Int J Clin Exp Med 2017;10(4):6883-6889
www.ijcem.com /ISSN:1940-5901/IJCEM0045270

Original Article

Role of simvastatin in endotoxemia-induced muscle injury

Hatice Yorulmaz[1], Elif Ozkok[2], Gulten Ates[3,4], Abdullah Aksu[5], Nuray Balkıs[5], Sule Tamer[3]

[1]Faculty of Medicine, Halic University, Istanbul, Turkey; [2]Department of Neuroscience, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Turkey; [3]Department of Physiology, Istanbul Medical Faculty, Istanbul University, Istanbul, Turkey; [4]Department of Physiology, Faculty of Medicine, TC Istanbul Yeni Yuzyil University, Istanbul, Turkey; [5]Department of Chemical Oceanography, Institute of Marine Sciences and Management, Istanbul University, Istanbul, Turkey

Received November 28, 2016; Accepted February 24, 2017; Epub April 15, 2017; Published April 30, 2017

Abstract: We aimed to investigate the role of prior treatment of simvastatin on cytokine response, energy levels, and apoptotic molecules on muscle tissue in rats treated with lipopolysaccharide (LPS) during the early phase of sepsis. Male Wistar albino rats (200-250 g) were divided into four groups: control, endotoxemia (20 mg/kg, i.p.), simvastatin (20 mg/kg, p.o.), and simvastatin + endotoxemia. Four hours after the beginning of the experiments, 8 rats from each group were sacrificied and gastrocnemius muscle tissue was dissected to examine for histologic changes using hematoxylin-eosin staining. The gene expressions of TNF-α, IL-10 and Bcl-2, Bax, and Caspase-3 mRNA levels were analyzed using real-time polymerase chain reaction. Creatine, creatine phosphate, adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) levels were investigated in muscle tissue using high performance liquid chromatography. ATP values were found low in the endotoxemia group and increased in the Simvastatin + endotoxemia group compared with the endotoxemia group ($P < 0.05$). Caspase-3, Bax, and TNF-α levels were significantly higher in the endotoxemia group than in the other groups ($P < 0.01$). In the simvastatin + endotoxemia group, Bcl-2 and TNF-α ($P < 0.05$), and IL-10 ($P < 0.01$) levels were higher than other groups. Muscle sections of the LPS group showed inflammation and atrophic areas. Tissue injury was reduced in the simvastatin + endotoxemia groups sections. Sepsis caused an increase of pro-inflammatory cytokine TNF-α and pro-apoptotic proteins; caspase-3 and Bax in muscle tissue may also have caused tissue damage. Pretreatment of simvastatin reduced muscle tissue damage by increasing levels of the anti-inflammatory cytokine IL-10 and the anti-apoptotic protein Bcl-2.

Keywords: Endotoxemia, energy, gene expression, muscle, simvastatin

Introduction

Systemic injection of lipopolysaccharide (LPS) to experimental animals is widely used *in vivo* model for the study of endotoxic shock and acute systemic inflammation. LPS activates the immune system, which leads to the release of endogenous proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 [1, 2]. Several lines of evidence indicate that these cytokines are important regulators of muscle protein balance [3]. Infusion of TNF-α in skeletal muscle decreased muscle protein synthesis. Infusion of TNF-α is capable of decreasing the rate of protein synthesis in heart and skeletal muscle [4-6].

Sepsis is often associated with a loss of muscle protein due to increased muscle proteolysis and a decrease in muscle protein synthesis [7, 8]. It was shown that endotoxemia caused disturbances in energy metabolism in skeletal muscle. Endotoxemia also leads to metabolic impairment, which results in an energy shortage [9-11]. Skeletal muscle mass, which accounts for 50% of body mass, is a key tissue in maintaining homeostasis in humans, and plays important roles in metabolism, mobility, and quality of life [12].

Apoptosis has been described as being associated with skeletal muscle atrophy [13]. Apoptosis is induced through two major signaling cascades, the extrinsic and intrinsic pathways.

