Nitric Oxide does not mediate Atrogin-1/MAFbx upregulation by inflammatory mediators

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Abstract

Accelerated proteolysis through the ubiquitin-proteasome system has been recognized as a major contributor to muscle wasting, a serious complication frequently associated with a number of inflammatory disorders. Muscle expression of atrogin-1/MAFbx, a rate-limiting ubiquitin ligase for muscle wasting, is upregulated in various inflammatory conditions, and is considered a therapeutic target for muscle wasting. As one of the free radicals whose production is elevated in inflammatory conditions, nitric oxide (NO) is implicated in the pathogenesis of muscle wasting. To understand how inflammatory mediators upregulate atrogin-1/MAFbx expression, we tested the hypothesis that NO mediates the upregulation of atrogin-1/MAFbx expression. C2C12 myotubes were incubated with a cocktail comprised of TNF-α, interferon γ and lipopolysaccharide (LPS), which stimulated NO production and atrogin-1/MAFbx expression. Pre-incubation of the myotubes with nitric oxide synthase (NOS) inhibitor L-NAME or S-ethylisothiourea (SETU) blocked the stimulation of NO production by the cocktail. However, the stimulation of atrogin-1/MAFbx expression was not disrupted. Intraperitoneal administration of LPS to mice resulted in elevated atrogin-1/MAFbx expression in gastrocnemius muscle. But, pretreatment of the mice with L-NAME did not alter LPS stimulation of atrogin-1/MAFbx expression. Therefore, NO does not mediate upregulation of atrogin-1/MAFb expression by inflammatory mediators.

Key Words: TNF-α, interferon γ, lipopolysaccharide, NOS inhibitors, muscle catabolism

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Muscle wasting is a major feature of the cachexia associated with such diseases as sepsis, cancer, AIDS, diabetes, uremia, congestive heart failure, and chronic obstructive pulmonary disease (COPD) [14;24;29]. In the diverse disorders that induce muscle wasting, a common theme is the existence of systemic inflammation indicated by elevated circulating inflammatory cytokines including TNF-α, IL-1, IL-6 and IFN-γ [24]. These inflammatory mediators have the capacity to accelerate muscle protein loss, particularly myosin loss [1;2;7;23]. Accelerated protein degradation by the ubiquitin proteasome pathway is considered a major contributor to muscle protein loss in various catabolic conditions [16;18]. Proteins degraded by the ubiquitin-proteasome pathway are first linked to a chain of ubiquitin molecules, which marks them for subsequent degradation by the 26S proteasome. The ubiquitin-ligases (E3-ligases) are responsible for the substrate specificity of this system [15]. Two muscle-specific E3-ligases, MAFbx (atrogin-1) and MURF-1, substantially increased in peripheral skeletal muscle during various muscle wasting conditions, are critical to muscle wasting [3;10]. Recent data indicate that inflammatory mediators stimulate the expression of atrogin-1/MAFbx [17;20] and MuRF1 [6]. Therefore, understanding the signaling mechanisms that mediate the upregulation of atrogin-1/MAFbx and MuRF1 by inflammatory conditions is desirable from the therapeutic point of view. Inflammatory conditions induce oxidative stress through the generation of such free radicals as reactive oxygen species (ROS) and NO [24]. ROS [5;11;23] and NO [5] have been linked to the pathogenesis of muscle wasting induced by inflammatory mediators. We have shown previously that hydrogen peroxide, one of the ROS generated in muscle, is capable of upregulating atrogin-1/MAFbx and MuRF1 gene expression [21]. At least two ROS-sensitive signaling pathways have been shown to stimulate muscle wasting by upregulating atrogin-1/MAFbx or MuRF1. NF-κB is a ROS-sensitive transcription factor that mediates TNF-α stimulation of muscle protein loss [22] via upregulating MuRF1 gene expression [6]. However, NF-κB does not mediate atrogin-1/MAFbx gene

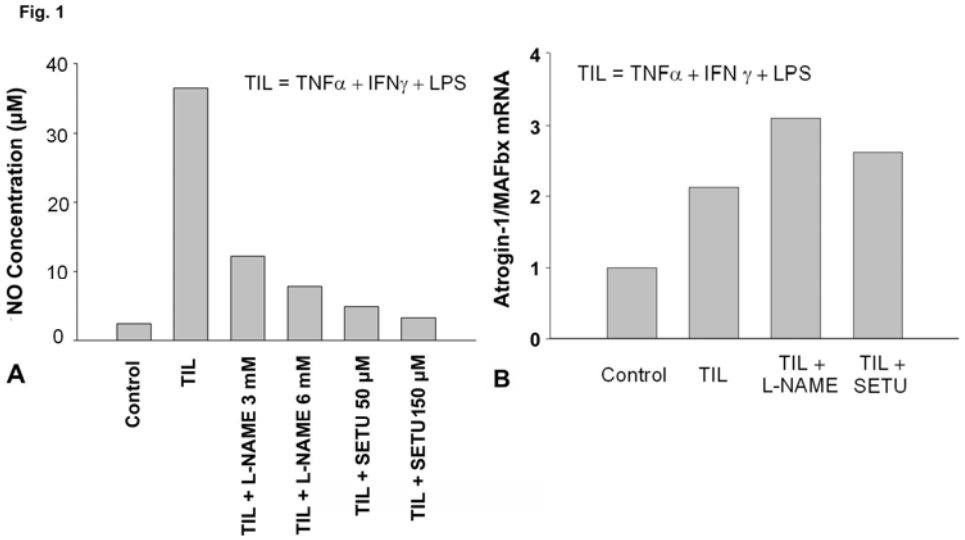

Fig. 1. *Atrogin-1/MAFbx upregulation by inflammatory mediators in C2C12 myotubes is not dependent on NO. C2C12 myotubes were pre-incubated with NOS inhibitor L-NAME (6 μM) or iNOS inhibitor SETU (150 μM) for 30 min before incubation with a mixture of TNF-α (10 ng/ml), IFNγ (10 units/ml) and LPS (100 μg/ml) for 24 h. NO released into the cell culture medium was determined (A). The atrogin-1/MAFbx mRNA in myotubes was measured by reverse transcription and real time PCR. The data was normalized to the GAPDH mRNA, and expressed as relative values in comparison to control in arbitrary unit (the value of control equals to 1) (B).*

upregulation [26]. Atrogin-1/MAFbx upregulation by TNF-α or hydrogen peroxide is mediated by the ROS-sensitive kinase p38 MAPK [20]. Although AKT-mediated phosphorylation of Foxo transcription factors regulates atrogin-1/MAFbx expression [26;27], in the muscle of mice received LPS which stimulates cytokine expression and glucocorticoid production, atrogin-1/MAFbx expression is correlated to p38 MAPK activity but not AKT activity [17]. These data highlight the role of ROS-sensitive signaling pathways in cachectic muscle wasting. However, how NO mediates muscle wasting remains largely unknown. We observed previously that curcumin, the yellow pigment isolated from turmeric, blocks LPS stimulation of atrogin-1/MAFbx upregulation, but not MuRF1 upregulation, and muscle protein loss while inhibiting p38 activation [17]. This observation supports a role of p38 in mediating atrogin-1/MAFbx upregulation. Nevertheless, considering that LPS stimulates NOS activity [13;25], and that curcumin is also known to inhibit NOS [4], it is necessary to investigate the potential involvement of NO in the upregulation of atrogin-1/MAFbx by inflammatory mediators. The present study is designed to test the hypothesis that NO mediates atrogin-1/MAFbx upregulation by inflammatory mediators.

Materials and Methods

Animal use

Experimental protocols were pre-approved by the Animal Protocol Review Committee of the Baylor Animal Program. Male adult ICR mice (~30 g) were preconditioned by i.p. injection of L-NAME (10 to 60 μg/kg) or an equal volume of PBS for 4 consecutive days. After the fourth injection of curcumin, LPS (1 mg/kg in 50 μl) or an equal volume of vehicle (PBS) was i.p. injected. The gastrocnemius muscle was collected at indicated times immediately after rapid euthanization of the mice.

