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# Contribution of iNOS/sGC/PKG pathway, COX-2, CYP4A1, and gp91$^{phox}$ to the protective effect of 5,14-HEDGE, a 20-HETE mimetic, against vasodilation, hypotension, tachycardia, and inflammation in a rat model of septic shock

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ABSTRACT

We have previously demonstrated that a stable synthetic analog of 20-hydroxyeicosatetraenoic acid (20-HETE), *N*-[20-hydroxyeicosa-5(*Z*),14(*Z*)-dienoyl]glycine (5,14-HEDGE), prevents vascular hyporeactivity, hypotension, tachycardia, and inflammation in rats treated with lipopolysaccharide (LPS) and mortality in endotoxemic mice. These changes were attributed to decreased production of inducible nitric oxide (NO) synthase (iNOS)-derived NO, cyclooxygenase (COX)-2-derived vasodilator prostanoids, and proinflammatory mediators associated with increased cytochrome P450 (CYP) 4A1-derived 20-HETE and CYP2C23-dependent antiinflammatory mediator formation. The aim of this study was to determine whether decreased expression and activity of iNOS, soluble guanylyl cyclase (sGC), protein kinase G (PKG), COX-2, gp91$^{phox}$ (NOX2; a superoxide generating NOX enzyme), and peroxynitrite production associated with increased expression of COX-1 and CYP4A1 and 20-HETE formation in renal and cardiovascular tissues of rats contributes to the effect of 5,14-HEDGE to prevent vasodilation, hypotension, tachycardia, and inflammation in response to systemic administration of LPS. Mean arterial pressure fell by 28 mmHg and heart rate rose by 47 beats/min in LPS (10 mg/kg, i.p.)-treated rats. Administration of LPS also increased mRNA and protein expression of iNOS and COX-2 associated with a decrease in COX-1 and CYP4A1 mRNA and protein expression. Increased NOS activity, iNOS-heat shock protein 90 complex formation (an index for iNOS activity), protein expression of phosphorylated vasodilator stimulated phosphoprotein (an index for PKG activity), gp91$^{phox}$, p47$^{phox}$ (NOXO2; organizer subunit of gp91$^{phox}$), and nitrotyrosine (an index for peroxynitrite production) as well as cGMP (an index for sGC activity), 6-keto-PGF$_{1\alpha}$ (a stable metabolite PGI$_2$) and PGE$_2$ levels (indexes for COX activity), and nitrotyrosine levels by LPS were also associated with decreased CYP hydroxylase activity as measured by 20-HETE formation from arachidonic acid in renal microsomes of LPS-treated rats. These effects of LPS, except iNOS mRNA and COX-1 protein expression, were prevented by 5,14-HEDGE (30 mg/kg, s.c.; 1 h after LPS). A competitive antagonist of vasoconstrictor effects of 20-HETE, 20-hydroxyeicosa-6(*Z*),

*Abbreviations:* 20-HEDE, 20-hydroxyeicosa-6(*Z*),15(*Z*)-dienoic acid; AA, arachidonic acid; 20-HETE, 20-hydroxyeicosatetraenoic acid; 5,14-HEDGE, *N*-[20-hydroxyeicosa-5(*Z*),14(*Z*)-dienoyl]glycine; 1,3-PBIT, phenylene-1,3-bis(ethane-2-isothiourea) dihydrobromide; cDNA, complementary deoxyribonucleic acid; cGMP, cyclic guanosine monophosphate; COX, cyclooxygenase; CYP, cyctochrome P450; EET, epoxyeicosatrienoic acid; ELISA, enzyme-linked immunosorbent assay; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; HR, heart rate; hsp, heat shock protein; IKK, IκB kinase; IκB, inhibitor of κB; IL, interleukin; iNOS, inducible nitric oxide synthase; i.p., intraperitoneally; LPS, lipopolysaccharide; MAP, mean arterial pressure; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase 1; mRNA, messenger ribonucleic acid; NF-κB, nuclear factor-κB; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; PG, prostaglandin; PKG, protein kinase G; p-VASP, phosphorylated vasodilator stimulated phosphoprotein; RT-PCR, reverse transcription-polymerase chain reaction; s.c., subcutaneously; sEH, soluble epoxide hydrolase; sGC, soluble guanylyl cyclase; TNF, tumor necrosis factor; VASP, vasodilator stimulated phosphoprotein.

