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ORIGINAL RESEARCH PAPER

Contribution of PPARα/β/γ, AP-1, importin-α3, and RXRα to the protective effect of 5,14-HEDGE, a 20-HETE mimetic, against hypotension, tachycardia, and inflammation in a rat model of septic shock

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Abstract

Objectives We have previously demonstrated that downregulation of the MyD88/TAK1-dependent signaling pathway associated with increased CYP4A1 expression and 20-HETE formation participates in the protective effect of *N*-(20-hydroxyeicosa-5[*Z*],14[*Z*]-dienoyl)glycine (5,14-HEDGE), a 20-HETE mimetic, against vascular hyporeactivity, hypotension, tachycardia, inflammation, and mortality in a rodent model of septic shock. The aim of this study was to determine whether increased renal and cardiovascular expression of PPARα/β/γ and RXRα associated with decreased expression and/or activity of AP-1 and importin-α3 participates in the protective effect of 5,14-HEDGE in response to systemic administration of lipopolysaccharide (LPS).

Methods Conscious male Wistar rats received saline (4 ml/kg) or LPS (10 mg/kg) at time 0. Blood pressure and heart rate were measured using a tail-cuff device. Separate groups of LPS-treated rats were given 5,14-HEDGE (30 mg/kg) 1 h after injection of saline or LPS. The rats were killed 4 h after saline or LPS administration and the kidney, heart, thoracic aorta, and superior mesenteric artery were collected for measurement of protein expression.

Results Blood pressure fell by 33 mmHg and heart rate rose by 72 beats/min at 4 h after LPS administration. In LPS-treated rats, tissue protein expressions of cytosolic/nuclear PPARα/β/γ and nuclear RXRα, in addition to nuclear translocation of PPARα/β/γ proteins, were decreased, while cytosolic/nuclear AP-1 subunit c-jun/phosphorylated c-jun and importin-α3 protein expression as well as their nuclear translocation were increased. The LPS-induced changes were prevented by 5,14-HEDGE.

Conclusions The results suggest that an increase in the expression of PPARα/β/γ and RXRα as well as a decrease in AP-1 and importin-α3 expression/activity participates in the protective effect of 5,14-HEDGE against hypotension, tachycardia, and inflammation during endotoxemia and thus have a beneficial effect in septic shock treatment.

Keywords Endotoxin · Septic shock · PPARα/β/γ · AP-1 · Importin-α3 · RXRα

Abbreviations

15d-PGJ$_2$	15-Deoxy-$\Delta^{12,14}$-prostaglandin J$_2$
20-HETE	20-Hydroxyeicosatetraenoic acid
5,14-HEDGE	*N*-(20-hydroxyeicosa-5[*Z*],14[*Z*]-dienoyl)glycine
AP	Activator protein
BSA	Bovine serum albumin
CD	Cluster of differentiation
COX	Cyclooxygenase
Crm	Chromosome region maintenance
CYP	Cyctochrome P450
DNA	Deoxyribonucleic acid
ERK	Extracellular signal-regulated kinase
HR	Heart rate
Ig	Immunoglobulin

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IKK	IκB kinase
IκB	Inhibitor of κB
IL	Interleukin
i.p.	Intraperitoneally
IL	Interleukin
IRAK	Interleukin-1 receptor-associated kinase
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
MD	Myeloid differentiation protein
MEK	Mitogen-activated protein kinase kinase
MKK	Mitogen-activated protein kinase kinase
miRNA	Microribonucleic acid
MyD88	Myeloid differentiation factor 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NES	Nuclear export signals
NF-κB	Nuclear factor-κB
NFAT	Nuclear factor of activated T cells
NLS	Nuclear localization signals
NO	Nitric oxide
PG	Prostaglandin
PPAR	Peroxisome proliferator-activated receptor
PPREs	Peroxisome proliferator-activated receptor response elements
RXR	Retinoid X receptor
s.c.	Subcutaneously
SEM	Standard error of means
STAT	Signal transducer and activator of transcription
TAK	Transforming growth factor-activated kinase
TIRAP	Toll-interleukin-1 receptor domain containing adaptor protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF receptor-associated factor

Introduction

20-Hydroxyeicosatetraenoic acid (20-HETE) is an ω-hydroxylation product of arachidonic acid that is produced by cytochrome P450 (CYP) enzymes, mainly by the CYP4A and CYP4F isoforms in the kidney, heart, liver, brain, lung, and vasculature [1–5]. In the vasculature, 20-HETE causes vasoconstriction in several vascular beds, including renal, aortic, mesenteric, and coronary arteries [6–8]. As opposed to its vasoconstrictor effect, 20-HETE has also been reported to produce species-specific vasodilation in renal and coronary arteries [9, 10]. In addition, 20-HETE has been shown to activate mitogen-activated protein kinase (MAPK) kinase (MEK) 1/extracellular signal-regulated kinase (ERK) 1/2/nuclear factor-κB (NF-κB) signaling and induce expression of cellular adhesion molecules and cytokines, thereby promoting inflammation [11–13]. CYP4A- and CYP4F-derived 20-HETE has been reported to be involved in lipopolysaccharide (LPS)-induced acute systemic inflammation as a proinflammatory mediator [14, 15].

Stimulation of host cells by LPS occurs through a series of interactions with several proteins including the LPS-binding protein, opsonic receptor cluster of differentiation (CD) 14, myeloid differentiation protein (MD)-2, and toll-like receptor (TLR) 4 [16–20]. In the myeloid differentiation factor 88 (MyD88)-dependent (canonical, classical) pathway, following the recognition of LPS, activation of inhibitor of κB (IκB) kinase (IKK) and MAPK pathways through TLR4/toll-interleukin (IL)-1 receptor domain containing adaptor protein (TIRAP)/IL-1 receptor-associated kinase (IRAK) 1/4/tumor necrosis factor (TNF) receptor-associated factor (TRAF) 6/transforming growth factor-activated kinase 1 (TAK1) cascade results in phosphorylation and degradation of IκB proteins by IKKs. Subsequent nuclear translocation of NF-κB and induction of another transcription factor, activator protein (AP)-1, by MAPKs (MEK1, MAPK kinase [MKK] 3/6, MKK4, ERK1/2, p38 MAPK, and c-jun N-terminal kinase 1/2), respectively, result in activation of proinflammatory mediator production [16–18, 20]. It has been reported that inhibition of the NF-κB and AP-1 pathways improves septic disturbances, restores normotension, ameliorates myocardial dysfunction and vascular derangement, inhibits proinflammatory gene expression, diminishes intravascular coagulation, reduces tissue neutrophil influx, prevents microvascular endothelial leakage, prevents multiple organ injury, and improves survival in rodent models of septic shock [17, 19, 21]. Therefore, the consequences of sepsis inducing septic shock, tissue injury, and multiple organ failure can be prevented by the development of targeted cell-specific approaches inhibiting activation of NF-κB and/or AP-1 pathways.

The peroxisome proliferator-activated receptor (PPAR) subfamily includes ligand-activated transcription factors and consists of three homologous members (PPARα, PPARβ [also known as PPARδ], and PPARγ) encoded by separate genes. PPARs are activated by heterodimerization with the retinoid X receptor (RXR) into biologically active transcription factors. PPAR–RXR heterodimers then bind to specific complementary deoxyribonucleic acid (DNA) sequences, known as peroxisome proliferator-activated receptor response elements (PPREs), in the promotor region of target genes, thereby acting as a transcriptional regulator [22–25]. In recent years, PPARs have been shown to possess profound antiinflammatory functions in a broad field of injury-associated conditions including sepsis, septic

shock, and multiple organ failure [24, 26]. On the other hand, PPAR activation in modulating immune response during sepsis and septic shock is still ambiguous and has been described as a "double-edged sword" [26].

Nucleoplasmic shuttling of macromolecules is a well-controlled process involving importins and exportins. These karyopherins recognize and bind to receptor-mediated intracellular signals through specific signal sequences that are present on cargo proteins and transport into and out of the nucleus through nuclear pore complexes. Nuclear localization signals (NLS) present on cargo molecules to be imported, while nuclear export signals (NES) on the molecules to be exported are recognized by importins and exportins, respectively. The classical NLS or NES are found on many transcription factors and molecules (e.g., NF-κB p50/p65, nuclear factor of activated T cells [NFAT], IκB, signal transducers and activators of transcription [STATs], and AP-1 subunit c-jun) that are involved in the pathogenesis of allergic and inflammatory conditions [27–29]. In light of the important role of these karyopherins in the nucleocytoplasmic transport of transcription factors including NF-κB, AP-1, and PPARα/β/γ during the inflammatory process, considering regulation of their expressions and/or functions could provide new opportunities for the development of effective intervention strategies for the treatment of sepsis and septic shock.

Our previous studies demonstrated that a stable synthetic analog of 20-HETE, *N*-(20-hydroxyeicosa-5[*Z*],14[*Z*]-dienoyl)glycine, 5,14-HEDGE, which mimics the effects of endogenously produced 20-HETE, prevents vascular hyporeactivity, hypotension, tachycardia, inflammation, tissue injury, and mortality in a rodent model of septic shock [30–34]. As a continuation of our previous studies, the present study was conducted to determine whether increased renal and cardiovascular expression of PPARα/β/γ and RXRα associated with decreased expression and/or activity of AP-1 and importin-α3 participate in the protective effect of 5,14-HEDGE against hypotension, tachycardia, and inflammation in LPS-treated rats.

Materials and methods

Endotoxic shock model

Experiments were performed on Wistar rats (male 200 to 350 g; $n = 24$) (Research Center of Experimental Animals, Mersin University, Mersin, Turkey) fed a standard chow. They were synchronized by maintenance of controlled environmental conditions throughout the experiments. The circadian rhythmicity of the animals was entrained by a standardized 12 h light and 12 h dark cycle. All experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Ethics Committee of Mersin University School of Medicine. Endotoxic shock was induced in rats as previously described by Tunctan et al. [35]. Rats were randomly divided into saline ($n = 6$), LPS ($n = 6$), saline + 5,14-HEDGE ($n = 6$), and LPS + 5,14-HEDGE ($n = 6$) groups. In the saline and 5,14-HEDGE groups, animals received saline (4 ml/kg, intraperitoneally [i.p.]) at time 0. Animals in the LPS and LPS + 5,14-HEDGE groups were treated with LPS (*Escherichia coli* LPS, O111:B4; Sigma Chemical Co., St. Louis, MO, USA) (10 mg/kg, i.p.; sub-lethal dose) at time 0. In the 5,14-HEDGE and LPS + 5,14-HEDGE groups, animals were treated with a stable synthetic analog of 20-HETE, 5,14-HEDGE (30 mg/kg, subcutaneously [s.c.]) [30–34] 1 h after injection of saline or LPS, respectively. 5,14-HEDGE was synthesized in the Department of Biochemistry University of Texas Southwestern Medical Center, Dallas, Texas, USA. Mean arterial pressure (MAP) and heart rate (HR) of the rats were measured using a tail-cuff device (MAY 9610 Indirect Blood Pressure Recorder System, Commat Ltd., Ankara, Turkey) during a control period at time 0 and 1, 2, 3, and 4 h. All rats survived the experiments. Rats were euthanized 4 h after the administration of saline or LPS, and kidney, heart, thoracic aorta, and superior mesenteric artery were collected from all animals. Cytosolic and nuclear fractions were prepared from freshly isolated tissues by Nuclear Extraction Kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions and stored at −80 °C for measurement of PPARα/β/γ, c-jun, phosphorylated c-jun, importin-α3, RXRα, and α-smooth muscle actin protein expression by immunoblotting as described below. Total protein in these fractions was determined by the Coomassie blue method using bovine serum albumin as standard.

