Differential Regulation of Skeletal Muscle Myofilament Protein Expression in Sepsis

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ABSTARCT

Sepsis is a clinical condition of dysregulated systemic inflammation and pervasive tissue damage. Patients with severe sepsis are prone to skeletal muscle dysfunction which is manifested as fiber atrophy and depressed contractile performance. The causes of sepsis-induced muscle atrophy remain under investigation. In this thesis, we hypothesize that sepsis triggers fiber atrophy in both the ventilatory and limb muscles and that fiber atrophy is the result of significant reduction in protein contents of various myofilament proteins including Myosin Heavy and Light Chain, Troponins and Tropomyosin. We also propose that the degree of myofiber atrophy and contractile protein degradation triggered by severe sepsis is related to muscle oxidative capacity with the diaphragm showing relatively lesser degree of atrophy and protein degradation than the limb muscles. Finally, we propose that reduction in myofilament protein contents of skeletal muscles is mediated through both increased proteolysis and reduction in mRNA expression of myofilament genes.

Sepsis was induced in adult C57/Bl6 mice by cecal ligation and perforation (CLP) technique. Control animals (Sham) were subjected to an identical surgical procedure with the exclusion of cecum perforation. Sham and CLP animals were investigated after 24, 48, and 96h post-surgical procedure and the diaphragm (DIA) and tibialis anterior (TA) muscles were quickly excised as representative of the ventilatory and limb muscles, respectively. Muscle atrophy was quantified by measuring myofiber diameter. Myofilament protein contents were measured with immunoblotting using antibodies selective to Myosin Heavy Chain, Myosin Light Chain, α-Actin, Troponin-T, Troponin-I, Troponin-C and Tropomyosin proteins. Expression of mRNA of various myofilament genes was measured with real-time PCR.

Sepsis induced significant atrophy of DIA and TA fibers in mice. The time course and the degree of atrophy in the two muscles differed significantly. While DIA atrophy

developed only after 24h of sepsis and was relatively mild in nature, atrophy of TA muscle fibers was detected after 24, 48, and 96h of sepsis and was relatively more severe than that of the DIA. Sepsis also triggered significant increase in the mRNA expression of two muscle-specific E3 ligases involved in protein ubiquitination (Atrogin-1 and MuRF1). The relative induction of Atrogin-1 and MuRF1 mRNA expression was stronger in the TA compared to the DIA. Sepsis was also associated with significant decreases in mRNA and protein levels of various myofilament contractile proteins in the DIA and TA. However, the time course and sensitivity to sepsis differed among various myofilament proteins with α-Actin and Troponin-I being less sensitive to the inhibitory effects of sepsis while mRNA and protein levels of Myosin Heavy Chain, Myosin Light Chain, Troponin-T, Troponin-C and Tropomyosin were strongly influenced by sepsis. Finally, reduction of Myosin Heavy Chain and Myosin Light Chain protein contents was sustained even after 96h of CLP while the decline in Troponin-T, Troponin-I, Troponin-C and Tropomyosin mRNA and protein levels in DIA and TA was restricted to 24 and 48h after induction of sepsis.

Our study is the first to show that sepsis triggers significant and more severe fiber atrophy in limb muscles compared to the DIA and that sepsis is associated with significant reductions in the contents of various myofilament proteins including those of thick contractile elements (Myosin Heavy and Light Chains) and regulatory proteins such as Troponins and Tropomyosin. Finally, our study indicates for the first time that decreased skeletal muscle myofilament protein contents in septic animals is mediated through increased proteolysis and decreased mRNA expression of these proteins.

RESUME

La septicémie est un état clinique d'inflammation systémique dérégulée menant à des lésions tissulaires omniprésente. Les patients qui développent les stages ultérieurs, sont sujets à un dysfonctionnement du muscle squelettique se traduisant par une atrophie et une détérioration de la contractilité du muscle. Les causes sous-jacentes à l'atrophie musculaire induite par la septicémie restent ambiguës et non entièrement exploré. Dans cette thèse, nous émettons l'hypothèse que atrophie du muscle squelettique induite par la septicémie est une résultante de la dégradation différentielles de protéines ciblant les diverses protéines contractiles myofibrillaires. Nous suggérons également que le degré d'atrophie des myofibre et le degré de dégradation des protéines contractiles généré en réponse à une septicémie ne sont pas uniformes dans toute la musculature : les muscles ayant une capacité oxydative relativement élevée tels que le diaphragme (DIA) devrait subir moins d'atrophie et moins d'activité protéolytique en comparaison aux muscles de capacité oxydative relativement faible tels que le jambier antérieur (JA).

La septicémie a été induite chez les souris adultes C57 / BL6 en employant la procédure de ligature et perforation du cæcum (LPC). Les animaux témoins (Sham) ont été soumis à une intervention chirurgicale identique à celle du groupe LPC à l'exclusion toutefois de la perforation du caecum. Les animaux Sham et LPC ont été étudiés 24h, 48h, 96h post-chirurgie. L'alimentation et la consommation d'eau ont été quantifiés sur une période post-chirurgicale de 96h. L'atrophie musculaire a ensuite été quantifiée par la mesure du diamètre des fibres musculaires. La dégradation différentielle des protéines contractile a été évaluée par mesure de l'expression de plusieurs protéines des fibres musculaires, notamment la troponine C (TNNC) Troponine I (TNNI), la troponine T (TNNT), MYL2 (f) MYL1 (f), S21 MYL3 (s), tropomyosine (TPM), l'actine sarcomérique, l'actinin, MyHC totale, MyHC IIB et MyHC IIA. L'expression des gènes

codant pour ces protéines a également été étudiée en quantifiant l'expression relative de leur ARNm par la méthode de PCR en temps réel.

La septicémie induit une atrophie significative des fibres du DIA et du JA chez la souris. Le cours du temps de l'atrophie musculaire dans les deux muscle diffère largement de sorte que l'atrophie du DIA se développe uniquement après 24h de septicémie tandis que dans le JA une importante atrophie des fibres persiste au cours des 24, 48 et 96h de la septicémie. La septicémie à également suscité une dimunition significative et différentielle du niveau d'expression de diverses protéines contractiles des myofilaments et conséquemment de leur ARNm spécifique, dans le DIA et JA. Cependant, le cours du temps et la sensibilité à la septicémie menant à une réduction dans l'expression protéique et génique, diverge entre plusieurs protéines des myofilaments. α-actine et Troponine-I semblait être moins sensibles aux effets inhibiteurs de la septicémie tandis que les niveaux d'ARNm et protéiques de la chaîne de myosine lourde, de la chaîne légère de la myosine, la troponine-T, Troponine-C et Tropomyosin sont fortement influencés par la septicémie. Enfin, la réduction du niveau d'expression des chaînes myosine lourde et légère est soutenue même après 96h post-LPC tandis que la baisse des taux de protéines et d'ARNm de la troponine-T, Troponine-I, troponine C et Tropomyosin s'est limité à 24h et 48h après l'induction de la septicémie.

Notre étude est la première extrapolant que la LPC déclenche une atrophie significative dans le muscle squelettique tout en explorant les mécanismes au niveau des protéines contractiles myofirillaire dans les muscles squeletique des membres inferieurs ainsi que dans les muscles respiratoires. En outre, cette étude démontre également que la dégradation différentielle de différentes protéines contractiles des myofilaments est induite dans une grande mesure dans les muscles des membres inférieurs ayant une capacité

d'oxydation faible par rapport aux muscles respiratoires ayant une capacité oxydative habituellement plus élevée.

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LIST OF ABBREVIATIONS

ATG:	Autophagy-related gene
ATP:	Adenosine triphosphate
Ca^{2+}:	Calcium (ion , free)
Caspase:	Cysteine-dependent aspartate-directed proteases
CLP:	Cecal ligation and perforation
CSA:	Cross-sectional area
DIA:	Diaphragm
DNHP:	2,4-dinitrophenylhydrazine
eNOS:	Endothelial nitric oxide synthase
ER:	Endoplasmic reticulum
HNE:	4-hydroxy-2-nonenal
Fbox32:	Atrogin-1 (gene)
ICU:	Intensive care unit
IL-1:	Interleukin-1
IL-1β:	Interleukin-1 beta
Il-6:	Interleukin-6
LPS:	Lipopolysaccharide
MYHC:	Myosin heavy chain
MYL:	Myosin light chain
MuRF-1:	Muscle RING-finger protein-1
nNOS:	Neuronal nitric oxide synthase
NO:	Nitric Oxide
PCR:	Polymerase chain reaction
PE:	Phosphatidylethanolamine
PEG-SOD:	Polyethylene glycol-superoxide dismutase
PVDF:	Polivinylidene difluoride
ROS:	Reactive oxygen species
RNS:	Reactive nitrogen species
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TA:	Tibialis anterior
TNF-α:	Tumor necrosis factor alpha

TNNC: Troponin C

TNNI: Troponin I

TNNT: Troponin T

TPM: Tropomyosin

UP: Ubiquitin proteasome

SECTION 1: LITERATURE REVIEW

1.1 Introduction

Sepsis constitutes a general term that includes an expansive spectrum of clinical syndromes characterized by a dysregulated systemic inflammatory host response towards an infection triggered mainly by endotoxin-producing gram negative bacteria in addition to parasites, viruses and fungi.[1-3] As a consequence of its complexity and perplexity, sepsis is widely documented throughout the literature as a major public health problem and as a chief cause of morbidity and mortality in intensive care units.[1,4] According to a 2015 statistical report performed by the World Health Organization; sepsis is associated with an estimated 30 million annual incidences worldwide while being responsible for 10,000 deaths and 30,000 hospitalizations annually in Canada alone. Septic shock survivors usually experience a preeminent risk of developing prolonged skeletal muscle weaknesses and dysfunctions that involve the ventilatory and limb muscles culminating in sustained mechanical ventilation, delayed weaning, amplified prevalence of respiratory failure, functional impairment, and diminished post-discharge recovery.[4,5,6] The underlying pathophysiological causes of sepsis-induced myopathy are attributed to several factors including mitochondrial dysfunction, depressed skeletal muscle energy biogenesis, excessive proteolysis, and abnormalities in sarcolemmal transmission and excitation-contraction coupling.[4,7] Despite the fact that skeletal muscle dysfunction as well as the attributed mechanisms of skeletal muscle atrophy and contractile protein degradation during sepsis have been comprehensively scrutinized throughout the literature; no data has ever addressed the status of the individual myofilament proteins and the contribution of the latter to the overall muscle atrophy and contractile dysfunction observed in sepsis. This thesis aims at assessing simultaneous changes in muscle fiber size, myofilament protein

contents and mRNA expression of various myofilament genes in the ventilatory and limb muscles during the course of severe sepsis in an animal model that simulates human sepsis.

1.2 Skeletal Muscle: Structure and Classification

Muscle mass constitutes 40-50% of the total human body and in mammals represents a locomotion agent, protein reservoir and one of the chief sites for metabolic control.[9,10] . Skeletal muscle is an exceedingly structured heterogeneous assemblage of myofibers bundled within sheaths of connective tissue.[10, 11] These myofibers, which result from the early fusion of muscle precursor cells referred to as myoblasts, in turn, encompass a high extent of further organization whereby each embraces an abundance of myofilament proteins arranged into myofibrils within the sarcoplasm.[11] Myofibrils themselves enclose contractile proteins amassed into basal repetitive structures known as sarcomeres serving as the elementary contractile units of the skeletal muscle. [10,11] Sarcomeric contraction is established via a regulated reversible interaction between peripheral Actin and central Myosin myofilament proteins.[11] Besides being recognized for its orchestrated structure; skeletal muscle displays key characteristic features of striation and multinucleation whereby myonuclei are localized under the basal lamina at the edge of the myofibers while the ATP-generating mitochondria, calcium releasing sarcoplasmic reticulum and organelles essential for either cell survival or myofiber contractility are dispersed in the surrounding sarcoplasm. [11, 14]

Based on histological data, mammalian skeletal muscles are a mixture of four different fiber types diverging at the level of their biochemical, structural, and functional features attributed to the specific myosin heavy chain isoform they express.[11] These four fiber types are further subcategorized into either slow-twitch (type I) fibers or fast twitch (type IIa , IIb or IIx) fibers conferring to their relative fiber shortening speed during contraction.[10,12,14] Slow-twitch skeletal muscle fibers display a characteristically red

appearance as a consequence of their dense capillary network and preeminent myoglobin content while also containing abundant mitochondria which make them more reliant on oxidative metabolism.[12,13] On the contrary , fast-twitch skeletal muscle fibers incorporate a minute number of both capillaries and mitochondria, and thus exhibit a white appearance and predominantly depend on glycolytic metabolism.[12 ,13] Moreover, based on the fact that myofiber content is a chief determinant of resistance to fatigue and speed of contraction, slow twitch fibers are recognized as more fatigue resistant than fast-twitch fibers upon electrical stimulation.[11,14] Accordingly, as illustrated in type IIA fibers which incorporate both dense capillary and mitochondria networks as well as glycolytic enzymes conferring them with a dual oxidative glycolytic metabolic capacity crowning in the advantage of being both fast and fatigue resilient; the greater the oxidative capacity of a fiber, the higher its resistance to fatigue [11,12,14] Similarly, type IIx fibers (not yet identified in humans) despite being the fastest amidst skeletal muscle fibers; are also the most effortlessly fatigued as a matter of their reliance on glycolytic metabolism. [11, 14,15] In addition to the previously discussed fiber type extensive variability perceived in Myosin Heavy Chain attributed gene expression; fiber type variances are also observed within the expression profile of supplementary muscle proteins epitomized by: Tropomyosin, Troponins, and Myosin Light Chains among others.

1.3 Skeletal Muscle Myofilament Proteins

As previously discussed, myofibers contain a vast number of essential contractile and regulatory myofilament proteins among which Myosin molecules consisting of both Myosin Heavy Chains and Myosin Light Chains, sarcomeric Actin, Troponins and Tropomyosins are of exceptional relevance .[14]

1.3.1 Myosin Heavy Chains

One of the chief components of the skeletal muscle fiber is the Myosin molecule which constitutes the motor contractile protein accountable for force production during muscle contraction.[10,16] The structural prototype of this indispensable skeletal muscle molecule depicts Myosin as a hexameric polypeptide entailing four light chains and two heavy chains whose amino terminal portions are disjointed into two elongated globular domains referred to as Myosin heads with an energy generating ATPase site embedded on one distal globular head and an Actin-binding region that undertakes slight conformational changes countenancing muscle contraction on the other head.[10,16] The latter conformational fluctuations are subsequently intensified and conveyed to the myosin carboxy-terminal portions which associate to form a thick α-helical coiled filament denoted as the myosin tail around which two alkali and two regulatory myosin light chains are wrapped.[10,16]

The skeletal Myosin Heavy Chain (MHC) gene family encompasses at least six MHC genes, each encoding a discrete isoform, some of which are still unidentified.[16, 17] The latter genes are positioned within two distinct clusters whereby chromosome 14 in both humans and mice houses the β/slow MHC gene while human chromosome 17 and mouse chromosome 11 embrace embryonic, neonatal and adult fast MHCS genes. [15-17] Despite the fact that the MHC isotype catalog is still partial and incomplete due to the fact that the majority of studies have concentrated on only particular muscle; four myosin heavy chain isotypes (MHC1, MHCIIa, MHCIIb, and MHCIIx) have been identified in small mammals, and have been allocated to the histochemically demarcated fiber types I, IIA, IIB and IIX, respetively.[15, 16] The functional role of these myosin isoforms has been thoroughly scrutinized in several investigations on mammalian single fibers whereby they have been demonstrated to differ in their explicit Actin-stimulated and Ca^{2+}-initiated ATPase activities which correlate with contraction speed dissimilarities.[15,16] As a

consequence, MHCs constitute the foremost determinants of ATP depletion, isomeric tension cost, and shortening velocities in skeletal muscles whereby MHCIIb epitomizes the fastest of four isoforms, MHCIIa exhibits a subordinate maximum contraction rate, while MHCIIx demonstrates a shortening velocity analogous to MHCIIa and a power output and curvature of the force velocity curve comparable to MHCIIb.[10,11,16,17] By comparison, slow MHC isozymes illustrate higher chemomechanical conversion competence, thus endowing them with superior tension preservation.[10, 11]

1.3.2 Myosin Light Chains

The MHC head-tail junction is associated with two small polypeptide units, recognized as Myosin Light Chains (MLCs), one belonging to the essential alkali/MLC1 family and one to the regulatory/MLC2 category.[10,15,16] These 17-23kDa MLC units stabilize the MHC α-helix and have been demonstrated to play a key role in the modulation of both the ATPase activity and actinomyosin interaction consequently regulating the speed of contraction. [17,18,19] Likewise MHCs, both MLC alkali and regulatory units exist in various fast (chiefly MLC1f and MC3f) and slow (essentially MLC2s) isoforms that are differentially expressed among the various fiber types.[16] Interestingly, based on the fact that each MHC is associated with two alkali and two regulatory MLCs and on the basis of three conceivable alkali MLC groupings; each fast MHC dimer has the potential to generate three diverse fast isomyosins (LC3f homodimer FM1, LC1f/LC3f heterodimer FM2 , and LC1f homodimer FM3).[15] At variance with MHCs, the numerous MLC isotypes are scattered on diverse chromosomes whereby each individual sarcomeric MLC isoform is encrypted by a distinctive gene except for the alkali MLC1f and MLC3f which originate from a single particular gene via alternative promoters and alternative exon splicing. [15,16]

1.3.3 Sarcomeric Actin

Sarcomeric Actin constitutes the major component of the thin myofibrillar filament and is associated with two regulatory proteins known as Troponin and Tropomyosin.[3] Upon receiving a stimulus in the form of an action potential; Ca^{2+} which is released from the sarcoplasmic reticulum binds to Troponin-C and via the contribution of Tropomyosin exposes the Myosin binding site embedded on the Actin molecule.[4] In the presence of ATP, Actin then indorses the binding to the Myosin head which in turn pulls the thin Actin filament along the thick Myosin filament allowing the shortening of the sarcomere.[4,10] Actin-based thin filaments comprise barbed ends which are attached to the Z-bands separating discrete sarcomeric units as well as pointed ends that are not bound to any specific structure.[4,20] At the termination of each sarcomeric unit, the latter ends are oriented in opposite directions in order to support the production of contractile forces via the unidirectional sliding of the Myosin motors.[20] Moreover, adjacent to the Actin barbed ends, α-Actinin crosslinks actin filaments at the Z-bands stabilizing the polarized conformation of the thin filaments.[20]

