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ORIGINAL ARTICLE

Protection by mTOR Inhibition on Zymosan-Induced Systemic Inflammatory Response and Oxidative/Nitrosative Stress: Contribution of mTOR/MEK1/ERK1/2/IKKβ/IκB-α/NF-κB Signalling Pathway

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Abstract— Mammalian target of rapamycin (mTOR), a serine/threonine kinase regulate variety of cellular functions including cell growth, differentiation, cell survival, metabolism, and stress response, is now appreciated to be a central regulator of immune responses. Because mTOR inhibitors enhanced the anti-inflammatory activities of regulatory T cells and decreased the production of proinflammatory cytokines by macrophages, mTOR has been a pharmacological target for inflammatory diseases. In this study, we examined the role of mTOR in the production of proinflammatory and vasodilator mediators in zymosan-induced non-septic shock model in rats. To elucidate the mechanism by which mTOR contributes to non-septic shock, we have examined the activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system caused by mTOR/mitogen-activated protein kinase kinase (MEK1)/extracellular signal-regulated kinase (ERK1/2)/inhibitor κB kinase (IKKβ)/inhibitor of κB (IκB-α)/nuclear factor-κB (NF-κB) signalling pathway activation. After 1 h of zymosan (500 mg/kg, i.p.) administration to rats, mean arterial blood pressure (MAP) was decreased and heart rate (HR) was increased. These changes were associated with increased expression and/or activities of ribosomal protein S6, MEK1, ERK1/2, IKKβ, IκB-α and NF-κB p65, and NADPH oxidase system activity in cardiovascular and renal tissues. Rapamycin (1 mg/kg, i.p.), a selective mTOR inhibitor, reversed these zymosan-induced changes in these tissues. These observations suggest that activation of mTOR/MEK1/ERK1/2/IKKβ/IκB-α/NF-κB signalling pathway with proinflammatory and vasodilator mediator formation and NADPH oxidase system activity contributes to systemic inflammation in zymosan-induced non-septic shock. Thus, mTOR may be an optimal target for the treatment of the diseases characterized by the severe systemic inflammatory response.

KEY WORDS: mTOR; zymosan; non-septic shock; systemic inflammatory response; rat.

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INTRODUCTION

Systemic inflammatory response syndrome (SIRS), actually having pro- and anti-inflammatory components, is an "inflammatory" response against infectious or non-

infectious invaders. It is believed that SIRS is a main detrimental process in the pathophysiology of sepsis, septic and non-septic shock, and finally multiple organ dysfunction syndrome (MODS) [1]. Non-septic shock is triggered by non-bacterial, non-endotoxic agents like zymosan. Zymosan, obtained from the cell wall of *Saccharomyces cerevisiae*, is a well-known reagent for examination of the inflammatory response. As zymosan is not degradable, phagocytosis by macrophages results in a prolonged inflammatory response [2]. The inflammatory cascade caused by zymosan is characterized by systemic hypotension, elevated nitric oxide (NO) levels, cellular infiltration, cyclooxygenase (COX) activity, and proinflammatory cytokine formation [3–5].

Zymosan, which is recognized by the innate immune system through Toll-like receptor (TLR)2 and TLR6 [6], induces systemic inflammation *via* many proinflammatory factors such as mitogen-activated protein kinases (MAPKs) and nuclear factor-kappa B (NF-κB) in macrophages [7]. Zymosan-induced activation of MAPK and NF-κB signalling in certain cells was studied previously [8, 9] in additon to zymosan administration that mimics the sign of MODS as well as it produces acute peritonitis especially in the lung, kidney, liver, and intestine following the systemic inflammatory response [5]. Therefore, it may also act on other tissues including cardiac, renal, and inflammatory cells. MAPKs are divided into three major subfamilies: the extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNK), and p38 [10]. MAPKs, a family of serine/threonine kinases, are involved in a variety of cellular processes including inflammation, cell growth/differentiation, and cell survival. These kinases play an important role in the release of inflammatory cytokines mediated by the systemic inflammatory response such as tumor necrosis factor (TNF)-α, NO, and COX-2 [11–14]. It is known that activation of ERK1/2 by its phosphorylation on threonine and tyrosine residues by an upstream kinase, MAPK kinase (MEK)1/2, results in degradation of inhibitor of κB (IκB) and activation of NF-κB [15]. Subsequent activation of NF-κB by ERK1/2 regulates transcription of number of genes including inducible NO synthase (iNOS) and COX-2 that are critical for the regulation of immunity and inflammation [16]. In our previous studies, we showed that MEK1/ERK1/2 pathway, as well as NF-κB activation, plays an important role in hypotension and vascular hyporeactivity to vasoconstrictors in endotoxemic rats [17, 18].

The disruption of the oxidant and antioxidant balance caused either by an increase of oxidants like reactive oxygen (ROS) and/or nitrogen species (RNS) or depletion of antioxidant mechanisms is reported to lead to oxidative stress in sepsis, septic, and non-septic shock [19]. ROS production involves in signalling pathways that regulate vascular tone as well as various cellular processes [20, 21] in physiological conditions and plays a critical role in host defense against pathogens, such as fungi, that is controlled by the NADPH oxidase system. NADPH oxidase enzymes are thought to exert proinflammatory roles *via* ROS induction. Moreover, cellular ROS production activates MAPKs, which in turn allow the nuclear translocation of NF-κB most likely through the documented inhibitor κB kinase (IKK)/IκB pathway [22]. Proinflammatory agents such as cytokines TNF-α and interleukin (IL)-8 and bacterial and fungal products as beta glucan and zymosan stimulate ROS production and induce the oxidative response in neutrophils [23, 24]. Generation of ROS is also associated with cell death which has been implicated in the pathophysiology of MODS and sepsis, and it may contribute to the immunological derangements [25, 26]. It is well known that zymosan by activating immune cells through pattern recognition receptors enhances the formation of intracellular and extracellular ROS which contributes to the pathophysiology of MODS [27, 28].

Mammalian target of rapamycin (mTOR), the downstream effector of the phosphatidylinositol 3-kinase family, regulates broad aspects of cellular function, including cell cycle progress, protein degradation, protein kinase C signalling, ribosome biogenesis, and transcription [29]. mTOR is essential for survival, cytokine production, and migration of neutrophils and mast cells [30, 31]. Moreover, the use of mTOR inhibitors as an immunosupressive agent indicates that mTOR plays an important role in the immune system [32]. Rapamycin, the prototype of selective mTOR inhibitor, enhances the anti-inflammatory activities of regulatory T cells and decreases the production of proinflammatory cytokines and chemokines by macrophages in addition to other immune cells [33]. Moreover, recent studies have implicated mTOR in inflammatory and autoimmune diseases [34]. In a previous study with lipopolysaccaride (LPS)-induced septic shock model in rats, we demonstrated that the selective mTOR inhibitor, rapamycin, reverses systemic hypotension, tachycardia, and inflammation *via* the activation of mTOR/IκB-α/NF-κB pathway and subsequently iNOS and COX-2 expressions and activities with formation of NO, peroxynitrite, prostacyclin, and TNF-α [35]. While mTOR is shown to have a proinflammatory effect, no studies have yet revealed the role of mTOR in the zymosan-induced inflammatory response manifested by hypotension and inflammation in non-septic shock model in rats. In this study, we have investigated the role of

mTOR//MEK1/ERK1/2/IKKβ/IκB-α/NF-κB signalling pathway activation which mediates the formation of pro-inflammatory and/or vasodilator mediators and activation of NADPH oxidase system provoking oxidative/nitrosative stress resulted in systemic inflammatory response induced by zymosan in rats.

MATERIALS AND METHODS

Materials

Rapamycin and zymosan (*Saccharomyces cerevisiae* product zymosan, Z4250) were purchased from Gold Biotechnology (St. Louis, MO, USA) and Sigma Chemical Company (St. Louis, MO, USA), respectively. Rat TNF-α, IL-1β, and NADPH oxidase enzyme-linked immunosorbent assay (ELISA) kits were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and Mybiosource (San Diego, CA, USA), respectively. rpS6, phosphorylated rpS6, ERK1/2, and IKKβ (Cell Signalling Technology, Danvers, MA, USA), MEK, phosphorylated MEK, phosphorylated ERK1/2, phosphorylated IKKα/β, NF-κB p65, phosphorylated NF-κB p65, IκB-α, phosphorylated IκB-α, nitrotyrosine, gp91^{phox}, p22^{phox}, COX-2 (Santa Cruz Biotechnology, TX, USA), and iNOS (BD Transduction Lab., San Jose, CA, USA) were obtained. Secondary antibodies (sheep anti-mouse IgG-horseradish peroxidase and goat anti-rabbit IgG-horseradish peroxidase) and ECL Prime Western Blotting Detection Reagents were also obtained from Amersham Life Sciences (Cleveland, OH, USA).

In Vivo Study

Animals

The male Wistar rats, weighing approximately 200–300 g (n = 32), were purchased from Research Center of Experimental Animals, Mersin University, Mersin, Turkey. All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rats were maintained on a 12-h light/dark cycle and fed with standard rat chow. The study protocols were approved by the Ethics Committee of Mersin University School of Medicine.

Zymosan-Induced Non-septic Shock in Rats

Rats were randomly divided into four groups containing control (n = 8), zymosan (n = 8), rapamycin (n = 8), and zymosan + rapamycin (n = 8). The non-septic shock was induced as previously described by Cuzzucrea et al. [5]. Control and rapamycin groups were injected intraperitoneally with saline (4 ml/kg) whereas zymosan and zymosan + rapamycin groups were treated intraperitoneally with zymosan (500 mg/kg) at time 0. In rapamycin and zymosan + rapamycin groups, rats were received rapamycin (1 mg/kg) intraperitoneally 1 h after injection of saline or zymosan. The dose of rapamycin 1 mg/kg used, that induces anti-inflammatory effect, was chosen according to our study and another study [35, 36]. Mean arterial pressure (MAP) and heart rate (HR) were measured by the tail-cuff device (MAY 9610 Indirect Blood Pressure Recorder System, Commat Ltd., Ankara, Turkey) at time 0 and 1, 2, 3, and 4 h. Following the zymosan or saline challenge after 4 h, the rats were euthanized, and blood samples, kidneys, hearts, thoracic aortas, and superior mesenteric arteries were harvested. Sera were separated from blood samples by centrifugation at 23,910×g for 15 min at 4 °C and stored at − 20 °C. Tissues were rapidly frozen in liquid nitrogen and stored at − 80 °C. Frozen tissues were pulverized into powder in liquid nitrogen and homogenized in 1–2 ml of an ice-cold 20 mM HEPES buffer (pH 7.5). Homogenates were centrifuged at 23,910×g for 10 min at 4 °C followed by sonication for 15 s on ice with 50 μl ice-cold Tris (50 mM, pH 8.0) and KCl (0.5 M). The samples were centrifuged at 23,910×g for 15 min at 4 °C and then supernatants were removed and stored at − 80 °C, until the measurement of β-actin, rpS6, MEK, IKKβ, ERK1/2, IκB-α, NF-κB p65, iNOS, COX-2, nitrotyrosine, gp91^{phox}, p47^{phox}, and p22^{phox} protein levels and/or activities and nitrite, IL-1β, and TNF-α protein levels in addition to NADPH oxidase activity. Amounts of proteins in the supernatants were determined using the Coomassie blue method [37].