Immunoblotting

Immunoblotting for PPARα/β/γ, c-jun, phosphorylated c-jun, importin-α3, RXRα, and α-smooth muscle actin proteins was performed according to the method described previously [30, 31, 33, 34]. Briefly, tissue homogenates (8–90 μg of protein) were subjected to a 10 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then proteins were transferred to a nitrocellulose membrane. The membranes were blocked with 5 % non-fat dry milk in Tris-buffered saline at room temperature for 1 h and incubated with the following primary antibodies in 5 % bovine serum albumin (BSA) (1:500) overnight at 4 °C: (1) PPARα rabbit polyclonal antibody (H-98) (sc-9000, Santa Cruz Biotechnology, Santa Cruz, CA, USA); (2) PPARβ mouse monoclonal antibody (F-10) (sc-74517,

Santa Cruz); (3) PPARγ mouse monoclonal antibody (E-8) (sc-7273, Santa Cruz); (4) c-Jun rabbit polyclonal antibody (H-79) (sc-1694, Santa Cruz); (5) phosphorylated c-Jun goat polyclonal antibody (Ser 63/73) (sc-16312; Santa Cruz); (6) KPNA3 rabbit polyclonal antibody (PA5-21035, ThermoFisher Scientific Inc., Waltham, MA, USA); and (7) RXRα rabbit polyclonal antibody (ΔN 197) (sc-774, Santa Cruz). The membranes were then incubated with goat anti-rabbit IgG-horseradish peroxidase (RPN4301, Amersham Life Sciences, Cleveland, OH, USA) for PPARα, c-jun, phosphorylated c-jun, importin-α3, and RXRα, and sheep anti-mouse IgG-horseradish peroxidase (RPN4201, Amersham) for PPARβ, PPARγ, and α-smooth muscle actin in 0.1 % BSA (1:1.000) at room temperature for 1 h. The blots were developed with enhanced chemiluminescence (ECL Prime Western Blotting Detection Reagent) (RPN2232, Amersham) according to the manufacturer's instructions. Immunoreactive proteins were visualized using a gel-imaging system (EC3-CHEMI HR imaging system; Ultra-Violet Products, UVP, Cambridge, UK). Densitometric analysis was performed with NIH image software (Image J 1.46r, Wayne Rasband, National Institute of Health, Bethesda, MD, USA). The membranes were reprobed with anti-α smooth muscle actin mouse monoclonal immunoglobulin (Ig) G (A2547, Sigma Chemical Co., St. Louis, MO, USA) (1:500 in 5 % BSA) as a loading control followed by incubation with sheep anti-mouse IgG-horseradish peroxidase (1:1000 in 0.1 % BSA). Relative densities of immunoreactive bands for PPARα/β/γ, c-jun, phosphorylated c-jun, importin-α3, RXRα, and α-smooth muscle actin proteins in each sample were normalized to the density of the corresponding bands for α-smooth muscle actin.

Statistical analysis

Data are expressed as means ± standard error of means (SEM). Data were analyzed by one-way ANOVA followed by Student–Newman–Keuls test for multiple comparisons, Kruskal–Wallis test followed by Dunn's test for multiple comparisons, and Student's t or Mann–Whitney U tests when appropriate. A p value <0.05 was considered to be statistically significant.

Results

Effect of 5,14-HEDGE on the cardiovascular response to LPS

LPS caused a gradual fall in MAP (Fig. 1a) and an increase in HR (Fig. 1b) over the 4 h course of the experiment ($p < 0.05$). The change in MAP and HR reached a maximum 4 h after the administration of LPS. MAP fell by

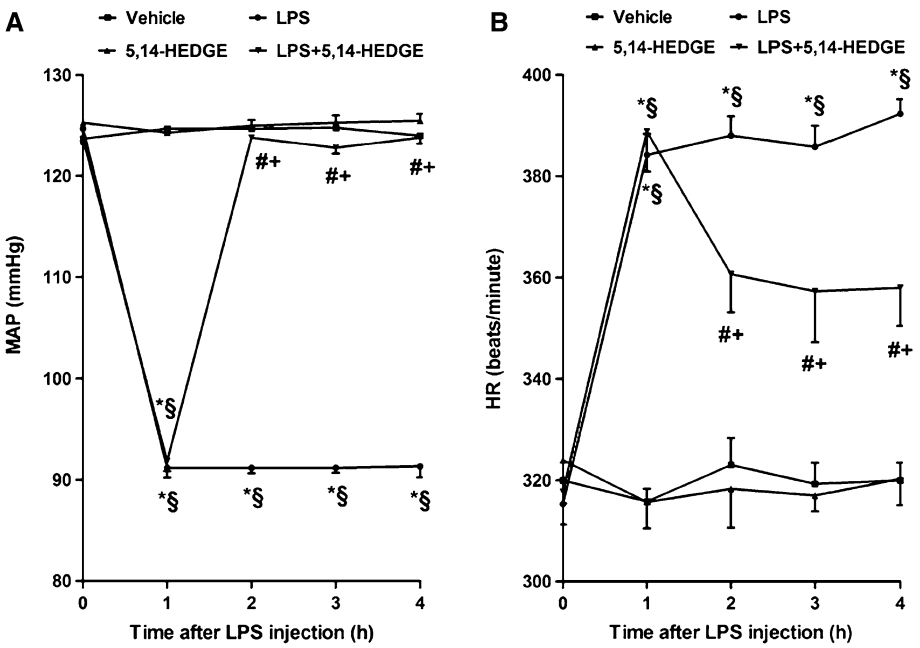

Fig. 1 Time course of the effects of 5,14-HEDGE on MAP and HR following administration of saline (vehicle) or LPS to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) was given 1 h after administration of saline (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.). Data are expressed as mean ± SEM of six animals. *Asterisk* significant difference from the corresponding value seen in rats treated with saline (vehicle) ($p < 0.05$). *Hash* significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$). *Section sign* significant difference from the time 0 h value within a group ($p < 0.05$). *Plus sign* significant difference from the time 1 h value within a group ($p < 0.05$)

33 mmHg and HR rose by 72 bpm in rats treated with LPS. A synthetic analog of 20-HETE, 5,14-HEDGE, which mimics the effects of endogenously produced 20-HETE, prevented the fall in MAP and the increase in HR in rats given LPS ($p < 0.05$). 5,14-HEDGE had no effect on MAP or HR in rats treated with vehicle ($p > 0.05$).

Effect of 5,14-HEDGE on LPS-induced decrease in expression and nuclear translocation of PPARα, PPARβ, and PPARγ in endotoxemic rats

To determine the effect of 5,14-HEDGE on LPS-induced changes in PPARs (transcriptional factors belonging to ligand-activated nuclear receptor superfamily) expression and their nuclear translocation, PPARα, PPARβ, and PPARγ proteins were measured in the cytosolic and nuclear fractions of the kidney, heart, thoracic aorta, and superior mesenteric artery of endotoxemic rats and the ratios of nuclear over cytosolic PPARα/β/γ proteins were calculated. LPS decreased the expression of cytosolic/nuclear PPARα/β/γ proteins in addition to calculated nuclear/cytosolic PPARα/β/γ protein ratios in the kidney (Figs. 2a, 3a, and 4a), heart (Figs. 2b, 3b, and 4b), thoracic aorta (Figs. 2c, 3c, and 4c), and superior mesenteric artery (Figs. 2d, 3d, and 4d) ($p < 0.05$). The decrease in cytosolic/nuclear PPARα/β/γ protein expression and calculated nuclear/cytosolic PPARα/β/γ protein ratios produced by LPS was prevented in the tissues of rats treated with 5,14-HEDGE ($p < 0.05$) (Figs. 2, 3, 4). 5,14-HEDGE had no effect on the basal expression of cytosolic/nuclear PPARα/β/γ proteins as well as nuclear/cytosolic PPARα/β/γ protein ratios in vehicle-treated rats ($p > 0.05$) (Figs. 2, 3 and 4).

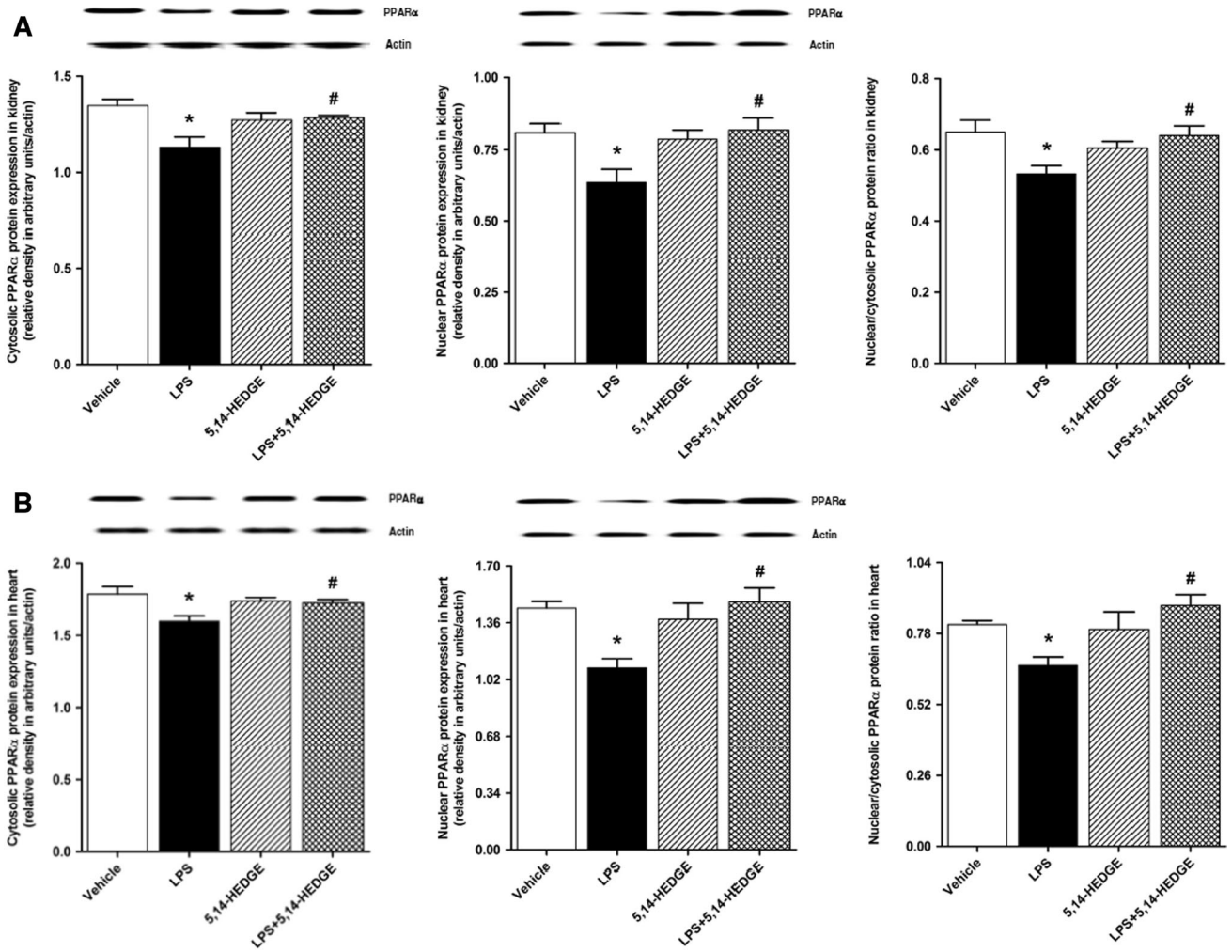

Fig. 2 Effects of 5,14-HEDGE on changes in cytosolic/nuclear PPARα protein expression and calculated ratios of nuclear/cytosolic PPARα protein in **a** kidney, **b** heart, **c** thoracic aorta (TA), and **d** superior mesenteric artery (SMA) measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) was given 1 h after administration of saline or LPS. PPARα protein expression in tissue homogenates was measured by immunoblotting. Data are expressed as mean ± SEM of four animals. *Asterisk* significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). *Hash* significant difference from the corresponding value seen in rats treated with LPS ($p < 0.05$)

