Free Radical Biology and Medicine 67 (2014) 30–40

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Genetic ablation of 12/15-lipoxygenase but not 5-lipoxygenase protects against denervation-induced muscle atrophy

Arunabh Bhattacharya [a,b,*], Ryan Hamilton [a,b], Amanda Jernigan [b], Yiqiang Zhang [b,c], Marian Sabia [b], Md. M. Rahman [b,d], Yan Li [b], Rochelle Wei [b], Asish Chaudhuri [b,e,f], Holly Van Remmen [a,b,c,f]

[a] Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA
[b] Sam and Ann Barshop Institute for Longevity and Aging Studies, University of Texas Health Science Center at San Antonio, San Antonio, TX 78245, USA
[c] Department of Physiology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA
[d] Department of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA
[e] Department of Biochemistry, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA
[f] Geriatric Research Education and Clinical Center, South Texas Veterans Health Care System, San Antonio, TX 78229, USA

ARTICLE INFO

Article history:
Received 28 June 2013
Received in revised form
30 August 2013
Accepted 1 October 2013
Available online 8 October 2013

Keywords:
12/15-Lipoxygenase
5-Lipoxygenase
NADPH oxidase
Denervation
Muscle atrophy
Free radicals

ABSTRACT

Skeletal muscle atrophy is a debilitating outcome of a number of chronic diseases and conditions associated with loss of muscle innervation by motor neurons, such as aging and neurodegenerative diseases. We previously reported that denervation-induced loss of muscle mass is associated with activation of cytosolic phospholipase A_2 ($cPLA_2$), the rate-limiting step for the release of arachidonic acid from membrane phospholipids, which then acts as a substrate for metabolic pathways that generate bioactive lipid mediators. In this study, we asked whether 5- and 12/15-lipoxygenase (LO) lipid metabolic pathways downstream of $cPLA_2$ mediate denervation-induced muscle atrophy in mice. Both 5- and 12/15-LO were activated in response to surgical denervation; however, 12/15-LO activity was increased ~2.5-fold versus an ~1.5-fold increase in activity of 5-LO. Genetic and pharmacological inhibition of 12/15-LO (but not 5-LO) significantly protected against denervation-induced muscle atrophy, suggesting a selective role for the 12/15-LO pathway in neurogenic muscle atrophy. The activation of the 12/15-LO pathway (but not 5-LO) during muscle atrophy increased NADPH oxidase activity, protein ubiquitination, and ubiquitin–proteasome-mediated proteolytic degradation. In conclusion, this study reveals a novel pathway for neurogenic muscle atrophy and suggests that 12/15-LO may be a potential therapeutic target in diseases associated with loss of innervation and muscle atrophy.

Skeletal muscle atrophy is a devastating outcome of a number of chronic diseases such as diabetes, cancer, and cystic fibrosis [1]. In addition, loss of innervation (or denervation) of skeletal muscle fibers by motor neurons as observed during aging and neurodegenerative diseases, such as familial amyotrophic lateral sclerosis (f-ALS)[1], leads to muscle atrophy [2–5]. We have previously shown that denervation-induced muscle atrophy is accompanied by an increase in the expression of cytosolic phospholipase A_2 ($cPLA_2$) [6], the rate-limiting step in arachidonic acid metabolism. Arachidonic acid released from membrane phospholipids by the action of $cPLA_2$ acts as a substrate for lipid metabolic pathways catalyzed by lipoxygenases (LOs), cyclooxygenase (COX), and cytochrome P450 [7]. The COX-2 pathway generates prostaglandins such as PGE_2, paracrine hormones that regulate a number of physiological and pathophysiological processes. It has been reported that genetic and pharmacological inhibition of COX-2 impairs skeletal muscle regeneration, suggesting that activation of the COX-2 pathway may be important in myogenesis [8,9]. However, the potential role of the 5- and 12/15-LO lipid metabolic pathways downstream of $cPLA_2$ in skeletal muscle physiology remains unknown.