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15(Z)-dienoic acid (30 mg/kg, s.c.; 1 h after LPS) reversed the effects of 5,14-HEDGE, except iNOS and COX-1 mRNA and protein expression as well as expression of CYP4A1 mRNA. These results suggest that increased CYP4A1 expression and 20-HETE formation associated with suppression of iNOS/sGC/PKG pathway, COX-2, and gp91$^{phox}$ participate in the protective effect of 5,14-HEDGE against vasodilation, hypotension, tachycardia, and inflammation in the rat model of septic shock.



## Introduction

The expression of inducible nitric oxide (NO) synthase (iNOS) is enhanced in many tissues in response to mediators released by the lipid A part of lipopolysaccharide (LPS), also known as endotoxin, which is the most potent microbial mediator in the pathogenesis of septic shock [1]. This leads to increased generation of NO, which contributes to a fall in blood pressure, vascular hyporeactivity, multiple organ failure, and high mortality rate that are associated with septic shock [1]. In many models, endotoxin-induced vascular hyporeactivity to vasoconstrictors is associated with an enhanced formation of NO within the blood vessels, involving activation of not only iNOS, but also endothelial NOS (eNOS) [2]. NO is a potent activator of soluble guanylyl cyclase (sGC) and exerts many of its effects by activating sGC, which produces cyclic guanosine monophosphate (cGMP) [3]. NO plays an important role in cGMP-mediated smooth muscle relaxation by activating protein kinase G (PKG) leading to phosphorylation of vasodilator stimulated phosphoprotein (VASP) [4,5]. NO also reacts with superoxide generated by mainly gp91$^{phox}$ (also known as NOX2) in the presence of p47$^{phox}$ (also known as NOXO2; organizer subunit of gp91$^{phox}$) to form peroxynitrite, a powerful oxidant and nitrating molecule, and subsequent reaction of peroxynitrite with proteins results in nitrotyrosine formation [6,7]. *In vivo*, peroxynitrite generation represents a NO-dependent pathogenic mechanism in conditions such as circulatory shock and chronic inflammatory diseases. In addition to NO, increased production of prostanoids by cyclooxygenase (COX)-2 has also been shown to contribute to systemic hypotension and related organ damage and decreased survival in animals and humans with sepsis [1]. Systemic blockade of iNOS or COX-2 opposes the fall in blood pressure in sepsis and septic shock [1]. This is not only due to withdrawal of the vasodilator effects of NO and prostanoids, but also associated with enhanced production of vasoconstrictor mediators including catecholamines, endothelin-1, and 20-hydroxyeicosatetraenoic acid (20-HETE) as well as activation of the renin-angiotensin system and increased sensitivity of baroreceptor reflex mechanisms [1].

20-HETE is an ω-hydroxylation product of arachidonic acid (AA) that is produced by cytochrome P450 (CYP) enzymes, mainly by the CYP4A and CYP4F isoforms in the kidney, heart, liver, brain, lung, and vasculature [1,8–10]. In the vasculature, 20-HETE causes vasoconstriction in several vascular beds, including renal, cerebral, aortic, mesenteric, and coronary arteries [11–15]. Activation of protein kinases, such as mitogen-activated protein kinase (MAPK),

