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Apocynin attenuates diaphragm oxidative stress and protease activation during prolonged mechanical ventilation

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Abstract

Objective—To investigate whether apocynin protects the diaphragm from wasting and oxidative stress during mechanical ventilation (MV).

Design—Prospective, randomized, controlled study.

Setting—Research laboratory.

Subjects—Adult female Sprague-Dawley rats.

Interventions—Rats were randomly assigned to one of five experimental groups: 1) acutely anesthetized control, 2) spontaneous breathing control, 3) spontaneously breathing control with administration of the nicotinamide adenine dinucleotide phosphate oxidase inhibitor, apocynin, 4) mechanically ventilated, and 5) mechanically ventilated with apocynin.

Measurements and Main Results—Apocynin attenuated MV-induced diaphragmatic oxidative stress, contractile dysfunction, and type I, type IIa, and type IIb/IIx myofiber atrophy. The apocynin-induced attenuation of MV-induced diaphragmatic atrophy and contractile dysfunction occurred in conjunction with a reduction in the small increase in nicotinamide adenine dinucleotide phosphate oxidase activity as well as the preservation of total glutathione levels, glutathione peroxidase protein abundance, and a decrease in the activation of the cysteine proteases, calpain-1 and caspase-3. Interestingly, independent of MV, apocynin increased diaphragmatic levels of calpastatin, an endogenous calpain inhibitor. Furthermore, treatment of skeletal muscle cells in culture (C2C12 myotubes) with apocynin resulted in an increase in both calpastatin mRNA levels and protein abundance.

Conclusions—Our results suggest that the protective effects of apocynin on the diaphragm during prolonged MV seem to be linked to both its functions as an antioxidant and role in cellular signaling regulating the cysteine protease inhibitor calpastatin.

Keywords

skeletal muscle; NADPH oxidase; protease; atrophy; oxidative stress; antioxidant

The first two authors contributed equally to this work.

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Mechanical ventilation (MV) is used to maintain adequate alveolar ventilation in patients who are incapable of doing so on their own. Evidence indicates that MV-induced oxidative stress in the diaphragm is a major contributor to diaphragmatic atrophy and contractile dysfunction (1,2) and leads to difficulties in removing patients from the ventilator (i.e., “weaning”). Therefore, circumventing reactive oxygen species (ROS) generation or enhancing the free radical scavenging capabilities of the diaphragm via the administration of antioxidant compounds possesses exciting potential for protecting the diaphragm during MV.

Our laboratory and others have suggested that oxidative stress is a critical upstream signaling event leading to the activation of cysteine proteases (i.e., calpains and caspases) involved in myofilament disassembly as well as the deactivation of endogenous calpain protease inhibitors (calpastatin) (2–6). The disassembly of sarcomeric protein organization is believed to be an initial and required step in the degradation of skeletal muscle proteins during disuse (6–8). Determining the sensitivity of these proteases or their endogenous inhibitors to disuse generated free radicals and/or antioxidant administration may be a key component in the understanding of inactivity induced free radical-mediated skeletal muscle dysfunction.

Recently, new light has been shed on the nonspecific free radical scavenging and cell signaling-related functions of antioxidant compounds (9,10). One compound in particular, apocynin, possesses inherent oxidant scavenging capabilities (10) as well as the potential to specifically inhibit nicotinamide adenine dinucleotide phosphate (NADPH) oxidase superoxide production (11). On the basis of these facts, we hypothesized that apocynin administration would alleviate oxidative stress, contractile dysfunction, and myofiber atrophy during MV. We further hypothesized that apocynin administration would be sufficient to decrease cysteine protease activation during MV. Apocynin attenuated MV-induced diaphragmatic oxidative stress, contractile dysfunction, and myofiber atrophy. Interestingly, apocynin increased expression of the cysteine protease inhibitor calpastatin independent of elevations in oxidative stress both *in vivo* and *in vitro*.

METHODS

Animals and Experimental Design

To test our hypotheses, adult (4 months) female Sprague-Dawley rats were randomly assigned to one of five groups (n = 8 per group): 1) acutely anesthetized control, 2) 18 hours anesthetized spontaneously breathing control (SB), 3) 18 hours anesthetized spontaneously breathing control with prior venous administration of the NADPH oxidase inhibitor, apocynin (SBA), 4) 18 hours mechanically ventilated (MV), and 5) 18 hours mechanically ventilated with prior venous administration of the NADPH oxidase inhibitor, apocynin. Experiments were conducted in accordance with the policies contained in the Guide for the Care and Use of Laboratory Animals and were approved by the University of Florida Animal Care and Use Committee.

