Title efficacy of phosphodiesterase 5 inhibitor on distant burn-induced muscle autophagy, microcirculation, and survival rate

Sachiko Hosokawa, Hiroaki Koseki, Michio Nagashima, Yoshihiro Maeyama, Kentaro Yomogida, Chelsea Mehr, Madeleine Rutledge, Hannah Greenfeld, Masao Kaneki, Ronald G. Tompkins, J. A. Jeevendra Martyn, and Shingo E. Yasuhara

Department of Anesthesiology and Critical Care and Pain Medicine, Shriners Hospital for Children, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts

Submitted 8 February 2013; accepted in final form 4 March 2013.


MUSCLE WASTING AND MUSCLE WEAKNESS ARE SEEN in many pathological states, including burns, trauma, sepsis, immobilization, and denervation (46). In severely burned patients, the muscles distant from the site of burns are often affected because of the systemic catabolic effects of burn (8, 40, 53). This catabolic state of muscle wasting greatly affects patients' morbidity and mortality. The mechanism underlying critical illness-induced muscle wasting reportedly involves ubiquitin/proteasome-mediated protein degradation (21) and/or apoptosis-mediated muscle mass loss (47, 49).

Cells under certain stresses often show autophagosomes inside the cytoplasm with engulfment of cellular components, including mitochondria and cytoplasm. Following the discovery of yeast mutants incapable of autophagy (44), a group of autophagy-related genes was identified. The autophagy pathway consists of two parts. Initially, during the activation phase, interactions of autophagy-related molecules lead to an orchestrated series of molecular and cellular events, including the formation of an isolation membrane, encapsulation of the cellular component, and the development of the autophagosome. The autophagosome vesicles then fuse with a lysosome to form an autophagolysosome, finally leading to degradation of the content (the maturation phase). Among the many autophagy-related molecules involved, Atg8/LC3 is one of the most widely used markers to detect autophagosomes by immunoblotting experiments and immunohistochemistry. This technique is based on the principle that LC3/Atg8 stays on the autophagosome membrane throughout the autophagy turnover cycle. Few research methods have been developed to analyze autophagy functions in live mice.

Nitric oxide increases intracellular level of cyclic guanosine monophosphate (cGMP) (1) in the target cells and leads to the activation of protein kinase G (PKG) (11). This chain of signal transduction is responsible for the relaxation of the vascular smooth muscle cells and vasodilation (19, 32, 33) as well as various cellular functions. Phosphodiesterase 5 inhibitors (PDE5Is) augment the vasodilative effect of nitric oxide by increasing the level of cGMP in the target organ and have been utilized for the treatment of male erectile dysfunction (36). Recent studies reported their beneficial impact in muscular dystrophy subjects (3, 51). One of the essential mechanisms where PDE5Is exert a cytoprotective function on skeletal muscles is considered through the improvement of circulatory disturbances (3, 28, 51). Although detailed analysis of the microcirculatory functions in the distant skeletal muscles after severe burn injury remains to be fully investigated, previous studies have reported disturbed circulation (16, 20, 23, 26, 37) and poor tissue oxygen extraction (7) in the skeletal muscles of burned subjects. There has been no report on the efficacy of PDE5Is on critical illness-induced muscle wasting and muscle autophagy or on microcirculation in muscles after burn injury.

In this article, we first document the involvement of muscle autophagy in burned subjects and evaluate whether tadalafil, a PDE5I, ameliorates muscle autophagy and muscle mass loss in burned mice. The effect of drug treatment on the survival rate and microcirculatory disturbance is also tested. In vivo confocal microscopy using autophagy reporter gene tandem fluorescent LC3 (tLC3) is utilized to provide a novel modality of imaging to quantify autophagy formation and maturation in the live mouse.

MATERIALS AND METHODS

Reagents. Antibodies against Atg8/LC3, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and α-tubulin were from MBL, Trevigen, and Abcam, respectively. All of the secondary antibodies and E64 were from EMD. Pepstatin A and the cell-staining dye...
PKH26L were from Sigma. Vectashield was from Vector Laboratories. CBQCA and SYBR Gold were from Life Technologies. tflC3 was obtained from Dr. T. Yoshimori.