Sarcomeric Actin filaments constantly undergo a dynamic exchange of Actin subunits within the filaments yet without modifying the overall sarcomeric structure since the consistent organization of Actin filaments conserving both uniform length and even polarity is critical for the contractile function of the striated muscle.[10,20] The multidimensional dynamics of sarcomeric Actin filaments along with their attributed regulatory mechanisms have been described in a number of reports whereby recent studies have advocated a relation between Actin dynamics and several muscle maladies.[20]

1.3.4 Troponins

Collectively, Troponins constitute an indispensable proteins that sensitize the actomyosin structure to Ca^{2+} whereby in association with Tropomyosin; Troponins play a

fundamental role in muscle activity and contractile force regulation by interrelating intracellular Ca^{2+} concentration shifts to contraction generation.[21,22,23] Functionally, upon release from the sarcoplasmic reticulum, Ca^{2+} binds to Troponin-C (TNNC) resulting in an affinity conversion culminating in the transferal of Troponin-I (TNNI) binding domain from an Actin-Tropomyosin affinity towards a TNNC affinity.[21-25] This binding amendment in addition to other supplementary alterations in the thin filament protein interactions release the inhibitory activity implemented by Troponin-I (TNNI), thus promoting the interaction of Actin with Myosin through both steric modifications in the Tropomysoin positioning and allosteric amendments in the thin filament both of which enhance strong cross bridge establishment and ultimately contraction.[21,22] As a result of these manifestations, Troponin components execute two essential mechanistic regulations summarized by inhibition of the contractile interaction of Myosin-Actin-Tropomyosin which is implemented by the inhibitory action of TNNI, and the discharge of the latter inhibition by TNNI itself through the binding of Ca^{2+} to TNNC.[21,22] Electron microscopy examinations show Troponin as an orderly distributed and structured regulatory complex that binds to precise regions of each Tropomyosin molecule at consistent intervals of 38 nm coinciding with each seventh Actin monomer along the thin filament.[22,23] Moreover, the Troponin complex has been demonstrated to structurally consist of three key subunits: contractile interaction inhibitory component TNNI which constrains Actin-Myosin cross bridges in a Ca^{2+} dependent manner, Tropomyosin-binding component TNNT which is responsible for fixating Troponin onto the thin filament by binding to Tropomyosin, and Ca^{2+} binding component TNNC.[21, 22,24] In humans, each of skeletal TNNI, TNNT and TNNC is expressed as fast and slow isoforms which during embryonic development are ubiquitously expressed throughout the entire musculature. In adults, the distribution of the former isoforms becomes differentially restricted to fast and slow muscles respectively.[22]

TNNI genes constitute paralogs designed by the triplication of the ancestor TNNI/TNNT gene pair and are organized in tandems with TNNT genes while human gene TNNC1 encoding the cardiac/slow TNNC isoform is located on chromosome 3 and TNNC2 coding the fast skeletal TNNC isoform is located on chromosome 20.[36] Moreover, numerous studies have described that both TNNT and TNNI have the potential of undergoing phosphorylation by several protein kinases and that posttranslational modifications impose considerable effects on the Troponin complex structure and properties.[23-28]

Extensive genetic studies have delineated a number of mutations in the genes coding for Troponin components, mainly TNNT and TNNI, which are involved in both the establishment of the three chief types of inherited cardiomyopathy and in the diagnostics of pathologies related to cardiomyocyte necrosis.[21-23] Besides, a spectrum of clinical and experimental studies have reported that cardiac Troponin levels are increased in the plasma during sepsis and could thus serve as an indication of myocardial dysfunction and poor outcome.[29] Accordingly, the Troponin complex has been considered as a key factor with can be utilized in medicine as target for cardiotonic drugs in the treatment of heart failure.[22,25,26] Yet, several aspects of the Troponin complex biology are still unclear especially within the context of skeletal muscle speed and force of contraction and attributed myopathies whereby the functional roles of Troponin are still not entirely understood. [26-28]

1.3.5 Tropomyosin

Tropomyosin (TPM) represents a ubiquitous α-helical coiled-coil protein assembled via the polymerization of α-helical dimers whereby the ends of each Tropomyosin dimer moderately overlay with adjacent dimers in a head-to-tail fashion resulting in two 40nm long fibrous strands along the Actin filament.[25,30,31] Tropomyosin

dimers occupy a locus in each Actin groove flanking seven Actin monomers via a non-covalent but rather flexible interaction blocking their corresponding Myosin binding sites [22,32,33,30] Tropomyosin's functional contributions in striated skeletal muscles have been thoroughly examined whereby besides its universal role contained in its ability to bind the Actin filaments enhancing their stability and regulating their interactions with other Actin binding proteins. It has also been characterized that Tropomyosin, in conjugation with the previously described Troponin complex, controls the cooperative binding of Myosin to Actin in a Ca^{2+} dependent fashion.[21,33,30] Tropomyosin belongs to an exceedingly conserved and miscellaneous genetic family. In mammals, TPM is encoded by four distinctive genes (TPM1, TPM2, TPM3, and TPM4) which via alternate promoters and alternative exon splicing can generate approximately 40 different mRNA variants expressed in developmental and tissue specific fashion.[30,32,33] For instance, three different Tropomyosin isotypes have been characterized in skeletal muscles: TPM1.1 (TMSKα), TPM1.2 (TMSKα1), and TPM2.2 (TPMSKβ) which are encoded by chromosomes 15 and 9, respectively.[31,30] Tropomyosin has been shown to be subject to phosphorylation, yet the effects of phosphorylation on the dynamic properties of muscle contraction and relaxation are still unidentified despite the fact that mutations in TPM have been acknowledged to constitute a plausible cause of cardio and skeletal myopathies.[31, 33]

1.4 Mechanisms of Skeletal Muscle Protein Degradation

Skeletal muscle atrophy is loss of muscle mass as a consequence of several conditions such as aging, muscular inactivity, immobilization, starvation, denervation and muscle unloading in addition to several pathological states including cachexia, cancer, AIDs, diabetes, and sepsis among many others.[20,35] Irrespective of the provoking events, it is well established that loss of muscle mass is characterized by a diminution in fiber diameter, decline in force production, descent in fatigue resistance, and prominently a

decrease in protein content accompanied by an escalation in protein degradation.[20,35,36] At least 60% of the overall muscle protein expanse is comprised by the myofibrillar proteins. These proteins are degraded by four proteolytic pathways: the ubiquitin-proteasome (UP) pathway, the Ca^{2+} dependent calpain pathway, the caspase pathway , and the autophagy-lysome pathway (ALP). [35-38]

1.4.1 Ubiquitin-Proteasome Pathway

The UP pathway is an adenosine triphosphate (ATP)-dependent multi-enzymatic system of targeted proteolysis and constitutes the foremost pathway through which skeletal muscle contractile proteins are disintegrated.[38-40] This intricate pathway degrade proteins in two discrete steps: an initial step comprising the covalent modifications of targeted proteins by ubiquitination and a second step involving the degradation of the ubiquitubinated proteins by the 26S proteasome.[40,41] Ubiquitination, a repetitive process requires three enzymes, an ATP-dependent activation of ubiquitin by the E1-ubiquitin activating enzymes; the transfer of the latter ubiquitin to the E2 ubiquitin-conjugating enzymes and finally propagation of ubiquitin to the E3 muscle specific ubiquitin-ligating enzymes which in skeletal muscles are represnted by the MAFbx32/Atrogin-1 and MuRF-1. These proteins covalently affix ubiquitin to the lysine (Lys48) residues of target proteins.[42-44,55] This process in which the E2-E3 enzyme coupling defines what protein category the ubiquitin machinery can perform on recurs several times until the initial substrate contains a poly-ubiquitin chain that permits recognition and subsequent degradation by the 26S proteasome.[38, 40-43] The 26S proteasome consists of a cylinder-shaped 20S proteolytic core associated with two 19S regulatory caps which recognize, bind, and facilitate the entry of substrate ubiquitin-tagged proteins into the proteolytic 20S core by engaging their ATPase activity to open the central channel, unfold the target protein, and eliminate its ubiquitin chain prior to further translocation within the 20S

core.[41,42,43] Once inside, proteins are truncated into small peptides by the 20S core chymotrypsin-like, trypsin-like, and caspase-like activities resulting in oligopeptides that are subsequently discharged into the cytoplasm for supplementary degradation by peptidases into amino acids.[41-43] Several potential stimuli have been recognized to induce skeletal muscle proteasome activity including pro-inflammatory cytokine TNF-α, oxidative stress and hypoxic stress.[45-47]

1.4.2 Calpain Pathway

The ubiquitin-proteasome pathway has been extensively confirmed to be indispensable for skeletal muscle proteolysis; however this catabolic pathway is restricted by the limitation of its inability to degrade myofibrillar proteins confined within integral sarcomeres.[48] Activation of both calpains and caspases has been documented to be critical for disruption of the rigid structure of the contractile apparatus.[49] Calpains are Ca^{2+}-dependent cysteine proteases that are principally activated in response to an increase in intracellular Ca^{2+} levels and Ca^{2+} triggers conformational changes that activate the catalytic domain of calpains leading eventually to selective protein cleavage in a site-specific fashion.[48-50] Calpains release myofilament proteins via the cleavage of sarcomeric components including Titin and Nebulin that function as important components that preserve the contractile unit's structural integrity.[49,51] Calpains exist in three main isoforms in skeletal muscles which include muscle-specific calpain 3 and ubiquitously expressed m-calpain and μ-calpain which under physiological conditions exhibit a relative degree of inactivity resulting from the endogenous inhibitor calpstatin.[50,51,52] Augmented calpain activity during sepsis appears to result in part from diminished calpstatin activity.[45,44] Under pathological states and in response to cellular damage, calpains are then activated; however their persistent activation triggers an unregulated proteolysis rate capable of provoking pervasive tissue damage and cell death.[53,54]A number of animal models have

been used to study calpains' prerequisite role in sepsis-induced myofilament protein degradation. For instance, Bhattacharya et al. demonstrated via a cegal ligation and perforation (CLP) model of sepsis that calpain was expressively higher in septic animal limb musculature; while Voisin et al. revealed, using a prolonged model of LPS-induced sepsis, that mRNA levels of m-calpain as well as proteasome proteolytic activity were considerably augmented in septic animal skeletal muscles.[56,57] Moreover, Supniski et al. revealed upon *E. coli* endotoxin administration that calpain activity was augmented while the force-generating capacity in the septic diaphragm was diminished.[60] Other investigations reported that exposure of C2Cl2 cells to a cytokine cocktail containing LPS, TNF-α, IL-1β and IFN-γ resulted in augmentation of calpain activity suggesting that calpain activation in septic skeletal muscles is promoted by pro-inflammatory cytokines.[61-63]

1.4.3 Caspase Pathway

Caspases are cysteine-aspartate proteases that constitute another family of enzymes that play an imperative role upstream of the ubiquitin-proteasome pathway in degrading cellular proteins during the process of apoptosis.[64] Caspases are capable of disassembling the organizational and functional components of a cell via the execution of a succession of proteolytic cleavages.[64] Several catabolic conditions have been demonstrated to stimulate either cytokine surges or insulin-resistance and cause activation of the apoptotic pathways in many tissues including skeletal muscle.[65,66] For instance, activation of caspase-3 which is known as the terminal protease of the caspase pathway has been detected in several pathological muscle wasting disorders such as ventilator-induced muscle weakness, cachexia, muscular dystrophy, oxidative stress, and cancer.[67-69] However based on the notion that skeletal muscle is multinucleated, caspase-3 activity may not be sufficient to evoke skeletal muscle cell death. Capase-3 may rather contribute to myofiber remodeling

and subsequent myofiber weakness as a result of myofilament protein cleavage.[65,66] Indeed, several studies have assessed the role of caspases in the release of myofilament proteins from intact myofibrillar contractile apparatus and disclosed that caspase-3 expedites muscle proteolysis by disintegrating the actomyosin complex into discrete myofibrillar proteins.[65] Furthermore, caspase-cleaved Actin fragments have been detected in the atrophied skeletal muscles in animal models of chronic uremian and diabetes.[65] In addition, caspase-3 has been reported to regulate sepsis-associated immune responses by inducing lymphocyte apoptosis. Interestingly, one study established that both endotoxin and CLP models of sepsis are associated with enhanced caspase-3 activation inside diaphragm muscle fibers which coincided with significant decline in diaphragm force-generating capacity.[66] Finally, upon administration of either a broad-spectrum caspase inhibitor or a discriminatory caspase-3 inhibitor; sepsis-induced diaphragm weakness was shown to be prevented.[66] Altogether these findings support the notion that caspase-3 activation may play a role in sepsis-induced skeletal muscle myofilament degradation.[66-71]

1.4.4 Autophagy-Lysome Pathway

Eukaryotic organisms are recognized to possess the evolutionarily conserved autophagy-lysosome pathway which constitutes a route through which cells breakdown and recycle organelles (mitochondria and peroxisomes), long-lived proteins, and bulk cytoplasmic portions.[72] This pathway employs a dual mechanism whereby cytosolic material is initially engulfed by double-membrane autophagosome which transport the cargo to the lysosomal lumen followed by degradation of the cargo by the lysosomal enzymes.[72] Basal levels of autophagy prevents the accumulation of damaged and dysfunctional proteins and organelles and is indispensable for the maintenance of intracellular homeostasis.[72] However, augmentation of autophagy above basal levels in response to microbial pathogens, cellular stress, and mitochondrial dysfunction may

contribute to the development of muscle wasting.[62,72] The functional implication of autophagy in skeletal muscle proteolysis has not yet been entirely understood. Recently the role of basal autophagy in skeletal muscle function was assessed by using transgenic mice with a muscle-specific ATG7 deletion and the authors have reported that these mice display several skeletal muscle morphological aberrations such as accumulation of impaired organelles and protein aggregates, diminished myofiber size, and misalignment of sarcomeric Z-bands.[74] Moreover, these pathological myofibers were documented to have depressed force generation and to develop oxidative stress and mitochondrial damage.[73,74] Additional studies have described that autophagy is activated in response to denervation as shown by a prompt induction of autophagosome formation and upregulation of mitophagy genes.[75] Our group has recently documented the extent of autophagy induction in response to acute starvation in mice and reported that oxidative stress capacity of skeletal muscles correlates negatively with the extent of autophagy.[76] Recent studies have revealed that autophagy inhibition triggers a compensatory upregulation of the proteasome proteolytic activity.[62,74] Overall, these studies confirm that basal level of autophagy acts as a pro-survival mechanism that is essential for the preservation of skeletal muscle mass through its removal of damaged organelles, inhibition of oxidative damage propagation, and prevention of apoptotic cell death.[74-76]

1.5 Sepsis

Sepsis depicts a broad clinical term that describes an intricate systemic host response to a severe infection instigated by viruses, fungi, parasites and most frequently endotoxin-producing gram-negative bacteria.[1,2,77,78] Under normal conditions, upon the exposure to injury or infection, the immune system launches a pro-inflammatory response at the site of trauma shortly followed by a balanced compensatory anti-inflammatory response.[2] In extreme cases where the host fails to eradicate or contain the infection, as in

the case of sepsis, pathogenic antigens as well as inflammatory mediators infiltrate the systemic circulation triggering a systemic dysregulated inflammatory response that eventually culminates in immune suppression as well as endothelial and epithelial cell damage.[2,78] Subsequently, disruption of the endothelial and epithelial barriers promotes dissemination of infection and inflammation into supplementary sites leading to severe sepsis or ultimately septic shock.[77,78] Severe sepsis is diagnosed when both organ dysfunction and insufficient blood flow are concomitant to the inflammatory response; a condition that can rapidly evolve into septic shock upon the development of acute circulatory failure.[1,79] In addition, mediators triggered by sepsis signaling cascade stimulate neutrophils which in turn exaggerate endothelial damage via the discharge of lysosomal proteases and free radical species.[77] Lipopolysaccharide (LPS), a key element of the gram-negative bacteria outer membrane, epitomizes a vigorous activator of the host immune response whereby its release into the circulatory system effectively prompts cascades of inflammatory mediators, cytokines, and chemokines accordingly leading to sepsis.[77-79]

Sepsis represents a life-threatening ailment and is a major cause of death in ICU.[2,80] The mortality rates of sepsis is related to the severity and the spectrum of the sepsis syndrome and can reach as high as 30% for sepsis, 50% for severe sepsis, and 80% for septic shock.[81] As a consequence of this relatively mortality rates, intense research efforts have been lunch which targeted the understanding of the underlying molecular mechanisms of sepsis and to facilitate the identification of biomarkers and establishment of effective therapeutic strategies designed to improve patient outcomes.[78,82]

Sepsis is usually investigated using animal models that simulate cardiovascular, metabolic, and immunological changes observed in human sepsis.[83] Two established murine models are characteristically used for sepsis investigations. The first model employs the exogenous administration of *E. coli* lipopolysaccharide (LPS) which activates

toll-like receptor 4 (TLR4) and triggers systemic transient and rapid cytokine surge of orders of magnitude higher than what is actually observed in clinical sepsis. Thus, this model is better employed to simulate extreme cases of human sepsis comparable to severe sepsis and septic shock.[78,84] T he second and most universally accepted experimental model of sepsis is the cecal ligation and perforation (CLP) model which produces clinically analogous poly-microbial states of sepsis resulting and a systemic cytokine profile of lower magnitude and longer duration than that associated with the LPS model.[78, 85] This relatively prolong time triggered by the CLP model mimics both human disease progression and replicates the cardiovascular and metabolic abnormalities that manifest throughout the progress of clinical sepsis.[78, 85]

