Valproic acid attenuates skeletal muscle wasting by inhibiting C/EBPβ-regulated atrogin1 expression in cancer cachexia

Sun R, Zhang S, Hu W, Lu X, Lou N, Yang Z, Chen S, Zhang X, Yang H. Valproic acid attenuates skeletal muscle wasting by inhibiting C/EBPβ-regulated atrogin1 expression in cancer cachexia. Am J Physiol Cell Physiol 311: C101–C115, 2016. First published April 27, 2016; doi:10.1152/ajpcell.00344.2015.—Muscle wasting is the hallmark of cancer cachexia and is associated with poor quality of life and increased mortality. Valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, has important biological effects in the treatment of muscular dystrophy. To verify whether VPA could ameliorate muscle wasting induced by cancer cachexia, we explored the role of VPA in two cancer cachetic mouse models [induced by colon-26 (C26) adenocarcinoma or Lewis lung carcinoma (LLC)] and atrophied C2C12 myotubes [induced by C26 cell conditioned medium (CCM) or LLC cell conditioned medium (LCM)]. Our data demonstrated that treatment with VPA increased the mass and cross-sectional area of skeletal muscles in tumor-bearing mice. Furthermore, treatment with VPA also increased the diameter of myotubes cultured in conditioned medium. The skeletal muscles in cachectic mice or atrophied myotubes treated with VPA exhibited reduced levels of CCAAT/enhancer binding protein beta (C/EBPβ), resulting in atrogin1 downregulation and the eventual alleviation of muscle wasting and myotube atrophy. Moreover, atrogin1 promoter activity in myotubes was stimulated by CCM via activating the C/EBPβ-responsive cis-element and subsequently inhibited by VPA. In contrast to the effect of VPA on the levels of C/EBPβ, the levels of inactivating forkhead box O3 (FoxO3a) were unaffected. In summary, VPA attenuated muscle wasting and myotube atrophy and reduced C/EBPβ binding to atrogin1 promoter locus in the myotubes. Our discoveries indicate that HDAC inhibition by VPA might be a promising new approach for the preservation of skeletal muscle in cancer cachexia.

VPA; muscle wasting; cancer cachexia; C/EBPβ; atrogin1

CACHEXIA IS A MULTIFACTORIAL syndrome associated with changes in many metabolic pathways. The syndrome is characterized by weight loss at the expense of skeletal muscle and body fat (3). Muscle wasting is a systemic response to various diseases, including cancer, sepsis, renal and cardiac failure, and acquired immune deficiency syndrome (AIDS) (11, 23). Millions of new cancer cases and cancer deaths occur worldwide (54), and more than 50% of cancer patients suffer from cancer cachexia, which is often accompanied by progressive atrophy of skeletal muscle (52).

In cancer cachexia, multiple factors can influence muscle mass, such as proteolysis-inducing factor, glucocorticoids, tumor necrosis factor, interleukin-6, and angiotensin II (53). Many of these factors induce muscle wasting by altering protein metabolism via protein synthesis and degradation pathways in muscle cells (28). Furthermore, the regulatory network constituted by these factors is intricate. One of the key signaling pathways responsible for enhancing protein synthesis in muscle is mediated by insulin-like growth factor 1 (IGF1). IGF1 binding to membrane receptors activates the Akt-mediated signaling pathway by increasing the level of phosphorylated Akt (p-Akt) (16, 45, 48). p-Akt promotes phosphorylation and activation of S6 kinase (S6) (14, 26), which is a downstream signaling molecule in the Akt signaling pathway. The activated kinase promotes muscle protein synthesis and inhibits proteolysis, ultimately leading to myofiber hypertrophy.

In contrast, members of the transforming growth factor-β (TGFβ) family, such as myostatin and activin A, inhibit muscle normal growth and promote muscle protein loss in disease states (63). Myostatin binds to cell surface receptors in muscle to suppress p-Akt, thereby leading to a reduction in the level of phosphorylated forkhead box O3 (p-FoxO3a) and accumulation of FoxO3a (22, 39, 55, 62). FoxO3a favors nuclear entry and activates muscle atrophy F-box (MAFbx), which is an atrophy-related ubiquitin ligase (47). Muscle RING finger 1 (MuRF1) and MAFbx (also called atrogin1) are two crucial ubiquitin ligases that promote the process of muscle wasting (7, 17). The myostatin-atrogin1 axis is involved in the degradation of several proteins, such as myoblast determination protein 1 (MyoD), myosin heavy chain (MyHC), and myosin light chain (MyLC), in muscle and is the key signaling pathway driving muscle atrophy (35, 36, 38). Previous studies evaluating the role of the myostatin-atrogin1 axis find that inhibiting myostatin, FoxO3a, and atrogin1 individually alleviates muscle wasting in mice (31, 44, 63). However, previous research shows that FoxO3a activity is not only inhibited by p-Akt but is also suppressed by peroxisome proliferator-activated receptor γ-coactivator 1α (PGC1α), which is downregulated in atrophied muscle (46). Additionally, inhibition of
myostatin has been shown to increase expression of PGC1α (32), and overexpression of PGC1α has been proven to prevent muscle wasting (10, 24, 30).

Atrogin1 is also regulated by CCAAT/enhancer binding protein-β (C/EBPβ), in addition to FoxO3a. Silencing C/EBPβ expression inhibits atrogin1 expression in myotubes with dexamethasone-induced atrophy (18). It was later demonstrated that C/EBPβ upregulated the expression of atrogin1 by binding to the C/EBPβ-responsive cis-element in the promoter of the atrogin1 gene in C2C12 myotubes (60).

Recently, accumulating evidence indicates that protein acetylation and deacetylation is involved in the process of muscle wasting. For example, muscle wasting caused by denervation or immobilization is associated with reduced protein acetylation (2). Emerging evidence has demonstrated that histone deacetylase 1 (HDAC1) is both necessary and sufficient for muscle wasting induced by nutrient deprivation and skeletal muscle disuse (6). In addition, a recent report shows that HDAC6 promotes skeletal muscle wasting induced by chronic angiotensin II signaling (13). Moreover, the study performed in rodent models of Duchenne muscular dystrophy (DMD) demonstrates that HDAC inhibitors are emerging candidate drugs for the treatment of muscular dystrophies (12).

To date, several HDAC inhibitors have been successfully used for the treatment of muscle wasting caused by multiple factors (5, 20, 50, 56–58). So far, only one HDAC inhibitor named AR-42 has been proved to prevent muscle wasting induced by cancer cachexia (56).

Valproic acid (VPA), an HDAC inhibitor, is a branched chain fatty acid used for treating bipolar disorders and epilepsy (19, 41). In early 2003, the drug was shown to represent a potential treatment for spinal muscular atrophy (50). The following year, one research group found that VPA was able to increase muscle cell size by inducing follistatin-mediated inhibition of the myostatin signaling pathway (25, 33). In addition, a prior report demonstrates that VPA promotes muscle hypertrophy by activating the Akt signaling pathway thereby ameliorating DMD (20). As VPA promoted muscle hypertrophy and alleviated muscle atrophy caused by another factor, VPA may also have the potential to attenuate cancer-induced muscle atrophy.

A previous report by Bonetto et al. (9) demonstrated by using the colon-26 (C26)-tumor model that VPA could effectively decrease muscle myostatin while increasing both the expression of follistatin and the inactivating phosphorylation of glycogen synthase kinase 3β (GSK3β), although it did not significantly prevent muscle wasting induced by cancer in mouse model. However, with the accumulative evidence suggesting the mounting therapeutic value of VPA in various systems, we are motivated to more systematically and comprehensively assess its effects in multiple cancer cachexia models and under alternative conditions. Consequently, in this study we found that higher doses and longer duration of VPA treatment could ameliorate muscle wasting in cancer cachexia. In addition, our findings further clarified and solidified the underlying mechanisms of VPA that would provide a basis for further investigation and validation of the values of VPA in muscle wasting prevention.

MATERIALS AND METHODS

Antibodies and other reagents. The following primary antibodies were used: anti-atrogin1 antibody (no. AP2041) purchased from ECM Biosciences (Versailles, KY); anti-PGC1α antibody (ab54481) purchased from Abcam (Cambridge, MA); anti-phospho-S6 (no. 4858), anti-S6 (no. 2317), anti-acetylated-lysine (ac-K) (no. 9441), anti-phospho-Akt (no. 4060), anti-Akt (no. 9272), anti-phospho-FoxO3a (no. 9466), and anti-FoxO3a (no. 2497) antibodies obtained from Cell Signaling Technology (Danvers, MA); and anti-p300 (sc-585), anti-C/EBPβ (sc-7962), anti-HDAC1 (sc-7872), anti-HDAC2 (sc-7899), and anti-HDAC3 (sc-11417) antibodies acquired from Santa Cruz Biotechnology (Santa Cruz, CA).

VPA was purchased from Sigma-Aldrich (St. Louis, MO). VPA was dissolved in corn oil at a concentration of 50 mg/ml for experiments with mice. VPA was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 2.5 M for experiments with cultured myotubes.

Cell culture and animal models. Colon-26 adenocarcinoma cells (C26 cells) (Medical Science Experimentation Center of Sun Yat-Sen University, Guangzhou, Guangdong, China) and Lewis lung carcinoma cells (LLC cells) (Shanghai Branch of Chinese Academy of Science, Shanghai, China) were cultured in Dulbecco’s modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 1% antibiotic and antimycotics (12, 20, 30, 44). Mice in the latter group were referred to as VPA mice. All animals were obtained from Beijing HFK Bioscience (Beijing, China). Before injection of C26 cells into CD2F1 mice (C26 model) or injection of LLC cells into C57BL/6 mice (LLC model), cells were counted and resuspended at 5 × 10⁷ cells/ml in sterilized phosphate buffer saline (PBS). The right flanks of mice were shaved and a subcutaneous injection of either 5 × 10⁶ C26 cells or LLC cells suspended in 100 μl sterilized PBS or 100 μl sterilized PBS without cells was administered. Eight-week-old female CD2F1 or male C57BL/6 mice were allocated randomly into one of three experimental groups: 1) control mice (CN mice) injected subcutaneously with PBS; 2) tumor-bearing mice (TB mice) injected subcutaneously with C26 cells or LLC cells received daily intraperitoneal injection of corn oil; and 3) tumor-bearing mice injected subcutaneously with C26 cells or LLC cells received daily intraperitoneal injection of VPA (200 mg/kg). Mice in the latter group were referred to as VPA mice. All animals received intraperitoneal injection starting the second day after tumor implantation. The animals were monitored daily and were euthanized on day 28 or 30 following tumor implantation. Quadriceps, tibialis anterior, soleus, and gastrocnemius muscles, hearts, epididymal fats, spleens, and tumors were immediately harvested and weighed. For subsequent studies, tibialis anterior muscles were fixed in 4% paraformaldehyde, and other tissues were immediately frozen in liquid nitrogen and stored at −80°C. All animal experiments were approved by the Institutional Animal Care and Use Committee at Tongji Medical College, Huazhong University of Science and Technology (21, 40, 42).