Mechanical Ventilation

Control animals acutely anesthetized with sodium pentobarbital (60 mg/kg interperitoneally) were killed immediately for tissue collection while the SB and MV animals were tracheostomized. The SB animals breathed spontaneously for the 18-hour duration while the MV animals were mechanically ventilated with a volume-driven ventilator (Inspira, Harvard Apparatus, Cambridge, MA) for the same duration (tidal volume, 0.55 mL/100 g; respiratory rate, 80 bpm; positive end-expiratory pressure, 1 cm H₂O) as previously reported (1).

Apocynin Administration

In the SBA and MV with apocynin administration animals, the NADPH oxidase inhibitor, apocynin, was dissolved in saline and administered via an IP injection before the experimental protocol (4 mg/kg body weight). This dosage has been previously used *in vivo* to inhibit NADPH oxidase activity in skeletal muscle (12).

Free Radical Producing Oxidase and Myeloperoxidase Activities

The activity of NADPH oxidase was measured by analysis of cytochrome *c* reductase in crude homogenates as previously described (13). The activity of xanthine oxidase was measured by the method of Westerfeld et al (14,15). Myeloperoxidase activity was determined as an indication of the level of neutrophil infiltration into the diaphragm as reported by Seekamp et al (16).

Total Glutathione and Protein Carbonyls

Total glutathione (GSH) was measured using a commercially available spectrophotometric kit (Cayman Chemical, Ann Arbor, MI). Protein carbonyls were measured in 40–50 mg total costal diaphragm muscle using a commercially available enzyme-lined immunosorbent assay (Biocell PC Test, Northwest Life Science Specialties, LLC, Vancouver, WA).

Diaphragmatic Contractile Properties

A muscle strip, including the tendinous attachments at the central tendon and rib cage (dimensions = 20 ± 3 mm), was dissected from the midcostal region. The strip was suspended vertically between two lightweight Plexiglas clamps with one end connected to an isometric force transducer (model FT-03, Grass Instruments, Quincy, MA) within a jacketed tissue bath and diaphragm skeletal muscle contractile properties were measured as previously described (17).

Myofiber Cross-Sectional Area and Morphologic Analyses

Sections from frozen diaphragm samples were cut at 8 μ m using a cryotome (Shandon, Pittsburgh, PA), stained for dystrophin protein (rabbit host, #RB-9024-R7, Lab Vision Corporation, Fremont, CA), myosin heavy chain Type I (mouse host, immunoglobulin M (IgM) isotype, A4.840, Developmental Studies Hybridoma Bank, Iowa City, IA [18]), and myosin heavy chain Type IIa (SC-71, mouse host, immunoglobulin G (IgG) isotype, a kind gift from Takao Sugiura, Laboratory of Biomechanics and Physiology, Faculty of Liberal Arts, Yamaguchi University, Yamaguchi, Japan [19]), and analyzed for myofiber cross-sectional analysis as previously described in detail (2,3).

Western Blotting

Protein extracts or cellular lysates were assayed using the Bradford method (Sigma, St. Louis, MO), separated by polyacrylamide gel electrophoresis via 4% to 15% gradient, and transferred to nitrocellulose membranes (100 V for 3 hours at 4°C) for Western blotting. As a verification of equal loading and transfer, the resulting transfer membrane was stained with Ponceau S, and each lane was analyzed using computerized image analysis and used to correct the Western Blot analysis for the total amount of protein in each lane, respectively (Scion Image, Frederick, MD) (data not shown). Membranes were probed for manganese superoxide dismutase (SOD-111; Stressgen; Victoria, BC, Canada), copper zinc superoxide dismutase (SOD-101; Stressgen), catalase (Ab16731; Abcam; Cambridge, MA), GSH peroxidase (Ab16798; Abcam), calpain-1 (#2556), cleaved caspase-3 (#9664), caspase-3 (#9665); (all purchased from Cell Signaling Technology, Carlsbad, CA), calpastatin (sc-20779; Santa Cruz Biotechnology, Santa Cruz, CA), or 4-hydroxynonenal (*trans*-4-

hydroxy-2-nonenal; 4-HNE, C₉H₁₆O₂) (ab46545; Abcam, Cambridge, MA) as described (20).