Western Blotting

rpS6, phosphorylated rpS6, MEK, phosphorylated MEK, ERK1/2, phosphorylated ERK1/2, phosphorylated IKKα/β, IKKβ, IκB-α, phosphorylated IκB-α, NF-κB p65, phosphorylated NF-κB p65, iNOS, COX-2, nitrotyrosine, gp91^{phox}, p47^{phox}, p22^{phox}, and β-actin proteins were performed according to the immunoblotting method described previously [35, 38, 39]. Briefly, an equivalent amount of total protein (93–120 μg) were loaded into each well and separated them by 10% SDS-polyacrylamide gel electrophoresis. After electrophoresis, separated proteins were transferred to a nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (TBST) buffer at room

temperature for 1 h and then incubated with specific primary antibodies in TBST including 5% bovine serum albumin (BSA) at 1:500–1:20,000 dilutions overnight at 4 °C. After incubation with secondary antibodies in TBST including 0.1% BSA at 1:1000 for 1 h at room temperature, protein bands were visualized with ECL Prime Western blotting detection reagent. The protein levels were analyzed by using ImageJ analysis software version 1.42r (National Institutes of Health, USA).

Measurement of IL-1β, TNF-α, and Nitrite Levels

IL-1β and TNF-α levels in tissue samples at 4 h were analyzed by ELISA following the kit manufacturer's instructions. Nitrite (a stable product of NO) levels in sera and tissue samples were measured using the diazotization method based on the Griess reaction, as an index for NOS-derived NO production [40, 41]. Briefly, samples (25 μl) were pipetted into 96-well microtiter plates, and an equal volume of Griess reagent (1% sulphanilamide and 0.1% *N*-1-naphthyl ethylenediamine dihydrochloride in 2.5% phosphoric acid) was added to each well. After incubation for 10 min at room temperature, absorbance was measured at 550 nm with a microplate reader. Standard curves were also constructed using sodium nitrite concentrations ranging from 0.25 to 100 μM.

Measurement of NADPH Oxidase Activity

NADPH oxidase activity in tissue samples at 4 h was analyzed by ELISA following the kit manufacturer's instructions.

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for multiple comparisons, the Kruskal-Wallis test followed by the Dunns test for multiple comparisons, and Student's *t* or Mann-Whitney *U* tests when appropriate. Quantitative data were presented as the means ± S.E.M. Statistically significance was accepted for *P* values < 0.05. GraphPad Prism 5.0 Version for Windows, GraphPad Software (San Diego, CA, USA), was applied in performing the statistical analyses.

RESULTS

Rapamycin Attenuates Cardiovascular Response to Zymosan

Administration of zymosan induced a significant decrease in MAP and increase in HR. These cardiovascular changes occurred within 1 h after zymosan administration and continued for 4 h (Fig. 1a, b) ($P < 0.05$). Rapamycin, a selective mTOR inhibitor, reversed these cardiovascular responses to zymosan ($P < 0.05$). Rapamycin alone did not alter the basal MAP or HR in any of the groups ($P > 0.05$).

Rapamycin Attenuates the Activation of rpS6 by Zymosan

As shown in Fig. 2, administration of zymosan activated mTOR signalling. Zymosan caused an increase in phosphorylation, but not change in expression, of rpS6 ($Ser^{240/244}$), a major downstream target of mTOR ($P < 0.05$) in the kidney (Fig. 2a), heart (Fig. 2b), thoracic aorta (Fig. 2c), and superior mesenteric artery (Fig. 2d) ($P < 0.05$). Rapamycin administration significantly reversed these changes induced by zymosan. Rapamycin alone did not alter the basal values of expression of these molecules in any of the groups (Fig. 2) ($P > 0.05$).

Rapamycin Attenuates Expression and Activities of MEK1, ERK1/2, IKKβ, IκB-α, and NF-κB p65 in Rats Treated with Zymosan

In order to evaluate whether MEK1/ERK1/2/IKKβ/IκB-α/NF-κB p65 signal transduction pathway is involved in zymosan-induced systemic inflammation, we measured expression and phosphorylation of these kinases in the kidney (Fig. 3a), heart (Fig. 3b), thoracic aorta (Fig. 3c), and superior mesenteric artery (Fig. 3d) of zymosan-treated rats ($P < 0.05$). Zymosan did not alter total protein expression of MEK1, ERK1/2, and IKKβ. Treatment of rapamycin reversed all these effects of zymosan. Rapamycin alone did not alter the basal levels of expression or activity of these signalling molecules in any of the groups (Fig. 3) ($P > 0.05$).

Rapamycin Attenuates Expression and/or Activities of iNOS and COX-2 in Addition to Production of Proinflammatory Cytokines TNF-α and IL-1β in Zymosan-Treated Rats

Administration of zymosan caused an increase in iNOS and COX-2 expression and nitrite levels in the kidney (Fig. 4a), heart (Fig. 4b), thoracic aorta (Fig. 4c), superior mesenteric artery (Fig. 4d), and/or sera (Fig. 4e) ($P < 0.05$). Rapamycin reversed all these increases in iNOS and COX-2 expression and nitrite levels ($P < 0.05$) in the tissues and/sera. Rapamycin alone did not alter the basal

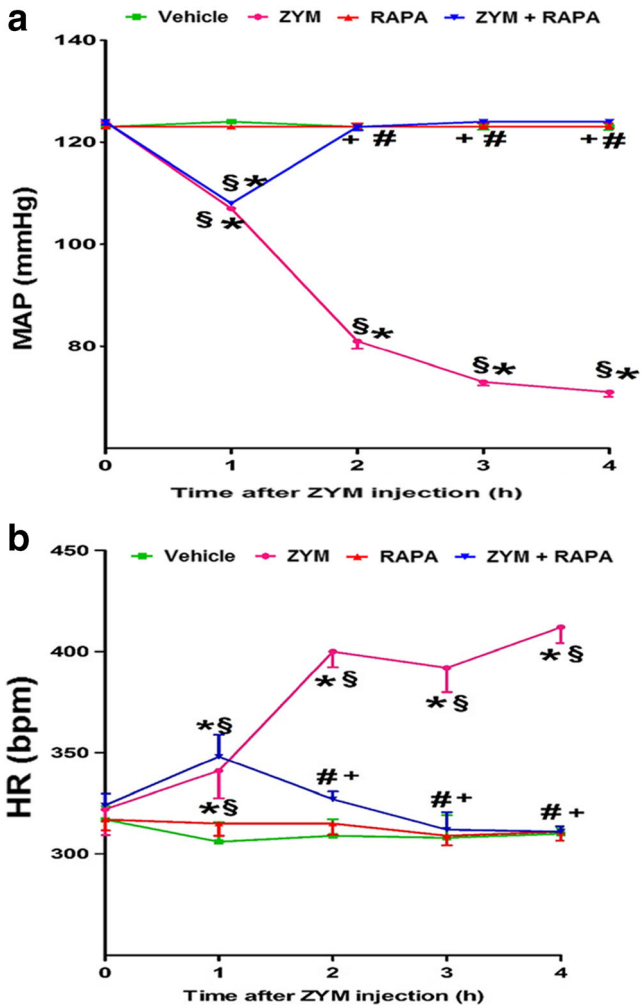


Fig. 1. The effect of mTOR inhibition on **a** mean arterial pressure (MAP) and **b** heart rate (HR) following administration of saline (vehicle) (4 ml/kg, i.p.) or zymosan (ZYM) (500 mg/kg, i.p.) to rats. Rapamycin (1 mg/kg, i.p.) was given 1 h after administration of saline or zymosan. MAP and HR were measured by tail-cuff method. The values are presented as the means ± S.E.M. ($n = 8$). *$P < 0.05$ *vs* control group; $^{\#}P < 0.05$ *vs* LPS-treated group; $^{\S}P < 0.05$ *vs* the time 0 h value within a group; $^{+}P < 0.05$ *vs* the time 1 h value within a group.

values of the expression of iNOX and COX-2 or nitrite levels in any of the groups (Fig. 4) ($P > 0.05$).

To determine the effect of rapamycin on cytokine production in zymosan-induced systemic inflammation, TNF-α and IL-1β levels were measured in the kidney (Fig. 4a), heart (Fig. 4b), thoracic aorta (Fig. 4c), and superior mesenteric artery (Fig. 4d). Zymosan caused an increase in TNF-α and IL-1β levels in all the tissues ($P < 0.05$). Rapamycin reversed all these increases in TNF-α and IL-1β levels (Fig. 4) ($P < 0.05$). Rapamycin alone had no effect on TNF-α and IL-1β levels in any of the treatment groups (Fig. 4) ($P > 0.05$).

