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Role of TLR4 in endoplasmic reticulum stress induced by physical exercise in skeletal muscle

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DEDICATION

"To my family and all the friends of GEFIME, with love, admiration and gratitude for all their understanding, affection, presence and tireless support throughout the period of execution of this work."

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I am grateful to God, who gave me the gift of life and guided me through paths that allowed me to enter the University of São Paulo (USP). Because of it, I had the opportunity to do my master's degree at this great university. Furthermore, none of this would have happened without the support and all the help from my parents. I thank my family for all the love and support they have given me along the way. I am grateful to my fiancée Aimee for being my foundation throughout the difficult times, for her patience when I had to work on days she wished she could have been with me, and for all her love and support over the years. I also thank Professor Adelino for his guidance and advice, both on my growth and knowledge in academic life and on decisions in my personal life. Still, I thank him for all of his patience and persistence to help me get the scholarship I so much needed. I am very grateful to my laboratory friends, who helped me whenever I needed. In particular, Ana Paula Pinto, who has been involved in the development of this project since before I started my master's degree; who has welcomed me and helped me at all the moments I needed. I'm lucky to be surrounded by such good people. I am grateful to Research Support Foundation of the State of São Paulo (FAPESP: 2020/04269-8) for the financial support. Finally, I thank all the people who have been part of my life since I was born. I am where I am today because of the people I've met in my life, no matter if they have played a big or a small role. Thanks.

RESUMO

MARAFON, Bruno Brieda. **Papel do TLR4 no estresse do retículo endoplasmático induzido pelo exercício físico no músculo esquelético.** 2022. 66f. Dissertação (Master) – Escola de Educação Física e Esporte de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 2022.

Muitas condições podem induzir o estresse do retículo endoplasmático (RE), como inflamação e exercício físico, por exemplo. O receptor tipo Toll 4 (TLR4) pode desencadear inflamação e eventos de estresse do RE. No entanto, ainda não existem dados na literatura sobre o papel do TLR4 no estresse do RE durante o exercício no músculo esquelético. Portanto, o presente estudo teve como objetivo investigar e verificar as respostas dos marcadores de estresse de RE em camundongos do tipo selvagem (WT) e *Tlr4* nocaute global (KO) após protocolos de exercício físico agudo e crônico. Camundongos machos WT e KO com oito semanas de idade foram submetidos a exercícios agudos (moderada ou alta intensidade) e crônicos (protocolo de 4 semanas). Em condições basais, os camundongos KO apresentaram desempenho inferior no teste do rotarod e maior fosforilação da proteína eIF2α em comparação aos animais WT. O exercício agudo de intensidade moderada aumentou a BiP e a proteína CHOP no grupo WT. Após o exercício agudo de alta intensidade, houve aumento nos níveis de RNAm de *Casp3* e *Ddit3* para os camundongos KO. O exercício agudo aumentou a Caspase-3 clivada/Caspase-3 no grupo KO, independentemente da intensidade do exercício. Em resposta ao exercício crônico, o grupo KO não apresentou melhora em nenhuma avaliação de desempenho. O protocolo crônico de 4 semanas não gerou alterações na CHOP, na razão p-eIF2α/eIF2α e Caspase-3 clivada/Caspase-3, mas reduziu a proteína BiP em relação ao grupo KO-Sedentário. Esses resultados demonstram que a deleção global de Tlr4 parece proteger os camundongos contra o estresse do RE, mas diminui seu desempenho. Ainda, parece que a razão Caspase-3 clivada/Caspase-3 pode ser ativada por outra via diferente do estresse de RE em animais *Tlr4* KO.

Palavras-chave: Apoptose, Exercício físico, Modelo Nocaute, Receptor Tipo Toll, TLR4.

ABSTRACT

MARAFON, Bruno Brieda. **Role of TLR4 in endoplasmic reticulum stress induced by physical exercise in skeletal muscle.** 2022. 66p. Thesis (Master) – School of Physical Education and Sport of Ribeirão Preto, University of São Paulo, Ribeirão Preto, 2022.

Many conditions can induce endoplasmic reticulum (ER) stress, such as inflammation and physical exercise. Toll-like Receptor 4 (TLR4) can trigger inflammation and ER stress events. However, there are still no data in the literature regarding the role of TLR4 in ER stress during exercise in skeletal muscle. Therefore, the current investigation aimed to verify the responses of ER stress markers in wild-type (WT) and *Tlr4* global knockout (KO) mice after acute and chronic physical exercise protocols. Eight-week-old male WT and KO mice were submitted to acute (moderate or high intensity) and chronic (4-week protocol) treadmill exercise. Under basal conditions, KO mice showed lower performance in the rotarod test, and increased eIF2α protein phosphorylation compared to WT animals. Acute moderate-intensity exercise increased BiP and CHOP protein in the WT group. After the acute high-intensity exercise, there was an increase in *Casp3* and *Ddit3* mRNA for the KO mice. Acute exercise increased the cleaved Caspase-3/Caspase-3 in the KO group regardless of exercise intensity. In response to chronic exercise, the KO group showed no improvement in any performance evaluation. The 4-week chronic protocol did not generate changes in CHOP, p-eIF2α/eIF2α, and cleaved Caspase-3/Caspase-3 ratio but reduced BiP protein compared to the KO-Sedentary group. These results demonstrate that the global deletion of *Tlr4* seems to protect the mice against ER stress but decreases their performance. The cleaved Caspase-3/Caspase-3 ratio may be activated by another pathway other than ER stress in *Tlr4* KO animals.

Keywords: Apoptosis, Knockout model, Physical exercise, Toll-like receptor, TLR4.

SUMMARY

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1. INTRODUCTION

Skeletal muscle is a highly organized tissue composed of a set of fascicles that have several bundles of muscle fibers. Each bundle contains several myofibrils with systematic arrangements of protein myofilaments that give the striated pattern to the skeletal muscle (MUKUND; SUBRAMANIAM, 2020). This arrangement is formed by darker bands, characterized by the presence of myosin and actin filaments, and lighter bands, characterized by the presence of actin filaments. Each myofibril is composed of numerous sarcomeres positioned in series, which allows actin filaments to slide over myosin filaments, resulting in muscle contraction (FRONTERA; OCHALA, 2015).

Muscle contraction occurs as a result of several biochemical reactions, ranging from stimulation of the nervous system, initiating an action potential that is transmitted to the muscle fiber, enabling the release of calcium (Ca^{2+}) from the cisterns of the sarcoplasmic reticulum (SR, endoplasmic reticulum of muscle cells), up to the hydrolysis of adenosine triphosphate (ATP) into ADP and inorganic phosphate, allowing the interaction between actin and myosin, generating the sliding between these myofilaments (FRONTERA; OCHALA, 2015; MUKUND; SUBRAMANIAM, 2020). In summary, these reactions transform chemical energy into mechanical energy and heat production. Thus, it is clear that skeletal muscle is an essential tissue for posture, locomotion, thermoregulation and metabolism (MCARDLE; KATCH; KATCH, 2010).

Additionally, skeletal muscle is the most abundant tissue in the human body and is considered one of the most metabolically active tissues of the body. Comprising approximately 40% and 30% of the total body weight of men and women, respectively, skeletal muscle contains 50-75% of all protein in the entire body (FRONTERA; OCHALA, 2015; JANSSEN; HEYMSFIELD; WANG; ROSS, 2000). Under normal conditions, the regulation of an individual's protein content is carried out by a dynamic and balanced process of protein synthesis and degradation, without significantly altering the muscle mass. When the organism is challenged, there is a change in this balance, which can shift this process more towards protein synthesis or towards protein degradation. Therefore, the pathways that regulate muscle protein content are extremely important for the human organism. A crucial organelle responsible for these events is the endoplasmic reticulum (ER) (BOHNERT; MCMILLAN; KUMAR, 2018).

The ER is an intracellular organelle responsible for several biological functions such as manipulation of Ca^{2+}, lipid synthesis, and translation of most body proteins, as well as folding

and post-translational modifications of proteins and formation of protein complexes (EIZIRIK; CARDOZO; CNOP, 2007). Therefore, newly synthesized proteins enter the ER lumen as unfolded polypeptide chains and must be properly folded before reaching their targets. Protein homeostasis imbalance leads to malformed proteins within the ER and their accumulation generates the "ER stress" (SCHRÖDER; KAUFMAN, 2005). To deal with this stress, eukaryotic cells have evolved the Unfolded Protein Response (UPR), which adjusts the cell's ability to fold proteins and attenuates the translation machinery, preventing the accumulation of unfolded proteins in the ER and ensuring the quality of secreted proteins (RON; WALTER, 2007). The UPR is comprised of three parallel paths. In each branch there is an integral protein resident in the ER membrane, namely Inositol-requiring enzyme 1 (IRE1), protein kinase R-like endoplasmic reticulum kinase (PERK) or activating transcription factor 6 (ATF6) (HETZ, 2012).

Some conditions such as disease, aging, high fat diet, inflammation and exercise can lead to ER stress and activate the UPR. Regarding physical exercise, some studies have verified the induction of some ER stress markers such as BiP, PERK, IRE1, CCAAT-enhancer-binding protein homologous protein (CHOP), X-box binding protein 1 splicing (*Xbp1s*) and Activating transcription factor 4 (ATF4) in skeletal muscle (PEREIRA; DA ROCHA; PINTO; PAULI *et al.*, 2016; WU; RUAS; ESTALL; RASBACH *et al.*, 2011). Thus, with the interest of clarifying the relationship between inflammation, exercise and ER stress, Pinto et al. used interleukin 6 (IL-6) knockout (KO) mice and wild-type (WT) mice models, subjecting them to an exhaustive acute physical exercise protocol. In skeletal muscle, BiP protein as well as the peIF2α/eIF2α ratio were attenuated in the IL-6 KO group. Therefore, IL-6 seems to be related to ER stress homeostasis (PINTO; DA ROCHA; KOHAMA; GASPAR *et al.*, 2018).

IL-6 is a pro-inflammatory cytokine that can be mediated by Nuclear Factor kappa B (NF-κB) (WELC; CLANTON, 2013). NF-κB is a transcription factor known to be involved in a wide variety of cellular processes such as the immunoinflammatory response, cytokine production, apoptosis, cell growth and development (GILMORE, 2006; WERNER; BARKEN; HOFFMANN, 2005). The activation of this important transcription factor is induced by Toll-like Receptor 4 (TLR4), a transmembrane protein related to the innate immune system (MEDZHITOV; PRESTON-HURLBURT; JANEWAY JR, 1997). TLR4 stimulation is performed by the lipid A subunit of lipopolysaccharide (LPS), which is almost entirely composed of fatty acids (LIEN; CHOW; HAWKINS; MCGUINNESS *et al.*, 2001). It is known that endurance exercise results in an extracellular increase in non-esterified fatty acids and therefore also leads to TLR4 activation (FRANCAUX, 2009). Acute aerobic exercise is

potentially inflammatory. Few studies involving acute physical exercise (APE) show anti-inflammatory effects, compared to chronic physical exercise (CPE), which leads to a decrease in these cell membrane receptors (CAVALCANTE; GREGNANI; HENRIQUE; ORNELLAS *et al.*, 2017). Thus, the decrease in TLR4 and TLR2 is often associated with the anti-inflammatory situation induced by chronic physical exercise (MA; HE; QIANG, 2013).

A recent study demonstrated that TLR4 deficiency protected rodents from high-fat diet (HFD)-induced obesity (PIERRE; DELDICQUE; BARBÉ; NASLAIN *et al.*, 2013). In the same study, WT mice after HFD increased levels of BiP, sXBP1 and CHOP in skeletal muscle, unlike what was reported in *Tlr4*$^{-/-}$ mice, in which the diet had no effect on ER stress markers. Thus, the authors report that TLR4 deficiency leads to protection of the mouse against ER stress (PIERRE; DELDICQUE; BARBÉ; NASLAIN *et al.*, 2013). However, there are still no data in the literature regarding the roles of TLR4 in the ER stress after physical exercise. Therefore, the aim of the study was to verify the responses of ER stress markers in TLR4 KO mice and WT mice after acute and chronic physical exercise. The thesis was divided into two manuscripts.

2. MANUSCRIPT 1

Review Article:

MARAFON, Bruno B. et al., Muscle endoplasmic reticulum stress in exercise. **Acta Physiologica**, v. 235, n. 1, p. e13799, 2022. (DOI: 10.1111/apha.13799).

Muscle endoplasmic reticulum stress in exercise

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ABSTRACT

The endoplasmic reticulum (ER) is an organelle responsible for the post-translational folding and modification of proteins. Under stress conditions, such as physical exercise, there is accumulation of misfolded proteins. The increased load of proteins in the ER results in ER stress, which activates the unfolded protein response (UPR). UPR is comprised of three parallel pathways, responsible for ensuring the quality of secreted proteins. Scientific studies show that resistance or endurance acute physical exercise can induce ER stress and activate the UPR pathways. On the other hand, regular moderate-intensity exercise can attenuate the responses of genes and proteins related to ER stress. However, these positive adaptations do not occur when exercise intensity and volume increase without adequate rest periods, which is observed in overtraining. The current review discusses the frontier-of-knowledge findings on the effects of different acute and chronic physical exercise protocols on skeletal muscle ER stress and its metabolic consequences.

INTRODUCTION

The most abundant tissue of the human body is skeletal muscle, comprising approximately 40% and 30% of the total body weight of men and women, respectively[1]. Skeletal muscle is a highly organized tissue, composed of systematic arrangements of protein myofilaments (i.e., myosin and actin) responsible for muscle contraction and relaxation, a result of the release of calcium ions (Ca^{2+}) from its reservoir and adenosine triphosphate hydrolysis. In addition, skeletal muscle can transform chemical energy into mechanical energy and heat production. Therefore, this tissue is essential for locomotion, posture, thermoregulation, and metabolism[2]. Skeletal muscle contains 50-75% of all proteins in the entire organism and, for this reason, uses several pathways that regulate the cellular protein content[2]. A crucial organelle responsible for these events is the endoplasmic reticulum (ER)[3].