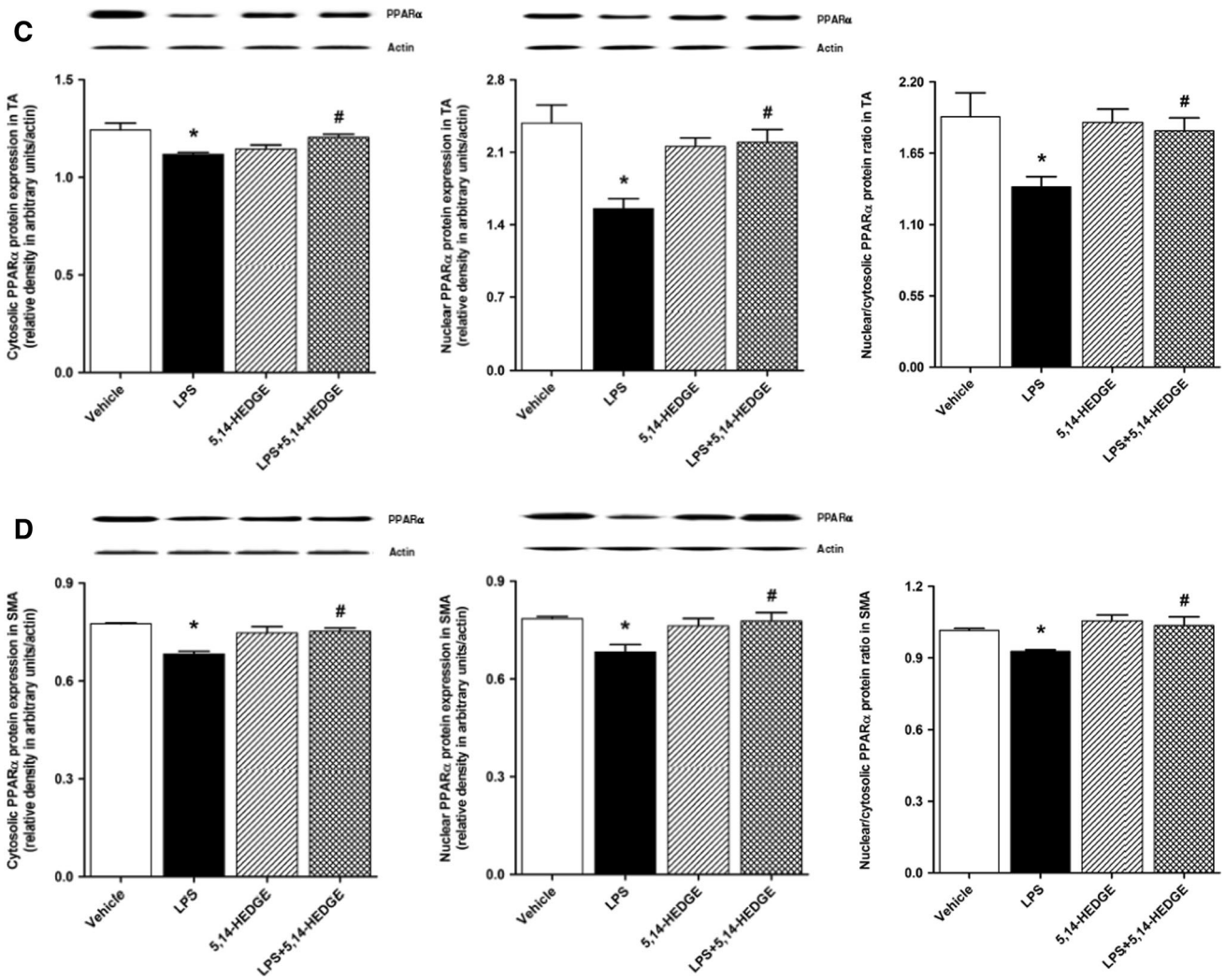

Fig. 2 continued

Effect of 5,14-HEDGE on LPS-induced increase in expression, activity, and nuclear translocation of AP-1

To determine the effect of 5,14-HEDGE on LPS-induced changes in AP-1 (a proinflammatory transcription factor) expression, activity, and its nuclear translocation, unphosphorylated and phosphorylated c-jun (at $Ser^{63/73}$) proteins were measured in the cytosolic and nuclear fractions of the kidney, heart, thoracic aorta, and superior mesenteric artery of endotoxemic rats and the ratios of nuclear over cytosolic unphosphorylated and phosphorylated c-jun proteins were calculated. LPS increased the expression of cytosolic/nuclear unphosphorylated and phosphorylated c-jun proteins (Fig. 5) as well as calculated nuclear/cytosolic unphosphorylated and phosphorylated c-jun protein ratios (Fig. 6) in the kidney (Figs. 5a, 6a), heart (Figs. 5b, 6b), thoracic aorta (Figs. 5c, 6c), and superior mesenteric artery (Figs. 5d, 6d) ($p < 0.05$). The increase in cytosolic/nuclear unphosphorylated and phosphorylated c-jun protein expression (Fig. 5) and calculated nuclear/cytosolic unphosphorylated and phosphorylated c-jun protein ratios (Fig. 6) produced by LPS was prevented in the tissues of rats treated with 5,14-HEDGE ($p < 0.05$). 5,14-HEDGE had no effect on basal cytosolic/nuclear unphosphorylated and phosphorylated c-jun proteins (Fig. 5) as well as nuclear/cytosolic unphosphorylated and phosphorylated c-jun protein ratios (Fig. 6) in vehicle-treated rats ($p > 0.05$).

Effect of 5,14-HEDGE on LPS-induced increase in expression and nuclear translocation of importin-α3 in endotoxemic rats

To determine the effect of 5,14-HEDGE on LPS-induced changes in importin-α3 (also known as karyopherin-α3, a nuclear import receptor responsible for nuclear transportation of transcription factors such as NF-κB, AP-1, and

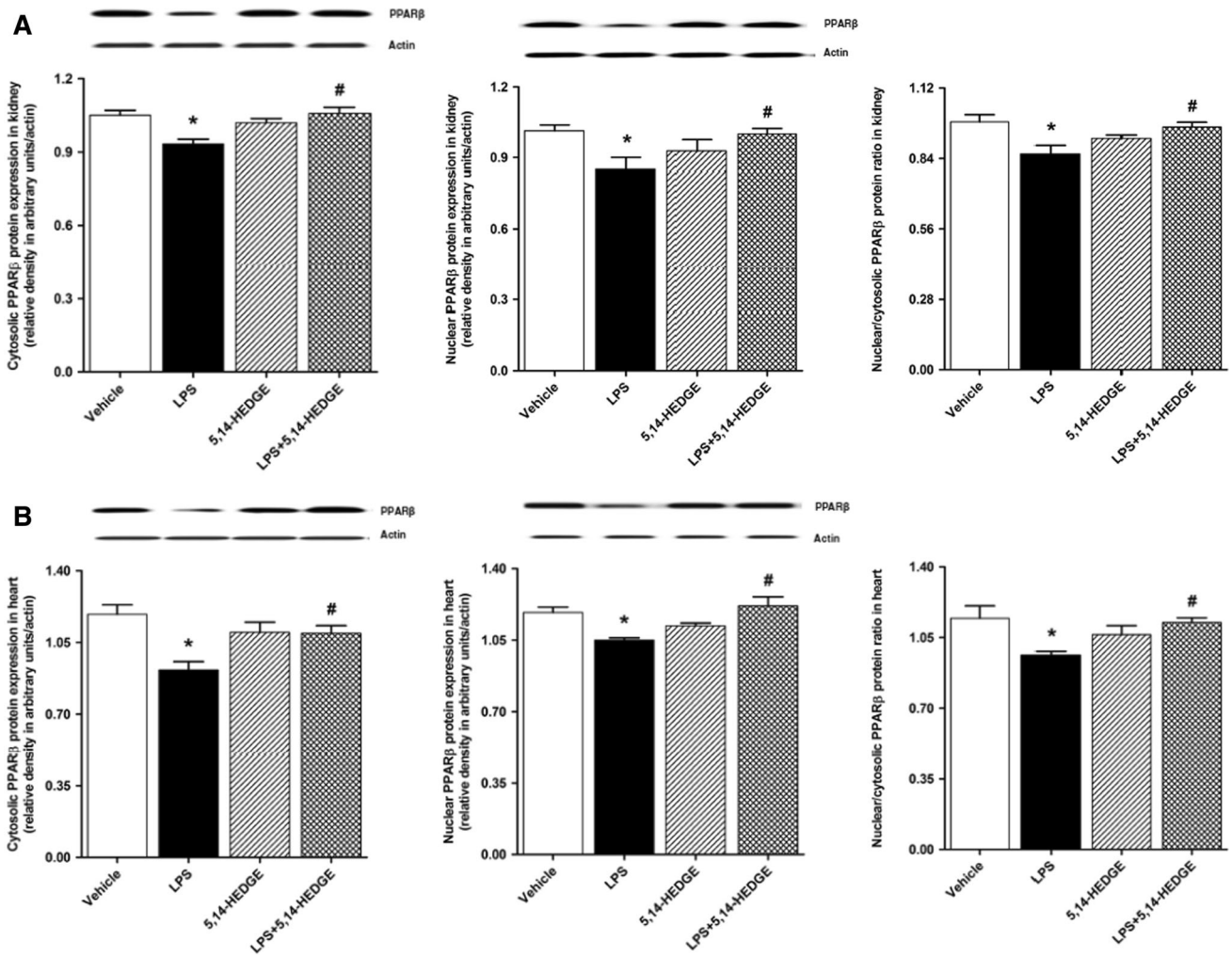

Fig. 3 Effects of 5,14-HEDGE on changes in cytosolic/nuclear PPARβ protein expression and calculated ratios of nuclear/cytosolic PPARβ protein in the **a** kidney, **b** heart, **c** thoracic aorta (TA), and **d** superior mesenteric artery (SMA) measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) was given 1 h after administration of saline or LPS. PPARβ protein expression in tissue homogenates was measured by immunoblotting. Data are expressed as mean ± SEM of four animals. *Asterisk* significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). *Hash* significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$)

PPARs) expression and its nuclear translocation, importin-α3 protein expression was measured in the cytosolic and nuclear fractions of the kidney, heart, thoracic aorta, and superior mesenteric artery of endotoxemic rats and the ratios of nuclear over cytosolic importin-α3 protein were calculated. LPS increased the expression of cytosolic/nuclear importin-α3 protein in addition to calculated nuclear/cytosolic importin-α3 protein ratios in the kidney (Fig. 7a), heart (Fig. 7b), thoracic aorta (Fig. 7c), and superior mesenteric artery (Fig. 7d) ($p < 0.05$). The increase in cytosolic/nuclear importin-α3 protein expression and calculated nuclear/cytosolic importin-α3 protein ratios caused by LPS were prevented in the tissues of rats treated with 5,14-HEDGE ($p < 0.05$) (Fig. 7). 5,14-HEDGE had no effect on the basal expression of cytosolic/nuclear importin-α3 protein as well as nuclear/cytosolic importin-α3 protein ratios in vehicle-treated rats ($p > 0.05$) (Fig. 7).

