Apocynin prevents cigarette smoking-induced loss of skeletal muscle mass and function by preserving proteostatic signalling

Stanley Chan[1], Ivan Bernardo[1], Chanelle Mastronardo[1], Huei Seow[1], Kevin Mou[1], Aleksandar Dobric[1], Kurt Brassington[1], Simone De Luca[1], Stavros Selemidis[1], Steven Bozinovski[1], and Ross Vlahos[1]

[1]RMIT University

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Abstract

Background and Purpose: Cigarette smoking (CS) is the major risk factor for developing COPD and related skeletal muscle dysfunction. It has been postulated that CS exposure may directly causes muscle dysfunction via the induction of oxidative stress. The present study examined the effect of a potent Nox inhibitor and ROS scavenger, apocynin on CS-induced muscle dysfunction. Experimental Approach: Male BALB/c mice were exposed to either room air (sham) or CS generated from 9 cigarettes per day, 5 days a week for 8 weeks with or without apocynin treatment (5 mg·kg-1 w/v, intraperitoneal injection). C2C12 myotubes exposed to either hydrogen peroxide (H2O2) or water-soluble cigarette smoke extract (CSE) with or without apocynin (500 nM), was set up as an experimental model in vitro. Key Results: Eight weeks of CS exposure caused significant lung inflammation and muscle dysfunction in mice; evidenced by a 10% loss in muscle mass and 54% loss in contractile function of tibialis anterior, attributable to altered myogenic homeostasis and protein oxidation. These effects were prevented by apocynin administration. In C2C12 myotubes, direct exposure to H2O2 or CSE caused myofiber wasting, which was associated with altered myogenic homeostasis marked by ˜50% loss in muscle-derived insulin-like growth factor (IGF)-1 and 1.5-fold increase in myostatin expression. Apocynin treatment completely attenuated CSE-induced Nox2 expression, preserving muscle-derived IGF-1 expression and downstream mammalian target of rapamycin (mTOR) signaling pathway, thereby preventing myofiber wasting. Conclusion and Implications: Targeted pharmacological inhibition of Nox-derived ROS may alleviate the lung and systemic manifestations in smokers with COPD.

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Steven Bozinovski, Ross Vlahos*

School of Health and Biomedical Sciences, RMIT University, BUNDOORA,

VIC 3083, Australia;

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***Author for correspondence**

Professor Ross Vlahos, PhD

School of Health and Biomedical Sciences

RMIT University, PO Box 71, Bundoora, VIC 3083 Australia

Tel: +61 3 9925 7362

Email: ross.vlahos@rmit.edu.au

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The authors declare no confliction of interest in this study.

What is already known:

- Skeletal muscle dysfunction is a major COPD comorbidity that predicts disease morbidity and mortality.
- Skeletal muscle dysfunction may be evoked by oxidative stress from cigarette smoke (CS) exposure.

What this study adds:

- By disrupting myogenic homeostasis, CS-induced oxidative stress causes skeletal muscle dysfunction.
- Apocynin preserved muscle mass and function against the detrimental oxidative effects of CS exposure

Clinical significance:

Targeting oxidative stress may improve pulmonary and systemic outcomes associated with COPD.

Abstract

Background and Purpose : Cigarette smoking (CS) is the major risk factor for developing COPD and related skeletal muscle dysfunction. It has been postulated that CS exposure may directly causes muscle dysfunction via the induction of oxidative stress. The present study examined the effect of a potent Nox inhibitor and ROS scavenger, apocynin on CS-induced muscle dysfunction.

Experimental Approach: Male BALB/c mice were exposed to either room air (sham) or CS generated from 9 cigarettes per day, 5 days a week for 8 weeks with or without apocynin treatment (5 $mg{\cdot}kg^{-1}$ w/v, intraperitoneal injection). C2C12 myotubes exposed to either hydrogen peroxide (H_2O_2) or water-soluble cigarette smoke extract (CSE) with or without apocynin (500 nM), was set up as an experimental model *in vitro* .

Key Results: Eight weeks of CS exposure caused significant lung inflammation and muscle dysfunction in mice; evidenced by a 10% loss in muscle mass and 54% loss in contractile function of tibialis anterior, attributable to altered myogenic homeostasis and protein oxidation. These effects were prevented by apocynin administration. In C2C12 myotubes, direct exposure to H_2O_2 or CSE caused myofiber wasting, which was associated with altered myogenic homeostasis marked by ˜50% loss in muscle-derived insulin-like growth factor (IGF)-1 and 1.5-fold increase in myostatin expression. Apocynin treatment completely attenuated CSE-induced*Nox2* expression, preserving muscle-derived IGF-1 expression and downstream mammalian target of rapamycin (mTOR) signaling pathway, thereby preventing myofiber wasting.

Conclusion and Implications: Targeted pharmacological inhibition of Nox-derived ROS may alleviate the lung and systemic manifestations in smokers with COPD.

Keywords: Chronic Obstructive Pulmonary Disease, NADPH oxidase, IGF-1, Antioxidants, Protein carbonylation.

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by progressive airflow limitation that is not fully reversible (Vogelmeier et al., 2017). Cigarette smoking (CS) is the major cause of COPD accounting for 80-90% of cases in industrialized countries (Vogelmeier et al., 2017). In addition to the pulmonary pathologies, COPD may also give rise to debilitating conditions (i.e. comorbidities) in extra-pulmonary tissues which may lead to a deterioration of function, quality of life and mortality (Fabbri & Rabe, 2007). Skeletal muscle dysfunction is considered to be one of the most common comorbidities that affects up to 40% of COPD patients (Passey, Hansen, Bozinovski, McDonald, Holland & Vlahos, 2016). Skeletal muscle dysfunction limits exercise performance and capacity, thereby is detrimental to the overall health of those suffering from COPD irrespective of the lung function decline (Swallow et al., 2007). In line with this, muscle dysfunction has also been demonstrated to be a major risk factor for future acute exacerbations and hospital readmission of COPD patients (Vilaro et al., 2010), suggesting skeletal muscle function may be a determinant of health outcomes in these patients.

Muscle dysfunction can be defined as the inability of a muscle to perform its task, as a result of reduced strength and/or endurance, leading to the manifestation of muscle weakness and fatigue (Yamano, Kawai, Minami, Hiraga & Miyata, 2010). By definition, muscle weakness (i.e. loss of strength) and fatigue (i.e. loss of endurance) are distinct conditions, however, the observation that a weak muscle becomes more easily fatigued have highlighted the inseparable nature of the two (Yamano, Kawai, Minami, Hiraga & Miyata, 2010). Indeed, both reduced force-generating capacity and fatigue resistance have been observed in limb muscles of COPD patients leading to exercise intolerance (Vogelmeier et al., 2017).