Burn/sham burn procedure. The protocol for the studies was approved by the Massachusetts General Hospital Animal Care Committee. Adult male C57BL10 mice of 25–30 g body wt were purchased from Jackson Laboratories (Bar Harbor, ME). The mice were anesthetized with pentobarbital sodium (65 mg/kg body wt) administered intraperitoneally and were divided into three groups (burn + PDE5I treatment, burn + no treatment, and sham burn control). Mice in the burn group received a third-degree thermal injury of 35% of total body surface area on the body trunk and the back according to a previously described protocol (41). Briefly, mice were immersed in 80°C water for 6 s on the back and both flanks and for 4 s on the abdominal side. Fluid resuscitation was performed by injecting 0.5 ml of normal saline. Butorphanol (2 mg/kg) was provided with saline as analgesia. Animals in the control group were given sham burns by immersion in the water at room temperature and were pair fed; all other procedures were conducted exactly the same for both groups of mice. Immediately after burn injury, either PDE5I (tadalafil, 0.3 mg/kg body wt) or water was administered via gastric tube, and the drug administration was continued through drinking water (0.01 mg/ml) until the termination of the experiment. A survival curve was drawn. In a parallel experiment, the animals were either utilized for in vivo microscopy or euthanized, and the hindlimb tibialis anterior muscle tissues were excised immediately for analysis on day 3 after burn injury.

Immunohistochemistry. Immunohistochemistry procedures followed the previously described standard cryosection method (49). On the day of harvest, mice were euthanized by an overdose of pentobarbital sodium, and tissues were harvested immediately. The muscle tissues were embedded in the OCT compound and cryosectioned at 8 μm. Sections were then fixed in 4% formalin in phosphate-buffered saline (PBS; pH 7.4) containing 2 mM EDTA for 2 h at 4°C. After brief rinsing, samples were postfixed in an acidic alcohol solution at −20°C for 5 min (acetic acid-ethanol, 1:2). Blocking in a 1% milk solution in PBS was followed by incubation with the primary antibody against LC3. The signal was visualized by incubation with the secondary antibody conjugated to Texas Red. Stained sections were mounted in Vectashield (Vector Laboratories) containing 4,6-diamidino-2-phenylindole dihydrochloride (DAPI).

Fig. 1. Autophagy is upregulated in distant muscle in burned animals. A: total homogenate of tibialis anterior muscle, a muscle distant from the site of burn injury, was separated on SDS-PAGE and blotted against the autophagy marker LC3. Muscles from burn mice (burn) and sham burn controls (sham) are compared. Samples from 4 mice in each group are documented. A, bottom: GAPDH bands are shown as the loading control. B: the amount of LC3I and LC3II was quantified by densitometry. The unit for the y-axis is the arbitrary densitometric unit. Note that the burn group shows significantly increased LC3I (open bars) and LC3II (gray bars) compared with the sham burn control group. C: the ratio between LC3I and LC3II was compared between burn and sham burn groups. The unit is the ratio for LC3II/LC3I. *P < 0.05, Student’s t-test (n = 4). The error bars represent SE of each group. MW, molecular weight.
nylindole (DAPI) for nuclei counterstaining. Images were captured using a Nikon Eclipse 800 microscope equipped with a SPOT charge-coupled device camera. All of the autophagosomes throughout the entire thickness of the section were visualized by scanning all the z planes by changing the focus depth.

Western blotting analysis. We followed the standard Western blotting method, as described previously (50). Briefly, muscle samples were homogenized in TBST (Tris-buffered saline with Triton X-100; 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, leupeptin, aprotinin, PMSF, 2% Triton X-100, and 1 mM DTT). The total homogenate after low-speed centrifugation (800 g, 10 min) was adjusted to the protein concentration, mixed, and boiled with Laemmli buffer, and equal amounts of total protein per lane were loaded onto 12% polyacrylamide gels and electrophoresed. After transfer to a nitrocellulose membrane, the samples were blocked and incubated with antibodies against LC3, GAPDH, or α-tubulin. Goat anti-mouse IgG was used as a secondary antibody. Membranes were washed with TBST and incubated in enhanced chemiluminescence detection reagents (Denville Scientific) to visualize the proteins of interest. The detected bands were quantified by Image J.

Autophagy turnover blockade. To block the turnover of autophagy, E64d and pepstatin A were injected intramuscularly at 50 μM and 50 μg/ml concentrations, respectively, twice at 30 and 60 min before the tissue was harvested. Control mice were given exactly the same treatment with the same solvent except for the lack of E64d and pepstatin A.

In vivo confocal microscopy. In vivo fluorescence microscopy imaging was based on the previously described method (30), with slight modifications. All mouse experiments were approved by the Institutional Review Board at Massachusetts General Hospital. Briefly, mice were anesthetized with pentobarbital sodium, intubated, and ventilated mechanically. Tibialis anterior muscles were exposed and observed with water-dipping objective lenses. A video-rate confocal microscope was designed and based on the model of Sanderson and Parker (38) with a Nikon Eclipse 50i platform. For observation of autophagosomes, a mammalian expression plasmid construct tFLC3 was injected into the muscles of anesthetized mice 3 days before observation. After DNA injection, transcutaneous electric pulses were applied by two stainless-steel external plate electrodes. Square wave electric pulses were generated by an electropulsator (BTX, San Diego, CA).