5- and 12/15-LO are members of the lipoxygenase family that convert arachidonic acid into lipid mediators such as leukotriene B4 (LTB_4) and 12(*S*)-hydroxyeicosatetraenoic acid (HETE) and 15 (*S*)-HETE, respectively. Evidence from several in vitro and in vivo studies has shown that activation of the 5- and 12/15-LO pathways is important in inflammation and, by extension, in inflammatory

Abbreviations: ChT, chymotrypsin-like; $cPLA_2$, cytosolic phospholipase A_2; NOX, NADPH oxidase; HETE, hydroxyeicosatetraenoic acid; LT, leukotriene; f-ALS, familial amyotrophic lateral sclerosis; LO, lipoxygenase; UPS, ubiquitin–proteasome system; GA, gastrocnemius; TA, tibialis anterior

* Corresponding author at: University of Texas Health Science Center at San Antonio, Barshop Institute for Longevity and Aging Studies, Department of Cellular and Structural Biology, 15355 Lambda Drive, San Antonio, TX 78245, United States. Fax: +1 210 562 6110.
E-mail address: bhattacharya@uthscsa.edu (A. Bhattacharya).

0891-5849/$ - see front matter © 2013 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.freeradbiomed.2013.10.002

diseases [10–15]. Moreover, the activation of these pathways has been linked to increases in intracellular oxidative stress [11,16]. In rodent models in which genes for 5- and 12/15-LO were either disrupted or overexpressed and using inhibitors specific for the pathways, the 5- and 12/15-LO pathways have been implicated in a number of pathological conditions including heart failure, atherosclerosis, type 2 diabetes, diabetic peripheral neuropathy, and neurodegenerative diseases such as Alzheimer disease and f-ALS [7,13,17–26]. In addition, the 12/15-LO pathway metabolite 15(*S*)-HETE has been shown to increase proteolytic degradation in cultured myotubes by activating the ubiquitin–proteasome system (UPS), suggesting that activation of the 12/15-LO pathway may potentially play a role in skeletal muscle atrophy [27]. However, no such evidence exists in vivo. Moreover, the role of the 5-LO pathway in skeletal muscle biology also remains unknown.

In this study, we show that activation of the 12/15-LO pathway is an important regulator of skeletal muscle atrophy during loss of muscle innervation. We find this effect to be specific for 12/15-LO, as the genetic and pharmacological inhibition of 5-LO does not affect denervation-induced muscle atrophy. We find that the protective effect of 12/15-LO inhibition during muscle atrophy is not mediated through reduction in the inflammatory response; rather, the inhibition of 12/15-LO during surgical denervation decreases NADPH oxidase (NOX) activity, protein ubiquitination, and UPS-mediated proteolytic degradation. These findings provide the first in vivo evidence that activation of the 12/15-LO pathway may have important implications for muscle degeneration during aging, neuromuscular diseases, and pathophysiological conditions associated with loss of neuromuscular junction integrity/denervation.

Materials and methods

Experimental animals

Mice were maintained under specific-pathogen-free conditions, housed three or four per cage, on a 12:12 (light:dark) cycle at 22 ± 2 °C and at $50 \pm 10\%$ relative humidity. 12/15-LO$^{-/-}$ and 5-LO$^{-/-}$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Male 4- to 6-month-old wild-type (WT), 12/15-LO$^{-/-}$, and 5-LO$^{-/-}$ mice on a C57BL/6 background were used for the denervation and inhibitor studies. Eight-month-old WT females were used for the denervation experiments and were fed ad libitum (AL) or dietary-restricted (DR; 40% less food than AL) from 2 months of age. The aging study was performed in young (6–8 months) and old (28–30 months) female mice. To harvest skeletal muscle, the mice were euthanized using a CO_2 chamber followed by cervical dislocation. All of the procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio and the Audie L. Murphy Veterans Hospital.