In the extrinsic pathway, caspase-3 becomes activated. The intrinsic pathway is the mitochondrial-mediated pathway of apoptosis. A balance exists between (a) antiapoptotic proteins such as Bcl-2, Bcl-XL, and several others, and (b) proapoptotic proteins such as Bim, Bax, and PUMA [14].

Statins have a number of beneficial effects including lipid-lowering and antiapoptotic effects, and reduce inflammation during sepsis [15]. Statins, particularly lipophilic statins (e.g., simvastatin, atorvastatin, cerivastatin, and lovastatin), also cause adverse effects in skeletal muscle ranging from mild to moderate muscle fatigue, weakness, and pain, to fatal rhabdomyolysis [16].

We aimed to investigate the effects of prior simvastatin treatment on cytokine and energy levels and apoptotic molecules on muscle tissue during early-phase sepsis.

Materials and methods

Animals

The Institutional Animal Ethics Committee of Istanbul University approved all animal experiments and procedures. (Resolution No: 2012/138). Thirty-two healthy male Wistar albino rats, weighing 200-250 g, were divided into four groups, (1) controls, (2) endotoxemia, (3) simvastatin, and (4) simvastatin + endotoxemia.

Experimental procedures

Rats in the endotoxemia group were intraperitoneally injected with 20 mg/kg LPS (Escherichia coli 0127:B8 (Sigma Aldrich, Product No: L5668) dissolved in 1 mL of sterile saline. Animals in simvastatin and simvastatin + endotoxemia group were treated with simvastatin (20 mg/kg) (Sigma Aldrich, Product No: S065-0000) via oral gavage for 5 days. In the simvastatin + endotoxemia group, LPS was given 1.5 h after the fifth dose of simvastatin. Animals in both the LPS and simvastatin + endotoxemia groups were sacrified 4 hours after LPS treatment, and gastrocnemius muscles were taken.

Gene expression procedure

After the rats were sacrificed, muscle tissue specimens were taken, and the tissues were ground into powder in liquid nitrogen. Total RNA was extracted using RNAzol RT reagent extraction with reference to the method on the kit. RNA was reverse transcribed into cDNA. The resulting cDNAs were amplified using polymerase chain reaction (PCR) with specific primersforratCaspase-3(forwardCCGACTTCCTGTATGCTTACTC; reverse CCACTCCCAGTCATTCCTTTAG), rat Bcl-2 (forward CAAGCCGGGAGAACAGGGTA; reverse CCCACCGAACTCAAAGAAGGC), rat Bax (forward CCGAGAGGTCTTCTTCCGTGTG; reverse GCCTCAGCCCATCTTCTTCCA), rat TNF-α (forward CACACGAGACGCTGAAGTAG; reverse GAGCAGAGGTTCAGTGATGTAG) and rat IL-10 (forward CTGCAGGACTTTAAGGGTTACT; and reverse GAGTGTCACGTAGGCTTCTATG). The upstream primer of housekeeping gene β-actin was 5'-AACCCT AAG GCC AAC CGT GAA AAG-3', and its downstream primer was 5'-TCATGAGGTAGTCTGTCAG-3'; the product length was 241 bp. For Caspase-3, Bcl-2, Bax, TNF-α, and IL-10, the PCR reaction was conducted with 45 cycles for the muscle. Each cycle consisted of 15 seconds at 95°C, 25 seconds at 54°C, and 30 seconds at 72°C. The amount of mRNA for each gene was normalized using β-actin, and the relative expression levels were calculated using $2^{-\Delta\Delta Ct}$ and expressed as fold changes as compared with those of the control samples as relative mRNA levels.

High-performance liquid chromatography (HPLC) analysis of creatine, creatine phosphate, AMP, ADP, ATP

Sample preparation: Muscle tissue samples weighing 200 mg were homogenized in 2 mL 0.42 M $HCLO_4$ using a homogenizer (Ultraturrax T25) for 30 sec. A 1.0 mL supernatant was taken for adjusting pH with 1.0 M K_2HPO_4 after centrifugation at 3000 rpm for 5 min.

Measurement of creatine, creatine phosphate, and high-energy compounds

Creatine, creatine phosphate, and adenine nucleotides were evaluated with C18 colon (5 μ, 250 mm × 4.6 mm, Nucleodur, USA) with isocratic elution using a KOH/KH_2PO_4 buffer (215 mM, pH 6.25), 3 mM tetrabutylammonium phosphate, and 5% acetonitrile ion-paired reverse-phase chromatography using high-pressure liquid chromatography (HPLC) (Agilent 1100, USA) at 214 nm. Creatine, creatine phosphate, and adenine nucleotides were calculated from their external standard curves from different concentrations [17].

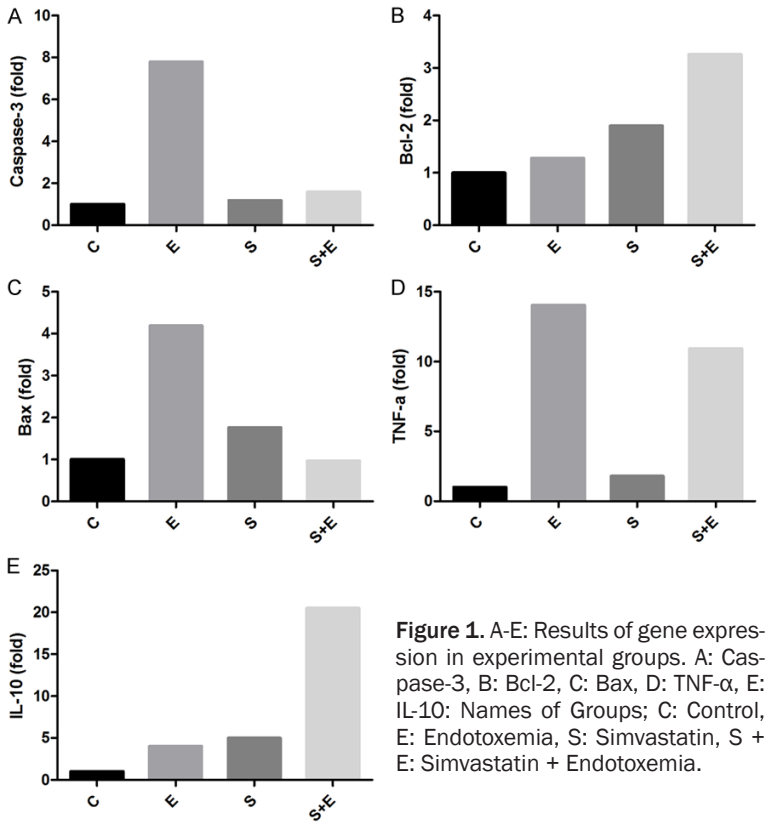

Figure 1. A-E: Results of gene expression in experimental groups. A: Caspase-3, B: Bcl-2, C: Bax, D: TNF-α, E: IL-10: Names of Groups; C: Control, E: Endotoxemia, S: Simvastatin, S + E: Simvastatin + Endotoxemia.

14.04-fold increase, the simvastatin group had a 1.8-fold increase, and the simvastatin + endotoxemia group had a 10.92-fold increase compared with the control rats.

The mean tissue concentrations of IL-10 gene expression in the simvastatin + endotoxemia group showed a 20.52-fold increase, the simvastatin group had a 4.98-fold increase, and the endotoxemia group had a 4.03-fold increase compared with the control rats.

The mean tissue concentrations of Bax gene expression in the endotoxemia group showed a 4.19-fold increase, the simvastatin + endotoxemia group had a 0.96-fold increase, and the simvastatin group had a 1.76 fold increase compared with the control rats.

Histologic procedures

The gastrocnemius muscle was fixed in 10% buffered formalin and embedded in paraffin wax. Five-micrometer-thick sections were placed on polylysine-coated slides and stained using hematoxylin and eosin (H&E). The stained sections were visualized and photographed under a light microscope at × 100 magnification (ECLIPSE 80i Nikon Corporation, JAPAN).

Statistical analyses

Statistical analyses were performed using SPSS 21 (Statistical Package for the Social Sciences Software, USA). Data are presented as mean values ± standard deviations. Groups of data were compared using analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. In all cases, $P < 0.05$ was set as the limit of significance.

Results

Gene expression results

The mean tissue concentrations of TNF-α gene expression in the endotoxemia group showed a

The mean tissue concentrations of Bcl-2 gene expression in the simvastatin + endotoxemia group were increased 3.26-fold, 1.9-fold in the simvastatin group, and 1.28-fold in the endotoxemia group as compared with the control rats.

The mean tissue concentrations of Caspase-3 gene expression were increased 7.79-fold in the endotoxemia group, 1.9-fold in the simvastatin group, and 1-59-fold in the simvastatin + endotoxemia group compared with control rats (**Figure 1A-E**).

Energy results

Creatine phosphate levels were decreased in the simvastatin and endotoxemia groups compared with the other groups ($P < 0.05$). In addition, creatine phosphate levels were increased in the simvastatin + endotoxemia group compared with the endotoxemia group ($P < 0.05$).

AMP levels were decreased in the experimental groups compared with the controls. AMP values were found lower in the simvastatin + endotoxemia group than in the other groups ($P < 0.01$). There were no changes in values of ADP for the

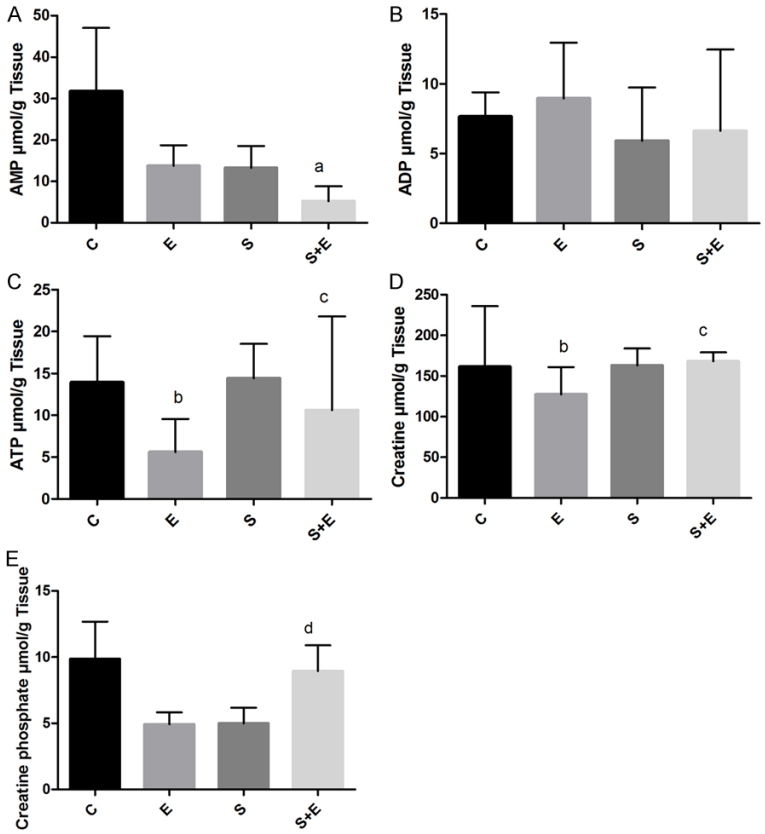

Figure 2. A-E: Results of HPLC. A: AMP, B: ADP, C: ATP, D: Creatine, E: Creatine Phosphate. a: Simvastatin + Endotoxemia group vs. other groups ($P < 0.01$); b: Endotoxemia group vs. control group ($P < 0.01$); c: Simvastatin + Endotoxemia group vs. Endotoxemia group ($P < 0.05$); d: Simvastatin + Endotoxemia group vs. other groups ($P < 0.05$). Names of Groups, C: Control, E: Endotoxemia, S: Simvastatin, S + E: Simvastatin + Endotoxemia.

experimental groups ($P > 0.05$). ATP and creatine values were found lower in the endotoxemia group than in the control group ($P < 0.01$), and also higher in the simvastatin + endotoxemia group compared with the endotoxemia group ($P < 0.05$) (**Figure 2A-E**).

Histopathologic findings

Classic skeletal muscle structure was seen in the simvastatin group. There was perivascular and perifascicular infiltration of inflammatory cells and atrophic areas in the endotoxemia group. Inflammation and atrophy in perivascular areas were reduced in the simvastatin + endotoxemia group (**Figure 3A-D**).

Discussion

Cytokines play a key role in early biochemical events in sepsis. LPS is a strong inducer of many cytokines including TNF-α and IL-6. It has been revealed that an increase of TNF-α leads to sepsis and organ failure, and decreases survival [18]. Induction of sepsis in animal models reduces muscle protein synthesis, the effects of which are prevented by prior injection of TNF inhibitors [19].

Also, proinflammatory cytokines in skeletal muscle may promote muscle wasting [20]. We demonstrated that TNF-α gene expression in the endotoxemia group was significantly increased compared with the control rats, and decreased in the simvastatin + endotoxemia group. Moreover, our data showed that IL-10 level was significantly increased by simvastatin administration in rats of the endotoxemia group.

Creatine phosphate, creatine, and ATP levels were increased in the simvastatin + endotoxemia group compared with the endotoxemia group. ATP depletion contribute to organ dysfunction in sepsis [21].

Statins have been associated with a decrease in CoQ10 synthesis, which leads to impaired oxidative phosphorylation and energy production [22]. Muscle high-energy phosphate concentrations were maintained during statin treatment in patients with hypercholesterolemia [23]. These results are supported by another clinical study in which no evidence of pathologic changes in muscle were detected after 18 weeks of treatment with simvastatin or pravastatin as judged by histologic examination of muscle tissue, and by measurements of exercise-induced release of creatine kinase or myoglobin [24].

AMP values were found low in the simvastatin + endotoxemia group. In this group, AMP may be shifted towards ATP-producing pathways (e.g.

