Exposure to cigarette smoke induces overexpression of von Hippel-Lindau tumor suppressor in mouse skeletal muscle

Vladimir T. Basic, Elsa Tadele, Ali Ateia Elmabsout, Hongwei Yao, Irfan Rahman, Allan Sirsjo, and Samy M. Abdel-Halim

1Department of Clinical Medicine, Örebro University, Örebro, Sweden; 2Department of Environmental Medicine, Lung Biology and Disease Program, University of Rochester Medical Center, Rochester, New York; and 3Division of Respiratory Medicine and Allergology, Department of Clinical Sciences, Danderyd Hospital, Stockholm, Sweden

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Basic VT, Tadele E, Elmabsout AA, Yao H, Rahman I, Sirsjo A, Abdel-Halim SM. Exposure to cigarette smoke induces overexpression of von Hippel-Lindau tumor suppressor in mouse skeletal muscle. Am J Physiol Lung Cell Mol Physiol 303: L519–L527, 2012. First published July 27, 2012; doi:10.1152/ajplung.00007.2012.—Cigarette smoke (CS) is a well-established risk factor in the development of chronic obstructive pulmonary disease (COPD). In contrast, the extent to which CS exposure contributes to the development of the systemic manifestations of COPD, such as skeletal muscle dysfunction and wasting, remains largely unknown. Decreased skeletal muscle capillarization has been previously reported in early stages of COPD and might play an important role in the development of COPD-associated skeletal muscle abnormalities. To investigate the effects of chronic CS exposure on skeletal muscle capillarization and exercise tolerance, a mouse model of CS exposure was used. The 129/SvJ mice were exposed to CS for 6 mo, and the expression of putative elements of the hypoxia-angiogenic signaling cascade as well as muscle capillarization were studied. Additionally, functional tests assessing exercise tolerance/endurance were performed in mice. Compared with controls, skeletal muscles from CS-exposed mice exhibited significantly enhanced expression of von Hippel-Lindau tumor suppressor (VHL), ubiquitin-conjugating enzyme E2D1 (UBE2D1), and prolyl hydroxylase-2 (PHD2). In contrast, hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) expression was reduced. Furthermore, reduced muscle fiber cross-sectional area, decreased skeletal muscle capillarization, and reduced exercise tolerance were also observed in CS-exposed animals. Taken together, the current results provide evidence linking chronic CS exposure and induction of VHL expression in skeletal muscles leading toward impaired hypoxia-angiogenesis signal transduction, reduced muscle fiber cross-sectional area, and decreased exercise tolerance.

Exposure to cigarette smoke (CS) is a well-known risk factor for the development of irreversible airway limitation in chronic obstructive pulmonary disease (COPD) (2, 12). However, the contribution of CS exposure to the development of the extrapulmonary complications of COPD remains largely unknown. A significant number of patients with COPD develop generalized weight loss and peripheral muscle wasting, collectively termed as pulmonary cachexia syndrome (PCS) (1, 10, 39, 46, 47). Development of PCS is directly correlated with the extent to which CS exposure contributes to the development of the respiratory changes (4, 30). In analogy, skeletal muscles from several murine models chronically exposed to CS exhibited patterns of morphological, metabolic, and functional changes similar to those observed in healthy smokers and patients with COPD (3, 13, 22, 29, 45).

Skeletal muscles of patients with COPD are characterized by several structural, metabolic, and functional alterations associated with the loss of muscle strength and endurance. These include loss of muscle mass and cross-sectional area (9, 16), fiber type shift toward the more fatigue-susceptible type-II glycolytic fibers (14, 20, 50), decrease in muscle oxidative capacity (25), and significantly decreased capillarization (20). Interestingly, fiber type shift, reduction in oxidative enzyme activity, and decreased muscle fiber diameter have been also observed in skeletal muscles of healthy smokers before development of COPD (23, 30, 51). Moreover, smokers exhibit lower peripheral muscle fatigueresistance than nonsmokers (51). The possibility of directly implicating chronic CS exposure to development of PCS in COPD is further supported by recent findings that CS induces direct oxidative damage of several important proteins involved in the regulation of muscle metabolism and bioenergetics and that these changes precede the respiratory changes (4, 30). In analogy, skeletal muscles from several murine models chronically exposed to CS exhibited patterns of morphological, metabolic, and functional changes similar to those observed in healthy smokers and patients with COPD (3, 13, 22, 29, 45).