The ER is an organelle responsible for the translation of most body proteins, post-translational folding and modification of proteins, and formation of protein complexes. Therefore, the newly synthesized proteins enter the ER lumen as unfolded polypeptide chains and must be adequately folded before reaching their targets[4]. Protein homeostasis imbalance leads to misfolded proteins inside the ER and their accumulation leads to "ER stress"[5]. To cope with this stress, eukaryotic cells develop the unfolded protein response (UPR), which adjusts the ability to fold proteins and attenuates the translation machinery, preventing further accumulation of unfolded/misfolded proteins in the ER and ensuring the quality of secreted proteins[6].

The ER normally processes the protein in an orderly manner. However, a simple Ca^{2+} depletion (when [Ca^{2+}] falls to 50-100μM) can impair the work of chaperones in the folding of misfolded proteins and lead to ER stress[7,8]. Therefore, depending on the condition, the UPR activation can produce beneficial or deleterious effects. For example, chronic ER stress contributes to skeletal muscle loss[9] and insulin resistance (IR), which can lead to type 2 diabetes mellitus (T2DM)[10]. Furthermore, high levels of UPR markers have been found in models of metabolic disorders[11,12] and aging[13,14]. Thus, several physiological and/or pathological stimuli can disrupt ER homeostasis. However, the deletion or inhibition of some of these markers also seems to harm skeletal muscle[15-17].

Therefore, many conditions can induce ER stress and activate UPR, one of which is physical exercise. Physical exercise or exercise is a planned, structured, and repetitive bodily movement that aims to improve or maintain physical fitness components[18]. Acute exercise increases the unspliced and spliced forms of X-box binding protein 1 (*Xbp1u, Xbp1s*) mRNAs, CCAAT enhancer-binding protein homologous protein (CHOP), and activating transcription

factor 4 (ATF4) protein. In addition, acute exercise can increase the protein levels of BiP (binding immunoglobulin protein), also known as GRP78 (78 kDa glucose-regulated protein), and other indicators of ER stress[17,19,20]. Therefore, physical exercise increases the levels of proteins and genes related to the protein-folding capacity of the ER in skeletal muscle.

It is already known that regular moderate-intensity exercise leads to adaptations that benefit the skeletal muscle against possible subsequent stresses. Wu *et al.*[17] demonstrated that chronic endurance exercise induces adaptations in mouse skeletal muscle and attenuates ER stress. However, when regular exhaustive exercise is performed without adequate rest periods, rodents fail to obtain these positive adaptations[21]. This brief review describes the responses of ER stress to acute and chronic physical exercise protocols in both humans and rodents, highlighting how intensity and volume generate positive adaptations to improve ER ability to cope with stress or damage.

ER STRESS AND UPR SIGNALING

The UPR is comprised of three parallel pathways. In each branch, there is an integral protein residing in the ER membrane, namely inositol-requiring enzyme 1 (IRE1; α and β subunit), protein kinase R-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6; α and β subunit)[22]. Under normal conditions, these three transmembrane proteins are associated with BiP in their intraluminal domains and are consequently inactive[23]. BiP is a chaperone protein involved in numerous functions, such as translocation and assistance in assembling and folding newly synthesized proteins. Therefore, under stress conditions, the accumulation of misfolded proteins and the increased load of proteins in the ER result in BiP dissociation from its sensors[6]. These events lead to the oligomerization and activation of PERK and IRE1α, and the translocation of ATF6α to the Golgi apparatus, where it will be processed into its active form[24].

Activation of the UPR aims to activate signaling pathways that will ensure cell survival. These pro-survival mechanisms include decreasing the influx of proteins to the ER, increasing protein components that ensure the folding and quality control of secreted proteins, and degrading misfolded proteins through ER-associated degradation (ERAD; pathway along which the misfolded proteins are transported from the ER to the cytosol for proteasomal degradation). When it is not possible to reduce the ER stress and restore protein homeostasis, the UPR follows another path, triggering apoptosis[25]. This section provides an overview of the signaling pathways after activation of the UPR. Figures 1 and 2 summarize these processes.

Protein kinase R-like endoplasmic reticulum kinase (PERK)

The cytoplasmic portion of PERK undergoes trans-autophosphorylation by oligomerization and its activation proceeds in the eukaryotic translation initiation factor 2 subunit-α (eIF2α) phosphorylation (peIF2α), leading to the transient attenuation of protein synthesis[26,27]. This phosphorylation limits the influx of newly synthesized proteins in the ER lumen, preventing its protein misfolding load. The peIF2α can also activate translation of selective mRNAs, including the ATF4 transcription factor that regulates the UPR-related genes, including growth arrest and DNA damage-inducible 34 (*Gadd34*) and DNA damage-inducible transcript 3 (*Ddit3*)[28]. *Gadd34* is upregulated to dephosphorylate eIF2α, comprising a negative feedback loop to reverse PERK-mediated translational attenuation[29]. *Ddit3* mRNA encodes the CHOP protein, a transcription factor that activates genes involved in apoptosis[30,31]. Therefore, sustained ATF4 expression contributes to the induction of apoptosis.

Inositol-requiring enzyme 1 (IRE1α)

As a type I transmembrane protein, IRE1α contains an amino (N) terminal in the luminal domain of ER, a transmembrane segment, and a cytoplasmic region. The cytoplasmic region encompasses a protein kinase domain followed by a carboxy (C) terminal extension[32]. In the presence of ER stress, BiP dissociates from IRE1α allowing its oligomerization, and activating its endoribonuclease domain. Then, IRE1α promotes unconventional mRNA splicing of *Xbp1u* mRNA, removing its intron, and splicing the exons to generate the *Xbp1s* mRNA. This mature mRNA is translated to produce a potent transcription factor, XBP1s protein, which positively modulates UPR-related genes linked to protein folding, secretion, ERAD, and lipid synthesis[22]. Under ER stress, XBP1s also modulate phospholipid synthesis, which is required for ER membrane expansion[33].

IRE1α can act beyond its nucleolytic activity. For example, accumulation of misfolded proteins in the ER can activate the c-Jun-N-terminal kinase (JNK), and this activation occurs through the interaction between IRE1α and tumor necrosis factor (TNF) receptor-associated factor 2 (TRAF2), a member of the TNF family.[34] The hyperactivation of JNK leads to suppression of insulin receptor signaling, systemic insulin resistance, which can lead to T2DM[10]. TRAF2 also plays an important role as an adapter molecule that recruits procaspase-12 and activates caspase-12, an essential protein for ER stress-induced cell-death[35]. Therefore, the IRE1α arm interacts with components of cell death independently of its RNase activity.

Activating transcription factor 6 (ATF6α)

ATF6α, unlike IRE1α and PERK, is a type II transmembrane glycoprotein. It is responsible for the positive regulation of chaperones, protein folding, and components of the ERAD pathway. ATF6α has a stress-sensitive C-terminal in the luminal domain and an N-terminal with a transcriptional factor in the cytosolic domain[36]. Under ER stress conditions, ATF6α exposes two Golgi-localization sequences within the ER-luminal domain, requiring its translocation to the Golgi apparatus, where it will be cleaved by resident proteases, releasing the 50 kDa N-terminal cytoplasmic portion (ATF6-N)[37]. The ATF6-N then moves to the nucleus to induce gene expression, such as heat shock protein family A member 5 (*Hspa5*; the gene that encodes the BiP protein) and *Xbp1u* mRNA, contributing to proteostasis and an increase in the regulatory production of the IRE1α arm[38].

Furthermore, ATF6α seems to be an important partner for the transcriptional peroxisome proliferator-activator receptor gamma coactivator-1 alpha (PGC-1α) in skeletal muscle[17]. As a member of the PGC-1 family and highly expressed in tissues with a high density of mitochondria, PGC-1α is a coactivator that binds to transcription factors and potentiates these transcription factors[39,40]. Transgenic expression of PGC-1α significantly induces chaperones, *Hspa5* and *Hsp90b1* (heat shock protein 90 beta family member 1; the gene that encodes GRP94) mRNAs, and the ER markers, *Atf3* and *Ddit3* mRNAs[17]. Moreover, PGC-1α can interact with and coactivate ATF6-N to regulate the UPR. Induction of the UPR genes by PGC-1α is almost completely abolished in the absence of ATF6α[17]. Therefore, increased PGC-1α promotes UPR gene expression, at least in part, through the coactivation of ATF6-N.

ER STRESS AND ACUTE PHYSICAL EXERCISE

Physical activity is defined as any bodily movement produced by skeletal muscles that requires energy expenditure. For this reason, everybody performs physical activity to sustain life[18]. When physical activity becomes structured and planned, using the correct energy pathways in order to improve or maintain the components of physical fitness, this is called physical exercise[18]. Stress can be caused by physical exercise, and scientific studies show that acute exercise, independent of the type, can induce ER stress and activate the UPR pathways[17,19,41-43]. When the exercise is performed only once, it is called acute physical exercise.

Regarding acute resistance exercise, there are only three articles reporting its effects on ER stress[41,43,44]. A session of knee extension exercise (75% of the maximum load) increased the protein contents of BiP, PERK, and IRE1α in young and older individuals, but did not find

differences in the eIF2α phosphorylation, or in *Atf4* and *Ddit3* mRNA expressions, compared to baseline levels[43]. It is known that PERK activation leads to eIF2α phosphorylation, which reduces the global protein synthesis to relieve the ER load[27]. In addition, peIF2α leads to the translation of ATF4, which is responsible for the transcription of *Ddit3* mRNA[6]. These results suggest that this type of exercise stimulates specific pathways that generate UPR proteins, but not the pathway that attenuates protein synthesis[43].

Similar results were found in other studies[41,44]. Hentila *et al.*[44] verified elevated contents of BiP, PERK, and ATF4 proteins, gene expressions of *Xbp1u* and *Xbp1s*, and no differences in *Ddit3* mRNA 48h after a 5 x 10 repetition maximum (RM) on a leg press device for young men. For the older male group, only the ATF4 protein was upregulated[44]. Similarly, in the tibialis anterior (TA) muscle of adult and older rats, acute unilateral electrical stimulation used to verify ER stress markers increased the BiP protein content at both ages and increased eIF2α phosphorylation in the older but not adult rats. Furthermore, the authors did not find differences in CHOP protein. However, the levels were higher in old rats[41].

Taken together, these investigations[41,43,44] show that acute resistance exercise leads to higher levels of BiP protein, a chaperone with the ability to properly fold newly synthesized or misfolded proteins in the ER lumen. The authors suggest that these increases are an attempt to relieve stress. The chronic activation of PERK increases the expression of CHOP through the ATF4 pathway[4]. The overexpression of CHOP protein induces apoptosis and, at least in some cellular contexts, inhibits expression of the gene that encodes the anti-apoptotic protein B-cell leukemia/lymphoma 2 (Bcl-2)[24]. Although PERK and ATF4 increased, CHOP and *Ddit3* levels were not modulated, indicating that this type of exercise probably does not induce apoptosis cellular signaling.

Aging is associated with a progressive decline in strength and muscle mass. Both begin to decay after 40 years of age and accelerate with aging[45]. Indeed, muscle mass can decline at a rate of almost 1% per year in aging, and muscle strength can decrease even faster[46-48]. Moreover, aging leads to impairment of mitochondria[49], and increases in arterial stiffness and cardiovascular disease[50], and in ER stress[14]. However, although the disuse of skeletal muscle leads to muscle atrophy, this loss in muscle mass can be reversed upon return to normal weight-bearing activity[51]. The authors suggest that age-related ER stress may limit protein synthesis[41] and the induction of UPR could be more important for maintaining skeletal muscle homeostasis than regulating muscle growth or strength adaptations in response to resistance exercise[44].

The transcription of *Xbp1u* is regulated by the ATF6-N in the cell nucleus. Therefore, IRE1α catalyzation removes a 26-nucleotide intron from this gene, creating the spliced form,

Xbp1s. In response to ultra-endurance exercise, ultramarathon runners presented increased mRNA levels of *Xbp1u* and *Xbp1s* 3 hours after 200km of running, suggesting that this exercise type leads to ER stress by activating ATF6-N and IRE1α. Despite the high exercise volume, the authors did not demonstrate activation of the PERK signaling pathway but verified higher expression of BiP protein content[20]. The authors suggest that increased BiP levels may be interpreted as an acute adaptation to alleviate the internal environment of the ER. The study was the first to demonstrate that endurance exercise activates ER stress. However, exercise intensity and food intake were not controlled, and there was no control group which did not perform the exercise.

When the exercise is performed until exhaustion and the animal is unable to continue the stimulus, some authors denominate this as an exhaustive exercise[17,19]. Performing exhaustive exercise also induces ER stress. Wu *et al*.[17] verified an increase in *Atf4*, *Ddit3*, *Hspa5*, and *Xbp1s* genes, as well as the protein content of BiP and eIF2α phosphorylation, in the quadriceps muscle of C57/Bl6 mice after exhaustive exercise (warm-up speed of 5m/min for 5 min with 5m/min increments every 5 min until exhaustion) on a treadmill (0% grade or inclined 10% uphill). Subsequent analyses indicated that similar activation of UPR occurs in the gastrocnemius muscle. At the same time, muscles not directly involved in the exercise (i.e., spine erector) are largely unaffected. Therefore, the authors consider that mechanical stress and local metabolic changes exerted by skeletal muscle samples have an essential role in UPR activation[17].

In addition, transcriptional PGC-1α, Hexokinase II (HKII), and pyruvate dehydrogenase kinase isozyme 4 (PDK4) were also increased to a similar extent as the UPR marker genes. These gene expressions are known to be induced by endurance and resistance exercise and directly regulate metabolic changes in skeletal muscle[52-55]. Thus, the authors suggested that the activation of the UPR in this context may have physiological consequences[17].

The contraction of the skeletal muscle produces and releases interleukin 6 (IL-6) into the circulation[56]. IL-6 can act as an anti-inflammatory cytokine by stimulating the appearance of other anti-inflammatory cytokines, such as IL-1 receptor antagonist (IL-1ra) and IL-10, and by suppressing the tumor necrosis factor-alpha (TNF-α)[57]. However, IL-6 is most often classified as a proinflammatory cytokine. For example, Kim *et al*.[20] observed that ultra-endurance exercise activated *Il6* and *Tnf* mRNA, inducing intense proinflammatory stress in skeletal muscle in humans.

To elucidate the relationship between inflammation and ER stress, Pinto *et al*.[19] investigated the impact of an acute session of exhaustive exercise on protein contents related to

ER stress in IL-6 knockout (IL-6 KO) mice. In this investigation, the authors observed no differences between the wild-type (WT) and IL-6 KO mice at the basal time. However, one hour after exercise, BiP protein levels were higher in the IL-6 KO group than the WT group. Furthermore, compared to WT mice, the oscillation patterns of BiP over time in the extensor digitorum longus (EDL) and soleus, as well as of the peIF2α/eIF2α ratio in soleus muscle, were attenuated in the IL-6 KO group. As the IL-6 KO mice exhibited attenuated ER stress[19], the authors suggest that IL-6 may be related to ER stress homeostasis. Future studies are necessary to understand the IL-6 and ER stress relationship.

Different intensities and volumes of treadmill running modulate the UPR, but according to Jamart *et al*.[42], this activation depends on nutritional condition. A single bout of treadmill running with low intensity (i.e., approximately 55% VO_{2max}) in the fed state increased the *Atf4* and *Xbp1s* expressions and eIF2α phosphorylation but decreased *Ddit3* expression. When the exercise was performed in the fasted state, no differences in *Xbp1s* and *Ddit3* expressions and eIF2α phosphorylation were visualized. For this reason, the authors conclude that low-intensity exercise cannot induce ER stress in fasted circumstances. Further studies should investigate the mechanisms responsible for the lack of UPR activation during exercise in the fasted state[42].

The previously mentioned studies indicated that acute physical exercise leads to ER stress and activates all UPR branch pathways. Independent of the exercise type, there is an increase in BiP protein and eIF2α phosphorylation. However, it seems that acute resistance exercise does not increase CHOP levels[41,43,44]. The authors mentioned that increased levels of BiP occur as an acute adaptation to properly folding proteins and alleviate the ER lumen, as well as that higher levels of eIF2α phosphorylation, serve to decrease global protein synthesis[17,20,43]. However, these increases cannot attenuate the activations of other genes and proteins related to ER stress. Table 1 summarizes the investigations regarding the effects of acute resistance exercise protocols on ER stress and UPR activation.

ER STRESS AND CHRONIC PHYSICAL EXERCISE

When acute physical exercise is performed regularly, it is called regular or chronic exercise. Regular moderate-intensity exercise induces adaptations, promoting benefits to the organism against possible subsequent stresses. For instance, endurance training triggers physiological and metabolic adaptations to increase the ability to sustain the highest speed for a given distance or time[58]. In comparison, resistance training leads to adaptations that shift the balance between protein synthesis and degradation, inducing skeletal muscle growth. When considering ER responses, these are not different. Several markers of ER stress are associated

with aging[13], T2DM[59], and muscle myopathies[60]. The literature shows that chronic exercise attenuates genes and protein levels related to ER stress[17,59,61], and can play an essential role in metabolic health. In contrast, the imbalance between high load exercise sessions and sufficient recovery periods (8-weeks of a running overtraining protocol as described in Pereira *et al.*[62]) does not allow positive adaptations to ER stress[21]. Therefore, does the ER stress adaptation depend on the exercise training intensity?

To the best of our knowledge, there is only one study comparing the influence of chronic exercise intensity on ER stress. In that investigation, Kim *et al.*[63] separated male Sprague-Dawley rats into low-intensity training (LIT), high-intensity training (HIT), and control groups. HIT presented lower levels of BiP content, and *Atf4* and *Ddit3* expressions compared to the control group. At the same time, there were no significant differences for the PERK, BiP, and CHOP contents, or *Atf4* and *Ddit3* expressions, between the LIT and control group. The authors suggest that regular exercise with adequate intensity may diminish apoptotic signaling and be considered a protective mechanism, generating muscle adaptation against ER stress[63].

Studies suggest that the development of IR in skeletal muscle may occur due to ER stress[64,65]. The IRE1α pathway of UPR can mediate the activation of JNK, a protein that inhibits the phosphorylation of insulin receptor substrate, mediating IR[66]. For example, mice fed a high-fat diet exhibit higher levels of BiP, IRE1α, and pPERK content, as well as *Xbp1s*, *Xbp1u*, *Atf4*, and *Ddit3* expression[64]. At the same time, a chemical chaperone drug, 4-phenylbutyrate (4-PBA), that inhibits ER stress has been found to improve insulin receptor signaling in diabetic mice[10]. Even though the ER stress adaptation occurred only in the HIT group, there were no differences in the plasma concentration of insulin and glucose between the trained mice. Furthermore, level in both trained groups were lower compared with control mice[63]. Taken together, LIT and HIT seem to be able to improve IR; however, more studies are needed to clarify the relationship between this improvement and ER stress.

As well as HIT exercise, moderate-intensity training seems to lead to adaptations in the ER pathway[17,59,61]. Similar increases of *Hspa5*, *Hsp90b1* (also known as glucose-receptor protein 94 - GRP94), and DnaJ homolog subfamily B member 9 (*Dnajb9*, also known as endoplasmic reticulum DnaJ homolog 4 - ERdj4) genes were observed in skeletal muscle of sedentary mice (naïve) and mice that underwent a previous training protocol (trained) after an equal distance treadmill exercise[17]. However, *Ddit3* and *Xbp1s* were not modulated in trained mice but were elevated in naïve mice. In addition, *Atf4* expression was markedly lower in the trained group[17]. These results suggest that moderate exercise may lead to adaptation of UPR by

downregulation of PERK and IRE1α pathways, protecting the skeletal muscle against further stress.

T2DM results in impairment in glucose tolerance and ER stress, and abnormal mitochondrial quality associated with reduced mitochondrial-associated membrane (MAM; zones of interaction between mitochondria and ER network). These events can impair energy metabolism in skeletal muscle[67]. Swimming moderate exercise showed attenuation of ER stress through ATF6α and pIRE1α branches in skeletal muscle of T2DM mouse but did not change levels of PERK phosphorylation in the gastrocnemius. Furthermore, this type of exercise significantly improved glucose tolerance and tended to alleviate mitochondrial dysfunction and decrease MAM caused by T2DM. In summary, the authors indicate that T2DM induced skeletal muscle ER stress, which was mitigated by regular moderate-intensity exercise[59].

The aging process is associated with molecular changes that promote muscle atrophy, an increase in ER stress markers, mitochondrial dysfunction, and activation of apoptosis pathways[3]. Aging also increases thioredoxin (TRX) isoform 1 protein (TRX-1; mainly located in the cytosol) and thioredoxin interacting protein (TxNiP) expression[61], two critical components of redox signaling and regulation of protection against oxidative stress[68]. Twenty-one months of wheel-running activity, despite not leading to significant differences in BiP protein, attenuated the levels of CHOP content in the skeletal muscle of old exercised mice compared to the sedentary group. These animals also demonstrated partly restored age-related decreases in skeletal muscle mass. In addition, the authors detected an increase in the TRX-1/TxNiP ratio in response to long-term exercise[61]. The TRX system, composed of NADPH, thioredoxin reductase (TrxR), and TRX (12 kDa ubiquitous protein), protects cells from oxidative stress through its disulfide reductase activity and exerts protective effects against apoptosis, whereas TxNiP inhibits the TRX system and promotes apoptosis[69]. In this regard, higher levels of TRX and a decrease in TxNiP expression could help prevent various pathologies. Belaya *et al.*[61] reported a negative and robust correlation between the TRX-1/TxNiP ratio and CHOP levels. Thus, the authors concluded that exercise might lead to ER stress adaptation in skeletal muscle, protecting this tissue against future stress.

Taken together, the previously mentioned investigations indicate that with proper intensity, long-term exercise may decrease apoptotic signaling in skeletal muscle and act as a protective mechanism for upcoming ER stress. The authors strongly suggest that moderate and high-intensity exercise can lead to ER adaptation and skeletal muscle protection from further stresses[17,63]. In contrast, mice performing an exhaustive treadmill running protocol presented activation of UPR markers after eight weeks of training, increasing ER stress. Even after two

weeks of complete rest, these rodents were not restored from the excessive training-induced ER stress[21]. As this same excessive exercise protocol led to DNA damage[70], hypertrophy inhibition[71], inflammation[72], and insulin signaling impairment[73] in skeletal muscle samples, the authors suggest that the imbalance between excessive training load and inadequate recovery may lead to a possible pathological condition of ER stress.

Only one investigation has verified the ER stress after resistance training. The authors selected young and older untrained men to participate in training twice per week for 21 weeks. Before and after this period, they collected vastus lateralis (VL) samples to verify ER stress markers. Unlike acute resistance exercise, the training program did not alter the relative content of UPR markers or ER chaperones in either group[44]. The training program increased muscle size, strength, and isometric force recovery, but these regulations were not associated with UPR. In response to resistance exercise, the authors suggest that UPR may be more important for maintaining skeletal muscle homeostasis than regulating muscle growth or strength adaptations[44].

It is essential to point out that an incremental load test should be applied before performing chronic physical exercise protocols to guarantee the same stressful stimulus for different experimental groups. Among the previously described investigations about training effects on skeletal muscle ER stress, only Pereira *et al.*[21] prescribed individualized exercise intensity in mice. Therefore, further studies aiming to investigate the impact of acute and chronic physical exercise on ER stress in skeletal muscle should be aware of this scientific literature gap. Table 2 summarizes the investigations regarding the effects of chronic physical exercise protocols on ER stress and UPR activation.

FINAL CONSIDERATIONS

It is known that the ER stress-activated UPR pathway is essential for skeletal muscle remodeling and homeostasis. While chronic activation of ER stress contributes to myopathy, atrophy, and IR, some evidence suggests that activation of the UPR pathway plays an important role in satellite cell homeostasis and myogenesis[10,11,15,16,74].

This review highlights that, regardless of type, acute physical exercise generates stress in the body, leading to the activation of all three arms of the UPR signaling pathway, either by the increase in BiP, ATF4, and peIF2α proteins or by the transcription of genes such as *Xbp1u*, *Xbp1s*, *Hspa5*, *Ddit3, Atf4,* and others. On the other hand, humans and animals that performed a chronic physical exercise program maintained the protein levels of BiP, PERK, IRE1α, CHOP, or even presented reductions in these proteins, and some ER stress-related genes, such

as *Ddit3*, *Atf4*, *Xbp1u,* and *Xbp1s*. Thus, chronic physical exercise can cause adaptation in the ER, protecting this organelle from becoming overloaded and avoiding cell death. The main findings presented in this review are summarized in figure 2.

In addition, the training intensity, volume, and proper recovery period seem to affect this adaptation. While moderate and high-intensity exercises led to beneficial adaptations to the ER, low-intensity stimulus seems to generate no changes in the molecular contents related to the ER compared to the sedentary group[17,59,61,63]. Furthermore, the imbalance between excessive training load and insufficient rest generates negative adaptation and induces pathological ER stress[21].

There are few studies about this topic and even less when considering ER stress in the skeletal muscle of humans. In addition, some of these scientific studies demonstrate limitations, such as no control of the food intake or the exercise intensity[20], nonexercised group or nontrained limb[20,44], and skeletal muscle of the animal model used (higher proportions of fast-twitch fibers compared to the same muscle of geriatric population)[41], for example. Therefore, further studies should pay attention to these current gaps. Furthermore, it is known that toll-like receptor 4 (TLR4) knockout mice are protected against ER stress[75], and physical exercise can reduce the TLR4 in the cell[76]. As inflammation seems to affect the ER stress related to physical exercise[19] and the TLR4 pathway can release pro-inflammatory cytokines[77], future studies should investigate the relationship between TLR4 and ER stress after exercise in skeletal muscle. In conclusion, there is a long way to go when investigating physical exercise and its relationship with the ER.

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Competing interests

The authors declare that they have no competing interests.