1.6 Skeletal Muscle Dysfunction during Sepsis

Skeletal muscles, besides their critical role in locomotion, contribute to homeostasis by providing an important protein and amino acid reservoir.[4,6] Catabolic conditions and persistent disease states which trigger elevated demand for amino acid supply through proteolysis are associated with significant protein recycling of skeletal muscles.[40,86] In septic humans and animals, there are several abnormalities that are detected both in the ventilatory and limb muscles. These abnormalities involve abnormal sarcolemmal integrity, severe muscle weakness, poor contractile performance as well as muscle fiber atrophy.[4,6, 40]

1.6.1 Sarcolemmal Integrity

Depressed skeletal muscle contractile performance in sepsis has been attributed to derangements at several subcellular sites within skeletal muscle fibers including loss of sarcolemmal membrane integrity, poor cell membrane excitability, impaired excitation-contraction, disruption of calcium homeostatsis and abnormalities in contractile protein interactions.[4,5] Loss of sarcolemmal integrity has been documented both in the ventilatory

and limb muscles by Lin et al. in both the LPS and CLP models of sepsis.[101] Lin et al and Benson et al reported that sepsis-induced derangements in skeletal muscle function may be related to abnormalities in intracellular Ca^{2+} levels which can influence muscle contractility and proteolysis.[102,103]

1.6.2 Muscle Weakness

In sepsis survivors, persistent skeletal muscle weakness conditions involving both the ventilatory and limb muscles has been reported.[4,5] This acquired weakness involving the ventilatory muscles contributes to prolonged mechanical ventilation, difficulty in weaning from mechanical ventilation while limb muscle weakness contribute to exercise limitations and poor quality of life frequently seen following ICU discharge.[3-5,87,88]

1.6.3 Muscle Force Generating Capacity and Contractility

Sepsis is known to depress force generating capacity and contractility of both the ventilatory and limb muscles mainly during the early phases of disease.[5] One of the first original investigations evaluating ventilatory muscle performance during sepsis was conducted by Hussain et al. in 1985 who reported that in an LPS-induced model of sepsis in dog that diaphragm contractile dysfunction develops rapidly and leads to ventilatory failure.[89] Subsequent studies by Lanone et al. on rectus abdominis muscle biopsies have documented the first evidence of sepsis-induced muscle contractile dysfunction in humans.[91] Another study has reported that septic patients' peripheral limb muscles also develop severe decline in force generating capacity compared to healthy subjects.[92]

1.6.4 Muscle Mass

Skeletal muscle wasting is a prominent feature of sustained models of sepsis.[4,5] Several animal studies have revealed a simultaneous muscle mass decline concomitant with depressed force generating capacity in both limb and ventilatory muscles during sepsis.[93,94] Sepsis-induced skeletal muscle atrophy has been attributed mainly to elevated

protein degradation that specifically target myofibrillar proteins such as Actin and Myosin. [95] Indeed, several studies have correlated myofibrillar protein degradation with an enhanced ubiquitin proteasome activity in both animal models, and septic patients suggesting that both myofibrillar protein contents and function are equally targeted during sepsis-induced myopathy.[96-100]

1.7 Mechanisms of Skeletal Muscle Dysfunction in Sepsis

Sepsis-induced skeletal muscle dysfunction has been attributed to several mechanisms including local and circulating pro-inflammatory cytokines, disproportionate free radical generation and enhanced muscle proteolysis.[4]

1.7.1 Systemic Inflammation

Sepsis initial stages are characterized by a massive systemic inflammation whereby inflammatory mediators subsequently trigger the release of several humoral mediators systems resulting in a vasculature damage, tissue hypoxia, and eventually organ failure.[105] Cytokine release comprises a customary step in host innate immunity; yet during severe sepsis, cytokine surge trigger secondary responses which further exaggerate tissue injury and dysfunction.[5] Indeed, levels of circulating cytokines and chemokines have been used as a prognostic indicators of patient outcome in terms of organ malfunction and mortality.[105] Consequently , based on the fact that skeletal muscle cells exhibit an intrinsic ability to both generate and respond to a broad spectrum of inflammatory stimuli, pro-inflammatory mediators are likely to contribute to sepsis-induced muscle dysfunction.[106]

In vitro studies have revealed that cultured human muscle cells express pro-inflammatory cytokines such as interleukin-1 (IL-1) and interleukin-6 (IL-6) and that exposure of these cells to these cytokines provokes further release of pro-inflammatory mediators.[106,107] Interestingly, following E. coli LPS administration, the diaphragm has been shown to be more prone to inflammatory offenses as a consequence of its increased

role as a producer of pro-inflammatory cytokines.[108] The pro-inflammatory cytokines IL-6 and TNF-α have been proposed as the main mediator of muscle dysfunction in sepsis. This is based on the observation that over-expression of IL-6 in a colon cancer murine model was shown to provoke severe muscle wasting and selective diminution in MHC expression.[109] In addition, significant reduction in total muscle protein and MHC contents was detected *in vitro* cultured muscle cells upon a prolonged exposure to tumor necrosis factor (TNF-α).[110-112] Furthermore, investigations on both intact muscles and myofiber bundles indicated that relatively short exposure to TNF-α triggers significant decline in skeletal muscle contractile performance.[113,114] Taken together, these findings support a strong role for pro-inflammatory cytokines in sepsis-induced skeletal muscle wasting and weakness.[115-117]

1.7.2 Free Radical and Oxidative Stress

Free radicals comprise a diverse class of molecules containing an odd number of electrons rendering them highly reactive.[118] Within living organisms, free radicals occur as either reactive oxygen (ROS) or nitrogen (RNS) species whereby superoxide anions (O_2^-) and nitric oxide (NO) are the most biologically relevant.[119] At relatively low levels, free radicals serve as secondary messengers in signal transduction pathways that are important in normal physiological functions such as maintenance of vascular tone, and blood oxygen levels.[119] Endogenous free radicals are generated by several enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase in addition to mitochondrial electron transport chain which is the main source of ROS inside living cells. In skeletal muscle fibers, NO is produced by nitric oxide synthase (NOS) isoforms including endothelial (eNOS) and neural (nNOS) isoforms.[120]

The redox state of a cell is determined by the balance between the rate of free radical generation and the levels of antioxidants. The condition of oxidative stress develops

when ROS exceeds the antioxidant capacity.[118,121] Excessive levels of free radicals interfere with normal skeletal muscle function through direct and indirect effects on protein structure and function including enhancement of protein degradation.[119 -124]

In skeletal muscles, ROS are generated mainly inside the mitochondria as a consequence of mitochondrial oxidative phosphorylation leading to premature electron leak from the respiratory chain and the univalent interaction of the electrons with molecular oxygen.[118,119,125] Under resting condition, ROS levels are needed for the promotion of muscle contractility whereby slight upsurges in ROS levels permit myofibers to attain optimal force production.[121] However, during oxidative stress, disproportionate ROS production results in elevated free radical concentrations damaging the force generating capacity of the muscle.[121] The role of ROS in contractile depression is supported by the observations that oxygen radicals target specific contractile proteins such as MHC, MLC, Tropomyosin and Actin.[126,127] Oxidative stress has also been shown to disturb the function of several mitochondrial enzymes involved in the citric acid cycle and the respiratory chain whereby complexes I, II, and V of the respiratory chain have been shown to be predominantly disposed to functional inhibition by ROS.[128] Moreover, in addition to protein alterations, increased ROS levels have been shown to target lipid peroxidation resulting in cytotoxic byproducts such as 4-hydroxy-2-nonenal (HNE) which further depresses muscle function by targeting enzymes involved in energy metabolism.[129,130] Interestingly, oxidation markers such as protein carbonylation and HNE have been detected abundantly in skeletal muscles in septic humans and animals and implicated in oxidative damage of myofilament and mitochondrial proteins.[127,130]

The involvement of ROS and RNS in sepsis-induced myopathy is supported by several studies in which antioxidant administration has been shown to avert muscle contractile dysfunction.[131-134] For instance, one such study aimed at comparing diaphragm

contractility of saline and endotoxin-injected hamsters in either the presence or absence of the antioxidant polyethylene glycol-superoxide dismutase (PEG-SOD) whereby despite the decline in diaphragmatic force generation induced by the endotoxin administration, synchronized antioxidant administration prohibited the decline in contractile function in septic animals.[131] Subsequent investigations defined the mechanism by which ROS and RNS influence contractile function in sepsis. Callahan *et al* employed skinned single fibers for the evaluation of ROS ability to target myofilament protein function.[135] These authors reported that endotoxin administration in conjugation with either a NOS inhibitor or PEG-SOD disallowed sepsis-induced diminutions in the inherent force generating capacity of the actomyosin complex.[135] In addition to functional deficiency at the myofilament level, ROS and RNS elicit structural modifications that render proteins more vulnerable to proteolytic degradation.[119] Furthermore, there is evidence that ROS and RNS activate the caspases and calpain proteolytic systems which constitute fundamental proteases for sarcomeric protein degradation in sepsis.[136,138]

1.7.3 Proteolysis

Muscle wasting is an important manifestation of a broad spectrum of catabolic and disease states including sepsis, cancer, fasting, and immobilization.[139] In healthy individuals skeletal muscle mass is determined by the balance between protein synthesis rates and protein degradation rates. In catabolic conditions, the rate of protein degradation exceeds the rate of protein synthesis leading to augmented skeletal muscle proteolysis. [140] Enhanced skeletal muscle proteolysis can be beneficial during early phases of sepsis and trauma whereby free amino acids provide other tissues with essential substrates for the sustenance of vital cellular processes.[141] However, prolonged muscle protein degradation results in detrimental outcomes whereby proteolytic processes target contractile proteins thereby contributing to the establishment of skeletal muscle weakness.[141-143] Various

components of the ubiquitin-proteasome pathway, including muscle specific E3 ligases and the 20S proteasome subunit, have been shown to be up-regulated in the skeletal muscle of animal models and patients with systemic inflammation including.[139,142,144]

1.8 Aims of This Study

Despite the recent progress in elucidating various aspects of skeletal muscle dysfunction in sepsis, there are several essential questions remain unanswered in this respect. For instance, it is unclear whether the degree of muscle atrophy that develops during the course of sepsis is similar among the ventilatory and limb muscles. In the majority of published studies, investigators have focused on measuring atrophy and contractile function in one limb or ventilatory muscle and it is unclear whether ventilatory muscles are affected by sepsis to a similar degree in terms of muscle atrophy to limb muscles. The first objective of the current study is to document the degree and the time course of muscle atrophy in the ventilatory and limb muscles in response to sepsis using an animal model that simulates human sepsis. To achieve this objective, we studied the diaphragm (DIA) as a representative of the ventilatory muscles and tibialis anterior (TA) as a representative of limb muscles. Fiber size of these muscles was monitored after 24, 48 and 96h of initiation of sepsis in a mouse model of CLP. The second objective of the current study is to assess the time course of skeletal muscle contents of various myofilament proteins. In previous studies, the main focus was to evaluate the expression of Myosin Heavy Chain and Actin as the main components of the contractile machinery. There is essentially no information whether the expression of other important regulatory myofilament proteins is affected by sepsis. To achieve this objective, we monitored protein levels of Myosin Heavy Chain, sarcomeric Actin, Myosin Light Chain, Troponin-T, Troponin-C, Troponin-I and Tropomyosin in the DIA and TA during the course of CLP-induced sepsis. Our third objective is to assess whether changes in skeletal muscle

contents of myofilament proteins during the course of sepsis is mediated in part by alterations in the transcription of myofilament genes. Previous studies have evaluated protein levels of Myosin Heavy Chain and Actin in the ventilatory and limb muscles in sepsis but provided no information whether changes in the levels of these proteins is solely the result of enhanced proteolysis and/or the result of decreased mRNA expression. To achieve this objective, we measured mRNA expression of Myosin Heavy Chain, sarcomeric Actin, Myosin Light Chain, Troponin-T, Troponin-C, Troponin-I, Tropomyosin in the DIA and TA during the course of CLP-induced sepsis.

SECTION 2: MATERIALS AND METHODS

2.1 Materials: Primary antibodies selective to Tropomyosin, Troponin-T, and α–Sarcomeric Actin were purchased from Sigma-Aldrich (Oakville,ON). Antibodies selective to Myosin Heavy and Light Chain isoforms (S21, MF20, 10F5, 2F7, MF5, and F310) were obtained from Developmental Studies Hybridoma Bank (Iowa,USA). Skeletal Troponin-I and Troponin-C antibodies were acquired from ThermoFisher Scientific (Rockford, IL).

2.2 Animal Protocols: Approval of the study was attained from the committee of animal experiments at McGill University and in accordance with the guidelines of the Canadian Council of Animal Care.

Cecal Ligation and Perforation: Adult male C57/BL6 mice (7-8 weeks) were anesthetized with isoflurane (3.5% to 4.5%) and 1 L/min O_2 flow. Mice were then subjected to a midline laparotomy whereby the cecum was cautiously isolated, ligated with a 4-0 silk tie at approximately 1 cm distance from the cecal tip and subsequently punctured with a 26 gauge needle. The puncture was made in a single pass through and through both sides of the bowel wall followed by the permeation of a trivial amount of fecal material into the peritoneal cavity. The abdominal cavity was then sealed in two layers using two 3-0 absorbable polyfilament interrupted sutures. Buprenorphine (0.05 to 0.2mg/kg of in 0.9% saline) was then administered s.c. either every 24h or upon requirement. Sham-operated mice were exposed to an identical procedure with the exception of cecum ligation and puncture. Mice were then sacrificed at time points coinciding with 24, 48 and 96h after the surgical procedure followed by the rapid excision of the diaphragm (DIA) and tibialis anterior (TA) muscles. Muscles were immediately frozen in liquid nitrogen and stored at -

80ºC for subsequent mRNA measurement and immunoblotting or immersed in cooled in isopentane and prepared for immunohistochemistry.

2.3 Immunohistochemistry and myofiber diameter measurements: Muscles frozen in liquid nitrogen-cooled isopentane were sectioned (10 μm cross-sections), fixed in 2% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 20 min and permeabilized in 0.2% Triton/PBS for 10 min. Sections were then washed with 100 mM Glycine/PBS, blocked for 30 min in blocking solution (PBS with 2% BSA, 0.2% Triton , and 0.05% Tween) and incubated with primary antibody selective to Laminin ((L9393, Sigma, 1:750) overnight at 4ºC. Sections were then washed three times in PBS/0.2% Triton/0.05% Tween and incubated with appropriate Alexa Fluor secondary antibody for 60 min at room temperature. Following three washes in PBS/0.2% Triton/0.05% Tween, samples were then counterstained with PBS containing 0.5 ng/ml of 4', 6-Diamino-2-Phenylindole for 5 min at room temperature, rinsed twice with double distilled water and finally mounted with aqueous based mounting medium (Aqua-mount Lerner Laboratories, or Immu-Mount Thermo Scienific). Images of DIA and TA cross-sections from five different mice per group were captured with Olympus 1X70 microscope at 20x magnification. Minimum Ferret diameters of a minimum of 600 fibers per muscle for DIA and 400 fibers per muscle for TA were measured from non-overlapping fields using Image J software (National Institutes of Health).

2.4 Immunoblotting: Frozen DIA and TA samples were homogenized in homogenization buffer (10 mM tris-maleate, 3 mM EDTA, 275 mM sucrose, 0.1 mM DTT, 2 μg/ml aprotinin, and 1 mg/100ml pepstatin A, pH7.2). Samples were then centrifuged at 5000 rpm for 10 min 4ºC whereby pellets were discarded and supernatants designated as crude homogenate. Total muscle protein levels corresponding to each sample were then determined using the Bradford protein assay technique. Crude homogenate samples (25-

50 μg/sample) were then mixed with SDS sample buffer boiled for 8 min at 95ºC and loaded onto tris-glycine sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) for electrophoresis separation. Proteins were then transferred by electrophoresis to polyvinylidene difluoride (PVDF) membranes and blocked with either 1% bovine serum albumin or milk for 1h at room temperature. PVDF membranes were subsequently incubated overnight at 4ºC with each of the primary antibodies of interest. Troponin-C (TNNC), Tropnin-I (TNNI) and Troponin-T (TNNT) proteins were detected at 8, 25 and 38 kDa, respectively. For the detection of Myosin Light Chain (MYL) isoforms, we used MF5 antibody to detect MYL isoform 2 (fast), F310 antibody for diction of MYLisoform 3 (fast, 21 kDa) and S21 antibody for the detection of MYL isoform 3 (slow, 64 kDa). Tropomyosin (TPM) was detected at 36 to 50 kDa while α–Sarcomeric Actin was detected at about 50 kDa. For the detection of total Myosin Heavy Chain protein, we used MF20 antibody. 10F5 and 2F7 antibodies were used to detect Myosin Heavy Chain type IIB and type IIA, respectively. After the incubation with primary antibodies, PVDF membranes were washed and re-incubated with horseradish peroxidase-conjugated secondary antibody for 1.5h at room temperature. Finally, specific proteins were detected via an enhanced chemiluminescence kit (ECL)(Millipore, Billerica, MA). Equal loading of proteins was confirmed by stripping each membrane and re-probing with anti-STAT3 antibody. In addition, PVDF membranes were stained with Coomassie Blue to confirm equal protein loading. Blots were then scanned with an imaging densitometer whereby the optical densities (OD) of relative protein bands were quantified using Gel-Pro Analyzer software (Media Cybernetics Inc., Rockville MD).

2.5 RNA extraction and Real-Time PCR: Total RNA was extracted from DIA and TA muscles via the employment of the GenElute^{TM} Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Oakville, ON). Quantification and purity assessment of the total RNA

were then performed by A260/A280 absorption approach. Total RNA (2 μg) was then reverse transcribed using a Superscript II Reverse Transcriptase Kit and random primers (Invitrogen Canada, Inc., Burlington, ON). Reactions were then incubated at 42ºC for 50 min and subsequently at 90ºC for 5 min. Real-time PCR detection of mRNA expression was accomplished using a Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Specific primers for various isoforms of Myosin Heavy Chain (*Myh*), Myosin Light Chain (*Myl*), α-*Actin*, Tropomyosin (*Tpm*), Troponin-T (*Tnnt*), Troponin-C (*Tnnc*), and Troponin-I (*Tnni*) are listed in Table 1. We also detected mRNA levels of two muscle-specific E3 ligases (Atrogin-1 and MuRF1) using specific primers (Table 1). *β-Actin* served as an endogenous control transcript. One μl of reverse-transcriptase reagent was added to 25 μl of SYBR Green (Qiagen Inc, Valencia, CA) master mix and 3.5 μl of each of 10 μM primers. The thermal profile was set as follows: 95ºC for 10 min; 40 cycles each of 95ºC for 15s; 57ºC for 30s; and 72ºC for 33s. All real-time PCR experiments were performed in triplicate. A melt curve analysis for each PCR experiment was accomplished in order to evaluate primer-dimer formation or presence of contamination. Cycle threshold (C_T) values were obtained and quantification of each target gene relative mRNA level was determined using the threshold cycle ($\Delta\Delta C_T$) method.