The presence of an intact skeletal muscle vasculature is crucial for responding to the various metabolic changes and local tissue insults, e.g. hypoxia (15). An important element in this adaptive mechanism is the hypoxia inducible factor-1α (HIF-1α) transcription factor acting as a molecular sensor capable of detecting a fine fluctuation in intracellular oxygen concentrations (41). During normoxia, HIF-1α levels are kept under tight control through hydroxylation by a family of prolyl hydroxylases (PHD1–4) (11) and subsequent ubiquitination and proteosomal degradation mediated by the von Hippel-Lindau tumor suppressor (VHL) protein, a member of RING finger E3-ligase family (21, 33). The latter involves binding of VHL/HIF-1α complex to ubiquitin-conjugating enzyme E2D1 (UBE2D1), which carries out multiple transfers of activated ubiquitin molecules to specific lysil residues within the HIF-1α structure (18, 36). Intracellular hypoxia inactivates VHL, thus allowing HIF-1α stabilization and transcriptional activation of a large panel of genes involved in metabolism, erythropoiesis, and angiogenesis including vascular endothelial growth factor (VEGF) (41). Beside involvement in transcriptional control of VEGF expression via HIF-1α pathway, VHL regulates VEGF expression on the posttranscriptional and translation levels (52). Recently, we have reported first evidence demonstrating VHL overexpression in skeletal muscles from patients with
COPD accompanied with decreased capillarization and muscle wasting (19). These changes were observed in patients with COPD with the mild, Stage-1 disease, where normal respiratory function has not been significantly compromised (19). This suggested that the observed abnormalities in skeletal muscle vascularity and hypoxia-angiogenic signaling are an early, primary event, rather than a secondary occurrence to development of COPD and its complications.

The objectives of the present investigation are specifically focused on the assessment of the effects of the chronic CS exposure on the expression of putative elements of the hypoxia-angiogenic signaling cascade and muscle capillarization in an experimental animal model. For this purpose, we have used a mouse model exposed to CS for 6 mo. The 129/SvJ mice strain used in this study was previously demonstrated to exhibit higher intrinsic resistance to CS-induced lung emphysema compared with other susceptible strains such as C57BL/6J and DBA/2 (17, 38, 54).

MATERIALS AND METHODS

Animals and CS exposure. The 129/SvJ mice (Jackson Laboratory, Bar Harbor, ME) were bred and maintained under specific pathogen-free conditions involving 12-h:12-h dark:light cycles inside adequate vivarium facilities at the University of Rochester. For CS exposure, 3R4F research cigarettes (University of Kentucky, Lexington, KY) were used to generate a mixture of sidestream smoke (89%) and mainstream smoke (11%) by a Teague smoking machine (Model TE-10; Teague Enterprises, Woodland, CA) at a concentration of ~100 mg TPM/m³ to avoid the possible toxicity to mice at a high concentration of long-term CS exposure (N = 6) (53, 54). The level of carbon monoxide in the chamber was 350 ppm. The 129/SvJ mice (8–10 wk old, 22–25 g body wt) received 5-h exposures per day, 5 days/wk for 6 mo, and were killed 24 h after the last CS exposure (53). No mouse mortality was found due to chronic CS exposure, but body weight was significantly reduced in 6 mo of CS-exposed mice (31.6 ± 1.16 g) compared with air-exposed mice (36.9 ± 1.01 g, n = 3, P < 0.05). Control mice (N = 8) were exposed to filtered air in an identical chamber according to the same protocol described for CS exposure. All animal procedures described in this study were approved by the University Committee on Animal Research Committee of the University of Rochester.

Exercise endurance determination. Exercise endurance in mouse was measured using a motorized rodent treadmill with an electric grid at the rear of the treadmill (Columbus Instruments, Columbus, OH) as described previously (28, 53). Run duration (min) and run distance (m, calculated from the run time and speed of the treadmill) were used as the parameters to reflect exercise tolerance. Mice were placed on the treadmill and allowed to adapt to the surroundings for 3–5 min before starting the exercise. The treadmill was started at a speed of 8.5 m/min with a 0° incline. After 9 min, the speed and incline were raised to 10 m/min and 5°, respectively. Speed was increased by 2.5 m/min every 3 min to a maximum of 40 m/min, and the incline increasing 5° every 9 min to a maximum of 15°. Exercise continued until mouse exhaustion, which is defined as an inability to maintain running despite repeated contact with the electric grid. At this stage, each mouse was immediately returned to its home cage.

