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Endotoxemia-induced muscle wasting is associated with the change of hypothalamic neuropeptides in rats

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ABSTRACT

In critical patients, sepsis-induced muscle wasting is considered to be an important contributor to complications and mortality. Previous work mainly focuses on the peripheral molecular mechanism of muscle degradation, however little evidence exists for the role of central nervous system in the process. In the present study, we, for the first time, characterized the relationship between muscle wasting and central neuropeptide changes in a septic model. Thirty-six adult male Sprague–Dawley rats were intraperitoneally injected with lipopolysaccharide (LPS) or saline. Twelve, 24 and 48 hrs after injection, skeletal muscle and hypothalamus tissues were harvested. Muscle wasting was measured by the mRNA expression of two E3 ubiquitin ligases, muscle ring finger 1 (MuRF-1) and muscle atrophy F-box (MAFbx), as well as 3-methyl-histidine (3-MH) and tyrosine release. Hypothalamic neuropeptides and inflammatory marker expressions were also measured in three time points. LPS injection caused an increase expression of MuRF-1 and MAFbx, and a significant higher release of 3-MH and tyrosine. Hypothalamic neuropeptides, proopiomelanocortin (POMC), cocaine- and amphetamine-regulated transcript (CART), agouti-related protein (AgRP) and neuropeptide Y (NPY) presented a dynamic change after LPS injection. Also, hypothalamic inflammatory markers, interleukin-1 β (IL-1β) and tumor necrosis factor α (TNF-α) increased substantially after LPS administration. Importantly, the expressions of POMC, AgRP and CART were well correlated with muscle atrophy gene, MuRF-1 expression. These findings suggest hypothalamic peptides and inflammation may participate in the sepsis-induced muscle wasting, but the exact mechanism needs further study.

1. Introduction

Severe sepsis and septic shock are important health hazard for human beings and are associated with substantial mortality and complications (Rivers et al., 2001). Sepsis can cause a profound alteration in metabolism, including loss of appetite, decreased anabolism and increased catabolism of fat and proteins, which all may contributed to the prolonged phase of critical illness. Importantly, unlike starvation or malnutrition, these metabolic changes can hardly be corrected by nutrition support alone. Although, much effort, such as growth factor (GH) treatment, glutamine administration and anti-inflammatory lipid supplementation 4 has been made to deal with the disorganized metabolism, the effectiveness is still far from satisfactory (Casaer and Van den Berghe, 2014).

Among those metabolic changes, protein catabolism and muscle wasting are thought to be the main obstacle to affect nutrition support and an important contributor to patients' morbidity and mortality (Zhou et al., 2010). According to the traditional view, muscle degradation in sepsis is regulated mainly by the peripheral action of circulating molecules, such as inflammatory cytokines and glucocorticoids. However, some experiments have shown that direct application of pro-inflammatory cytokines gained opposite results in skeletal muscle explants, implying that the in-vivo catabolic results of cytokine may not solely dependent on peripheral organs (Moldawer et al., 1987).

On the other hand, studies have shown central inflammatory cytokines can be induced by peripheral inflammation in a septic model (Laye et al., 1994). Besides, central administration of several cytokines can recapitulate the response of peripheral LPS stimulation. In addition, using bone marrow transplant methods, Wisse et al. have demonstrated central inflammation, not circulating cytokines, was essential to elicit anorexia in response to peripheral LPS stimulation (Wisse et al., 2007). Collectively, these evidences implied the importance of central nervous system (CNS) in the regulation of metabolism under pathological conditions.

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Due to the anatomic position, the hypothalamic arcuate nucleus (ARC) is considered to be an important site in the CNS to regulate metabolism and energy homeostasis (Arora and Anubhuti, 2006). ARC consists of two populations of neurons, POMC and AgRP neurons (Oyama et al., 2010). The former expresses anorexigenic peptides, POMC and CART. POMC can be cleaved into α-melanocyte-stimulating hormone (α-MSH), which derives its anorectic effects by binding to type-3 melanocortin receptor (MC3-R) and MC4-R. AgRP neuron expresses orexigenic peptides AgRP and NPY, which stimulate feeding and decrease metabolism by acting as an inverse agonist of the MC4-R (Krasnow and Marks, 2010). Both neurons of the ARC are subject to pro-inflammatory cytokines. Studies have shown cytokines interleukin (IL)-1β, and IL-7 increase POMC mRNA expression and inhibits AgRP gene expression, which lead to anorexia and weight loss (Macia et al., 2010; Scarlett et al., 2007, 2008).

However, most studies of the ARC focus on anorexia and obesity. Little evidence exists for the central role in the sepsis-induced hypercatabolism, especially muscle wasting. Therefore, we hypothesized the hypothalamic neuropeptides may participate in the process of muscle wasting during sepsis. In the present study, we, for the first time, characterized the relationship between muscle wasting and neuropeptides changes in a septic model. We found that muscle degradation was in parallel with the changes of central neuropeptides, including POMC, AgRP and CART, suggesting that muscle wasting may be regulated by these central peptides, especially POMC.

2. Materials and methods

2.1. Animals

Thirty-six adult male Sprague–Dawley rats (200 ± 20 g) were used in this study. The animals were kept under regular lighting conditions (light cycle 6.00–18.00) in a temperature-controlled environment with free access to standard rodent chow and tap water. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Nanjing University and Jinling Hospital. The animals were allowed to acclimatize to their environment for 7 days before start of the study. Rats were fasted for 12 h before the experiment.