Cultured Myogenic Cell Line

Myoblasts derived from mouse skeletal muscle (C2C12 cells; American Type Culture Collection, Rockville, MD) were cultured on six-well dishes in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, 1% penicillin/streptomycin, and 0.1% fungi-zone at 37°C in the presence of 5% CO₂ until 90% confluence was reached. Differentiation was then initiated by differentiation medium: Dulbecco's modified Eagle's medium supplemented with 2% heat-inactivated horse serum, 1% penicillin/streptomycin, and 0.1% fungizone at 37°C in the presence of 5% CO₂ for 4 days. Apocynin treatment of C2C12 cells involved dilution in differentiation media to final desired concentrations (100 nM, 250 nM, 500 nM, and 1 μM) and standard incubation (37°C in the presence of 5% CO₂) for 18 hours. H₂O₂ treatment of C2C12 cells involved dilution in differentiation media to final desired concentrations 0 μM (Con), 25 μM, 100 μM, and 500 μM H₂O₂ for 1, 2, 4, 6, and 8 hours. Treatment of cells with both apocynin (500 nM) and H₂O₂ involved incubation of fully differentiated C2C12 myotubes in apocynin-treated Dulbecco's modified Eagle's medium for 12 hours and subsequent addition of H₂O₂ to a final concentration of 100 μM for 6 additional hours. Cells were rinsed 2× in ice-cold 1× phosphate-buffered saline and scraped for protein isolation in 130 μL nondenaturing lysis buffer (1% Triton X-100, 300 mM NaCl, 50 mM Tris-base, 5 mM EDTA, 3.1 mM sodium azide, 95 mM NaF, 22 μM Na₃VO₄), vortexed, incubated at 4°C for 25 minutes, and centrifuged at 1000 × g for 5 minutes. Supernatants were assayed by Western blot analysis as described. Cells harvested for RNA isolation were rinsed 2× in ice-cold 1× phosphate-buffered saline and scraped in TRIzol Reagent (Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. Total RNA (5 μg) was then reverse transcribed using the Superscript III First-Strand Synthesis System for reverse transcriptase polymerase chain reaction (Life Technologies) using oligo(dT)20 primers and the protocol outlined by the manufacturer.

Real-Time Polymerase Chain Reaction

One microliter of cDNA was added to a 25-μL polymerase chain reaction reaction for real-time polymerase chain reaction using Taqman chemistry and the ABI Prism 7000 Sequence Detection System (ABI, Foster City, CA). Relative quantitation was performed using the comparative computed tomography method (ABI, User Bulletin no. 2) with the calibrator sample (β-Glucuronidase; GenBank NM_Y00717, NM_M13962) for comparison of every unknown sample's gene expression. Fivefold dilution curves were assayed on selected samples to confirm the validity of this quantitation method for each gene. Calpastatin (GenBank NM_009817.1) mRNA transcripts were assayed using predesigned rat primer and probe sequences commercially available from Applied Biosystems (Assays-on-Demand).

Statistical Analysis

Comparisons between the groups for both the diaphragm and C2C12 measurements were made by a one-way analysis of variance and, when appropriate, a Tukey Honestly Significant Difference test was performed *post hoc*. Significance was established at $p < 0.05$. Values are reported as mean ± SE.

RESULTS

Systemic and Biological Response to MV

Heart rate (HR), systolic blood pressure (BP), arterial pH, the partial pressures of O₂ and CO₂, lactate, sodium (Na⁺), potassium (K⁺), calcium (Ca⁺⁺), glucose, and body weights

were maintained during the SB and MV protocol (Table 1). None of the SB or MV animals tested positive for Gram-positive or Gram-negative bacteria, and there were no visual abnormalities of the lungs or peritoneal cavity.