Myofiber cross-sectional area. To determine the myofiber cross-sectional area (CSA), hematoxylin and eosin (H&E) staining was performed on a middle cross section of the tibialis anterior. Images were acquired using a digital camera and were quantified using ImageJ software (National Institutes of Health, Bethesda, MD). Within each section, 5 view fields with 100 myofibers per field were measured (60).

Immunofluorescence. To visualize the outlines of myofibers, 10-μm sections were obtained from the middle cross section of the tibialis anterior. The sections were incubated with Alexa Fluor 350-conjugated wheat germ agglutinin (Invitrogen, Carlsbad, CA) at 37°C for 2 h and subsequently washed in PBS. Images were acquired using a digital camera (6). Representative view fields were elected and shown.

Real-time PCR. RNA was extracted from quadriceps muscles using TRIzol reagent (Invitrogen). Reverse transcription of mRNA was performed using a Revert Aid First-Strand cDNA Synthesis Kit (Thermo Scientific, Shanghai, China) and anti-HDAC3 (sc-11417) antibodies acquired from Santa Cruz Biotechnology (Santa Cruz, CA).
Fisher Scientific, Rockford, IL). Quantitative real-time PCR was performed with 2× SYBR Green Mix (Thermo Fisher Scientific) using a Roche LightCycler 480 system (Roche, Mannheim, Germany). The primer sequences are as follows: atrogin1: forward: 5'-ACA-CATCTTTATGCACACTTG-3'; reverse: 5'-TCTCATCAGGATCAGCACCACACCAC-3'; myostatin: forward: 5'-AGTGGATCTAAATGAGGCGAGT-3'; reverse: 5'-GTTCCAGGGCCGACCTTAC-3'; MuRF1: forward: 5'-AGCATAAGATCGCTGTGACCA-3'; reverse: 5'-CCAGACCGCTCCAACAACT-3'; FoxO3a: forward: 5'-GCAAGCGG-CGTAGTACTCGGA-3'; reverse: 5'-CGGAGGCGGAGTGTTATCC-3'; PGC1α: forward: 5'-AACCACACCCCCAGGATCAGA-3'; reverse: 5'-GAGGTCAATGAAGGGTGTCATTG-3'. The data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

Western blotting analysis. The quadriceps muscles were homogenized, and total protein was extracted using RIPA protein lysis buffer (P1003; Beyotime Institute of Biotechnology, Nantong, Jiangsu, China) with freshly added 1% protease inhibitor cocktail and 1 mM phenylmethylsulphonyl fluoride (PMSF). A total of 80 μg of protein was subjected to a 10% SDS-PAGE gel to separate the proteins by gel electrophoresis and then transferred onto polyvinylidene fluoride (PVDF) (0.45 μm; Millipore, Boston, MA) membranes. The membranes were blocked for 1 h at 37°C in 5% (wt/vol) nonfat dried skim milk (blocking buffer) and incubated with primary antibodies in blocking buffer overnight at 4°C. The membranes were washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Invitrogen) in blocking buffer for 2 h at room temperature. Finally, the membranes were washed before detection. Proteins were detected using the electrochemiluminescence (ECL) reagents (Thermo Fisher Scientific) and visualized using the GelDoc XR Gel Documentation System (Bio-Rad, San Francisco, CA). Quantitative analyses of protein expression were performed using ImageJ software (37).

Myogenic cell culture and differentiation. Murine C2C12 myoblasts (American Type Culture Collection, Manassas, VA) were cultured in growth medium (DMEM supplemented with 10% FBS) at 37°C and 5% CO2. When myoblasts reached 80–90% confluence, cell differentiation and myotube formation were induced by incubation in differentiation medium (DMEM supplemented with 0.5% FBS) for 6 days. C26 cells or LLC cells cultured for 48 h were centrifuged, and the supernatant was diluted at a 1:3 ratio with fresh growth medium as the conditioned medium. The growth medium was used as the control. The use of VPA in conditioned medium was indicated (37, 60).

Analysis of myotube viability. The myotube viability was determined by the dimethyl thiazolyl diphenyl tetrazolium (MTT) assay (Sigma-Aldrich). C2C12 cells were seeded at a density of 1,000 cells per well containing 100 μl of growth medium in 96-well plates. Cell differentiation and myotube formation were induced in differentiation medium for 6 days. VPA was added to the medium with increasing concentrations (2.5, 5, 10, 20, and 40 mM). After 24 or 48 h, 10 μl of MTT (5 mg/ml) were added to the medium and then the myotubes were incubated at 37°C for 4 h. The medium was then removed, and 150 μl of DMSO were added to dissolve the precipitate. The optical density values were tested at a wavelength of 490 nm.

Diameter of C2C12 myotubes. For morphometric studies, images of C2C12 myotubes were obtained using brightfield microscopy. Within each group, the diameters in a total of 100 myotubes (10 view fields with 10 myotubes per field) were measured using ImageJ software (8).