Rapamycin Attenuates Zymosan-Induced Increase in NADPH Oxidase Activity, $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, and Nitrotyrosine Expression

As shown in Fig. 5, zymosan administration induced a significant increase in NADPH oxidase activity and expression of $p22^{phox}$, $gp91^{phox}$, and $p47^{phox}$, which are the subunits of NADPH oxidase system, and nitrotyrosine protein levels I kidney (Fig. 5a), heart (Fig. 5b), thoracic aorta (Fig. 5c), and superior mesenteric artery (Fig. 5d) ($P < 0.05$). Zymosan increased $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, and nitrotyrosine protein levels as well as NADPH oxidase activity in the tissues ($P < 0.05$). Rapamycin administration

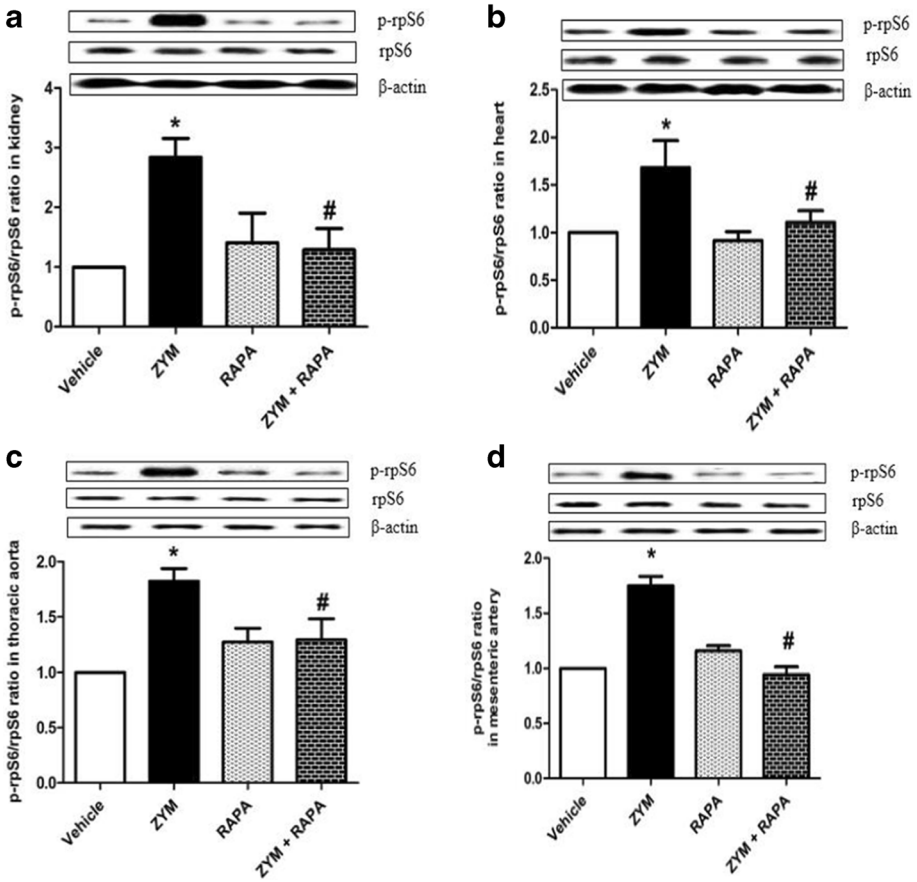


Fig. 2. The effect of rapamycin on changes in rpS6 expression and phosphorylation at $Ser^{240/244}$ in the **a** kidney, **b** heart, **c** thoracic aorta, and **d** superior mesenteric artery measured 4 h following saline (vehicle) (4 ml/kg, i.p.) or zymosan (ZYM) (500 mg/kg, i.p.) administration to rats. Rapamycin (1 mg/kg, i.p.) was given 1 h after administration of saline or zymosan (ZYM). rpS6 and p-rpS6 protein levels in the tissue homogenates were measured by immunoblotting. Density of bants was analyzed with ImageJ 1.42 software. The values are presented as the means ± S.E.M. ($n = 4$). *$P < 0.05$ *vs* control group; $^{\#}P < 0.05$ *vs* zymosan-treated group.

reversed all these increases in expression and activity of NADPH oxidase (Fig. 5) ($P < 0.05$). Rapamycin alone had no effect on basal level of NADPH oxidase activity or expression of its subunits and nitrotyrosine expression in any of the treatment groups (Fig. 5) ($P > 0.05$).

DISCUSSION

The present study provides the first evidence that rapamycin, a selective mTOR inhibitor, reverses the decrease in MAP, increase in HR, the systemic inflammatory response, and tissue damage as indicated by the inflammatory markers and oxidative/nitrossative stress, related to mTOR/MEK1/ERK1/2/IKKβ/IκB-α/NF-κB pathway activation in zymosan-induced rat model of non-septic shock. This evidence is based on our findings that in systemic inflammatory response and tissue damage related to zymosan-induced non-septic shock in rats, selective inhibition of mTOR by rapamycin normalized the decrease in MAP with the increase in HR and reduced iNOS and COX-2 expression as well as TNF-α and IL-1β levels, besides NADPH oxidase system activity regarding the reduced activity of mTOR/MEK1/ERK1/2/IKKβ/IκB-α/NF-κB signal transduction pathway in the kidney, heart, thoracic aorta, and superior mesenteric artery, and/or sera.

Numerous studies have shown that sustained inflammatory responses, sepsis, and septic shock result in tissue and organ damage with a high incidence of mortality [42]. Initiation and development of SIRS are mainly controlled

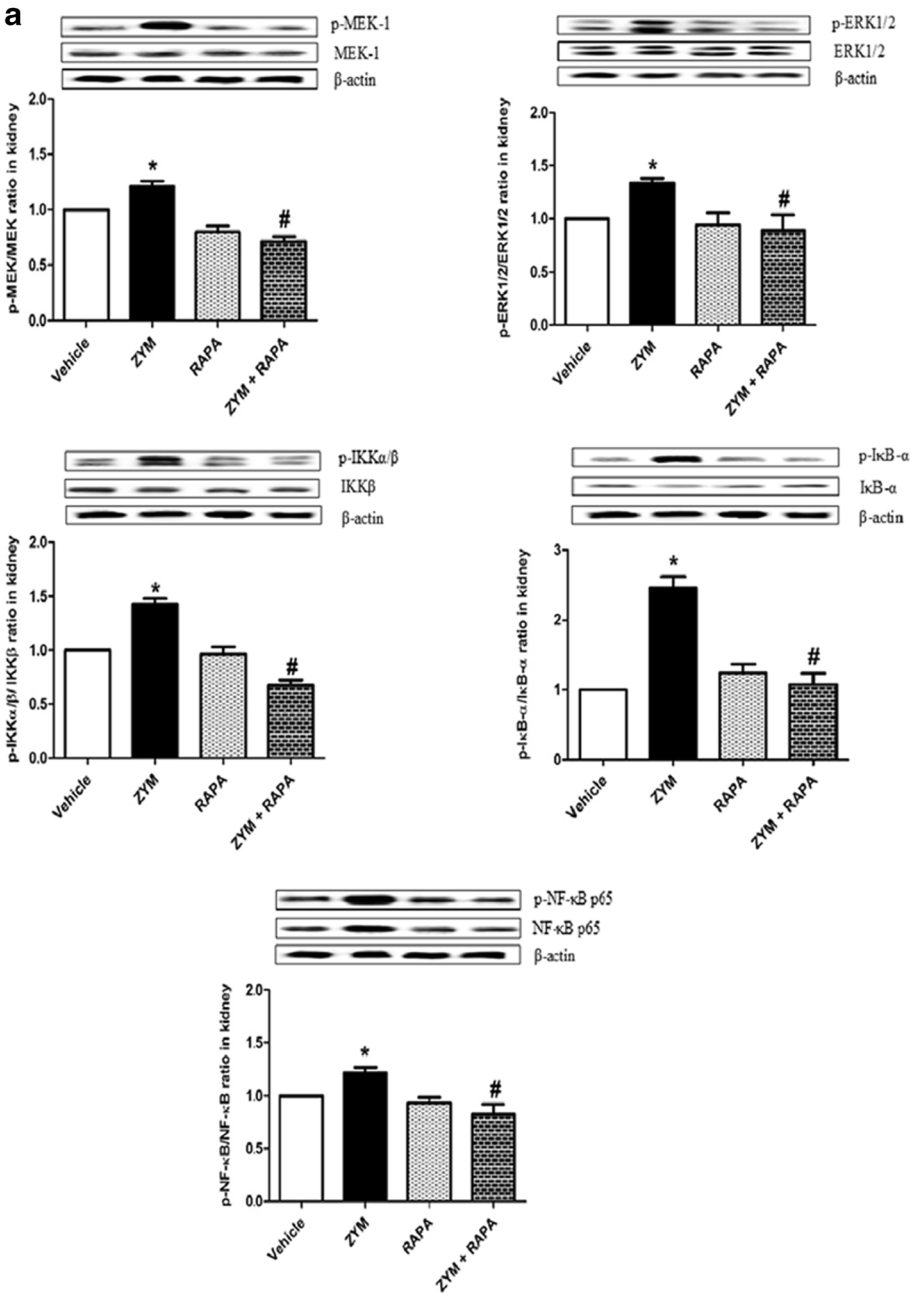
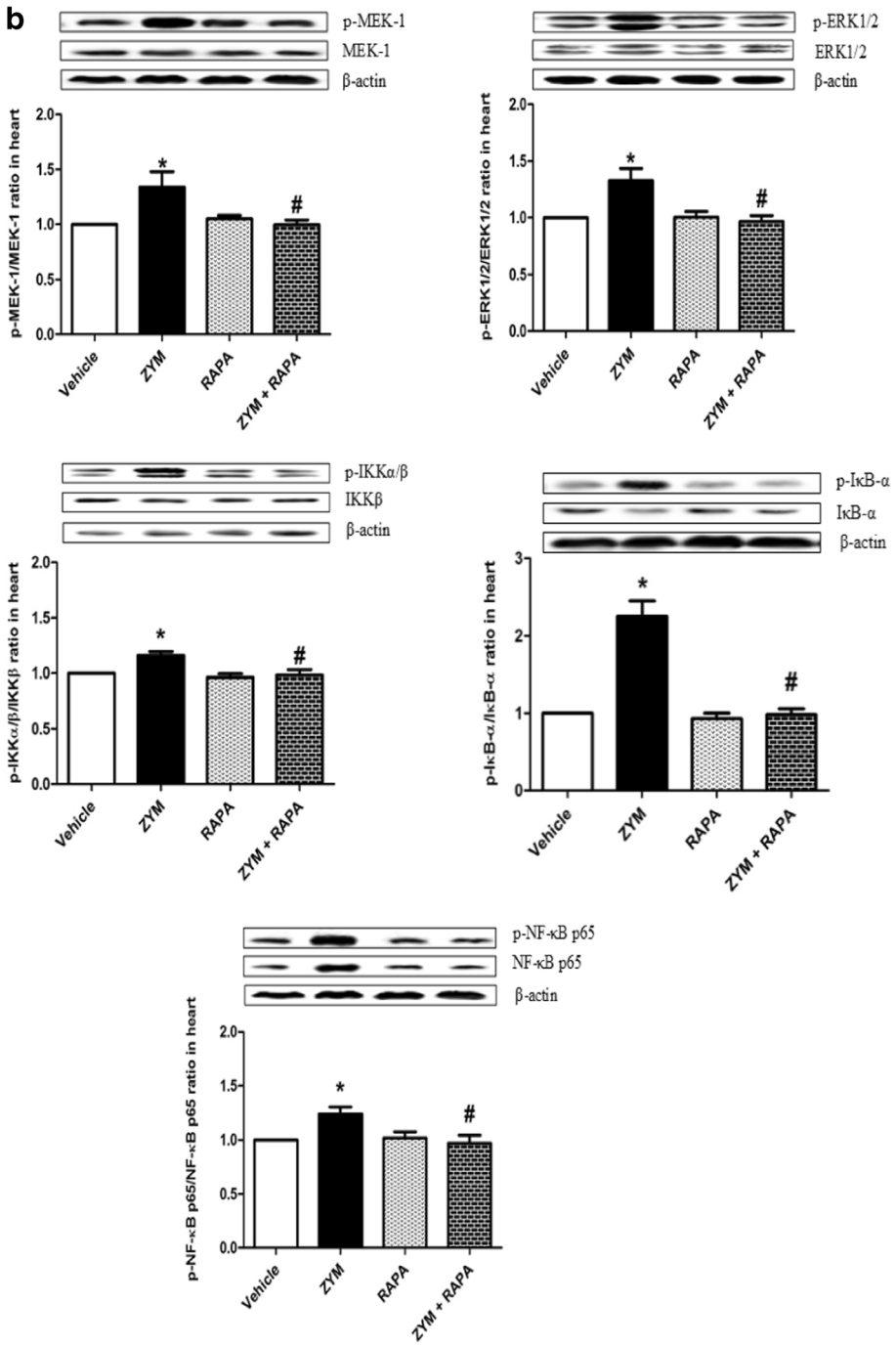