Muscle biopsies. Animal models were killed 24 h after the last CS exposure. Mice were anesthetized using 100 mg/kg pentobarbital sodium (Abbot Laboratories, Abbot Park, Illinois). Gastrocnemius muscle specimens (~150 mg) in both legs were dissected, cleaned for fat and connective tissue, placed into sealed vials, snap frozen in liquid nitrogen, and stored at −80°C until analysis.

RNA isolation. Muscle specimens were frozen in the liquid nitrogen, cut in pieces, and disrupted using Micro-Dismembrator II (B Braun, Melsungen, Germany). Total RNA was isolated from the obtained homogenized material with the RNEasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA) according to the instructions specified by the manufacturer. The total RNA was quantified by using the Nanovue Plus spectrophotometer (GE Healthcare, Buckinghamshire, UK). Isolated total RNA was stored on −80°C until further use.

Real-time PCR analysis. Total RNA (0.75 μg) was reverse-transcribed to cDNA by using SuperScript II first-strand synthesis kit (Invitrogen, Carlsbad, CA) following detailed instructions described by the manufacturer. Synthesized complementary DNA was stored on −20°C until further use. Quantitative RT-PCR gene expression analysis was performed on ABI Prism Sequence Detection System 7900HT (PE Applied Biosystems, Foster City, CA). Genes targeted in the expression analysis including HIF-1α (catalogue no. Mn00468869_m1), VEGF (catalogue no. Mn00437304_m1), PHD2 (catalogue no. Mn00459770_m1), UBE2D1 (catalogue no. Mn01172638_m1), and VHL (catalogue no. Mn00494136_m1) were provided as Assay-on-Demand by Applied Biosystems. Gene expression analysis was normalized to the expression levels of β-microglobulin (catalogue no. Mn00432100_m1) gene expression. The probes were labeled using FAM as the reporter dye and TAMRA as the quencher dye.

![Fig. 1. Increased expression levels of von Hippel-Lindau tumor suppressor (VHL) in skeletal muscles of cigarette smoke (CS)-exposed mice. A: qRT PCR analysis of VHL mRNA levels (Control: Median = 3.91, interquartile range (IQR) = 3.31–4.3; N = 8 vs. CS: Median = 5.58, IQR = 4.6–5.9; N = 6; **P = 0.0055); B: representative Western blot showing VHL protein expression levels. C: quantification of VHL protein expression levels by densitometry. VHL density signals were normalized to α-tubulin signal values (Control: Median = 1.41, IQR = 0.53–2.1 vs. CS: Median = 3.04, IQR = 2.77–3.98; N = 6, *P = 0.016).](http://ajplung.physiology.org/DownloadedFrom)
Each sample was analyzed in duplicate under the following conditions: 2 min at 50°C, 10 min at 95°C, 15 s at 95°C, and 1 min at 60°C. PCR amplification was correlated against a standard curve. Reactions were performed in MicroAmp optical 96-well reaction plates (PE Applied Biosystems).

Western blot analysis. The excised muscles were homogenized in the ice cold lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA) containing a protease inhibitor cocktail and centrifuged for 10 min on 10,000 g on 4°C. Supernatant was separated from the pellet and used in further steps. Concentration of total protein was determined using Bradford assay (Sigma Aldrich, Taufkirchen, Germany). Equal amounts of total protein (30 μg) were separated under reducing conditions using 7.5%, 10%, and 12% SDS page and transferred onto nitrocellulose membrane (Amersham) in a transblot electrophoretic transfer cell (Bio-Rad Laboratories, Hercules, CA). Membranes were probed overnight at 4°C using rabbit polyclonal anti-HIF-1α (Novus Biologicals, Cambridge, UK) in 1:1,000 dilution, rabbit polyclonal anti-VEGF (Santa Cruz Biotechnology), rabbit polyclonal anti-UBE2D1 in 1:1,000 dilution (Abnova, Taipei, Taiwan), and rabbit polyclonal anti-α-tubulin in 1:10,000 (Abnova) used as loading control. Presence of nuclear protein yield was assessed and confirmed using rabbit polyclonal anti-TBP in 1:1,000 dilution (Abnova) used as nucleolar loading control (unpublished results). Membranes were developed using an enhanced chemiluminescence system (Amersham) and exposed to Hyperfilm enhanced chemiluminescence (Amersham). Densitometric analysis was performed using NIH software package Image J (ImageJ 1.32; NIH, Bethesda, MD).