DNA and protein concentration. The DNA and protein content in the total homogenate were measured according to previously reported methods (15), with minor modification. Tibialis anterior muscle samples were homogenized in TBST buffer. Samples were centrifuged at 10,000 g for 10 min, and the supernatant was used for protein analysis. The DNA concentration was measured using a spectrophotometer (Bio-Rad, Hercules, CA). The protein concentration was determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL). The protein concentration was also determined with the Lowry method (13). The protein concentration was determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL). The protein concentration was determined using a Lowry method (13). The protein concentration was determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL).

Fig. 2. Autophagy blockade experiment confirms upregulated autophagy turnover in burned mice. A: before the samples were harvested, autophagy turnover was blocked by E64d and pepstatin A injection and compared with the control sample in the same burn/sham burn group (DMSO/vehicle injection) for 60 min under anesthesia. Samples from representative 6 individual mice (2 control and 4 drug injection mice) are shown for each group [burn (B) or sham burn control (S)]. B: bottom: GAPDH signals are shown as the loading control. B: autophagy turnover blockade markedly increased the amount of LC3II in burned mice but not in sham burn controls. The unit for the x-axis is the arbitrary densitometric unit. C: E64d plus pepstatin A (E + A) increased the ratio of LC3II/LC3I in burns, suggestive of increased autophagy turnover. The unit is the ratio for LC3II/LC3I. *P < 0.05, Student’s t-test (n = 3–5). The error bars represent the SE of each group.
800 g. The pellet was rehomogenized in TBST. This centrifugation-rehomogenization step was repeated three times, and samples are finally combined with the supernatant lysate. Thus the total prepared homogenate was served for protein and DNA concentration measurement without centrifugation. Protein concentration was measured by the CBQCA staining method and DNA concentration by the SYBR Gold staining method according to the manufacturer’s directions (Life Technologies).

Blood flow analysis. Red blood cells (RBCs) were collected from BL10 mice, washed, and stained by PKH26L staining dye according to the previously reported method (3). Fifty microliters of the stained RBCs were injected through the penile vein of each mouse. The tibialis anterior muscle was exposed for observation of the RBC flow based on the in vivo microscopy according to the previously described method, with minor modification (3, 4). The image data recorded at 120 frames/s were analyzed by automated blood flow analysis software (KEIO-IS2) (43). After the experiment, blood was collected from each mouse, and the ratio of stained/nonstained RBCs was documented with hematocytometer. The absolute RBC flux was calculated based on the ratio and the result from KEIO-IS2 analysis. The validity and accuracy of automated analysis was confirmed by the linear correlation between the result from manual and automated counting of 20 randomly selected capillary flows ($r^2 = 0.943$ for velocity and 0.933 for RBC flux).

Wound healing. In this experiment, only the backside of the body trunk was burned to monitor the chronological changes in the size of the burn lesion. A third-degree burn injury was administered by dipping the backside of the mouse in 80°C water for 5 s, and the size of burn was measured. The changes in the size of the skin lesion were measured each day in the three groups: sham burn control, burn with tadalafil treatment, and without tadalafil treatment.

Statistics. The comparison of means was performed with one-way ANOVA or t-test for multiple two-group comparisons. The decrease from basal level in body weight or the wound size was assessed by one-way repeated-measures ANOVA. For the survival curve analysis, log-rank test was used. For all tests, significance was accepted when $P < 0.05$.

RESULTS

Burn injury increases autophagy in skeletal muscles. After burn injury, systemic loss of muscle mass occurs (18, 45). To examine whether autophagy is involved, we induced a whole body burn injury. On day 3 after burn injury, the hindlimb muscle, tibialis anterior, was harvested, which is distant from the site of the burn on day 3 after burn, and was evaluated for the autophagy marker LC3 by Western blotting.

