (IMD). Myotubes were photographed at  $\times 20$  magnification following increasing incubation time with CS: (a) Control (Sham air exposure for 6 h), (b) 6 h with IMD, (c) 6 h. (d) Following incubation with CS, cell lysates were prepared and subjected to Western blot analysis using antibodies against MyHC and actin proteins. (e) Changes

in myotube diameters are expressed as percent of the diameter in sham-air control myotubes. Results are relative to control and expressed as means  $\pm$  SE of three different experiments. \*p < 0.05 vs. control myotubes. (f) MyHC and actin protein levels were normalized by total protein densitometry detected by Ponceau S staining and expressed relative to the corresponding value of sham-air exposed control myotubes. Results are relative to control and expressed as mean  $\pm$  SE of three different experiments. \*p < 0.05 versus control myotubes

(Fig. 2.3a). Furthermore, to examine the effects of CS exposure on MAFbx/atrogen-1 and MuRF1 protein levels, myotubes were exposed to CS at 50 mmHg for increasing incubation time periods in the absence or presence of IMD-0354. Control myotubes were exposed to air at the same level of negative pressure and incubated for 6 h. Protein levels were examined by Western blot using appropriate antibodies. Pretreatment with IMD-0354 inhibited the MuRF1 activation, as opposed

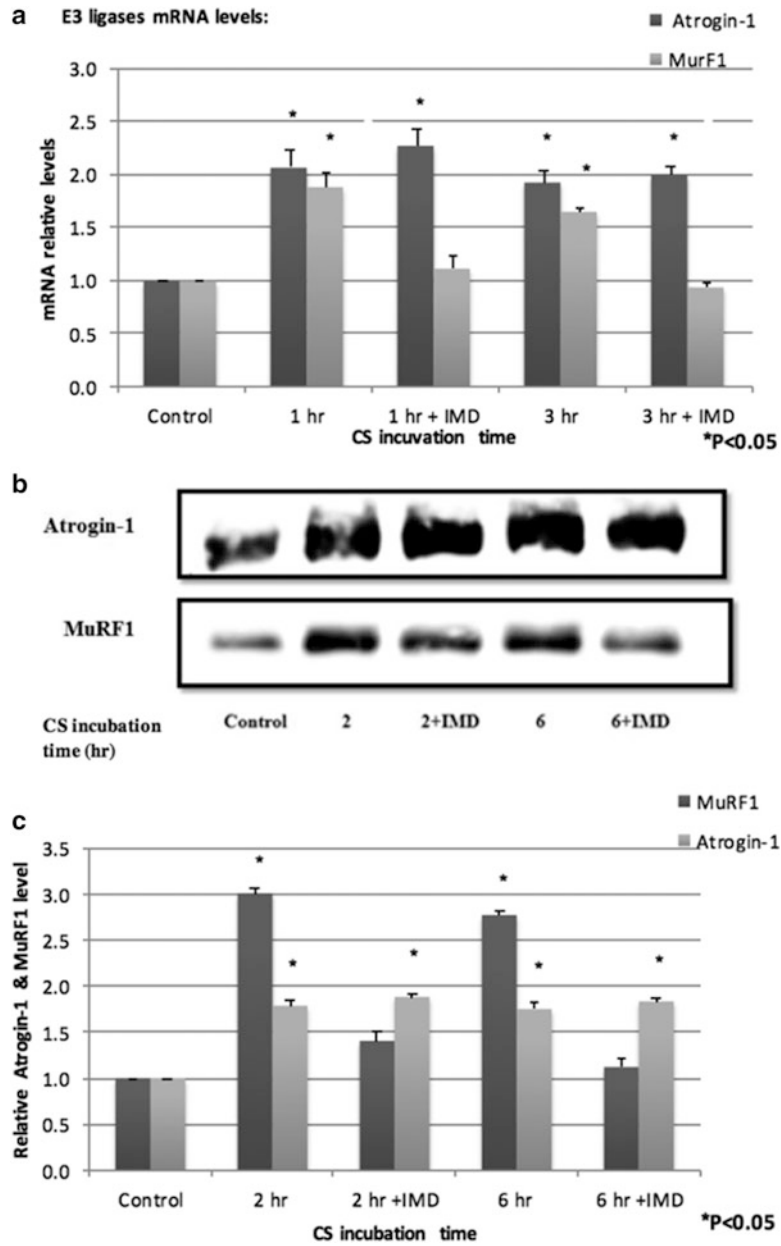
to the lasting MAFbx/atrogen-1 activation (Fig. 2.3b, c).