Determination of muscle fiber cross-sectional area. Transverse sections (5 μm) were cut from the mid-belly of the gastrocnemius muscle on a cryostat at −22°C using Leica CM1850 cryostat attached to positively charged glass slides (Superfrost; MenzelGläser, Braunschweig, Germany). Hematoxylin and eosin (H & E) staining was performed on sections from six animals per group (N = 6) to assess general fiber morphology and measure fiber cross-sectional area. Digital photographs were taken from each H & E section at ×10 magnification using a Magnafire digital camera and software (Optronics, Galena, CA) with 30-ms exposure. Cross-sectional area for the muscles (CSA) was determined from the composite images of the entire muscle cross-section. More than 300 individual fibers per each

![Fig. 2. Chronic CS exposure increases expression levels of ubiquitin-conjugating enzyme E2D1 (UBE2D1) in mice skeletal muscles. A: qRT PCR analysis of UBE2D1 mRNA levels. **P < 0.001 (Control: Median = 3.62, IQR = 2.98–4.5, N = 7 compared with the CS: Median = 5.68, IQR = 5.17–5.95; N = 5; **P = 0.0057). B: representative Western blot showing UBE2D1 protein expression levels. C: quantification of UBE2D1 protein expression levels by densitometry. UBE2D1 density signals were normalized to α-tubulin signal values (Control: Median = 0.86, IQR = 0.66–1.02 compared with the CS: Median = 1.77, IQR = 1.4–1.94; N = 6; *P = 0.03).](http://ajplung.physiology.org/)

![Fig. 3. Increased expression levels of prolyl hydroxylase-2 (PHD2) in skeletal muscle of CS-exposed mice. qRT PCR analysis of PHD2 mRNA levels. *P < 0.05 (Control: Median = 22.87, IQR = 19.13–24.06, N = 7 vs. CS: Median = 30.13, IQR = 28.27–35.9, N = 6; *P = 0.038). B: representative Western blot showing PHD2 protein expression levels. C: quantification of PHD2 protein expression levels (Control: Median = 0.92, IQR = 0.75–1.1 vs. CS: Median = 2.22, IQR = 1.39–3.1, N = 6; *P = 0.021).](http://ajplung.physiology.org/)
cross-section have been included in the CSA evaluation. With the aid of an image morphometry program (ImageJ 1.32j), the outline of the individual fibers was traced and parameters such as fiber area expressed in $\text{mm}^2$, smallest and biggest fiber diameter, and fiber cross-sectional roundness index were assessed for each individual fiber. The roundness index (recorded as a value between 0 and 1) is the ratio of the cell area relative to the area of a circle that fully enclosed that cell; circular cells have a value approaching 1, whereas noncircular cells have smaller values. Cut point roundness index for fiber inclusion in CSA evaluation was 0.8.

Immunohistochemistry and capillary measurements. Serial transverse sections, 5 $\mu$m in thickness, were cut at $-22^\circ$C using Leica CM1850 cryostat attached to positively charged glass slides (Superfrost). Before primary antibody incubation, the sections were incubated in 2.5% BSA for 5 min. Capillaries were identified by staining with monoclonal antibody detecting CD31 (MO823; Dako, Glostrup, Denmark) in 1:100 dilution and diaminobenzidine.

Capillary analysis was performed using light microscope (Olympus BX60) connected to a computerized image system (Cell Images). Capillary-to-fiber ratio parameter was determined by analyzing number of capillaries surrounding more than 200 individual fibers per section belonging to each animal ($N = 5$). Briefly, digital photographs of four to six randomly chosen cross-sectional areas were taken at $\times40$ magnification, area perimeter outlined using free-hand selection tool, and total number of fibers and surrounding capillaries determined. Quality of cross-sections was assessed by measuring roundness index of individual fibers with the aid of an image morphometry program (ImageJ 1.32j). Only fibers entirely included in outlined area and with roundness index $\geq 0.8$ have been included in analysis.