In the burned muscle, both LC3I and LC3II were increased compared with sham burn control on day 3 after burn injury (Fig. 1). In tissues where autophagy is actively induced, LC3I is converted to LC3II. Thus, in the acute phase of autophagy

![Image](image_url)

Fig. 3. Immunohistochemistry against LC3 confirms autophagy upregulation, which is reverted by phosphodiesterase 5 inhibitor. A–C: tibialis anterior muscles were harvested and cryosectioned. After fixation, the sections were stained by the antibody against an autophagosome marker, LC3, and Alexa Fluor 594-conjugated secondary antibody. Nuclei are counterstained in blue by 4,6-diamidino-2-phenylindole. The red punctuate staining indicating autophagosome was compared among sham burn control (A), burned mouse without treatment (B), and burned mouse treated with tadalafil (C). White scale bars at the lower right corner represent 10 μm. D and E: both the no. of cells with increased autophagosomes (D) and the average no. of autophagosomes per cross-sectional area of a myocyte (E) were increased in the tibialis anterior muscle with distant burn (black bars) compared with sham burn control (open bars). Tadalafil treatment (gray bars) reduced autophagosome staining in the burned mice compared with nontreated burned animals. **$P < 0.01$; *$P < 0.05$. 

AJP-Endocrinol Metab • doi:10.1152/ajpendo.00078.2013 • www.ajpendo.org
upregulation, the ratio of LC3II/LC3I is known to increase with more LC3I converted to LC3II. However, in the chronic phase, de novo synthesis of LC3I can overcome its decrease. Pathological or experimental conditions developing downstream blockade of autophagy turnover can also be disguised as LC3II upregulation by piling up the LC3 species. To examine whether the observed upregulation of LC3I and LC3II in burns represents the increased turnover of autophagy, protease inhibitors E64 (E) and pepstatin A (A) were injected intramuscularly. Blocking autophagy turnover (E + A) significantly increased the ratio of LC3II/LC3I by accumulating the relative amount of LC3II in burned muscle (Fig. 2), confirming the hypothesis that autophagy turnover is increased.

Observations from fluorescence microscopy studies were consistent with the Western blotting data. Tibialis anterior muscles were cryosectioned, and the amount of autophagosomes (Fig. 3, A–C, red punctate staining) was quantified by immunohistochemistry against LC3. Compared with sham burn controls, the numbers of autophagosomes were increased in burned mice. Both the amount of autophagosomes in each myofiber and the numbers of autophagosome-enriched myofibers showed a significant increase in the burned mice (Fig. 3, D and E). Some amount of autophagy was detected in the sham burn control mice, consistent with the notion that there is a basal level of turnover of the cytosol and organelles.

**PDE5I decreases the amount of autophagy after burn injury.** Previous reports demonstrated that the PDE5I, including tadalafil and sildenafil, ameliorated muscle damage and improved muscle function in muscular dystrophy models (3, 22, 28, 34, 51) possibly through diverse mechanisms (51). As shown in Fig. 3, the number of dots representing autophagosomes increased in the burned mice, consistent with the result from Western blotting. This increase was ameliorated by the treatment with tadalafil. In burned mice treated with tadalafil, the number of autophagosome-enriched cells, as well as the number of autophagosomes in each cell, was decreased, suggesting that PDE5I treatment resulted in the reduction of autophagy.

**Does PDE5I block autophagy maturation, induction, or further upstream events?** Our results demonstrated that PDE5I reduces the amount of autophagy; however, the mechanism remains obscure. Experiments were designed to evaluate whether tadalafil reduces the numbers of autophagosomes by directly blocking the autophagy signal and thus inhibiting their maturation or by blocking the upstream events that otherwise

---

**Fig. 4.** Tandem fluorescent LC3 (tLC3) documents upregulation of autophagy turnover in the distant burn muscle. Three days before the experiment, tLC3 was transiently transfected in the tibialis anterior muscle of BL10 control mice by electroporation. A burn injury of 35% BSA was created on the body trunk away from the muscles of analyses by our standard method (48, 49). On day 3 after burn, in vivo confocal microscopy monitored the activation and maturation of autophagosomes. In sham burn mice, there was not a prominent induction of autophagosomes, as would be detected by both green (GFP; A) and red fluorescent protein (RFP; B) double-positive dots. Rather, only a basal level of RFP-only dots (red dots in C), which represent mature autophagolysosomes, was observed. In burns, a prominent upregulation of GFP-positive (D) and RFP-positive (E) dots was observed. The overlay image (F) revealed that both GFP + RFP double-positive dots for premature autophagosomes and RFP dots only for mature autophagolysosomes were increased. The area in F enclosed by dotted box is magnified as shown in J–L. Arrowheads and arrows point to RFP-only or GFP + RFP double-positive dots, respectively. The white bars at bottom right in C, F, and I represent the scale bar (5 μm). Note that the striated patterns of GFP and RFP derive from the background localization of the free LC3 in the cytosol and are a normal cytosolic distribution. These striations correspond to the “I bands” of the skeletal muscle. Instead, punctate dots are the typical autophagosome formation. In all the images presented here, only parts of the myofiber cells are shown. Complete myofibers are much longer along the axis perpendicular to the striation. However, the distribution of autophagosomes was uniform along the axis of the fibers, and the shown images represent the actual autophagosome occurrence rate in each group. The overall transfection efficiency (fluorescence intensity, the ratio of expression) was equal among all 3 groups. PDE5I, phosphodiesterase 5 inhibitor.
would lead to the induction of autophagy. The autophagy reporter gene tflC3 was transiently transfected in the tibialis anterior muscle and observed by an in vivo confocal microscope. This transgene reporter construct is widely used to assess the turnover of autophagy or to distinguish the premature and mature autophagosomes (24, 25). Autophagosomes in the early phase, immediately after induction and formation, exhibit both green and red fluorescence from green (GFP) and red fluorescent protein (RFP) tandemly conjugated to an autophagosome marker, LC3. When mature autophagosomes