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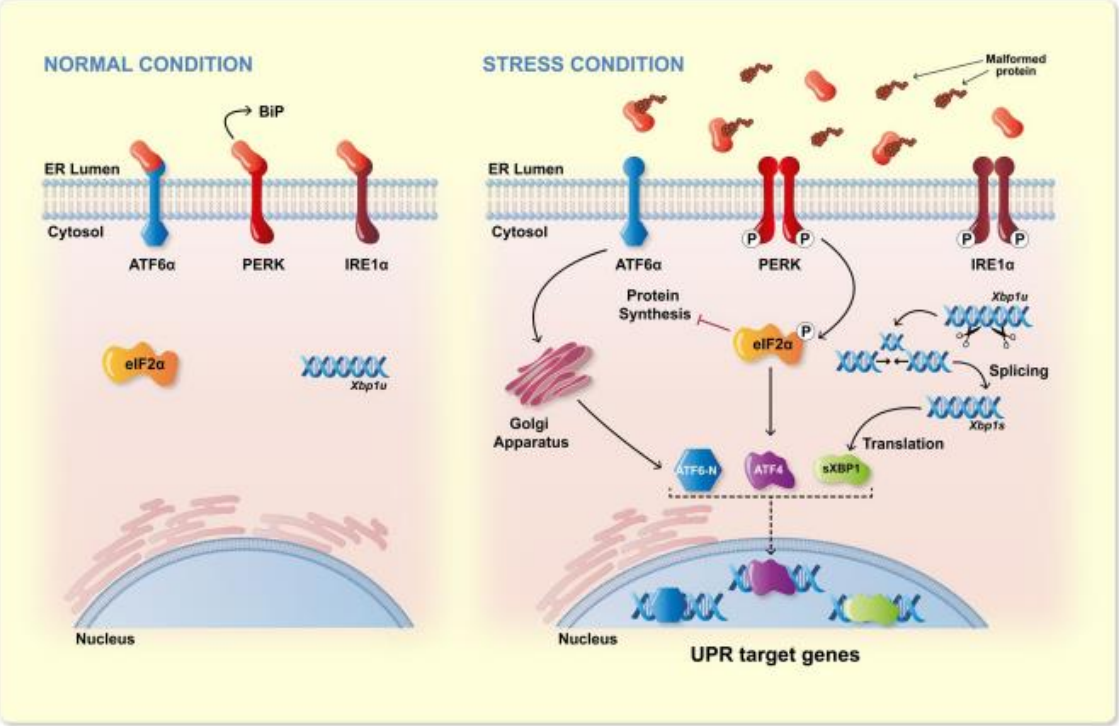

Figure 1. Schematic model of the ER stress mechanism. Under normal conditions, three transmembrane proteins are associated with BiP in their intraluminal domains and are consequently inactive. Under stress conditions, the accumulation of malformed proteins in ER results in BiP dissociating from its sensors. These events lead to the oligomerization and activation of PERK and IRE1α and translocation of ATF6α to the Golgi apparatus, where it will be cleaved by resident proteases, releasing the 50 kDa N-terminal cytoplasmic portion (ATF6-N). Activation of PERK leads to eIF2α phosphorylation, responsible for the inhibition of protein synthesis and activation of ATF4. IRE1α promotes the endonucleolytic cleavage of *Xbp1u* mRNA, removing the intron and splicing the exons. The mature mRNA (*Xbp1s*) is translated to produce a potent transcription factor, the sXBP1. ATF6-N, ATF4, and sXBP1 enter the nucleus to transcript ER stress-related genes. If the UPR cannot reestablish homeostasis, undergoing prolonged ER stress, the cells will undergo apoptosis.

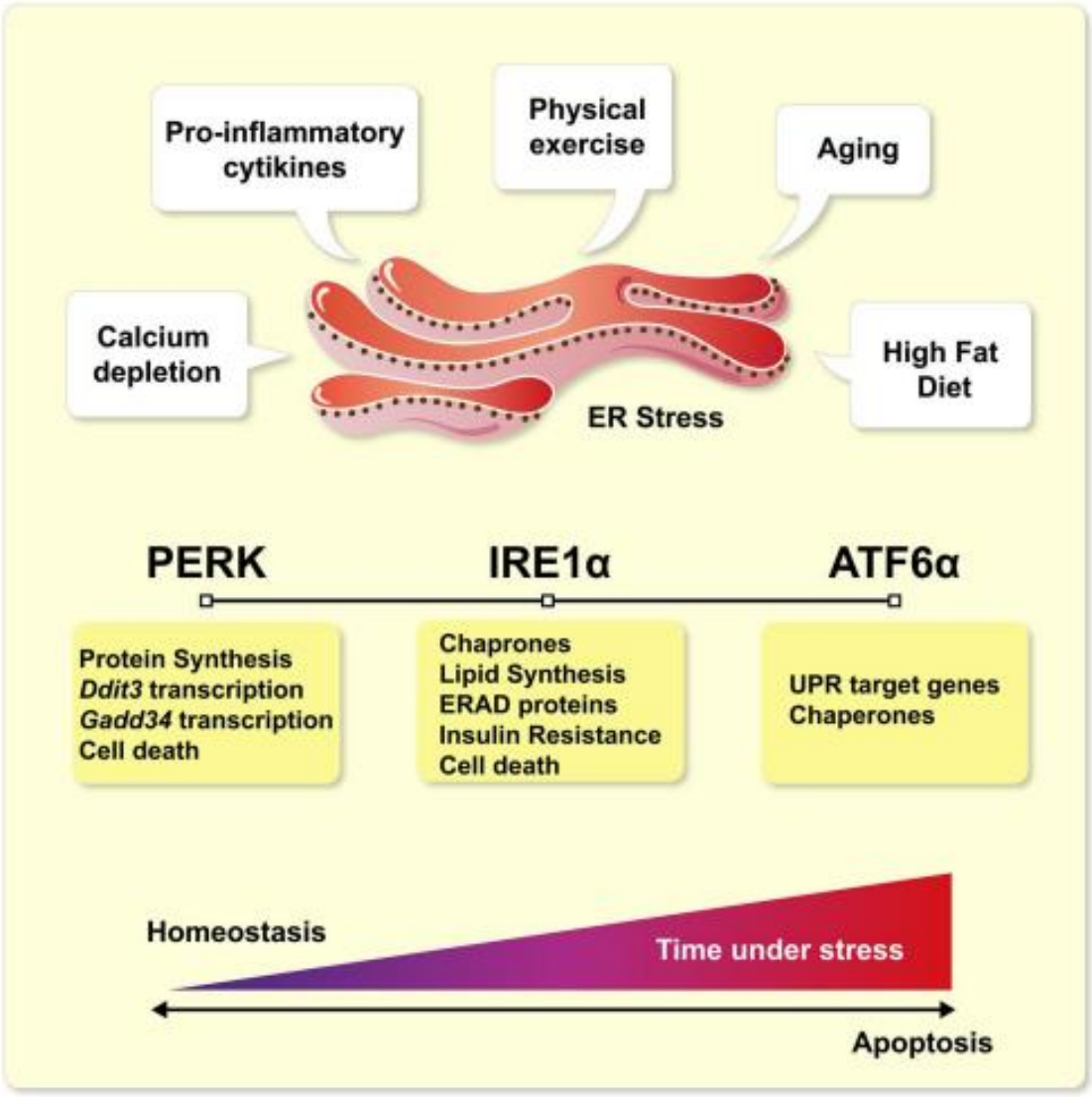

Figure 2. Schematic representation summarizing the ER stress and consequences of the UPR activation. Conditions such as calcium depletion, pro-inflammatory cytokines, aging, high-fat food, and exercise lead to ER stress. These conditions activate the UPR response through PERK, IRE1α, and ATF6α transmembrane proteins to restore homeostasis. If the stress is prolonged, represented by the red color, signaling pathways will be activated to transcription genes and encoding proteins related to cell death.

Table 1. Main results regarding acute physical exercise and ER stress.

Reference	Sample	Exercise Protocol	Main Results
West et al., 2019	- Adult (10 months) and Old (30 months) male Fischer 344-Brown Norway rats. - Tibialis anterior (TA) muscles were removed 6, 18, and 48h after exercise.	- Acute unilateral electrical stimulation of the sciatic nerve. - After stimulation, animals were kept in their cages with *ad libitum* access to food.	- ↑ BiP 48h after electrical stimulation in adult and old. - ↔ CHOP in adult and old, but higher in old rats. - ↑ peIF2α 18h after stimuli in old rats.
Pinto et al., 2019	- C57BL/6 mice and IL-$6^{-/-}$ mice (8 wks old): - 22±2°C on 12:12 light-dark inverted cycle. - Extensor digitorum longus (EDL) and soleus muscle were extracted before exercise, 1h, and 3h after exercise.	- Incremental load test to compare the exhaustive time between the WT and IL-6 KO groups. - Acute exhaustive exercise on the treadmill. - 22m/min, 10° uphill inclination for 90 min.	- CHOP and cleaved/total caspase12 ratio in EDL as well as ATF6 and CHOP in soleus were sensitive to the acute exercise protocol. - Compared to the WT, the oscillation patterns over time of BiP in EDL and soleus as well as of peIF2α/eIF2α in soleus were attenuated for the IL-6 KO group.
Hentila et al., 2018	- Untrained young (26±4 years) and older men (61±6 years). - Young group: exercised and nonexercised control. - Vastus Lateralis (VL) muscles were extracted.	- Resistance exercise (RE). - 5x10 repetition of 1RM performed on a leg press device.	RE (Young): - ↑ PERK, ↑ ATF4, ↑ BiP, ↑ pJNK, ↑ *Xbp1u* and ↑ *Xbp1s.* - ↑ *Ddit3* 1 hour after RE, but 48h Ddit3 decreased back to the baseline. RE (Old) - ↑ ATF4, ↔ PERK, ↔ BiP, ↔ IRE1α, ↔ PDI proteins.
Ogborn et al., 2014	- Younger (21±3 years) and older (70±4 years) untrained men. - VL muscles were extracted 3h, 24h, and 48h after exercise.	- Program for knee extensors. - Unilateral 1 RM protocol for both the leg press and knee extension. - Immediately after 1RM protocol → 4x10 repetitions of each exercise at 75% of 1RM with 2' between each bout.	- ↑ *Xbp1u*, ↑ *Xbp1s*, ↑ *Xbp1s/Xbp1u* ratio, ↑ *Ire1α,* and ↑ *Atf6* mRNAs after exercise. - ↑ BiP, ↑ PERK, ↑ IRE1α, ↔ peIF2α proteins after exercise. - ↔ *Ddit3*, ↔ *Atf4*, ↔ *Gadd34* mRNA 48h after exercise. - ↑ *Eif2s1* mRNA 24h and 48h after exercise. - No differences between ages.

Jamart et al., 2013	- Female C57BL6 mice (12 wks old). - 22°C on a 14h:10h light-dark cycle. - 4 groups (n = 9 per group): 1) Fed+Rest; 2) Fed+Run; 3) Fasted+Rest; e 4) Festad+Run.	- Three preliminary exercise bouts at 8 m/min for 10 min for familiarization with treadmill running. - Exercise group: ran for 90min, 10m/min (low intensity ± 55% VO_{2max}). - Fasted group were deprived of food access during the dark cycle. - Fasted+Run started the exercise 8 h after the beginning of food deprivation.	(Fed+Run) - ↑ *Atf4* and ↑ *Xbp1s* mRNA. - ↑ peIF2α. - ↓ *Ddit3* mRNA. (Fasted+Run) - ↑ *Atf4* mRNA. - ↔ *Ddit3* and ↔ *Xbp1s* mRNA. - ↔ peIF2α.
Wu et al., 2012	- C57BL/6 wild-type mice. - 3 groups: control (CT, sedentary mice), naïve (N; untrained mice), trained (T; trained mice). - Five hours after the acute session, quadriceps were extracted.	Treadmill running - 0% grade or 10% uphill. - warm up speed of 5m/min. - Every 5 min, speed increased by 5m/min until exhaustion or maximal speed 20m/min or 25m/min was reached.	- ↑ *Hspa5*, ↑ *Gadd34* mRNA, ↑ *Ddit3*, ↑ *Xbp1s*, ↑ *Atf3*, ↑ *Atf4* mRNA levels after exercise. - ↔ *Hsp90b1* and ↔ *Xbp1u* mRNA levels after exercise. - ↑ BiP and ↑ peIF2α levels after exercise.
Kim et al., 2011	- 8 men ± 44 years old. - Well-prepared ultramarathon runners. - VL muscle was extracted before and 3h after the race (same leg).	- Ultramarathon exercise. - 200km run.	- ↔ *Atf4* and ↔ *Ddit3* mRNA. - ↑ *Xbp1s* and ↑ *Xbp1u* mRNA. - ↑ BiP protein. - ↔ PDI, ↔ Calnexin.

Legend: ↑ = increase, ↓ = decrease, and ↔ = no difference.

Table 2. Main results regarding chronic physical exercise and ER stress.

Reference	Sample	Exercise Protocol	Main Results
Zhang et al., 2020	- 39 clean-grade male C57BL/6 mice (age, 4 weeks). - 4 groups: control (C), exercised (E), diabetic sedentary (D), and diabetic exercised (DE) group. - After exercise, animals fasted for 12h then gastrocnemius muscle was extracted.	- Swimming (depth of 50cm; 30±2°C). - E and DE swam for short periods every day for 5 days to get acclimated to swimming. - The mice swam for 1h per day, 5 days per week for 6 weeks.	- pIRE1α, ATF6, caspase 12 protein: ↑ D compared to C. - pIRE1α, ATF6, caspase 12 protein: ↓ DE compared to D. - pPERK protein did not change in either group.
Belaya et al., 2018	- 22 male ICR mice (Three-months-old). - 3 groups: young (Y), old sedentary (OS), and old exercise (OE). - TA and soleus muscles were extracted at 3 months of age (Y) and 24 months of age (OS and OE).	- The OE group had free access to an activity wheel (628mm circumference, 50mm wide running surface of wire mesh).	TA muscle - BiP, CHOP, PDI: ↔ OE compared to OS. Soleus muscle - BiP, PDI: ↔ OE compared to OS. - CHOP: ↓ OE compared to OS.
Memme et al., 2016	- 72 male Sprague-Dawley (SD) rats (12-18 wks of age). - Wire electrodes were used for electrical stimulation. - TA and EDL muscles were extracted 3h after contractile activity.	- Electrical stimulation of the common peroneal nerve for 3h/day followed by 21h of recovery. - 7 days of exercise.	- *Atf4* mRNA: ↑ days 2 and 3, returning to control levels by day 5. - *Xbp1s* mRNA: ↑ days 1-3; ↓ day 7. - *Hspa5*, *Ddit3* mRNA: ↑ throughout days 1-7. - CHOP protein: ↑ day 7. - BiP protein: remain unchanged until day 7.
Pereira et al., 2015	- 48 male C57BL/6 mice (age, 8 weeks). - 4 groups: control (CT), overtraining (OTR), OTR downhill running (OTR/down), and OTR uphill running (OTR/up). - After a short period of 6h, the EDL and soleus muscles were extracted.	- Incremental load test to prescribe the intensity of OT protocols. - 8-week running protocol; Increases in volume after week 4 and intensity after week 6. - Grade of treadmill: OTR (0%), OTR/up (14%) and OTR/down (-14%). - Performance evaluations were applied.	EDL - OTR/down: ↑ pPERK/PERK ↑ BiP, ↑ ATF6 ↑ pIRE1α/IRE1α, ↑ peIF2a/eIF2a compart to CT, end of wk 8. Soleus - In general, the protein levels were higher in OT groups compared to CT.

Kim et al., 2014	- 21 male Sprague-Dawley rats. - 3 groups: control (CT), low-intensity training (LIT), and high-intensity training (HIT). - After 3 days of training, the gastrocnemius muscle was extracted.	- Running adaptation (15 min, increasing speed from 0~15m/min, once per day for 5 days. - Protocol: 60 min of treadmill running, with 10° inclination, 5 days/week, for 5 weeks. Speeds: LIT (20m/min) and HIT (34 m/min).	- PERK protein: ↔ across the 3 groups. - BiP protein: ↓ HIT compared to CT. - *Atf3* mRNA: ↑ LIT, ↔ HIT compared to CT. - *Atf4* mRNA: ↓ HIT, ↔ LIT compared to CT. - *Ddit3* mRNA: ↓ HIT compared to CT and LIT. - CHOP protein: ↔ across the 3 groups.
Yang et al., 2014	- 5 and 8-week-old SD rats. - 3 groups: control, normal diet; high fat diet (HFD); HFD + exercise. - Soleus and gastrocnemius muscles were extracted.	- 12 weeks after introducing the HFD. - 8-week treadmill protocol: 60 min at 26m/min, 5 days/week.	- HFD increased BiP and CHOP. - Exercise prevented HFD-induced ERS: (↓ BiP and ↓ CHOP).
Wu et al., 2012	- C57BL/6 wild-type mice. - 3 groups: control (CT, sedentary mice), Naïve (N; untrained mice), Trained (T; trained mice). - After 5h after acute session, quadriceps were extracted.	Moderate Training - T: 5 sessions of 1h treadmill run/wk, 4 weeks. - N and T: ran one bout of equal distance.	- *Hspa5*, *Grp94*, *ERdj4*: ↑ similar extent in N and T. - *Ddit3*, *Xbp1* mRNA: ↑ 3-fold in T, ↑ 20-fold in N compared to CT. - *Atf4* mRNA: ↓ T compared to CT.

Legend: ↑ = increase, ↓ = decrease, and ↔ = no difference.

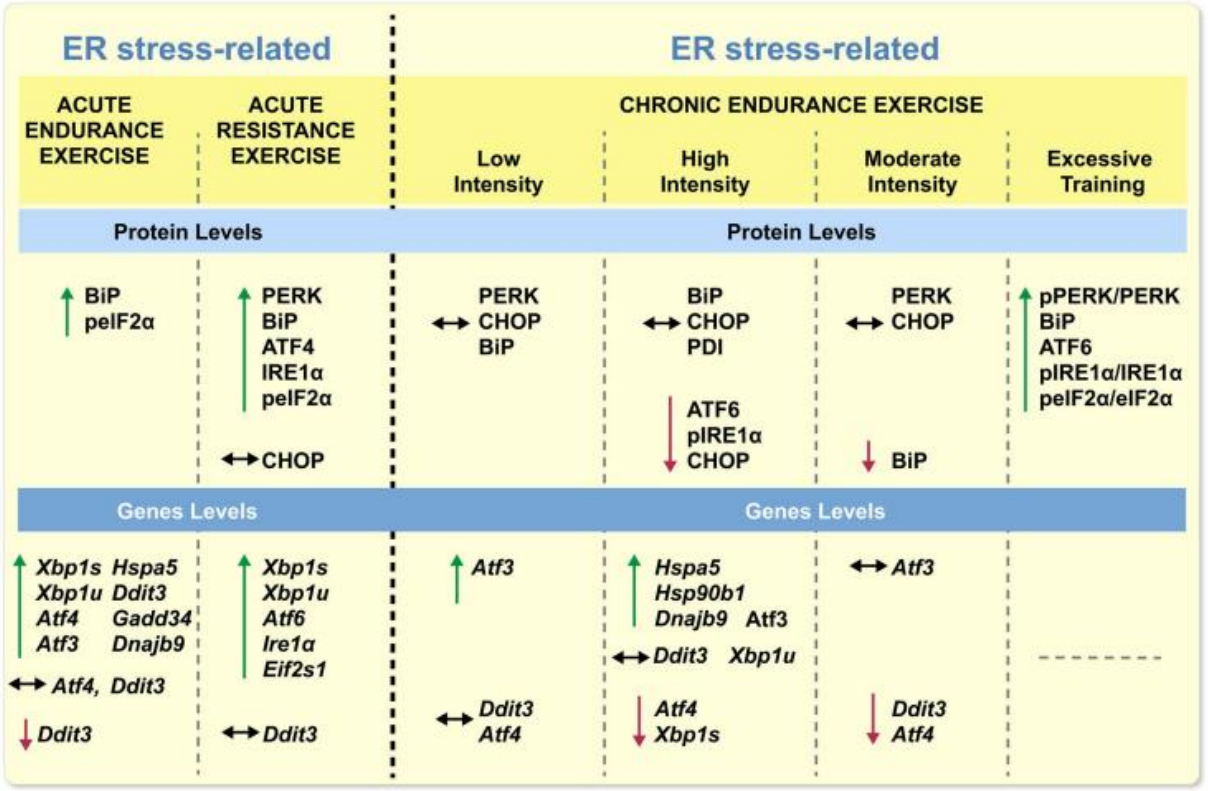

Figure 3. Schematic representation summarizing the main effects of acute and chronic physical exercise protocols on ER stress and UPR markers.

3. MANUSCRIPT 2

Original Paper:

Genetic ablation of Toll-like Receptor 4 protects against physical exercise-induced endoplasmic reticulum stress in skeletal muscle of mice.

(Submitted to the journal ***Cell Biochemistry and Function***).

Genetic ablation of Toll-like Receptor 4 protects against physical exercise-induced endoplasmic reticulum stress in skeletal muscle of mice

Running Title: *Tlr4* ablation protects against ER stress

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ABSTRACT

Many conditions can induce endoplasmic reticulum (ER) stress, such as inflammation and physical exercise. Toll-like Receptor 4 (TLR4) can trigger inflammation and ER stress events. However, there are still no data in the literature regarding the role of TLR4 in ER stress during exercise in skeletal muscle. Therefore, the current investigation aimed to verify the responses of ER stress markers in wild-type (WT) and *Tlr4* global knockout (KO) mice after acute and chronic physical exercise protocols. Eight-week-old male WT and KO mice were submitted to acute (moderate or high intensity) and chronic (4-week protocol) treadmill exercise. Under basal conditions, KO mice showed lower performance in the rotarod test, and increased eIF2α protein phosphorylation compared to WT animals. Acute moderate-intensity exercise increased BiP and CHOP protein in the WT group. After the acute high-intensity exercise, there was an increase in *Casp3* and *Ddit3* mRNA for the KO mice. Acute exercise increased the cleaved Caspase-3/Caspase-3 in the KO group regardless of exercise intensity. In response to chronic exercise, the KO group showed no improvement in any performance evaluation. The 4-week chronic protocol did not generate changes in CHOP, peIF2α/eIF2α, and cleaved Caspase-3/Caspase-3 ratio but reduced BiP protein compared to the KO-Sedentary group. These results demonstrate that the global deletion of *Tlr4* seems to protect the mice against ER stress but decreases their performance. The cleaved Caspase-3/Caspase-3 ratio may be activated by another pathway other than ER stress in *Tlr4* KO animals.

Keywords: Apoptosis, Knockout model, Physical exercise, Toll-like receptor, TLR4.

SIGNIFICANCE STATEMENT

The endoplasmic reticulum (ER) is responsible for translating and folding most of the proteins in our body. Protein homeostasis imbalance leads to malformed proteins in the ER lumen and their accumulation leads to ER stress. It is known that Toll-like Receptor 4 (TLR4) can trigger inflammation and TLR4 global knockout (KO) mice are protected against ER stress. Furthermore, physical exercise can reduce the TLR4 in the cell. As inflammation seems to affect the physical exercise-related ER stress, the present study investigated the relationship between TLR4 and ER stress after physical exercise in skeletal muscle.

INTRODUCTION

The post-translational folding and modification of proteins and the formation of protein complexes are some of the many functions of the endoplasmic reticulum (ER) (Eizirik, Cardozo & Cnop, 2007). The imbalance in protein homeostasis can lead to unfolded/misfolded proteins inside the ER, which accumulation induces a condition known as ER stress (Schröder & Kaufman, 2005). In turn, eukaryotic cells developed the unfolded protein response (UPR) to deal with stress, comprised of three parallel pathways. In each branch, there is an integral protein residing in the ER membrane, namely inositol-requiring enzyme 1 (IRE1α), protein kinase R-like endoplasmic reticulum kinase (PERK) or activating transcription factor 6 (ATF6) (Hetz, Zhang & Kaufman, 2020). These pathways adjust the ability to fold proteins, ensuring the quality of secreted proteins (Ron & Walter, 2007).

Under normal conditions, these three transmembrane proteins are associated with BiP (binding immunoglobin protein), also known as 78 kDa glucose-regulated protein (GRP78), in their intraluminal domains and consequently inactive (Gardner et al., 2013). Under stress conditions, the accumulation of misfolded/unfolded proteins and the increased load of proteins in the ER results in BiP dissociating from its sensors (Ron and Walter, 2007). These events lead to the oligomerization and activation of PERK and IRE1, as well as the translocation of ATF6 to the Golgi complex, which is processed into its active form. Thus, complex signaling pathways are required and triggered to relieve the stress (Kim, Xu & Reed, 2008).

A simple Ca^{2+} depletion can impair the work of BiP in the folding of unfolding protein (High et al., 2000; Primm, Walker & Gilbert, 1996). Therefore, conditions such as physical exercise can induce ER stress and activate UPR (Pinto et al., 2018; Kim et al., 2011; Wu et al., 2011; Pereira et al., 2016). Physical exercise is defined as a structured and planned body movement produced by skeletal muscles that use the correct energy pathways in order to improve or maintain the components of physical fitness (Caspersen, Powell & Christenson, 1985). Physical exercise increases the levels of ER stress markers such as BiP protein, genes levels of unspliced and spliced X-box binding protein 1 (*Xbp1u*, *Xbp1s*), activating transcription factor 4 (*Atf4*), heat shock protein family A member 5 (*Hspa5*; gene that encode BiP protein) and DNA damage-inducible transcript 3 (*Ddit3*; gene that encode CHOP protein) (Kim et al., 2011; Wu et al., 2011). Some of these results come together with higher interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) levels in skeletal muscle, which may explain the exercise-induced ER stress (Kim et al., 2011; Pinto et al., 2018). Pinto *et al*. (Pinto et al., 2018) tested wild-type and interleukin 6 (IL-6) KO mice in an exhaustive acute exercise session to study this hypothesis. The BiP protein and the phosphorylated and total eukaryotic translation

initiation factor 2α (eIF2α) (peIF2α/eIF2α) ratio were attenuated in the skeletal muscle of the IL-6 KO group. Thus, IL-6 seems to be related to the homeostasis of ER stress (Pinto et al., 2018).

Regarding its pro-inflammatory role, IL-6 can be mediated by the nuclear factor-kappa B (NF-κB) (Welc & Clanton, 2013). NF-κB is involved in various cellular processes, such as immunoinflammatory response, cytokine production, apoptosis, cell growth, and development (Gilmore, 2006; Werner, Barken & Hoffmann, 2005). The activation of this critical transcription factor is induced by the Toll-like Receptor 4 (TLR4), a transmembrane protein related to the innate immune system (Medzhitov, Preston-Hurlburt & Janeway Jr, 1997). TLR4 stimulation can be mediated by the lipid subunit A of the lipopolysaccharide (LPS), which is in part composed of fatty acids (Lien et al., 2001). It is known that endurance exercise raises the non-esterified fatty acids (NEFAs) extracellular levels, recognizing and activating TLR4 (Zbinden-Foncea et al., 2012). The decrease in TLR2/4 is often associated with the anti-inflammatory impact of exercise (Ma, He & Qiang, 2013).

Pierre *et al*. (Pierre et al., 2013) verified that the wild-type (WT) mice had higher BiP, *Xbp1s*, and *Ddit3* levels in skeletal muscle samples after a high-fat diet (HFD). At the same time, ER stress markers were not affected in *Tlr4*$^{-/-}$ mice. Thus, the authors concluded that TLR4 deficiency protected rodents from HFD-induced ER stress (Pierre et al., 2013). However, there are still no data in the literature regarding the functions of TLR4 in exercise-induced ER stress. It is known that acute physical exercise leads to ER stress and activates UPR markers, while chronic exercise can cause adaptation in the ER, protecting this organelle from future stresses (Marafon et al., 2022). Therefore, this study aimed to verify the responses of ER stress and UPR markers in wild-type (WT) and *Tlr4*$^{-/-}$ (knockout; KO) mice after acute and chronic physical exercise.

MATERIALS AND METHODS

Experimental design

The present study consisted of two experiments. In the first experiment, the responses of the ER stress markers in C57BL/6 wild-type (WT) and *Tlr4* global knockout (KO; *Tlr4*$^{-/-}$) mice after acute moderate or high-intensity physical exercise protocols (APE) were investigated. In the second experiment, the responses of the ER stress markers in WT and *Tlr4* KO mice after chronic physical exercise protocol (CPE) were investigated. The mice were maintained in cages (i.e., two or three rodents per cage) in a ventilated rack (INSIGHT™,

Ribeirão Preto, São Paulo, Brazil), on a 12:12-h light-dark inverted cycle (light: 6 PM to 6 AM, dark: 6 AM to 6 PM), under controlled temperature (22±2 °C) with food (Nuvilab® CR1; Sogorb Indústria e Comércio Ltda, São Paulo, Brazil) and water provided *ad libitum*. The experimental procedures were approved by the Ethics Committee of the University of São Paulo (I.D 2016.5.82.90.7) and followed by the Brazilian College of Animal Experimentation (COBEA). A schematic representation of experimental procedures is shown in figure 1.