Statistical analysis. Obtained data for each experimental group were not normally distributed. The results were represented as median [interquartile range, (IQR)] as determined using Mann-Whitney $U$ rank test for unpaired data. Differences were considered significant at $P < 0.05$. Statistical analysis was performed with SPSS v.16 Statistics Software.

RESULTS

CS exposure increases skeletal muscle levels of VHL, UBE2D1, and PHD2. VHL mRNA expression levels were significantly increased in skeletal muscles from mice exposed to CS relative to

Fig. 4. CS exposure destabilizes hypoxia-inducible factor-1α (HIF-1α) protein. A: representative Western blot showing HIF-1α protein expression levels. B: quantification of HIF-1α protein expression levels by densitometry. HIF-1α density signals were normalized to $\alpha$-tubulin signal values. (Control: Median = 1.07, IQR = 0.74–1.64 vs. CS: Median = 0.39, IQR = 0.3–0.47, $N = 6$; *$P = 0.027$). C: qRT PCR analysis of HIF-1α mRNA values (Control: Median = 11.83, IQR = 11.04–12.91, $N = 6$ vs. CS: Median = 14.06, IQR = 13.27–15.39, $N = 8$, *$P = 0.045$).

Fig. 5. Decreased vascular endothelial growth factor (VEGF) abundance in skeletal muscles of CS-exposed mice. A: qRT PCR analysis of VEGF mRNA levels (Control: Median = 10.37, IQR = 7.28–13.82, $N = 8$ vs. CS: Median = 9.16, IQR = 8.31–11.42, $N = 6$; $P = 0.75$). B: representative Western blot showing VEGF protein expression levels. C: quantification of VEGF protein expression levels by densitometry. VEGF density signals were normalized to $\alpha$-tubulin signal values (Control: Median = 14.9, IQR = 12.27–17.05 vs. CS: Median = 9.55, IQR = 8.92–9.82, $N = 6$; *$P = 0.037$).
the matching control group (Control: Median = 3.91, IQR = 3.31–4.3; N = 8 vs. CS: Median = 5.58, IQR = 4.6–5.9; N = 6; **P = 0.0055, Fig. 1A). Similarly, Western blot analysis demonstrated an increase in VHL protein levels in skeletal muscles from CS-exposed animals compared with the matching controls (Control: Median = 1.41, IQR = 0.53–2.1 vs. CS: Median = 3.04, IQR = 2.77–3.98; N = 6; *P = 0.016) (Fig. 1, B and C).

An important element of VHL-regulated ubiquitination machinery is UBE2D1 ubiquitin-conjugating enzyme, belonging to the E2 family of ubiquitin ligases. UBE2D1 is required for supply and transfer of the activated ubiquitin molecules to the VHL/target protein complex, therefore facilitating the process of ubiquitination and degradation of VHL target proteins (18, 36). The results of this study demonstrated statistically significant overexpression of UBE2D1 on both, mRNA (Control: Median = 3.62, IQR = 2.98–4.5, N = 7 compared with the CS: Median = 5.68, IQR = 5.17–5.95; N = 5; **P = 0.0057) (Fig. 2A) and protein level (Control: Median = 0.86, IQR = 0.66–1.02 compared with the CS: Median = 1.77, IQR = 1.4–1.94; N = 6; *P = 0.03) (Fig. 2, B and C).

VHL-mediated polyubiquitination and subsequent proteosomal degradation of intracellular HIF-1α is facilitated via its O2-dependent hydroxylation by a family of PHDs (PHD1–4) (6). PHD2, a key member of this family, is responsible for maintaining basal levels of HIF-1α in normoxia and was selected for analysis (11). Our results demonstrated a significant increase in PHD2 mRNA levels (Fig. 3A) in muscles from CS-exposed mice compared with controls (Control: Median = 22.87, IQR = 19.13–24.06, N = 7 vs. CS: Median = 30.13, IQR = 28.27–35.9, N = 6; *P = 0.038). In this regard, 6 mo...
of CS exposure significantly elevated PHD2 protein levels in gastrocnemius muscle of exposed animals (Control: Median = 0.92, IQR = 0.75–1.1 vs. CS: Median = 2.22, IQR = 1.39–3.1, N = 6; *P = 0.021) (Fig. 3, B and C).

