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Induction of MafBx and Murf ubiquitin ligase mRNAs in rat skeletal muscle after LPS injection

Mischaël J.M. Dehoux, Ronald P. van Beneden, Laura Fernández-Celemín, Pascale L. Lause, Jean-Paul M. Thissen*

Unité de Diabétologie et Nutrition, Université catholique de Louvain, B-1200 Brussels, Belgium

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Abstract MafBx and Murf are two new rat E3 ubiquitin ligases induced in muscle atrophy. Our goal was to investigate whether lipopolysaccharide (LPS) injection, a model of muscle catabolism, is associated with increased expression of MafBx and Murf. LPS (750 μg/100 g body weight) induces MafBx and Murf mRNA (respectively, 23-fold and 33-fold after 12 h; $P < 0.001$). A transient induction of tumor necrosis factor-α mRNA (21-fold; $P < 0.001$ at 3 h) and a decrease of insulin like growth factor-I mRNA (50%; $P < 0.001$ at 6 h), two potential regulators of the ubiquitin–proteasome system were also demonstrated. In summary, MafBx and Murf mRNA are up-regulated in response to LPS and might play a role in the muscle proteolysis observed.

Key words: Muscle; Ubiquitin ligase; Tumor necrosis factor-α; Insulin like growth factor-I; Lipopolysaccharide; Real-time quantitative polymerase chain reaction

1. Introduction

Muscle wasting is a common complication of many hypercatabolic states such as sepsis, cancer and polytrauma [1,2]. Although reduced protein synthesis contributes to the decrease in muscle protein content [3–5], increased myofibrillar protein breakdown seems to play the major role in this muscle loss [6]. Myofibrillar protein degradation caused by sepsis is thought to be mediated essentially through the activation of the ubiquitin–proteasome proteolytic system. In this process, the ubiquitin (Ub) is activated by an ubiquitin-activating enzyme (E1 family) [7] and conjugated through the ubiquitin-conjugating enzymes (E2 family) [8] to the protein to be degraded. In most cases, the presence of a third protein, the ubiquitin protein ligase (E3 family) [7,9], is required to stabilize the protein–Ub complex. The muscle expression of ubiquitin, $E2_{14kDa}$ and E3-α has been reported to be increased during sepsis [10–12]. Two new ubiquitin ligases have been recently identified, the mouse atrophy gene-1 [13] (atrogin-1) also described as the rat muscle atrophy F-box (MafBx) [14], and the rat muscle RING finger 1 (Murf) [14]. By contrast to the ubiquitin [15] and $E2_{14kDa}$ [16], these enzymes are muscle specific and therefore are thought to be specifically involved in myofibrillar protein degradation. The MafBx and Murf ligases are indeed strongly increased during muscle atrophy such as following prolonged immobilization, denervation, treatment with glucocorticoids and under weighting by suspension [14]. However, the regulation of these two new ubiquitin ligases by sepsis has not yet been assessed. Therefore, as a first step in this direction, we used a classical rat lipopolysaccharide (LPS) model mimicking sepsis to investigate whether the acute inflammation caused by LPS is associated with an increased expression of MafBx and Murf in the skeletal muscle. Although it may be argued that intraperitoneal injection of LPS is not a proper model of sepsis, the method is nonetheless an accepted model of septic shock [17]. Because muscle proteolysis, in particular activation of the ubiquitin–proteasome system, has been reported to be regulated by tumor necrosis factor (TNF)-α and insulin like growth factor (IGF)-I, we assessed in parallel the expression of these two molecules into the muscle [18–21].

2. Materials and methods

2.1. Experimental design

Fifty male Wistar rats (8 weeks old, 218 ± 9 g, Katholieke Universiteit of Leuven, Leuven, Belgium) were maintained for 1 week under standardized conditions of light (12 h light/12 h dark cycle) and temperature (22 ± 2°C). Access to animals chow was available only between 6:00 p.m. and 9:00 a.m., whereas access to water was unrestricted. The morning of the eighth day, the rats were randomly divided in three different groups: control (non-injected, $n = 4$), LPS ($n = 30$) and saline ($n = 16$). The LPS and saline groups were injected intraperitoneally respectively with LPS (750 μg/100 g body weight, Sigma, St. Louis, MO, USA) or an equivalent volume of saline buffer. The control group was immediately sacrificed (0 h). The LPS and the time-matched saline groups were sacrificed after 1, 3, 6 and 12 h. Tibialis anterior muscles were dissected, snap frozen in liquid nitrogen and stored at −80°C until processing.