2.6 Statistical analyses: Statistical analysis of the data was performed with SAS Statistical package (SAS Institute, Cary, NC) and Sigma Stat software. Comparisons between the Sham and CLP groups at a given time point (24, 48 and 96h after completion of the surgical procedures) were performed with One-Way Analysis of Variance followed by a Tukey post hoc test. A p value <0.05 was considered as significant. Data are expressed as means ± standard errors of the means. For each group (Sham and CLP groups at 24, 48 and 96 post-surgical procedure), six or five animals were used to detect protein and mRNA levels of a given myofilament gene.

SECTION 3: RESULTS

3.1 Changes in myofiber diameter: Myofiber diameter was measured in the DIA and TA muscles of the sham and the CLP groups in order to quantify sepsis-induced muscle atrophy. In the DIA, myofiber diameter was significantly decreased after 24h of CLP-induced sepsis, as compared to the sham group; however, no significant differences in DIA myofiber diameter were observed between the CLP and sham groups at 48h and 96h (Figure 1A). In the TA, CLP-induced sepsis also elicited a significant decrease in myofiber diameter after 24h, 48 and 96h following the CLP procedure (Figure 1B). These findings demonstrate that CLP-induced sepsis produces transient atrophy in the DIA and persistent atrophy in the TA.

3.2 Expression of muscle-specific E3 ligases: Figure 2 illustrates changes in mRNA expression of muscle-specific E3 ligases, *Atrogin-1* (*Fbox32*) and *MuRF1* (*Trim36*) in the DIA and TA in the CLP groups. In the DIA, *Atrogin-1* and *MuRF1* mRNA expression increased significantly after 24 and 96h but not after 48h of CLP-induced sepsis compared to the Sham group. In the TA, *Atrogin-1* and *MuRF1* mRNA expression increased significantly after 24, 48 and 96h of CLP-induced sepsis compared to the Sham group (Figure 2). The relative rise in *Atrogin-1* and *MuRF-1* mRNA expression in the TA was larger than that observed in the DIA (Figure 2).

3.3 Changes in Myosin Heavy Chain and Sarcomeric Actin:

Diaphragm: To investigate the effects of sepsis on Myosin Heavy Chain and Sarcomeric Actin expressions, we detected mRNA levels of *Myh1* (type IIx), *Myh2* (type IIa), *Myh4* (type IIb), *Myh7* (type I) and α-*Actin* in the DIA and TA using real-time PCR. In the DIA, mRNA levels of *Myh2* declined significantly after 24h of CLP-induced sepsis compared to the Sham group while those of *Myh1*, *Myh4* and *Myh7* remained unchanged (Figure 3). After 48 and 96h of CLP-induced sepsis, mRNA levels of the four isoforms of *Myh*

declined significantly compared to the Sham group (Figure 3). *α-Actin* mRNA levels were not altered by CLP-induced sepsis at the three time points (Figure 3). To investigate whether these changes in mRNA levels of *Myh* isoforms and *α-Actin* were associated with alterations in protein levels, we used immunoblotting and antibodies that detect total MYHC, MYHCIIA, IIB and α-ACTIN. Protein levels of total MYHC, type IIA and type IIB declined significantly after 24, 48 and 96h of CLP-induced sepsis as compared to the Sham group (Figure 4). α-ACTIN protein levels declined significantly only after 96h of CLP-induced sepsis compared to the Sham group (Figure 4).

Tibialis Anterior: In the TA, mRNA levels of *Myh1* declined significantly after 24h ad 48h and those of *Myh2*, *Myh4* and *Myh7* declined significantly only after 24h of CLP-induced sepsis compared to the Sham group (Figure 5). After 96h of CLP-induced sepsis, mRNA levels of the *α-Actin* mRNA levels were not altered by CLP-induced sepsis at the three time points (Figure 5). Immunoblotting revealed that protein levels of MYHC, type IIA and type IIB declined significantly after 24h and 96h but not at 48h of CLP-induced sepsis as compared to the Sham group (Figure 6). α-ACTIN protein levels in the CLP group remained similar to those detected in the Sham (Figure 6).

3.4 Changes in Myosin Light Chain isoforms:

Diaphragm: Detection of mRNA of Myosin Light Chain isoforms 1(*Myl1-1f* and *Myl1-3f*) and 3 (*Myl3-1s*) using real-time PCR revealed significant decrease in the levels of these isoforms after 48 and 96h but not after 24h of CLP-induced sepsis compared to the Sham group (Figure 7). Immunoblotting with antibodies selective to MYL1 (f), MYL2 (f) and MYL3(s) proteins revealed significant decreases in their levels after 24, 48 and 96h of CLP-induced sepsis compared to the Sham group (Figure 8).

Tibialis Anterior: Figure 9 illustrates that mRNA levels of *Myl1-1f*, *Myl1-3f* and *Myl3-1s* in the TA declined significantly after 24, 48 and 96h of CLP-induced sepsis as compared to

the Sham group except for *Myl3-1s* after 96h of sepsis (Figure 9). Immunoblotting with antibodies selective to Myosin Light Chain isoforms proteins revealed a similar pattern to that of mRNA of these isoforms with the MYL1, MYL2 and MYL3 declining significantly after 24, 48 and 96h of CLP-induced sepsis compared to the Sham group except for MYL3 after 96h of sepsis (Figure 10).

3.5 Changes in Troponins and Tropomyosin:

Diaphragm: We detected the mRNA levels of the fast and slow isoforms of Troponin-T (*Tnnt*), Troponin-I (*Tnni*), and Troponin-C (*Tnnc*) using real-time PCR. Expression of the fast and slow isoforms of *Tnnt* declined significantly after 24 and 48h but not after 96h of sepsis (Figure 11). The expression of the fast and slow isoforms of *Tnni* and slow isoform of *Tnnc* declined significantly only after 48h of sepsis while the expression of the fast isoform of *Tnnc* declined significantly after 24, 48 and 96h of sepsis (Figure 11). Figure 12 shows mRNA expression of Tropomyosin 1α (*Tmp-1*), 2β(*Tpm-2*) and 3γ(*Tpm-3*) in the DIA of the CLP and Sham groups. The expression of these isoforms of Tropomyosin declined significantly only after 48h of CLP-induced sepsis compared to the Sham group (Figure 12). Figure 13 illustrates the changes in protein levels of Troponins and Tropomyosin isoforms in the DIA of the Sham and the CLP groups. TNNT protein levels declined significantly after 24 and 48h but not after 96h of CLP-induced sepsis compared to the Sham group (Figure 13). Protein levels of TNNI, TNNC and TPM declined significantly in the CLP group only after 48h of sepsis compared to the Sham group (Figure 13).

Tibialis Anterior: Figure 14 illustrates the changes in mRNA levels of Troponins in the TA of the Sham and the CLP groups. Expression of the fast and slow isoforms of *Tnnt* declined significantly after 24 and 48h but not after 96h of sepsis (Figure 13). The expression of the fast and slow isoforms of *Tnnc* declined significantly only after 24h of

sepsis while the expression of the fast and slow isoforms of *Tnni* remained unchanged in the CLP group compared to the Sham group (Figure 14). Figure 15 shows mRNA expression of *Tpm-1*, *Tpm-2* and *Tpm-3* isoforms in the TA of the Sham and the CLP groups. The expression of these isoforms declined significantly after 24, 48 and 96h of sepsis except for *Tpm-2* and *Tpm-3* where their levels at 96h of sepsis remained similar to the Sham group (Figure 15). Figure 16 illustrates the changes in protein levels of Troponins and Tropomyosin isoforms in the TA of the Sham and the CLP groups. TNNT and TPM protein levels declined significantly after 24 and 48h but not after 96h of CLP-induced sepsis compared to the Sham group (Figure 16). Protein levels of TNNC declined significantly in the CLP group only after 24h of sepsis compared to the Sham group (Figure 16). There was no effect of CLP-induced sepsis on TNNI expression in the TA (Figure 16).

SECTION 4: DISCUSSION

The main findings of this study are: 1) CLP-induced sepsis triggers significant atrophy of DIA and TA fibers in mice. The time course of atrophy in the two muscles differs with DIA atrophy developing only after 24h of sepsis while TA showed significant fiber atrophy after 24, 48 and 96h of sepsis; 2) Sepsis elicits significant and differential decreases in mRNA and protein levels of various myofilament proteins in the DIA and TA; however, the time course and the sensitivity to sepsis-induced decline in expression differ among various myofilament proteins. α-ACTIN and TNNI appear to be less sensitive to the inhibitory effects of sepsis while mRNA and protein levels of MYHC, MYL, TNNT, TNNC and TPM are strongly affected by sepsis; 3) Reduction of MYHC and MYL protein contents persists even after 96h of sepsis while the decline in TNNT, TNNI, TNNC and TPM mRNA and protein levels in the DIA and TA is limited to 24 and 48h after the induction of sepsis.

Skeletal Muscle Atrophy in Sepsis: Loss of skeletal muscle mass is a common observation in patients with sepsis.[145] The clinical implications of muscle atrophy include weakness and fatigue as well prolonged hospitalization and rehabilitation. When muscle atrophy involves the diaphragm, the consequences are even more severe and include prolonged periods of ventilatory support and difficulty in weaning from mechanical ventilation. Our animal model of sepsis (CLP) reproduces skeletal muscle atrophy feature of human sepsis as indicated by significant fiber atrophy in the DIA and TA; however, our study reveals that the time course and the severity of fiber atrophy differ among these two skeletal muscles. While fiber atrophy in the DIA is transient (detectable only after 24h) and relatively mild in nature, more severe atrophy develops in the TA that persist even after 96h of sepsis (Figure 1). To understand these differences in the development of fiber atrophy between the DIA and TA muscles, one needs to identify the precise mechanisms

behind the development of fiber atrophy. It has been well established that skeletal muscle mass is determined to a large extent by the balance between protein synthesis and degradation. Sepsis is a catabolic disease state that is associated with significant increase in proteolysis inside skeletal muscles which precipitates skeletal muscle atrophy if sustained for prolonged periods of time.[146] To determine whether enhanced protein degradation is involved in the development of fiber atrophy in the DIA and TA, we assessed the expression of two muscle-specific E3 ligases (*Atrogin-1* and *MuRF1*) that are known to be important in promoting myofilament protein ubiquitin conjugation and enhanced degradation by the 26S proteasome system. We found that *Atrogin-1* and *MuRF1* mRNA levels are significantly upregulated in the DIA and TA in the CLP groups (Figure 2).This finding suggests that protein ubiquitination and degradation by the 26S proteasome is significantly enhanced in skeletal muscles of septic animals, an observation which is in accordance with previous studies that documented enhanced protein degradation in septic humans and animals.[147,148] Interestingly, we found that the relative degree of *Atrogin-1* and *MuRF1* induction in TA of septic animals is greater than that of the DIA (Figure 2), an observation that may explain the relatively more severe fiber atrophy in the TA compared to the DIA (Figure 1). We also observed that time course of *Atrogin-1* and *MuRF1* mRNA induction occurs in a biphasic manner in response to sepsis, where an early induction after 24h of sepsis is followed by a relative decline in expression after 48h and a second phase of induction evident after 96h of sepsis (Figure 2). The mechanisms behind this biphasic changes in *Atrogin-1* and *MuRF1* expression remain unclear. We speculate that these muscle-specific E3 ligases catalyze their own ubiquitination[149] and consequently, it is possible that the initial rise in *Atrogin-1* and *MuRF1* expression (24h of sepsis) could have resulted in enhanced ubiquitination of these proteins and reduction in their activities and

that this development will require a second round of increased transcription of these E3 ligases (96h of sepsis).

In the current study, we provide several novel observations regarding skeletal myofilament protein expression in sepsis. First, reduction in skeletal muscle myofilament protein contents is observed as early as 24h and is evident event after 96h of sepsis. Reduced protein contents not only involve MYHC as previous studies have suggested but also include MYL, TNNT, TNNC, TNNI and TPM. In the current study, we detected total and isoforms IIA and IIB MYHC protein levels using immunoblotting with selective antibodies. We are unable to find an antibody that selectively and reliably detects MYHC type I isoform. Our finding of significant decline in MYHC protein levels in the DIA and TA of septic animals is in accordance with previous studies in septic rats and mice.[145,151] The fact that we observed significant reduction in MYHC protein levels in the DIA after 48 and 96h of sepsis despite the fact that fiber atrophy is no longer detectable in the DIA at these time points (Figures 1 and 4) suggests that significant reduction in myofilament protein content may be present even though muscle fiber size may appear to be normal.

Skeletal Muscle Protein breakdown: Our observation of significant reduction in myofilament protein contents in the DIA and TA can be attributed to increased breakdown of these proteins. As discussed above, the induction of *Atrogin-1* and *MuRF1* expression in the DIA and TA of septic animals indicate that protein ubiquitination is activated in septic muscles and that proteasome proteolytic pathway is responsible for myofilament protein degradation; however, there is evidence that the proteasome is not capable of degrading intact myofibrils. Indeed, incubation of ovine skeletal muscle with pure proteasome didn't elicit myofibrillar breakdown.[152] Similarly, isolated skeletal muscle proteasome has been shown to degrade free Myosin and Actin but was incapable of degrading intact myofibrils.[153] These observations suggest that myofibrils are degraded initially by other

proteolytic systems such as calpains and caspases and that the released myofilament proteins are then degraded by the proteasome. This was in fact observed by Williams et al.[48] who found significant increase in the release of myofilament proteins from skeletal muscles of septic rats and that this release was Ca^{2+}-dependent and involves mainly muscles rich in fast-twitch muscle fibers. These authors concluded that release of myofilament protein is an initial and rate-limiting component of sepsis-induced muscle proteolysis. Additional studies have provided indirect and direct evidence of increased calpain activity and expression in septic skeletal muscles. For instance, Fischer and colleagues[155] reported increased calpain gene expression which was associated with increased proteolysis in septic rats. Williams *et al.*[149] also provided indirect evidence of enhanced calpain activity in septic skeletal muscles in rats. Direct evidence of increased calpain activity and expression in skeletal muscle during sepsis was described by several authors. Bhattacharyya *et al.*[156] reported that calpain activity as determined by measuring the degradation of the calpain substrate azocasein, rose by about 70% in septic rats. In a second study, Voisin *et al.*[57]reported that 6 days after i.v. injection of live *E. coli* bacteria, significant increase m-calpain mRNA expression was observed. Furthermore, Wei *et al.* [58] found that the degradation of different calpain-specific substrates was increased in skeletal muscles of septic rats suggesting that sepsis triggers significant increase in skeletal muscle calpain activity. Interestingly, the increase in this activity appears to be not mediated by actual increase in μ- and m-calpain activity but by inhibition of calpastatin activity. Supinski and Callahan[138] in a more recent study documented a significant increase in calpain activity, protein levels of active μ- and m-calpains in the DIA of septic mice. To examine the importance of calpain activation in skeletal muscle dysfunction in sepsis, these authors administered calpain inhibitor and reported that this inhibitor prevented sepsis-induced diaphragm contractile dysfunction.[138]

Another proteolytic pathway that is likely to be involved in the release of myofilament proteins from intact myofibrils in sepsis is caspase-3. Hotchkiss and colleagues[158] were the first to document increased caspase-3 activity in the tissues of humans with severe sepsis. Du et al.[159] confirmed that caspase-3 is capable of degrading actomyosin and that limited actomyosin cleavage by caspase-3 stimulates myofilament protein degradation by the proteasome. Supinski and colleagues [60,138] reported that *E. coli* LPS administration markedly increases caspase-3 activation in the diaphragm and that administration of a broad-spectrum caspase inhibitor or selective caspase-3 inhibitor attenuated the effects of LPS-induced sepsis on diaphragm force generation.

The fourth pathway that is responsible for protein and organelle recycling in skeletal muscles is the autophagy pathway. Autophagy is an evolutionarily conserved process by which cells can break down and recycle long-lived proteins, organelles and bulk portions of cytoplasm.[161] This pathway involves a dual mechanism whereby cytosolic cargo such as proteins and organelles is first delivered to the lysosomal lumen, after which it is degraded by lysosomal enzymes. Although autophagy has been described in all cells, its role in skeletal muscle protein degradation has largely been ignored. Recent work has demonstrated that basal autophagy is critical to muscle homeostasis, since it is responsible for the removal of protein aggregates and damaged mitochondria.[162] The kinetics of autophagy is different in skeletal muscles under stress as compared to other tissues. Most tissues undergo transient induction of autophagy in response to stress stimuli and the process only lasts for a few hours. In contrast, persistent generation of autophagosomes continues for days in skeletal muscles.[163] This prolonged induction is made possible by a unique FoxO transcriptional program that works to replenish the short-lived proteins that are required for autophagosome-lysosome fusion.[164] In catabolic conditions, Sandri and colleagues have shown that stimulation of mitochondrial fission and remodelling induces

autophagy, activates the proteasomal pathway, and causes muscle atrophy.[165] They also reported that BNIP3 is an important inducer of mitochondrial autophagy downstream from FoxO3a.[164] Taken together, these results indicate that mitochondria are both a trigger for, and a target of, autophagy in skeletal muscles. So, although there has been some progress in understanding the role of autophagy in skeletal muscle proteolysis, its contributions to sepsis-induced skeletal muscle protein degradation remain unclear. In our recent study, we documented that autophagy is significantly induced in the DIA and limb muscles of mice injected with *E. coli* LPS and that this induction was associated with significant morphological and functional mitochondrial dysfunctions. Interestingly, we found that the degree of autophagy induction and the extent of mitochondrial morphological injury are relatively milder in the DIA as compared to limb muscles. Despite the documentation of enhanced autophagy in skeletal muscles of septic animals, it remains unclear whether autophagy is involved in the degradation of myofilament proteins and additional studies are need to elucidate the relationship between the induction of proteasome, calpain and caspase proteolytic systems and the upregulation of autophagy in septic skeletal muscles.

