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RESEARCH

mTOR inhibition modulates apoptosis and oxidative stress in hindlimb ischemia/reperfusion injury

mTOR inhibisyonu, arka bacak iskemisi/reperfüzyon zedelenmelerinde apoptozu ve oksidatif stresi düzenler

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Abstract

Purpose: Ischemia/reperfusion (I/R)-induced injuries represent serious clinical events regarding profound target organ destructions followed by remote organ complications due to the loss of oxidant/antioxidant balance and apoptosis. Recent studies examining the mammalian target of rapamycin (mTOR) during I/R injury in different organs have remained a matter of debate. The current study aimed to explore further the protective and underlying antiapoptotic and antioxidant mechanisms of mammalian target of rapamycin (mTOR) inhibition in hindlimb (HL) schemia/reperfusion (I/R)injury.

Materials and Methods: Occlusion of bilateral hindlimbs for 4 h with tourniquets was carried out under anesthesia to induce I/R for 4 h in rats. Rapamycin (1 mg/kg) or saline (4 mL/kg) was injected intraperitoneally 1 h before reperfusion. Gastrocnemius muscle, kidney, and blood were collected at the end of the experiments for analysis. Muscle and kidney damages were evaluated by measuring protein expression and/or phosphorylation of eukaryotic initiation factor 4E-binding protein 1 (4EBP1), ribosomal protein S6 (rpS6), B-cell lymphoma 2 (Bcl-2), caspase-3, and Bcl-2-associated X protein (Bax) with NADPH oxidase level and total antioxidant capacity in tissues or sera.

Results: I/R-induced organ damages were demonstrated by enhanced phosphorylation and/or expression of rpS6, 4EBP1, caspase-3, and Bax with a significant reduction in Bcl-2 accompanied by a decreased total antioxidant capacity and increased level of NADPH oxidase.

Öz

Amaç: İskemi/reperfüzyon (İ/R)'nun neden olduğu zedelenmeler, oksidan/antioksidan dengenin bozulması ve apopitoz nedeniyle hedef organlarda ve takip eden uzak organlarda gelişebilecek komplikasyonlar ile ilgili ciddi klinik olayları temsil eder. İ/R zedelenmeleriyle ilgili yapılan son çalışmalarda farklı organlarda gelişebilecek İ/R kaynaklı zedelenmelerde rapamisinin memeli hedefi (mTOR)'un etkileri halen tartışılmaya devam etmektedir. Bu çalışmada arka bacak İ/R zedelenmesinde mTOR inhibisyonunun olası koruyucu etkisi ve bu etkisine aracılık edebilecek antiapoptotik ve antioksidan etki mekanizmalarının araştırılması amaçlandı.

Gereç ve yöntem: Wistar sıçanlar dört gruba ayrıldı. Sıçanlarda arka bacak İ/R zedelenmesini oluşturmak için anestezi altında her iki arka ekstremitelerine turnike uygulanarak 4 saat iskemi ve ardından turnikeler açılarak 4 saat reperfüzyon uygulandı. Reperfüzyondan 1 saat önce rapamisin (1 mg/kg) veya salin (4 mL/kg) periton içine uygulandı. Deneylerin sonunda gastroknemius kası, böbrek ve kanları alındı. Kas ve böbrek dokularında İ/R'de gelişebilecek zedelenmeler ribozomal protein S6 (rpS6), ökaryotik başlatma faktörü 4E-bağlayıcı protein 1 (4EBP1), kaspaz-3, Bcl-2 ile ilişkili X proteini (Bax) ve B-hücreli lenfoma (Bcl)-2'nin protein ekspresyonu ve/veya fosforilasyonu ile NADPH oksidaz seviyesi ve toplam antioksidan kapasitesi ölçülerek değerlendirildi.

Bulgular: İ/R'nin neden olduğu organ zedelemeleri artan rpS6, 4EBP1, kaspaz-3, Bax ve azalan Bcl-2 ekspresyon ve/veya fosforilasyonu ile buna eşlik eden toplam antioksidan kapasitede azalma ve NADPH oksidaz

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Administration of rapamycin, an inhibitor mTOR, protected against I/R-mediated injuries.
Conclusion: Our findings suggest that the activation of mTOR signaling plays a crucial role in HL I/R-triggered organ damages presumably through the activation of apoptosis as a result of oxidant/antioxidant imbalance.

Keywords:. Apoptosis, ischemia/reperfusion injury, mTOR, oxidative stress

seviyesinde artma ile gösterildi. Bir mTOR inhibitörü olan rapamisin uygulaması, İ/R zedelenmesine aracılık eden etkileri ortadan kaldırdı.
Sonuç: Bulgularımız, mTOR sinyal iletisinin etkinleşmesinin oksidan/antioksidan dengedeki bozulmanın sonucu olarak apopitozun etkinleşmesi aracılığıyla arka bacaklarda İ/R'nin neden olduğu doku ve organ zedelenmelerinde önemli bir rol oynadığını göstermektedir.