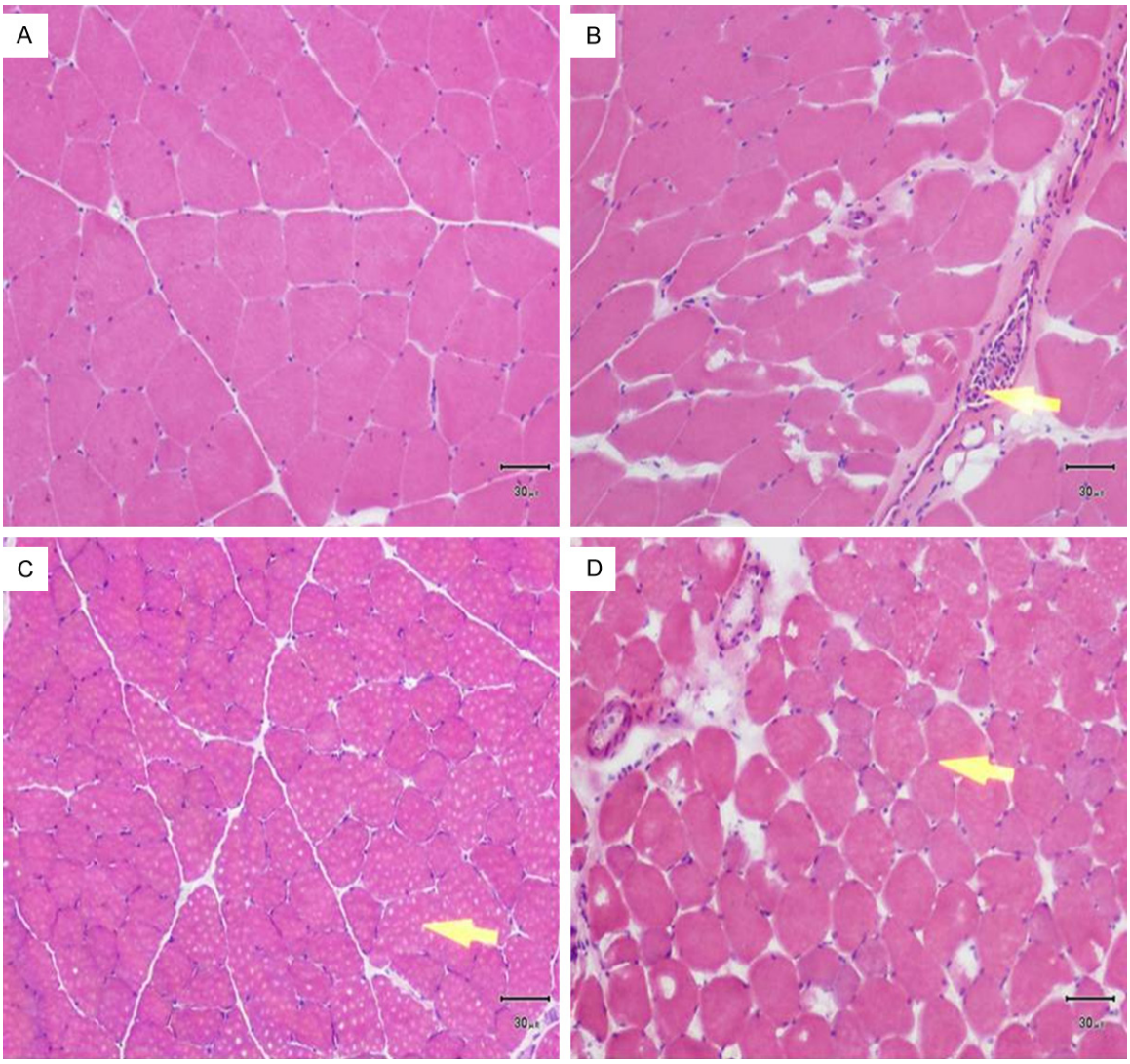

Figure 3. A-D: Section of liver tissue stained with H&E. (A) Section of liver tissue from control group, × 100 magnification (B). Section of liver tissue from LPS group, × 100 magnification (C). Section of liver tissue from Simvastatin group, × 100 magnification (D). Section of liver tissue from Simvastatin + LPS group, × 100 magnification.

oxidative phosphorylation). Therapies should be aimed towards increasing ATP production (e.g. substrate availability, mitochondrial functional capacity).

Statins demonstrate a concentration-dependent adverse effect on muscle cell viability and promote cell disruption via proteolysis and apoptosis [25]. The mechanism of apoptosis induced by endotoxin injection has been postulated as Akt pathway activation, Bcl-2 family induction or release of inflammatory cytokines [26].

A study showed that Bcl-2 inhibited cell apoptosis through various ways: Bax-mediated apoptosis, inhibition of calcium ion release in endoplasmic reticulum [27]. Pretreatment with simvastatin protected against alpha-toxin-induced sepsis associated with reduced p53, TNF-alpha, apoptosis, and necrosis [28]. Fu et al showed that simvastatin inhibited sepsis-induced endothelial cell apoptosis by upregulating the Bcl-2 gene and downregulating the Bax gene. The authors suggested that simvastatin could inhibit apoptosis of endothelial cells induced by sepsis through Bcl-2 expression upregulation and downregulation of Bax and Caspase-3, which may be one of the mechanisms for simvastatin to treat sepsis. Consistent with Fu et al's results, in this study Bax and Caspase-3 gene expression in endotoxemia group showed an increase, and there was

decreased expression in the simvastatin + endotoxemia group. Simvastatin treatment increased Bcl-2 gene expression in rats of the endotoxemia group [29].