Fig. 3. The effect of rapamycin on changes in MEK-1, ERK1/2, IKKβ, IκB-α, and NF-κB p65 expression and phosphorylation in the **a** kidney, **b** heart, **c** thoracic aorta, and **d** superior mesenteric artery measured 4 h following saline (vehicle) (4 ml/kg, i.p.) or zymosan (ZYM) (500 mg/kg, i.p.) administration to rats. Rapamycin (1 mg/kg, i.p.) was given 1 h after administration of saline or zymosan (ZYM). MEK-1, p-MEK-1, ERK1/2, p-ERK1/2, IKKβ, p-IKKα/β, IκB-α, p-IκB-α, NF-κB p65, and p-NF-κB p65 protein levels in the tissue homogenates were measured by immunoblotting. Density of bants was analyzed with ImageJ 1.42 software. The values are presented as the means ± S.E.M. ($n = 4$). $^{*}P < 0.05$ *vs* control group; $^{\#}P < 0.05$ *vs* zymosan-treated group.

by the balance of pro- and anti-inflammatory mediators [43]. Significant advancements in SIRS pathophysiology might be attributed to the well-prepared SIRS models induced by zymosan [44, 45]. The beginning of the



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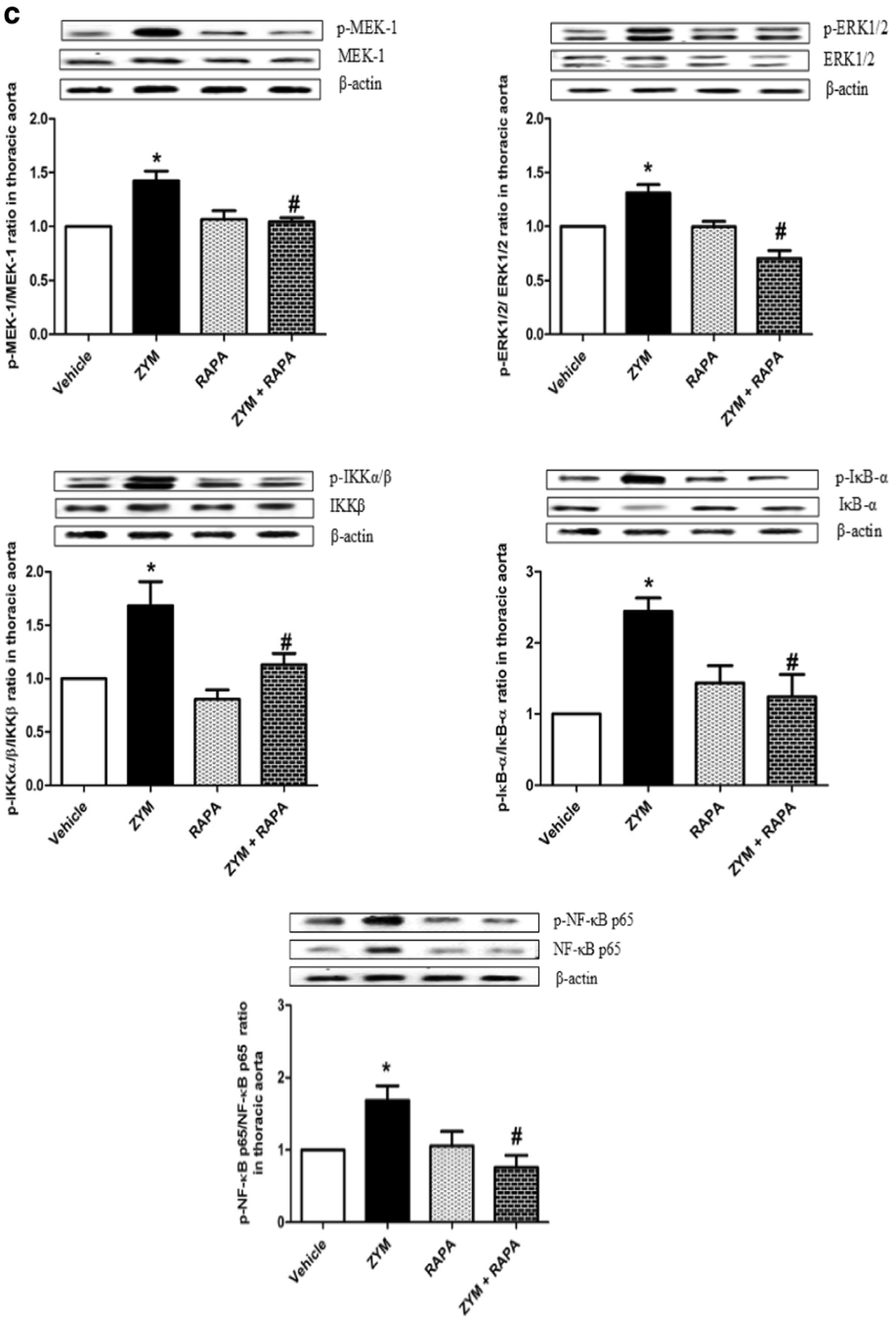
inflammatory processes in SIRS and non-septic shock induced by zymosan is reported to be associated with increased NO formation, COX-2 activity, and proinflammatory cytokine production [5, 45]. Several studies have shown the involvement of MAPKs, including p38 MAPK and ERK1/2 MAPK to increased iNOS expression after induction of these proteins by cytokine [46–48]. In our previous study, we demonstrated the decrease in arterial



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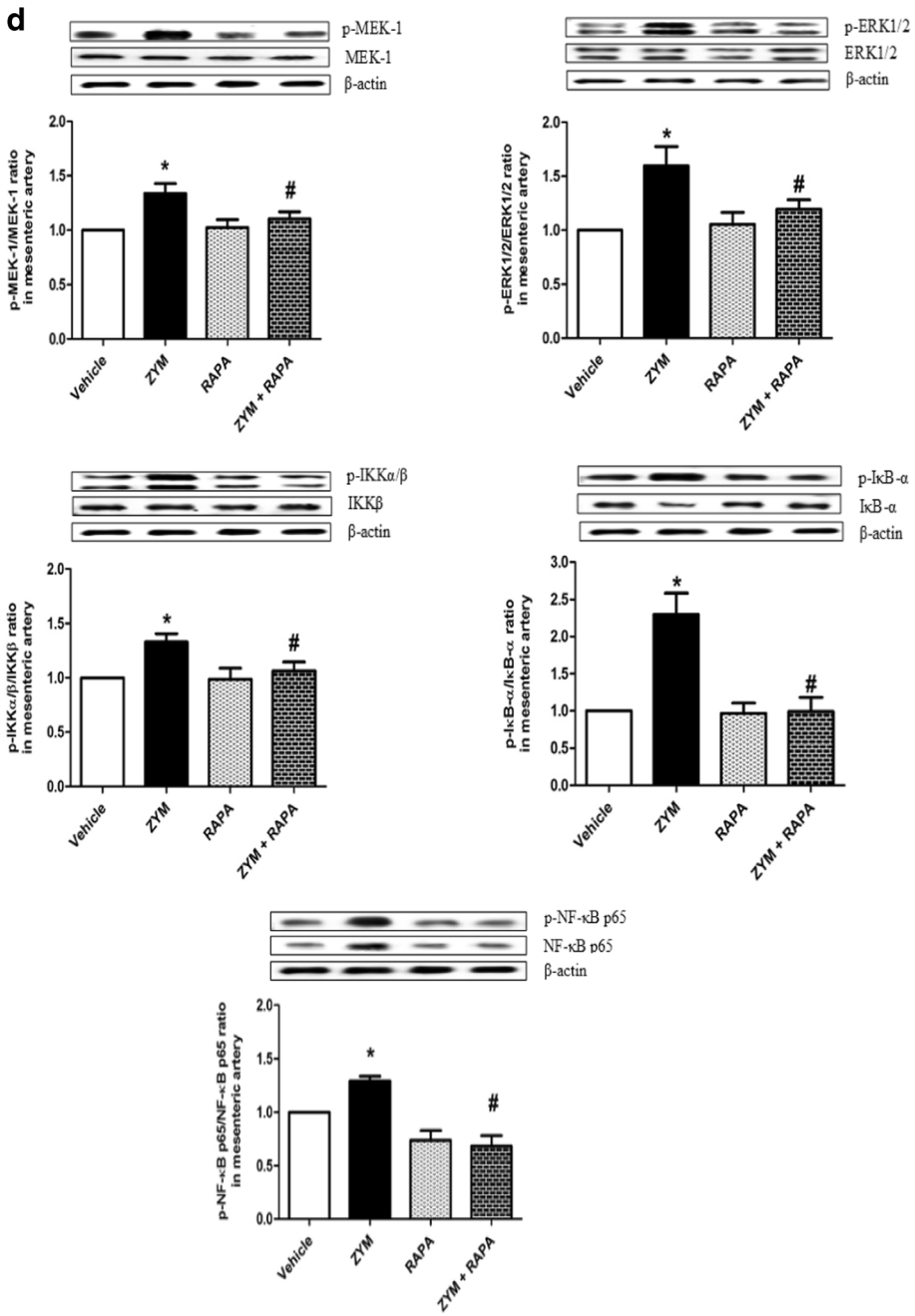
blood pressure and vascular reactivity to vasoconstrictor agents was associated with increased iNOS expression and activity associated with a rise in the expression of phosphorylated ERK1/2 in endotoxemic rats [18]. Although zymosan significantly increased phosphorylation of ERK1/2 and p38 MAPK, the role of mTOR in zymosan-induced SIRS has not yet been clarified. With other studies, based on our findings, these zymosan-induced phenomena seem to depend on the activation of the mTOR/MEK1/ERK1/2/IKKβ/IκB-α/NF-κB signal transduction