![Fig. 5. Quantification of autophagosomes by cytofluorogram. The microscopic images from tflC3 experiments were fed into Image J colocalization plugin (6) for cytofluorogram analyses after segmentation of the images by fluorescence intensity and the area size to distinguish between autophagosome-associated and nonassociated LC3. This quantification demonstrated that, compared with the sham burn control (A), distant burn injury (B) increases both mature (RFP positive and GFP negative, encircled by thin black line) and premature (RFP and GFP positive, encircled by thick dotted gray line) autophagosomes, suggesting that burn injury increases both induction and turnover of autophagy. Sham burn data revealed only a basal level of mature autophagosomes and a negligible amount of active induction. C: PDE5I treatment decreased both mature and premature autophagosome fractions. The numbers (×10⁴) above each enclosure (thin black and thick dotted gray lines for mature and premature autophagosomes, respectively) indicate the densitometry of autophagosomes in each microscopic image (arbitrary unit).]

![Fig. 6. Quantification of LC3I/LC3II in tibialis anterior muscle with or without tadalafil treatment. A: total homogenate of tibialis anterior muscle was separated on SDS-PAGE and blotted against the autophagy marker LC3. Muscles from burned mice with (burn + tadalafil) or without tadalafil treatment (burn) on day 3 after burn injury were compared. Representative samples from 3 mice in each group are presented. A, bottom: α-tubulin bands are shown as the loading control. B: the amount of LC3I and LC3II was quantified by densitometry. The unit for the y-axis is the arbitrary densitometric unit. Note that the tadalafil treatment group shows significantly less LC3II (gray bars) compared with the group without treatment. C: the ratio between LC3I and LC3II (LC3II/LC3I) is shown and compared between the burned mice with and without treatment. *P < 0.05, Student’s t-test (n = 4). The error bars represent SE of each group.]

AJP-Endocrinol Metab • doi:10.1152/ajpendo.00078.2013 • www.ajpendo.org
fuse with lysosome to form autophagolysosomes, the intravesicular pH drops. Because only the green, but not red, fluorescence fades due to the low pH susceptibility of the GFP molecule (27), the mature autophagosomes are labeled red. The number of both premature and mature autophagosomes increased in the burned animals compared with sham burn controls (Figs. 4 and 5), suggesting that both induction and turnover are augmented. PDE5I treatment decreased both the premature and mature autophagosomes (Figs. 4 and 5), consistent with the hypothesis that PDE5I blocks either induction or rather the upstream events but not the downstream maturation process.

To confirm the finding from immunohistochemistry and in vivo microscopy that tadalafil treatment results in a decrease in autophagy in the distant muscle after burn injury, Western blotting of the tibialis anterior muscle homogenate was performed against LC3 (Fig. 6A). The amount of LC3II was reduced significantly (7.12 × 10^5 vs. 4.48 × 10^5) in the samples treated with tadalafil with decreased LC3II/LC3I ratio (1.53 vs. 0.83) compared with that in the nontreated burned mouse (P < 0.05; Fig. 6, B and C). This result of Western blotting validates that tadalafil treatment ameliorated the burn-induced skeletal muscle autophagy. When LC3I and LC3II bands from sham burn control mice treated with or without tadalafil were compared, neither LC3I [2.12 × 10^4 vs. 2.83 × 10^4 arbitrary units (AU)] nor LC3II [3.40 × 10^4 vs. 3.64 × 10^4 AU] showed significant difference. Tadalafil treatment alone has minimal impact on autophagy in the sham burn control mice.