The observations that even a single session of smoking was sufficient to result in decreased exercise capacity (Hirsch, Sue, Wasserman, Robinson & Hansen, 1985), and that non-symptomatic smokers more often complain of fatigue than non-smokers (Corwin, Klein & Rickelman, 2002), have led to the concept that CS may directly impact on muscle function. In non-symptomatic smokers and patients with COPD, Barreiro *et al* . (Barreiro et al., 2010) demonstrated that CS exposure directly elicits oxidative stress in the vastus lateralis muscle which may contribute to atrophy and dysfunction. Importantly, the same study also reported no significant rise in muscle inflammation amongst smokers and COPD patients, thus confirming the direct effects of CS exposure on muscle dysfunction which may be exerted through an oxidative stress-driven mechanism that is independent of inflammation.

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) is a multimeric enzyme that catalyzes the formation of reactive oxygen species (ROS) - superoxide anion (O_2^-) which is the parent species amongst a family of molecules that ultimately contribute to oxidative stress (Griffith, Pendyala, Hecker, Lee, Natarajan & Thannickal, 2009). Not only O_2^- in itself is a potent oxidant, but it can be converted into hydrogen peroxide (H_2O_2), which is a more influential form of ROS in in terms of redox signaling with a longer half-life (Griffith, Pendyala, Hecker, Lee, Natarajan & Thannickal, 2009). The role of Nox-derived ROS has long been recognized in the pathogenesis of COPD. However, deletion of *Nox2* or its catalytic subunit, $p47^{phox}$, was found to result in greater lung inflammation and alveoli destruction in mice exposed to CS, despite showing decreased ROS production (Yao et al., 2008). This suggests normal expression of Nox2 is essential for maintaining redox and immune homeostasis.

In skeletal muscle, a functional Nox enzyme complex has been detected at the plasma membrane during muscular contraction, suggesting its active involvement in muscle function (Sakellariou et al., 2013). However, the exact role of Nox-derived ROS in CS-induced muscle dysfunction remains unclear. Given the detrimental effects of genetic disruption of Nox, the present study opted a pharmacological inhibitor approach, using apocynin. Apocynin inhibits Nox activation by blocking the cytosolic to membrane translocation of $p47^{phox}$ and $p67^{phox}$, thereby disrupting the assembly of the active enzyme complex (Johnson et al., 2002). Furthermore, apocynin has also been shown to act as a scavenger for O_2^- and other ROS (Heumuller et al.,

2008). For these reasons, the present study aimed to examine the role of Nox-derived ROS in CS-induced muscle dysfunction using apocynin. We hypothesize that inhibition of Nox-derived ROS would attenuate lung inflammation and muscle dysfunction induced by CS exposure.

Materials and methods

Mice

Male BALB/c mice (7 weeks of age) were obtained from the Animal Resource Centre (Perth, Australia). Mice were housed in micro-isolator cages at 21°C on a 12-hour day/night cycle with free access to food and water. After four days of acclimatization, mice were randomly assigned to room air (sham) and cigarette smoke (smoke) exposure groups with or without daily supplementation of apocynin (5 $mg{\cdot}kg^{-1}$) via intraperitoneal (i.p.) injection. The vehicle groups were injected with saline (solvent of apocynin). Mice were weighed three times a week with daily monitoring. All experiments were conducted in accordance with the Australian Code of Practice for the Care of Experimental Animals, the ARRIVE Guidelines and with RMIT University Animal Ethics Committee approval (Animal Ethics Application Number 1521).

Cigarette smoke exposure and muscle function analysis

Mice were placed in 18L perspex chambers and exposed to CS from three cigarettes (Winfield Red, 16 mg or less of tar, 15 mg or less of carbon monoxide, 1.2 mg or less of nicotine; Philip Morris, Australia) spaced evenly over 1 hour and carried out three times per day (09:00, 12:00, and 15:00 h), five days a week (Monday to Friday) for 8 weeks. The sham mice were handled identically and exposed to room air. We have previously shown that this CS exposure protocol in male Balb/C mice replicates key clinical traits of human COPD, including lung inflammation and pathology (emphysema , mucous hypersecretion, impaired lung function), increased lung and systemic oxidative stress and comorbidities including skeletal muscle dysfunction (Austin, Crack, Bozinovski, Miller & Vlahos, 2016; Chan et al., 2020; Vlahos & Bozinovski, 2014). At the end of protocol, *in situ* muscle function analysis was performed as previously described (Chan et al., 2020). In brief, mice were anaesthetised with ketamine (80 mg/kg BW) /xylazine (16mg/kg BW) and small incisions were then made on the skin to expose the tibialis anterior (TA) muscle taking care not to damage the fascia. The mouse was secured on the heated platform (37^{o}C) of an*in situ* contractile apparatus (809B *in situ* Mouse Apparatus, Aurora Scientific, Canada) with a pin behind the patellar tendon and a foot clamp. The distal end of the TA was tied firmly to a lever arm attached to an isometric force transducer. Two fine electrodes (3-5mm apart) were inserted into the belly of the TA muscle. The muscle was stimulated by two field stimulating platinum electrodes coupled to an amplifier. The TA muscle was contracted via square wave (0.2 ms) pulses at 10 V from the stimulator (701C stimulator, Aurora Scientific, Canada). Forces were converted to a digital signal and recorded by DYNAMIC MUSCLE ANALYSIS 611A^{TM} (Aurora Scientific, Canada). Optimum muscle length (Lo) was first determined by eliciting twitch contractions by incrementally adjusting muscle length with a micromanipulator until a repeatable maximum peak twitch force was obtained. Optimal muscle length (L_o) was measured with precision digital calipers from the beginning of the distal tendon to the insertion of the TA at the base of the knee. Subsequently, the TA was stimulated at 100 Hz tetanic contraction, followed by a 2 min rest interval, and then twitch contraction. Comparable twitch forces pre and post 100 Hz stimulation indicated that the knots were both secure and unlikely to slip during the remaining protocol. If a decrease in twitch force was observed, the muscle was incrementally tensioned and stimulated between 2 min rest intervals until peak twitch force (Pt) was re-established. To establish the force frequency relationship, the TA was stimulated supramaximally (10 V) for 500 ms at 10, 20, 30, 40, 50, 80, 100, 150, 200, 250, 300 Hz, with a 2 min rest interval in-between.