Experiment 1

Experimental Animals

Eight-week-old C57BL/6 WT (n = 15) and *Tlr4*$^{-/-}$ (n = 15) mice from the Central Animal Facility of the Ribeirão Preto campus from the University of São Paulo (USP) were used for the WT and KO APE groups. To determine the exercise intensity of the APE protocol, the animals performed the incremental load test (ILT) on the treadmill after one day of rest after adaptation.

Incremental load test (ILT)

The animals were adapted to exercise on a treadmill (INSIGHT™, Ribeirão Preto, São Paulo, Brazil) for five days, 10 min.day^{-1} at 6 m.min^{-1}. The ILT test started at an initial speed of 6 m.min^{-1}, with 0% of inclination, and with increments of 3 m.min^{-1} each 3 min until the voluntary exhaustion of mice. When the mice became exhausted before finishing the stage, the exhaustion velocity (EV) was corrected as proposed by Kuipers *et al*. (Kuipers et al., 1985) and used for prescribing intensities of the acute physical exercise protocols.

Acute Physical Exercise (APE)

While physical exercise performed in intensities below the maximal lactate steady state (MLSS) corresponds to the moderate-intensity (Da Silva et al., 2010), a physical exercise conducted above the MLSS intensity corresponds to the high-intensity. Ferreira *et al*. (Ferreira et al., 2007) verified that the intensity corresponding to 60% of EV obtained in the incremental load test was similar to the MLSS intensity. Herein, were used two different physical exercise intensities: 1) moderate-intensity exercise (i.e., MLSS intensity; 60% of EV during 22 min and 30s); 2) high-intensity exercise (i.e., above MLSS intensity; 75% of EV until exhaustion).

For the APE protocol, the animals were randomly divided into six groups: WT-Control mice (WTC; sedentary mice, n=5); KO-Control mice (KOC; *Tlr4*$^{-/-}$ sedentary mice, n=5); WT-Moderate-intensity exercised mice (WTM; WT mice that performed the moderate APE protocol, n=5); KO-Moderate-intensity exercised mice (KOM; *Tlr4*$^{-/-}$ mice that performed the moderate APE protocol, n=5); WT-High-intensity exercised mice (WTH; WT mice that

performed the high APE protocol, n=5); KO-High-intensity exercised mice (KOH; *Tlr4*$^{-/-}$ mice that performed the high APE protocol, n=5).

Experiment 2

Experimental Animals

Eight-week-old C57BL/6 WT (n=10) and *Tlr4*$^{-/-}$ (n=10) mice from the Central Animal Facility of the Ribeirão Preto campus from the University of São Paulo (USP) were used for the WT and KO CPE groups. For the CPE protocol, the animals were randomly divided into four groups: WT-Sedentary mice (WTS; sedentary mice, n=5); KO-Sedentary mice (KOS; *Tlr4*$^{-/-}$ sedentary mice, n=5); WT-Trained mice (WTT; WT mice that performed the CPE protocol, n=5); KO-Trained mice (KOT; *Tlr4*$^{-/-}$ mice that performed the CPE protocol, n=5). To prescribe the exercise intensity of the CPE protocol, the animals performed the incremental load test (ILT) on the treadmill. Also, these animals underwent performance evaluations to verify the pre-and post-protocol performance conditions.

Performance evaluations

The performance evaluations were applied before (i.e., after 48 h of acclimation) and 48 after the CPE. The tests used were the rotarod test, ILT (performed 4 h after the rotarod test – described in 2.2.2), and the exhaustive test (performed 24 h after the ILT).

Rotarod test

After 48 hours of acclimation and after 48 hours of the last session of the CPE protocol, motor balance and coordination were evaluated with the rotarod test (Junior et al., 2012). The device (INSIGHT®, Ribeirão Preto, São Paulo, Brazil) was programmed for an initial speed of 1 revolution per minute (rpm) and a final speed of 40 rpm, which was reached 300 s after the beginning of the movement. The acceleration throughout the test was constant, with three attempts per animal, with an interval of 3 minutes between each effort, recording the time in seconds until each animal fell. After 4 hours of the rotarod test, the rodents performed the ILT.

Exhaustive test

Twenty-four hours after performing the ILT, the mice ran at a speed of 36 m.min^{-1} with an inclination of 8% until voluntary exhaustion, which occurred when they touched the end of the treadmill 5 times within a 1 min interval.

Chronic physical exercise protocol

The chronic physical exercise protocol was based on the investigation of Vicente *et al*. (de Vicente et al., 2021). This protocol consisted of 3 days of treadmill running per week for

four weeks. There was 48 h of rest between each exercise day. Table 1 summarizes the CPE protocol characteristics.

Table 1. Chronic physical exercise protocol.

Week	Intensity (%EV)	Volume (min)	Daily Sessions	Inclination (%)	Interval between session (h)
1	60	15	1	0	48
2	60	30	1	0	48
3	60	45	1	0	48
4	60	60	1	0	48

Legend: EV: Exhaustion velocity in the incremental load test.

Skeletal Muscle Extraction

Two hours after the APE protocols and one week after the CPE protocol, the animals were anesthetized through the intraperitoneal administration of xylazine (10 $mg.kg^{-1}$ body weight) and ketamine (100 $mg.kg^{-1}$ body weight) mixed in the same syringe. The loss of pedal reflexes assessed anesthesia control (Pauli et al., 2008). Subsequently, the skeletal muscles (gastrocnemius) were removed, isolated, and stored in the freezer at a temperature of -80 °C for gene expression analysis by the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and protein analysis using the immunoblotting technique.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total skeletal muscle RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) and subsequently quantified in a NanoDrop2000c spectrophotometer (Thermo Scientific). According to the manufacturer's instructions, the cDNA was synthesized with 600 ng of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Then, quantitative real-time PCR was performed in the StepOne Plus PCR System (Applied Biosystems) to analyze the relative expression of mRNA of the following genes associated with proteins in parentheses: *Hspa5* (BiP), *Ddit3* (CHOP), *Casp3* (Caspase-3). The primers' sequences are presented in table 2. *Gapdh* was used as reference genes for data normalization. Each amplification reaction took place in the standard cycling in the following cycles: 10 minutes at 95°C and another 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C.

Relative quantification was calculated by the $2^{-\Delta\Delta CT}$ method using Thermo Fisher Cloud Software, RQ version 3.7 (Life Technologies Corporation, Carlsbad, CA, USA).

Table 2. The primers design.

Gene	Forward	Reverse
Ddit3	ATCTTGAGCCTAACACGTCGAT	GACCAGGTTCTCTCTCCTCAG
Hspa5	GTGTGTGAGACCAGAACCGT	GCAGTCAGGCAGGAGTCTTA
Casp3	AGCTTGGAACGGTACGCTAA	CCAGAGTCCACTGACTTGC
Gapdh	AAGAGGGATGCTGCCCTTAC	CGGGACGAGGAAACACTCTC

Legend: *Ddit3*: DNA Damage Inducible Transcript 3; *Hspa5:* Heat Shock Protein Family A (Hsp70) Member 5; *Casp3:* Caspase-3; *Gapdh:* Glyceraldehyde-3-phosphate dehydrogenase.

Immunoblotting

The immunoblotting technique was executed to determine the protein concentrations in the skeletal muscle samples as previously described (Da Rocha et al. 2016). The antibodies used were BiP (SC33757), GADD153/CHOP (SC71136), eIF2α (SC11386), GAPDH (SC365062) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Caspase-3 (9665), Cleaved Caspase-3 (9664), and p-eIF2α (9721) from Cell Signaling Technology (Cell Signaling Technology, MA, USA). Routine chemical reagents were purchased from Sigma Chemical Corporation (St. Louis, MO, USA).

Statistical Analysis

Results were expressed as mean ± standard error of the mean (SEM). Levene's test was used to verify the homogeneity of variances, and the Shapiro–Wilk W-test was used to check data normality. The unpaired Student's t-test examined the genetic composition (WT or KO) effects on ER stress in the basal conditions. The two-way analysis of variance (ANOVA) was used to compare the other parameters between exercise protocols and genetic groups. Bonferroni's post hoc test was performed when the two-way ANOVA indicated significance. All analyses were bilateral, and the significance level was fixed at $p \leq 0.05$.

RESULTS

Experiment 1

Ddit3 and Casp3 were increased after the high-intensity APE

No statistical differences were observed for *Hspa5* (Figure 2A), *Ddit3* (Figure 2B), and *Casp3* (Figure 2C) mRNA levels comparing the WT and *Tlr4* KO mice at basal conditions. However, the *Ddit3* and *Casp3* mRNA levels increased in the KO-High group compared to their control state. Moreover, the levels of *Ddit3* mRNA were higher for the KO-High than the KO-Moderate group.

TLR4 deletion seems to protect the mice from ER stress

Figure 3 shows the representative bands of each protein with their respective endogenous (Figure 3A, D, G and J), as well as the graphs about the protein responses related to the ER stress in the control condition (Figure 3B, E, H and K) and after the APE protocols (Figure 3C, F, I, and L). The BiP, CHOP, and cleaved-Caspase-3/Caspase-3 proteins were not different between WT and *Tlr4* KO mice at basal conditions (Figure 3B, E, and H). However, the p-eIF2α/eIF2α was higher in the KO compared to the WT group (Figure 3K).

The BiP and CHOP protein levels were increased in WT-Moderate compared to the WT-Control group (Figure 3C and F). Also, the WT-Moderate had higher BiP levels than the WT-High group (Figure 3C). Furthermore, the WT-Moderate had higher BiP and CHOP levels than the KO-Moderate group. However, there were no differences in BiP and CHOP proteins (Figure 3C and F) between the *Tlr4* KO mice under different experimental conditions (i.e., Control, Moderate, and High). The KO-Moderate and KO-High had higher levels of cleaved-Caspase-3/Caspase-3 than their control group. The KO-High group had higher cleaved-Caspase-3/Caspase-3 levels than the WT-Moderate group (Figure 3I). The p-eIF2α/eIF2α were higher for the WT-High group than for the WT-Control and WT-Moderate. The KO-High group had a lower p-eIF2α/eIF2α ratio than the WT-High group (Figure 3L).

Experiment 2

Tlr4 KO mice had lower performance levels than WT animals

There was no difference in the ILT and exhaustive test between the WT and KO groups pre-exercise (Figure 4A and B). The WT increased their performance in the exhaustive test post-exercise compared to pre-exercise (Figure 4B). The KO group had lower performance levels (i.e., ILT, rotarod test, and exhaustive test) post-exercise than the WT post-exercise

(Figure 4A, B, and C). Regarding the rotarod test, *Tlr4* KO mice also had lower values than the WT group pre and post-exercise (Figure 4C).

ER stress-related genes after CPE

There was no statistical difference in *Hspa5* (Figure 5A), *Ddit3* (Figure 5B), and *Casp3* (Figure 5C) mRNA levels between WT and KO mice for both sedentary and trained conditions in skeletal muscle.

ER stress-related proteins after CPE

Figure 6 shows the representative bands of each protein with their respective endogenous (Figure 6A, C, E, and G), as well as the graphs regarding the protein responses related to ER stress in sedentary and trained conditions (Figure 6B, D, F, and H). The KO-Trained group had lower BiP levels than the KO-Sedentary group (Figure 6B). The WT-Trained group had lower CHOP levels than the WT-Sedentary group (Figure 6D). The KO-trained group had higher cleaved Caspase-3/Caspase-3 than WT-Trained (Figure 6F). There were no differences in CHOP and peIF2α/eIF2α levels between *Tlr4* KO mice sedentary and trained (Figure 6D and H).

DISCUSSION

Recently, Marafon and coworkers (Marafon et al., 2022) reviewed the frontier-of-knowledge data regarding the impact of acute and chronic physical exercise protocols on skeletal muscle ER stress and its metabolic consequences, highlighting that the global deletion of *Tlr4* protected against HFD-induced ER stress (Pierre et al., 2013). It is known that inflammation can affect the relationship between physical exercise and ER stress (Pinto et al., 2018). Based on the fact that the TLR4 pathway can release pro-inflammatory cytokines (Lu, Yeh & Ohashi, 2008), we investigated the relationship between TLR4 and ER stress in skeletal muscle after acute and chronic exercise protocols.

Genetic ablation of *Tlr4* prevents the triggering of ER stress after APE

To verify the impact of *Tlr4* ablation in skeletal muscle ER stress responses, we compared genes and proteins of this molecular pathway between WT and *Tlr4* KO mice at basal conditions. There were no significant differences in ER stress (*Hspa5* mRNA levels and BiP protein) and apoptosis markers (*Casp3* and *Ddit3* mRNA levels, as well as CHOP and cleaved Caspase-3/Caspase-3 proteins) between these experimental groups in the skeletal muscle. These data agree with Pierre *et al*. (Pierre et al., 2013), who verified that *Tlr4* KO mice fed with

standard chow showed no difference compared to the WT group in BiP protein and *Ddit3* mRNA levels, as well as in *Xbp1u* and *Xbp1s* mRNA levels in the gastrocnemius muscle.

However, in this same study (Pierre et al., 2013), the authors found no difference in phosphorylated eIF2α, unlike our current results. Besides some differences between the experimental designs (14-16-week-old mice and 14/10 h light/dark cycle), it is essential to note that Pierre and coworkers (Pierre et al., 2013) did not measure the ratio between phosphorylated eIF2α and total eIF2α as we did. The eIF2α phosphorylation is responsible for the transient decrease in cellular protein synthesis (Hummasti & Hotamisligil, 2010; Harding, Zhang & Ron, 1999). Therefore, when the organism is under stress and proteins accumulate within the ER, this phosphorylation limits the flux of proteins entering the organelle lumen. The protein responsible for eIF2α phosphorylation is PERK, one of the first pathways activated with ER stress (Ron & Walter, 2007; Shi et al., 1998). In the present investigation, the acute high-intensity exercise increased peIF2α/eIF2α compared to the control group. This result is similar to other studies in which a significant increase in eIF2α phosphorylation was observed in mice performing acute sessions of exhaustive (Wu et al., 2011) and low-intensity exercise (Jamart et al., 2013) in the skeletal muscle.