**PDE5I improves burn-induced muscle mass loss and body weight loss.** To investigate whether increased turnover of autophagy in distant burned muscle leads to the changes in physiological parameters, muscle weight and body weight were compared between burn injury and sham burn control on day 7 after burn injury. Burn injury caused a decrease in the total body weight (Fig. 7A) and in the muscle weight compared with sham-burn control (Fig. 7B). Tadalafil treatment mitigated these decreases. Accordingly, total protein content was differ-

---

**Fig. 7. Physiology data in burned mice improve with tadalafil treatment.** A: body weight changes are shown. The body weight of each mouse on day 0 was regarded as 100% or the baseline before burn injury or drug treatment. Burn injury causes decrease in body weight (burn + water; □). Tadalafil treatment of burned mice (burn + tadalafil; ■) ameliorated the body weight change; n = 7–9 for each group. * and **Statistically significant difference between sham and burn without treatment (P < 0.05 and P < 0.01); #tadalafil treatment showed improvement compared with burned mice without treatment (P < 0.05). B: wet weight of tibialis anterior muscles on day 7 after burn injury is shown. Burn (gray bars) with no treatment shows significantly smaller muscle size compared with sham burn control (open bars). Tadalafil treatment ameliorated the size decrease; n = 18–22 for each group. *P < 0.05; **P < 0.01. The error bars represent SE of each group. C: the concentrations of DNA and protein were measured in the homogenate of muscle samples in burn and sham burn groups with or without tadalafil treatment on day 7 after burn injury. The ratio of protein to DNA concentration was compared between sham and burn and between treatment (tadalafil) and no treatment (no drug); n = 10–15 for each group. *P < 0.05; **P < 0.01. The error bars represent the SE of each group.
ent in all the three groups (10.7, 8.3, and 9.8 mg for the sham burn, burn, and burn + tadalafil group, respectively). But the ratio of total protein content to the muscle weight was not different among sham burn control, and burn with tadalafil treatment, burn or without tadalafil treatment (0.259, 0.263, and 0.250 mg/mg, respectively), suggesting that the muscle weight loss was due mainly to the loss of protein content. To confirm this, the protein/DNA ratio was measured. Burn injury caused a decrease in the ratio compared with sham burn control (247.0 ± 7.8 vs. 305.3 ± 12.9 mg/mg, P < 0.01; Fig. 7C), suggesting that protein content per cell is decreased. Tadalafil treatment alleviated this change (270.8 ± 7.3 mg/mg, P < 0.05; Fig. 7C).

**PDE5I improves burn survival rate.** To examine the effect of tadalafil on the prognosis after burn injury, a translational experiment was performed. This intervention was particularly important since autophagy is generally accepted as a cytoprotective mechanism. Previous studies suggest that autophagy itself, as well as its blockade, can be either beneficial or a detriment to the survival of cells. As shown in Fig. 8, PDE5I improved the burn survival rate despite the fact that it prevents autophagy (Figs. 3–6).

**PDE5I mitigates perturbed muscle microcirculation after burn injury.** One of the major pharmacological functions of PDE5Is on skeletal muscle is the augmentation of blood flow. Previous studies attributed the cytoprotective effect of tadalafil on muscular dystrophy models at least partly to the improvement of muscle blood flow (3, 28). To analyze the effect of burn injury on the tissue microcirculation in the muscles, in vivo microscopic blood flow analysis was performed.

In burned mice, there was less perfused capillary compared with sham burn control (Fig. 9, A–D). Tadalafil treatment alleviated this microcirculatory disturbance after burn injury. The average capillary RBC flux of the control mouse was 2,737.0 ± 392.2 (no. of RBCs passing by per minute per capillary) in sham burn control, 990.3 ± 106.6 in burned mice, and 1,530.2 ± 186.9 in the treated group (P < 0.001 for sham vs. burn, P < 0.05 for burn vs. burn + tadalafil; Fig. 9E). The range of average capillary flux number matches well with previous studies [8,000–10,000 for primary arteriole feeding several capillaries (3), 1,800 in rats (10), and 1,200–2,000 in hamsters (5)].

Next, to examine whether burn injury affects the flow velocity of muscle microcirculation, velocity map was drawn with KEIO-IS2 analysis (Fig. 10, A–C). The muscles of burned mice had more capillaries with slower velocity and fewer ones with higher velocity compared with sham burn control (Fig. 10D). The average flow velocity was slower in burned mice (985.6 vs. 677.7 μm/s, P < 0.01; Fig. 10E). This change was ameliorated by tadalafil treatment (889.6 μm/s, P < 0.05).