As contractile force is closely related to muscle mass which is directly proportional to the cross-sectional area (CSA) of myofibers and the intrinsic properties of the contractile machinery within the muscle (Hakim, Wasala & Duan, 2013). To differentiate whether the observed muscle weakness induced by CS was attributed to reduced muscle mass and/or an impaired excitation–contraction coupling, the maximal contractile force at 120 Hertz is normalized to the whole-muscle CSA to produce the specific muscle force. CSA can be

approximated from the gross mass and L_0 of the muscle, together with the muscle density (~1.06 g·cm^{-3}) (Close, 1972). Hence, the following equation is used:

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`image1.emf` available at https://authorea.com/users/356126/articles/487396-apocynin-prevents-cigarette-smoking-induced-loss-of-skeletal-muscle-mass-and-function-by-preserving-proteostatic-signalling

Immediately following the contraction protocol, the mouse was removed from the apparatus and euthanized with an overdose of anaesthetic (Sodium pentobarbitone; 240mg/kg: Virbac Pty. Ltd., Australia) via an i.p. injection to continue with tissue collection and the procedures described below. Muscle mass was measured using an analytical balance after the tendons and other non-muscle tissues have been removed and after brief contact with absorbent material such as filter paper to remove excess solution.

Tissue collection

The lungs were lavaged *in situ* using 0.4ml of ice-cold PBS and three subsequent repeats of 0.3ml PBS, with a return of approximately 1ml of bronchoalveolar lavage fluid (BALF) per mouse as previously published (Chen et al., 2006; Vlahos et al., 2006). 20µL of BALF was diluted 1:1 with Acridine Orange and the total number of viable cells counted on a standard Neubauer haemocytometer under fluorescent light on an Olympus BX53 microscope (Olympus, Japan). To differentiate cell populations in BALF, cytocentrifuge preparations (Shandon Cytospin 3, 400 rpm, 10 min) were performed using approximately 5x10^4 cells from BALF. Once dried, cells were fixed with Shandon Kwik-Diff fixative (Thermo Fischer Scientific, USA) and subsequently stained with Hemacolor® Rapid Red and Blue dye (Merck, Germany) as per manufacturers' instructions, mounted with Enetellan® new (Merck, Australia). Cell types (macrophages, lymphocytes, and neutrophils) were identified according to standard morphological criteria and at least 500 cells per slide were counted. After the lavage procedure, 10ml of PBS was used to clear the lungs of blood via a right ventricular perfusion of the heart. Lungs were then weighed, snap frozen in liquid nitrogen and stored at -80°C until required. Lower limb muscles were removed tendon to tendon from each mouse. The muscles were weighed, snap frozen in liquid nitrogen and stored at -80°C until required.

Quantitative real-time PCR

Total RNA was extracted from tissues and C2C12 myotubes using RNeasy kits (Qiagen, USA), reverse transcribed using High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific, USA) before real time PCR analysis using QuantStudio 7 (Thermo Fisher Scientific, USA). All reactions were performed in triplicate using Taqman Fast Advanced Master Mix and pre-developed gene expression assays (Table 1) except for *Igf1-eb*(Thermo Fisher Scientific) and data obtained were normalized against GAPDH as reference gene prior to analysis using the delta delta CT method as previously described (Vlahos, Stambas, Bozinovski, Broughton, Drummond & Selemidis, 2011).

Table 1. List of gene expression assays

Gene name	Abbreviation	Taqman assay ID
Granulocyte-macrophage colony stimulating factor 2	*Gmcsf*	Mm01290062_m1
Chemokine (C-C motif) ligand 2	*Ccl2*	Mm00441242_m1
Chemokine (C-X-C motif) ligand 2	*Cxcl2*	Mm00436450_m1
Tumor necrosis factor	*Tνφα*	Mm00443258_m1
Insulin like growth factor Ea	*Igf-ea*	Mm00710307_m1
Insulin like growth factor Eb	*Igf-eb*	AIKALFT
Myostatin	*Mstn*	Mm01254559_m1
Cytochrome b-245, beta polypeptide (NADPH oxidase 2)	*Nox2*	Mm01287743_m1
Glutathione peroxidase 1	*Gpx1*	Mm00656767_g1
Interleukin-6	*Il-6*	Mm00446190_m1

Gene name	Abbreviation	Taqman assay ID
F-box protein 32 (MAFbx)	*Fbxo32*	Mm00499523_m1

Cell culture and intervention protocols

C2C12 murine myoblasts (American Type Culture Collection, CRL-1772) were cultured in growth medium consisting of Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, USA) supplemented with 1% penicillin/streptomycin (100 units/mL penicillin and 100 μg/mL streptomycin; Thermo Fisher Scientific, USA) and 10% fetal bovine serum (FBS; Thermo Fisher Scientific, USA). Cells were cultured in a T-75 culture flask at a density of 5 x 10^3 viable cells/cm2 and were passaged at 70-80% confluence. Flasks were kept in a humidified incubator at 37°C with the supplementation of 5% CO_2. To induce differentiation, confluent monolayers of C2C12 myoblasts were cultured in differentiation medium (DM) consist of DMEM supplemented with 1% penicillin/streptomycin and 2% horse serum (Thermo Fisher Scientific, USA) and the DM was changed daily. All experiments were performed on day 6 when most myoblasts have fused to form mature myotubes.

The gas phase of cigarette smoke, otherwise known as cigarette smoke extract (CSE), was prepared by bubbling 1 cigarette (Winfield Red, Phillip Morris International, Australia) through 25 mL of pre-warmed DM at a rate of 5 $mL{\cdot}sec^{-1}$ to produce 100% CSE stock solution. The stock solution was sterile filtered and serially diluted with pre-warmed DM to obtain concentrations required for experimentation. Hydrogen peroxide (H_2O_2; Chem-Supply, Australia) was prepared in sterile water resulting in a 3,000 μM stock solution. The stock solution was serially diluted with pre-warmed DM to obtain the required concentrations for experimentation. Apocynin (Sigma-Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA) to give a stock solution of 500μM (stock solution). The stock solution was diluted with pre-warmed DM to give a final administering concentration of 500nM (68). To ensure bioavailability, apocynin was pre-incubated for 30 min prior to administration of the respective oxidative insults (H_2O_2 or CSE), recapitulating that in our animal model.