Diaphragm Antioxidant Capacity and Oxidative Stress

GSH is the major non-protein thiol in cells and is considered to be the most important intracellular anti-oxidant. Protein carbonyl levels were measured as a general index of protein oxidation in the diaphragm. MV resulted in a significant reduction in the amount of reduced GSH (Fig. 1A) and an increase in protein carbonyl formation in the diaphragm (Fig. 1B). Apocynin administration prevented both the MV-induced decrease in GSH as well as the increase in protein oxidation. Copper zinc superoxide dismutase, manganese superoxide dismutase, catalase, and GSH peroxidase protein expressions were also analyzed via Western blotting in the diaphragm (Table 2). Catalase and GSH peroxidase protein abundances decreased 75% and 39%, respectively, with MV. Apocynin administration rescued only the protein abundance of GSH peroxidase.

Diaphragm Contractile Dysfunction and Atrophy

MV resulted in a significant reduction in the specific force of the diaphragm compared with all other groups at all stimulation frequencies except 15 Hz (Fig. 2). However, treatment with apocynin attenuated the MV-induced contractile dysfunction of the diaphragm. Similarly, MV resulted in significant atrophy of Type I, Type IIa, and Type IIb/IIx diaphragm myofibers, and apocynin administration attenuated atrophy in all myofiber types (Fig. 3B).

Diaphragm Sources of Oxidants

NADPH oxidase activity (superoxide production in nM/mg protein) was not altered from Con (2.53 ± 0.02 nM/mg) by SB (2.55 ± 0.01 nM/mg) or SBA (2.54 ± 0.04 nM/mg) treatment groups. MV (2.65 ± 0.03 nM/mg) slightly increased (5%) NADPH oxidase activity and apocynin administration during MV (2.54 ± 0.01 nM/mg) attenuated this small increase. There were no alterations in diaphragm myeloperoxidase activity (Units/gram wet weight) with SB (1.33 ± 0.10 U/gww), SBA (1.21 ± 0.05 U/gww), MV (1.22 ± 0.05 U/gww), or MV with apocynin administration (1.19 ± 0.06 U/gww) treatment groups when compared with Con (1.17 ± 0.07 U/gww).

Xanthine oxidase activity was measured both for its role as a separate potential source of oxidants in the diaphragm during MV and its purported link to NADPH oxidase activity (21,22). Xanthine oxidase activity (Units/gram protein) was not altered from Con (6.74 ± 0.63 U/g protein) values following SB (5.98 ± 0.24 U/g protein) or SBA (6.34 ± 0.51 U/g protein). Xanthine oxidase activity increased 27% with MV (8.56 ± 0.74 U/g protein). Apocynin administration during MV did not restore xanthine oxidase activity (8.41 ± 0.56 U/g protein) to control values.

Diaphragm Protease Activation

We assessed the protein abundance of cleaved and activated calpain 1 and caspase-3 cysteine proteases in the diaphragm after ventilation (4) (Fig. 4A). The abundance of active calpain-1 (Fig. 4B) and caspase-3 (Fig. 4C) increased 60% and 210%, respectively, with MV. Apocynin administration attenuated the MV-induced increases in both calpain-1 and caspase-3 activation. Interestingly, apocynin administration to either spontaneously breathing or mechanically ventilated animals resulted in 104% and 105% increases, respectively, in the expression of the endogenous calpain inhibitor calpastatin (Fig. 4D).

Apocynin Treatment of C2C12 Myotubes

To further investigate the protective role of apocynin in skeletal muscle, we treated fully differentiated C2C12 myotubes with varying concentrations of apocynin. Using this myotube model of culture allowed for the isolation and careful manipulation of individual actions of apocynin, devoid of potentially interfering ventilation-induced stimuli in the whole diaphragm. Apocynin (concentrations ranging from 100 nM to 1 μ M) administration does not contribute to oxidative injury in these cells by assessment of the lipid peroxidation by-product, 4-HNE (data not shown). We chose 4-HNE as our measure of oxidative stress in the C2C12 myotube model due to the small amount of cellular protein material that is harvested per experiment and the minimal requirements of absolute protein required for quantification using this method. Next, we examined calpastatin protein abundance in myotubes treated with these varying concentrations of apocynin (Fig. 5A). Calpastatin protein expression increased (60% to 120%) with all apocynin treatment concentrations (Fig. 5B). This increase in calpastatin protein seems to be, at least in part, transcriptionally regulated as calpastatin mRNA abundance increased 35% with 18 hours of apocynin treatment (500 nM; Fig. 5C). These results verified that apocynin administration increases calpastatin protein abundance and mRNA expression in fully differentiated skeletal muscle myotubes. Finally, we also determined whether apocynin could protect differentiated C2C12 myotubes from oxidative injury when exposed to a H₂O₂ challenge. Cells were treated with 0 μ M (Con), 25 μ M, 100 μ M, or 250 μ M H₂O₂ for 1, 2, 4, 6, or 8 hours. Treatment of myotubes with 100 μ M H₂O₂ for 6 hours resulted in an increase in 4-HNE modified proteins and apocynin did not attenuate this increase (Fig. 6A). Calpastatin protein abundance, assessed by Western blot (Fig. 6B), was increased by treatment with apocynin and this outcome was not influenced by exposure to H₂O₂ (Fig. 6C).