Cell Culture

Myotubes were cultured from the murine skeletal muscle-derived C2C12 myoblast line

(American Type Culture Collection, Rockville, Md.) as described previously [24]. Briefly, C2C12 cells were cultured in DMEM supplemented with 10% fetal calf serum at 37ºC in the presence of 5% CO2. Myoblast differentiation was initiated by replacing the growth medium with differentiation medium: DMEM supplemented with 2% horse serum. Differentiation was allowed to continue for 96 hr before experimentation (changing to fresh medium at 48 hr).

Determination of NO levels

C2C12 myotubes were pre-incubated with NOS inhibitor L-NAME or iNOS inhibitor SETU for 30 min before incubation with a mixture of TNF-α (10 ng/ml), IFNγ (10 units/ml) and LPS (100 μg/ml) for 24 h. Cell culture medium was collected and NO released into the medium was determined in a 96-well plate by mixing 100 μl medium with 50 μl of 2X Griess solution A and 50 μl of 2X Griess solution B [12], incubating at room temperature for 10 min, and measuring optical density

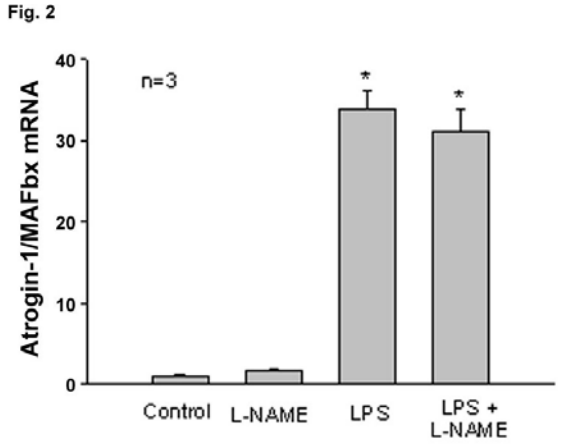

Fig. 2. *NO does not mediate LPS upregulation of atrogin-1/MAFbx in mouse muscle.*

at 543 nm with a microplate reader (SpectraMAX Gemini). NO concentration was derived from standard curve. Male mice (8 week-old ICR) were given L-NAME in drinking water at 1mg/ml (freshly prepared daily). After 4 days, LPS (1 mg/kg in 50 µl) or PBS (control) was administered via i.p. Gastrocnemius was collected 12 h later and analyzed for atrogin-1/MAFbx mRNA level by real time PCR after reverse transcription. The data was normalized to GAPDH mRNA and expressed in arbitrary unit (the value of control equals to 1). Data were analyzed with ANOVA. Tukey's multiple comparison test found difference between control and LPS-treated mice (*, $p < 0.01$). But L-NAME did not alter LPS effect ($p > 0.05$).

RT-PCR analysis of the atrogin-1/MAFbx mRNA

Total RNA was extracted from C2C12 myotubes or mouse gastrocnemius muscle using the TriZol reagent (Invitrogen). The atrogin-1/MAFbx mRNA was analy zed by reverse transcription and real time PCR using the SYBR Green detection method in a MyiQ PCR instrument (BioRad). Data was normalized to the levels of GAPDH mRNA. PCR primers for the mouse atrogin-1/MAFbx gene were 5'-CACATTCTCTCCTG GAAGGGC-3' and 5'-TTGATA-AAGTCTTGA GGGG-AA-3', and that for the mouse GAPDH gene were 5'- CATGGCCTTCCGTGTTCCTA-3' and 5'-GCGGCACGTCAGATCCA-3'.