**The effect of PDE5I on wound healing.** It is possible that the effect of PDE5I on the improved survival after burn injury is through the improvement of wound healing. To analyze the impact of tadalafil on wound healing, the size of skin lesion was monitored after burn injury and compared between the tadalafil-treated and nontreated groups. The wound size was 87.2 ± 6.9 (SE) and 81.6 ± 11.0% on day 3 and 59.5 ± 5.5 and 63.7 ± 5.7% on day 7 in burned mice with or without tadalafil treatment, respectively (n = 6). Baseline 100% was the skin lesion area size of the initial burn injury on day 0. There was no statistical difference in the size of the skin lesion between the two groups ≥7 days after burn injury.

**DISCUSSION**

*Increased autophagy turnover is involved in burn-induced muscle wasting.* This is the first detailed report of autophagy occurring in the skeletal muscle after burn injury. This increase persisted for ≥7 days after burn injury. Measuring either the raw numbers of autophagosomes by morphological experiment or the raw amount of LC3I and LC3II is a widely used technique in assessing the extent of autophagy occurring in a tissue. In many cases, acute induction of autophagy causes conversion of LC3I to LC3II, yielding increase in the LC3II/I ratio. In some cases, however, the data from such analyses can disguise the actual rate of autophagy turnover. For example, if autophagosome maturation is inherently blocked, premature autophagosomes will accumulate and both the amount of autophagosomes and the level of LC3II appear to be increased despite the significant blockade of autophagy turnover (31). Likewise, under the situation where autophagy is constantly upregulated, the feedback mechanism may increase the gene induction of LC3 as a whole and result in the increase in LC3I species, complicating the interpretation of LC3II/LC3I ratio. Thus, it has been a common practice to perform a blocking experiment where autophagy turnover will be impeded to evaluate the impact on the changes in the LC3II/LC3I ratio or to quantify both the premature and mature autophagosomes (31). However, it has been technically difficult to conduct similar experiments using whole animals. There have been attempts where pharmacological reagents were administered via the systemic route, including intraperitoneal injection, for a prolonged period. However, in those previous studies, the possibility of artifactual induction of secondary autophagy upregulation cannot be ruled out due to nonphysiological stress from prolonged drug treatment and gastrointestinal or systemic complications from intraperitoneal injections. In this study, we directly injected the autophagy

---

**Fig. 8.** Kaplan-Meier survival curve of the postburn mouse with or without PDE5I treatment. Immediately after burn injury or sham burn treatment, drug treatment (tadalafil) was started and continued until the end of the experiment. The burned group with vehicle treatment (solid line; n = 19) resulted in a high rate of early burn-related deaths. Tadalafil treatment (dashed line; n = 17) improved the survival ratio. All of the sham burn control group (dashed/dotted line; n = 8) survived. *Significant difference between water and tadalafil, P < 0.05 by log-rank test.

AJP-Endocrinol Metab • doi:10.1152/ajpendo.00078.2013 • www.ajpendo.org
blocking drugs (E64 plus pepstatin A) intramuscularly, limited the incubation period to 1 h, and obtained data supporting increased autophagy turnover.

To confirm the upregulation of autophagy, we utilized an in vivo confocal microscopic assay with tfLC3 to rule out the blockade of autophagy turnover. If autophagy maturation was inherently blocked, there would be a lack of matured population of autophagosomes, and only the premature population would be increased remarkably (25). The fact that both premature and mature populations showed significant increase confirms the increased upregulation of autophagy turnover. To our knowledge, this study is the first in vivo demonstration using tfLC3 that muscle autophagy is upregulated.

Biological consequences of autophagy and the potential mechanisms of PDE5I treatment. Previous investigations have documented both protective and destructive effects of autophagy to cells. Autophagy is generally accepted to be involved in cell survival mechanisms by clearing disturbed cytosolic components and organelles, sequestering the stress mediators that would otherwise be released from such organelles, and degrading and recycling the old cellular components (13). There are also assumptions that uncontrolled increases in autophagy are related to a detrimental outcome in some cells, although in many studies it is unclear whether the observed increase in autophagy was the cause or result of the stress leading to cell death (42). Despite conflicting interpretations, previous data suggest that a pharmacological approach could result in the reduction of both autophagy and cell death (52). In our study, PDE5I decreased the rate of autophagy (Figs. 3 and 4) but had a beneficial effect on the reversal of muscle atrophy and the burn survival rate (Fig. 8). Thus PDE5I could be a potential candidate for drug intervention in burned patients. Our in vivo confocal microscopy data suggested that PDE5I blocks either the induction of autophagy or further upstream events.