DISCUSSION

Overview of Principle Findings

Several new and important findings emerged from these experiments. First, these results demonstrate that the *in vivo* administration of apocynin attenuates diaphragmatic oxidative stress, contractile dysfunction, and myofiber atrophy resulting from 18 hours of prolonged mechanical ventilation. Furthermore, this apocynin-mediated protection of the diaphragm may not be due to a singular role as an inhibitor of NADPH oxidase, but may also include both its inherent antioxidant properties and its involvement in cellular signaling regulating the protease inhibitor calpastatin. A brief discussion of these key results follows.

Apocynin Alleviates Oxidative Stress During MV

The main objective of this study was to test the hypothesis that apocynin, an exogenous compound with the ability to inhibit NADPH oxidase and scavenge radicals, could attenuate MV-induced diaphragmatic oxidative injury, contractile dysfunction, and myofiber atrophy. Our data indicate that NADPH oxidase activity is increased with 18 hours of MV, but only to a small degree (+5%). Thus, it seems unlikely that this small MV-induced increase in diaphragmatic NADPH oxidase activity is a major contributor to MV-induced oxidative stress in the diaphragm with 18 hours of MV. Our findings do not, however, preclude a potential role for NADPH oxidase activity in oxidant production at earlier time points of MV (i.e., <18 hours).

Note that ROS can be produced by both phagocytic and nonphagocytic isoforms of NADPH oxidase (23). This is of particular interest in studies using apocynin in skeletal muscle as recent studies demonstrate the specificity of this compound in the inhibition of NADPH oxidase only in cells that both generate large amounts of ROS and express myeloperoxidase (10). Nonstimulated resident immune cells exist in skeletal muscle and when stimulated

contribute to inflammatory signaling and the sequestration of other immune cells from the peripheral circulation (24–27). However, during disuse, locomotor skeletal muscle inflammatory pathways including neutrophil, macrophage, and mast cell infiltration, myofiber necrosis, and inflammatory cytokine gene expression are not increased (27,28). Similarly, our laboratory has shown that prolonged MV does not result in the infiltration of neutrophils, phagocytic or nonphagocytic macrophages into the diaphragm (29). In addition, our current data demonstrate that myeloperoxidase activity is not altered in the diaphragm during MV, which further indicates a lack of neutrophil infiltration (16). Collectively, these results suggest that resident immune cells remain un-stimulated during disuse in skeletal muscle; although it cannot be completely ruled out that they may contribute to oxidant production during MV.

Logically, apocynin-induced protection against oxidative stress can be achieved by one of three mechanisms: 1) a reduction in ROS production; 2) an increase in antioxidant protection; or 3) some combination of 1 and 2. In this regard, apocynin treatment does not seem to reduce ROS production by inhibiting xanthine oxidase activity (10). However, Hemuller et al (10) have recently demonstrated that apocynin acts as a scavenger for peroxide-dependent ROS formed by pyrogallol, xanthine/xanthine oxidase, or potassium peroxide. The molecular structure of apocynin contains a phenol group with potential oxidant scavenging capacity (30), suggesting its possible role as an oxidant scavenger. Although its inability to scavenge O_2^- has been demonstrated, its ability to scavenge H_2O_2 has yet to be definitively proven (11,31). Our study also suggests that apocynin assists in the maintenance of antioxidants as indicated by higher levels of both total GSH and GSH peroxidase within the diaphragm of MV animals. Apocynin-induced increases in these diaphragmatic antioxidants provides a plausible explanation for the protection against oxidative injury at 18 hours of MV. The possibility that apocynin is a clinically useful antioxidant via either its direct scavenging capacity or stimulation of oxidant scavengers warrants further investigation to determine the exact biochemical mechanisms of action of this compound.