Oxyblots and Western blots

˜20mg of muscle tissue was homogenized in 500 μL of RIPA lysis buffer containing 1% protease inhibitors cocktail. For cell experiments, C2C12 myotubes in 6-well plates were collected and homogenised in 100 μL of RIPA lysis buffer. The samples were then centrifuged for 10 min at 14000 x g rpm, 4°C. The supernatant was collected for immediate use or stored at -80°C. Protein concentrations were determined through use of commercially available colorimetric bicinchonnic acid (BCA) protein kit (Thermo Fisher Scientific, USA) to standardize the loading amount for the SDS-PAGE. SDS-PAGE was conducted as previously described (Chan et al., 2013) with specific antibodies against Phospho-eIF2α (Ser51, Cell Signaling Technology, USA #3398), Phospho-S6 Ribosomal Protein (Ser235/236, Cell Signaling Technology, USA #4858), Phospho 4E-BP1 (Thr37/46, Cell Signaling Technology, USA #2855), Fbx32/MAFbx (Abcam, USA ab168372), 19S proteasome (Abcam, USA ab20239), LC3A/B (Cell Signaling Technology, USA #12741), p62 (Cell Signaling Technology, USA #23214), actin (Cell Signaling Technology, USA #8457).

For oxyblots, the extracted protein samples were derivatised and stabilised using the OxyBlot Protein Oxidation Detection kit (Merck, Massachusetts, USA) for immunoblot detection of carbonyl groups, according to manufacturer's instructions. For cell experiments, the protein samples were solubilised by boiling in 1x Laemmli sample buffer containing 10% 2-mercaptoethanol for 10 min. The samples were then loaded into 10% acrylamide gel for SDS-PAGE and immune-detection using the chemiluminescence method as previously described (Chan et al., 2013). Densitometry analysis was performed using the ImageLab software (Bio-Rad Laboratories).

Immunofluorescence and myotube diameter analyses

C2C12 myotubes grown on coverslips coated with Matrigel matrix basement membrane (Sigma-Aldrich, USA). At the end of experiment, the myotubes were fixed in 4% paraformaldehyde in 1X PBS at room

temperature for 30 min and the excess was quenched with 300μM glycine. The fixed myotubes on coverslips were blocked and permeabilised in 10% bovine serum albumin, 2% Triton-X in 1X PBS for 1 hr at room temperature. The coverslips were then rinsed with 0.1% Tween 20 in 1X PBS and incubated overnight at 4°C with fluorophore conjugated antibodies against skeletal muscle myosin (F59 clone; AlexaFluor 488, Santa Cruz Biotechnology, USA). Unbound antibodies were removed by rinsing the coverslips in PBST, excessive moisture was removed before mounting in Fluoroshield with DAPI (Sigma-Aldrich, USA). Fluorescence images were captured using VS120 Olympus Virtual Slide Microscope (Olympus Life Science, Australia). The captured images were analysed using the Olympus cellSens software (Olympus Life Science, Australia). A minimum of 270 myotube diameters were measured for each condition.

Cell viability assay

The CellTiter 96 Aqueous One Solution (MTS) Cell Proliferation Assay (Promega, Australia) was used to determine cell viability of C2C12 myotubes, according to manufacturer's instructions. Briefly, C2C12 myotubes stimulated with cigarette smoke extract (CSE) or hydrogen peroxide (H_2O_2) were washed and incubated in media containing MTS reagent at 37°C and 5% CO_2 for 1 hour. Absorbance was then recorded at 490nm using a plate reader (CLARIOstar Monochrome Microplate Reader; BMG Labtech, Australia). To validate the specificity of the assay, MTS reagent was added to unseeded culture plate containing CSE or H_2O_2 for 1 hr before absorbance reading. No significant absorbance changes were observed in response to these stimuli verifying the reliability and specificity of the assay.

Enzyme-linked immunosorbent assay (ELISA)

Mature IL-6 and IGF-1 released by the C2C12 myotubes were quantified using commercially available ELISA kits: murine IL-6 ELISA Kit (Thermo Fisher, USA) and murine IGF-1 DuoSet ELISA Kit (R&D Systems, USA) as per manufacturer's instructions. Briefly, plates were pre-coated with capture antibody and then blocked with a universal diluent. Antibody standards were serially diluted in the universal diluent, constructing a 7-point curve with a universal buffer as blank. Cell supernatant (undiluted) was then added in duplicates into the appropriate wells and agitated on a Thermomixer (Eppendorf, Germany) at 800rpm for [?]2 hr at room temperature. Wells were thoroughly washed with 0.05% Tween 20 in 1X PBS (PBST) before the detection antibody was added and agitated for 1 hr at 800rpm at room temperature. After washing, a developing solution with reporter enzyme and substrate was added and agitated for a further 1 hr at room temperature. Absorbance was then recorded at 450nm using a plate reader (CLARIOstar Monochrome Microplate Reader; BMG Labtech, Australia).

Data and Statistical Analysis

All data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). Data are presented as mean + standard errors of the mean (SEM). Statistical differences between treatments were determined by analysis of variance (ANOVA) followed by Tukeys multiple comparison post-hoc tests where appropriate. One-way ANOVA were used for three of more unmatched groups. Two-way ANOVA were used to analyse data when response was influenced by two independent factors of interest. All statistical analyses were performed using GraphPad PrismTM for Microsoft Windows(r) (Versions 8, Graphpad software(r), USA) where $p < 0.05$ was accepted as significant for all cases.

Materials

Winfield red cigarettes (Phillip Morris, Australia); apocynin (Sigma Aldrich, Australia); Ketamine/Xylazine (Virbac, Australia); acridine orange/ethidium bromide (Invitrogen, USA); Kwik-Diff$^{(r)}$reagent 1, fixative (Thermo Fisher Scientific, USA); RNeasy Mini Kit (Qiagen, Germany); High Capacity RNA-to-cDNA kit (Thermo Fisher Scientific, USA); pre-developed TaqMan primers (Thermo Fisher Scientific, USA), C2C12 murine myoblasts (American Type Culture Collection, USA; CRL-1772); cell culture reagents (Thermo Fisher Scientific, USA); H_2O_2 (Chem-Supply, Australia); antibody for immunofluorescence (Santa Cruz Biotechnology, USA); Fluoromount-G^{TM}, with DAPI (Thermo Fisher Scientific, USA); MTS Cell Prolif-

eration Assay (Promega, Australia); murine IL-6 ELISA Kit (Thermo Fisher, USA); murine IGF-1 DuoSet ELISA Kit (R&D Systems, USA); Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, USA), phosphorylation-specific, actin antibodies and p62 for western blots (Cell Signaling Technology, USA); all other antibodies for western blots (Abcam, USA); SuperSignal West Femto Maximum Sensitivity Substrate for chemiluminescence detection (Thermo Fisher Scientific, USA).