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pathway. More recently, it has been postulated that MAPK, activated by phosphorylation *via* a specific upstream phosphorylation cascade like MEK1, such as ERK1/2 and p38 MAPK may play a role in the generation of proinflammatory cytokines and free radicals which have been implicated in the pathogenesis of several inflammatory diseases [49, 50]. Zymosan, through TLR2/6 heterodimer, activates the MAPK signalling pathway that acts as upstream of NF-κB, which causes translocation of NF-κB to the nucleus [51–56]. During the basal conditions, NF-κB exists in the cytoplasm and associated with an inhibitory protein, IκB-α. IκB-α phosphorylation allows activation and translocation of NF-κB into the nucleus and regulates gene transcription of several proinflammatory mediators. This

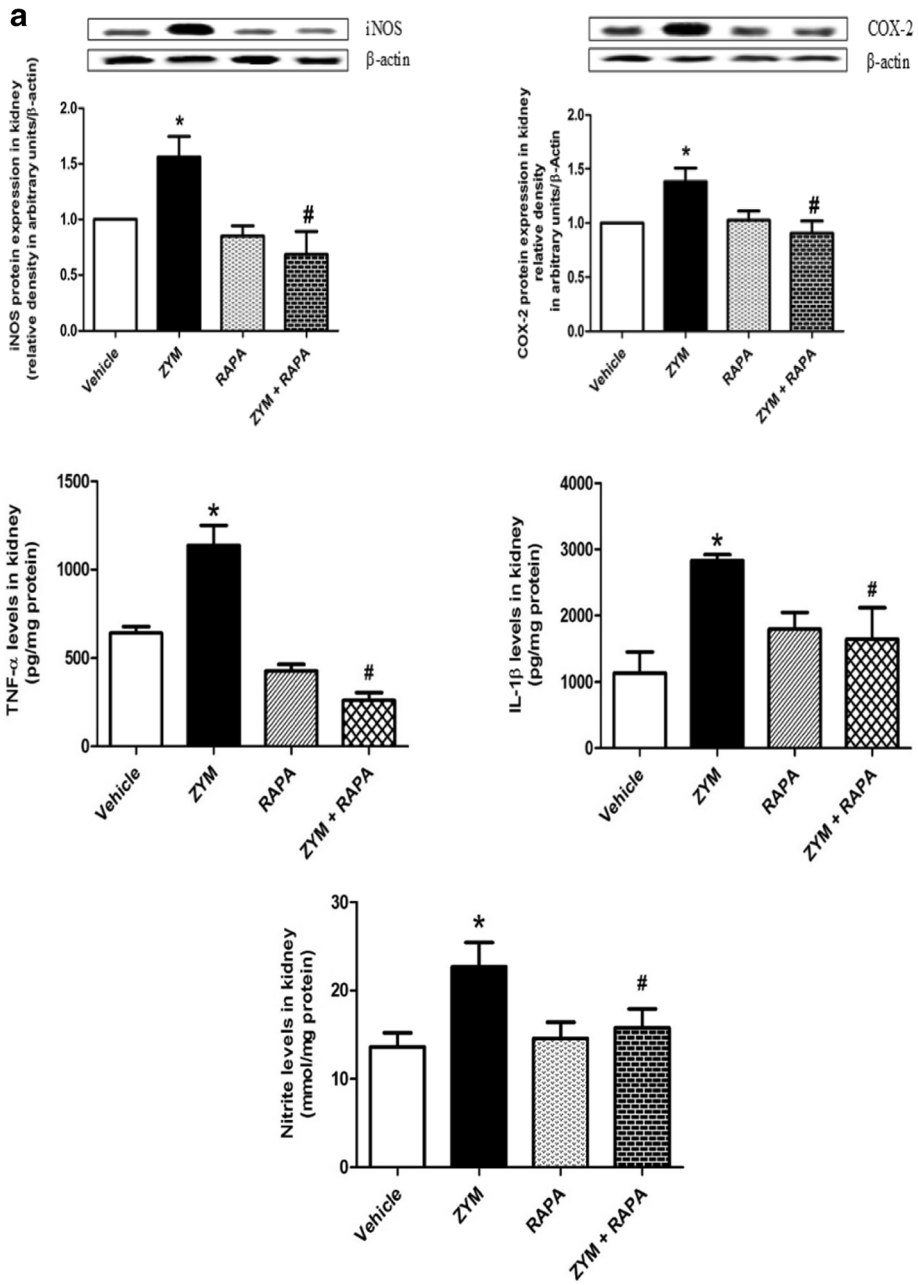
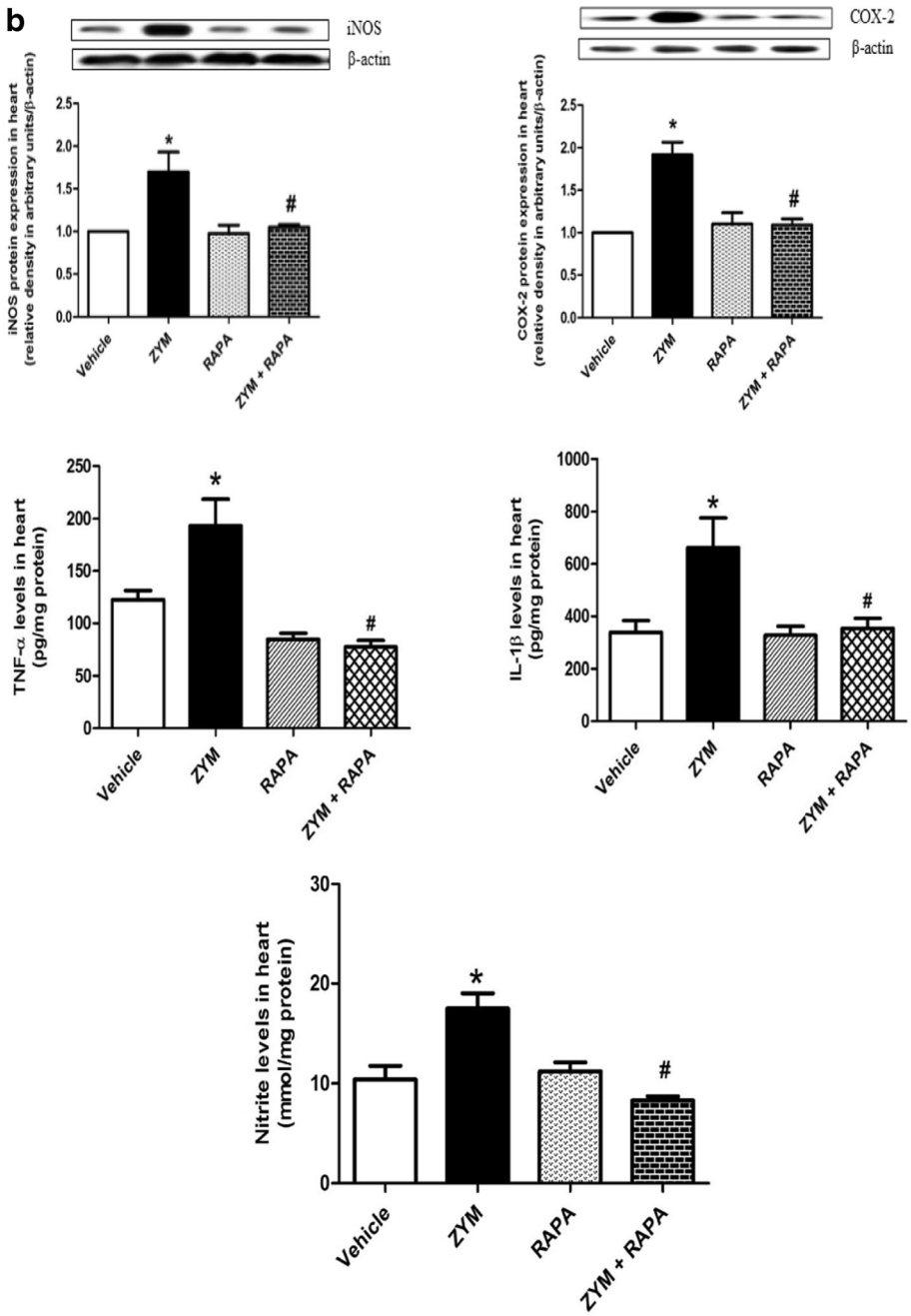


Fig. 4. The effect of rapamycin on changes in expression and/or activity of iNOS and COX-2 as well as levels of TNF-α and IL-1β in the **a** kidney, **b** heart, **c** thoracic aorta, and **d** superior mesenteric artery measured 4 h following saline (vehicle) (4 ml/kg, i.p.) or zymosan (ZYM) (500 mg/kg, i.p.) administration to rats. Rapamycin (1 mg/kg, i.p.) was given 1 h after administration of saline or zymosan (ZYM). iNOS and COX-2 protein levels in the tissue homogenates were measured by immunoblotting. Nitrite levels were measured by using the diazotization method based on the Griess reaction. TNF-α and IL-1β levels were measured by ELISA kit. The values are presented as the means ± S.E.M. ($n = 4–5$). $^{*}P < 0.05$ *vs* control group; $^{\#}P < 0.05$ *vs* zymosan-treated group.

phosphorylation is performed by an IKK complex, which consists of a dimer of two catalytical subunits, IKKα and IKKβ in response to inflammatory stimulus [57–59]. It has been clearly demonstrated in the other studies that NF-κB regulates transcription of a vast range of genes encoding for proinflammatory cytokines and mediators such as