2.2. Real-time quantitative polymerase chain reaction (RTQ-PCR)

Total RNA was prepared from snap frozen tissue samples (400 mg) using TRIzol® (Gibco Life Technologies, Paisley, UK). RNA was quantified by spectrophotometry ($\lambda = 260$ nm) and its concentration adjusted to 0.25 μg/μl using RNase free water. Reverse transcription (RT) was performed using the GeneAmp PCR system 2400 (Applied Biosystem, Foster City, CA, USA) with 1 μg of total RNA in a reaction volume of 20 μl, containing 7.5 μM random hexamers, RT buffer 1×, 9 mM dithiothreitol, 220 μM of each deoxyribonucleotide triphosphate (dNTP), 20 U of ribonuclease inhibitor (Applied Biosystem) and 50 U of reverse transcriptase (Superscript®, Gibco BRL). Final RT product was adjusted to 40 μl using RNase free water. RTQ-PCR primers were designed (Primer Express Software, Applied Biosystem) for rat Ub, $E2_{14kDa}$, MafBx, Murf, IGF-I, TNF-α and GAPDH. The primers (Table 1) were produced on an automated

*Corresponding author. Fax: (32)-2-7645418.
E-mail address: thissen@diab.ucl.ac.be (J.-P.M. Thissen).

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Table 1
Gene sequences used as forward and reverse primers for RTQ-PCR

Gene	Sequence 5′–3′	Amplicon (bp)
Ubiquitin	Forward primer GAT CCA GGA CAA GGA GGG C	71
	Reverse primer CAT CTT CCA GCT GCT TGC CT	
$E2_{14kDa}$	Forward primer TCC TGC AGA ACC GAT GGA G	77
	Reverse primer CGG CTC ATC CAA CAG AGA CTG	
MafBx	Forward primer CCA TCA GGA GAA GTG GAT CTA TGT T	75
	Reverse primer GCT TCC CCC AAA GTG CAG TA	
Murf1	Forward primer TGT CTG GAG GTC GTT TCC G	59
	Reverse primer ATG CCG GTC CAT GAT CAC TT	
IGF-I	Forward primer GCT ATG GCT CCA GCA TTC G	63
	Reverse primer TCC GGA AGC AAC ACT CAT CC	
TNF-α	Forward primer GCC ACC ACG CTC TTC TGT CT	101
	Reverse primer GTC TGG GCC ATG GAA CTG AT	
GAPDH	Forward primer TGC ACC ACC AAC TGC TTA	72
	Reverse primer GGA TGC AGG GAT GAT GTT C	

Last column indicates the length of amplicon (bp).

synthesizer (Applied Biosystem) according to the manufacturer's protocol. Sybr Green® RTQ-PCR analyses were carried out using the GeneAmp 5700 (Applied Biosystem). RTQ-PCR was carried out using the following cycle parameters: 10 min at 95°C, followed by 40 cycles of 1 min at 60°C and 15 s at 95°C. For each gene, RTQ-PCR was conducted in duplicate with a 25 μl reaction volume of 5 ng of cDNA, 2.5 μl Sybr Green® buffer, 250 μM dNTP, 3 mM $MgCl_2$, 400 nM of each primers and 0.625 U amp Taq Gold® Polymerase (Applied Biosystem). To ensure the quality of the measurements, each plate included for each gene a negative control (sample replaced by RNase free water) and a positive control consisting in a pool of cDNA from positive samples. The threshold cycle (Ct) from a positive sample was used to calculate the inter-assay coefficient of variation (CV). For each gene, the CV was calculated as standard deviation/mean of the Ct determined on five different plates and with different mixes. The CVs obtained were 4%, 5%, 8%, 6%, 8%, 7% and 6%, for Ub, $E2_{14kDa}$, MafBx, Murf, IGF-I, TNF-α and GAPDH, respectively.