Higher phosphorylation of eIF2α leads to increased ATF4, which activates UPR target genes, including the growth arrest and DNA damage-inducible 34 (*Gadd34*) and *Ddit3*. The first one encodes the regulatory subunit of protein phosphatase PP1C complex that dephosphorylates eIF2α and serves as a negative feedback loop to reverse the translation attenuation (Novoa et al., 2001). The *Ddit3* mRNA encodes the CHOP protein, which is involved in apoptosis (Zinszner et al., 1998; Wang et al., 1998). Despite the significant increase in the peIF2α/eIF2α ratio, the *Ddit3* and CHOP were not altered in our study. These results agree with others that verified higher p-eIF2α without significant differences in *Ddit3, Atf4,* and *Gadd34* mRNA levels, as well as in CHOP protein in skeletal muscle (Jamart et al., 2013; Kim et al., 2011; West et al., 2018). In contrast, Wu *et al*. (Wu et al., 2011) found an increase in BiP protein content and *Hspa5,* and *Ddit3* mRNA levels after exhaustive exercise in the quadriceps muscle.

Despite some similarities described above, the differences in the results of BiP, *Hspa5,* and *Ddit3* can probably be explained by the intensity and/or volume of physical exercise protocols performed in each investigation. While these other studies used light and exhaustive intensities (Wu et al., 2011), ultramarathon (Kim et al., 2011), and electrical stimulation (West et al., 2018), we used moderate and high-intensity exercises. Still, the time of muscle extraction

after exercise may also have influenced the results shown here. Therefore, further studies should be conducted to address these topics and standardize the factors mentioned above.

To the best of our knowledge, this is the first study verifying the effects of physical exercise on ER stress in the skeletal muscle of *Tlr4* KO mice. Compared to *Tlr4* KO mice in the sedentary state, those performing moderate and high-intensity exercises did not have the ER stress (BiP and peIF2α/eIF2α proteins) and cell apoptosis markers (CHOP protein) modulated. Furthermore, the acute moderate-intensity exercise increased the BiP and CHOP proteins in the WT mice, which was not observed in the *Tlr4* KO group performing the same exercise intensity. These results demonstrate that *Tlr4* deletion seems to protect animals from ER stress. In accordance, Vicente *et al*. (de Vicente et al., 2020) did not verify significant changes in BiP and p-PERK/PERK proteins in the heart of *Tlr4* KO animals submitted to physical exercise. As previously stated, Pierre e*t al*. (Pierre et al., 2013) demonstrated that *Tlr4* was involved in protecting mice against HFD-induced ER stress.

Interestingly, the cell apoptosis-related genes (*Ddit3* and *Casp3* mRNA levels) increased after the acute high-intensity exercise in KO mice. Furthermore, regardless of exercise intensity, the cleaved Caspase-3/Caspase-3 ratio was elevated after the APE. Caspase-3 is a member of the caspase family. Fourteen caspases have been identified and can be divided into three categories: inflammatory mediator, an activator of apoptosis, and executor of apoptosis (Fan et al., 2005). Caspase-3 is in the latter category and can be activated by ER stress through caspase-12 (Zhang et al., 2016). However, as the APE did not modulate ER stress-related proteins, this activation appears to come from another signaling pathway, such as the death signal-induced pathway, mediated by the death receptor pathway, and/or the pathway mediated by stress-induced mitochondria (Fan et al., 2005). Further investigations should evaluate these pathways to elucidate these adaptations.

Experiment 2

When acute physical exercise is performed regularly, it is called chronic physical exercise (Caspersen, Powell & Christenson, 1985), which is very beneficial for maintaining or improving various components of human physical fitness. Indeed, after the CPE protocol, the WT animals increased the exhaustive test performance and had an increasing tendency in the incremental load and rotarod and tests. Regarding the KO group, these animals did not improve any performance evaluations. In addition, KO mice showed lower performance compared to the WT group after CPE. Interestingly, these data did not agree with other studies using *Tlr4*$^{-/-}$ mice (Zhu et al., 2016; Okun et al., 2012; Okun, Griffioen & Mattson, 2011). However, these

investigations used 15 to 20 minutes of rest between attempts in the rotarod test, while the present study used only 3 minutes. In addition, Zhu *et al*. (Zhu et al., 2016) found that *Tlr4* KO mice displayed lower balance, motor control, and motor coordination than WT rodents. In this way, impaired motor coordination and the difference between resting time in the rotarod test may be why the *Tlr4* KO mice did not improve in the rotarod test compared to WT mice.

A recently published review showed several studies indicating that chronic physical exercise can lead to ER stress adaptation in skeletal muscle, maintaining or decreasing protein levels of PERK, IRE1α, CHOP, and BiP, as well as reducing mRNA levels of *Ddit3*, *Atf4*, *Xbp1u,* and *Xbp1s* in animals and/or humans (Marafon et al., 2022). Thus, CPE leads to the protection of the ER against new stress, preventing possible cell apoptosis. Our data agree with these studies once the BiP protein, cleaved Caspase-3/Caspase-3 ratio, and p-eIF2α/eIF2α levels did not change between the WT-Sedentary and WT-Trained groups, at the same time, the CHOP protein had its levels diminished, reinforcing the possible ER adaptation.

Up to today, this is the first investigation reporting the responses of ER stress markers in skeletal muscle after chronic physical exercise in *Tlr4* KO animals. The CPE protocol did not generate changes in CHOP protein, p-eIF2α/eIF2α, and cleaved Caspase-3/Caspase-3 ratios but reduced BiP protein compared to the KO-Sedentary group. In addition, there was no difference in the mRNA levels of *Hspa5*, *Ddit3,* and *Casp3*. Similar results were found in heart tissue, where the authors visualized the *Ddit3* and *Casp3* mRNA levels, as well as CHOP, ATF4, Caspase-3, and p-eIF2α/eIF2α protein levels, were not different between the KO-Sedentary and KO-Trained animals (de Vicente et al., 2021). These data demonstrate that *Tlr4* deletion protects mice from ER stress in skeletal muscle.

In summary, in response to different intensities of acute physical exercise, there were no changes in the protein content of BiP, CHOP, and p-eIF2α/eIF2α ratio in *Tlr4* KO mice. Furthermore, after the chronic exercise protocol, BiP was downregulated, and there was no change in CHOP, p-eIF2α/eIF2α, and cleaved Caspase-3/Caspase-3 ratio levels for the KO group. These results demonstrate that the global deletion of *Tlr4* seems to protect the animal against ER stress. However, acute exercise upregulated the cleaved Caspase-3/Caspase-3 ratio, independent of the intensity. This result demonstrates that this protein may be activated by another pathway other than ER stress. Future studies are needed to evaluate the roles of TLR4 in skeletal muscle adaptation and the relation between ER stress and apoptosis. Figure 7 summarizes the main findings of the present study.

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Conflict of Interest Statement

The authors declare that they have no competing interests.

Data Availability Statement

All data support the conclusions are presented in the manuscript. Additional information will be made available by the corresponding author upon request.

Author's Contributions

Conceptualization: BBM, LGV and ASRS; Data curation: BBM, APP, LGV and ALR; Funding acquisition: FMS, ERR, LPM, DEC, JRP and ASRS; Methodology: BBM, APP, LGV and ALR; Supervision: ASRS; Visualization: BBM, APP, LGV, ALR, FMS, ERR, LPM, DEC, JRP and ASRS; Roles/Writing - original draft: BBM, APP and ASRS; Writing - review & editing: BBM, APP, LGV, ALR, FMS, ERR, LPM, DEC, JRP and ASRS.

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FIGURES

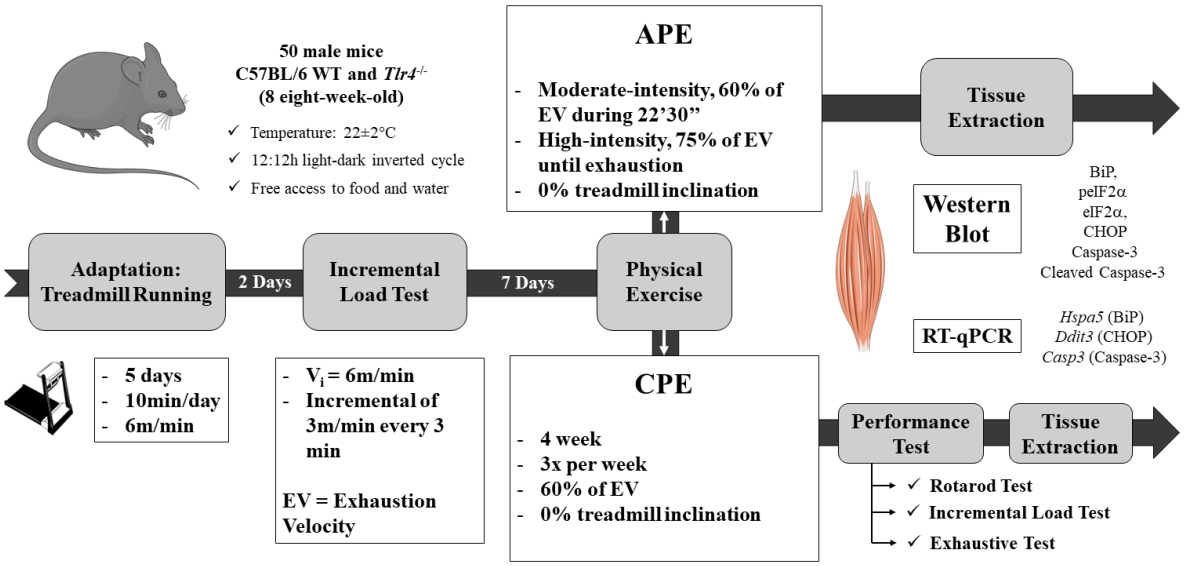

Figure 1. Schematic representation of experimental procedures. APE: acute physical exercise; CPE: chronic physical exercise.

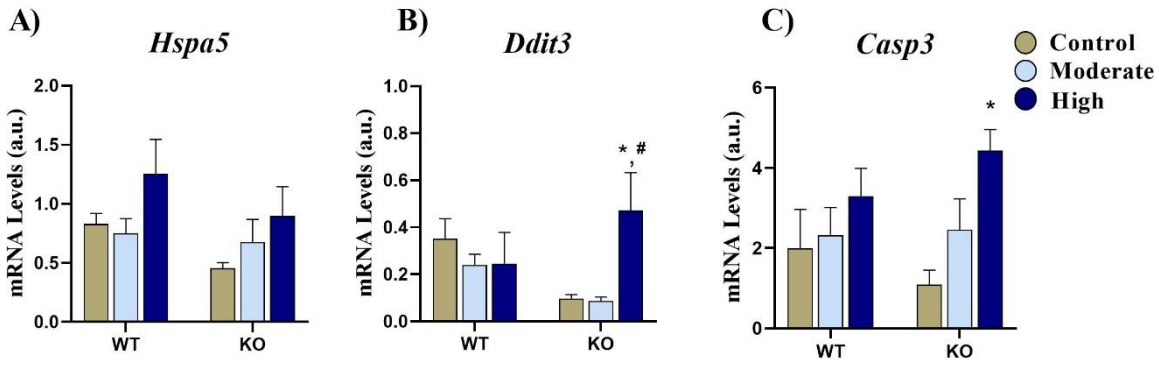

Figure 2. mRNA levels of (A) *Hspa5*, (B) *Ddit3*, and (C) *Casp3* of control and APE groups (i.e., moderate, and high-intensity exercises). The data correspond to the mean ± SEM of n = 5/group. (*p<0.05 *versus* KO-Control; #p <0.05 *versus* KO-Moderate).

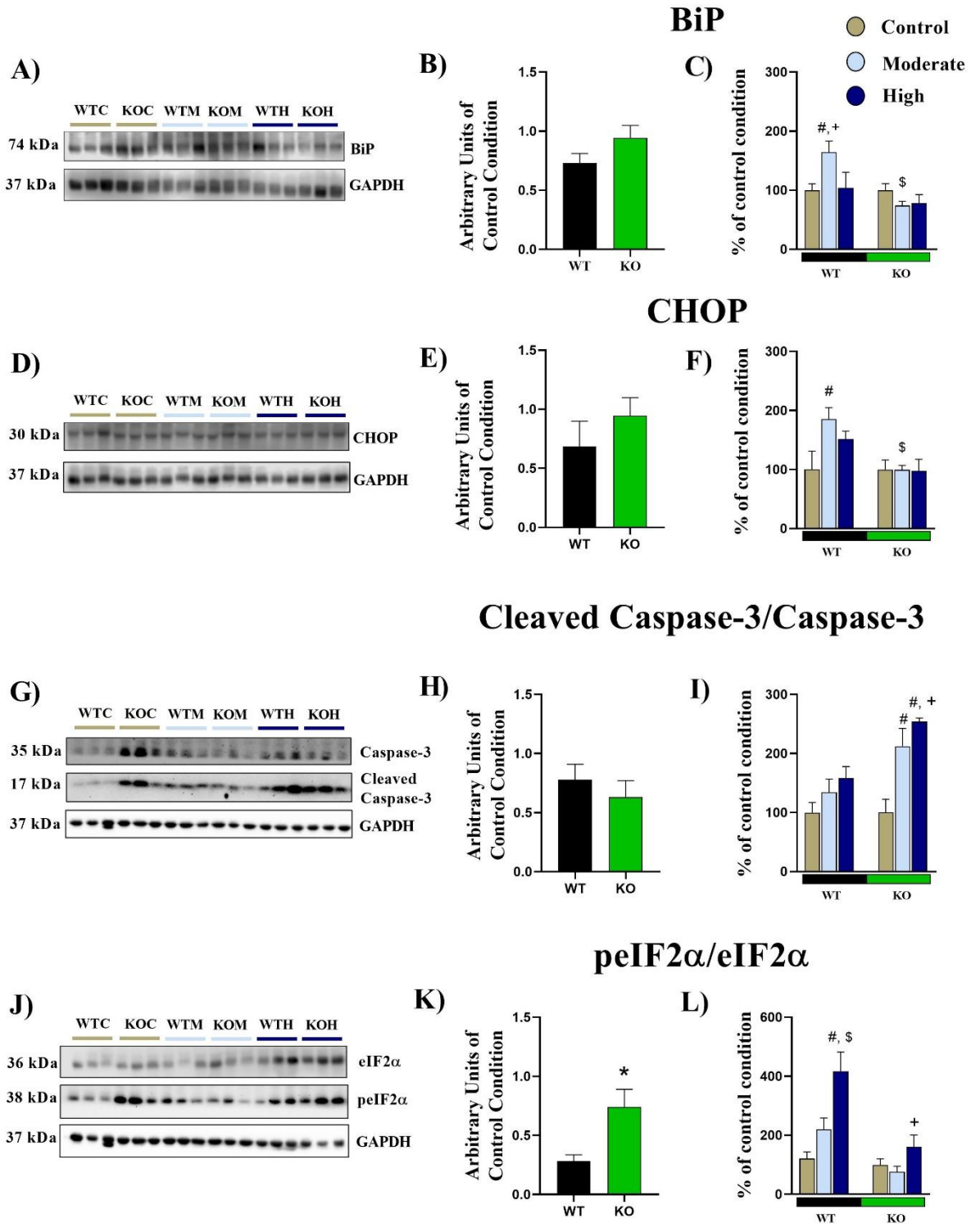

Figure 3. (A, D, G, J) Representative bands for each protein; (B, E, H, K) BiP, CHOP, cleaved Caspase-3/Caspase-3, and p-eIF2α/eIF2α protein levels in arbitrary units of the control condition of WT and KO groups; (C, F, I, L) Percentage of protein levels to baseline protein condition after moderate and high-intensity exercise of WT and KO groups. The data correspond to the mean ± SEM of n = 5/group. (*p<0.05 *versus* WT; #p <0.05 *versus* control