Anahtar kelimeler: Apopitoz, iskemi/reperfüzyon zedelenmesi, mTOR, oksidatif stres

INTRODUCTION

Skeletal muscle ischemia/reperfusion (I/R) injury depending on the severity and duration of the ischemia leads to the development of postoperative complications after free flap transfer, trauma, and vascular surgery[1-3]. In addition to local responses following the restoration of blood flow to ischemic tissues, I/R injury may paradoxically result in remote organ failure and even death[4]. The I/R injury-induced kidney damage is one of the most common complications during reperfusion[5-7]. It is clearly implicated that oxidative stress and apoptosis are the key pathophysiological processes contributing to both local and systemic injury caused by I/R[8-13]. Apart from the complicated pathophysiological mechanism, I/R damage is still the most serious type of vascular disease and requires more research[14-16]. Thus, there is no specific treatment available against organ protection related to this complex phenomenon. A novel pharmacological approach is required to target the processes mentioned above to minimize I/R injury and, subsequently, multiple organ damage.

The mammalian target of rapamycin (mTOR) operates cell survival, growth, and proliferation[17, 18]. mTOR is composed of distinct complexes such as mTOR complex (mTORC) 1 and mTORC2. Among them, mTORC1 is the best characterized functional complex that integrates upstream signaling molecules via mTOR-controlled downstream targets by regulating phosphorylation of eukaryotic initiation factor 4E-binding protein 1 (4EBP1) and ribosomal protein S6 (rpS6)[19]. Selective mTOR inhibitor rapamycin has been widely used as an immunosuppressive drug to prevent transplant rejection and stent-induced coronary restenosis[20]. However, given the potential antioxidant, antiinflammatory, and antiapoptotic effects of mTOR inhibition in several ischemic diseases, there is increasing focus on the mTOR signaling pathway as the novel therapeutic target for different types of I/R injuries[21-24]. However, controversial results have been reported regarding the beneficial or detrimental role of mTOR in the heart, liver, and kidney after I/R[19,21-24]. We have recently demonstrated that the preventative role of rapamycin on hindlimb (HL) I/R-induced changes is caused by reduced inflammation and oxidative/nitrosative stress primarily as a result of mTOR/inhibitor κB (IκB)/nuclear factor-κB (NF-κB) p65 signaling pathway activity[25]. However, there is no data about the exact function of mTOR in HL or remote organ ischemic injuries linked to apoptosis and oxidant/antioxidant imbalance.

Therefore, we sought to investigate the contribution of downstream targets of mTOR mediating the protective or counterproductive action against apoptosis and oxidative stress stimulated by the HL I/R model in rats. Furthermore, we aimed to explore the effects of the mTOR inhibition on local and systemic complications in the kidney and gastrocnemius muscle, mainly focusing on the inhibition of 4EBP1 phosphorylation about apoptosis together with total antioxidant capacity.

MATERIALS AND METHODS

Chemicals

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, antioxidant assay ELISA kits, and Bovine serum albumin (BSA) were supplied by MyBioSource (MBS2602768; San Diego, USA), Cayman Chemical (709001; Ann Arbor, USA), and Sigma Chemical Co. (St. Louis, USA) respectively. Anti-rpS6 (5G10) and anti-phospho-rpS6 (D68F8) (Cell Signalling Technology, Danvers, USA), anti-4EBP1 (SC-9977) and anti-phospho-4EBP1 (SC-271947), anti-caspase-3 (SC-56053), anti-Bcl-2-

associated x protein (Bax) (SC-23959), and anti- B-cell lymphoma (Bcl)-2 (SC-7382) were obtained from Santa Cruz Biotechnology (Texas, USA). Antibodies for α-actin (A2172) and β-actin (A2547) were bought from Sigma Chemical Co. (St. Louis, USA). Additionally, we obtained goat antimouse (ab6808) and antirabbit (ab6721) IgG-horseradish peroxidase (HRP) secondary antibodies from Abcam (Cambridge, UK).

Rat classifications and drug administrations

This study's methods were approved by the Mersin University Experimental Animals Local Ethics Committee (decision date 31/07/2017 and number 16). In accordance with the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, male Wistar albino rats weighing 200–300 g were purchased from the Research Center of Experimental Animals, Mersin University (Mersin, Turkey). Before the experiments, rats were housed under continuously monitored settings with a standardized 12 h light/dark cycle and free access to standard food and tap water.