Effect of 5,14-HEDGE on LPS-induced decrease in RXRα expression in endotoxemic rats

To determine the effect of 5,14-HEDGE on LPS-induced changes in expression of RXRα (a nuclear receptor acting as a ligand-dependent transcription factor that modifies the expression of genes such as CYPs), RXRα protein expression was measured in the nuclear fractions of the kidney, heart, thoracic aorta, and superior mesenteric artery of endotoxemic rats. LPS decreased the expression of RXRα protein in the kidney (Fig. 8a), heart (Fig. 8b),

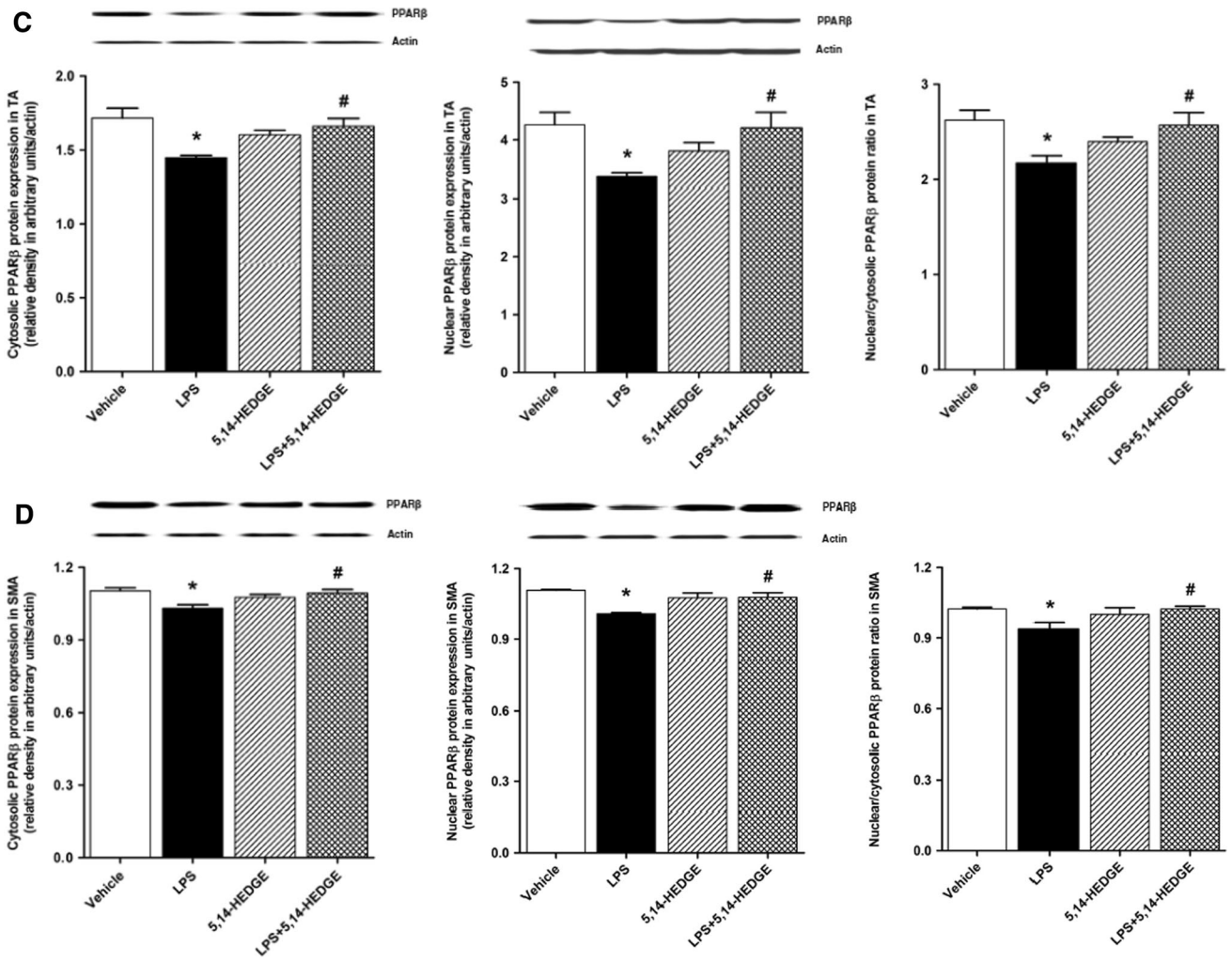

Fig. 3 continued

thoracic aorta (Fig. 8c), and superior mesenteric artery (Fig. 8d) ($p < 0.05$). The decrease in nuclear RXRα protein expression produced by LPS was prevented in the tissues of rats treated with 5,14-HEDGE ($p < 0.05$) (Fig. 8). 5,14-HEDGE had no effect on the basal expression of nuclear RXRα protein in vehicle-treated rats ($p > 0.05$) (Fig. 8).

Discussion

The results of the present study indicate that increased cytosolic/nuclear AP-1 subunit c-jun/phosphorylated c-jun and importin-α3 protein expression associated with their translocation into the nucleus as well as decreased expression of cytosolic/nuclear PPARα/β/γ and nuclear RXRα in addition to nuclear translocation of PPARα/β/γ proteins participate in the fall in blood pressure, tachycardia, and inflammation in rats treated with LPS. These data also demonstrate that 5,14-HEDGE, a 20-HETE mimetic, prevents hypotension, tachycardia, and inflammation which may be due to increased expression and nuclear import of PPARα/β/γ associated with RXRα expression as well as decreased AP-1 and importin-α3 expression/activity and their nuclear translocation, leading to vasodilatory and inflammatory mediator production in the rat model of septic shock.

During the early phase of LPS-induced endotoxemia, compensatory mechanisms, including (1) activation of the renin–angiotensin–aldosterone system, (2) increased sensitivity of baroreceptor reflex mechanisms, and (3) overproduction of endothelin-1 and catecholamines (which are known to activate phospholipase A_2 and cause release of arachidonic acid from tissue lipids resulting in increased prostaglandin synthesis and production of reactive nitrogen [e.g., nitric oxide (NO) and peroxynitrite] and oxygen [e.g.,

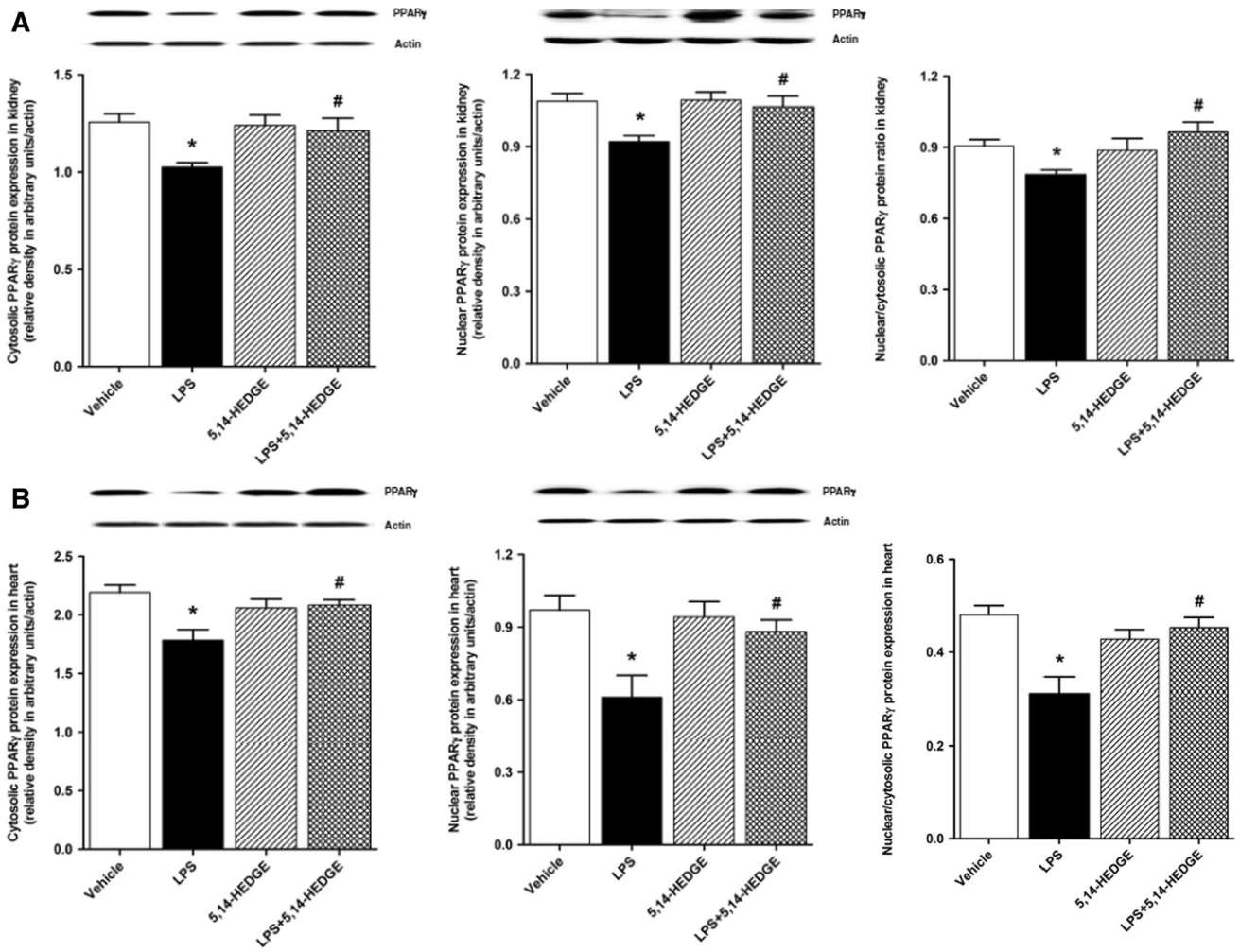

Fig. 4 Effects of 5,14-HEDGE on changes in cytosolic/nuclear PPARγ protein expression and calculated ratios of nuclear/cytosolic PPARγ protein in the **a** kidney, **b** heart, **c** thoracic aorta (TA), and **d** superior mesenteric artery (SMA) measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) was given 1 h after administration of saline or LPS. PPARγ protein expression in tissue homogenates was measured by immunoblotting. Data are expressed as mean ± SEM of four animals. *Asterisk* significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). *Hash* significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$)

superoxide] species) have also been reported to be responsible for the changes in the formation of vasoregulatory molecules that could contribute to the fall in blood pressure [4]. In the present study, we measured changes in the expression and/or activity of PPARα/β/γ, AP-1 subunit c-jun, importin-α3, and RXRα in cytosolic and/or nuclear fractions of renal and cardiovascular tissues of endotoxemic rats at 4 h after LPS administration. LPS produced a fall in MAP and an increase in HR within 1 h that was sustained for 4 h. Administration of 5,14-HEDGE prevented the LPS-induced fall in blood pressure and increase in HR within 1 h. Furthermore, 5,14-HEDGE given 1 h after LPS prevented (1) the decreases in the expression of nuclear/cytosolic PPARα, PPARβ, and PPARγ proteins as well as nuclear RXRα protein, (2) elevated amounts of both unphosphorylated and phosphorylated c-jun ($Ser^{63/73}$) proteins in addition to importin-α3 protein in the cytosolic and nuclear fractions of the kidney, heart, thoracic aorta, and superior mesenteric artery of rats.

NF-κB has been implicated in the regulation of multiple biological phenomena and disease states, including inflammation and septic shock. Activation of MyD88/TAK1/IKKβ/IκB-α pathway in response to LPS has been reported to act as an important regulator of NF-κB p65 activation and increased expression of NF-κB p65-dependent proinflammatory genes (e.g., inducible NO synthase [iNOS], cyclooxygenase-2 [COX-2], acute phase proteins, and proinflammatory cytokines) [17, 19]. On the other hand, it has been demonstrated that NF-κB subunits p65 (RelA; encoded by the RELA gene) and p50 (generated from its precursor, p105/NF-κB1, encoded by the NFKB1 gene) have critical inhibitory functions during the systemic

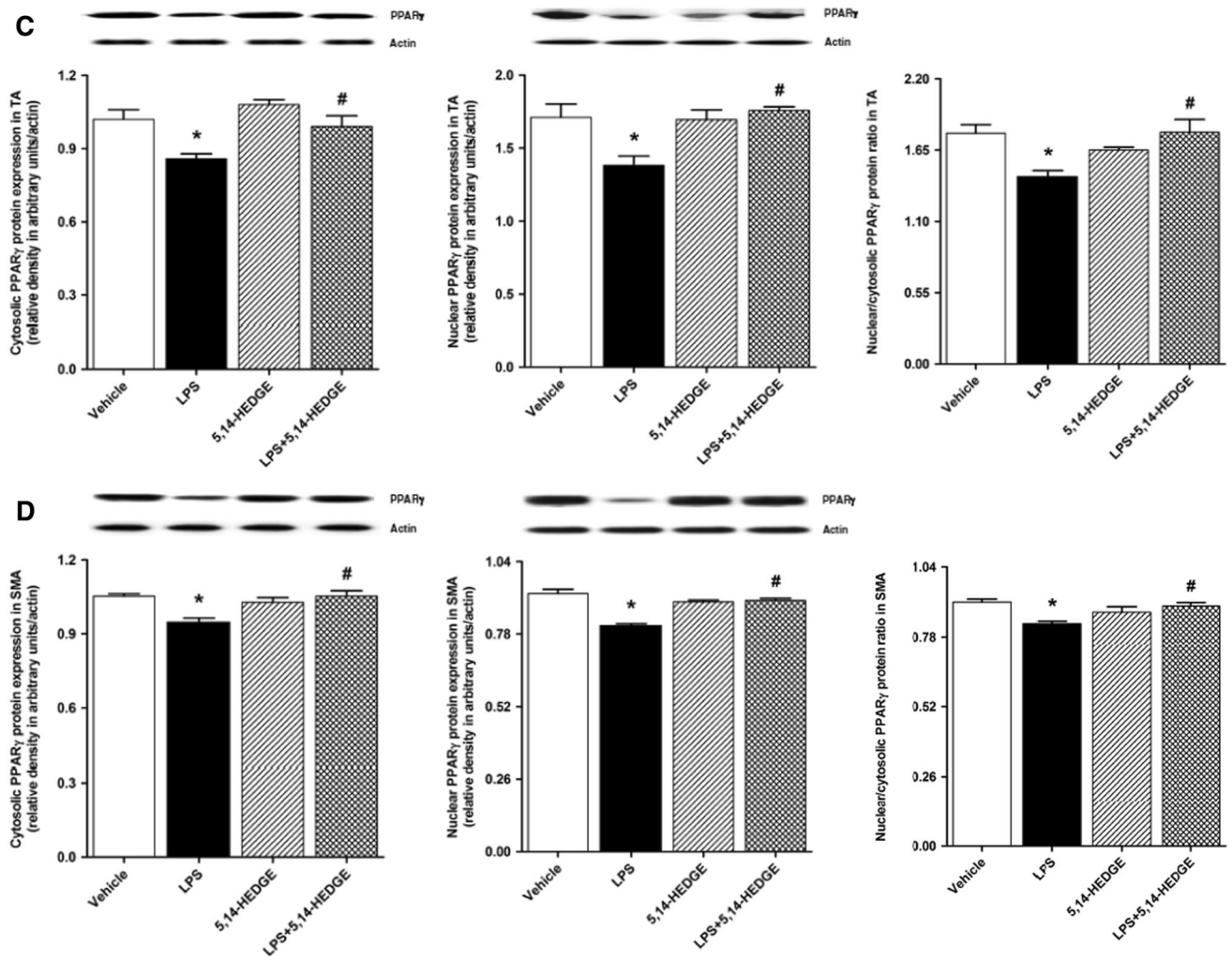

Fig. 4 continued

response to LPS and raise the possibility that these functions could be essential in preventing mortality associated with systemic inflammatory response syndrome [36]. NF-κB activation is tightly regulated mainly through its subcellular localization [19, 37–41]. The karyopherins, importins α3 and α4, have been shown to be responsible for the location of TNF-α-stimulated active subunits of NF-κB p65 to the nucleus from the cytoplasm and subsequent export back into the cytoplasm with the support of exportin, chromosome region maintenance (Crm) 1 [42–44]. Furthermore, it has been reported that exposure to LPS in ex vivo or in vitro conditions results in increased nuclear NF-κB p65 protein levels associated with decreased amounts of cytosol, suggesting increased nuclear translocation of NF-κB p65 during endotoxemia [45–49]. Moreover, Ishizuka et al. [12] demonstrated that 20-HETE activates the MEK1/ERK1/2/NF-κB pathway and increases nuclear translocation of NF-κB p65 leading to expression of proinflammatory mediators in human endothelial cells. In bovine endothelial cells, Cheng et al. [11] reported that tyrosine kinase or MEK1/ERK1/2 inhibitors prevent the effects of 20-HETE on the decrease in NO production associated with increased NF-κB activity through the IKKβ/IκB-α pathway. As repressors of central inflammatory transcription factors like NF-κB and AP-1, PPARs regulate development, differentiation, and cellular bioenergetics by modulating glucose and lipid metabolism as well as inflammatory response. It is well known that natural and synthetic PPAR ligands (e.g., nitro-fatty acids, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 [15d-PGJ_2] long-chain polyunsaturated fatty acids such as arachidonic, linoleic, docosahexaenoic, and eicosapentaenoic acids, fibrates, and thiazolidinediones) possess antiinflammatory activity in several inflammatory conditions [22–25, 50]. Members of the PPAR family display significant differences in tissue distribution, biological functions, and ligand recognition; however, they share structural and biochemical properties that allow them to associate with the RXR generating

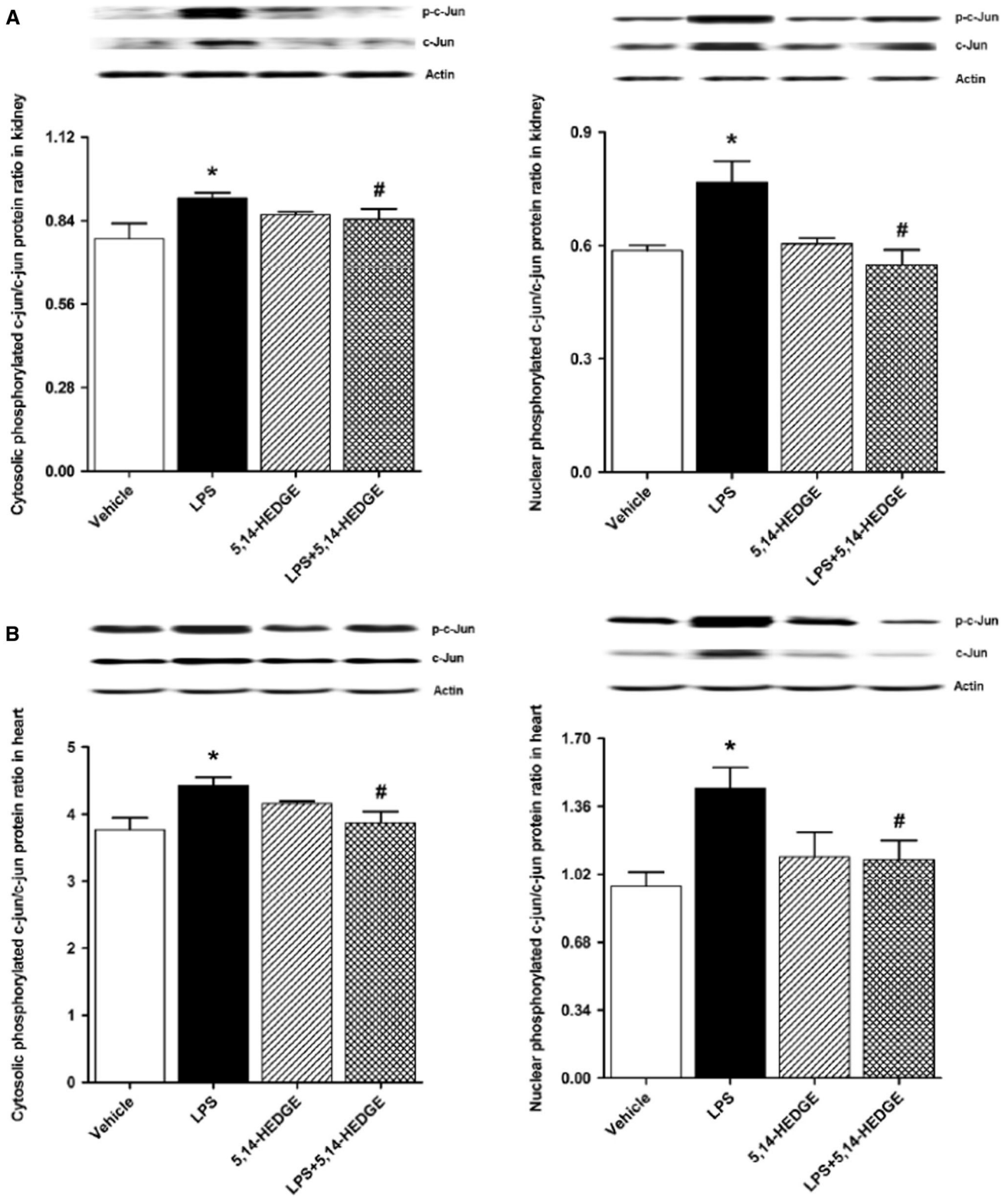

Fig. 5 Effects of 5,14-HEDGE on changes in unphosphorylated and phosphorylated c-jun (p–c-jun) protein expression in cytosolic and nuclear fractions of the **a** kidney, **b** heart, **c** thoracic aorta (TA), and **d** superior mesenteric artery (SMA) measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) was given 1 h after administration of saline or LPS. Unphosphorylated and phosphorylated c-jun protein levels in tissue homogenates were measured by immunoblotting. Data are expressed as mean ± SEM of four animals. *Asterisk* significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). *Hash* significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$)