Results

Apocynin treatmentattenuates the pro-inflammatory lung response induced by CS exposure

Mice displayed no significant difference in starting body weight and food intake. However, CS exposure concomitantly reduced body weight gain (˜7% loss) and food intake (˜17% loss) which were unaffected by apocynin (5 mg kg^{-1}) administration, suggesting apocynin did not impact on growth or appetiteof these mice at the administered dosage (Figure 1A-B). In line with the reduced body weight gain, tissue mass of testicular (30%) and retroperitoneal (38%) white adipose tissue (WAT), heart (9%) and spleen (21%) were also reduced by CS exposure; however this was prevented by apocynin treatment, except for the heart (Table 2). To examine whether apocynin treatment was effective in attenuating the direct impact of CS on immune cell recruitment to the lung, we performed differential cell count analyses on the bronchoalveolar lavage fluid (BALF). CS exposure caused a 3.7-fold increase in total cell infiltration which was attributed to a marked increase in the number of macrophages, neutrophils and lymphocytes (Figure 1C-F). In line with this, CS exposure caused a marked increase in gross lung weight (Table 2) and the expression of key pro-inflammatory cytokines/chemokines in the lungs, including granulocyte–macrophage colony-stimulating factor (*Gmcsf*), CC-chemokine 2 (*Ccl2*), C-X-C motif ligand 1 (*Cxcl2*) and *Tνφa*(Figure 1G-J). Apocynin treatment significantly attenuated the CS-induced BALF cellularity evidenced by a 28% reduction in total cell counts (Figure 1C), 50% reduction in neutrophil counts (Figure 1E) and 86% reduction in lymphocyte counts in the CS-exposed mice (Figure 1F), without significant alterations in macrophage counts (Figure 1D). Accordingly, the CS-induced expression of *Ccl2* , *Cxcl2* ,*Tνφa* in the lungs were significantly attenuated by 84% (Figure 1H), 27% (Figure 1I) and 51% (Figure 1J), respectively; while the expression of *Gmcsf* remained elevated despite apocynin treatment (Figure 1G). This lung inflammation-attenuating effect of apocynin appeared to be specific to the CS exposure, as no significant effects in BALF cellularity (Figure 1C-F) and gene expression (Figure 1G-J) were observed in the sham-exposed mice.

Apocynin treatment prevents loss of skeletal muscle function caused by CS exposure

Eight weeks of CS exposure resulted in a loss of skeletal muscle mass in mice similar to that observed in human smokers, as evidenced by a 10% reduction in gross weight of the tibialis anterior (TA) muscle (Figure 2A) which is a prime mover of the hind limb, predominated by fast-twitch myofibers. CS exposure also caused an ˜8% reduction in the weight of soleus (Table 2) which is a slow-twitch fibers predominated muscle of the hind limbs (Timson, Bowlin, Dudenhoeffer & George, 1985), suggesting the muscle wasting effect of CS exposure is unbiased by fiber composition in our model. In addition to the loss of muscle mass, CS exposure also resulted in a significant reduction in contractile force (Figure 2B) and maximum contraction rate (Figure 2D) of the TA muscles which translated to a 54% decrease in specific force generated (Figure 2C), suggesting CS exposure caused muscle weakness. In addition to preventing the loss of TA mass, apocynin treatment attenuated the CS-induced skeletal muscle weakness as evidenced by the improved contractile force, maximum contraction rate and specific force (Figure 2A-D).

We next conducted qPCR analyses to examine the molecular changes within the TA muscles. In line with the loss of mass and function, CS exposure resulted in a 50% reduction in *Igf1-eb* (a precursor isoform of muscle-derived IGF-1; Figure 2E) and a 2-fold increase in *Mstn*(myostatin; Figure 2F) expression which were completely prevented by apocynin treatment. *Tνφa* expression remained unaltered regardless of CS exposure or apocynin treatment, suggesting the CS-induced phenotypical and molecular changes are unlikely to involve myocellular inflammation. Lastly, our oxyblot analysis revealed a 2.3-fold increase in protein carbonylation of TA muscles following CS exposure which was completely prevented by apocynin treatment (Figure 2 H-I),

suggesting the protective effects of apocynin *in vivo* may be related to its ability to antagonize the oxidative burden evoked by CS exposure.

Exposure to oxidative insult (H_2O_2) or cigarette smoke extract (CSE) results in a similar degree of wasting in C2C12 myotubes without cellular inflammation

To further dissect the importance of oxidative stress on muscle dysfunction, we compared the direct impact of a ROS, $H_2O_2$and CSE on C2C12 myotubes. Both H_2O_2 and CSE exposure resulted in a similar dose-dependent reduction in myotube size (Figure 3A–B). Unlike that of the higher dose, the reduction in myotube size induced by low doses of H_2O_2 (5 μM) or CSE (10%) did not impact cell viability (Figure 3C and H). In line with the presence of oxidative stress, exposure to H_2O_2(10 and 100 μM) elicited a robust expression of *Nox2* which is involved in the formation of ROS in muscle (Figure 3D) (Sakellariou et al., 2013). Unlike that of H_2O_2, no significant induction of *Nox2* expression was observed under sub-maximal dosages of CSE (Figure 3I). Meanwhile, the expression of glutathione peroxidase 1 (*Gpx1*), a detoxifying enzyme that scavenges H_2O_2, was unaltered by either of the stimuli (Figure 3E and J). Noteworthy, the maximal concentration of either H_2O_2 or CSE was capable of eliciting a cellular inflammatory response evidenced by the increased*Il-6* gene expression (Figure 3F and K) and release of IL-6 (Figure 3G and L), but not at their respective sub-maximal concentrations. This suggests oxidative stress accounts for some of the deleterious effects of CSE and this oxidative stress-driven myofiber wasting can occur without any detectable cellular inflammation recapitulating that of our *in vivo* model.

CSE-driven myofiber wasting suppresses myogenic factor production without impacting on atrophy related genes

In humans, smoking has been demonstrated to hamper muscle protein synthesis and increase the expression of genes associated with defective muscle maintenance such as *Mstn* and Muscle atrophy F-box (*MAFbx)* (Petersen et al., 2007). Indeed, direct exposure of myotubes to sub-maximal concentrations of H_2O_2 also resulted in a significant induction of both *Mstn* (Figure 4A) and *MAFbx* (Figure 4B), while the production and release of IGF-1, a potent driver of protein synthesis and myogenesis (Florini, Ewton & Coolican, 1996), were concomitantly suppressed (Figure 4C-E). Like that of H_2O_2, direct exposure to CSE also suppressed the production and release of IGF-1 (Figure 4H-J), regardless of concentration. However, the expression of *Mstn* (Figure 4F) and*MAFbx* (Figure 4G) remained largely unaltered, suggesting the deleterious effects of CSE exposure on myofiber wasting may predominantly lie in suppression of IGF-1 mediated protein synthesis.