Rats were randomly classified into the vehicle, I/R, rapamycin (RAPA), and I/R+rapamycin (I/R+RAPA) groups (n=8 in each group). The latex tourniquets were applied to the rat hindlimb roots using the previously reported technique for HL I/R modeling[26,27]. Briefly, rats were anesthetized with ketamine (90 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.), and then blood flow was blocked in both hindlimbs by placing the tourniquet on proximal to trochanter major in I/R and I/R+RAPA groups. The latex tourniquets were removed following 4 h of ischemia and then animals were subjected to reperfusion for 4 h. The RAPA (1 mg/kg) was intraperitoneally (i.p.) administered at 1 h before reperfusion in the RAPA and I/R+RAPA groups, and saline (4 ml/kg) was administered i.p. in the vehicle and I/R groups at the same schedule with RAPA[25].

After the reperfusion period, the animals were euthanized with a high i.p. dose of ketamine injection. The gastrocnemius muscle, kidney, and blood samples were harvested from all groups for the determination of protein amount, immunoblotting, and biochemical analysis, respectively. Blood samples were obtained from the abdominal aorta, centrifugated at 23,910 x *g* for 15 min at 4˚C to separate the sera, and stored at -20˚C for measurement of total antioxidant capacity. Tissues were immediately cooled in liquid nitrogen and kept at -80˚C for the following processing. Frozen tissues were subjected to liquid nitrogen and crushed by a pestle in a supercooled mortar for the homogenization procedure. Then, homogenates were extracted using a lysis buffer that contained protease and phosphatase inhibitors. Following the centrifugation at 23,910 x *g* for 10 min at 4˚C, the supernatant was collected and sonicated. The lysates were centrifuged at 23,910 x *g* for 15 min at 4°C to collect supernatants and then chilled at -80°C until the experiment was conducted. Using the Bradford method, the amount of total protein in the supernatant fractions was ascertained[28].

Immunoblotting

The expression of rpS6, phosphorylated rpS6, 4EBP1, phosphorylated 4EBP1, caspase-3, Bax, Bcl-2, α-sarcomeric and β-actin proteins were detected by immunoblotting as previously described[25,27,29-31]. An equivalent amount of total protein was dissolved in 4x Laemmli sample buffer and separated by 10% or 12% sodium dodecil sulfate polyacrylamide gel electrophoresis followed by electrophoretically transferred to nitrocellulose membranes. The membranes were blocked by 5% dry milk dissolved in Tris-buffered saline (TBS)/Tween 20 solution (TBST) for 1 hour at room temperature and washed with TBST.

The membranes were then treated with primary antibodies against the aforementioned proteins in TBST containing 5% bovine serum albumin (BSA) overnight at 4°C with gentle shaking. The protein bands were then detected using a highly sensitive chemiluminescence reagent after membranes were exposed to their respective HRP-conjugated secondary antibodies in TBST containing 0.1% BSA for 2 h at room temperature.

After washing with TBST, membranes were stripped (Restore™ Western Blot Stripping Buffer) for target proteins and reprobed with α-actin or β-actin (as a loading control) antibodies overnight at 4°C followed by incubation with their respective HRP-conjugated secondary antibodies (1:1000 in TBST including 0.1% BSA). Image J (1.46r) was used to perform a densitometric analysis of the target protein bands, and the relative densitometric intensities of the target proteins were standardized to α-actin and β-actin in both tissues[25, 27].

Estimation of NADPH oxidase concentration and total antioxidant capacity

NADPH oxidase levels in tissue samples and total antioxidant capacity in the sera were determined using ELISA kits. Antioxidant Assay Kit and Rat NADPH Oxidase Elisa Kit were performed following the instructions provided by the manufacturers.

Statistical analysis

Quantitative data were reported as the mean ± S.E.M. GraphPad Prism 5.0 Version for Windows, GraphPad Software (San Diego, CA, USA) was applied in performing the statistical analyses. Statistical analyses between the groups were carried out using one-way analysis of variance (ANOVA) in all parameters after assessing the conformity of the data in the study to the normal distribution. Differences between the groups were determined using post hoc the Student-Newman-Keuls test in all parameters, and they were considered statistically significant at $P < 0.05$. Statistical significance was accepted for P values < 0.05.