Regulation of Myofilament Gene Expression: Despite increasing interest in identifying the mechanisms of muscle atrophy in sepsis, investigators have so far focused on increased myofilament protein degradation as the main mechanism behind loss of skeletal muscle mass in septic humans and animals. However, our study reveals that reduced myofilament protein contents in septic skeletal muscles may not be entirely due to increased degradation. Indeed, we found mRNA expression of several myofilament genes with the exception of *α-Actin* is significantly attenuated in the DIA and TA during the course of CLP-induced sepsis and that this attenuation coincides with reduced myofilament protein contents. This observation implies that the reduction in myofilament protein levels in septic skeletal muscles may be mediated in part by decreased synthesis as a result of

decreased transcription of myofilament genes. The mechanisms responsible for the downregulation of myofilament mRNA expression in septic muscles remain unclear. We speculate that pro-inflammatory cytokines including tumor necrosis factor (TNF-α) and interferon gamma (IFN) which are upregulated in septic skeletal muscles are involved in the downregulation of myofilament mRNA expression particularly that of Myosin Heavy Chain isoforms. This speculation is based on in-vitro studies in cultured C2C12 cells in which exposure to a combination of TNF-α and IFN results in significant downregulation of *Myh* mRNA without affecting the expression of other myofilament genes.[166] In-vivo administration of TNF-α has also been shown to trigger significant downregulation of skeletal muscle *Myh* mRNA expression.[166] This inhibitory effect of TNF-α and IFN on *Myh* mRNA expression is believed to be mediated through activation of the NFκB transcription factor which selectively inhibits the expression of the myocytes regulatory factor MyoD.[167] MyoD is a positive regulator of *Myh* mRNA expression.[168] The transcription of other myofilament genes including *Tnni*, *Tnnc*, *Tnnt* and *Tpm* are regulated by several transcription factors including myocytes regulatory factors (MyoD, Myogenin, Myf5), Pax3, Pax7, Myocyte Enhancer Factors (MEF2), nuclear factor of activated T cells (NFAT), JunB and Serum Response Factor (SRF).[169-171] It is possible that downregulation of myofilament gene expression in septic skeletal muscles is mediated through selective inhibition and/or enhanced degradation of the above-described transcription factors. It is also possible that alterations in microRNA (miRNA) expression also contribute to the downregulation of myofilament gene expression in septic muscles. Several miRNAs including miR-1, miR-133, miR-206 and miR-208 are selectively expressed in skeletal muscle fiber.[169] These myo-specific miRNAs provide additional epigenetic control mechanisms regulating the expression of myofilament genes and alterations in the expression of these miRNAs may contribute to the observed downregulation of

myofilament gene expression in septic muscles. Clearly, additional studies are needed to further explore the molecular mechanisms behind the distinct transcriptional regulation of myofilament genes during the course of sepsis.

In addition to mRNA transcription, myofilament protein synthesis is also regulated at the post-transcriptional level through the balance between two opposing pathways; the Insulin Growth Factor like 1 (IGF)/AKT/mTOR pathway acting as a positive regulator, and the myostatin/Smad2-3 pathway acting as a negative regulator.[172] Protein kinase B (AKT) is activated downstream from the PI-3 kinase pathway which, in turn, is activated downstream from IGF1 and insulin receptor.[172] AKT regulates protein synthesis primarily through activation of complex 1 of the mammalian target of rapamycin (mTORC1). mTORC1 activates protein translation through phosphorylation of the ribosomal protein S6 kinases (S6K1 and 2) and inhibition of 4E-BP1 (repressor of the cap-binding protein eIF4E).[172] In addition to promoting protein synthesis, both the AKT and mTORC1 pathways inhibit protein breakdown through inhibition of the transcription factors FOXOs which are required for the transcription of Atrogin-1, MuRF1 and several autophagy-related genes.[173,174] The mTORC1 pathway also inhibits autophagy through selective phosphorylation-dependent inhibition of ULK1 protein activity which is critical for the initiation of autophagosome formation.[156] In the current study, it is possible that the observed decline in myofilament protein contents in the DIA and TA in septic mice is mediated in part through inhibition of the AKT and mTORC1 pathways and that inhibition of these two pathways results in both attenuation of myofilament protein synthesis and activation of proteasome and autophagy proteolytic pathways. Our recent findings of significant attenuation of both AKT phosphorylation and mTORC1 activity in the DIA and TA of septic mice lend further credence to this proposal.[175]

Regulation of Skeletal Muscle Contractility in Sepsis: Two main mechanisms have been proposed to explain sepsis-induced skeletal contractile muscle dysfunction, namely, oxidative stress and increased protein degradation.

Reactive oxygen species, including O_2^- , H_2O_2, and HO^-, are produced at relatively low rates in resting muscle fibres.[176-181] Increased ROS levels have been extensively documented in the ventilatory and limb muscles of humans and animals with sepsis.[182-186,188] Increases in ROS production and/or decreases in antioxidant levels result in accumulation of ROS and the development of oxidative stress. Oxidative stress alters skeletal muscles in sepsis by inducing mitochondrial dysfunction[153] and by modifying critical proteins such as creatine kinase and myosin, increasing their degradation and inhibiting their activity.[191,192,194] Moreover, there is strong evidence that oxidative stress in septic muscles enhances protein degradation by stimulating calpains, caspases, and proteasomes.[60,138,195] Several authors have shown that pre-treatment of septic animals with antioxidant enzymes and free radical scavengers reduces oxidative stress and improves muscle function.[186,187,197]

It has been well established that skeletal muscle atrophy, or loss of muscle mass, develops in sepsis and that it is mediated in part by increased protein degradation. For example, in septic rats, total protein and myofibrillar protein degradation rose by 50% and 440%, respectively.[98] This observation was associated with upregulation of protein ubiquitination and enhanced activity of the 20S proteasome in the DIA and limb muscles.[196,198,199] Decreased contractile function, increased protein degradation, and muscle atrophy have also been reported in the ventilatory and limb muscles of LPS- and CLP-treated animals.[201-203] Taken together, these observations provide strong evidence that contractile dysfunction, increased protein degradation, and atrophy are important manifestations of sepsis-induced muscle dysfunction. Despite this strong documentation of

muscle atrophy in sepsis, investigators have played down the role of loss of muscle mass as a cause of contractile dysfunction in sepsis primarily because specific force (normalized per cross sectional areas) generation by skeletal muscles is significantly reduced along with reduction in absolute force generation.[4] The current study reveals several importance observations that challenge this view and emphasize the importance of reduction in myofilament protein contents as an important mechanism of impaired skeletal muscle contractile function in sepsis. Firs, the timing of significant reduction in the expression of several myofilament proteins (first observed after 24h of CLP-induced sepsis) coincides with significant reduction in the contractility of the DIA and limb muscles.[138] Second, reduction in myofilament protein levels occurs even when muscle fiber size remains normal (as in the case of the DIA after 48 and 96h of CLP-induced sepsis). This observation indicates that normalization of muscle isometric force generation per cross sectional areas doesn't exclude the possible contribution of low myofilament protein contents to the decline in force generation in septic muscles. Third, sepsis triggers significant decline not only in MYHC protein levels but also in the levels of other myofilament proteins including MYL, TNNT, TNNC and TPM. This observation implies that impaired skeletal muscle force generation in sepsis may be caused by reduced myofilament protein contents involving not only Myosin and Actin but also the regulatory myofilament proteins such as TPM and Troponins. Reduced MYHC protein content is likely to contribute to decreased muscle force generation as a result of reduced total number of Myosin crossbridges. TPM is a dimeric coiled-coil Actin-binding protein that acts as a "gatekeeper" of thin filament Ca^{2+} activation and is an important regulator of active force generation in striated sarcomeres. Significant reduction in the levels of TPM after 24 and 48h of sepsis in TA and after 48h of sepsis in the DIA of septic animals is expected to significantly impair Myosin-Actin interaction in these muscles. We also found

that TNNT and TNNC contents are significantly attenuated in the DIA and TA of septic animals (Figures 13 and 16). Troponin complex is composed of three subunits which interact strongly with each other: the Ca^{2+} binding TNNC, the inhibitory TNNI and the TPM-binding TNNT.[204]. Under basal intracellular Ca^{2+} levels, the interaction between Myosin and Actin is inhibited. Upon Ca^{2+} release from the sarcoplasmic reticulum, Ca^{2+} binds to specific sites of TNNC leading to the exposure of hydrophobic residues on this protein and the promotion of strong interactions between TNNC, TNNI and TNNT and the translocation of Troponin-Tropomyosin complex away from Myosin-binding sites on Actin resulting eventually in the cyclic interaction between Myosin heads and Actin.[204] TNNI is the inhibitory subunit of the Troponin complex responsible for inhibiting Myosin Mg^{2+}-ATPase activity. The binding of Ca^{2+} to TNNC improves the interaction of TNNC with TNNI resulting in the dissociation of TNNI from Actin and thereby enabling the interaction between Myosin and Actin. Reduced TNNT, TNNC and TNNI protein contents in the DIA and TA of septic animals (Figures 13 and 16) is expected to disrupt the interactions between Troponin complex and TPM and to the reduction of Ca^{2+} sensitivity of the contractile myofilaments leading eventually to impairment of Myosin-Actin interaction and depressed active force generation by the sarcomeres.

In summary, our study indicates that sepsis triggers significant fiber atrophy which is transient in the DIA but more severe and persistent in the TA. We also found that sepsis is associated with significant reduction in the contents of various myofilament proteins of the DIA and TA and that this reduction is mediated through downregulation of myofilament gene expression, inhibition of myofilament protein synthesis and enhanced degradation of these proteins by proteasome and possibly by the calpain and caspase-3 systems.

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SECTION 6:TABLE1

Table 1: Primers used for real-time PCR experiments to detect mRNA expression of various myofilament genes and E3 ligases in the DIA and TA of the Sham and the CLP groups

Name	Primers	Accession #
Myosin heavy polypeptide 1 (*Myh1*)	F-GCGAATCGAGGCTCAGAACAA R-GTAGTTCCGCCTTCGGTCTTG	NM_030679
Myosin heavy polypeptide 2 (*Myh2*)	F-ACTTTGGCACTACGGGGAAAC R-CAGCAGCATTTCGATCAGCTC	NM_001039545
Myosin heavy polypeptide 4 (*Myh4*)	F-TTTGCTTACGTCAGTCAAGGT R-AGCGCCTGTGAGCTTGTAAA	NM_010855
Myosin heavy polypeptide 7 (*Myh7*)	F- CCTGCGGAAGTCTGAGAAGG R- CTCGGGACACGATCTTGGC	NM_080728
Myosin Light polypeptide 1 (*Myl1-1f*)	F-AACACTCTGGGTCCACCCTC R-TGACACTTGGAAGAGCAGTGTGA	NM_021285
Myosin Light polypeptide 1 (*Myl1-3f*)	F-TGC TGA CCA GAT TGC CGA CT R-AGG ACG TCT CCC ACC TGA CT	NM_001113387
Myosin Light polypeptide 3 (*Myl3-1s*)	F-TTCGGGAAGGAGTGGTTCGG R-GCAGGGGCCAGGAAAGACTA	NM_010859
Troponin-T (*Tnnt*, f)	F-AACAGATTGGCGGAGGAGAA R-TTGGCCAGGTAGCTGCTGTA	L48988
Troponin-T (Tnnt, s)	F-AGCGCTTCAGAACGGAAAAG R-GGCGTCATCCTCTGCTCTCT	AJ131711
Troponin-I (*Tnni*, f)	F-TGCAGAAGAGCAGCAAGGAG R-GGTCCCGTTCCTTCTCAGTG	NM_00940
Troponin-I (*Tnni*, s)	F-GGAGTGTTGGGAGCAGGAAC R-GAGCTCTCGGCACAAGTCCT	NM_021467
Troponin-C (*Tnnc*, f)	F-CCTTTGACATGTTCGATGCTG R-TCGATGATGGCATCCAATTC	M57590
Troponin-C (*Tnnc*, s)	F-CCTGAGGAGCTGCAGGAGAT R-TTCCCTTTGCTGTC TCCTT	NM_009393.2
Tropomyosin-1 (*Tpm1*)	F-GGCTGAGCTCTCAGAAGGCA R-TCAGCCCGAGTTTCAGCCTC	NM_001164248
Tropomyosin-2 (*Tpm2*)	F-GAGCACCAGCTAGCCACGTT	NM_009416

	R-GGGCTTCCGGAGTAGAAGAGC	
Tropomyosin-3 (*Tpm3*)	F-TGGACCACGCCCTCAATGAC R-GAATCCAGAGCGAGTGGGGT	NM_001293748
Actin, α	F-CACTTCCTACCCTCGGCACC R-GTAGGAGAGCACCGGCTTGT	NM_001272041.1
Atrogin-1 (Fbox32)	F-TGGGTGTATCGGATGGAGAC R-TCAGCCTCTGCATGATGTTC	AF441120
MuRF-1	F-AGAAGCTGGGCTTCATCGAG R-TGCTTGGCACTTGAGAGGAA	DQ229108
β-Actin	F-AACCGTGAAAAGATGACCCAG R-CACAGCCTGGATGGCTACGTA	NM_007393

SECTION 7: FIGURES

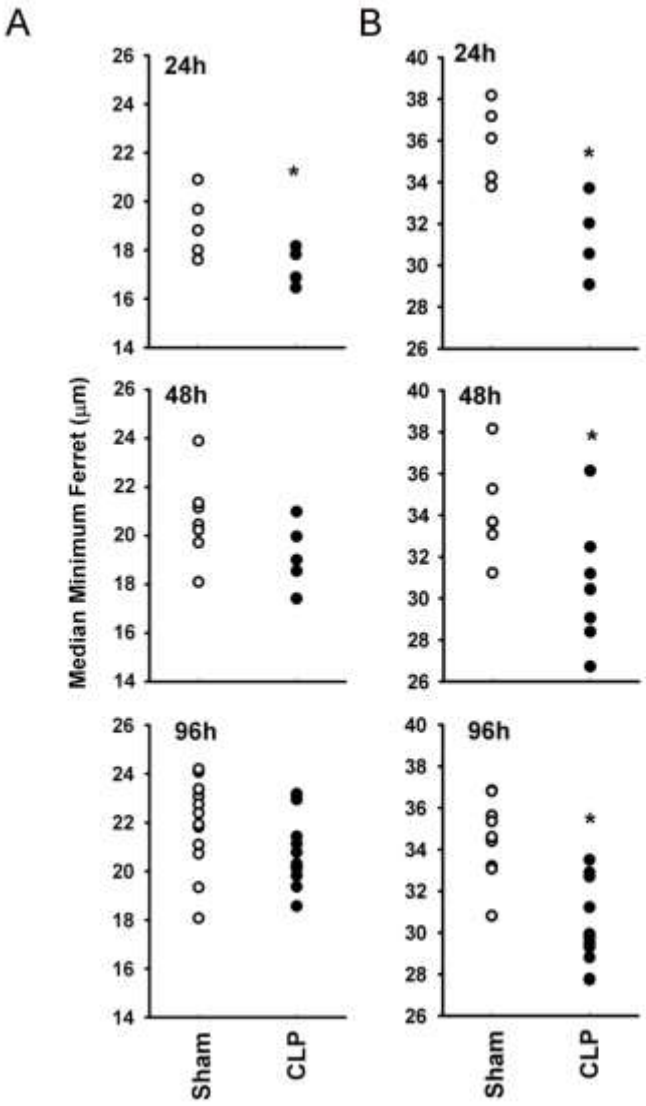

FIGURE 1

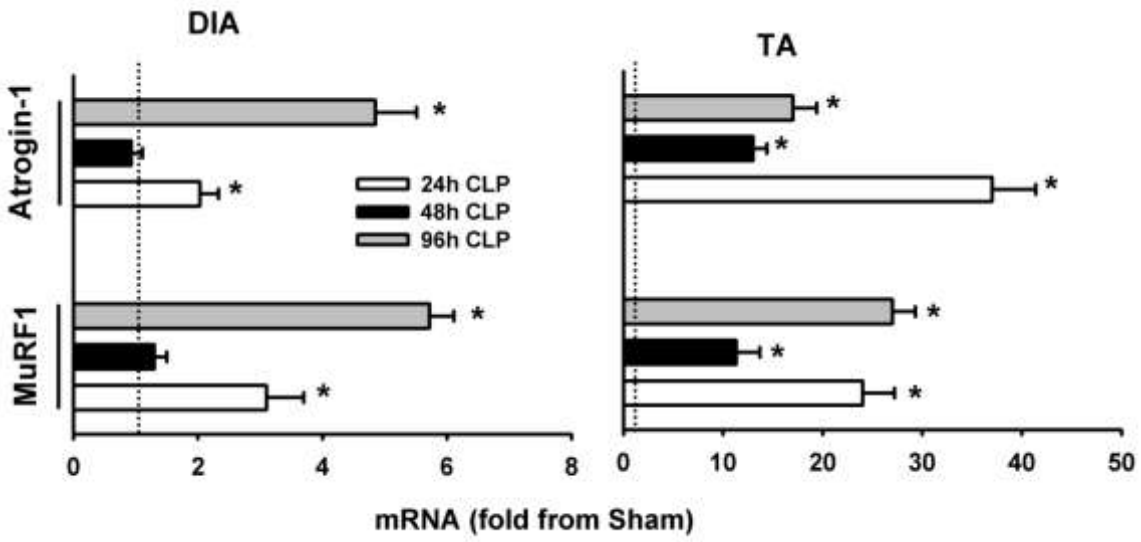
DIA
TA
Atrogin-1
MuRF1
24h CLP
48h CLP
96h CLP
0
2
4
6
8
0
10
20
30
40
50
mRNA (fold from Sham)