Apocynin prevents the suppression of myogenic factor expression and myofiber wasting induced by CSE and H_2O_2

Under unstimulated conditions, apocynin treatment had no effect on myotube diameters (Figure 5A and F). However, treatment with apocynin prevented myofiber wasting (i.e. reduction in myofiber size) elicited by different concentrations of H_2O_2 (Figure 5A) or CSE (Figure 5F). Apocynin treatment completely attenuated the up-regulated expression of *Nox2* (Figure 5B and G) and *Il-6*(Figure 5C and H) driven by H_2O_2 or CSE. Moreover, apocynin normalized the expression of *Igf1-ea and Igf1-eb* , suggesting apocynin was able to antagonize the oxidant-dependent and -independent effects of CSE on myofiber wasting.

The protective effects of apocynin is attributed to a preserved proteostatic signaling

The main function of muscle-derived IGF-1 is to promote protein synthesis and muscle growth via the action of an intracellular signal transducer, mTOR (Nicklin et al., 2009). Given the expression of muscle-derived IGF-1 was found to be suppressed by CS (Figure 2E) and CSE (Figure 4C-E and H-J) exposure, we reasoned whether the key signal transduction pathways responsible for maintaining balance between protein synthesis and breakdown (i.e. proteostatic signaling) were impacted. H_2O_2 exposure concentration-dependently ablated the phosphorylation level of S6 ribosomal protein and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1; Figure 6A, C-D), which are the key downstream effectors of mTOR (Schiaffino & Mammucari, 2011). The phosphorylation status of a key repressor of protein synthesis, eukaryotic translation initiation factor 2A (eIF2α· Figure 6A and B), was found to be increased by 5 to 15-fold, suggesting

a global inhibition of protein synthesis. In line with the mRNA expression (Figure 4A), H_2O_2 increased the protein abundance of MAFbx (˜50%), a muscle specific E3 ubiquitin ligase (Figure 6A and E). Furthermore, a significant increase in abundance of the 19S proteasome (S5a), a regulatory subunit of the 26S proteasomal complex, was observed following exposure to 100 μM of H_2O_2 (Figure 6A and F), suggesting the activation of the ubiquitin-proteasome system (UPS). H_2O_2exposure also resulted in the activation of autophagic pathway evidenced by the conversion of LC3A/B-I to LC3A/B-II (Figure 6A and G-H) and decrease in p62 abundance (Figure 6A and I). Apocynin treatment maintained phosphorylated S6 ribosomal protein expression against 10 μM of H_2O_2 but not 4E-BP1 or eIF2α (Figure 6A-D). Meanwhile, no significant effects were detected for the activation of UPS and autophagic pathways (Figure 6A, E-I) suggesting the protective effects of apocynin are unlikely to be modulated through protein degradative pathways.

Meanwhile, exposure of myotubes to submaximal concentrations of CSE did not evoke the phosphorylation of eIF2α (Figure 7A-B) or decrease the phosphorylated S6 ribosomal protein expression (Figure 7A and C), although a concentration-dependent reduction in the phosphorylation levels of 4E-BP1 was observed (Figure 7A and D). Like that of H_2O_2, CSE exposure increased abundance of MAFbx (Figure 7A and E), however no detectable changes in 19S proteasome protein were observed until the maximal concentration (100%) of CSE was used (Figure 7A and F). Likewise, exposure to the maximal concentration of CSE resulted in the activation of autophagic pathway evidenced by the LC3A/B-I to LC3A/B-II conversion (Figure 7A and G-H) and decrease in p62 abundance (Figure 7A and I), but no significant effects were observed under submaximal conditions (10-20% of CSE). Apocynin treatment preserved the phosphorylation of 4E-BP1 without affecting that of eIF2α or S6 ribosomal protein (Figure 7A and B-D). Apocynin treatment completely blocked the enrichment of 19S proteasome elicited by maximal concentration of CSE (Figure 7A and F). To our surprise, the conversion of LC3A/B-I to LC3A/B-II which was undetectable at submaximal CSE concentrations under vehicle condition, became apparent starting at 20% CSE concentration, suggesting apocynin may selectively enhance cellular autophagic response in the CSE-exposed myotubes.

Discussion

The present study found that apocynin treatment was effective in attenuating lung inflammation and prevented the skeletal muscle dysfunction resulting from CS exposure. Our molecular analysis found that the CS-induced muscle dysfunction is attributed to oxidative stress and impaired muscle derived-IGF-1 expression which leads to a disruption of proteostatic signalling. Apocynin effectively modulated oxidative stress, thereby preserving muscle derived-IGF1 expression and the downstream proteostatic signalling in myofibers, protecting them from the damaging effects of CS/CSE exposure.

In the lungs, CS exposure elicited an abnormal inflammatory response, which may promote mucous metaplasia and lung destruction leading to the manifestation of chronic bronchitis and emphysema (O'Donnell, Breen, Wilson & Djukanovic, 2006). Neutrophils have been suggested to be a key driver of these deleterious effects in the lungs, by secreting a number of proteases, such as matrix metalloproteinases and neutrophil elastases (Vlahos et al., 2006). These proteases degrade components of the pulmonary extracellular matrix leading to the destruction of the lung parenchyma (Vlahos et al., 2006). Meanwhile, neutrophilic proteases may perpetuate lung inflammation by acting on proteinase-activated receptors (PARs)(Jenkins et al., 2006; Scotton et al., 2009). Destruction of the lung parenchyma and persistent inflammation not only drives the development of airflow limitation and emphysema, but also compromises the integrity of epithelial lining of the airway (Vlahos et al., 2006). This increases lung permeability allowing for the overspill of pro-inflammatory mediators into the systemic circulation, which has been postulated to be a key mechanism for the onset of skeletal muscle dysfunction (Bernardo, Bozinovski & Vlahos, 2015; Passey, Hansen, Bozinovski, McDonald, Holland & Vlahos, 2016).