RESULTS

Rapamycin inhibited HL I/R-induced changes in expression and/or activity of rpS6, 4EBP1, Caspase-3, Bax, and Bcl-2

To establish the role of mTOR in the HL I/R-induced injury, we assessed the phosphorylation of rpS6 and 4EBP1 proteins (commonly used markers of mTOR activity) in the kidney and muscle of rats following I/R insult. The phosphorylation of rpS6 at Ser240/244 and 4EBP1 at Thr45 was increased in both muscle (Figs. 1A, 1B) and kidney (Figs. 1C, 1D; $P < 0.05$) following I/R. However, total levels of 4EBP1 and rpS6 were unchanged between groups (Figs. 1B-D; $P > 0.05$). At 3rd h post-ischemia, the administration of rapamycin resulted in decreased phosphorylation of rpS6 and 4EBP1 when compared with the I/R group (Figs. 1A-D) ($P < 0.05$). In comparison to the rats administered with a vehicle, there was no significant difference in the rapamycin alone group (Figs. 1A-D) ($P > 0.05$).

Rapamycin inhibited apoptosis following HL I/R-induced injury

To further explore the mechanism of mTOR inhibition in HL I/R-induced apoptosis, we evaluated the expression of cleaved caspase-3, Bax, and Bcl-2 in the gastrocnemius muscle and kidney following HL I/R insult. The expression of cleaved-caspase-3 and Bax was increased; in contrast, Bcl-2 expression was reduced in both muscle (Figs. 2A-2C) and kidney (Figs. 2D-2F; $P < 0.05$) following I/R. Rapamycin treatment significantly reduced cleaved caspase-3 expression (Figs. 2A, 2D; $P < 0.05$) in parallel with decreased protein expression of Bax in all tissues (Figs. 2B, 2E; $P < 0.05$). Conversely, rapamycin led to a substantial increase in the Bcl-2 expression (Figs. 2C, 2F; $P < 0.05$). At 3rd h post-ischemia, administration of rapamycin reversed the effect of I/R insult by restoring the expression of cleaved-caspase-3, Bax, and Bcl-2 to nearly control values when compared with the I/R group (Figs. 2A-F) ($P < 0.05$). In comparison to the rats administered with a vehicle, there was no significant difference in the rapamycin alone group (Figs. 2A-F) ($P > 0.05$).

Rapamycin provided protection against oxidative stress

To examine the contribution of oxidant/antioxidant balance in the protective effect of rapamycin on HL I/R-induced target and distant organ injury, two often used biomarkers of oxidative status, NADPH oxidase enzyme, and total antioxidant capacity were measured in the gastrocnemius muscle and kidney or serum samples following I/R insult. HL I/R caused an increase in NADPH oxidase levels (a powerful oxidant enzyme) in muscle (Fig. 3A) and kidney (Fig. 3B; $P < 0.05$) with a slight decrease in systemic total antioxidant capacity (Fig. 3C; $P < 0.05$). All of these I/R-induced changes in oxidant/antioxidant balance were prevented by rapamycin administration (Figs. 3A-C; $P < 0.05$). In comparison to the rats administered with a vehicle, there was no significant difference in the rapamycin alone group (Figs. 3A-C) ($P > 0.05$).