Results

To test our hypothesis we first tried to determine whether atrogin-1/MAFbx upregulation is dependent on NO production stimulated by inflammatory mediators in C2C12 myotubes. In a preliminary study, we observed that a cocktail of TNF-α, IFN-γ and LPS was more potent in stimulating NO production than TNF-α combined with IFN-γ in C2C12 myotubes (data not shown). In C2C12 myotubes that were incubated with the TNF-α, IFN-γ and LPS cocktail for 24 hours NO production increased 37-fold (Figure 1A), while the level of atrogin-1/MAFbx mRNA expressed by the myotubes increased by 100% (Figure 1B). Preincubation of myotubes with the NOS general inhibitor L-NAME or the inducible NOS (iNOS)-specific inhibitor S-ethylisothiourea (SETU) blocked NO production stimulated by the cocktail in a dose-dependent manner, suggesting that the cocktail stimulated primarily iNOS activity (Figure 1A). However, the NOS inhibitors failed to block this increase (Figure 1B). This result indicates that NO does not mediate atrogin-1/MAFbx upregulation by the inflammatory mediators in myotubes.

Then, we tried to determine whether Atrogin-1/MAFbx upregulation is dependent on NO in mouse muscle in vivo. LPS is known to stimulate muscle NO production [9] and atrogin-1/MAFbx expression [8;17]. To test the effect of NO production on atrogin-1/MAFbx expression, mice were administered with L-NAME or vehicle (PBS) for 4 days so that a steady serum level of the NOS inhibitor could be established. Subsequent administration of LPS resulted in a 33-fold increase in atrogin-1/MAFbx mRNA level. L-NAME did not block the increase in atrogin-1/MAFbx expression (Figure 2). This result is consistent with the observation made in myotubes above. Thus, we conclude that NO exerts its catabolic effect on muscle through a mechanism that does not involve the upregulation of atrogin-1/MAFbx expression.

Discussion

A role of NO in the pathogenesis of muscle wasting was indicated by the observation that inhibition of NOS blocks TNF-α induced muscle mass loss in mice [5]. In addition, NOS inhibition prevents muscle protein loss induced by disuse [28] which is generally non-inflammatory. Therefore, NO is likely involved in a general mechanism of muscle catabolism utilized by both inflammation and non-inflammation induced muscle catabolism. Although NO inhibits muscle protein synthesis [9], the possibility exists that it may also stimulate proteolysis. Since atrogin-1/MAFbx is upregulated in inflammatory disease- as well as disuse-induced muscle catabolism [3;10;19] by multiple signaling pathways such as Foxo transcription factors [26;27] and p38 MAPK [20], there could be additional signaling molecules that regulate its expression. Considering that MuRF1 upregulation by LPS in mouse muscle is not inhibited by curcumin [17] which inhibits NOS [4], the present study focuses on the potential role of NO in mediating atrogin-1/MAFbx upregulation by inflammatory mediators. In testing the hypothesis that NO stimulates atrogin-1/MAFbx expression., we treated C2C12 myotubes with a cocktail of TNF-α, IFN-γ and LPS which was more potent than TNF-α alone in stimulating NO production. Yet, this cocktail did not exert a greater stimulation on atrogin-1/MAFbx expression in comparison to

previously observed effect of TNF-α alone [20]. In addition, the stimulation of atrogin-1/MAFbx expression by this cocktail was not affected when NO production is blocked. Furthermore, the data from mice received LPS and NOS inhibitor verified in vivo that there is not a causal relationship between NO elevation and atrogin-1/MAFbx upregulation. Therefore, we conclude that NO does not mediate atrogin-1/MAFbx upregulation by inflammatory mediators.

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References

[1] Acharyya, S., Ladner, K. J., Nelsen, L. L., Damrauer, J., Reiser, P. J., Swoap, S., and Guttridge, D. C. J Clin Invest 2004; 114: 370-378.

[2] Argiles, J. M., Busquets, S., and Lopez-Soriano, F. J. Int J Biochem Cell Biol 2005; 37: 2036-2046.

[3] Bodine, S. C., Latres, E., Baumhueter, S., Lai, V. K., Nunez, L., Clarke, B. A., Poueymirou, W. T., Panaro, F. J., Na, E., Dharmarajan, K., Pan, Z. Q., Valenzuela, D. M., DeChiara, T. M., Stitt, T. N., Yancopoulos, G. D., and Glass, D. J. Science 2001: 294: 1704-1708.