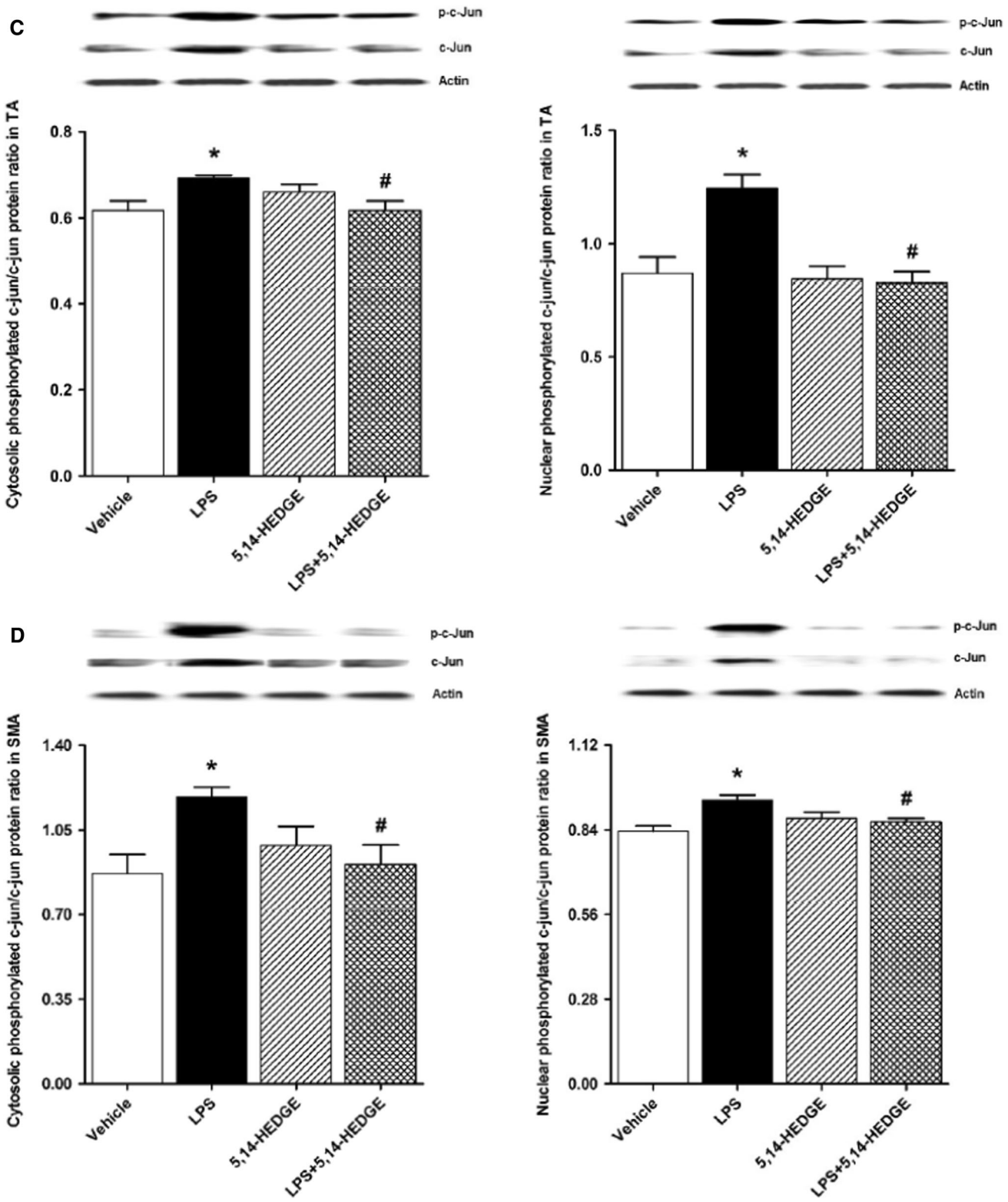

Fig. 5 continued

PPAR–RXR heterodimers. These heterodimers bind to regulatory DNA regions in the promoter of their target genes, PPREs. The transcription of PPAR target genes is modulated by the presence of the ligand. In the absence of ligands, PPAR–RXR heterodimers bind to PPREs adopting a conformation that favors the binding of corepressor

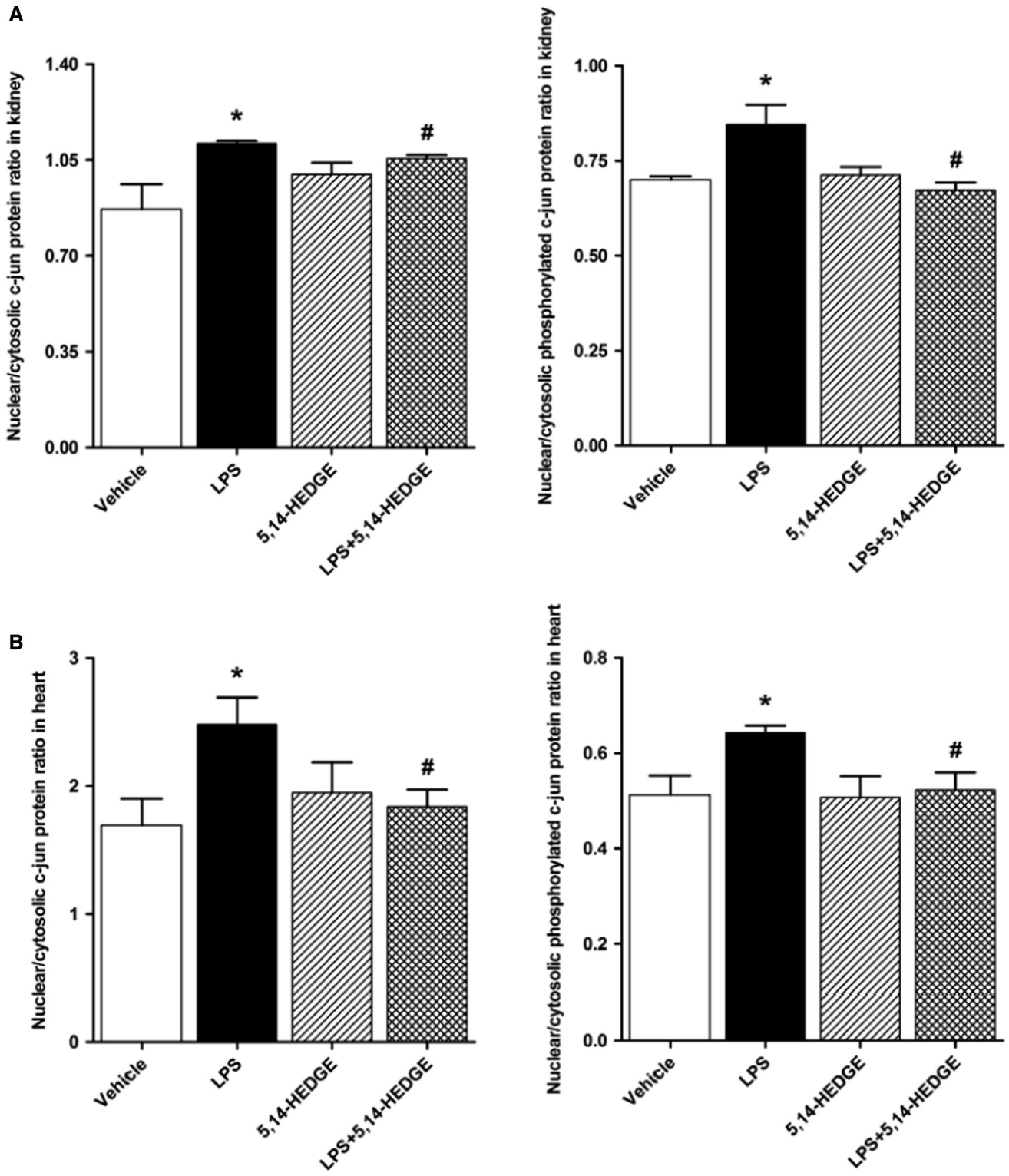

Fig. 6 Effects of 5,14-HEDGE on changes in calculated ratios of nuclear/cytosolic unphosphorylated and phosphorylated c-jun proteins in the **a** kidney, **b** heart, **c** thoracic aorta (TA), and **d** superior mesenteric artery (SMA) measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) was given 1 h after administration of saline or LPS. Unphosphorylated and phosphorylated c-jun (at $Ser^{63/73}$) protein levels in tissue homogenates were measured by immunoblotting. Data are expressed as mean ± SEM of four animals. *Asterisk* significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). *Hash* significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$)

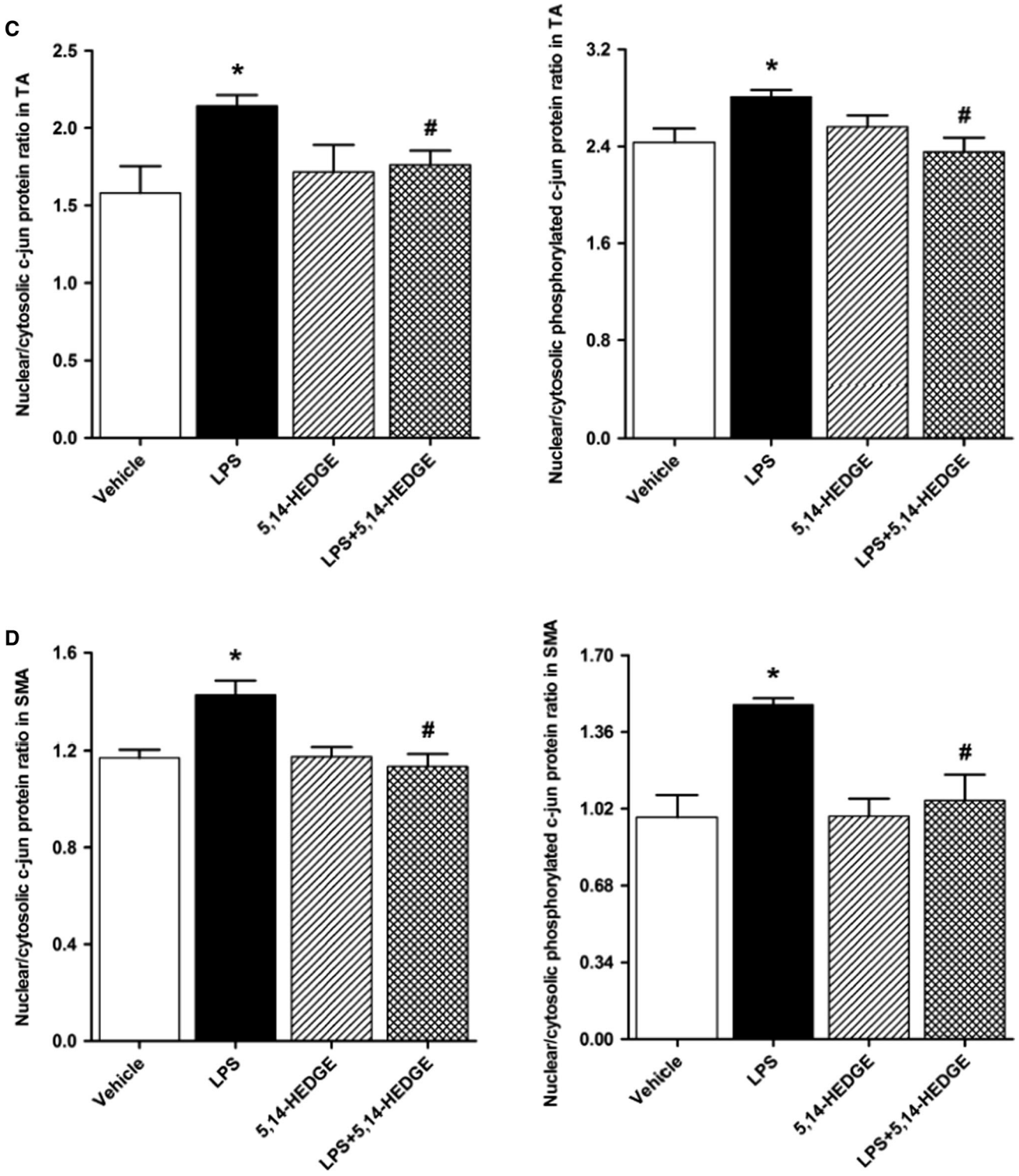

Fig. 6 continued

molecules, leading to inhibition of gene transcription. In contrast, upon PPAR ligand binding, the heterodimer undergoes conformational changes that promote the dissociation of corepressors and the recruitment of coactivators, inducing the subsequent activation of gene transcription. Activation of PPARs may also result in the repression of gene expression through indirect regulatory mechanisms. The role of PPARs and protective properties of PPAR agonists have been examined in multiple animal models of sepsis and septic shock [24]. In addition, PPARα