2.2. Study protocol

Animals were randomized into two groups, LPS group and control group. The LPS group was injected intraperitoneally with 10 mg/kg LPS (Escherichia coli serotype 055:B5, Sigma, St. Louis, MO). The control group received an intraperitoneal injection with an equal volume of sterile saline. Rats were then returned to the animal facility. LPS injection was widely used to study muscle wasting in sepsis for the controllability, reproducibility and representativeness (Holecek, 2012), thus we adopted this model as previously described (Chen et al., 2011). At 12, 24 and 48 h after injection, rats were killed with an overdose of phenobarbital sodium and the hypothalamus of brain was rapidly dissected and stored at –80 °C until analysis. The gastrocnemius and Extensor Digitorum Longus (EDL) muscles were isolated and weighed. Gastrocnemius muscles were frozen immediately in liquid nitrogen for the analysis of MuRF-1 and MAFbx expression. The EDL muscles were used for protein breakdown studies as will be described later.

2.3. Rate of protein turnover

Rate of protein breakdown was measured as previously described (Balasubramaniam et al., 2009; Chen et al., 2011). In brief, fresh EDL muscle was fixed via the tendons to aluminum wire supports at resting length, and preincubated in oxygenated medium (95% O2-5% CO2): Krebs–Henseleit bicarbonate buffer (pH7.4) containing 5 mM glucose, 0.1 U/ml insulin, 0.17 mM leucine, 0.1 mM isoleucine, and 0.20 mM valine. After 1 h preincubation, muscles were transferred to fresh medium of identical composition and incubated for a further 2 h with 0.5 mM cycloheximide. The degradation rates of total and myofibrillar proteins were determined by release in the medium of free tyrosine and 3-MH, respectively, and expressed as nanomoles of tyrosine/3-MH in medium/2·h^{-1} g·muscle^{-1}. Muscle was also homogenized in 0.4 mM perchloric acid to determine tissue free tyrosine and 3-MH. The net production of free tyrosine was calculated as the amount of tyrosine released into the medium plus the increase in tissue free tyrosine during incubation. Net 3-MH production was calculated as the amount of 3-MH in the medium minus the decrease in tissue free 3-MH before and after incubation. Levels of both tyrosine and 3-MH in medium or tissue samples were determined by high-performance liquid chromatography (HPLC).

Table 1
The primer sequences.

Gene		Primers
MuRF1	Forward	5′-GGACGGAAATGCTATGGAGA-3′
	Reverse	5′-AACGACCTCCAGACATGGAC-3′
MAFbx	Forward	5′-CCATCAGGAGAAGTGGATCTATGTT-3′
	Reverse	5′-ATGACGTG AAACCCCCTTCG-3′
POMC	Forward	5′-CCTCCTGCTTCAGACCTCCA-3′
	Reverse	5′-GGCTGTTCATCTCCGTTGC-3′
AgRP	Forward	5′-TGAAGGGCATCAGAAGGT-3′
	Reverse	5′-CACAGGTCGCAGCAAGGT-3′
CART	Forward	5′-CCGAGCCCTGGACATCTA-3′
	Reverse	5′-GGAATGCGTTTACTCTTGAGC-3′
NPY	Forward	5′-CTGACCCTCGCTCTATCC-3′
	Reverse	5′-GGTCTTCAAGCCTTGTTCT-3′
IL-1β	Forward	5′-TTCAAATCTCACAGCAGCAT-3′
	Reverse	5′-AGGTCGTCATCATCCCAC-3′
TNF-α	Forward	5′-CCACGCTCTTCTGTCTACTG-3′
	Reverse	5′-GCTACGGGCTTGTCACTC-3′
GAPDH	Forward	5′-GCAAGTTCAACGGCACAG-3′
	Reverse	5′-GCCAGTAGACTCCACGACAT-3′

2.4. Real time polymerase chain reaction (RT-PCR) RNA preparation and analysis of gene expression in the muscle and hypothalamus

The total RNA from the sorted cerebral tissues and skeletal muscle were both isolated using Trizol reagent. Once isolated, 5 μg of total RNA was reverse transcribed to yield cDNA. For each sample, 1 μl cDNA was added to a 50 μl PCR containing 0.5 μl primer, 8 μl SYBR green I premix and 11.3 μl ddH2O. The PCR temperature profile consisted of a single cycle at 95 °C for 10 min; 40 cycles for 15 s at 95 °C, for 15 s at 60 °C, and for 20 s at 72 °C (extension), and a final cycle at 72 °C for 2 min. Real-time quantitative PCR was performed using a Rotor Gene 3000 system (Corbet, Australia). Gene expression was analyzed using the Rotor-Gene Real-Time Analysis Software 6.1. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal control gene in order to normalize the PCRs for the amount of RNA added to the reverse transcription reactions. The primer sequences are shown in Table 1.

2.5. Statistical analyses

Data were expressed as means ± standard error (SE), and statistical analysis was performed using ANOVA. All data were analyzed with SPSS software (version 17.0, Chicago, IL). Pearson correlation coefficients were used to assess the relationships between muscle MuRF1 expression and central POMC, AgRP, CART and NPY expression. A P value of <0.05 was considered statistically significant.