2.3. Statistical analysis and results presentation

Results were expressed using the comparative cycle threshold (Ct) method as described in the User Bulletin #2 from the manufacturer (Applied Biosystem). The ΔCt values were calculated in every sample for each gene of interest as followed: $Ct_{\text{gene of interest}} - Ct_{\text{reporter gene}}$ with GAPDH as the reporter gene. The calculation of the relative changes in the expression level of one specific gene (ΔΔCt) was performed by subtraction of the ΔCt from the control group (0 h, used as a calibrator) to the corresponding ΔCt (at different time) from the LPS or the saline groups [22]. The values and ranges given in Fig. 1 were determined as followed: $2^{-\Delta\Delta Ct}$ with ΔΔCt+S.E.M. and ΔΔCt−S.E.M., where S.E.M. is the standard error of mean of the ΔΔCt value (User Bulletin #2, Applied Biosystem).

Statistical analysis for the gene expression levels assessed by RTQ-PCR was performed using an unpaired *t*-test to compare the ΔCt values from the LPS and saline groups at each time point. Differences were considered statistically significant for $P < 0.05$.

3. Results

Our data show that LPS injection stimulates the muscle gene expression of all the four components of the ubiquitin–proteasome system investigated. After LPS injection, the mRNA levels of these four genes (Ub, $E2_{14kDa}$, MafBx and Murf) increased gradually with time, but with different amplitude and kinetics (Fig. 1). The ubiquitin mRNA levels raised as early as 3 h after LPS injection to reach a maximal value at 12 h, the last time point investigated (8.3-fold induction vs saline, $P < 0.001$). In contrast, the increase in $E2_{14kDa}$ mRNA levels was only observed 12 h after LPS injection (3.0-fold, $P < 0.001$). But, the most dramatic changes were observed for the two recently described ubiquitin ligases, MafBx and Murf. LPS injection caused a rapid and gradual increase in MafBx (1.6-fold, $P < 0.001$ at 3 h and 4.3-fold, $P < 0.001$ at 6 h) with a maximum at 12 h, the last point examined (23-fold induction; $P < 0.001$). The Murf mRNA stimulation by LPS was parallel to that of MafBx mRNA, but even stronger with a maximal induction of 33.1-fold at 12 h ($P < 0.001$). In an attempt to define the mediators responsible for these changes, we assessed in parallel the gene expression levels of IGF-I and TNF-α in muscle after LPS injection. These two factors have indeed been demonstrated to regulate the gene expression of several components of the ubiquitin–proteasome system in the muscle [23–26]. IGF-I mRNA levels progressively declined in response to LPS injection with a nadir at 6 h and 12 h (−50%, $P < 0.001$ and −60%, $P < 0.001$). In contrast, TNF-α mRNA increased rapidly (peak at 1 h) and dramatically (21-fold, $P < 0.001$) to persist at lower levels until 12 h, the last point examined (3.6-fold, $P < 0.001$) (Fig. 1).

4. Discussion

Our study showed that inflammation induced by LPS injection dramatically induces the muscle gene expression of MafBx and Murf, two recently described ubiquitin ligases considered to play a major role in muscle atrophy [14]. As previously reported [10,12], sepsis increases other components of the ubiquitin–proteasome system, namely Ub itself and $E2_{14kDa}$. However, the role of their induction in the acceleration of proteolysis caused by sepsis is uncertain. In contrast, a number of findings indicate that the two E3 ligases (MafBx and Murf) play a major role in the skeletal muscle proteolysis during muscle atrophy. First, the expression of MafBx and Murf, which is increased in most models of muscle atrophy, is limited to skeletal muscle [14], in contrast to ubiquitin and $E2_{14kDa}$ [15]. Second, MafBx and Murf knockout mice are more resistant than there wild littermates to muscle atrophy following denervation, a classical model of muscle atrophy [14]. In contrast, muscle proteolysis caused by fasting is not attenuated in $E2_{14kDa}$ knockout mice [27]. Finally, the overexpression of MafBx in C_2C_{12} myotubes induces cell atrophy [14]. Together with our findings, these observations indicate that the dramatic increase in the expression of these two ubiquitin ligases might contribute to the muscle proteolysis caused by sepsis.