**CS exposure decreases stability of HIF-1α protein.** Enhanced VHL and PHD2 activity strongly suggested the possibility of increased HIF1-protein degradation and reduced availability in skeletal muscles of CS-exposed animals. Indeed, a significant decrease in HIF-1α protein levels was observed in CS-exposed animals relative to controls (Control: Median = 1.07, IQR = 0.74–1.64 vs. CS: Median = 0.39, IQR = 0.3–0.47, N = 6; *P = 0.027) (Fig. 4, A and B). By contrast, qRT-PCR analysis of HIF-1α mRNA expression level revealed mild but statistically significant increase in expression (*P = 0.045) in muscles of CS-exposed animals (Median = 11.83, IQR = 11.04–12.91, N = 6) relative to the control (Median = 14.06, IQR = 13.27–15.39, N = 8).

**Decreased VEGF expression is observed in CS-exposed mice.** Decreased HIF-1α protein expression is expected to exert a negative impact on downstream gene expression including VEGF, which is central in neocapillary formation and maintenance of adult tissue vascularization. Indeed, VEGF mRNA levels demonstrated a tendency toward decreased expression in CS-exposed mice (Control: Median = 7.28–13.82, N = 8 vs. CS: Median = 9.16, IQR = 8.31–11.42, N = 6; P = 0.75) (Fig. 5A). Likewise, immunoblot analysis detected a statistically significant decrease in VEGF protein levels in skeletal muscles from CS-exposed mice relative to the control (Control: Median = 12.27–17.05 vs. CS: Median = 9.55, IQR = 8.92–9.82, N = 6; *P = 0.037) (Fig. 5, B and C).

**CS exposure reduces muscle fiber cross-sectional area.** Statistically significant reduction in muscle fiber cross-sectional area was observed in gastrocnemius muscles of 129 SvJ mice after 6 mo of chronic CS exposure (Control: Median = 2,771.16, IQR = 2,462.83–2,914.93 vs. CS-exposed animals: Median = 2,429.3, IQR = 2,360.46–2,454.91; N = 6, *P = 0.025) (Fig. 6, A–C).

**Muscle capillarization is impaired in CS-exposed mice.** To determine the impact of decreased HIF-1α and VEGF expression on muscle capillarization, capillary density was analyzed by anti-CD31 immunostaining, and capillary-to-fiber ratio was assessed in CS-exposed animals and matching controls. As expected, capillary-to-fiber ratio was decreased by 33% in skeletal muscles from CS-exposed mice relative to controls (Control: Median = 1.91, IQR = 1.77–2.13, vs. CS: Median = 1.42, IQR = 1.37–1.55, N = 5; *P = 0.014) (Fig. 6, D–F).

**Chronic CS exposure decreases exercise tolerance in mice.** Exercise capacity is an important parameter to evaluate and determine limitations of skeletal muscle function, which is known to be impaired in patients with COPD (31). Exercise capacity was, thus, evaluated in CS-exposed mice vs. controls. We found that run time was significantly decreased in CS-exposed mice compared with controls (Control: Median = 34.2, IQR = 33.35–35 vs. CS: Median = 30.9, IQR = 30.35–31.75, N = 4; *P = 0.029). Moreover, the mouse run distance was also reduced in CS-exposed vs. control mice (Control: Median = 614, IQR = 584–640 vs. CS: Median = 536, IQR = 507.567, N = 4; *P = 0.03) (Fig. 7).

**DISCUSSION**

The results of the present study demonstrated overexpression of VHL as well as other elements in the ubiquitination cascade including PHD2 and UBE2D1 in skeletal muscles of mice exposed to CS for 6 mo. This provides first evidence that chronic exposure of normal mice to CS directly enhances VHL expression in skeletal muscles. The present results are in agreement with and further strengthen our recent findings in skeletal muscles of patients with COPD (19). In the latter study, VHL overexpression was observed already at the early (Stage-1) of the disease, before development of significantly impaired pulmonary function (19). It is unclear whether an early VHL overexpression also occurs in lungs from mice exposed to CS, this needs to be addressed in future studies specifically addressing this point. Hence, our findings in humans (19) together with the results of the present study indicate that enhanced VHL expression in skeletal muscles might be an early, primary event induced by chronic CS exposure rather than a secondary occurrence to development of COPD.