In this study, apocynin failed to attenuate oxidative stress induced by direct H_2O_2 challenge of skeletal muscle cells *in vitro*. Note, however, that the failure of apocynin to attenuate the modification of myotube proteins by 4-HNE is not entirely indicative of its inability to provide protection or act as an antioxidant. The concentration of H_2O_2 added to the media of our cell culture experiments (100 μ M) may exceed the radical scavenging abilities of this compound, resulting in an inability to prevent the damaging lipid modifications that occur with high levels of oxidants. In addition, previous studies suggest that apocynin's antioxidant properties are conveyed through inhibiting H_2O_2 signaling interactions, including cytochrome-P450 (32), thromboxane synthase inhibition (33), the synthesis of the endogenous antioxidant GSH (34), and the inhibition of H_2O_2 -induced p38 mitogen-activated protein kinase, Akt, and extracellular regulated kinase 1/2 activity (10). Further, our *in vivo* data demonstrate that apocynin may also serve as an antioxidant in the diaphragm during MV through the maintenance of higher levels of the endogenous antioxidants, GSH and GSH peroxidase. Therefore, the apocynin-induced increases in GSH suggest that the protection it confers may not be due solely to the ability of apocynin to scavenge radicals.

Apocynin Attenuates Contractile Dysfunction and Atrophy

The production of oxidants in the diaphragm during MV initiates critical catabolic signaling involved in the progression of skeletal muscle atrophy and contractile dysfunction (1,2,4). Our laboratory and others have suggested that the oxidative stress that occurs during skeletal muscle disuse is a critical component of the activation of cysteine proteases (i.e., calpains

and caspase-3) involved in the release of myo-filaments (2–5). This postulate was based on previous reports indicating that 1) skeletal muscle disuse results in an increase in oxidative stress and intracellular free calcium (35–38); 2) prolonged MV promotes the activation of calcium-activated neutral proteases (calpains, caspases) in the diaphragm (36,38); and 3) the alleviation of MV-induced oxidative stress attenuates diaphragm contractile dysfunction and myofiber atrophy during MV (1,2). The attenuation of oxidative stress by apocynin occurred concomitantly with the protection against MV-induced diaphragmatic contractile function and myofiber size. Consequently, apocynin administration also attenuated the MV-induced activation of calpain-1 and caspase-3 proteases. However, the link between apocynin protection and protease activity may not be entirely explained by the alleviation of oxidative injury.

One of the most interesting and novel findings of this study is that apocynin administration promoted an increase in diaphragm calpastatin protein abundance. Calpastatin is an endogenous and specific inhibitor of calpains in skeletal muscle (6) and its abundance increases in the diaphragm in both SB and MV animals treated with apocynin. We interpret these results as evidence that apocynin promotes calpastatin protein accumulation independent of the redox status of the muscle fiber. These *in vivo* findings were corroborated *in vitro*, with the treatment of C2C12 myotubes to varying concentrations of apocynin resulting in an increase in calpastatin protein levels independent of whether the cells were exposed to an oxidant (i.e., H₂O₂) challenge. This increase in calpastatin cellular protein abundance seems to be regulated transcriptionally, as the mRNA expression of calpastatin also increased in the myotubes with apocynin administration. The mammalian calpastatin gene possesses a promoter region that includes cyclic adenosine monophosphate (cAMP) response element motifs, which may be initiated by β receptor occupancy, cAMP formation, protein kinase A activation, and phosphorylation of cAMP response element binding (39–42). Additional work is required to delineate which transcription factors and signaling pathways are responsible for the induction of calpastatin protein abundance and gene expression by apocynin in skeletal muscle.

CONCLUSIONS

Our study revealed that the *in vivo* administration of apocynin attenuates diaphragmatic oxidative stress, contractile dysfunction, and myofiber atrophy associated with 18 hours of MV. The mechanisms of apocynin-induced diaphragmatic protection during MV are not clear, but seem to be related to the alleviation of oxidative stress (inhibition of a minor increase in NADPH oxidase activity, induction of antioxidants, and potential scavenging capabilities) and involvement in signaling regulating the calpain inhibitor, calpastatin. Our discovery that apocynin administration is sufficient to induce antioxidant and endogenous calpastatin expression could lead to pharmacologic therapies targeted at the maintenance of protein balance in skeletal muscle during wasting conditions.