FIGURE 2

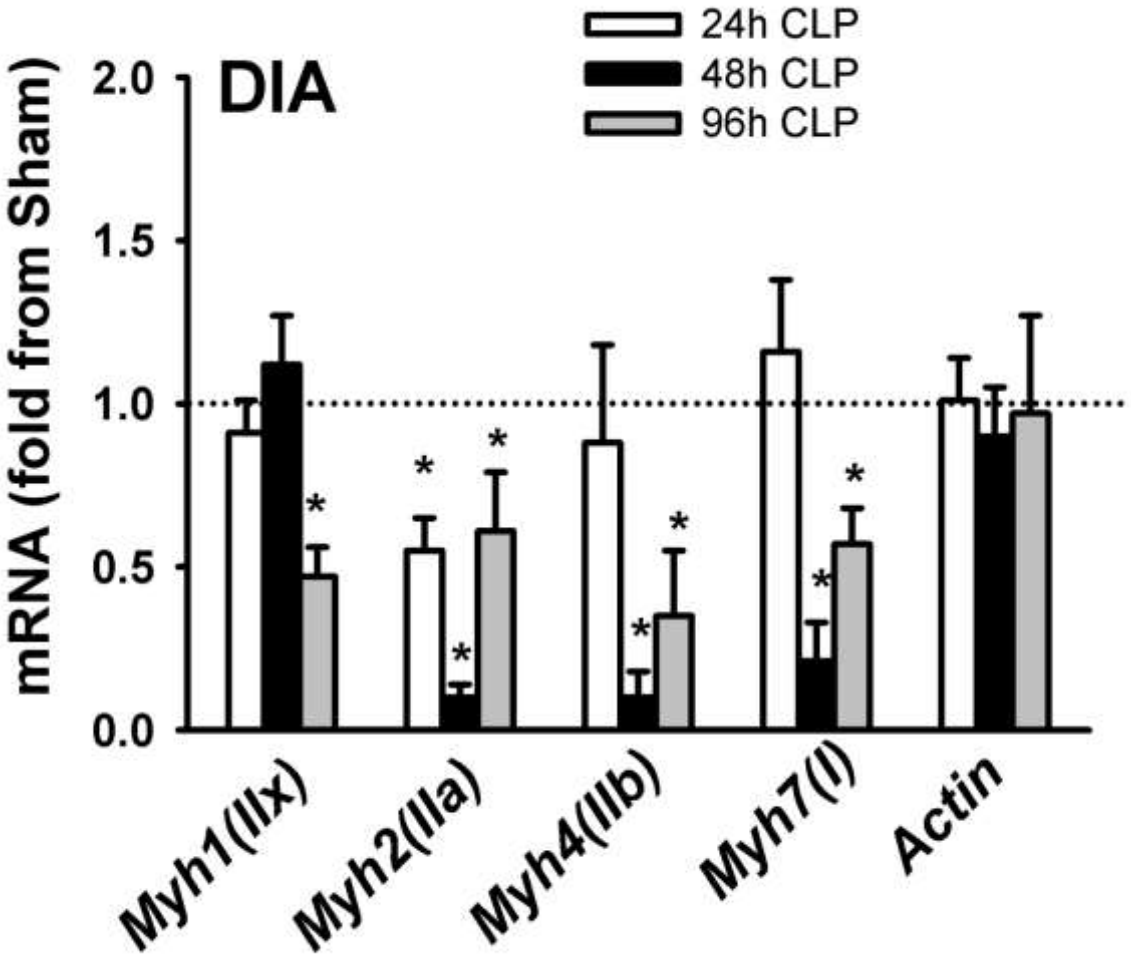
DIA
24h CLP
48h CLP
96h CLP
mRNA (fold from Sham)
2.0
1.5
1.0
0.5
0.0
Myh1(IIx)
Myh2(IIa)
Myh4(IIb)
Myh7(I)
Actin

FIGURE 3

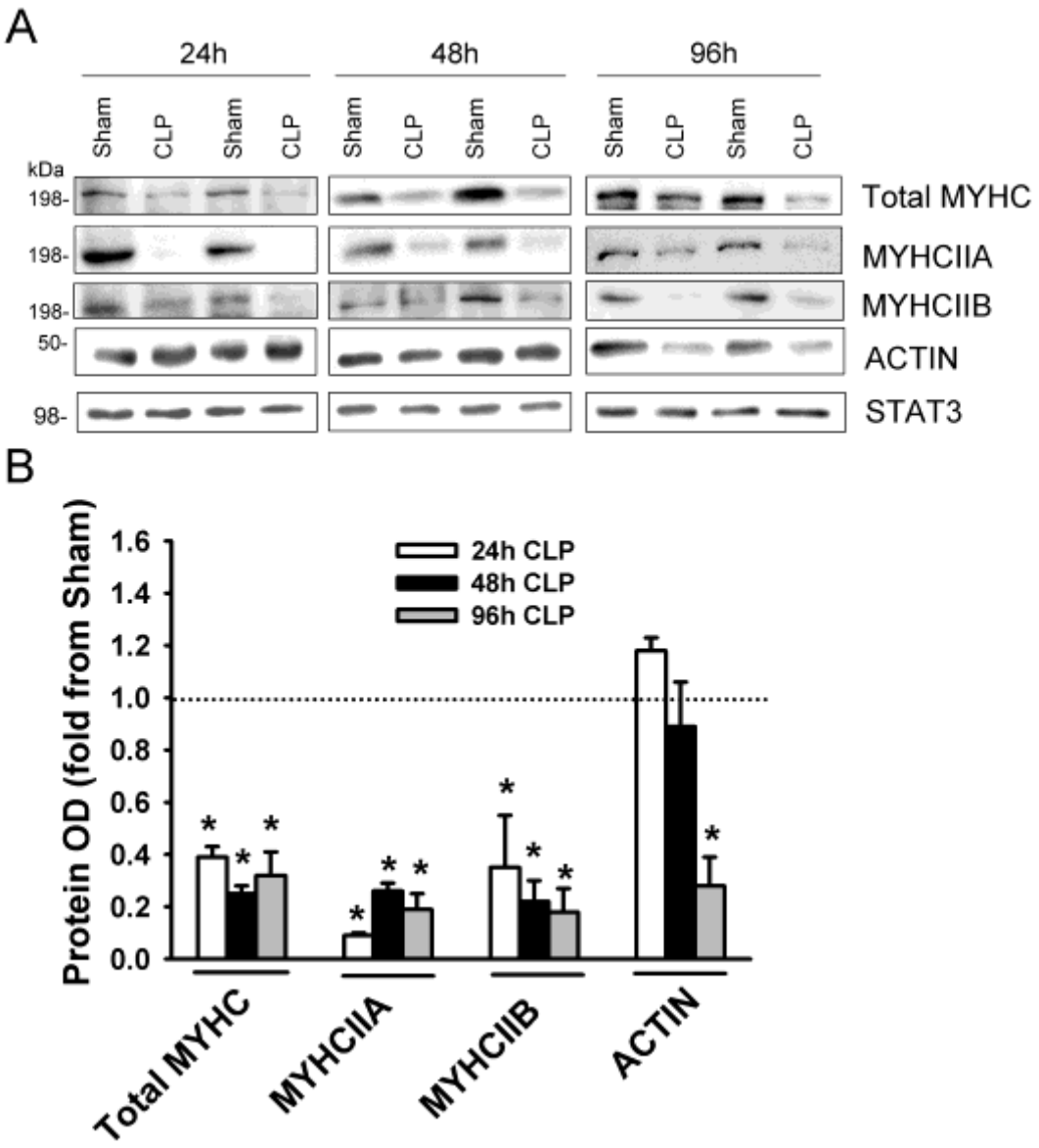
A
24h
48h
96h
Sham
CLP
Sham
CLP
Sham
CLP
Sham
CLP
Sham
CLP
Sham
CLP
kDa
198-
198-
198-
50-
98-
Total MYHC
MYHCIIA
MYHCIIB
ACTIN
STAT3
B
Protein OD (fold from Sham)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
24h CLP
48h CLP
96h CLP
Total MYHC
MYHCIIA
MYHCIIB
ACTIN

FIGURE 4

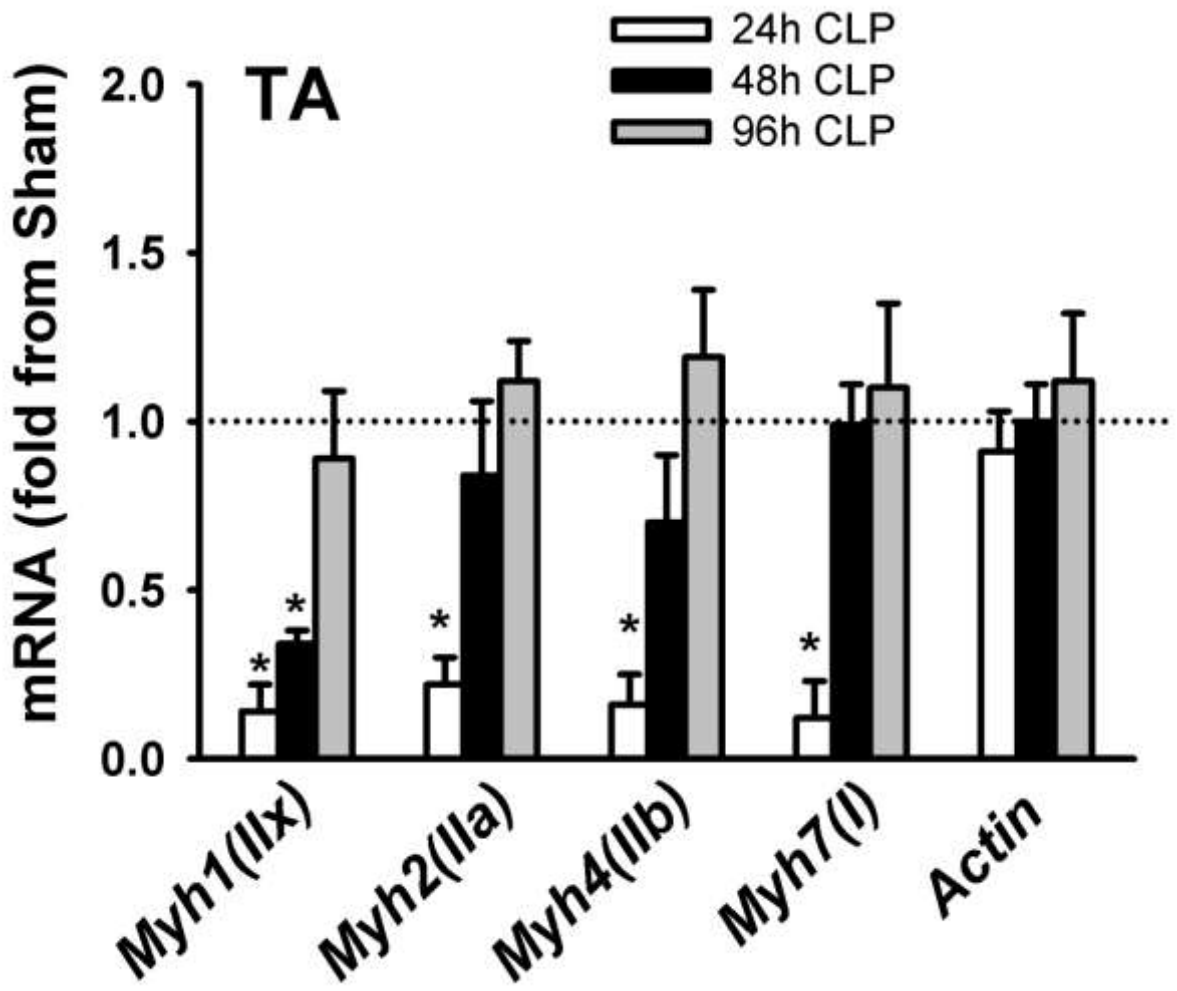
TA
24h CLP
48h CLP
96h CLP
mRNA (fold from Sham)
2.0
1.5
1.0
0.5
0.0
*
*
*
*
Myh1(IIx)
Myh2(IIa)
Myh4(IIb)
Myh7(I)
Actin

FIGURE 5

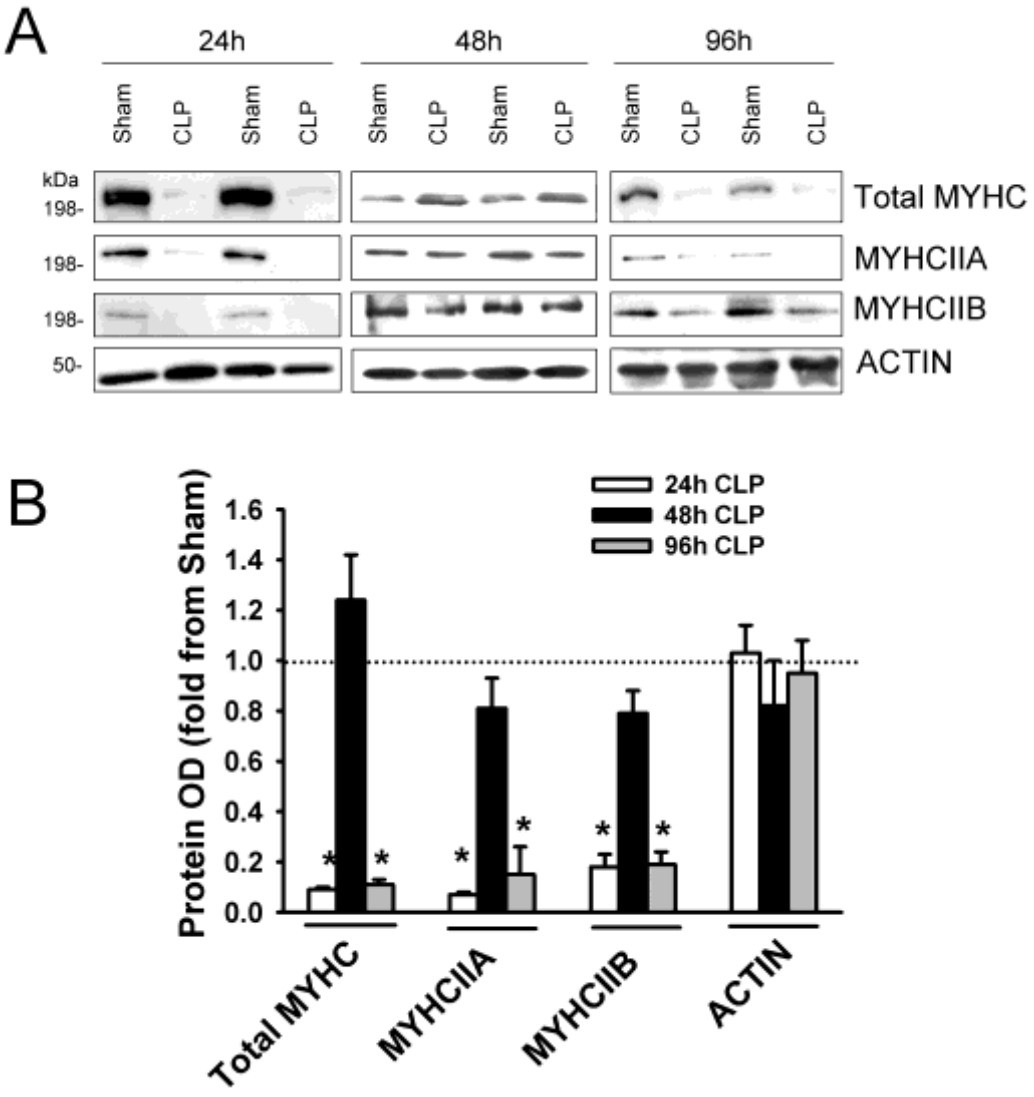
A
24h
48h
96h
Sham
CLP
Sham
CLP
Sham
CLP
Sham
CLP
Sham
CLP
Sham
CLP
kDa
198-
198-
198-
50-
Total MYHC
MYHCIIA
MYHCIIB
ACTIN
B
24h CLP
48h CLP
96h CLP
Protein OD (fold from Sham)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Total MYHC
MYHCIIA
MYHCIIB
ACTIN

FIGURE 6

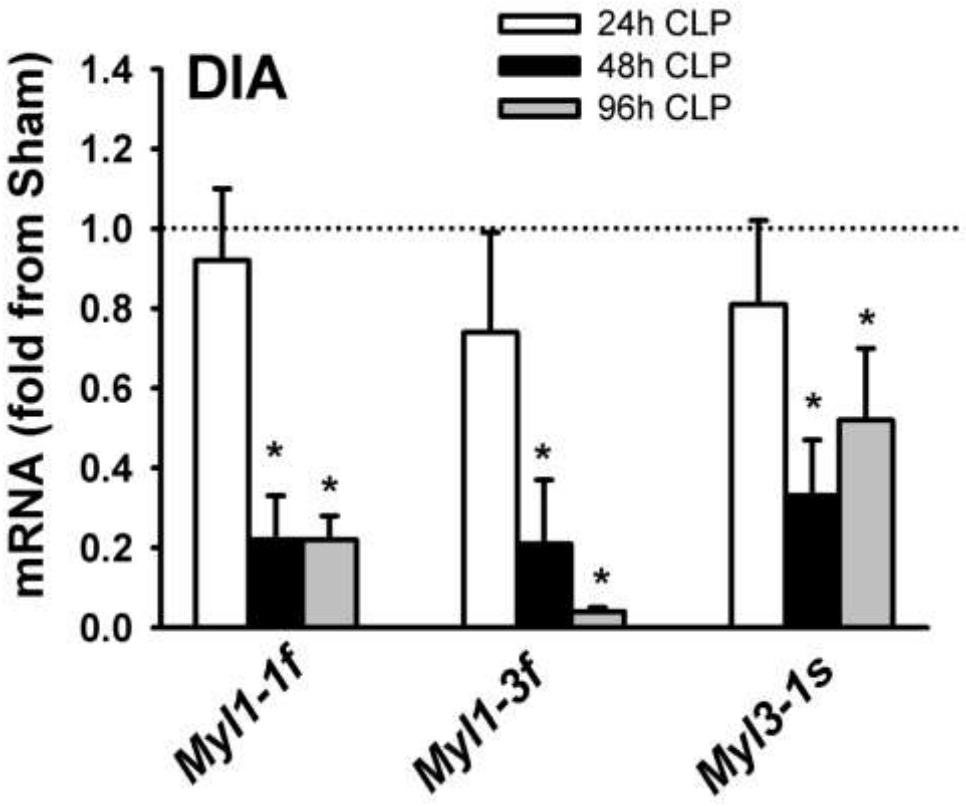
DIA
24h CLP
48h CLP
96h CLP
mRNA (fold from Sham)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Myl1-1f
Myl1-3f
Myl3-1s