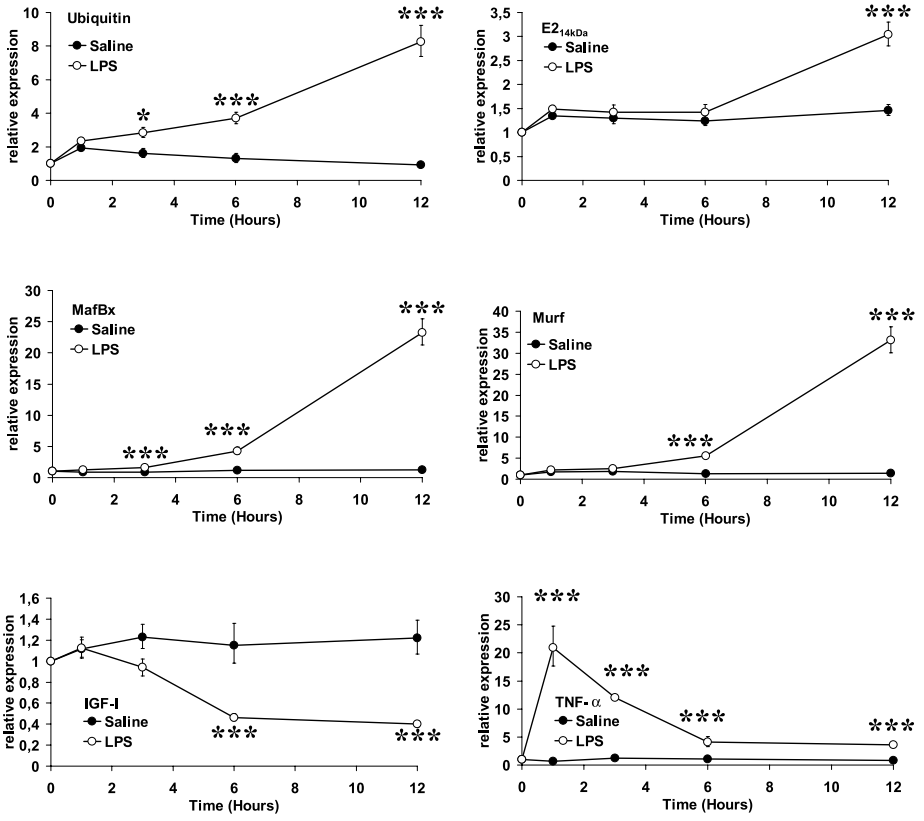

Fig. 1. Effect of LPS injection on several components of the ubiquitin–proteasome system gene expression levels in rat tibialis anterior muscle. Male Wistar rats were injected with 750 μg/100 g body weight of LPS (open circles, $n = 7$–8 at each time point) or saline solution (filled circles, $n = 4$ at each time point) and killed 1 h, 3 h, 6 h and 12 h after LPS injection. Results are expressed as mRNA relative expression and are means ± S.E.M. RNA quantification was performed as indicated in Section 2. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs time-matched saline.

The mechanisms of the activation of muscle MafBx and Murf by LPS are not yet unravelled. It is unlikely that these changes may be due to the anorexia caused by LPS, since both LPS-injected and saline-treated animals fasted throughout the day of the experience. Previous reports showed that TNF-α is able to stimulate proteolysis and the muscle Ub gene expression [25,26,28]. Furthermore, inhibition of TNF-α by torbafylline inhibits enhanced skeletal muscle ubiquitin–proteasome-dependent proteolysis in septic rats [29]. Therefore, given its strong induction in the muscle of LPS-treated rats, TNF-α could be responsible for the increase in MafBx and Murf mRNA observed in this catabolic model. IGF-I has been reported to inhibit muscle proteolysis in several catabolic animal models [30,31]. Since IGF-I mRNA levels are decreased in the muscle of LPS-treated rats [21], it could be hypothesized that the reduction of the muscle IGF-I can be at least partially responsible for the increase in MafBx and Murf. Although IGF-I blunts the increased expression of several components of the ubiquitin–proteasome system such as Ub and $E2_{14kDa}$ in septic rats [32], the possibility for IGF-I to inhibit the induction of these two new ubiquitin ligases by LPS is still unknown. Finally, because the activation of the ubiquitin–proteasome system by sepsis is glucocorticoid-dependent [33,34], it is likely that induction of MafBx and Murf by LPS may be mediated by increased glucocorticoids. This hypothesis is supported by the recent observation that exogenous dexamethasone strongly induces the muscle expression of these two ligases in mice [14]. Further experiments will have to delineate the role of IGF-I, TNF-α and glucocorticoids in the induction of these E3 ligases during sepsis. In conclusion, we showed that LPS injection in rat causes induction of MafBx and Murf, two recently described ubiquitin ligases specifically expressed in muscle and required for skeletal muscle atrophy. Further experiments will have to test whether their induction is mediated by the parallel decrease in IGF-I and increase in TNF-α expression into muscle.

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