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Figure 1. Effects of apocynin administration on diaphragm oxidative stress during MV. *A*, total glutathione (mmol/g wet weight) in diaphragm skeletal muscle from acutely anesthetized control (*Con*), 18-hour spontaneous breathing (*SB*), 18-hour spontaneous breathing with apocynin administration (*SBA*), 18-hour mechanically ventilated (*MV*), and 18-hour MV with apocynin administration (*MVA*) treatment groups. *B*, protein carbonyl formation in diaphragm skeletal muscle. Values are mean $\pm$ SE, n = 8/group. #Significantly ($p < 0.05$) different from all other groups.

Figure 2.

Apocynin's effects on the force–frequency relationship for *in vitro* diaphragm strips immediately following 18 hours of MV. Values are mean $\pm$ SE, $n = 8$ /group. #Significantly ($p < 0.05$) different from all other groups at stimulation frequency. *Con*, control; *SB*, spontaneously breathing; *SBA*, spontaneously breathing with apocynin administration; *MV*, mechanically ventilated; *MVA*, mechanically ventilated with apocynin administration.

Figure 3.

Fiber cross-sectional area (CSA) in diaphragm skeletal muscle myofibers expressing myosin heavy chain (MHC) I (Type I), MHC IIa (Type IIa), and MHC IIb/IIx (Type IIb/IIx). A, representative fluorescent staining of MHC I (blue), MHC IIa (green), and dystrophin (red) proteins in diaphragm from control (Con), 18-hour mechanical ventilation (MV) and 18-hour mechanical ventilation with apocynin administration (MVA). B, Mean CSA (μm^2) of Type I, Type IIa, and Type IIb/IIx diaphragm skeletal muscle myofibers. Bar = 30 μm . Values are mean $\pm$ SE, n = 8/group. Values are mean $\pm$ SE. #Significantly ($p < 0.05$) different from all other groups within MHC type. SB, spontaneously breathing; SBA, spontaneously breathing with apocynin administration.

Figure 4.

Calpain-1 and caspase-3 protease activation and calpastatin protein abundance in diaphragm skeletal muscle. *A*, representative Western blots for the analysis of intact and cleaved calpain-1 and intact and cleaved caspase-3 proteases as well as calpastatin protein. *B*, fold induction of cleaved calpain-1. *C*, fold induction of cleaved caspase-3. *D*, fold induction of calpastatin. Values are corrected for ponceau stained total protein within lane and normalized to acutely anesthetized control (*Con*) and presented as mean $\pm$ SE, $n = 8$ /group. #Significantly ($p < 0.05$) different from all other treatment groups. *Significantly ($p < 0.05$) different from *Con*. †Significantly ($p < 0.05$) different from spontaneously breathing (*SB*). ‡Significantly ($p < 0.05$) different from mechanical ventilation (*MV*). *SBA*, spontaneously

breathing with apocynin administration; *MVA*, mechanically ventilated with apocynin administration.

Figure 5. Calpastatin protein abundance and gene expression in apocynin-treated C2C12 myotubes. C2C12 myotubes were differentiated for 4 days and administered 0 nM (control, 3Con3), 100 nM, 250 nM, 500 nM, and 1 μM apocynin (final concentrations) for 18 hours. *A*, representative Western blots for the analysis of calpastatin protein. *B*, fold induction of calpastatin protein. Values are normalized to 0 nM (Con) concentration of apocynin and presented as mean ± SE, n = 6/treatment. *Significantly ($p < 0.05$) different from 0 nM (Con). *C*, Calpastatin gene expression. Values are mRNA corrected for β-glucuronidase and normalized to Con cells and presented as mean ± SE, n = 6/treatment.

Figure 6.