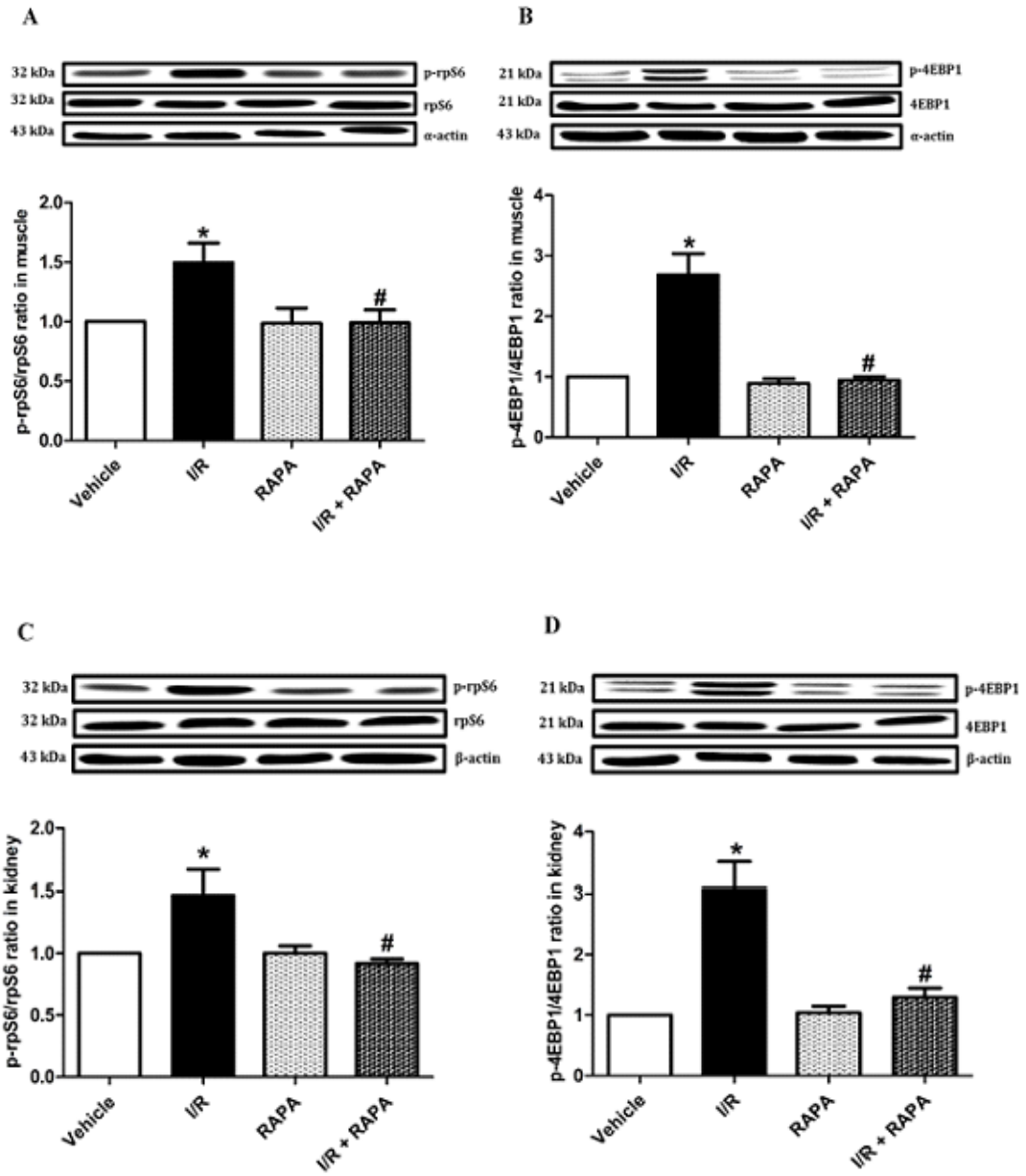

Figure 1. Protective effects of mTOR inhibition via inhibiting two major targets during HL I/R injury. Representative immunoblots and densitometry analysis of phosphorylated and total rpS6 and 4EBP1 in tissue lysates from gastrocnemius muscle (A, B) and kidney (C, D) of rats subjected to 4 h of ischemia and 4 h of reperfusion in the vehicle, hindlimb I/R, rapamycin, and I/R+rapamycin groups. Rapamycin (1 mg/kg, i.p.) was administered 1 h before reperfusion. Immunoblotting was performed to detect rpS6, p-rpS6, 4EBP1, and p-4EBP1 protein levels in tissue lysates. Phosphorylation is represented as a ratio of phospho/total protein levels. All data are presented as means±S.E.M of 4 animals from each group. The asterisk (*) indicates a significant difference from the corresponding value versus the vehicle, and the hash (#) indicates a significant difference from the corresponding value versus the I/R group (P <0.05).