FIGURE 7

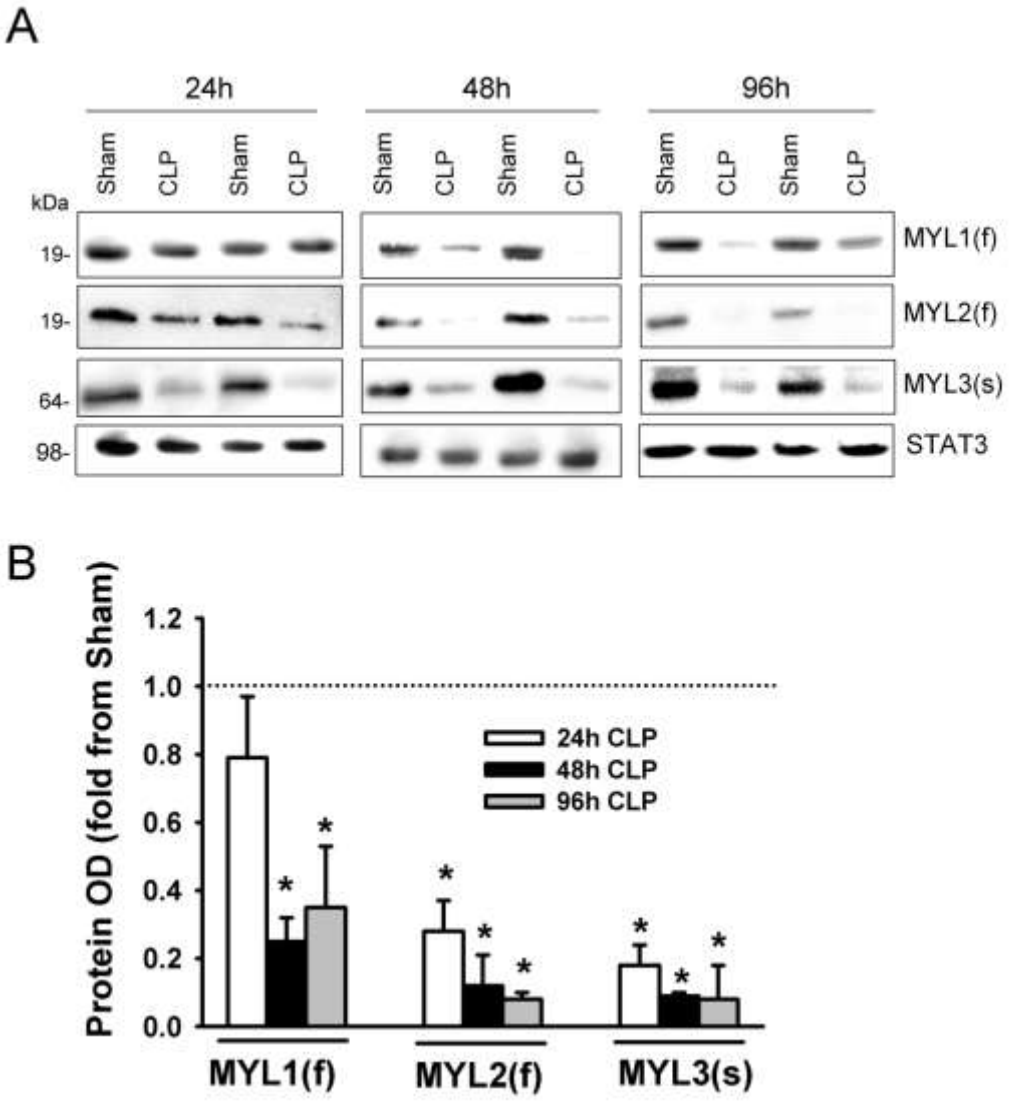
A
24h
48h
96h
Sham
CLP
Sham
CLP
Sham
CLP
Sham
CLP
Sham
CLP
Sham
CLP
kDa
19-
19-
64-
98-
MYL1(f)
MYL2(f)
MYL3(s)
STAT3
B
Protein OD (fold from Sham)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
24h CLP
48h CLP
96h CLP
MYL1(f)
MYL2(f)
MYL3(s)

FIGURE 8

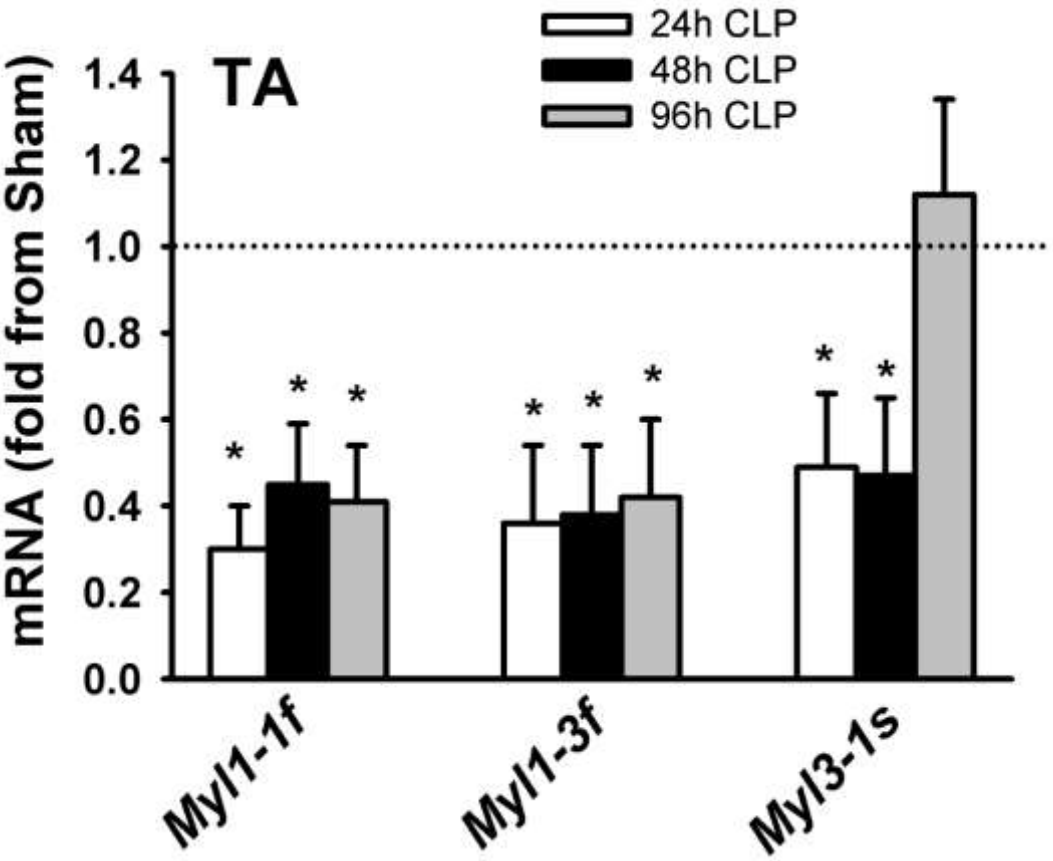
TA
24h CLP
48h CLP
96h CLP
mRNA (fold from Sham)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Myl1-1f
Myl1-3f
Myl3-1s

FIGURE 9

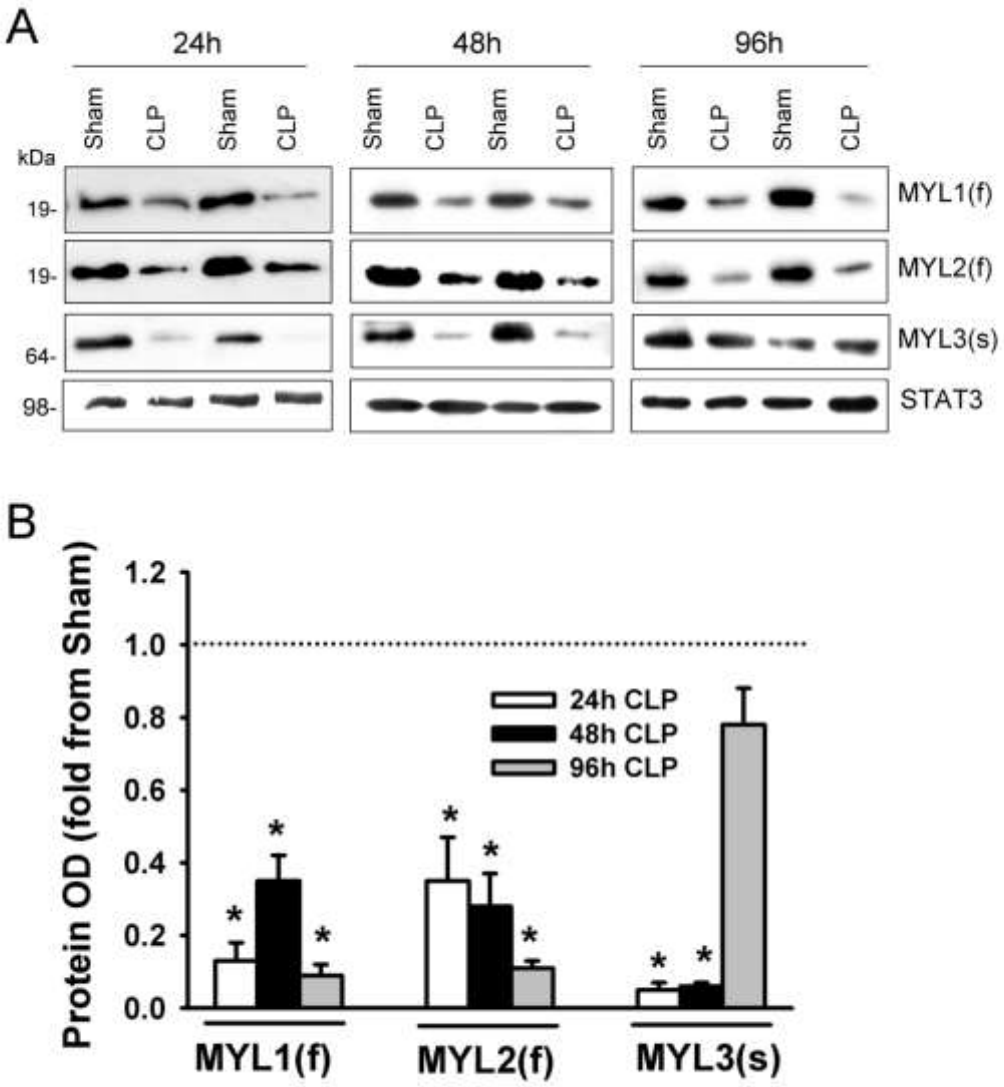
A
24h
48h
96h
Sham
CLP
Sham
CLP
Sham
CLP
Sham
CLP
Sham
CLP
Sham
CLP
kDa
19-
19-
64-
98-
MYL1(f)
MYL2(f)
MYL3(s)
STAT3
B
Protein OD (fold from Sham)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
24h CLP
48h CLP
96h CLP
MYL1(f)
MYL2(f)
MYL3(s)

FIGURE 10

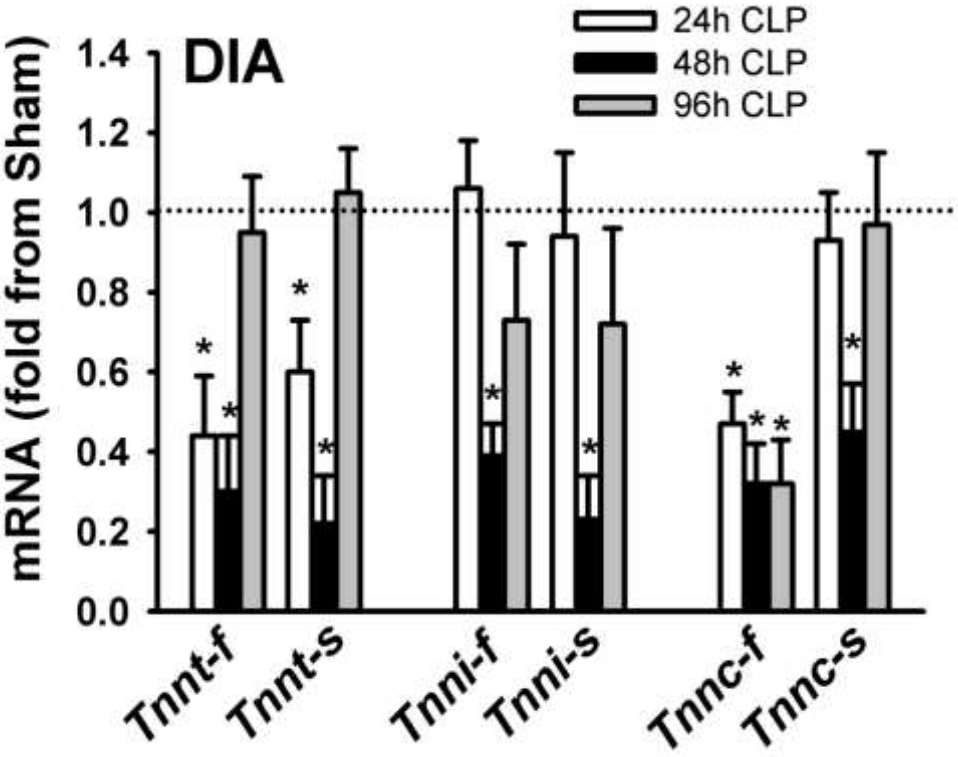
DIA
24h CLP
48h CLP
96h CLP
mRNA (fold from Sham)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Tnnt-f
Tnnt-s
Tnni-f
Tnni-s
Tnnc-f
Tnnc-s

FIGURE 11

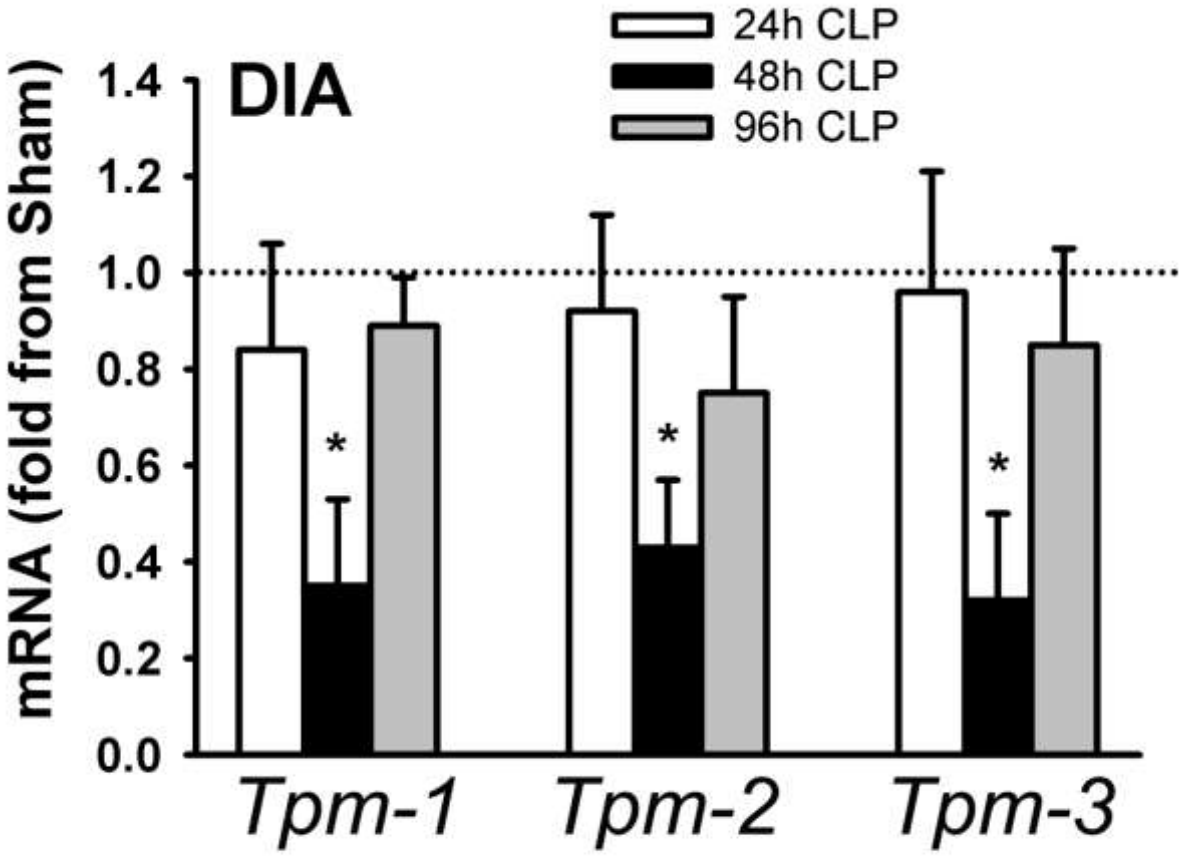
DIA
24h CLP
48h CLP
96h CLP
mRNA (fold from Sham)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
Tpm-1
Tpm-2
Tpm-3