Calpastatin protein abundance in hydrogen peroxide (H_2O_2)-treated C2C12 myotubes. C2C12 myotubes were differentiated for 4 days and administered 0 μ M (control, [Con]), 25 μ M, 100 μ M, and 500 μ M H_2O_2 (final concentrations) for 1, 2, 4, 6, and 8 hours. **A**, fold induction α,β -unsaturated hydroxynonenal modification of proteins. Values are normalized to 0.3M (Con) concentration of H_2O_2 and presented as mean $\pm$ SE, $n = 6$ /treatment. *Significantly ($p < 0.05$) different from 0 μ M (Con). †Significantly ($p < 0.05$) different from apocynin (Apo). **B**, representative Western blots for the analysis of calpastatin protein (P) in Con, 500 nM apocynin for 18 hours (Apo), 100 μ M H_2O_2 (final concentration) for 6-hours, or 500 nM apocynin for 18 hours and 100 μ M H_2O_2 (final concentration) for 6 hours (Apo +

H₂O₂). C, Fold induction calpastatin protein. C2C12 myotubes were untreated (Con) or treated with 500 nM apocynin for 18 hours (Apo), 100 μ M H₂O₂ (final concentration) for 6 hours (H₂O₂), or 500 nM apocynin for 18 hours and 100 μ M H₂O₂ (final concentration) for 6 hours (Apo + H₂O₂). Values are normalized to Con and presented as mean $\pm$ SE, n = 6/treatment. *Significantly ($p < 0.05$) different from Con. †Significantly ($p < 0.05$) different from Apo. ‡Significantly ($p < 0.05$) different from H₂O₂.

Table 1

Arterial blood analysis during spontaneous breathing and mechanical ventilation

	pH	pCO ₂ (mm Hg)	pO ₂ (mm Hg)	Sodium (Na ⁺) (mM)	Potassium (K ⁺) (mM)	Calcium (Ca ⁺⁺) (mM)	Glucose (mg/dL)
SB	7.35 ± 0.01	48.7 ± 2.3	62.6 ± 2.9	144.9 ± 0.50	3.47 ± 0.11	1.08 ± 0.02	95.3 ± 4.48
SBA	7.31 ± 0.01	54.2 ± 2.5	71.9 ± 5.1	142.4 ± 0.57	3.55 ± 0.13	1.08 ± 0.03	89.2 ± 5.22
MV	7.46 ± 0.01	31.9 ± 1.4	68.5 ± 1.6	143.6 ± 0.53	3.45 ± 0.14	1.05 ± 0.02	96.9 ± 5.37
MVA	7.41 ± 0.01	35.0 ± 1.7	69.9 ± 2.7	146.5 ± 1.14	3.15 ± 0.09	1.04 ± 0.01	76.3 ± 4.52

SB, spontaneously breathing; SBA, spontaneously breathing with apocynin administration; MV, mechanically ventilated; MVA, mechanically ventilated with apocynin administration.

Values are expressed as mean ± SE of the prearterial, midarterial, and postarterial blood gas analyses from 18-hour SB, 18-hour SBA, 18-hour MV, and 18-hour MVA treatment groups.

Table 2

Antioxidant enzyme expression

	CuZnSOD	MnSOD	Catalase	GPX
Con	1.00 ± 0.09	1.00 ± 0.06	1.00 ± 0.07	1.00 ± 0.16
SB	1.16 ± 0.05	1.07 ± 0.15	1.04 ± 0.12	0.83 ± 0.12
SBA	1.17 ± 0.05	0.86 ± 0.07	0.91 ± 0.12	1.17 ± 0.22
MV	1.06 ± 0.06	0.88 ± 0.09	0.25 ± 0.07 ^{a,b,c}	0.61 ± 0.15 ^{a,c}
MVA	1.12 ± 0.13	0.86 ± 0.06	0.22 ± 0.06 ^{a,b,c}	0.95 ± 0.03 ^{a,c,d}

CuZnSOD, copper zinc superoxide dismutase; MnSOD, manganese superoxide dismutase; GPX, glutathione peroxidase; Con, control; SB, spontaneously breathing; SBA, spontaneously breathing with apocynin administration; MV, mechanically ventilated; MVA, mechanically ventilated with apocynin administration.

^aSignificantly different ($p < 0.05$) from Con;

^b significantly different from SB;

^c significantly different from SBA;

^d significantly different from MV.

Expression of the major cellular antioxidants-during mechanical ventilation and ventilation with apocynin administration. CuZnSOD, MnSOD, catalase, and GPX antioxidant expressions were measured via Western blot analysis in acutely anesthetized Con, 18-hour SB, 18-hour SBA, 18-hour MV, and 18-hour MVA treatment groups. Values are expressed mean ± SE fold differences in integrated optical density measurements normalized to Con.