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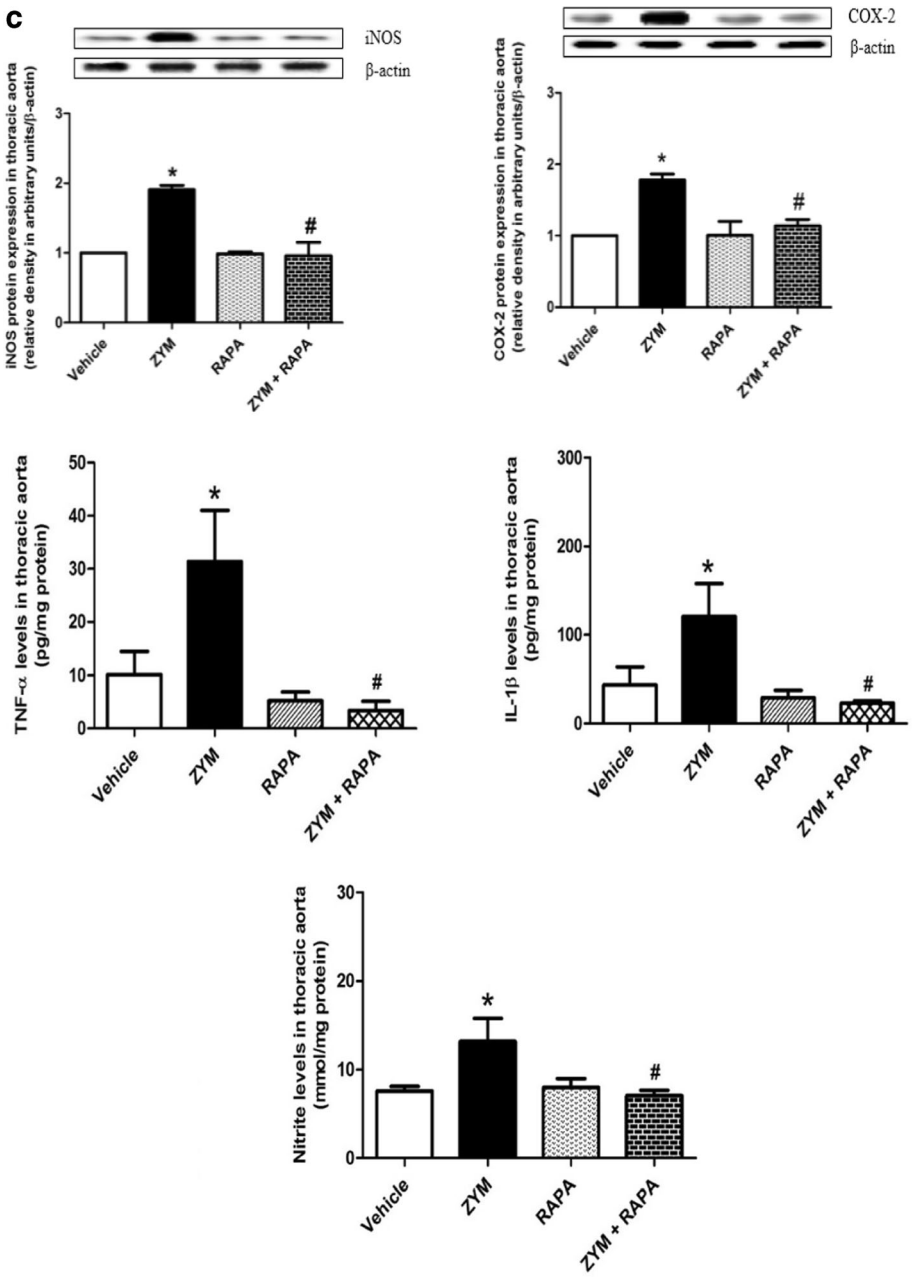
TNF-α, IL-1β, iNOS, and COX-2 which trigger the systemic inflammatory response [60–64]. Lee et al. [49] have shown the expression of activated MAPKs such as ERK1/2 and p38 MAPK may play a key role in the production of proinflammatory cytokines and free radicals. The phosphorylation of ERK1/2 and MEK that is a well-known immediately upstream of ERK1/2 kinase was shown to enhance in zymosan-induced shock which was inhibited by PD98059, a selective MEK inhibitor in mice. Moreover, it was determined that zymosan-induced non-septic shock associated with increased degradation of IκB and activation following translocation of the p65 subunit of NF-κB reversed by PD98059 [10]. In the light of these observations, MAPK could play a central role in proinflammatory



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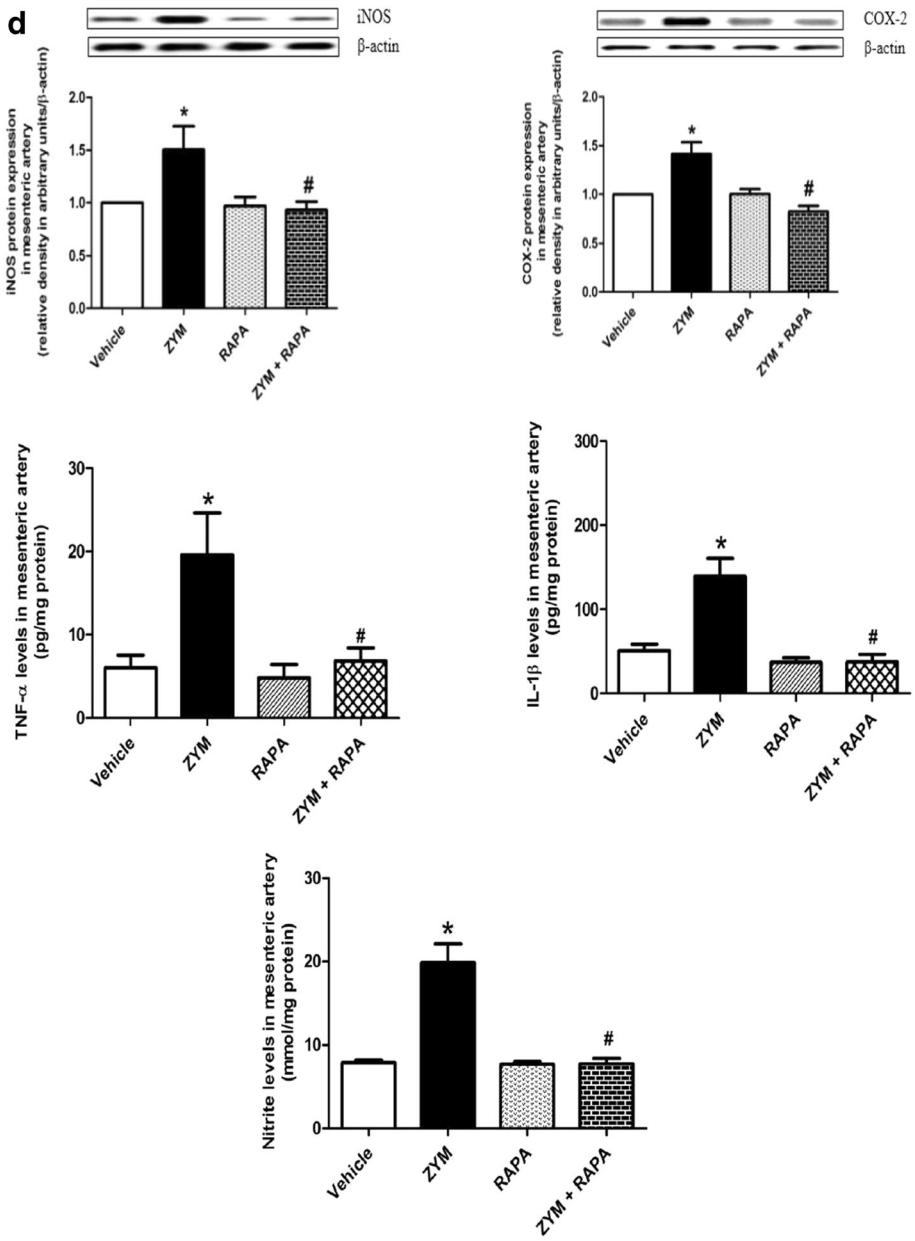
effects of zymosan. In our previous study, we showed that MEK1/ERK1/2/IKKβ/IκB-α/NF-κB pathway activation and proinflammatory cytokine production contribute to LPS-induced vascular hyporeactivity regarding hypotension with related tachycardia and inflammatory response in endotoxemic rats and mortality in mice. The Ras-ERK and PI3K (phosphatidylinositol 3-kinase)-mTOR signalling pathways are the cell's chief mechanisms for controlling cell survival, differentiation, proliferation, metabolism, and motility in response to different stimulus and they might intersect to regulate each other and co-regulate downstream functions by positively or negatively regulating each other's activity [65]. A phase I clinical trial with an mTORC1 inhibitor revealed an upregulation of ERK activity in metastatic breast cancer patients [66]. In a resveratrol-inhibited activation of BV-2 cells by LPS, a



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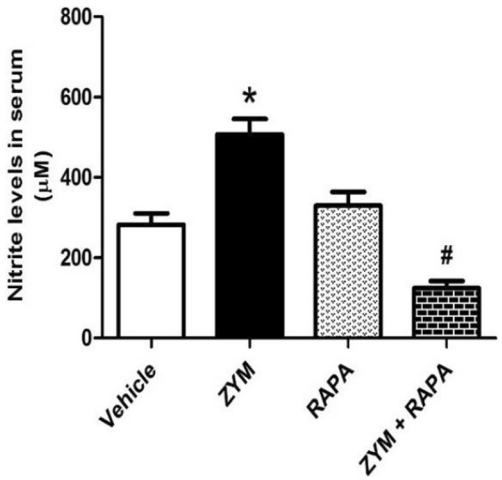
group of researcher hypothesized to determine the upstream regulators of MAPK signalling and they showed pretreated with rapamycin partly reversed the effect of resveratrol-inhibited phosphorylation of ERK1/2, JNK, and p38 MAPK. Resveratrol also significantly attenuates overactivation of microglial cells by repressing expression levels of neurotoxic proinflammatory mediators and cytokines *via* activation of mTOR signalling pathway [67]. In another study, mTOR inhibitor rapamycin dramatically prevented Ang II-induced increases in marked reduction of phosphorylated levels of p-mTOR and p-ERK1/2 [68]. In the present study, the reversal effect of rapamycin on increased mTOR/MEK1/ERK1/2/IKKβ/IκB-α/NF-κB pathway activation and enhanced production of proinflammatory and vasodilator mediators in renal and cardiovascular tissues of rats induced by zymosan seems to be related to decreased mTOR activity.