[4] Brouet, I. and Ohshima, H. Biochem Biophys Res Commun 1995; 206: 533-540

[5] Buck, M. and Chojkier, M. EMBO J 1996; 15: 1753-1765.

[6] Cai, D., Frantz, J. D., Tawa, N. E., Jr., Melendez, P. A., Oh, B. C., Lidov, H. G., Hasselgren, P. O., Frontera, W. R., Lee, J., Glass, D. J., and Shoelson, S. E. Cell 2004; 119: 285-298.

[7] Chang, H. R. and Bistrian, B. JPEN J Parenter Enteral Nutr 1998; 22: 156-166.

[8] Dehoux, M. J., van Beneden, R. P., Fernandez-Celemin, L., Lause, P. L., and Thissen, J. P. FEBS Lett 2003; 544: 214-217.

[9] Frost, R. A. and Lang, C. H.X. J Anim Sci 2003; 86: E84-E93.

[10] Gomes, M. D., Lecker, S. H., Jagoe, R. T., Navon, A., and Goldberg, A. L. Proc Natl Acad Sci USA 2001; 98:14440-14445.

[11] Gomes-Marcondes, M. C. and Tisdale, M. J. Cancer Lett 2002; 180: 69-74.

[12] Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R. Anal Biochem 1982; 126: 131-138.

[13] Griffiths, M. J., Liu, S., Curzen, N. P., Messent, M., and Evans, T. W. Am J Physiol 1995; 268: L509-L518.

[14] Hasselgren, P. O. Biochem.Soc.Trans. 1995; 23: 1019-1025.

[15] Hershko, A. and Ciechanover, A. Annu Rev Biochem 1998; 67: 425-479.

[16] Jagoe, R. T. and Goldberg, A. L. Curr.Opin.Clin.Nutr.Metab Care 2001; 4: 183-190.

[17] Jin, B. and Li, Y. P. J Cell Biochem 2007; 100: 960-969.

[18] Kandarian, S. C. and Jackman, R. W. Muscle Nerve (2006; 33: 155-165.

[19] Lecker, S. H., Jagoe, R. T., Gilbert, A., Gomes, M., Baracos, V., Bailey, J., Price, S. R., Mitch, W. E., and Goldberg, A. L. FASEB J 2004; 18: 39-51.

[20] Li, Y. P., Chen, Y., John, J., Moylan, J., Jin, B., Mann, D. L., and Reid, M. B. FASEB J 2005; 19: 362-370.

[21] Li, Y. P., Chen, Y., Li, A. S., and Reid, M. B. Am.J Physiol Cell Physiol 2003; 285: C806-C812.

[22] Li, Y. P. and Reid, M. B. Am J Physiol 2000; 279: R1165-R1170.

[23] Li, Y. P., Schwartz, R. J., Waddell, I. D., Holloway, B. R., and Reid, M. B. FASEB J 1998; 12: 871-880.

[24] Moylan, J. S. and Reid, M. B. Muscle Nerve 2007; 35: 411-429.

[25] Ravindranath, T. M., Goto, M., Bakr, S., and Ramasamy, R. Biol Neonate 2003; 84: 319-324.

[26] Sandri, M., Sandri, C., Gilbert, A., Skurk, C., Calabria, E., Picard, A., Walsh, K., Schiaffino, S., Lecker, S. H., and Goldberg, A. L. Cell 2004; 117: 399-412.

[27] Stitt, T. N., Drujan, D., Clarke, B. A., Panaro, F., Timofeyva, Y., Kline, W. O., Gonzalez, M., Yancopoulos, G. D., and Glass, D. J. 2004; Mol Cell 14: 395-403.

[28] Suzuki, N., Motohashi, N., Uezumi, A., Fukada, S., Yoshimura, T., Itoyama, Y., Aoki, M., Miyagoe-Suzuki, Y., and Takeda, S. J Clin Invest 2007;, 117: 2468-2476.

[29] Tisdale, M. J. J Natl Cancer Inst 1997; 89: 1763-1773.