Short interfering RNA transfection. Short interfering RNA (siRNA) against C/EBPβ and the negative control oligonucleotides were synthesized by GenePharma (Shanghai, China). The synthesized siRNA oligonucleotides specific for C/EBPβ were as follows: C/EBPβ siRNA-1: 5'-ACAAGGCCAAGAGCGGCAA-3'; C/EBPβ siRNA-2: 5'-GGCAAGAUAACAGAGGGCAGG-3'; and C/EBPβ siRNA-3: 5'-GGAACUUUGUACGACGCU-3'. siRNA transfection was performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol. After 48 h, the myotubes were harvested for further study.

Chromatin immunoprecipitation. C2C12 myotubes were fixed in 1% formaldehyde for 10 min at room temperature and washed twice with ice-cold PBS. The myotubes were lysed in lysis buffer [5 mM ethylene diamine tetraacetic acid (EDTA), 50 mM Tris-HCl (pH 8.0)], 1% sodium dodecyl sulfate (SDS), 1% protease inhibitor cocktail, and 1 mM PMSF. The lysate was sonicated four times (10 s with a 30-s pause between sonications) on ice. The lysate was clarified by centrifugation and diluted 10-fold in dilution buffer [1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl (pH 8.0)], 1% protease inhibitor cocktail, and 1 mM PMSF. The lysate was precleared with 45 μl Protein A Agarose/Salmon Sperm DNA (no. 16-157C) for 1 h at 4°C. Twenty microliters of each lysate were extracted for use as an input control. The remaining lysate was immunoprecipitated with primary antibody against C/EBPβ or normal mouse IgG (sc-2025; Santa Cruz Biotechnology) overnight at 4°C followed by addition of Protein A Agarose/Salmon Sperm DNA for 1 h at 4°C. The immunoprecipitates were harvested and washed sequentially with TSE I buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, and 20 mM Tris-HCl (pH 8.0)], TSE II buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, and 20 mM Tris-HCl (pH 8.0)], Buffer III [0.25 M LiCl, 1% NP40, 1% deoxycholic acid, 1 mM EDTA, and 10 mM Tris-HCl (pH 8.0)], and TE buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA]. DNA was eluted from the immunoprecipitates with elution buffer (1% SDS and 0.1 M NaHCO3). The eluted DNA was reverse crosslinked at 65°C for 45 min followed by purification using a PCR purification kit (BSC03M1; Bioer Technology, Hangzhou, Zhejiang, China). The purified DNA was used as a template for PCR amplification of a 176-bp fragment using primers for the C/EBP-binding motif in the promoter region of the atrogin1 gene (forward primer: CCTTTGTGTGGCGGAAGAG; reverse primer: CGAGGGTCACGGGGAACATC). PCR products were analyzed by agarose gel electrophoresis (60).

Colomin immunoprecipitation. C2C12 myotubes were washed twice with ice-cold PBS and lysed in Trition-Lysis buffer [20 mM Tris-HCl (pH = 7.5), 150 mM NaCl, 5 mM EDTA, 2 mM diethiothreitol (DTT), 0.5% Triton X-100, 1% protease inhibitor cocktail, and 1 mM PMSF]. The lysate was centrifuged at 12,000 g for 10 min at 4°C. Supernatant was precleared with 20 μl Protein A/G PLUS-Agarose (sc-2003; Santa Cruz Biotechnology) for 1 h at 4°C. The supernatant was incubated with primary antibody against C/EBPβ or normal mouse IgG overnight at 4°C, which was followed by incubation with 20 μl Protein A/G PLUS-Agarose for 1 h at 4°C. The immunoprecipitates were collected by centrifugation, washed five times with PBS, and boiled in 2× SDS sample buffer. After centrifugation, the supernatant was collected and analyzed by western blotting (60).

Statistical analysis. All values are represented as the mean ± SE unless otherwise stated. Differences between group means were determined using ANOVA with Tukey’s post hoc comparison or Student’s t-test with Graphpad Prism 5. A two-sided P < 0.05 was considered statistically significant.

RESULTS

VPA reduces loss of skeletal muscle mass caused by cancer-induced cachexia in mouse models. Loss of body weight and organ mass is a characteristic symptom of cancer-induced cachexia in mice. The body weights of tumor-bearing mice (TB mice) in the colon-26 adenocarcinoma-induced cancer cachectic model (C26 model) and the Lewis lung carcinoma-induced cancer cachectic model (LLC model) did not decline from baseline (Fig. 1, A and B). However, the tumor-free body weight and organ mass of mice in the VPA treatment group was significantly higher than that of the control group (Fig. 1, C and D). The tumor-bearing mice treated with VPA remained more active and lived longer than the control group (Fig. 2, A–D).

To further verify the anti-cachectic effect of VPA, we measured theatty phospholipid content of tissues in tumor-bearing mice. The phospholipid content of the gastrocnemius muscle, quadriceps muscle, and diaphragm of the control group was significantly lower than that of the VPA treatment group, while that of the liver was significantly higher (Fig. 3, A and B). VPA treatment also significantly reduced the levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β in tumor-bearing mice (Fig. 3, C–E). These results indicate that VPA can effectively reduce the loss of body weight and organ mass caused by cancer cachexia in mice.
Fig. 1. Valproic acid (VPA) partially restored the tumor-free body weights and tissue mass in the colon-26 (C26) and Lewis lung carcinoma (LLC) models. 

A: the body weights of CD2F1 mice (tumor-bearing group vs. VPA group, *P < 0.05). 

B: the body weights of C57BL/6 mice. 

C: the tumor-free body weights of CD2F1 mice. 

D: the tumor-free body weights of C57BL/6 mice. 

E and F: the mass of quadriceps, tibialis anterior, soleus, and gastrocnemius muscles in the C26 model (E) and the LLC model (F), respectively. 

G and H: the mass of hearts, epididymal fats, spleens, and tumors in the C26 model (G) and the LLC model (H), respectively; n = 6–8 per group. 

A–D: the weights were recorded every 2 days and normalized to the percentage of their initial body weights. *P < 0.05, **P < 0.01, ***P < 0.001.
Fig. 2. Middle cross section of the tibialis anterior derived from mice in each group. A and B: representative images of an hematoxylin and eosin (H&E)-stained middle cross-section of the tibialis anterior from mice in the C26 model (A) and the LLC model (B), respectively. Bar = 100 μm. C and D: representative images of a middle cross section of the tibialis anterior from mice in the C26 model (C) and the LLC model (D) incubated with Alexa Fluor 350-conjugated wheat germ agglutinin to allow for visualization of muscle fiber membranes (blue). Bar represents = 50 μm. E and F: the average cross-sectional area (CSA) of myofibers in the tibialis anterior from mice in the C26 model (E) and the LLC model (F), respectively. G and H: analyses of the size distribution of myofibers in the tibialis anterior derived from mice in the C26 model (G) and the LLC model (H), respectively. G and H: P value was calculated by Mann-Whitney’s U-test. ***P < 0.001.
weights in both models of TB mice decreased by ~20% compared with the control mice (CN mice) (Fig. 1, C and D). The tumor-free body weights of mice that received intraperitoneal injection of VPA (VPA mice) increased (by ~6.5% compared with TB mice) in the C26 model but did not increase in the LLC model (Fig. 1, C and D).

Body composition was subsequently analyzed in these two tumor animal models. The mass of quadriceps, tibialis anterior, soleus, and gastrocnemius muscles from TB mice decreased by ~30% compared with the CN mice. VPA slowed the loss of quadriceps, tibialis anterior, and gastrocnemius mass by ~10–15% compared with the TB mice in both models (Fig. 1, E and F).

The heart mass in TB mice decreased, and VPA reversed this effect in the C26 model (Fig. 1G). However, the mice in each group of the LLC model exhibited similar heart mass (Fig. 1H). The decrease in fat mass was not affected by VPA in either model (Fig. 1, G and H). The spleen mass in both TB and VPA mice increased (compared with the CN mice) in both models, but no significant difference was observed between TB and VPA mice in either model (Fig. 1, G and H). To monitor the potential effect of VPA on the growth of tumors, we also measured the tumor mass. As shown in Fig. 1, G and H, VPA treatment did not affect the mass of C26 or LLC tumors.

Collectively, these results indicated that treatment with VPA was able to reduce the loss of skeletal muscle without significant effects on other organs in the C26 and LLC models.

VPA attenuates muscle fiber wasting induced by cancer cachexia. Histologically, the effect of VPA was demonstrated by an increase in the fiber size of the tibialis anterior in VPA mice compared with the TB mice (Fig. 2, A–D). VPA mice exhibited an increase in mean fiber CSA of the tibialis anterior (Fig. 2, E and F). Notably, VPA slowed the rate of all tibialis anterior fibers CSA loss induced by C26 or LLC tumors (Fig. 2, G and H).

VPA suppresses molecules promoting protein degradation and activates molecules enhancing protein synthesis in atrophied muscle in mice. Multiple lines of evidence have shown that the ubiquitin-proteasome system mediates muscle protein catabolism during cancer cachexia. In atrophied muscles, multiple components of this pathway are induced, specifically the muscle-specific ubiquitin ligases, atrogin1, and MuRF1. To determine whether VPA regulates these critical components associated with muscle wasting, we examined the mRNA and protein levels of these components in quadriceps muscles derived from mice in each group.

The mRNA levels of atrogin1, but not of MuRF1, were markedly increased in TB mice compared with the CN mice in both models (Figs. 3A and 4A). Western blotting analysis further revealed that protein levels of atrogin1 were also increased (Figs. 3, B and C, and 4, B and C). VPA treatment reduced the magnitude of these changes in both models (Figs. 3, B and C, and 4, B and C).

Fig. 3.Expression of the genes involved in muscle wasting in the quadriceps muscles from mice in the C26 model. A: the mRNA levels of atrogin1, myostatin, muscle RING finger 1 (MuRF1), forkhead box O3 (FoxO3a), and peroxisome proliferator-activated receptor γ-coactivator 1α (PGC1α) in the quadriceps muscles from mice in the C26 model. B: the protein levels of PGC1α, p-FoxO3a, FoxO3a, atrogin1, p-Akt, Akt, p-S6, S6, histone deacetylase 1 (HDAC1), HDAC2, HDAC3, and CCAAT/enhancer binding protein beta (C/EBPβ) in the quadriceps muscles from mice in the C26 model. C–E: the densitometric analyses of PGC1α, p-FoxO3a, FoxO3a, atrogin1 (C), p-Akt, Akt, p-S6, S6 (D), HDAC1, HDAC2, HDAC3, and C/EBPβ (E) based on Western blotting in the C26 model. A, C, D, and E: the data are expressed as relative fold change compared with the corresponding matched controls. *P < 0.05, **P < 0.01, ***P < 0.001.
The mRNA levels of myostatin and PGC1α were not significantly different among each group in the C26 model (Fig. 3A). Although the mRNA level of myostatin was increased and the mRNA level of PGC1α was decreased in TB mice compared with the CN mice in the LLC model, treatment with VPA could not reverse these changes (Fig. 4A). Although the protein levels of PGC1α were decreased in both models of TB mice, VPA treatment restored PGC1α protein level in the C26 model but not in the LLC model (Figs. 3, B and C, and 4, B and C).

We found that the mRNA and protein levels of FoxO3α were higher in TB mice compared with the CN mice but VPA treatment did not reduce FoxO3α mRNA or protein levels in either model (Figs. 3, A–C, and 4, A–C). These findings indicated that FoxO3α was activated in atrophied muscle and was not suppressed by VPA. Consequently, the decrease in atrogin1 expression in VPA mice might be mediated by pathways independent of FoxO3α inhibition.

To test the hypothesis, we measured protein levels of C/EBPβ, a protein that is also known to regulate atrogin1 expression. Our data showed that C/EBPβ levels increased in TB mice and decreased in both models of VPA mice (Figs. 3, B and E, and 4, B and E). As VPA is an HDAC inhibitor, we evaluated HDAC levels in muscles derived from mice in each group. The result showed that no obvious difference was observed in the three major HDAC proteins (HDAC1, HDAC2, and HDAC3) between TB and VPA mice, with the exception of the decrease in HDAC2 levels in VPA mice in the LLC model (Figs. 3, B and E, and 4, B and E). Furthermore, we examined the levels of acetylated proteins in the muscles and did not find differences between TB and VPA mice in the C26 model (see Fig. 7, A and C).

To fully understand protein metabolism in atrophied muscle, we subsequently explored the protein anabolism in muscles derived from mice in each group. We found that protein anabolism was also affected by VPA treatment. In previous studies, VPA was reported to increase Akt activity (20, 51). We evaluated protein levels of p-Akt and total Akt in the quadriceps muscles. Western blotting analysis revealed an increase in total Akt levels with an accompanying decrease in the ratio of p-Akt/Akt in the TB mice in both models. VPA treatment reversed the p-Akt/Akt ratio in the two models (Figs. 3, B and D, and 4, B and D). Moreover, the levels of phosphorylated S6 (p-S6), a downstream signaling molecule in the Akt signaling pathway, and the p-S6/S6 ratio decreased in TB mice in the C26 model but not in the LLC model (Figs. 3, B and D, and 4, B and D). VPA reversed the change in p-S6/S6 ratio in the C26 model (Fig. 3, B and D).

Taken together, the results suggested that VPA treatment attenuated protein catabolism and enhanced protein anabolism in the atrophied muscles evaluated in our experiments.
VPA ATTENUATES CANCER-INDUCED MUSCLE ATROPHY

Fig. 5. VPA reduced the wasting of C2C12 myotubes. A: the viability of C2C12 myotubes treated for 24 or 48 h with DMSO or increasing doses of VPA was determined by MTT assay. B and C: C2C12 myotubes cultured in cell conditioned medium (CCM; B) or LLC cell conditioned medium (LCM; C) were treated for 24 or 48 h with DMSO or increasing doses of VPA. Representative micrographs were acquired and shown. Bar = 100 μm. D and E: the diameter of myotubes in CCM for 24 h (D) or 48 h (E) treatments, respectively. F and G: the diameter of myotubes in LCM for 24 h (F) or 48 h (G) treatments, respectively; n = 4. D–G: the diameter of myofibers in the treated groups was normalized to the percentage of the average diameter of myofibers in the corresponding control (CN) groups. *P < 0.05, **P < 0.01, ***P < 0.001.
VPA reduces myotube atrophy induced by conditioned medium. In addition to establishing mouse models for these studies, we also established in vitro cell models to examine the effect of VPA on atrophied myotubes. To determine if VPA interferences with C2C12 myotube atrophy induced by conditioned medium, we incubated myotubes treated with or without VPA in C26 cell conditioned medium (CCM) or LLC cell conditioned medium (LCM). Based on two previous studies (25, 59), we initially monitored the doses of VPA and found that the high doses (for example, 20 or 40 mM) had significant cytotoxicity (Fig. 5A). Therefore, we elected intermediate concentrations of VPA (2.5, 5, and 10 mM) for the following studies that carried minimal cytotoxicity. Consistent with our expectations, myotube atrophy induced by conditioned medium was reversed by VPA upon 48-h treatment (Fig. 5, B, C, E, and G). The diameter of myotubes in each group changed slightly compared with the control group at 24 h (Fig. 5, B, C, D, and F). Therefore, we treated myotubes with VPA for 48 h in the following study.

VPA suppresses molecules promoting protein degradation and activates molecules enhancing protein synthesis in atrophied myotubes. As VPA was able to relieve myotube atrophy induced by conditioned medium, we predicted that VPA directly affected the proteins associated with wasting in myo-

Fig. 6. Protein levels of the molecules involved in the wasting of C2C12 myotubes cultured in CCM. C2C12 myotubes cultured in CCM were treated with DMSO or increasing doses of VPA for 48 h. A and B: the protein levels (A) and the corresponding densitometric analyses (B) of atrogin1, PGC1α, p-FoxO3a, and FoxO3a in C2C12 myotubes. C and D: the protein levels (C) and the corresponding densitometric analyses (D) of p-Akt, Akt, p-S6, and S6 in C2C12 myotubes. E and F: the protein levels (E) and the corresponding densitometric analyses (F) of HDAC1, HDAC2, HDAC3, and C/EBPβ in C2C12 myotubes. The data are expressed as relative fold change compared with the CCM groups. CN group vs. CCM group, CCM group vs. 10 mM VPA group, *P < 0.05, **P < 0.01, ***P < 0.001.
tubes. We characterized the expression of the molecules involved in the process.

We first examined the molecules involved in protein catabolism in myotubes. As shown in Figs. 6 and 7, treatment of atrophied myotubes with VPA reduced the increase in atrogin1 protein levels induced by both conditioned media (CCM and LCM). However, VPA did not detectably alter the levels of PGC1α, p-FoxO3α, or FoxO3α (Figs. 6, A and B, and 7, A and B).

Similar to what was observed in atrophied muscles in mice, treatment of atrophied myotubes with VPA dramatically reduced the increase in C/EBPβ protein levels induced by both conditioned media (Figs. 6, E and F, and 7, E and F). The protein levels of HDACs were not detectably affected by VPA, with the exception of a decrease in HDAC1 level in the myotubes treated with CCM (Figs. 6, E and F, and 7, E and F). The levels of acetylated proteins in the myotubes treated with CCM decreased, while treatment with VPA restored the levels to about the normal levels (Fig. 8, B and D).

We also found that protein anabolism was altered by VPA in atrophied myotubes. Treatment with VPA increased the...
level of p-Akt and the p-Akt/Akt ratio (Figs. 6, C and D, and 7, C and D). The level of p-S6 and the p-S6/S6 ratio increased when the myotubes cultured in conditioned medium were treated with VPA (Figs. 6, C and D, and 7, C and D).

Collectively, these results supported that VPA treatment suppressed protein catabolism and enhanced protein anabolism in atrophied myotubes.

The expression of atrogin1 is downregulated by VPA via inhibition of C/EBPβ binding to the atrogin1 promoter. In the previous study, C/EBPβ was found to bind to the C/EBPβ-binding motif in the atrogin1 promoter and thereby promote expression of atrogin1 (60). Since the changes in the levels of atrogin1 and C/EBPβ were more obvious in CCM than that in LCM, we next explored the underlying mechanism using CCM.

To validate the regulation of atrogin1 by C/EBPβ, we next designed three independent siRNAs targeting C/EBPβ and selected the one which was most effective (siRNA-1) for the following studies (Fig. 9A). Our data showed that the decrease in C/EBPβ resulted in reduction of atrogin1 (Fig. 9, B and C).

To elucidate the molecular mechanism of VPA inhibition of atrogin1 expression, we then evaluated C/EBPβ binding to the atrogin1 promoter in C2C12 myotubes using the chromatin immunoprecipitation (ChIP) assay. As shown, C/EBPβ binding to the atrogin1 promoter increased when myotubes were exposed to CCM, and treatment with VPA reversed this change (Fig. 9D).

To further investigate the underlying mechanism, we examined the interaction between C/EBPβ and certain general transcriptional cofactors involved in HAT or HDAC activities. Using coimmunoprecipitation assay, we found that HDAC2 and HDAC3 had almost no interaction with C/EBPβ. Although p300 interacted with C/EBPβ, the interaction between the two molecules was not obviously affected by VPA. Interestingly, VPA significantly inhibited the interaction of HDAC1 with C/EBPβ, and this was associated with substantial increase in C/EBPβ acetylation (Fig. 9E).

Taken together, these data supported the proposal that VPA inhibits the interaction between HDAC1 and C/EBPβ to increase the acetylation of C/EBPβ and ultimately reduces C/EBPβ binding to the putative binding motif present in the atrogin1 promoter.

DISCUSSION

In this study, we found that VPA attenuated muscle wasting in cachectic mice and cultured myotubes. There were five aspects of this research we would like to emphasize.

Different mechanisms exist in different models of cancer cachexia. We used two different mouse models of cancer cachexia (the C26 and LLC models) and two cell models (C2C12 myotubes were treated with CCM or LCM) to address our hypothesis. Interestingly, the overall variations in terms of VPA responsiveness were different in the in vivo studies compared with the in vitro ones. The method based on conditioned medium to induce myotube atrophy was a relatively simple approach, and VPA could effectively reverse myotube atrophy induced by both conditioned media. However, VPA only exhibited modest effects on muscle