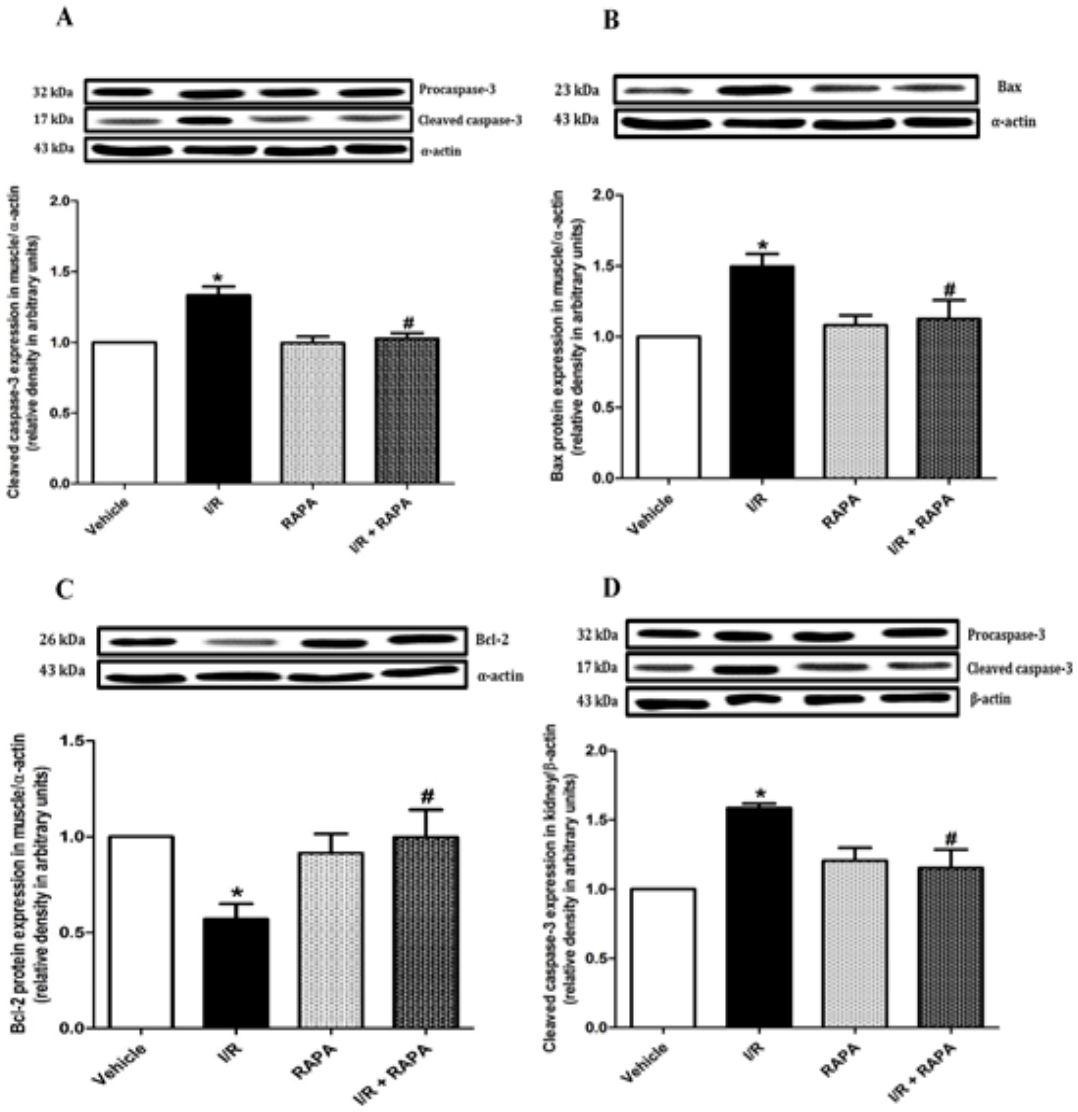

Figure 2. Protective effects of mTOR inhibition against apoptosis induced by HL I/R injury. Representative immunoblots and densitometry analysis of main proapoptotic and antiapoptotic markers cleaved caspase-3, Bax, and Bcl-2 in tissue lysates from gastrocnemius muscle (A, B, C) and kidney (D, E, F) of rats subjected to 4 h of ischemia and 4 h of reperfusion in the vehicle, HL I/R, rapamycin, and I/R+rapamycin groups. Rapamycin (1 mg/kg, i.p.) was administered 1 h before reperfusion. Immunoblotting was performed to detect cleaved caspase-3, Bax, and Bcl-2 protein levels in tissue lysates. All data are presented as means±S.E.M of 4 animals from each group. The asterisk (*) indicates a significant difference from the corresponding value versus the vehicle, and the hash (#) indicates a significant difference from the corresponding value versus the I/R group (P <0.05).

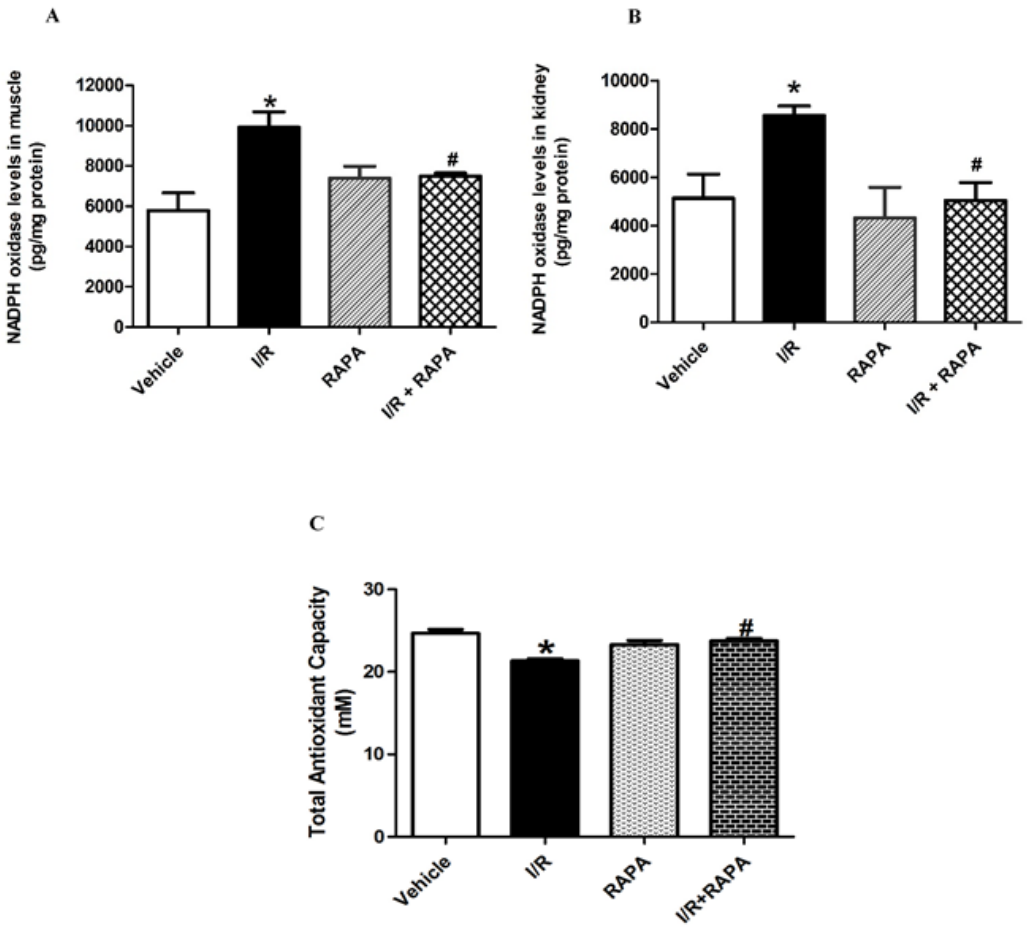

Figure 3. Protective effects of mTOR inhibition via balancing oxidative status induced by HL I/R injury. The levels of NADPH oxidase in tissue lysates from gastrocnemius muscle (A), kidney (B), and total antioxidant capacity in serum (C) from blood samples of rats subjected to 4 h of ischemia and 4 h of reperfusion in the vehicle, HL I/R, rapamycin, and I/R+rapamycin groups. Rapamycin (1 mg/kg, i.p.) was administered 1 h before reperfusion. NADPH oxidase levels in tissue lysates and total antioxidant capacity in sera were measured using commercial ELISA kits. All data are presented as means±S.E.M of 4 animals from each group. The asterisk (*) indicates a significant difference from the corresponding value versus the vehicle, and the hash (#) indicates a significant difference from the corresponding value versus the I/R group (P <0.05).

DISCUSSION

The present study's key findings include the fact that mTOR, a serine-threonine kinase enzyme, contributes to HL I/R-induced (1) increase in the expression and/or phosphorylation of mTOR downstream targets; rpS6 and 4EBP1, (2) induction of apoptotic process as shown by increased expression of cleaved caspase-3, Bax with a diminished expression of Bcl-2, (3) a decrease in total antioxidant capacity, and (4) an enhanced NADPH oxidase level. Recently, we showed for the first time that the specific inhibition of mTOR with rapamycin had a protective effect against HL I/R-associated inflammation and oxidative/nitrosative stress through the suppression of mTOR/IκB-α/NF-κB p65 signaling following occlusion of bilateral hindlimbs[25]. This study further demonstrated that

mTOR inhibition promoted significant benefits for HL I/R-induced injury in the target and distant organs by modulating the apoptotic process in parallel to oxidative stress.

The pathophysiological events associated with the development of HL I/R injury are very complex processes consistent with oxidative stress, inflammation, and apoptosis[32]. Increasing evidence has been reported that mTOR signaling pathway plays an essential role in the pathophysiological processes induced by I/R[23,24,33-35], but little is known about its role in HL I/R injury. Due to the controversial results obtained with the contribution of mTOR in the I/R injury, we examined the role of mTOR through phosphorylation of rpS6 and 4EBP1. We hypothesized for the first time whether it regulates apoptosis besides oxidative stress in response to I/R by regulating these main targets. Rapamycin was shown to inhibit the activation of mTOR pathway and had a protective effect on cerebral I/R injury in rats by regulating apoptosis[32]. Also, previous research proved that deletion of mTORC1 protects against HL ischemic damage in diabetic mice mainly through suppression of oxidative stress and inflammation[36]. It has been reported that phosphorylation of 4E-BP1 was increased above the control level during short-term reperfusion with ischemic animals[37]. In the present study activation of the mTOR pathway was shown by increased expression of phosphorylated rpS6 and 4EBP1 after HL I/R injury. Treatment with rapamycin 1 h before reperfusion markedly decreased the phosphorylation of these mTOR-mediated pathway targets suggesting that rapamycin alleviates target and remote organ damages from I/R-induced injury, at least partially via inhibition of these mTOR downstream effectors. Likewise, phosphorylation of 4EBP1 was significantly mitigated by rapamycin treatment in a kidney I/R injury model[38]. Contrary to this report, the positive aspect of mTOR activity during the reperfusion phase was demonstrated previously[39]. Consistently, cardiac-specific mTOR overexpression was shown to reduce chronic cardiac remodeling after I/R[40]. It is plausible that the significance of the mTOR signaling pathway in I/R injury is still a debatable issue because contradictory reports have been observed from different animal strains, I/R models, affected organs, and the time of drug administration.

It is increasingly clear that skeletal muscle cell apoptosis may occur during peripheral vascular diseases and surgeries, subsequently leading to an I/R injury[41,42]. Prevention of apoptosis is linked to inhibition of 4EBP1 phosphorylation, serving as a marker of blocked mTOR activity[38]. This conclusion is supported by the findings that the HL I/R damage caused changes in the expression of proapoptotic and antiapoptotic proteins that are regulated by mTOR inhibition with rapamycin treatment in concurrence with significantly decreased 4EBP1 phosphorylation. Although previous studies documented the antiapoptotic effects of rapamycin in different injury models[43-46], our results are the first evidence suggesting this effect of rapamycin in HL I/R injury. Moreover, we found that apoptosis was increased in the I/R group, as evidenced by increased caspase-3 activity and expression of Bax while decreased expression of Bcl-2 following bilateral occlusion of HL. Supporting this view, a recent report showed that I/R led to a substantial downregulation of Bcl-2 along with the upregulation of active (cleaved) caspase-3[38]. Following spinal I/R injury, treatment with rapamycin decreased mitochondrial apoptosis-related protein as evidenced by the decrease in expressions of Apaf-1, caspase-3, and caspase-9 accompanied by reduced Bax translocation and the release of the cytochrome c from the mitochondria[45]. Yin et al[46], found that preconditioning with rapamycin reduces transient focal cerebral I/R injury via inhibiting apoptotic response. While I/R-induced tubular apoptosis drives the severity of kidney damage caused by oxidative stress due to a burst of ROS production as well as a decrease in antioxidants[47], our experiments showing the antiapoptotic effect of rapamycin could be the consequence of the restoration of oxidant/antioxidant status in HL I/R injury.

It has been indicated that NADPH oxidase activation is the initial source of superoxide production during I/R[27,48]. To the best of our knowledge, it has not been validated if mTOR exerts its critical roles on HL I/R injury via regulating oxidant/antioxidant balance except in our current work. It is apparent from the present study that I/R-induced gastrocnemius muscle and kidney injury are supported biochemically by elevated NADPH oxidase levels in these tissues beside diminished total antioxidant capacity in sera. Also, rapamycin administration reduced oxidative stress and restored antioxidant status in these tissues and sera, respectively. Our previous study on the same model revealed that mTOR inhibition could exert a protective effect on muscle and renal failure, possibly through a mechanism involving decreased

expression of gp91^{phox}, catalase activity, and lipid peroxidation[25]. It is noteworthy that rapamycin markedly reduced the over-activated gp91^{phox} and inhibited superoxide production following myocardial I/R[49]. Rapamycin-induced autophagy was attributed as a key antioxidant defense system for the removal of ROS-induced damaged/misfolded macromolecules[50] but still needs further explorations. Our data imply that mTOR inhibition with rapamycin has antioxidant properties and restored antioxidant status by the previous studies[49, 50].

In conclusion, this is the first study that highlighted the crucial role of the downstream targets of the mTOR pathway for organ protection against apoptosis and oxidative stress during HL I/R injury. Although mTOR has a theoretically attractive role as a therapeutic target in I/R, potential future treatments targeting mTOR should be tested against a wide variety of I/R injuries. On the other hand, clinical trials with mTOR inhibitors are limited to cancer and amyotrophic lateral sclerosis, still it deserves much attention as a potential treatment for I/R in the future. However, because of the limitations of this work, which include the lack of an immunohistological examination to demonstrate tissue injury, more research is necessary to clarify the mechanism of the pathogenic processes and organ damage that underlie HL I/R and its relationship to mTOR.

Author Contributions: Concept/Design : ZP, DSG, SSF; Data acquisition: ZP, DSG, MTR, SPS, OV, NS, SSF; Data analysis and interpretation: ZP, DSG, MTR, SSF; Drafting manuscript: ZP, DSG, MTR, SSF; Critical revision of manuscript: ZP, DSG, SPS, OV, NS, BT; Final approval and accountability: SSF; Technical or material support: ZP, DSG, MTR, SPS, OV, NS, SSF; Supervision: KUM; Securing funding (if available): n/a.

Ethical Approval: The experimental protocol was approved by the Mersin University Experimental Animals Local Ethics Committee with the number 2017/16 (31/07/2017).

Peer-review: Externally peer-reviewed.

Conflict of Interest: The authors declare that they have no conflict of interest.

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