Recently, several independent studies reported the efficacy of PDE5Is in Duchenne muscular dystrophy (DMD) models (3, 22, 28). The mechanism of action of PDE5Is in muscle fiber

Fig. 9. Red blood cell (RBC) flux analysis of the skeletal muscle microcirculation. A–C: RBC flux through each blood vessel is analyzed based on computer-assisted blood flow analysis software (KEIO-IS2). RBC number maps are shown for sham burn control (burn –, tadalafil –) and burned mouse with (burn +, tadalafil +) or without tadalafil treatment (burn +, tadalafil –). The flow traces drawn in the image represent RBC flux through each blood vessel and are color coded according to the amount of flow traffic. The color bar to the right of A–C represents the absolute nos. of RBCs flowing through each blood vessel per minute. Hot colors represent higher numbers, and dark colors represent lower numbers. One representative image from each group is presented; n = 6–9. The white size scale bar at bottom right of each image corresponds to 100 μm. B: frequency distribution histogram of the capillary RBC flux is shown. The y-axis is the number of capillaries. The x-axis shows the range of RBC flux of each subgroup (nos. of RBCs flowing through per minute per capillary). C: total RBC flux is compared among the 3 groups; sham burn control (burn –, tadalafil –) and burned mice with (burn +, tadalafil +) or without treatment (burn +, tadalafil –); n = 5–7. *P < 0.05; ***P < 0.001. The error bars represent the SE of each group.

AJP-Endocrinol Metab • doi:10.1152/ajpendo.00078.2013 • www.ajpendo.org
protection was attributed to or can be speculated to involve vascular modulation, mitochondrial protection, or reduction of the redox stress (51), among many others.

Among the suggested mechanisms, studies of pharmacological intervention aimed at the improvement of blood flow dysfunction in DMD (3, 28) have demonstrated that an abnormal response of blood flow, or functional ischemia, is one of the essential causes of DMD (3, 51), because mitigating functional ischemia by pharmacological intervention, including phosphodiesterase 5 inhibition, blocked muscle cell death in the murine equivalent of DMD. Similarly, the current study showed that distant burn injury caused disturbed microcirculation in the skeletal muscle and upregulation of autophagy turnover away from the site of burn lesion. Tadalafil alleviated both tissue ischemia and autophagy, ameliorated muscle mass loss, and resulted in better survival. However, it remains unclear whether disturbed microcirculation is the cause of burn-induced muscle autophagy. Considering that oxidative stress is a strong inducer of autophagy in various tissues, including the brain and the heart (9, 29), it is possible that tissue hypoperfusion and/or poor oxygen extraction by the tissue after burn injury are playing a role in the induction of autophagy and that part of the efficacy of tadalafil is through the mitigation of the oxidative stress resulting from the perturbed microcirculation.

This study does not answer whether blockade of PDE5 by tadalafil directly inhibits myocyte autophagy. PDE5I increases intracellular cGMP and activates PKG in the target cell. Previous studies have shown that cGMP (17) or PKG activation (39) does not directly inhibit autophagy. Considering that direct inhibition of the canonical pathway of autophagy can lead to the induction of myopathy (14), the PDE5I therapy may be beneficial if it functions on the reversal of oxidative stress or the potential upstream inducer of autophagy.

Recently, there have been reports that blockade of PDE5 facilitates angiogenesis and stimulates wound healing (12, 35). However, in our experiment there was no difference in the speed of wound healing of the skin lesion until 7 days after burn injury between tadalafil-treated and nontreated groups. This does not exclude the possibility that, in the deeper tissue,
the wound healing is facilitated but not reflected by the measurement from outside or the possibility that longer observation yields different speed of wound healing at later phase of recovery after burn injury. It is also possible that, with milder burn injury, tadafalir might improve the wound healing in the affected skin lesion. However, considering that the improvement of survival of the burned mice by tadafalir administration is observed by day 7, it is unlikely that the potential effect of tadafalir on wound healing in the skin lesion plays a major role in improving the mouse survival under the current experimental conditions.

In summary, the current study is consistent with the hypothesis that PDE5i improves skeletal muscle microcirculation and ameliorates the oxidative stress induced by a distant burn injury. Thus it is suggested that the alleviation of skeletal muscle cellular stress leads to the decrease in muscle autophagy and results in the concomitant improvement in the survival rate.

ACKNOWLEDGMENTS

We thank Bob Crowther, Minoru and Yutaka Tomita, and Istvan Schiszler for technical assistance. Microscopy was performed using the Shriners Chronic Morphology Facility.

GRANTS

This work is supported by a Shriners Research grant.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