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The cellular mechanism of these cardiovascular responses to zymosan-induced systemic inflammation is reported to be due to overproduction of proinflammatory and vasodilator mediators [69]. iNOS with its product NO, COX-2, and prostanoids and proinflammatory cytokine production are also involved in the pathogenesis of the zymosan-induced shock with inflammation, leading to tissue damage [3, 70]. Cuzzocrea et al. [71] demonstrated that 18 h after zymosan injection to Sprague Dawley rats, it decreased the blood pressure associated with the increased nitrite and nitrate levels as well as increased iNOS expression. In fact, besides NO, several proinflammatory lipid mediators such as COX metabolites also have been proposed as responsible for the zymosan-induced non-septic shock [63, 72]. There is overwhelming evidence that COX-2 expression is increased in chronic inflammatory diseases and animal models of inflammation [73–76]. Furthermore, there is a significant increase in the production of prostanoids in zymosan-induced peritonitis [77, 78], indicating the contribution of COX-2 in zymosan-induced inflammatory responses [79]. Indeed, previously as shown by our group, overproduction of NO by iNOS and vasodilator prostanoids by COX-2 contributes to the refractory hypotension in LPS-treated rats following by induction of mTOR/IκB-a/NF-κB pathway [33]. In addition to iNOS and COX-2, the release of TNF-α and IL-1β is known to play an important role in the pathophysiology of non-septic shock [69]. Furthermore, TNF-α stimulates the release of COX-derived products that contribute to vascular damage in shock [80]. We observed in the present study that enhanced TNF-α and IL-1β production is correlated with mTOR/MEK1/ERK1/2/IKKβ/IκB-α/NF-κB signalling pathway activation which was suppressed by rapamycin in zymosan-induced non-septic shock. As we have previously demonstrated, increased activity of MEK1/ERK1/2/IKKβ/IκB-α/NF-κB pathway and associated production of proinflammatory cytokines, TNF-α, and IL-8 involves in the vascular hyporeactivity to vasoconstrictor agents and decreased blood pressure in endotoxemic rats [38]. Thus, the harmony between previously published studies and our present results could be attributed that production of NO, vasodilator prostanoids with proinflammatory, and also vasodilator mediators following activation of mTOR/MEK1/ERK1/2/IKKβ/IκB-α/NF-κB pathway might be the cause of the sustained fall in blood pressure and systemic inflammatory response in non-septic shock.

Under systemic inflammatory conditions, the ROS-generating NADPH oxidase system is overexpressed in a variety of cells and tissues. It is believed that the management of oxidative stress is an important strategy in controlling the development and progression of many diseases and the NADPH oxidase inhibitors have the potential to control oxidative stress [81]. ROS and peroxynitrite cause cellular injury and necrosis *via* several mechanisms including peroxidation of membrane lipids, protein denaturation, and DNA damage [28]. Zymosan has been described to exert a major effect on ROS production, and zymosan by itself induces ROS production in monocytes *via* activating NADPH oxidase system [23, 24]. Maalej et al. [82] clearly demonstrated that zymosan stimulates neutrophil NADPH oxidase activation and also suggested that a protein tyrosine kinase ERK1/2 is involved in zymosan-induced ROS production in human neutrophils. In the present study, we demonstrated the beneficial role of rapamycin in zymosan-induced non-septic shock with severe systemic inflammatory response as its treatment decreased the development of oxidative/nitrosative stress which was associated with reduced NADPH oxidase system activation and expression of the subunits of these enzyme systems such as $p22^{phox}$, $p47^{phox}$, and $gp91^{phox}$ induced by zymosan administration. Recent reports have demonstrated that zymosan-induced MODS is related to the formation of proinflammatory and vasoactive mediators including NO, and the products of COX metabolism can

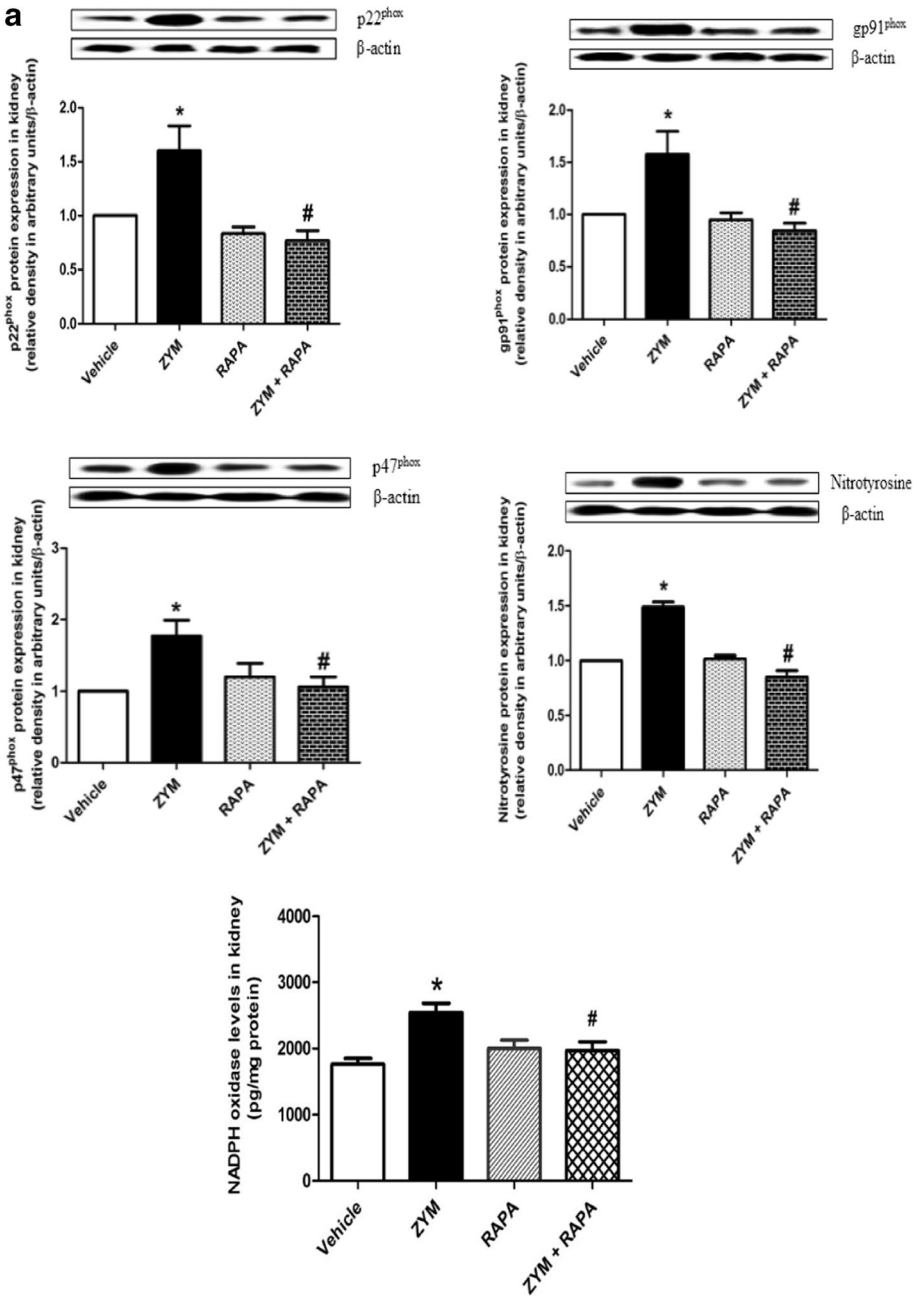
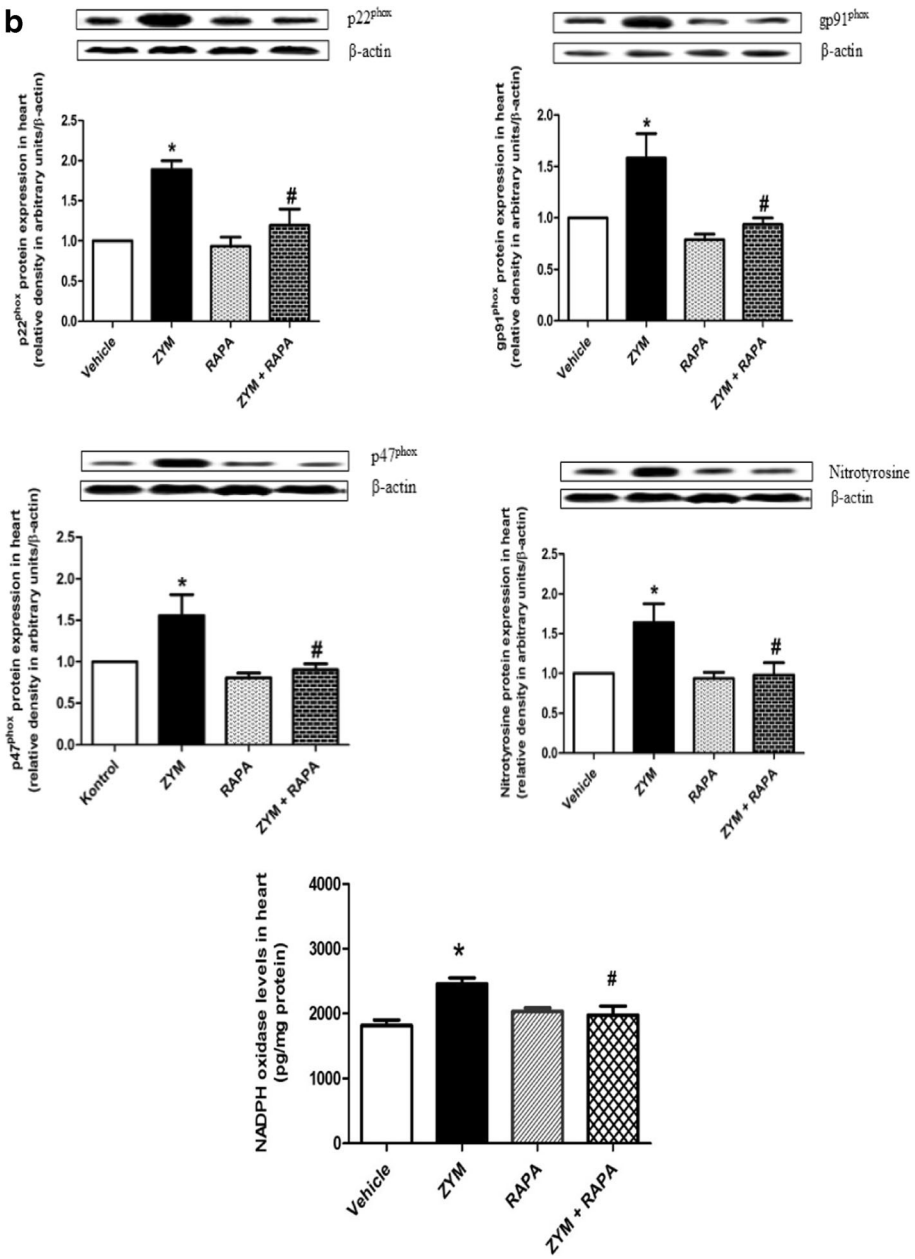