Indeed, skeletal muscle dysfunction was observed following 8 weeks of CS exposure, characterized by the loss of mass and contractile function (Figure 2A-D). In patients with COPD, muscle dysfunction is most frequently reported in the lower limbs than the upper limbs (Gea, Pasto, Carmona, Orozco-Levi, Palomeque & Broquetas, 2001; Man et al., 2003), suggests leg muscles are more susceptible to dysfunction in patients

with COPD. Strikingly, symptoms of muscle weakness, which are hallmarks of functional impairment, have been reported in smokers without detectable decline in respiratory function (Maltais et al., 2014). This not only suggests that CS may directly impair leg muscle function, but also that the onset of limb muscle dysfunction may well precede that of respiratory symptoms. On this note, impaired quadricep function was detected in asymptomatic smokers with matching physical activity levels to non-smokers, which may be attributed to an acute toxicity of CS exposure on oxygen delivery and mitochondrial function (Wust, Morse, de Haan, Rittweger, Jones & Degens, 2008).

In addition to exerting acute toxicity, our study suggests that muscle loss and dysfunction may also arise from chronic oxidative stress elicited by repeated CS exposure. It is understood that CS represents an external source of oxidants ($>10^{16}$ free radicals per puff) which exert adverse effects on tissues through oxidative damage of biological structures (Bartalis, Chan & Wooten, 2007). Moreover, CS also activates inflammatory cells of the airway and lungs which may enhance oxidant production in pulmonary and extra-pulmonary tissues. Through these sources, chronic CS exposure generates transient and repeated bouts of oxidative stress which may modify key proteins involved in muscle metabolism or function, leading to the manifestation of muscle dysfunction seen in patients with COPD (Barreiro et al., 2010). Indeed, our results demonstrated the presence of oxidative stress and increased protein oxidation following CS exposure. This took place independent of muscle inflammation but was linked to an altered myogenic homeostasis characterised by a blunted expression of IGF-1 and increased expression of myostatin, suggesting a disrupted proteostasis. In C2C12 myotubes, we found that oxidative stress suppressed mTOR-driven protein synthesis, while activating the UPS degradative pathway resulting in myofiber wasting. Myostatin is a member of the transforming growth factor beta (TGF-β) family and a potent inducer of muscle atrophy. By inhibiting myogenic signalling, myostatin activates the UPS pathway through Forkhead box class O 3a (FoxO3a), thereby promoting the expression of the muscle-specific ubiquitin ligases: Muscle RING finger 1 (MuRF1) and MAFbx, resulting in a net loss of muscle protein and atrophy (Zhou et al., 2010). In muscle, Sriram *et al* (Sriram et al., 2011) demonstrated that oxidative stress is a potent stimulator of myostatin expression. Intriguingly, the same study also showed that myostatin itself also causes oxidative stress via the action of Nuclear Factor Kappa B (NFϰB) and Nox2, meaning that a self-perpetuated mechanism may exist to sustain protein degradation in atrophic muscles. Nevertheless, these findings highlight the instrumental role of oxidative stress in CS-induced myostatin expression and muscle loss observed in our study.

In accordance with this, attenuation of oxidative stress by apocynin markedly ameliorated the CS-induced lung inflammation and muscle dysfunction. In the muscle, apocynin prevented the induction of myostatin and its inhibitory effects on myogenic signalling, thereby preserving muscle proteostasis. In human COPD patients, muscle loss has been postulated to be a result of unintended weight loss due to malnutrition (Collins, Yang, Chang & Vaughan, 2019). Our *in vivo*data certainly reflects an association between loss of TA mass with reduced weight gain and food intake by CS exposure. However, apocynin treatment was able to preserve muscle mass and function despite both weight gain and food intake remaining suppressed, suggesting that the CS-induced muscle loss is unlikely a result of simple weight loss from malnutrition. Moreover, loss of muscle mass was mainly observed in the TA and soleus muscles, but not the gastrocnemius and plantaris, highlighting the selective nature of CS-induced muscle loss. While malnutrition and weight loss may be a major contributor to muscle loss in advanced COPD where respiratory function is severely compromised, they are unlikely to be accountable for the direct effects of CS induced muscle loss observed in this study.

Another interesting finding of the present study is that the impaired contractile function by CS exposure was only partially improved by apocynin, despite a fully preserved muscle mass. This apparent mismatch raises an important notion that muscle mass and function may not always correlate in a linear fashion in patients with COPD, unlike that in healthy individuals. In agreement with this, Mantoani *et al*(Mantoani et al., 2017) reported no correlations between muscle mass and muscle function assessed by quadriceps maximal voluntary contraction, although baseline physical activity was found to be related to greater muscle strength. In addition to its deleterious effects on muscle mass, CS exposure has been shown to directly impair excitation-contraction coupling (Nogueira et al., 2018) suggesting muscle contractile apparatus are sensitive to redox modifications. Barreiro *et al* . (Barreiro et al., 2010) reported that a number of muscle

proteins involved in force generation are subjected to post-translational oxidative modifications, including ATP synthase and actin. Oxidative modifications of protein, such as carbonylation, may result in loss of protein function and accelerated degradation by the UPS (Barreiro et al., 2010) which may offer an explanation for the impaired contractile function observed in our study. Collectively, these findings suggest that the relationship between muscle mass and function is unlikely to be linear, particularly in smokers or patients with COPD. Future studies should be mindful of factors that may influence this relationship, such as muscle of interest, the type of assessment chosen, age, sex and disease severity of the test subject, when designing interventional trials for COPD patients aiming to examine muscle changes.

Since muscle mass and function may be disconnected in the context of COPD, the finding that not all leg muscles display susceptibility to CS-induced muscle loss would prompt a new set of research questions on: 1) whether strength is preserved in muscles that are seemingly unaffected by mass loss; and 2) what effect does apocynin have on the contractile function of these muscles? Due to the limitation of the present study, we are unable to shed further light on these questions.

Regarding apocynin, it seems to act as a prodrug, which must be initially oxidized into its dimeric form, diapocynin, in order to be active (Johnson et al., 2002). Supporting this, Ximenes *et al.*(Ximenes, Kanegae, Rissato & Galhiane, 2007) reported the isolation of diapocynin in apocynin-treated neutrophils, and that the purified forms of diapocynin have been suggested to be more effective than apocynin itself (Kanegae et al., 2010; Mora-Pale, Weiwer, Yu, Linhardt & Dordick, 2009). Despite the controversies regarding its potency and selectivity as a Nox inhibitor, apocynin remains one of the most promising drugs for experimental models of disease involving ROS since its characterization in 1994.