of the same group; $p<0.05 *versus* WT-Moderate; +p<0.05 *versus* WT-High;). WTC: WT-Control mice; KOC: KO-Control mice; WTM: WT-Moderate-intensity exercised mice; KOM: KO-Moderate-intensity exercised mice; WTH: WT-High-intensity exercised mice; KOH: KO-High-intensity exercised mice.

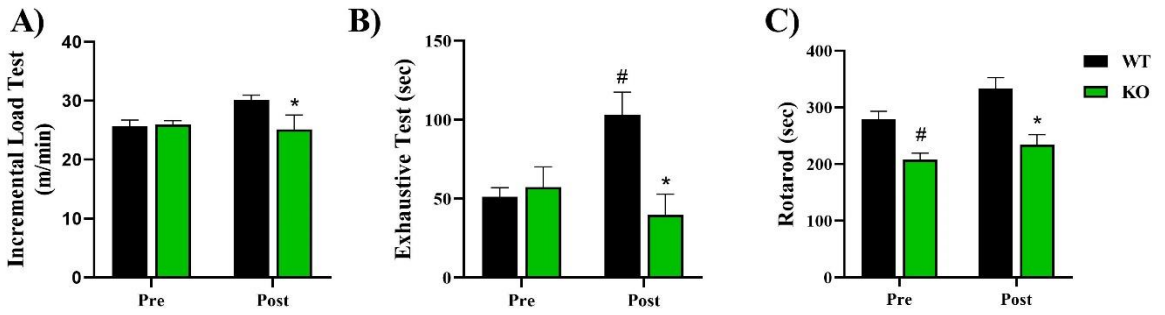

Figure 4. (A) Incremental load test to determine the exhaustion velocity (EV) in the pre and post CPE; (B) Exhaustive test in pre and post CPE; and (C) Rotarod test to determine the motor balance and coordination in pre and post CPE. The data correspond to the mean ± SEM of n = 5/group. (*p<0.05 *versus* WT-post; #p <0.05 *versus* WT-pre).

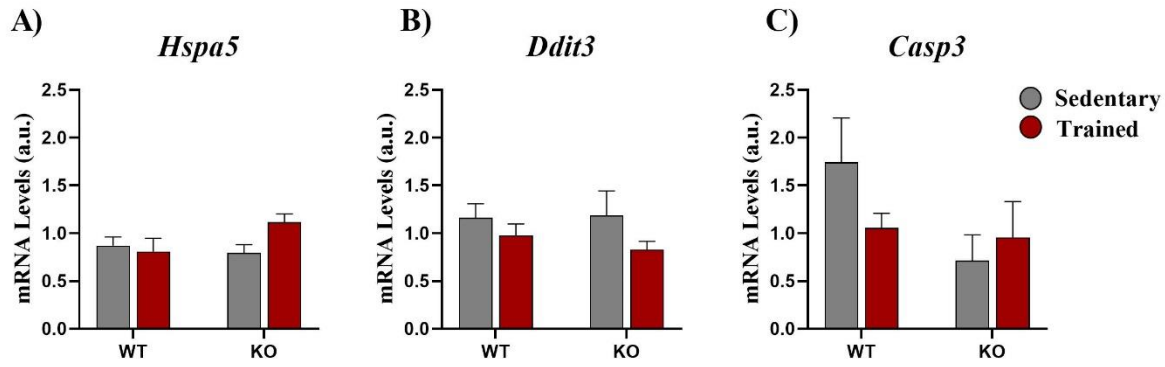

Figure 5. mRNA levels of (A) *Hspa5;* (B) *Ddit3;* and (C) *Casp3* of sedentary and trained mice. The data correspond to the mean ± SEM of n = 5/group.

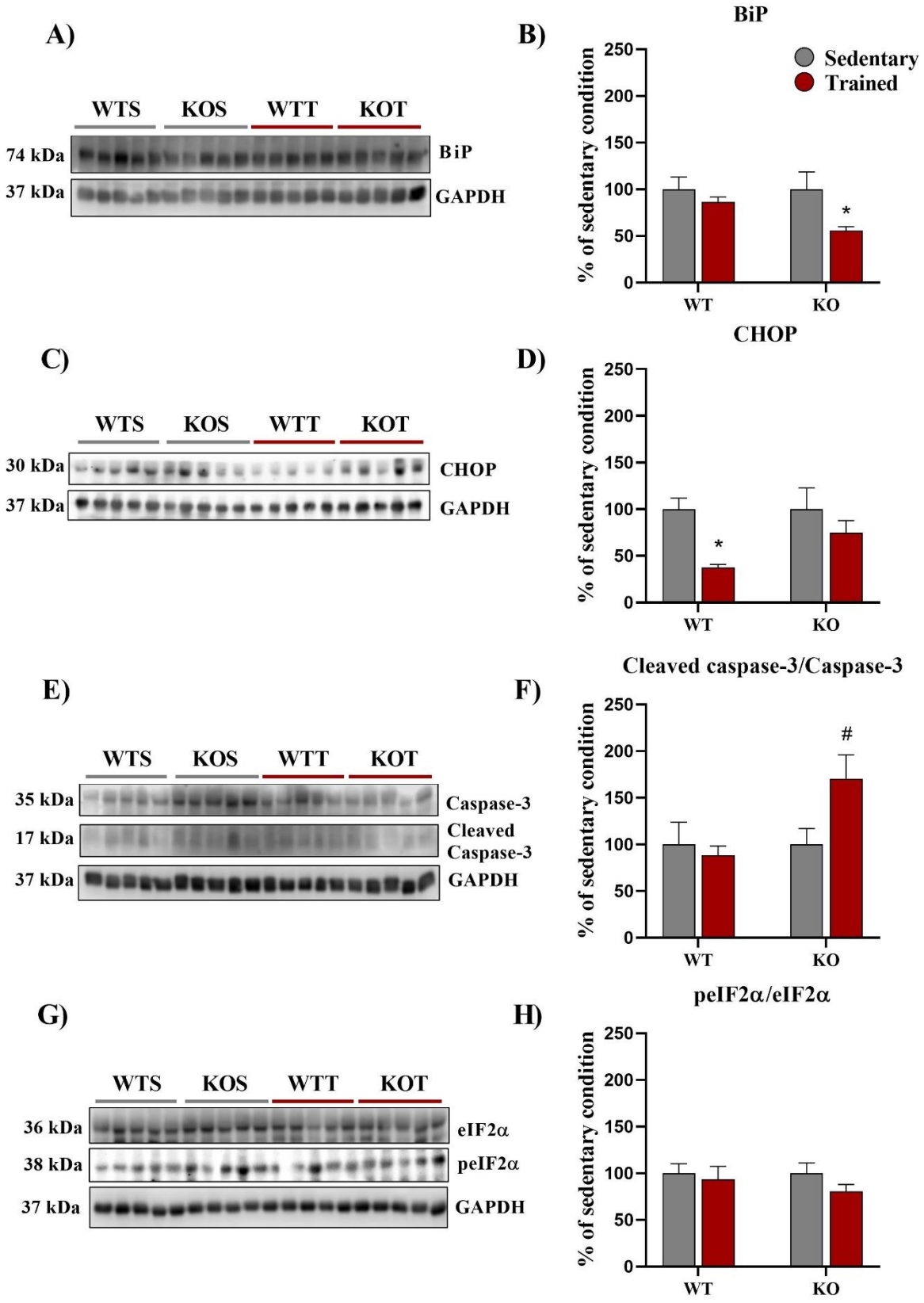

Figure 6. (A, C, E, G) Representative bands for each protein; (B, D, F, H) BiP, CHOP, cleaved Caspase-3/Caspase-3, and p-eIF2α/eIF2α protein levels in arbitrary units of the sedentary

condition of WT and KO groups. The data correspond to the mean ± SEM of n = 5/group. (*p<0.05 *versus* sedentary of the same group; #p <0.05 *versus* WT-Trained). WTS: WT-Sedentary mice; KOS: KO-Sedentary mice; WTT: WT-Trained mice; KOT: KO-Trained mice.

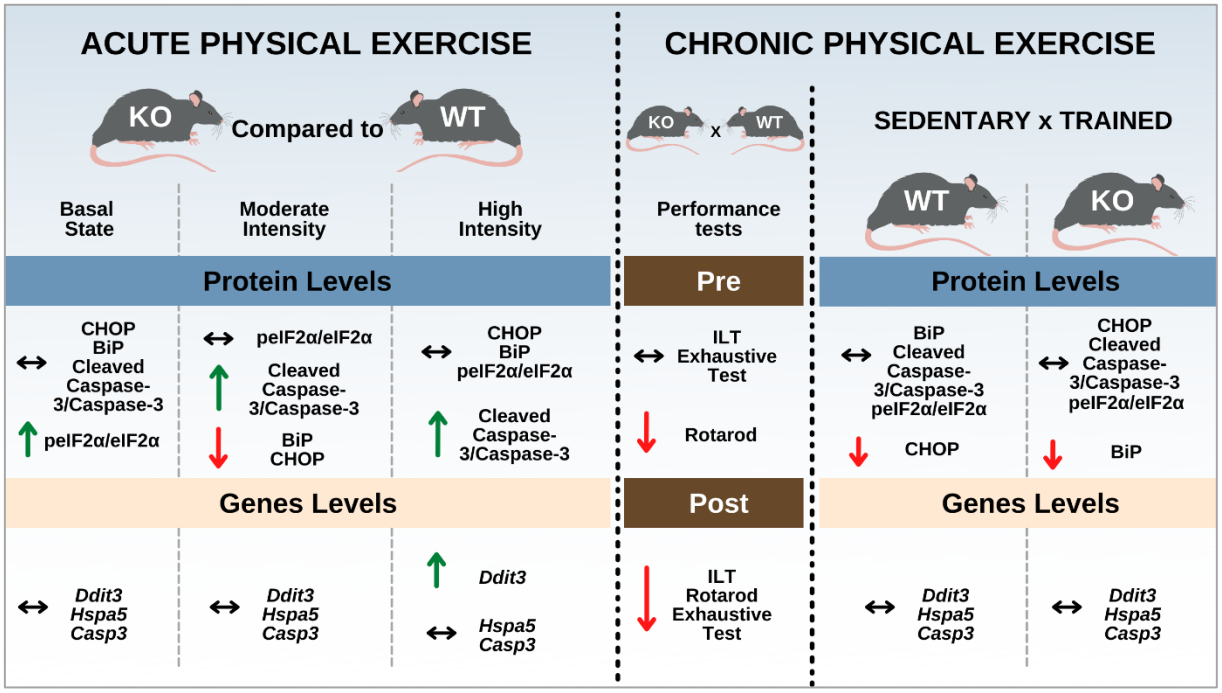

Figure 7. Schematic representation summarizing the main findings of the present study.

4. CONCLUSION

In summary, it is known that the ER stress-activated UPR pathway is essential for skeletal muscle remodeling and homoeostasis. Acute physical exercise, regardless of the type, generates stress in the body, leading to the activation of all three arms of the UPR signaling pathway. On the other hand, chronic physical exercise maintains or reduces some of the protein and genes levels related to ER stress. Thus, chronic physical exercise can cause adaptation in the ER, protecting this organelle from being overloaded and preventing cell death.

In response to different intensities of acute physical exercise, there were no changes in the protein content of BiP, CHOP, and p-eIF2α/eIF2α ratio in *Tlr4* KO mice. Furthermore, after the chronic exercise protocol, BiP was downregulated, and there was no change in CHOP, p-eIF2α/eIF2α, and cleaved Caspase-3/Caspase-3 ratio levels for the KO group. Similar to Pierre *et al.* (PIERRE et al., 2013), these results demonstrate that the global deletion of *Tlr4* seems to protect the animal against ER stress. However, acute exercise upregulated the cleaved Caspase-3/Caspase-3 ratio, independent of the intensity. This result demonstrates that this protein may be activated by another pathway other than ER stress. Future studies are needed to evaluate the roles of TLR4 in skeletal muscle adaptation and the relation between ER stress and apoptosis.

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