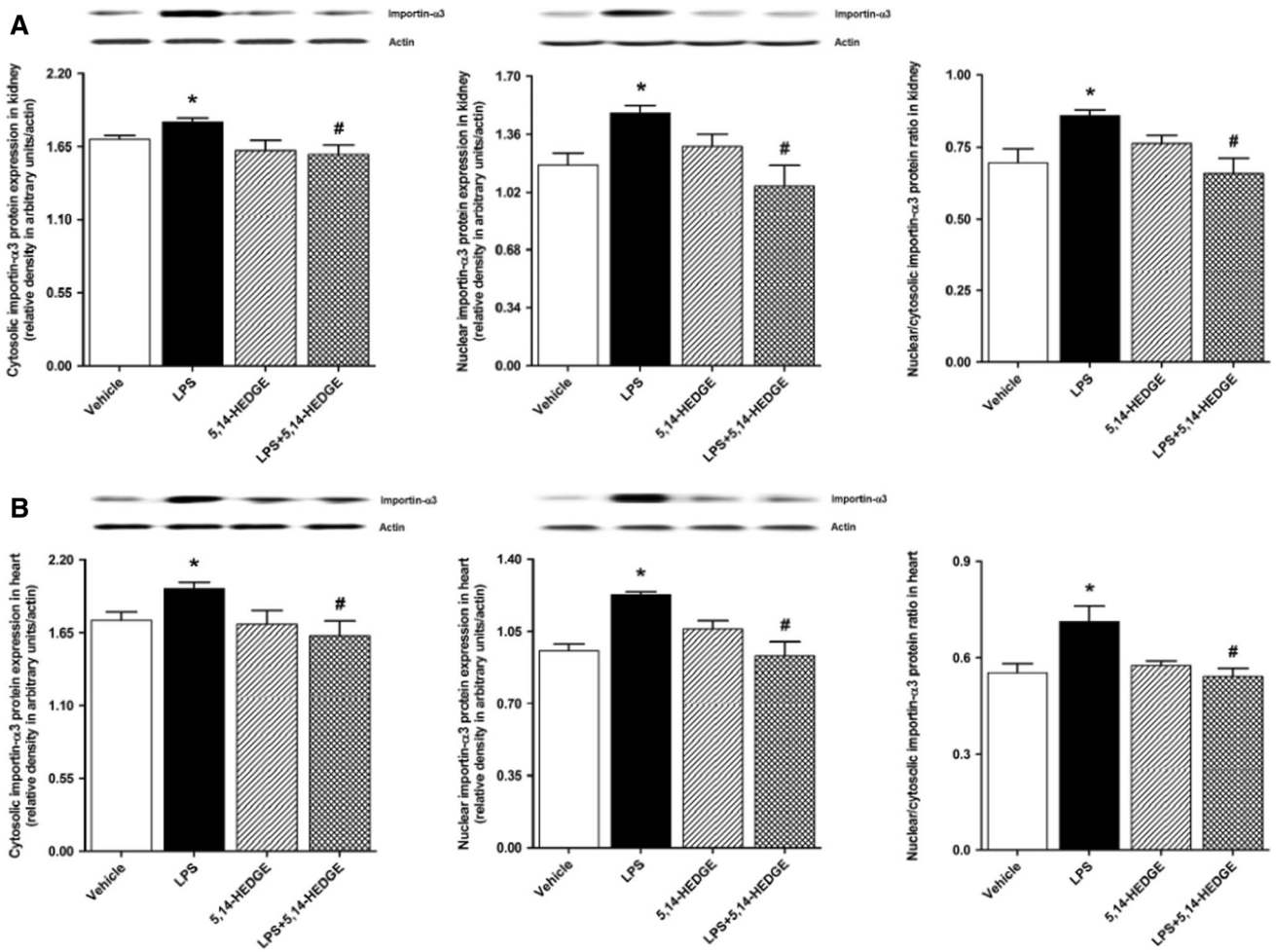

Fig. 7 Effects of 5,14-HEDGE on changes in cytosolic/nuclear importin-α3 protein expression and calculated ratios of nuclear/cytosolic importin-α3 protein in the **a** kidney, **b** heart, **c** thoracic aorta (TA), and **d** superior mesenteric artery (SMA) measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) was given 1 h after administration of saline or LPS. Importin-α3 protein expression in tissue homogenates was measured by immunoblotting. Data are expressed as mean ± SEM of four animals. *Asterisk* significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). *Hash* significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$)

and PPARγ have been reported to regulate the expression of some isoforms of CYP4A family having the PPRE in the promotor area of the genes and 20-HETE formation in the rat kidney and liver [51–55]. A previous report also demonstrates that PPARα mediates the inhibitory effect of 20-HETE on COX-2 expression through a negative cross talk between PPARα and the COX-2 promotor in vascular smooth muscle cells [56]. Activation of PPARα/β/γ have also been shown to inhibit inflammatory responses by preventing the activation of nuclear transcription factors such as NF-κB, NFAT, AP-1, and STATs in response to a variety of inflammatory stimuli, including TLR ligands and cytokines [22–26]. Furthermore, PPARα has been shown to be involved in the induction of CYP4A during endotoxemia [57]. Importins (e.g., α, β, and 7) and exportins (e.g., Crm1 and calreticulin) have also been reported to be involved in the nucleocytoplasmic shuttling of PPARα/β/γ [58–60] and AP-1 subunits [61–63]. In addition, MEK1 and ERK1/2 as well as COX-2-derived PGD_2 and 15d-PGJ_2 have been shown to mediate nucleocytoplasmic shuttling of PPARγ [64, 65]. COX-2-derived PGI_2 has also been reported to be involved in nuclear translocation of PPARα [66]. Moreover, it has been demonstrated that the stimulation of RAW264 cells by LPS- or TNF-α causes selective nitration of PPARγ which in turn inhibits its ligand-dependent translocation from the cytosol into the nucleus [67]. It has been also reported that endotoxin increases nuclear translocation of NF-κB and importin-α3 associated with cytosolic IκB-α degradation [68]. Collectively, the results of the present study together with our previous observations [30–34] suggest that 5,14-HEDGE prevents LPS-induced vascular hyporeactivity, hypotension, tachycardia, inflammatory response, and tissue injury in endotoxemic rats and mortality in mice by (1) increasing

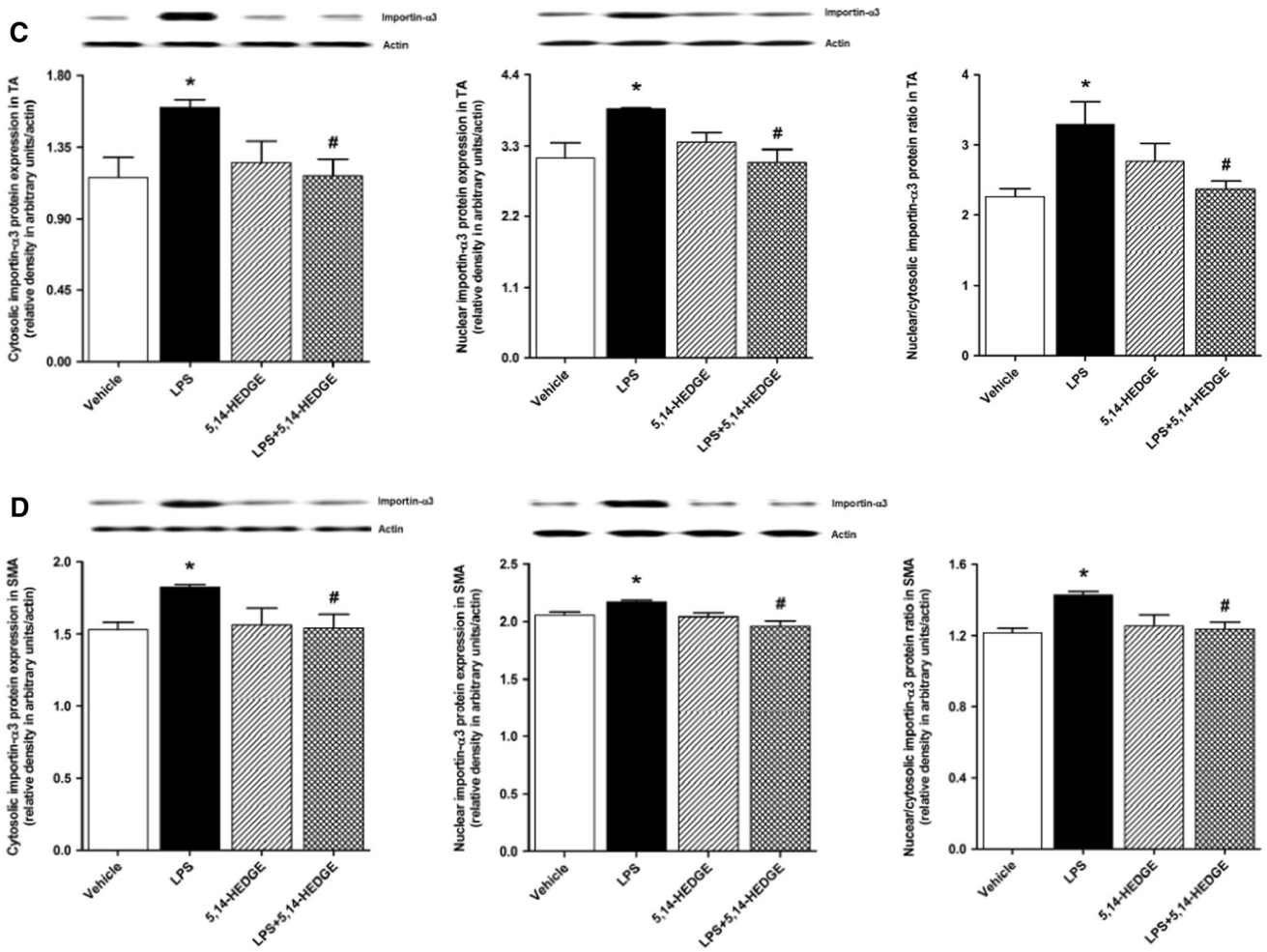

Fig. 7 continued

CYP4A1, CYP2C23, PPARα/β/γ, and RXRα expression together with the production of vasoconstrictor eicosanoids (i.e., 20-HETE) and antiinflammatory mediators (i.e., epoxyeicosatrienoic acids), (2) suppressing both MyD88/TAK1/IKKβ/IκB-α/NF-κB and MyD88/TAK1/MEK1/ERK1/2/AP-1 pathways, in addition to soluble epoxide hydrolase activity associated with importin-α3 expression, (3) inhibiting iNOS, soluble guanylyl cyclase, protein kinase G, COX-2, $gp91^{phox}$ (NOX2, a superoxide generating NOX enzyme), and $p47^{phox}$ (NOXO2, organizer subunit of $gp91^{phox}$) expression/activity associated with formation of superoxide, NO, peroxynitrite, and vasodilator prostanoids (i.e., PGI_2 and PGE_2), (4) reducing production of proinflammatory cytokines (i.e., TNF-α and IL-8), and circulating microribonucleic acids (miRNAs) (i.e., miR-150, miR-223, and miR-297). In the present study, 5,14-HEDGE also prevented the increase in both calculated nuclear/cytosolic unphosphorylated and phosphorylated c-jun protein ratios in addition to importin-α3 protein as well as the decrease in calculated nuclear/cytosolic PPARα, PPARβ, and PPARγ protein ratios in renal and cardiovascular tissues of endotoxemic rats. Therefore, our findings suggest that 5,14-HEDGE prevents nuclear translocation of both unphosphorylated and phosphorylated c-jun via importin-α3 as well as phosphorylation of c-jun in the nucleus through the MyD88/TAK1/MEK1/ERK1/2 pathway in addition to PPARα/β/γ during endotoxemia. It is also likely that 5,14-HEDGE increases CYP4A1 expression and 20-HETE formation by triggering the expression and nuclear translocation of PPARα/β/γ proteins and their interaction with RXRα/PPRE. On the other hand, additional experiments need to be conducted to demonstrate the validity of these hypotheses. Whether the changes in the signaling molecules are caused by an increase in blood pressure by 5,14-HEDGE also remains to be determined. Further characterization of the molecular mechanisms of the effects of 5,14-HEDGE on the molecules (e.g., importin-α3 and Crm1) responsible for nuclear import and export of IκB-α, NF-κB p65, AP-1 subunits c-fos and c-jun, and PPARα/β/γ as well as PPARα/β/γ/RXRα/PPRE interaction will provide the framework for extension of this work into understanding the role of the