FIGURE 12

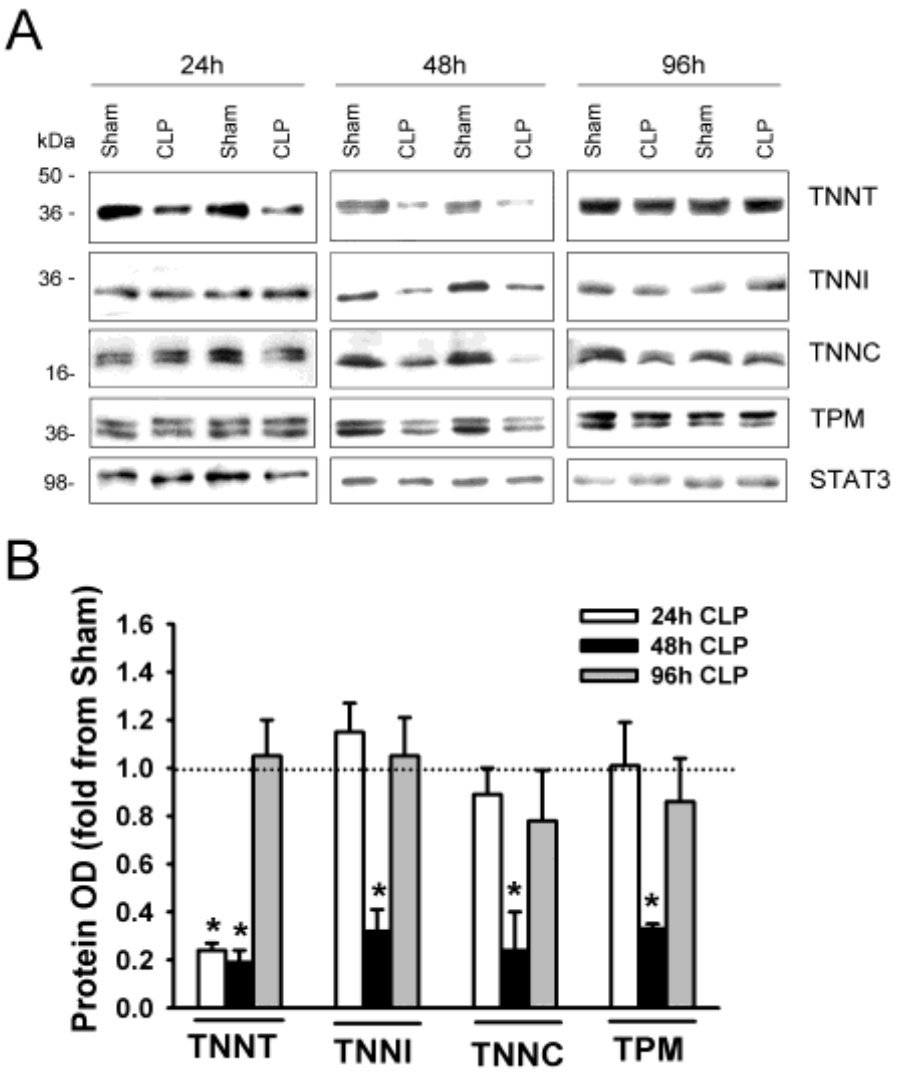
A
24h
48h
96h
Sham
CLP
Sham
CLP
Sham
CLP
Sham
CLP
Sham
CLP
Sham
CLP
kDa
50 -
36 -
36 -
16-
36-
98-
TNNT
TNNI
TNNC
TPM
STAT3
B
Protein OD (fold from Sham)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
24h CLP
48h CLP
96h CLP
TNNT
TNNI
TNNC
TPM

FIGURE 13

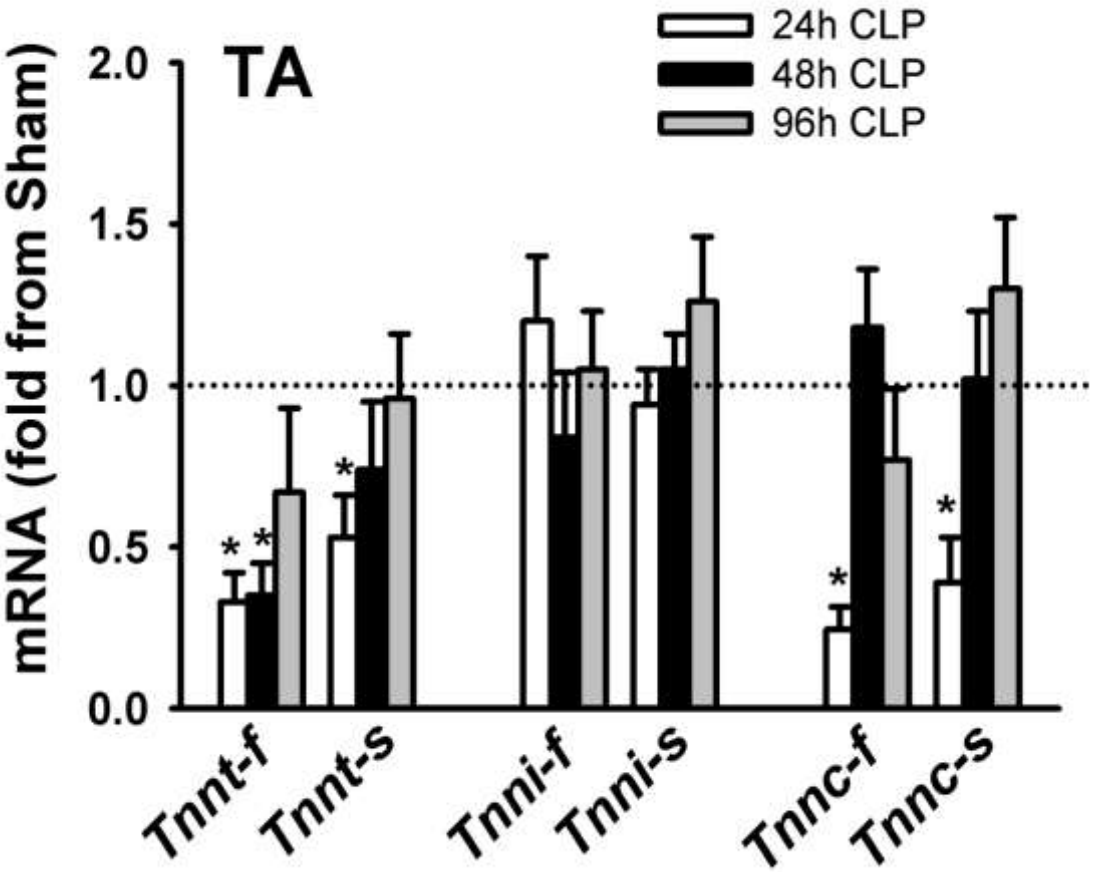
TA
24h CLP
48h CLP
96h CLP
mRNA (fold from Sham)
2.0
1.5
1.0
0.5
0.0
*
*
*
*
*
Tnnt-f
Tnnt-s
Tnni-f
Tnni-s
Tnnc-f
Tnnc-s

FIGURE 14

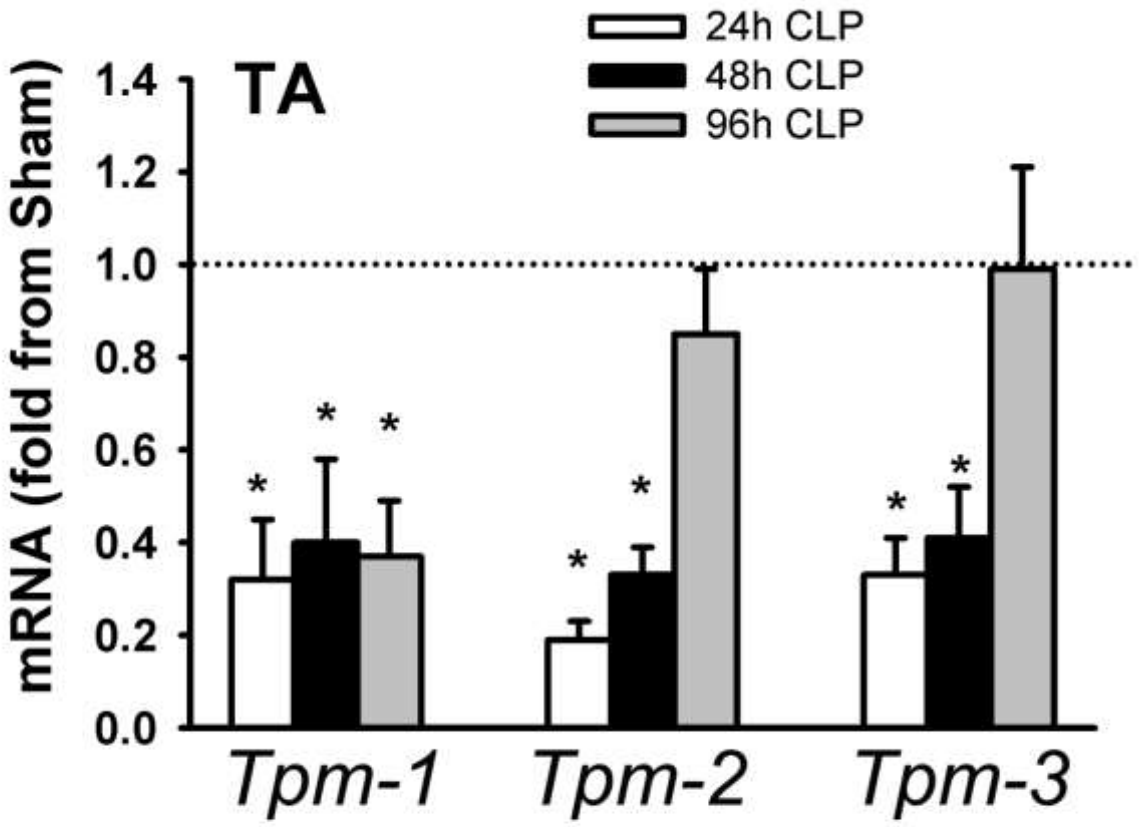
TA
24h CLP
48h CLP
96h CLP
mRNA (fold from Sham)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Tpm-1
Tpm-2
Tpm-3

FIGURE 15

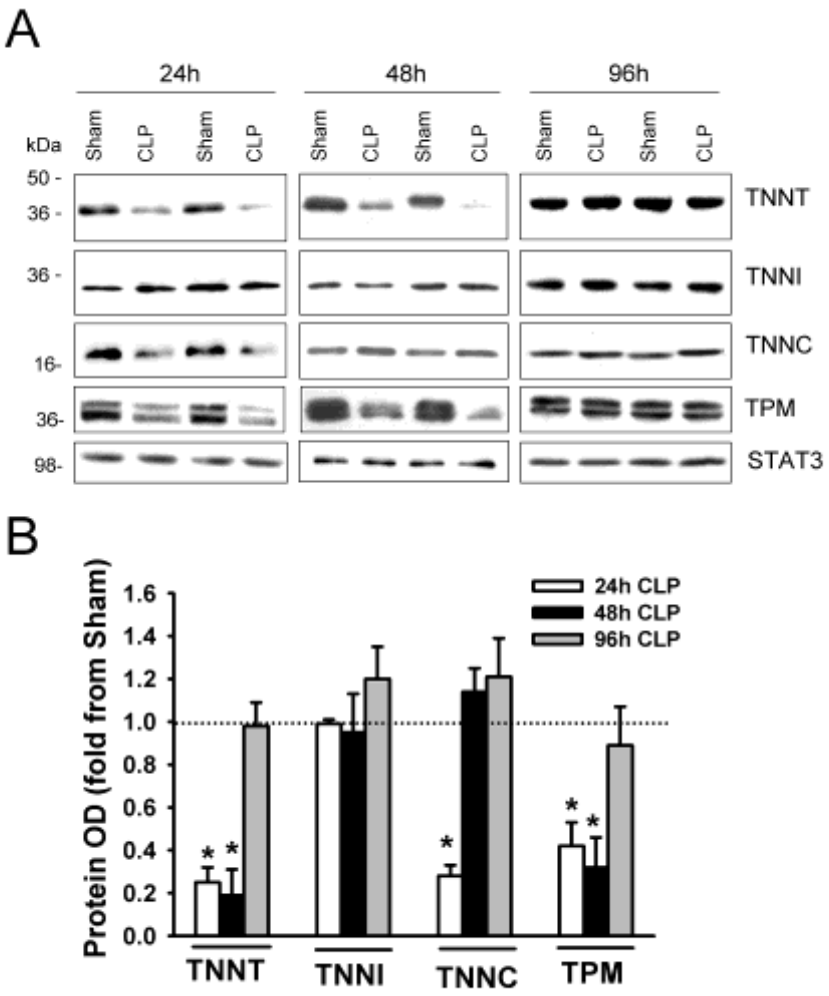
A
24h
48h
96h
kDa
Sham
CLP
Sham
CLP
Sham
CLP
Sham
CLP
Sham
CLP
Sham
CLP
50 -
36 -
36 -
16-
36-
98-
TNNT
TNNI
TNNC
TPM
STAT3
B
Protein OD (fold from Sham)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
24h CLP
48h CLP
96h CLP
TNNT
TNNI
TNNC
TPM

FIGURE 16

SECTION 8: FIGURE LEGENDS

Figure 1:

A-B: Median values of minimum ferret of DIA (A) and TA (B) cross sections measured after 24, 48 and 96h in the Sham and the CLP groups. *P<0.05 compared to the Sham group.

Figure 2: mRNA expression of *Atrogin-1* and *MuRF1* E3 ligases in the DIA and TA of the CLP groups. Values (means±SEM) and are expressed as fold change from corresponding Sham groups. *p<0.05, compared to the Sham groups.

Figure 3: mRNA expression of Myosin Heavy Chain isoform *Myh1* (type *IIx*), *Myh2* (type *IIa*), *Myh4* (type *IIb*) and *Myh7* (type *I*) as well as *α-Actin* in the DIA of the CLP groups. Values (means±SEM) and are expressed as fold change from corresponding Sham groups. *p<0.05, compared to the Sham groups.

Figure 4:

A: Representative immunoblots of total Myosin Heavy Chain (MYHC), Myosin Heavy Chain type IIA, type IIB, α-ACTIN and STAT3 (equal loading indicator) in the DIA of the Sham and the CLP groups.

B: Optical densities of total MYHC, Myosin Heavy Chain type IIA, type IIB and α-ACTIN proteins in the DIA of the CLP groups. Values (means±SEM) are expressed as fold change relative to the corresponding Sham group. *p<0.05, compared to the Sham groups.

Figure 5: mRNA expression of Myosin Heavy Chain isoform *Myh1* (type *IIx*), *Myh2* (type *IIa*), *Myh4* (type *IIb*) and *Myh7* (type *I*) as well as *α-Actin* in the TA of the CLP groups. Values (means±SEM) and are expressed as fold change from corresponding Sham groups. *p<0.05, compared to the Sham groups.

Figure 6:

A: Representative immunoblots of total MYHC, MYHCIIA, type IIB, α-ACTIN and STAT3 (equal loading indicator) in the TA of the Sham and the CLP groups.

B: Optical densities of total MHC, MYHCIIA, type IIB and α-ACTIN proteins in the TA of the CLP groups. Values (means±SEM) are expressed as fold change relative to the corresponding Sham groups. *p<0.05, compared to the Sham groups.

Figure 7: mRNA expression of Myosin Light Chain isoform *Myl1* (type 1 fast), *Myl1* (type 3 fast) and *Myl3* in the DIA of the CLP groups. Values (means±SEM) and are expressed as fold change from corresponding Sham groups. *p<0.05, compared to the Sham groups.

Figure 8:

A: Representative immunoblots of MYL1 (fast), MYL2 (fast) and MYL3 (slow) and STAT3 (equal loading indicator) in the DIA of the Sham and the CLP groups.

B: Optical densities of MMYL1 (fast), MYL2 (fast) and MYL3 (slow) in the DIA of the CLP groups. Values (means±SEM) are expressed as fold change relative to the corresponding Sham groups. *p<0.05, compared to the Sham groups.

Figure 9: mRNA expression of *Myl1* (type 1 fast), *Myl1* (type 3 fast) and *Myl3* in the TA of the CLP groups. Values (means±SEM) and are expressed as fold change from corresponding Sham groups. *p<0.05, compared to the Sham groups.

Figure 10:

A: Representative immunoblots of MYL1 (fast), MYL2 (fast) and MYL3 (slow) and STAT3 (equal loading indicator) in the TA of the Sham and the CLP groups.

B: Optical densities of MYL1 (fast), MYL2 (fast) and MYL3 (slow) in the TA of the CLP groups. Values (means±SEM) are expressed as fold change relative to the corresponding Sham groups. *p<0.05, compared to the Sham groups.

Figure 11: mRNA expression of the fast and slow isoforms of Troponin-T (*Tnnt*), Troponin-I (*Tnni*) and Troponin-C (*Tnnc*) in the DIA of the CLP groups. Values (means±SEM) and are expressed as fold change from corresponding Sham groups. *p<0.05, compared to the Sham groups.

Figure 12: mRNA expression of Tropomyosin 1(α), 2 (β) and 3 (γ) isoforms in the DIA of the CLP groups. Values (means±SEM) and are expressed as fold change from corresponding Sham groups. *p<0.05, compared to the Sham groups.

Figure 13:

A: Representative immunoblots of TNNT, TNNI, TNNC, TPM and STAT3 (equal loading indicator) proteins in the DIA of the Sham and the CLP groups.

B: Optical densities of TNNT, TNNI, TNNC and TPM in the DIA of the CLP groups. Values (means±SEM) are expressed as fold change relative to the corresponding Sham groups. *p<0.05, compared to the Sham groups.

Figure 14: mRNA expression of the fast and slow isoforms of Troponin T (*Tnnt*), Troponin I (*Tnni*) and Troponin C (*Tnnc*) in the TA of the CLP groups. Values (means±SEM) and are expressed as fold change from corresponding Sham groups. *p<0.05, compared to the Sham groups.

Figure 15: mRNA expression of TPM1, 2 and 3 isoforms in the TA of the CLP groups. Values (means±SEM) and are expressed as fold change from corresponding Sham groups. *p<0.05, compared to the Sham groups.

Figure 16:

A: Representative immunoblots of TNNT, TNNI, TNNC, TPM and STAT3 (equal loading indicator) in the TA of the Sham and the CLP groups.

B: Optical densities of TNNT, TNNI, TNNC and TPM in the TA of the CLP groups. Values (means±SEM) are expressed as fold change relative to the corresponding Sham groups. *p<0.05, compared to the Sham groups.