Denervation surgery

Surgical sciatic nerve transection was performed using constant-flow isoflurane inhalation anesthesia. In each hindlimb (at the level of femur), a small incision was made, and the sciatic nerve was isolated [6,28]. In the left leg, the sciatic nerve was severed and a 5-mm section of nerve was removed. The ends of the nerve were folded back and closed with reabsorbable sutures to prevent nerve regrowth. The skin incisions were closed with wound clips. The contralateral limb served as the intra-animal control. All the experiments were performed in gastrocnemius (GA) and tibialis anterior (TA) muscles (the muscles innervated by the sciatic nerve), 7 days postdenervation.

Inhibitor injection protocol

To study the effects of the pharmacological inhibition of 12/15-LO and 5-LO on denervation-induced muscle atrophy, mice were subjected to denervation surgery and treated with intraperitoneal injections of dimethyl sulfoxide (DMSO; vehicle), PD146176, baicalein, or zileuton (4 mg/kg body wt, in DMSO), on the day of the denervation surgery (day 0, right after surgery) and thereafter at days 2, 4, and 6 postsurgery. Twenty-four hours after the final injection, the mice were sacrificed for collection of muscles for various assays.

NADPH oxidase activity

GA muscles were homogenized in 10 volumes of isotonic ice-cold phosphate buffer (pH 7.4) containing a protease inhibitor cocktail. The homogenates were centrifuged first at 1000*g* and thereafter at 15,000*g* (10 min at 4 °C) in a refrigerated centrifuge (Beckman Coulter 22R centrifuge, Fullerton, CA, USA) and the supernatants were assayed for NOX activity. Cytochrome *c* (100 μmol/L) and NADPH (100 μmol/L) were added in the presence or absence of superoxide dismutase (SOD; 200 U/ml) and incubated at 37 °C for 15 min. Cytochrome *c* reduction was measured by reading the absorbance at 550 nm for 5 min in a spectrophotometer. Superoxide production was calculated (in nanomoles per milligram of protein) as the difference between absorbance with and without superoxide dismutase on the basis of the extinction coefficient of cytochrome *c* (21.0 mmol/L/cm).

RNA isolation and QRT-PCR

RNA was extracted from homogenized GA muscles using TRIZOL reagent (Invitrogen) and an RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. The quantification of mRNA expression was performed using the SYBR green dye method on a sequence detection system (Model 7300; Applied Biosystems). One microgram of purified RNA was used to synthesize first-strand cDNA by reverse transcription using an oligo(dT) primer (Applied Biosystems) and the Omniscript reverse transcription kit (Qiagen). The first-strand cDNA reaction (0.5 μl) was subjected to real-time PCR amplification using gene-specific primers. Approximately 25 μl of reaction volume was used for the real-time PCR assay, which consisted of 2×12.5 μl Brilliant SYBR green QPCR master mix (Applied Biosystems), 400 nM primers (0.5 μl each from the stock), 11 μl water, and 0.5 μl of template. The thermal conditions consisted of an initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min, and for a final step, a melting curve of 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s. All reactions were performed in duplicate to reduce variation. Data normalization was accomplished using the endogenous control (β-actin), and the normalized values were subjected to a $2^{-\Delta\Delta C_t}$ formula to calculate the fold change between the control and the experimental groups. The following primers were used: atrogin-1, sense, GTCGCAGCCAAGAAGAGAAAGA, antisense, TGCTATCAGCTCCAACAGCCTT; MuRF-1, sense, TAACTGCATCTCCATGCTGGTG, antisense, TGGCGTAGAGGGTGTCAAACTT; MCP-1, sense, GCTGTTCACAGTTGCCGGCTG, antisense, CAGCAGGTGAGTGGGGCGTT; CD-68, sense, GGCCGTTACTCTCCTGCCATCCT, antisense, TGGGCCTGTGGCTGGTCGTA; and β-actin, sense, AATCGTGCGTGACATCAAAGAG, antisense, GCCATCTCCTGCTCGAAGTC.

Western blotting

Gastrocnemius or quadriceps muscle homogenates were prepared in radioimmune precipitation assay buffer (50 mM Tris–HCl buffer with 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium