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Ayse Karabulbul
Mersin Üniversitesi
Meryem Temiz-Resitoglu
Mersin Üniversitesi
Zainab Sabrie
Mersin Üniversitesi
Esraa Ebraheem Al-Jomaa
Mersin Üniversitesi
Taskin Kalkan
Mersin Üniversitesi
Tuba Isik
Mersin Üniversitesi
Ekrem Can Yaylim
Mersin Üniversitesi
Hatice Oruc-Demirbag
Mersin Üniversitesi
Nehir Sucu
Mersin Üniversitesi
Kafait U. Malik
University of Tennessee Health Science Center
Seyhan Sahan-Firat
seyhansahan06@gmail.com

Mersin Üniversitesi

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New Perspective on Inflammation and Oxidative Stress Following Peripheral Ischemia/Reperfusion Injury: The Role of Selective mTORC2 Inhibition

Ayse Karabulbul[1], Meryem Temiz-Resitoglu[1], Zainab Sabrie[1,2], Esraa Ebraheem Al-Jomaa[1], Taskin Kalkan[1], Tuba Isik[1], Ekrem Can Yaylim[3], Hatice Oruc-Demirbag[3], Nehir Sucu[4], Kafait U. Malik[5], Seyhan Sahan-Firat[1]

[1] Department of Pharmacology, Faculty of Pharmacy, Mersin University, Mersin, Türkiye

[2] Faculty of Medicine and Health Sciences, SIMAD University, Mogadishu, Somalia

[3] Department of Histology and Embryology, Faculty of Medicine, Mersin University, Mersin, Türkiye

[4] Department of Cardiovascular Surgery, Faculty of Medicine, Mersin University, Mersin, Türkiye

[5]Department of Pharmacology, Addiction Science and Toxicology, College of Medicine, University of Tennessee, Health Science Center, Memphis, TN, USA.

Corresponding author

Correspondence to Seyhan Sahan-Firat | Email: seyhansahan06@gmail.com

Abstract

Background Ischemia/reperfusion (I/R) injury is a major pathological condition that contributes to local and distant organ damage through inflammatory and oxidative stress mechanisms. Although mammalian target of rapamycin complex 1 (mTORC1) has been widely studied in inflammation, the role of mTOR complex 2 (mTORC2) in I/R injury remains unclear.

This study aimed to investigate the contribution of mTORC2 in hindlimb I/R-induced injury in the kidney and gastrocnemius muscle tissues using the selective mTORC2 inhibitor JR-AB2-011.

Methods and Results Rats were assigned to control, dimethyl sulfoxide (DMSO), I/R, JR-AB2-011, and I/R + JR-AB2-011 groups. JR-AB2-011 was administered one hour before reperfusion. Kidney and gastrocnemius muscle tissues were collected for molecular analysis.

Hindlimb I/R significantly increased in the kidney and gastrocnemius muscle rictor expression and protein kinase B (Akt) phosphorylation, indicating mTORC2 activation, which JR-AB2-011 prevented. I/R also activated the IκB kinase (IKKα)/inhibitor κB (IκBα)/nuclear factor-κB (NF-κB) signaling pathway, along with elevated levels of tumor necrosis factor (TNF)-α and interleukin (IL)-1β. In addition, oxidative stress markers, including $gp91^{phox}$ and $p22^{phox}$ expression, superoxide production, matrix metalloproteinase (MMP9) expression, and myeloperoxidase (MPO) levels, were significantly increased following I/R and attenuated by JR-AB2-011 treatment.

Conclusions mTORC2 contributes to inflammatory and oxidative stress responses following hindlimb I/R in both the locally affected gastrocnemius muscle and the remote kidney. Selective inhibition of mTORC2 by JR-AB2-011 attenuates NF-κB-mediated inflammatory signaling and oxidative stress, suggesting a potential therapeutic target for limiting both local and remote consequences of I/R injury.

Keywords ischemia/reperfusion; mTORC2; inflammation; oxidative stress; JR-AB2-011; DHE

Abbreviations

I/R, ischemia/reperfusion; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; ROS, reactive oxygen species; Akt, protein kinase B; DMSO, dimethyl sulfoxide; DHE, dihydroethidium; MPO, myeloperoxidase; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance; SEM, standard error of the mean.

Introduction

Restoration of blood flow to ischemic tissues is one of the therapeutic interventions in numerous medical disorders such as trauma, vascular surgery, acute limb ischemia, myocardial infarction, and ischemic stroke [1]. While restoration of the blood supply is required for the viability of the tissue, reperfusion paradoxically causes tissue injury worse than ischemia itself due to excessive production of reactive oxygen species (ROS), inflammatory factors, and mitochondrial dysfunction. Hence, ischemia-reperfusion (I/R) injury continues to be an important medical problem contributing to both local and systemic tissue damage [2]. Hindlimb I/R serves as a validated animal model that resembles lower limb ischemia during vascular surgery and traumatic injuries [3]. Although skeletal muscles are the main tissues affected, reperfusion triggers inflammatory cascade involving cytokine secretion, leukocyte activation, oxidative stress, and endothelial dysfunction [4,5]. This causes damage to tissues

outside of the ischemic limb. The kidneys are especially prone to I/R injury because of their high metabolic activity and extensive microcirculation. Inflammation of the kidneys after the hindlimb I/R injury results in the development of acute kidney injury, which significantly increases morbidity and mortality. Consequently, reducing damage to the remote kidneys has emerged as an important therapeutic target in hindlimb I/R experimental and clinical studies [6]. Among the intracellular signaling mechanisms activated during I/R injury, the NF-κB pathway is critical for regulating the inflammatory process. In its unactivated form, NF-κB is localized in the cytoplasm and held by IκB, activation of IKK leads to IκB phosphorylation and degradation, resulting in the entry of NF-κB into the nucleus where it triggers transcription of proinflammatory cytokine genes such as TNF-α and IL-1β [7-9]. Beyond its role in inflammation, NF-κB also contributes to oxidative stress synthesis of ROS producing enzymes [10]. Indeed, our earlier investigations in the rat hindlimb I/R model indicated involvement of mTOR/IκB-α/NF-κB signaling mechanism in muscle and remote kidney damage due to increased inflammation and oxidative/nitrosative stress [11-13].

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that is involved in the regulation of cell growth, metabolism, proliferation, and survival. It functions as a catalytic component of two distinct multiprotein complexes, known as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC2 phosphorylates Akt at position Ser^{473}, and regulates multiple downstream molecules responsible for cell survival [14]. Furthermore, accumulating data suggest that mTORC2 may regulate inflammatory response and oxidative stress [15-19]. Importantly, our group has recently shown that mTORC2 inhibition can reduce the IL-1β stimulated inflammatory response in human chondrocytes [20]. Zymosan-induced hypotension and systemic inflammation through the regulation of IKKα/IκB-α/NF-κB pathway [21]. These results support the notion that mTORC2 is involved in regulating inflammatory response. Although the participation of mTORC2 in the regulation inflammation is becoming clearer, its role in peripheral I/R injury has not yet been addressed. Therefore, the goal of the current study was to investigate the role of mTORC2 in the local gastrocnemius muscle and remote kidney responses to hindlimb I/R, with particular emphasis on inflammatory signaling and oxidative stress.

Materials and Methods

Reagents

JR-AB2-011 was obtained from Aobious (Gloucester, MA, USA). Primary antibodies against rictor, Akt, phosphorylated Akt (Ser^{473}), IKKα, phosphorylated IKKα, IκB-α, phosphorylated IκB-α (Ser^{32}), NF-κB p65, phosphorylated NF-κB p65, TNF-α, IL-1β, MMP-9, $gp91^{phox}$, $p22^{phox}$, α-sarcomeric actin, and β-actin were purchased from Santa Cruz Biotechnology Inc. Secondary antibodies (goat anti-rabbit IgG–HRP conjugate, RPN4301, and sheep anti-mouse IgG–HRP conjugate, RPN4201) were purchased from Amersham (GE Healthcare, United Kingdom). We used ECL Prime Western Blotting Detection Reagents (RPN2232, Cytiva Life Sciences, Amersham, United Kingdom) for chemiluminescent detection.

Animals

Experiments were carried out using male Wistar rats (250–300 g) obtained from the Research Center of Experimental Animals, Mersin University, Mersin, Turkey. Rats were housed under standard laboratory conditions with a 12-hour light/dark cycle and allowed free access to standard chow and tap water. Experimental procedures

were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Mersin University Experimental Animals Local Ethics Committee approved all experimental procedures performed on the rats in this study.

Experimental Design

Five groups of rats (n = 6) were randomly assigned for this experiment: control, I/R, JR-AB2-011, I/R + JR-AB2-011, and DMSO. The rats in the control group received no treatment. Animals in the JR-AB2-011 group received only the selective mTORC2 inhibitor JR-AB2-011 (1 mg/kg, i.p.). The dosing of JR-AB2-011 was based on results from our earlier investigation [21]. The hindlimb I/R model was used as previously described [22,23]. I/R was achieved by the placement of elastic tourniquets bilaterally on both hindlimbs under ketamine (90 mg/kg, i.m.)/xylazine (10 mg/kg, i.m.) anesthesia. Ischemia was sustained for 4 hours, followed by 4 hours of reperfusion after tourniquets removal. In the I/R + JR-AB2-011 group, JR-AB2-011 (1 mg/kg, i.p.) was administered 1 hour before the onset of reperfusion. DMSO-treated animals received the vehicle solution (DMSO dissolved in physiological saline). The animals were euthanized with ketamine/xylazine anesthesia upon completion of the experimental protocol. Gastrocnemius muscle and kidneys were removed and stored at −80°C until biochemical and molecular analysis. Blood samples were drawn from the vena cava caudalis, allowed to clot at room temperature for 30 minutes, then defibrillated. The blood samples were centrifuged at 23,910 × g for 15 minutes at 4°C. The serum was collected and stored at −20°C until analysis. Kidney and gastrocnemius muscle were homogenized using cold RIPA lysis buffer (pH 7.5) containing sodium orthovanadate, sodium fluoride, leupeptin, and aprotinin. Supernatants were prepared by centrifuging the homogenates at 23,910 × g for 10 minutes at 4°C. The supernatants were combined with Tris/KCl buffer and sonicated (2 x 15 seconds) using a water-bath sonicator. After sonication, the samples were centrifuged again at 23,910 × g for 15 minutes at 4°C. The supernatants were collected and stored at −80°C until further use. The total protein concentration in tissue homogenate supernatants was determined using the Bradford protein assay [24].

Western Blot Analysis

The expression and/or phosphorylation of rictor, Akt, IKKα, IκB-α, NF-κB p65, TNF-α, IL-1β, MMP9, $gp91^{phox}$, $p22^{phox}$, α-sarcomeric actin, and β-actin proteins were detected by Western blotting according to our previous studies [11, 21, 25-29]. Equivalent amounts of proteins were separated through sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using resolving and stacking gels of appropriate composition and transferred to nitrocellulose membranes using a semi-dry transfer apparatus. The transfer efficiency was validated by Ponceau S staining. The membranes were blocked with non-fat dry milk in Tris-buffered saline containing 0.05% Tween-20 (TBST) and incubated overnight at 4ºC with primary antibodies. Post-incubation, the membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature. Protein bands were visualized using enhanced chemiluminescence (ECL) reagent and a gel documentation system. Band intensities were measured densitometrically using ImageJ software (version 1.46r). Protein expression levels were standardized against β-actin or α-sarcomeric actin, and the results were expressed in relative density units/mg total protein.

DHE ROS staining

Unfixed kidney and gastrocnemius muscle tissues were embedded in optimal cutting temperature (OCT) compound, rapidly frozen, and sectioned at a thickness of 10 μm using a cryostat (Leica CM1900, Leica GmbH,

Germany). The sections were allowed to equilibrate to room temperature. A 10 mM stock solution of dihydroethidium (DHE; ab145360, Abcam) was prepared in dimethyl sulfoxide (DMSO) and diluted in PBS to a final working concentration of 10 μM immediately before use. The sections were incubated with this DHE solution for 30 min at 37°C, protected from light. Following DHE incubation, the sections were washed three times with PBS and mounted with an aqueous antifade mounting medium containing DAPI (Fluoroshield Mounting Medium with DAPI, ab104139, Abcam) for nuclear counterstaining.

For quantitative analysis, DHE fluorescence images were acquired using a Nikon Ti fluorescence microscope (Nikon, Japan) with a 20× objective at excitation wavelengths of 544 nm. Five randomly selected, non-overlapping fields were imaged from each animal under identical acquisition settings for all experimental groups. DHE fluorescence intensity (relative intensity) was quantified from these images using ImageJ software (version 1.54d). Representative images were additionally acquired using the TRITC and DAPI channels.

Measurement of Myeloperoxidase (MPO) Activity

The levels of myeloperoxidase in serum and tissue homogenate were estimated using commercially available myeloperoxidase enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol.

Statistical Analysis

Data are presented as mean ± SEM. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls multiple comparison test. Student's t-test was used for comparisons between two groups when appropriate. Statistical analyses were conducted using GraphPad Prism, and P values <0.05 were considered statistically significant.

Results

JR-AB2-011 inhibits I/R-induced increase in rictor expression and Akt phosphorylation

The protein expression of rictor and Akt was assessed in kidney and gastrocnemius muscle to investigate the change in mTORC2 activation. I/R led to an increase in rictor protein expression, the primary mTORC2 component ($P < 0.05$; Fig. 1A). Additionally, I/R increased phosphorylation of Akt (Ser^{473}), downstream target of mTORC2 ($P < 0.05$; Fig. 1B), without altering its protein expression within the kidney and gastrocnemius muscle tissues. JR-AB2-011 prevented these I/R induced alterations (Fig. 1; $P < 0.05$). JR-AB2-011 alone or its vehicle (DMSO) did not alter these molecules' expression within any of the experimental groups (Fig. 1; $P > 0.05$).

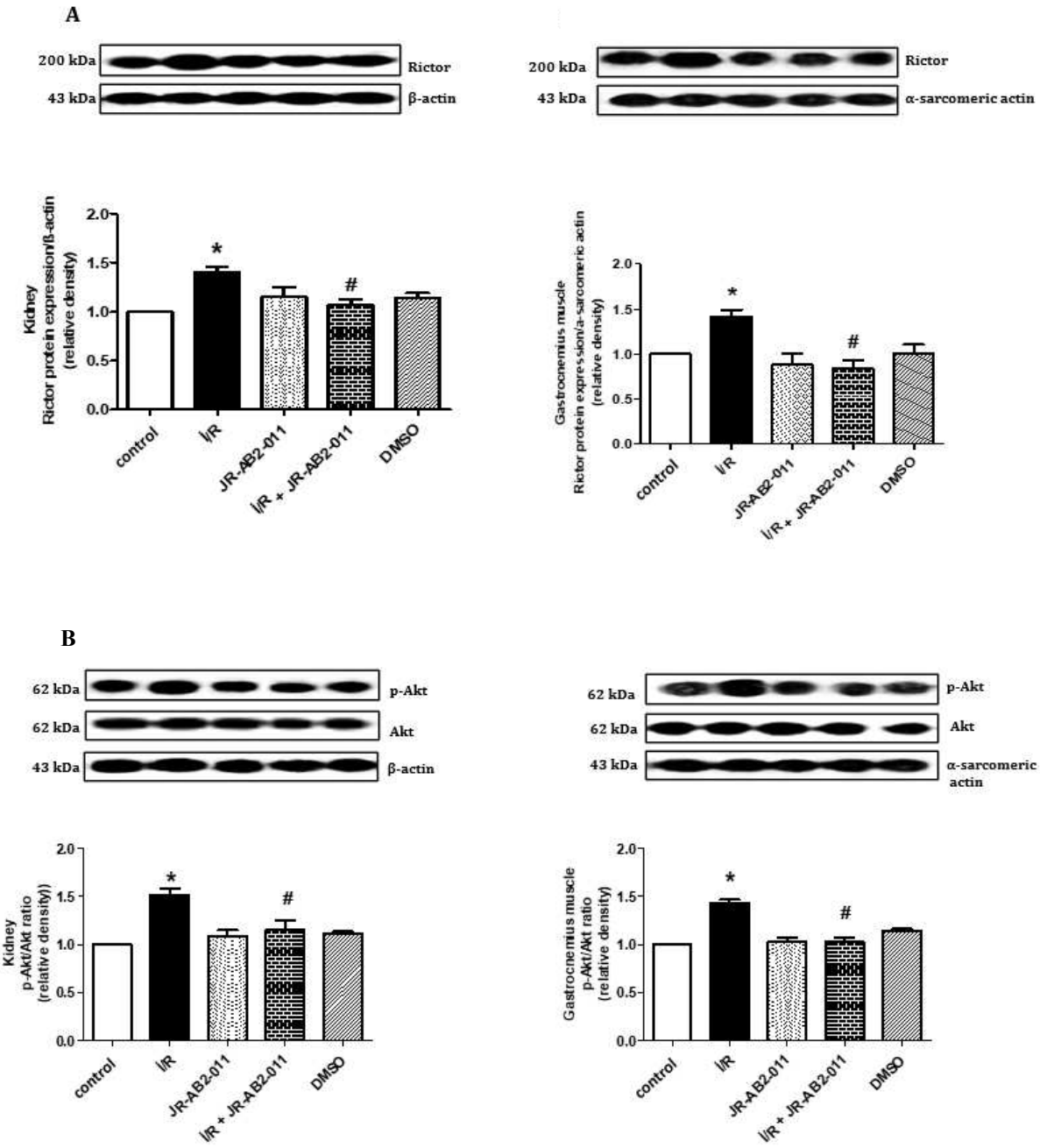
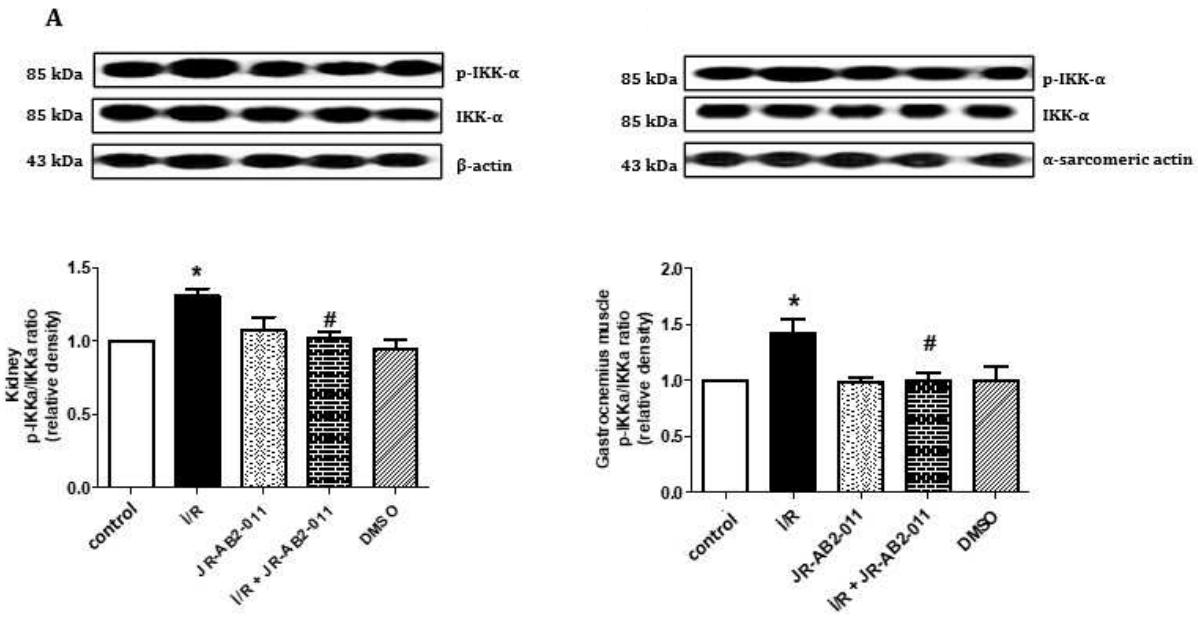


Fig. 1 JR-AB2-011 prevented I/R-induced increase in A) rictor and B) Akt protein expression and/or phosphorylation in kidney and gastrocnemius muscle tissues of rats measured after 8 hours following saline (4 ml/kg, i.p.), DMSO (4 ml/kg, i.p.), or I/R induction (4 hours of reperfusion following 4 hours of ischemia) in rats. JR-AB2-011 (1 mg/kg, i.p.) was administered 1 hour before reperfusion. Rictor and Akt protein expression was measured in tissue homogenates by Western blotting analysis. Bands density was analyzed by ImageJ 1.54d software. Data are expressed as means ± SEM (n = 4). * $P < 0.05$ vs. control group; $^{\#}$ $P < 0.05$ vs. I/R group

JR-AB2-011 inhibits I/R-induced increase in IKKα, IκB-α, and NF-κB p65 expression

To examine the contribution of mTORC2 to the IKKα/IκB-α/NF-κB p65 signaling pathway, our investigation involved assessing the impact of mTORC2 on the phosphorylation of IKKα, IκB-α, and NF-κB p65 and their non-phosphorylated form in response to I/R. I/R caused an increase in the phosphorylated form of IKKα (Fig. 2A), NF-κB p65, and phosphorylated NF-κB p65 (Fig. 2C) protein expressions; whereas it caused a decrease in the expression of IκB-α (Fig. 2B), in contrast to the group treated with JR-AB2-011 ($P < 0.05$, Fig. 2). JR-AB2-011 alone or its vehicle (DMSO) did not alter both the phosphorylation and expression of these proteins (Fig. 2; $P > 0.05$).



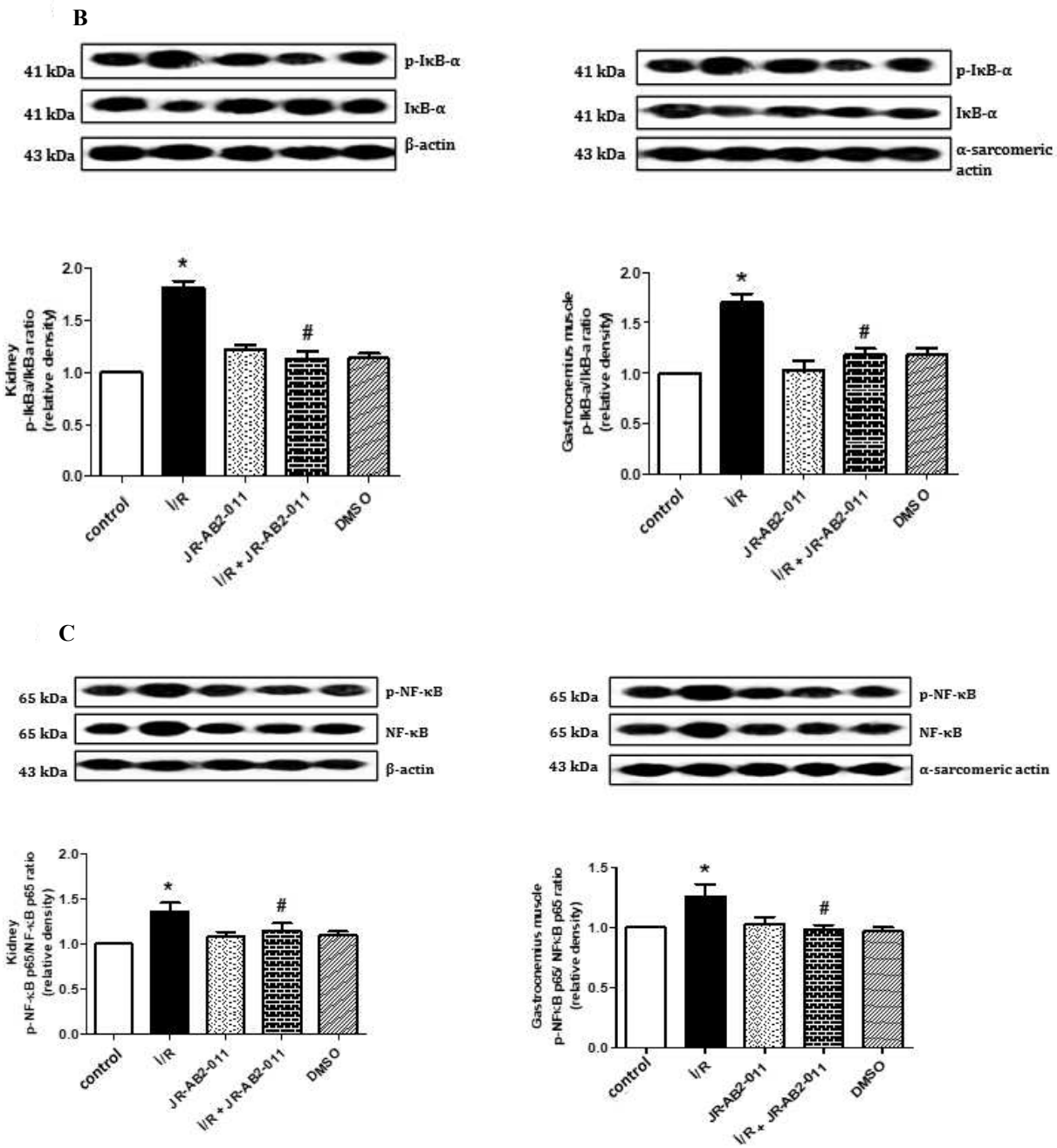
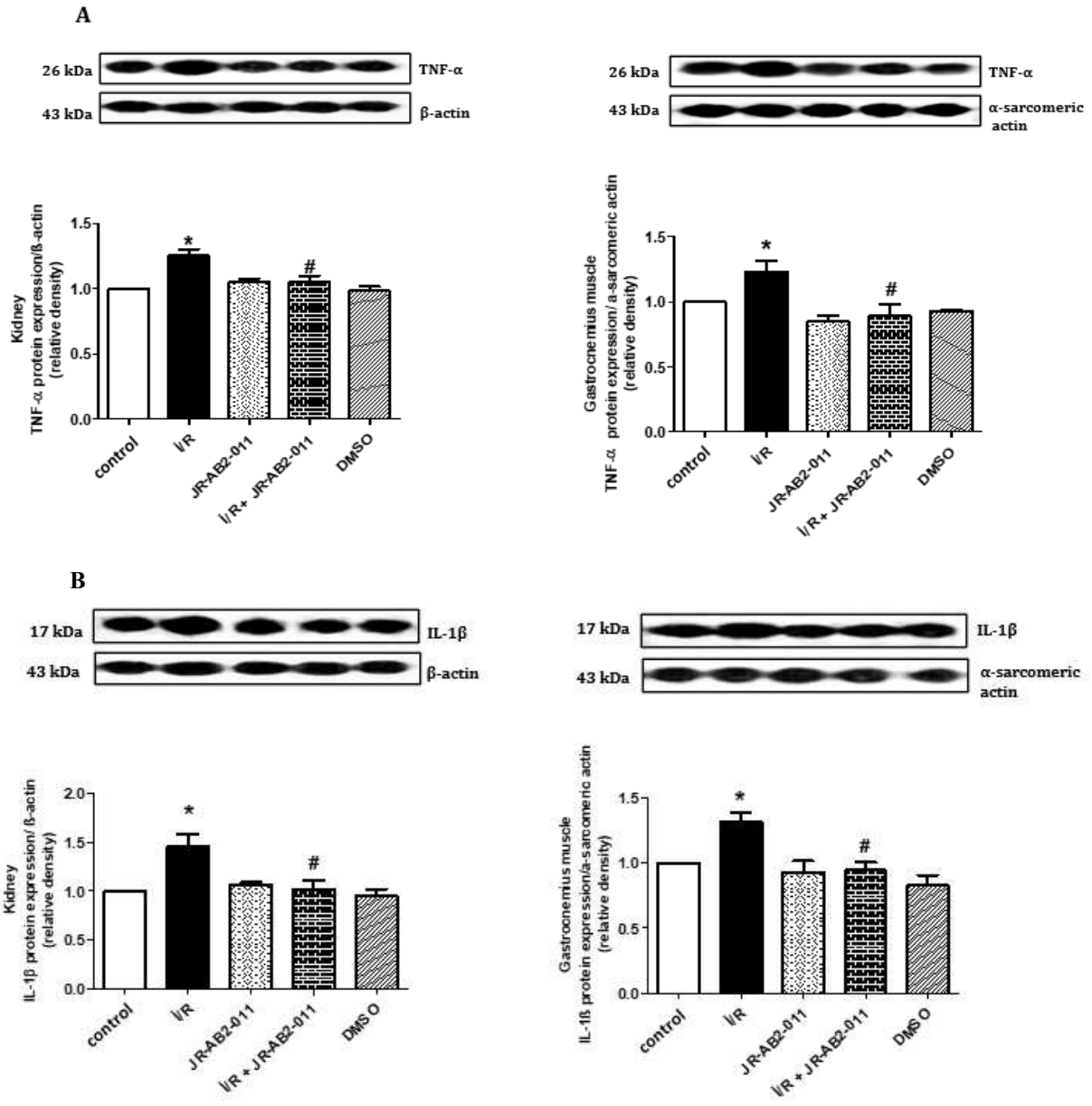


Fig. 2 JR-AB2-011 prevented I/R-induced changes in IKKα, IκB-α, and NF-κB p65 protein expression and phosphorylation in kidney and gastrocnemius muscle tissues of rats measured after 8 hours following saline (4 ml/kg, i.p.), DMSO (4 ml/kg, i.p.) or I/R induction (4 hours of reperfusion following 4 hours of ischemia) in rats. JR-AB2-011 (1 mg/kg, i.p.) was administered 1 hour before reperfusion. IKKα, IκB-α, and NF-κB protein expression were measured in tissue homogenates by western blotting analysis. The density of bands was analyzed by Image J 1.54d software. Data are expressed as means ± SEM (n = 4). * $P < 0.05$ vs. control group; $^{\#}$ $P < 0.05$ vs. I/R group

JR-AB2-011 inhibits the increase in the expression of TNF-α, IL-1β, and MMP9, in addition to MPO levels in rats subjected to I/R injury

We evaluated the consequences of mTORC2 inhibition on inflammatory markers by assessing its capacity to decrease TNF-α, IL-1β, and MMP9 expression, in addition to MPO levels in the kidney, gastrocnemius muscle, and serum 8 hours after I/R induction. In Fig. 3, an increase in TNF-α (Fig. 3A), IL-1β (Fig. 3B), MMP9 (Fig. 3C), and MPO levels (Fig. 3D) was observed in the above-mentioned tissues and serum. Treatment with JR-AB2-011 effectively prevented this increase. Moreover, no difference was observed in groups treated with JR-AB2-011 alone or its vehicle (DMSO) compared to their corresponding control groups in terms of TNF-α, IL-1β, and MMP9 expression, and MPO levels.



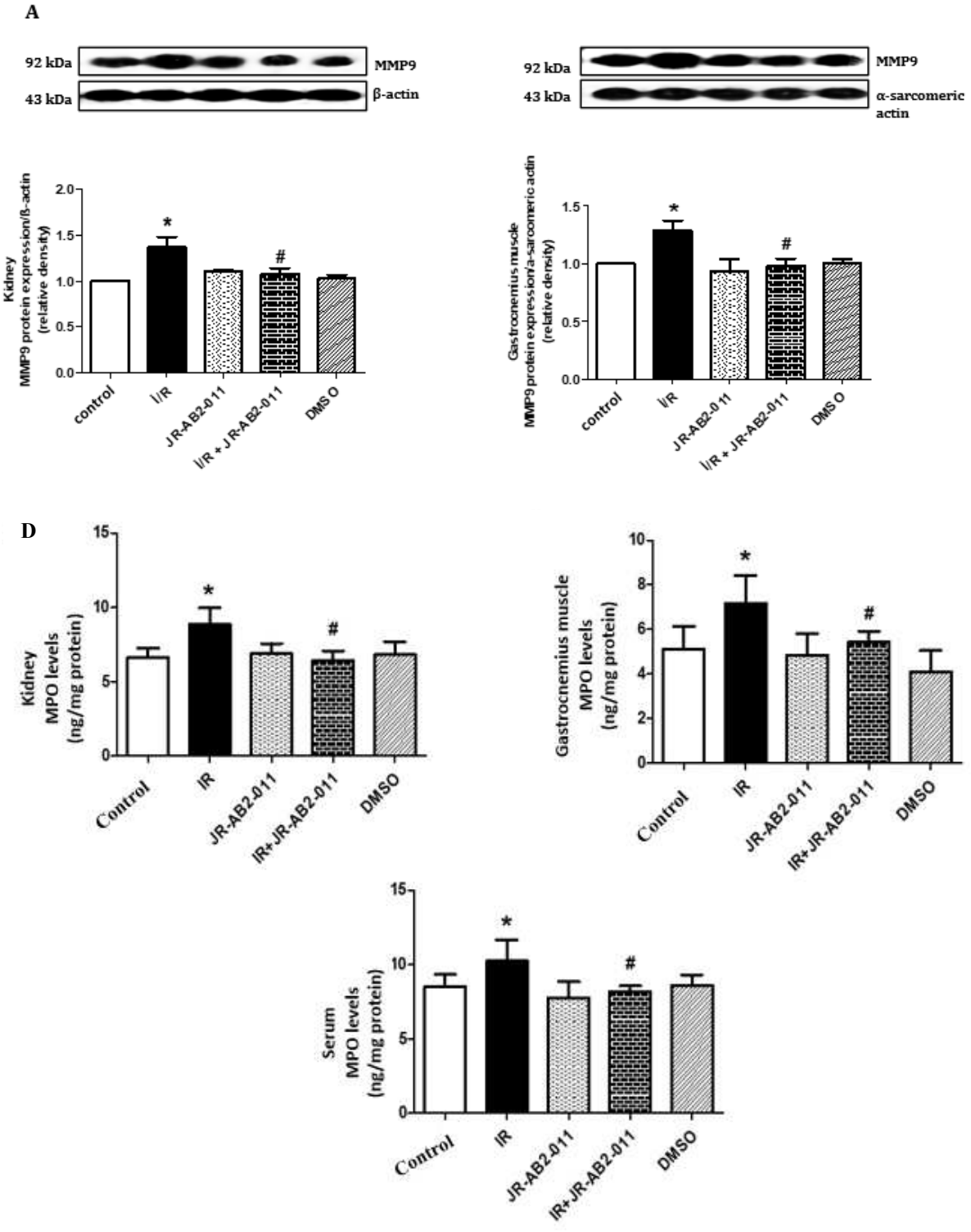
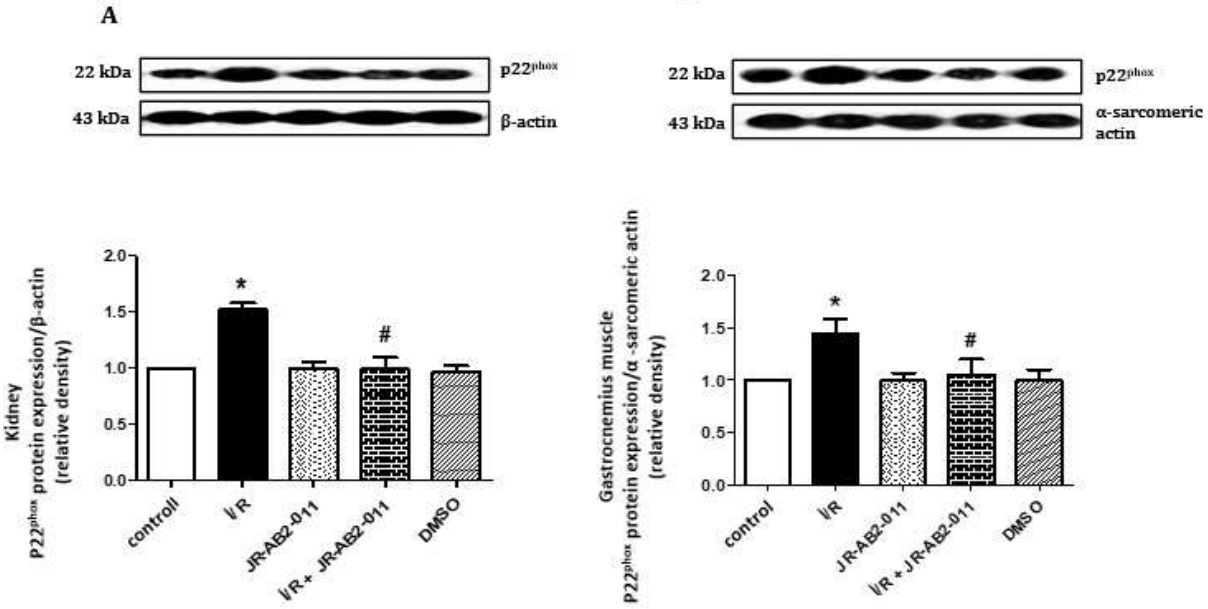


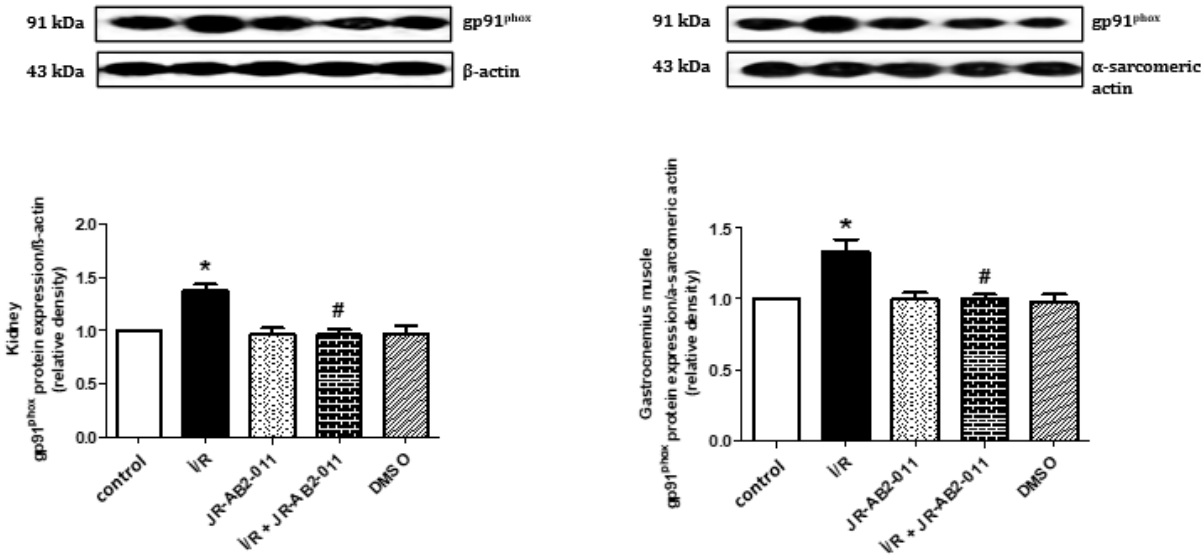
Fig. 3 JR-AB2-011 prevented the I/R -induced increase in protein expression of inflammatory markers. Expression of A) TNF-α, B) IL-1β, C) MMP9 in kidney and gastrocnemius muscle, and D) levels of MPO in kidney, gastrocnemius muscle, and sera of rats measured after 8 hours following saline (4 ml/kg, i.p.), DMSO (4 ml/kg, i.p.), or I/R induction. JR-AB2-011 (1 mg/kg, i.p.) was administered 1 hour before reperfusion. The protein expression of TNF-α, IL-1β, and MMP9 was detected by western blotting analysis. The density of bands was analyzed using ImageJ 1.54d software. MPO levels were measured by ELISA kit. Data are expressed as means ± SEM (n = 4). *P < 0.05 vs. control group; #P < 0.05 vs. I/R group

JR-AB2-011 prevents the I/R-induced increase in $p22^{phox}$ and $gp91^{phox}$ expression and superoxide formation

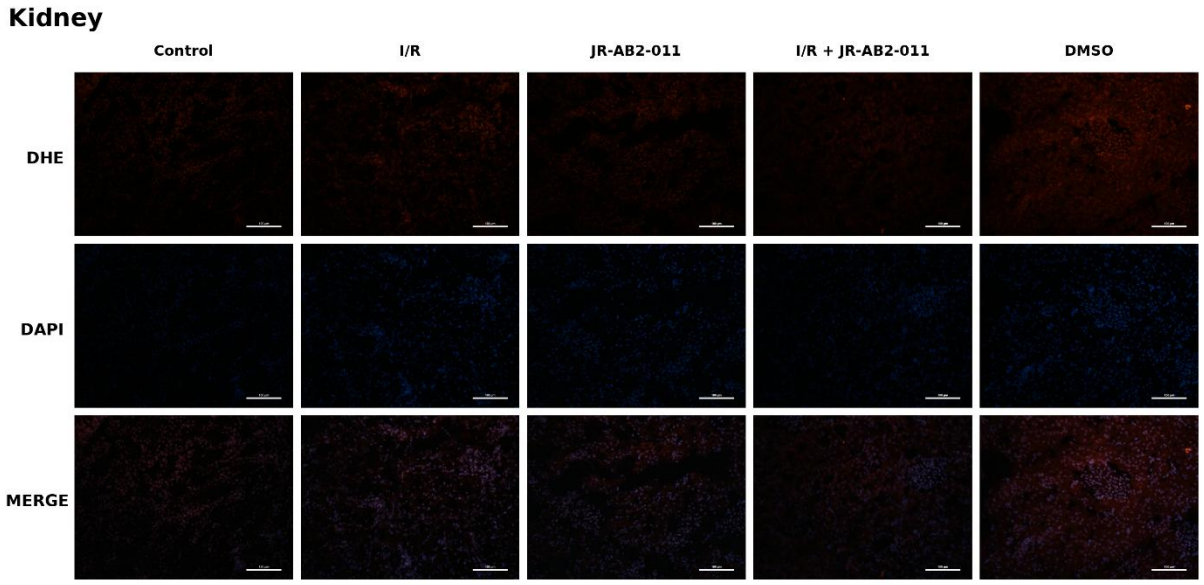
We assessed the impact of mTORC2 inhibition on oxidative stress-related markers by measuring its ability to decrease the expression of NADPH oxidase subunits $p22^{phox}$ and $gp91^{phox}$, as well as superoxide formation in kidney and gastrocnemius muscle tissues 4 hours after reperfusion following 4 hours of ischemia. In Fig. 4, an increase in $p22^{phox}$ (Fig. 4A) and $gp91^{phox}$ (Fig. 4B) expression was observed in the above-mentioned tissues following I/R injury. Treatment with JR-AB2-011 effectively prevented this increase. Furthermore, I/R injury significantly increased superoxide formation, as detected by DHE fluorescence staining (Fig. 4C), while treatment with JR-AB2-011 reduced I/R-induced superoxide generation. Moreover, groups treated with JR-AB2-011 alone or its vehicle (DMSO) showed no difference in $p22^{phox}$, $gp91^{phox}$ expression, or superoxide formation compared with their corresponding control groups.



B


91 kDa
gp91phox
43 kDa
β-actin
91 kDa
gp91phox
43 kDa
α-sarcomeric actin
Kidney
gp91phox protein expression/β-actin (relative density)
2.0
1.5
1.0
0.5
0.0
*
#
control
I/R
JR-AB2-011
I/R + JR-AB2-011
DMSO
Gastrocnemius muscle
gp91phox protein expression/α-sarcomeric actin (relative density)
1.5
1.0
0.5
0.0
*
#
control
I/R
JR-AB2-011
I/R + JR-AB2-011
DMSO

C


Kidney
Control
I/R
JR-AB2-011
I/R + JR-AB2-011
DMSO
DHE
DAPI
MERGE

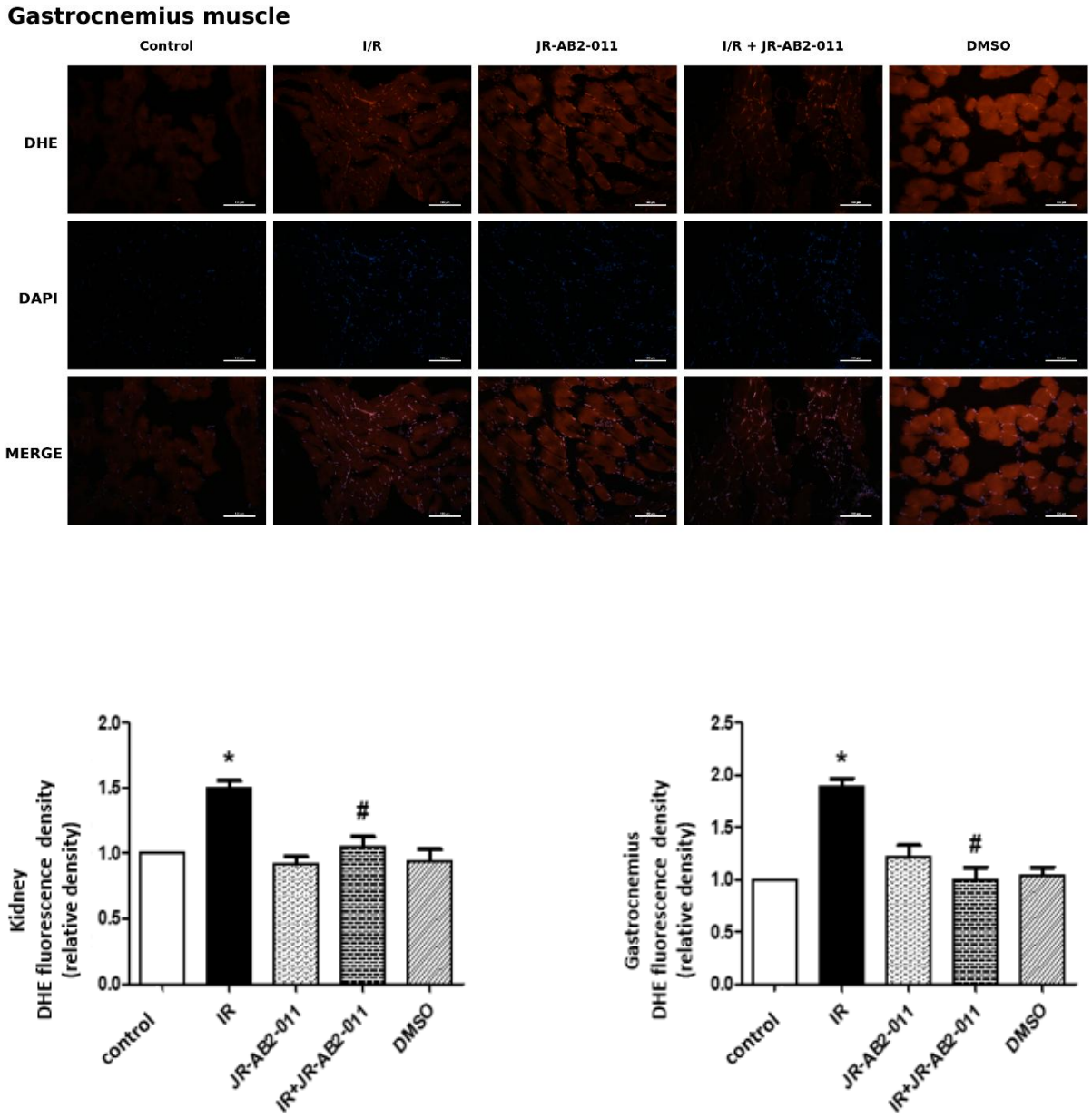


Fig. 4 JR-AB2-011 prevents I/R-induced oxidative stress by reducing NADPH oxidase subunit expression and superoxide accumulation. Expression of A) $p22^{phox}$ and B) $gp91^{phox}$ in kidney and gastrocnemius muscle tissues, and C) superoxide accumulation detected by DHE fluorescence staining with DAPI nuclear counterstaining in kidney and gastrocnemius muscle sections of rats, measured after 4 hours of reperfusion following 4 hours of ischemia. Rats received saline (4 ml/kg, i.p.), DMSO (4 ml/kg, i.p.), or underwent I/R injury. JR-AB2-011 (1 mg/kg, i.p.) was administered 1 hour before reperfusion. The protein expression of $p22^{phox}$ and $gp91^{phox}$ was detected by Western blotting analysis. The density of bands was analyzed using ImageJ 1.54d software. DHE fluorescence images were acquired at 20× magnification, and fluorescence intensity was quantified using ImageJ 1.54d software. Data are expressed as means ± SEM (n = 4). $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. I/R group

Discussion

The main finding of this study is that mTORC2 is involved in the regulates inflammatory and oxidative stress responses associated with I/R injury to peripheral organs. This statement is based on results showing that the mTORC2-selective inhibitor JR-AB2-011 provided pronounced protective effects against I/R injury in the kidney and gastrocnemius muscle. Indeed, mTORC2 signaling was impaired by decreased rictor levels and Akt phosphorylation, along with inhibition of the IKKα/IκB-α/NF-κB cascade and a reduced inflammatory response, as manifested by decreased TNF-α and IL-1β levels. In addition, JR-AB2-011 reduced neutophil-mediated inflammation and extracellular matrix remodeling as shown by the decreased MPO and MMP9 expression. Moreover, JR-AB2-011 inhibited oxidative stress by suppressing NADPH oxidase-associated proteins $p22^{phox}$ and $gp91^{phox}$ expression and reducting superoxide formation, as visualized by DHE staining. Taken together, these findings indicate that mTORC2 mediates an upstream signaling that links inflammatory and oxidative stress responses after I/R injury to peripheral organs.

Although the contribution of mTORC1 to peripheral I/R-associated injury has been extensively investigated [11–13,30], the role of mTORC2 in I/R-related pathological responses remains poorly understood. Previous studies have linked mTORC2 signaling to the regulation of inflammatory and oxidative stress responses [15–17,31]. Our previous studies further demonstrated that selective mTORC2 inhibition attenuated IL-1β-induced inflammatory responses in human chondrocytes and protected against zymosan-induced systemic inflammation by suppressing rictor expression and Akt phosphorylation [20,21]. However, whether mTORC2 is involved in the local and remote tissue responses induced by hindlimb I/R has not been established. In the present study, hindlimb I/R significantly increased rictor expression and Akt phosphorylation at Ser^{473} in both the gastrocnemius muscle, representing the local ischemic tissue, and the kidney, representing a remote organ affected by hindlimb I/R. Treatment with JR-AB2-011 prevented these alterations in both tissues, indicating that mTORC2 signaling is activated during both the local muscular and remote renal responses to hindlimb I/R. These findings identify mTORC2 activation as an important component of the pathological response to hindlimb I/R in both local and remote tissues.

Previous studies have demonstrated an important role for NF-κB signaling in I/R-induced inflammatory responses [32–34]. In the present study, hindlimb I/R increased activation of the IKKα/IκB-α/NF-κB pathway in both the gastrocnemius muscle and kidney. The simultaneous activation of mTORC2/Akt and IKKα/IκB-α/NF-κB signaling, together with their attenuation by JR-AB2-011, suggests that mTORC2 contributes to NF-κB activation during hindlimb I/R. This effect was observed in both the locally affected gastrocnemius muscle and the remote kidney, indicating that mTORC2-associated inflammatory signaling may contribute to both local and distant responses to hindlimb I/R. These findings are consistent with previous evidence linking mTORC2/Akt signaling with IKKα/NF-κB activation [15,34,35].

NF-κB activation promotes the production of pro-inflammatory cytokines such as TNF-α and IL-1β, which contribute to the amplification of inflammatory responses during I/R injury [32,36]. In the present study, hindlimb I/R increased TNF-α and IL-1β expression in both the gastrocnemius muscle and kidney, whereas JR-AB2-011 markedly reduced these responses. Together with the inhibition of IKKα/IκB-α/NF-κB signaling, these findings suggest that mTORC2 contributes to cytokine production during hindlimb I/R through regulation of NF-κB

signaling. The changes observed in both the locally affected muscle and the remote kidney further indicate that this pathway may participate in both local and distant inflammatory responses following hindlimb I/R.

Neutrophil activation and extracellular matrix remodeling are important components of I/R-induced inflammatory injury [37–40]. In the present study, hindlimb I/R increased MPO and MMP9 expression in both the gastrocnemius muscle and kidney, whereas JR-AB2-011 markedly reduced these changes. The increase in MPO most likely reflects enhanced neutrophil-associated inflammatory activity, while the increase in MMP9 is consistent with inflammatory cell migration and extracellular matrix remodeling. The parallel reduction of MPO and MMP9 together with suppression of NF-κB signaling by JR-AB2-011 suggests that mTORC2 may contribute to these responses through regulation of inflammatory and immune-cell activation. The presence of these changes in both the locally affected muscle and the remote kidney further supports the involvement of mTORC2 in both local and distant inflammatory responses following hindlimb I/R.

Oxidative stress is an important component of I/R-induced tissue injury, and NADPH oxidase is a major source of ROS during inflammatory responses [41,42]. In the present study, hindlimb I/R increased $p22^{phox}$ and $gp91^{phox}$ expression together with superoxide formation in both the gastrocnemius muscle and kidney, whereas JR-AB2-011 markedly attenuated these changes. These findings suggest that mTORC2 contributes to NADPH oxidase-associated oxidative stress following hindlimb I/R. The simultaneous increase in MPO, NADPH oxidase subunits, and superoxide formation also suggests that inflammatory and immune-cell activation may contribute to ROS generation during reperfusion. Since ROS can further enhance NF-κB signaling [43], the parallel suppression of oxidative stress and inflammatory signaling by JR-AB2-011 suggests an interaction between these processes downstream of mTORC2 in both local muscle and remote kidney responses. Taken together, our findings suggest that mTORC2 contributes to the coordination of inflammatory and oxidative stress responses following hindlimb I/R in both the gastrocnemius muscle and remote kidney. Activation of mTORC2/Akt was associated with activation of the IKKα/IκB-α/NF-κB pathway and increased TNF-α and IL-1β expression. This inflammatory response was accompanied by increased MPO and MMP9, suggesting enhanced immune-cell-associated inflammation and extracellular matrix remodeling, together with increased $p22^{phox}$, $gp91^{phox}$, and superoxide formation. The attenuation of these changes by JR-AB2-011 suggests that mTORC2 is an important upstream component linking NF-κB-mediated inflammation, immune-cell-associated responses, and oxidative stress during hindlimb I/R. The occurrence of these alterations in both the locally affected gastrocnemius muscle and the remote kidney further indicates that this signaling mechanism may contribute to both local and distant consequences of hindlimb I/R.

Conclusion

Overall, this study demonstrates that mTORC2 contributes to the inflammatory and oxidative stress responses induced by hindlimb I/R in both the locally affected gastrocnemius muscle and the remote kidney. Selective inhibition of mTORC2 with JR-AB2-011 attenuated activation of the IKKα/IκB-α/NF-κB pathway, reduced TNF-α and IL-1β expression, and decreased MPO, MMP9, NADPH oxidase-associated proteins, and superoxide formation. These findings suggest that mTORC2 acts as an important upstream component linking inflammatory signaling, immune-cell-associated responses, and oxidative stress during hindlimb I/R. Thus, mTORC2 may represent a potential therapeutic target for limiting both local and remote consequences of reperfusion injury.

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Statements and Declarations

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Ethical approval All experimental procedures were approved by the Local Ethics Committee for Animal Experiments of Mersin University (Decision date 22/11/2024).

Data availability The authors state that all the information needed to support the study's findings is included in the paper.

Consent to participate Not applicable (animal study; no human participants).

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Author contributions:

AK: conceptualization, experimental studies, data analysis, reviewing, and editing. MTR: experimental studies, data analysis, reviewing, and editing. ZS: literature research, manuscript drafting, and writing, EEA: experimental studies, reviewing and editing, TK: experimental studies, reviewing and editing. TI: experimental studies, reviewing and editing, ECY: experimental studies, reviewing and editing, HOD: experimental studies, data analysis, reviewing and editing, NS: experimental studies, data analysis, reviewing and editing, KUM: reviewing and editing, SSF: conceptualization, study design, experimental studies, data analysis, supervision, reviewing and editing.