## 4 Discussion

CS is known to be one of the environmental stimuli that causes NF- $\kappa$ B activation (Ahn and Aggarwal 2005). Thus, it was interesting to explore the effects of CS on NF- $\kappa$ B activation in



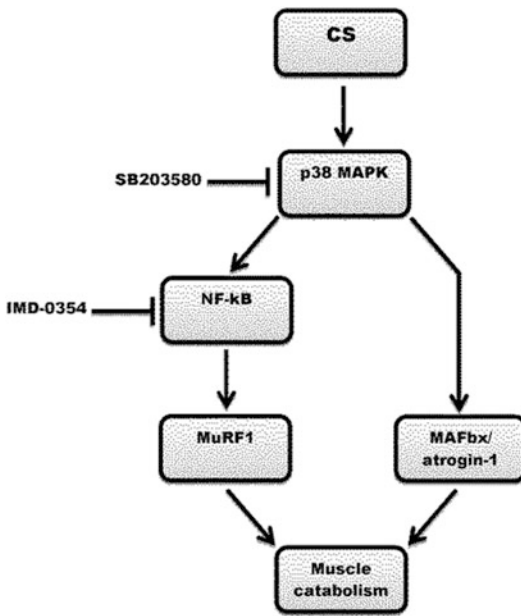
**Fig 2.3** NF- $\kappa$ B inhibition prevents CS-induced upregulation of MuRF1, but not that of MAFbx/atrogin-1. (a) Myotubes were exposed to CS at negative pressure of 50 mmHg in the presence or absence of 10  $\mu$ M of NF- $\kappa$ B inhibitor IMD-0354 (IMD) and incubated for increasing time periods. Sham-air exposed myotubes served as control. Following incubation with CS, total RNA was isolated and subjected to reverse transcription and qPCR analysis to determine the expression of MAFbx/atrogin-1 and MuRF1. Data were normalized by GAPDH expression and are relative to the corresponding value of sham air exposed control myotubes. (b) Following incubation with CS cell lysates were prepared and subjected to Western blot analysis using antibodies against MAFbx/atrogin-1 and MuRF1 proteins. (c) Proteins level were normalized by total protein densitometry detected by Ponceau S staining and expressed relative to the corresponding value of sham-air exposed control myotubes. Results are expressed as means  $\pm$  E of three different experiments. \* $p < 0.05$  vs. control myotubes



muscle myotubes. In a recent study, we have shown that the first step of CS induced muscle catabolism is oxidative stress (Hasnis et al. 2007). Consequently, p38 MAPK phosphorylation was triggered, which resulted in upregulation of

muscle specific E3s. In the current study, it was found that CS stimulates I $\kappa$ B degradation and p65 translocation from the cytoplasm into the nucleus.

Recently, it has been shown that CS exposure causes MyHC degradation in skeletal myotubes



**Fig. 2.4** Induction of muscle specific E3 ligases by CS

in vitro (Liu et al. 2011). In the present study, the involvement of NF-κB activation in catabolic effects of C2 myotubes exposure to CS was explored by examining changes in myotube diameters and levels of the most important contractile proteins, MyHC, and actin in the absence or presence of NF-κB inhibitor IMD-0354. Our study demonstrate that prolonged CS exposure resulted in MyHC degradation and reduction in myotube diameters. Furthermore, NF-κB inhibition resulted in a maintained level of MyHC and myotube diameters following prolonged incubation with CS.

Previous studies have shown that exposure of skeletal muscle cells to various ROS and RNS promotes muscle catabolism through activation of the ubiquitin proteasome system (UPS) (Bar-Shai and Reznick 2006) and upregulation of MAFbx/atrogen-1 and MuRF1 (Li et al. 2003). The UPS and the two muscle-specific E3s MAFbx/atrogen-1 and MuRF1 play an important role in the process of muscle wasting degradation (Meng and Yu 2010). Our results demonstrate that upregulation of MuRF1, but not that of MAFbx/atrogen-1 in skeletal myotubes exposed to vapor phase CS was abolished by NF-κB

inhibition. These findings demonstrate the role of NF-κB in muscle specific E3 MuRF1 expression and muscle wasting caused by CS exposure. p38 MAPK was previously suggested to trigger the upregulation of MAFbx/Atrogen-1 (Glass 2005; Li et al. 2005). Also, pretreatment with p38 MAPK inhibitor SB203580 blunted the increase in MAFbx/atrogen-1 gene expression. MuRF-1 transcription is believed to be driven by the activation of NF-κB (Cai et al. 2004; Glass 2005; Meng and Yu 2010). In our study, the observed upregulation of MuRF1 following exposure to CS was demonstrated to be a result of direct activation of NF-κB by CS. Another possibility for triggering upregulation of MuRF1 following CS exposure is a biochemical cross-talk between p38 MAPK and the NF-κB pathways. CS activates p38 MAPK, which in turn may activates transcriptional activity of NF-κB leading to MuRF1 upregulation in C2 myotubes (Rom et al. 2012). This possible cross-talk was demonstrated previously by exposing C2C12 myotubes to the inflammatory IL-1 (Li et al. 2009). IL-1 exposure to C2C12 caused p38 MAPK and NF-κB activation resulting in overexpression of both MAFbx/atrogen-1 and MuRF1 leading to loss of myofibrillar proteins and wasting of myotubes (Li et al. 2009).

In conclusion, we show that exposure of vapor phase CS to cultured skeletal myotubes caused atrophy and degradation of muscle proteins via activation of NF-κB pathway and upregulation of MuRF1 in addition to the well known involvement of CS-induced p38 MAPK phosphorylation. Within the limitation of an in vitro study, our findings provide an extra possible molecular mechanism for the complex catabolic effects of CS in skeletal muscle (Fig. 2.4).

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

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