Fig. 5. The effect of rapamycin on changes in expression and/or activity of NADPH oxidase, $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, and nitrotyrosine levels in the **a** kidney, **b** heart, **c** thoracic aorta, and **d** superior mesenteric artery measured 4 h following saline (vehicle) (4 ml/kg, i.p.) or zymosan (ZYM) (500 mg/kg, i.p.) administration to rats. Rapamycin (1 mg/kg, i.p.) was given 1 h after administration of saline or zymosan (ZYM). NADPH oxidase levels were measured by ELISA kit. $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, and nitrotyrosine protein levels in the tissue homogenates were measured by immunoblotting. Density of bants was analyzed using ImageJ 1.42 software. The values are presented as the means ± S.E.M. ($n = 4–5$). $^{*}P < 0.05$ *vs* control group; $^{\#}P < 0.05$ *vs* zymosan-treated group.

cause a significant change in vascular reactivity [5, 83–85]. NO also reacts with superoxide produced by $gp91^{phox}$ and $p47^{phox}$ resulting in nitrotyrosine formation indicating NO-dependent pathogenic



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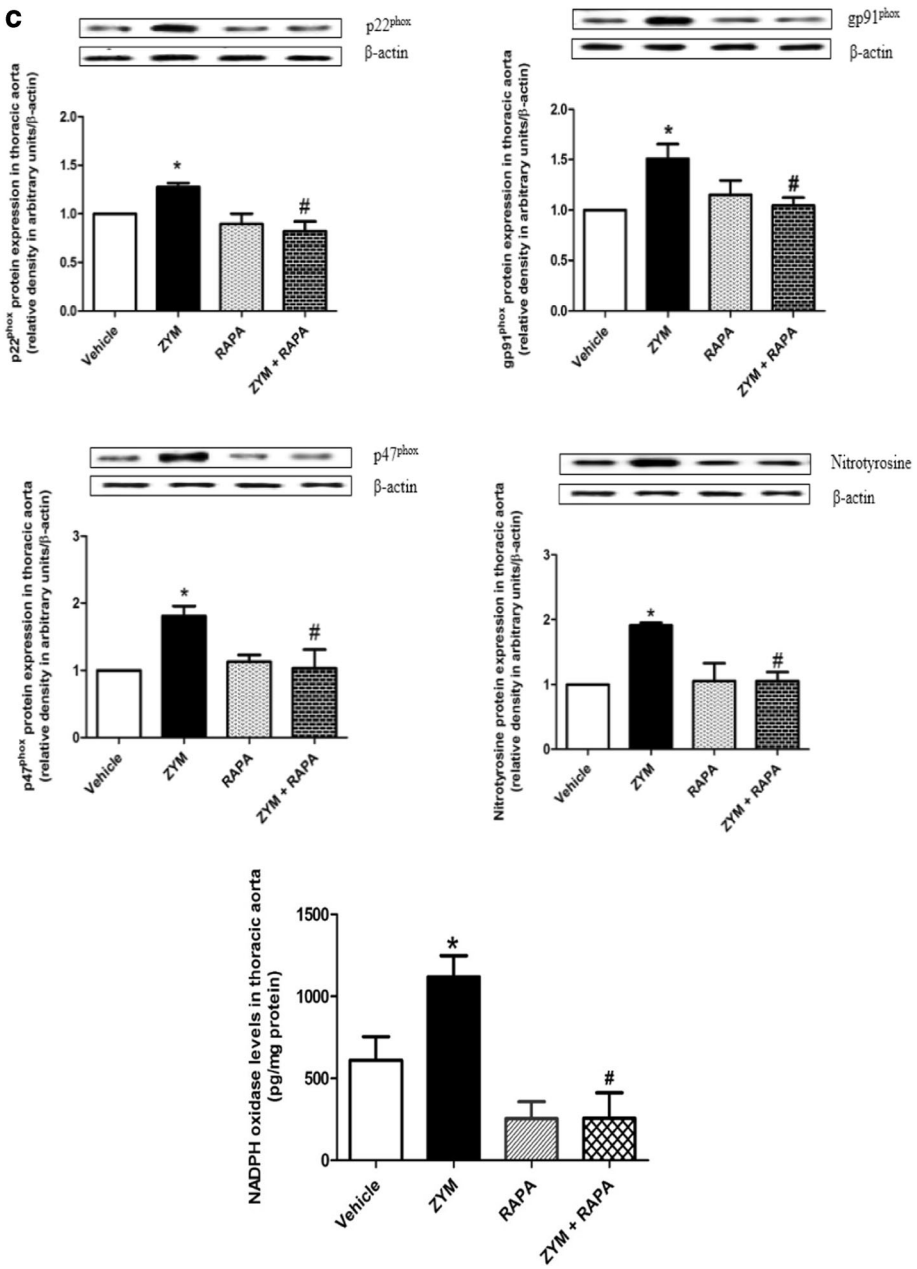
mechanism in circulatory shock and chronic inflammatory diseases [86, 87]. Nitrotyrosine formation is known as a specific marker for the formation "footprint" of peroxynitrite and indicator of especially the increased nitrosative stress [88]. It has been reported that NO-derived peroxynitrite formation plays a major role in vascular-resistant hyporeactivity to vasopressor agents in endotoxemic rats [89–91]. In the present study, zymosan caused a significant increase in nitrotyrosine protein expression in cardiac, renal, and vascular tissues which was prevented by rapamycin. Our group previously reported that increased formation of peroxynitrite contributes to the decrease in blood pressure associated with oxidative/nitrosative stress and NO formation by MEK1/ERK1/2/iNOS pathway in endotoxemic rats [19]. These



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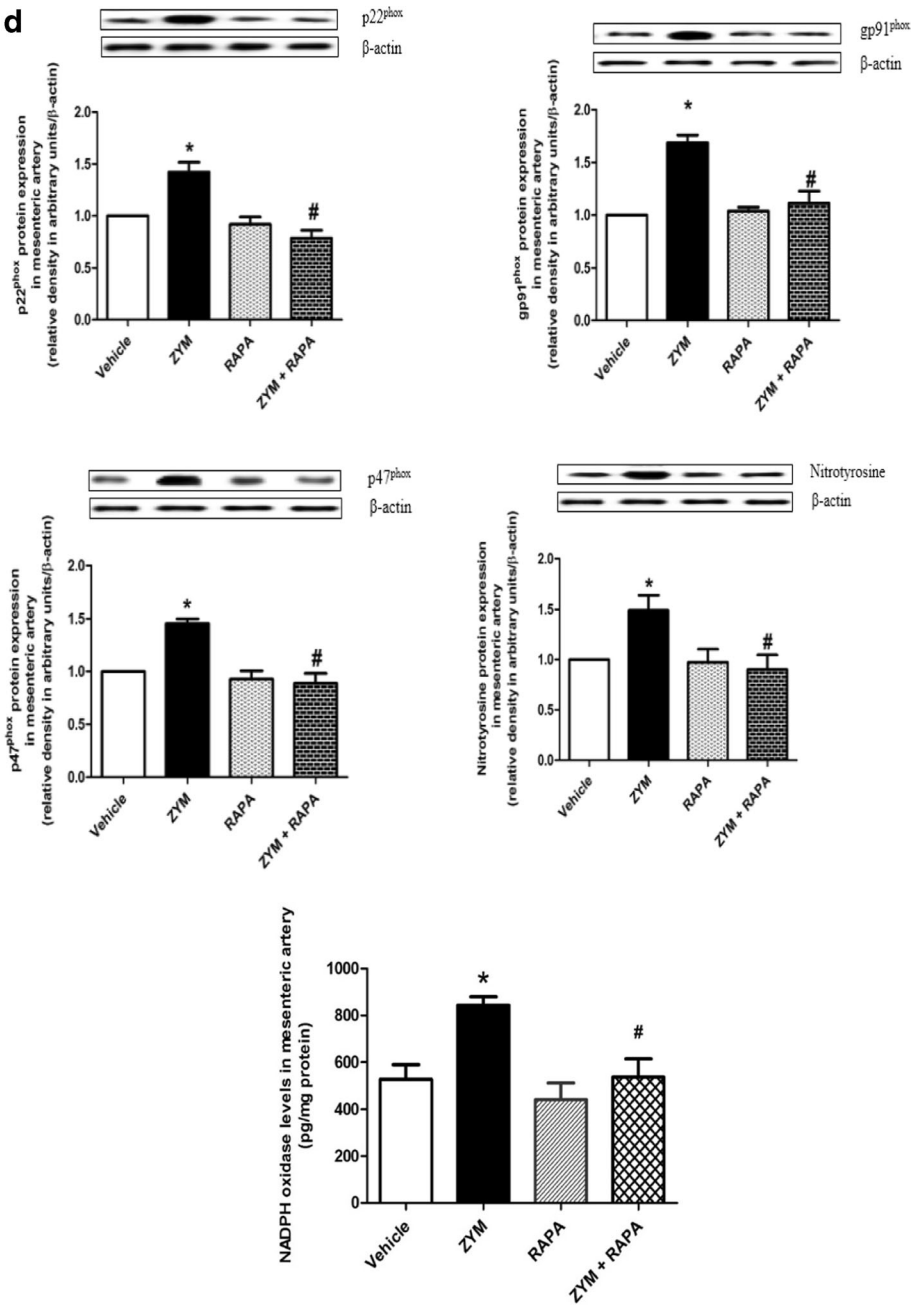
results may be evaluated as the increased production of peroxynitrite by mTOR/MEK1/ERK1/2/IKKβ/IκB-α/NF-κB pathway activation might contribute to the hypotension and systemic inflammation during zymosan-induced shock.

In conclusion, the present study provides the first evidence that mTOR contributes to systemic inflammation and related tissue damage in zymosan-induced non-septic shock characterized by hypotension, inflammation, and oxidative/nitrosative stress. This is most likely due to increased activation of mTOR/MEK1/ERK1/2/IKKβ/IκB-α/NF-κB signalling pathway with proinflammatory and vasodilator mediator formation and



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NADPH oxidase system activation. We are unable to evaluate ideal timepoint for checking this pathway activation due to limited literature. Although additional studies are necessary to evaluate activation of mTOR/MEK1/ERK1/2/IKKβ/IκB-α/NF-κB signalling pathway in different time points by using various inhibitors related to these signalling pathways, it is possible that mTOR inhibition could serve as an important novel approach for the treatment of the diseases characterized by systemic inflammation combined with MODS such as sepsis, septic, and non-septic shock.

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COMPLIANCE WITH ETHICAL STANDARDS

All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study protocols were approved by the Ethics Committee of Mersin University School of Medicine.

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

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