Perivascular and perifascicular infiltration of inflammatory cells and atrophic areas were observed in the endotoxemia group. The effects were less prominent with simvastatin pre-treatment.

In this study, we found that sepsis caused an increase in the pro-inflammatory cytokine TNF-α and pro-apoptotic proteins; Caspase-3 and Bax in muscle tissue may also cause tissue damage. Simvastatin reduced muscle tissue damage with decreased levels of TNF-α, Bax, Caspase-3, and increased IL-10 and Bcl-2.

Acknowledgements

Our study was granted from Istanbul University Research Projects (Project No: 42019). We would like thank Mr. David F Chapman for editing the language of the manuscript. Also Hatice Taşlı for preparing the muscle sections.

Disclsoure of conflcit of interest

None.

Address correspondence to: Dr. Elif Ozkok, Department of Neuroscience, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Vakif Gureba Cad, 34280 Istanbul, Turkey. Tel: 90 (0212) 4142000/33355; E-mail: eozkok34@hotmail.com

References

[1] Ando H, Takamura T, Ota T, Nagai Y, Kobayashi K. Cerivastatin improves survival of mice with lipopolysaccharide induced sepsis. J Pharmacol Exp Ther 2000; 294: 1043-1046.

[2] Schulte W, Bernhagen J, Bucala R. Cytokines in sepsis: potent immunoregulators and potential therapeutic targets an updated view. Mediators Inflamm 2013; 2013: 165974.

[3] Schiaffino S, Dyar KA, Ciciliot S, Blaauw B, Sandri M. Mechanisms regulating skeletal muscle growth and atrophy. FEBS J 2013; 280: 4294-314.

[4] Lang CH, Frost RA, Nairn AC, MacLean DA, Vary TC. TNF decreases myocardial and skeletal muscle protein synthesis via alterations in translation initiation but not elongation. Am J Physiol Endocrinol Metab 2002; 282: E336-E347.

[5] Li YP and Reid MB. Effect of tumor necrosis factor-α on skeletal muscle metabolism. Curr Opin Rheumatol 2001; 13: 483-487.

[6] Dogra C, Changotra H, Wedhas N, Qin X, Wergedal JE, Kumar A. TNF-related weak inducer of apoptosis (TWEAK) is a potent skeletal muscle wasting cytokine. FASEB J 2007; 21: 1857-1869.

[7] Holecek M, Tilser I, Skopec F, Sprongl L. Leucine metabolism in rats with cirrhosis. J Hepatol 1996; 24: 209-216.

[8] Holecek M, Sprongl L, Skopec F, Andrys C, Pecka M. Leucine metabolism in TNF-α- and endotoxin-treated rats: contribution of hepatic tissue. Am J Physiol 1997; 273: E1052-E1058.

[9] Giannesini B, Izquierdo M, Dalmasso C, Le Fur Y, Cozzone PJ, Verleye M, Le Guern ME, Gillardin JM, Bendahan D. Endotoxemia causes a paradoxical intracellular pH recovery in exercising rat skeletal muscle. Muscle Nerve 2007; 36: 505-514.

[10] Singer M. Mitochondrial function in sepsis: acute phase versus multiple organ failure. Crit Care Med 2007; 35 Suppl: S441-448.

[11] Fredriksson K, Hammarqvist F, Strigard K, Hultenby K, Ljungqvist O, Wernerman J, Rooyackers O. Derangements in mitochondrial metabolism in intercostal and leg muscle of critically ill patients with sepsis induced multiple organ failure. Am J Physiol Endocrinol Metab 2006; 291: E1044-1050.

[12] Rennie MJ, Wackerhage H, Spangenburg EE, Booth FW. Control of the size of the human muscle mass. Annu Rev Physiol 2004; 66: 799-828.