Fig. 8. VPA restored the expression of the acetylated proteins in the C2C12 myotubes, but not in the quadriceps muscles from mice. A: the expression of the acetylated proteins in quadriceps muscles from mice in the C26 model. B: the expression of the acetylated proteins in C2C12 myotubes treated with CCM. C and D: the densitometric analyses of the acetylated molecules based on Western blotting of the quadriceps muscles from mice in the C26 model (C) and C2C12 myotubes treated with CCM (D) (n = 3), respectively. *P < 0.05, ***P < 0.001.
wasting in mice. VPA was used with the same dose and administration route in a previous study, and 252- and 25.7- M concentrations in VPA-treated mice were detected after 30 and 120 min postinjection, respectively (15). Therefore, we could estimate the VPA concentrations in the plasma of our animal models would be in a similar range. This blood level of VPA is lower than the VPA concentration in the myotube medium. These data suggest that VPA effects would be restricted by effective dose and more complicated environmental factors in the animal studies. Consistently, distinct cancer cachectic mouse models appear to be associated with different molecular mechanisms of cachexia. For example, VPA restored the ratio of p-S6/S6 in muscles derived from the C26 model of mice but not the LLC model. Furthermore, VPA treatment completely restored the levels of acetylated proteins in C2C12 myotubes but not in muscles from the animal models. These discrepancies may arise from a difference in VPA half-life between in vivo and in vitro systems and further determining VPA concentrations in the plasma vs. culture medium will help to clarify this issue. Nevertheless, VPA significantly reversed the increase in both atrogin1 and C/EBPβ protein expression in both animal and cell models. These results suggest that atrogin1 and C/EBPβ are the outstanding molecular determinants across various cachectic models.

A mechanistic link may exist between atrogin1 gene expression and C/EBPβ acetylation. In the past, research evaluating the regulation of atrogin1 expression mainly focused on FoxO3a (47). Currently, many more molecules known to promote muscle wasting have become candidate targets for cancer cachexia therapy. In recent years, accumulating research has demonstrated that C/EBPβ plays an important role in regulating the expression of atrogin1 (60, 61). Although some researchers predicted that increasing C/EBPβ expression alone was not sufficient to augment atrogin1 expression, they conceded that muscle wasting was at least partly affected by C/EBPβ. C/EBPβ is an important leucine-zipper transcription factor associated with cellular differentiation and proliferation. The transcriptional activity of C/EBPβ is affected by acetylation resulting from its interaction with p300 or HDAC1 (1, 27, 49). The acetylation of C/EBPβ increased following VPA treatment in our ex-
peperiments, which may underlie the inhibitory effect of VPA on atrogin1 gene transactivation.

In particular, VPA functions to decrease the binding between HDAC1 and C/EBPβ, indicating that at least in our current models HDAC1 is a functional mediator of VPA in muscle wasting.

Collectively, our data showed that C/EBPβ was not only a prominent molecule promoting the process of muscle wasting but also a target of VPA. These results further suggested that targeting C/EBPβ and HDAC1 might serve as a novel approach for the treatment of muscle wasting in cancer cachexia.

VPA has the potential to promote protein synthesis in atrophied muscles and myotubes. In our experiments, VPA not only inhibited the functions of molecules involved in protein catabolism but also activated molecules enhancing protein anabolism in atrophied muscle and myotubes. The Akt signaling pathway that was repressed by cancer cachexia could be re-activated by VPA. Consistently, VPA also reactivated S6, an effector in protein synthesis and a downstream mediator in the Akt signaling pathway. These results indicated that a more general function of VPA might relieve muscle wasting.

**Doses and duration of VPA may dictate the final outcomes on muscle wasting.** An independent research group has also investigated previously if VPA treatment could prevent cancer cachexia in the C26 mouse model (9). They concluded that VPA does not attenuate muscle wasting induced by a C26 tumor. The discrepancy might well be due to the differences in doses and duration of VPA used in their experiments and ours. In their experiment, mice received VPA at a dose of 100 mg·kg⁻¹·day⁻¹, whereas a dose of 200 mg·kg⁻¹·day⁻¹ was used in our research, a dose that has been used in a previous report for renal carcinoma (29). In addition, studies have demonstrated that the dose of 200 mg/kg corresponds to the human equivalent of 32 mg/kg, which is below the 60 mg/kg limit considered safe and well tolerated in humans (4, 34, 43). Another difference between our research and the previous report was the duration of time in the experiments (9). The C26 model in the previous study was maintained for only 13 days, but the animal models in our research were extended for 30 days on VPA treatment.

The usage of VPA needs be further studied. In our research, the data showed that VPA attenuated muscle wasting induced by cancer cachexia. Although the magnitude of the overall attenuation in mice was modest, the treatment with VPA was able to keep muscle loss at bay and have potential to improve the movement ability of mice with cancer cachexia. Nevertheless, the VPA dosage and administration route can be further optimized and its usage in combination with additional cachexia-targeting compounds to treat cancer-induced muscle wasting remains to be tested.

In conclusion, our study indicates that VPA can be a compensatory approach to reduce and possibly ameliorate muscle wasting in cancer cachexia.

ACKNOWLEDGMENTS

The manuscript was also read by Dr. Ke Chen (Department of Urology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology). All animal experiments were performed under the guidance of veterinarian Chunheng Zhou in the Animal Experimental Laboratory (temperature: 22 ± 2°C; relative humidity: 40~70%; light: darkness = 12:12; noise <60 dB), the Institutional Animal Care and Use Committee at Tongji Medical College, and Huazhong University of Science and Technology.

GRANTS

This study was supported by the National Natural Science Foundation of China (NSFC) Grant 81272560, Open Research Foundation of the State Key Laboratory of Virology of Wuhan University Grant 2014KF007, Hubei Province Scientific and Technical Project Grant 2011CDB366, and Hubei Provincial Health Project Grant WJ2015MB0120 (to H. Yang). This research was also supported by National NSFC Grants 30872924, 81072095, and 81372760, Program for New Century Excellent Talents in University from the Department of Education of China (NCET-08-0223), and National High Technology Research and Development Program of China (863 Program) (2012AA021101) (to X. Zhang).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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