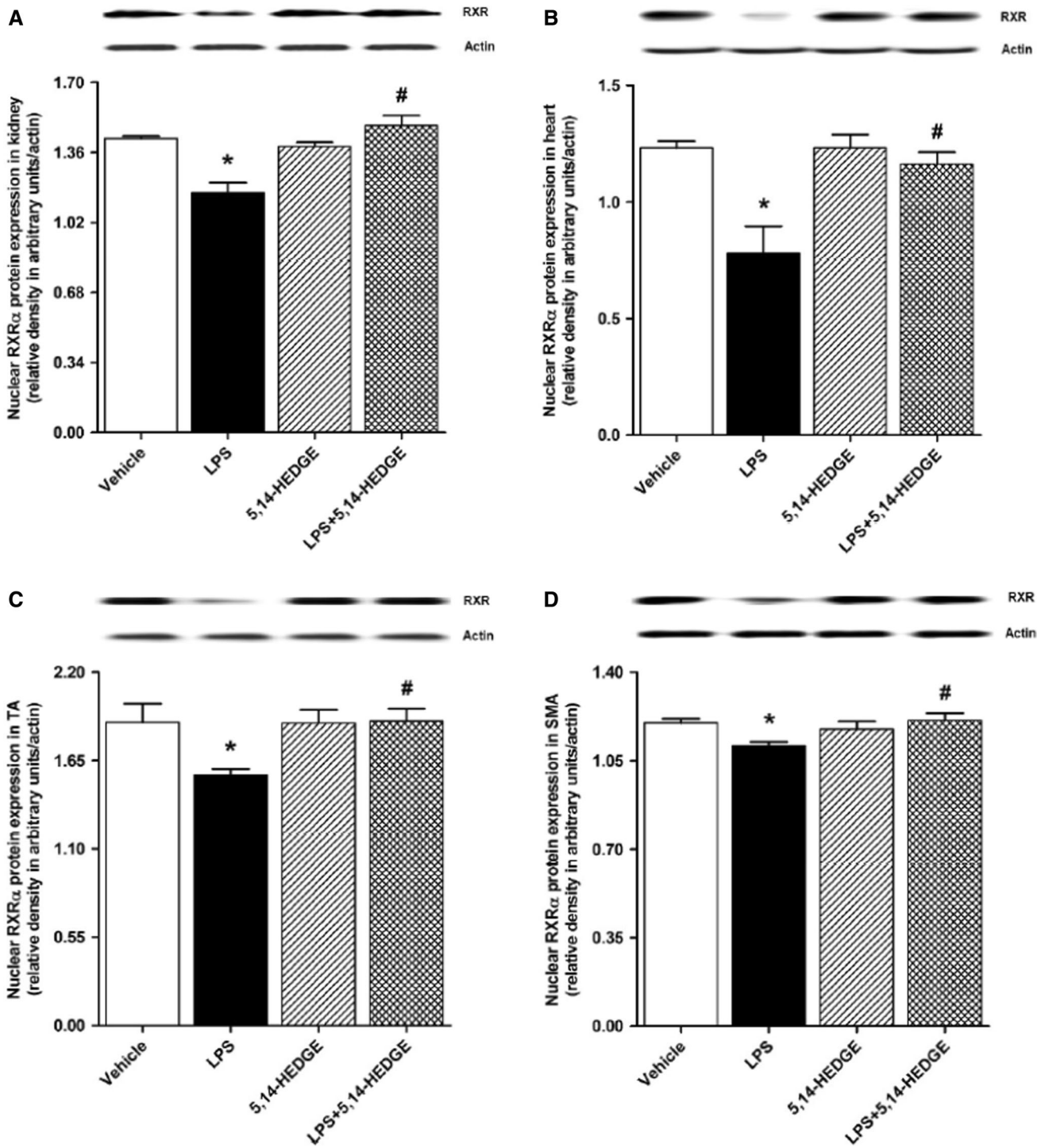

Fig. 8 Effects of 5,14-HEDGE on changes in nuclear RXRα protein expression in the **a** kidney, **b** heart, **c** thoracic aorta (TA), and **d** superior mesenteric artery (SMA) measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) was given 1 h after administration of saline or LPS. RXRα protein expression in tissue homogenates was measured by immunoblotting. Data are expressed as mean ± SEM of four animals. *Asterisk* significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). *Hash* significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$)

MyD88/TAK1/IKKβ/IκB-α/NF-κB and MyD88/TAK1/MEK1/ERK1/2/AP-1 pathways in the protective effect of 5,14-HEDGE against vascular hyporeactivity, hypotension, tachycardia, inflammation, tissue injury, multiple organ failure, and mortality in the rat model of septic shock. Further studies are also required to determine the

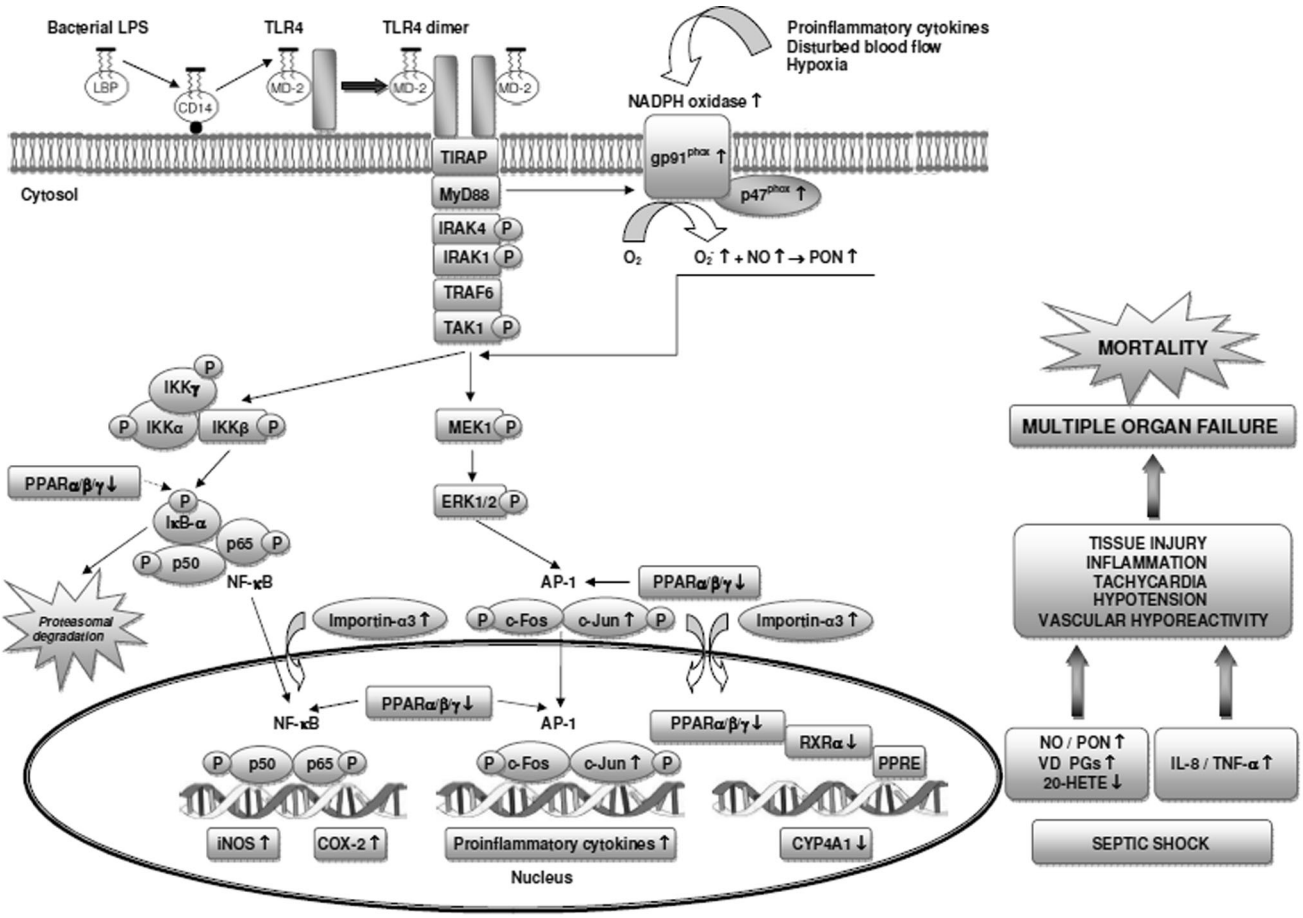

Fig. 9 Schematic diagram showing the involvement of PPARα/β/γ, AP-1, importin-α3, and RXRα that are responsible for changes in production of vasoactive mediators (i.e., NO, PGs, and 20-HETE) by certain enzymes (i.e., iNOS, COX-2, and CYP4A1) and formation of proinflammatory mediators (i.e., IL-8 and TNF-α) in endotoxin-induced vascular hyporeactivity, hypotension, tachycardia, inflammation, tissue injury, multiple organ failure, and mortality based on the results of the current study and our previous findings. *Up arrow* increase, *down arrow* decrease

progression of changes in the signaling molecules, especially for PPARα/β/γ, AP-1, importin-α3, and RXRα at different time points.

In conclusion, the results of the present study together with our previous observations [30–34] provides evidence that endotoxin, the lipid A part of LPS which is the most potent microbial mediator of the pathogenesis of sepsis and septic shock, (1) upregulates MyD88/TAK1/IKKβ/IκB-α/NF-κB and MyD88/TAK1/MEK1/ERK1/2/AP-1 pathways, (2) enhances NADPH oxidase activity and peroxynitrite formation, increases expression and activity of transcription factors (i.e., NF-κB p65 and AP-1 subunit c-jun) and their translocation into the nucleus via importin-α3, (3) decreases expression of PPARα/β/γ and RXRα and their nuclear translocation, and (4) causes changes in the production of vasoactive mediators (i.e., NO, PGs, and 20-HETE) by certain enzymes (i.e., iNOS, COX-2, and CYP4A1) and formation of proinflammatory mediators (i.e., IL-8 and TNF-α) leading to vascular hyporeactivity, hypotension, tachycardia, inflammation, tissue injury, multiple organ failure, and mortality in the rodent model of septic shock (Fig. 9). Furthermore, the endotoxin-induced changes are prevented by 5,14-HEDGE, a 20-HETE mimetic. Hence, we speculate that the antihypotensive effect of 5,14-HEDGE is due to its 20-HETE mimetic activity and inhibition of vasodilatory mediator formation as well as antiinflammatory effect due to decreased proinflammatory mediator production. The current management of septic shock relies on immediate treatment with antibiotics and strong supportive care to control hypotension, tachycardia, cardiac output, and tissue oxygenation to maintain organ function. However, the failure of conventional therapy is that the pathophysiology of septic shock is the result of a highly complex set of processes in which the host response becomes dysregulated and causes cellular damage, tissue damage, and ultimately organ failure. In

light of the important role of 20-HETE in the regulation of cardiovascular hemostasis and inflammatory processes, further studies with 20-HETE mimetics in experimental models of endotoxemia could provide a novel approach to treat hypotension, tachycardia, inflammation, and mortality which lead to multiple organ failure and death in septic shock.

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