VHL, a member of the E3 ubiquitin ligase family, tightly regulates intracellular levels of HIF-1α through the mechanism that includes transfer of activated ubiquitin molecules from...
UBE2D1-conjugating enzyme to lysil residues of HIF-1α protein marking it for rapid proteasomal degradation (18, 21, 33, 36). HIF-1α availability within the cell is further regulated by the family of PHD1–4, which catalyze HIF-1α hydroxylation in positions 402 and 564 required for VHL-HIF-1α recognition and interaction during the ubiquitination process (6, 11). Overexpression of VHL, UBE2D1, and PHD2 observed in the present study was accompanied by significantly decreased HIF-1α protein expression in skeletal muscles of CS-exposed animals. This can account for the impaired transduction of the HIF-1α signal toward VEGF expression, resulting in decreased skeletal muscle capillarization. Of interest, our results demonstrate a small but statistically significant increase in HIF-1α mRNA expression in CS-exposed animals, whereas the protein expression levels were significantly decreased. The mechanisms behind this finding are unclear. However, increased HIF-1α mRNA expression in skeletal muscles has been previously reported after short intermittent hypoxic training and in ischemic muscle tissue (8, 48) and represents a compensatory response to inadequate fiber perfusion and O2 availability in the muscle tissue. The findings of the present study provides further evidence to the ongoing debate (7) that increased ubiquitination might be an important mechanism mediating development of systemic complications in COPD.

VEGF plays a central role in the maintenance of adult skeletal muscle capillarity (34). Decreased VEGF expression in skeletal muscles of CS-exposed animals in the present study was accompanied with an ~33% decrease in muscle capillarization. This is in an agreement with a previous report demonstrating significant capillary loss in the gastrocnemius-specific VEGF-deficient mice (34, 35, 42). Moreover, decreased VEGF expression and inhibition of VEGF-dependent signaling have been associated with a substantial endothelial cell apoptosis (42, 49, 54), which might represent potential mechanism underlying capillary loss observed in CS-exposed animals in the present study. Previously, significantly decreased capillarization was reported only in the oxidative soleus muscle but not the glycolytic extensor digitorum longus in the CS-exposed C57Bl/6J mice (44). A likely explanation to the apparent discrepancy between the two studies may be the three-times longer CS exposure period in our study (24 vs. 8 wk). In this study, VEGF mRNA expression was not significantly decreased. The mechanisms behind the discrepant levels of mRNA and protein expression in this study cannot be readily explained. However, as observed in our human study (19), decreased VEGF protein levels, which represent the active signaling component, can be explained by the concurrently decreased HIF-1 and increased VHL protein levels. In this regard, VEGF mRNA steady-state levels do not necessarily mirror transcriptional rate, especially in hypoxia (24, 43). This suggestion is also supported with the recent findings by Xin et al. (52), which provided evidence that VHL regulates post-transcriptional levels of VEGF.

Parallel to the disturbed hypoxia/angiogenic signaling in CS exposed mice, a significant decrease in skeletal muscle exercise tolerance was also observed. Previously, reduced exercise tolerance was demonstrated in mice with specific loss of HIF-1α in the skeletal muscle (25). Moreover, skeletal muscle capillary adaptation to exercise training was shown to be highly dependent on myocyte-expressed VEGF (33). However, additional mechanisms, such as CS-induced oxidative damage of proteins important for muscle bioenergetics and metabolism or inflammatory processes, might also play a role in development of skeletal muscle dysfunction and reduced exercise tolerance in the present study animals (3, 4, 22, 44).

Taken together, the results of the present study provide first evidence that chronic CS exposure significantly enhances elements of the ubiquitination cascade including VHL, UBE2D1, and PHD2. This has adversely affected transduction of the HIF-1α signal toward VEGF expression and capillary formation. Impaired hypoxic signaling in the CS-exposed mice was simultaneously accompanied by morphological skeletal muscle changes and decreased exercise capacity. The findings of this study are in agreement with those reported in skeletal muscles from patients with COPD and PCS (19) and further identify activation of the ubiquitin-proteolysis cascade as an important mechanism mediating decreased skeletal muscle capillarization due to CS exposure.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES
9. Engelen MP, Schols AM, Does JD, Wouters EF. Skeletal muscle weakness is associated with wasting of extremity fat-free mass but not...
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