[13] Tews DS and Goebel HH. Apoptosis-related proteins in skeletal muscle fibers of spinal muscular atrophy. J Neuropathol Exp Neurol 1997; 56: 150-6.

[14] Pinheiro da Silva F and Nizet V. Cell death during sepsis: integration of disintegration in the inflammatory response to overwhelming infection. Apoptosis 2009; 14: 509-521.

[15] Koh KK, Sakuma I, Quon MJ. Differential metabolic effects of distinct statins. Atherosclerosis 2011; 215: 1-8.

[16] Bellosta S and Corsini A. Statin drug interactions and related adverse reactions. Expert Opin Drug Saf 2012; 11: 933-46.

[17] Shellvold OF, Jnge P, Aarstad K. High performance liquid chromatography: a rapid isocratic method for determination of creatine compounds anda denine nucleotides in the myocardial tissues. J Mol Cell Cardiol 1986; 18: 517-527.

[18] Dofferhoff AS, Bom VJ, de Vries-Hospers HG, van Ingen J, vd Meer J, Hazenberg BP, Mulder PO, Weits J. Patterns of cytokines, plasma endotoxin, plasminogen activator inhibitor, and

acutephase proteins during the treatment of severe sepsis in humans. Crit Care Med 1992; 20: 185-192.
[19] Cooney R, Kimball SR, Eckman R, Maish G 3rd, Shumate M, Vary TC. TNF binding protein ameliorates inhibition of skeletal muscle protein synthesis during sepsis. Am J Physiol 1999; 276: E611-619.
[20] Cooney RN, Kimball SR, Vary TC. Regulation of skeletal muscle protein turnover during sepsis: mechanisms and mediators. Shock 1997; 7: 1-16.
[21] Carré JE and Singer M. Cellular energetic metabolism in sepsis: the need for a systems approach. Biochim Biophys Acta 2008; 1777: 763-771.
[22] Vaklavas C, Chatzizisis YS, Ziakas A, Zamboulis C, Giannoglou GD. Molecular basis of statin-associated myopathy. Atherosclerosis 2009; 202: 18-28.
[23] Laaksonen R, Jokelainen K, Laakso J, Sahi T, Harkonen M, Tikkanen MJ, Himberg JJ. The effect of simvastatin treatment on natural antioxidants in low-densitylipoproteins and high-energy phosphates and ubiquinone in skeletal muscle. Am J Cardiol 1996; 77: 851-854.
[24] Contermans J, Smit JW, Bär PR, Erkelens DW. A comparison of the effects of simvastatin and pravastatin monotherapy on muscle hissslogy and permeability in hypercholesterolaemic patients. Br J Clin Phurmacol 1995; 39: 135-141.
[25] Skottheim IB, Gedde-Dahl A, Hejazifar S, Hoel K, Asberg A. Statin induced myotoxicity: the lactone forms are more potent than the acid forms in human skeletal muscle cells in vitro. Eur J Pharm Sci 2008; 33: 317-325.
[26] Jacob A, Hensley LK, Safratowich BD, Quigg RJ, Alexander JJ. The role of the complement cascade in endotoxin-induced septic encephalopathy. Lab Invest 2007; 87: 1186-1194.
[27] Schulz JB, Weller M, Moskowitz MA. Caspases as treatment targets in stroke and neurodegenerative diseases. Ann Neurol 1999; 45: 421-429.
[28] Buerke U, Carter JM, Schlitt A, Russ M, Schmidt H, Sibelius U, Grandel U, Grimminger F, Seeger W, Mueller-Werdan U, Buerke M. Apoptosis contributes to septic cardiomyopathy and is improved by simvastatin therapy. Shock 2008; 29: 497-503.
[29] Fu H, Wang QS, Luo Q, Tan S, Su H, Tang SL, Zhao ZL, Huang LP. Simvastatin inhibits apoptosis of endothelial cells induced by sepsis through upregulatingthe expression of Bcl-2 and downregulating Bax. World J Emerg Med 2014; 5: 291-297.