In summary, we show that Nox-driven oxidative stress may be an underlying mechanism for the skeletal muscle loss and dysfunction caused by CS exposure. The induction of oxidative stress disrupts proteostasis by dampening myogenic signalling and enhancing UPS activation, resulting in muscle loss. Meanwhile, the oxidative modification of muscle proteins may also give rise to contractile impairment. By inhibiting Nox-driven oxidative stress, apocynin treatment attenuated lung inflammation and preserved myofibrillar proteostasis, thereby preventing muscle loss and dysfunction. Therefore, targeted inhibition of oxidative stress may be utilized to improve pulmonary and systemic outcomes associated with COPD.

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Table 2. Summary of tissue weight expressed as mean ± SEM

Data are expressed as mean ± SEM.

* $p< 0.05$ compare to the relevant Sham; analyzed with two-way ANOVA with multiple comparisons and Tukey post-hoc test.

Figure legends

Figure 1 . Effectof apocynin on body weight, food intake and lung inflammation induced by CS exposure. Mice were exposed to CS (smoke) or room air (sham) for 8 weeks with or without i.p. injection of apocynin (5 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$) or vehicle (saline). Progressive body weight of CS-exposed (smoke) and room air-exposed (sham mice with or without apocynin (**A**) and average food intake (**B**) across the experimental period. Total number of cells (**C**), macrophage (**D**), neutrophils (**E**) and lymphocytes (**F**) in BALF. Quantitative PCR was performed to assess the expression of *Gmcsf* (**G**), *Ccl2*(**H**), *Cxcl2* (**I**), and *Tνφa* (**J**) in homogenized lung tissues. Data are expressed as mean + SEM (n= 8-10 mice per group) and analyzed by two-way ANOVA with multiple comparisons and Tukey post-hoc test. $*p< 0.05$ denotes differences from the relevant sham group; $+p< 0.05$ denotes difference between the compared groups.

Figure 2 . Effect of CS exposure on tibialis anterior (TA) muscle weight, contractile performance and homeostatic changes . TA muscle weight (**A**), maximum contractile force (**B**), specific force at 120 Hertz (**C**), and maximum contraction rate measurements (**D**) were analyzed at the end of the experimental period. Quantitative PCR was performed to assess the expression of*Igf1-eb* (**E**), *Myostatin* (**F**) and*Tνφa* (**G**) in homogenized TA muscle. Total oxidized proteins (carbonylation; **H**) in the TA muscle was detected using the Oxyblot method and analyzed for densitometry (**I**). Data are expressed as mean + SEM (n= 8-10 mice per group, Oxyblot analysis was conducted on n= 5 mice per group) and analyzed by two-way ANOVA with multiple comparisons and Tukey post-hoc test. $*p< 0.05$ denotes differences from the relevant sham group; $+p< 0.05$ denotes difference between the compared groups.

Figure 3 . Effect of H_2O_2- and CSE-exposure on C2C12 myotube size, viability and cellular stress response. C2C12 myotubes were exposed to increasing concentrations of either H_2O_2 (**A**) or CSE (**B**) for 24 hours. Cell viability was assessed using the MTS assay following H_2O_2- (**C**) or CSE- (**H**) exposure. Quantitative PCR was performed to assess the expression of*Nox2* (**D** & **I**), *Gpx1* (**E** &**J**) and *Il-6* (**F** & **K**). IL-6 released into the medium in response to H_2O_2(**G**) or CSE (**L**) was quantified using ELISA. For myotube size assessments, data are represented as mean + SEM of 3 independent experiments (n = 270 myotubes counted per condition), other data are represented as mean + SEM of 3 independent

experiments (n = 7-9 per condition). *$p<$ 0.05 denotes differences from the relevant sham group; +$p<$ 0.05 denotes difference between the compared groups. Scale bars = 100 μm (**A** & **B**).

Figure 4 . **Effect of H_2O_2- and CSE-exposure on C2C12 myotubes proteostassis.** C2C12 myotubes were exposed to increasing concentrations of either H_2O_2 or CSE for 24 hours. Quantitative PCR was performed to assess the expression of *MAFbx*(**A** & **F**), *Mstn* (**B** & **G**),*Igf-1ea* (**C** & **H**) and *Igf-1eb* (**D**& **I**). Mature IGF-1 released into the medium in response to H_2O_2 (**E**) or CSE (**J**) was quantified using ELISA. Data are represented as mean + SEM of 3 independent experiments (n = 7-9 per condition). *$p<$ 0.05 denotes differences from control (i.e. concentration zero).

Figure 5 . **Effect of apocynin on C2C12 myotubes size and cellular stress.** C2C12 myotubes were exposed to increasing concentrations of either H_2O_2 or CSE with or without apocynin (500 nM) for 24 hours. Changes in myotube diameters were quantified (**A** & **F**) from 3 independent experiments (n = 270 myotubes counted per condition). Quantitative PCR was performed to assess the expression of *Nox2* (**B** &**G**), *Il-6* (**C** & **H**), *Igf1-ea*(**D** & **I**) and *Igf1-eb* (**E** &**J**). Data are represented as mean + SEM of 3 independent experiments (n = 7-9 per condition unless otherwise stated). *$p<$ 0.05 denotes differences from vehicle control (i.e. concentration zero); +$p<$ 0.05 denotes difference between the compared.

Figure 6 . **Effect of H_2O_2 on C2C12 myotubes proteostasis** . C2C12 myotubes were exposed to increasing concentrations of H_2O_2 with or without apocynin (500 nM) for 24 hours. At the end of experiment, samples were harvest for western blotting analysis. Representative images of the western blots (**A**) and their respective densitometry analyses (**B** –**I**). Data are represented as mean + SEM of 3 independent experiments (n = 6 per condition), with open bar represents vehicle conditions and closed bar represents apocynin conditions. *$p<$ 0.05 denotes differences from vehicle control (i.e. concentration zero); +$p<$ 0.05 denotes difference between the compared.

Figure 7. Effect of CSE on C2C12 myotubes proteostasis. C2C12 myotubes were exposed to increasing concentrations of CSE with or without apocynin (500 nM) for 24 hours. At the end of experiment, samples were harvested for western blotting analysis. Representative images of the western blots (**A**) and their respective densitometry analyses (**B** – **I**). Data are represented as mean + SEM of 3 independent experiments (n = 6 per condition), with open bar represents vehicle conditions and closed bar represents apocynin conditions. *$p<$ 0.05 denotes differences from vehicle control (i.e. concentration zero); +$p<$ 0.05 denotes difference between the compared.

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