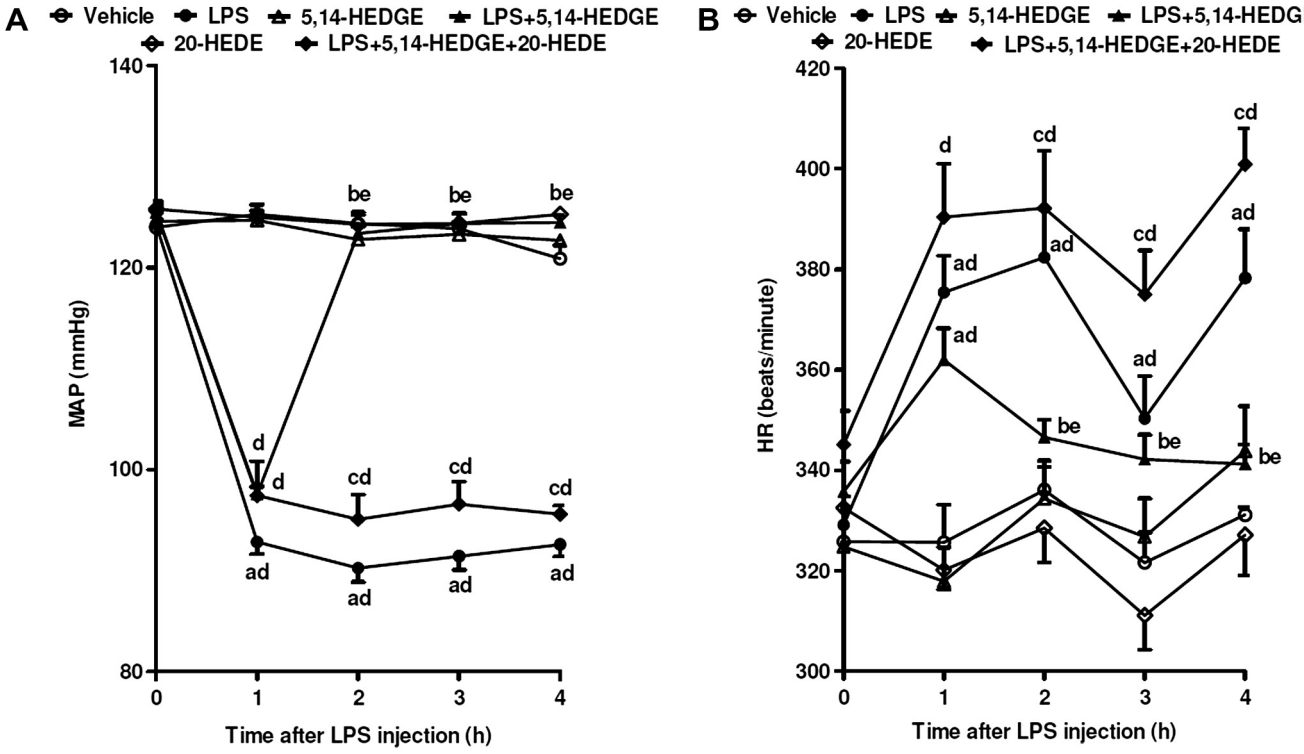


**Fig. 1.** Time course of the effects of 5,14-HEDGE and 20-HEDE on (A) MAP and (B) HR following administration of saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) and/or 20-HEDE (30 mg/kg, s.c.) were given 1 h after administration of saline or LPS. Data are expressed as means ± S.E.M. of 10 animals. [a]Significant difference from the corresponding value seen in rats treated with saline (vehicle) ($p < 0.05$). [b]Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$). [c]Significant difference from the corresponding value seen in the rats treated with LPS and 5,14-HEDGE ($p < 0.05$). [d]Significant difference from the time 0 h value within a group ($p < 0.05$). [e]Significant difference from the time 1 h value within a group ($p < 0.05$).

MAPK kinase (MEK), and extracellular signal-regulated kinase (ERK) which contribute to the regulation of vascular tone, have been shown to mediate the vasoconstrictor effect of 20-HETE [16–18]. As opposed to its vasoconstrictor effect, 20-HETE has also been reported to produce vasodilation in the vasculature including renal and coronary arteries [19–21]. These vasodilatory responses of 20-HETE have been attributed to NO release [22], conversion of 20-HETE to 20-OH-$PGE_2$ and 20-OH-$PGF_{2\alpha}$ by COX [13,19,23], and increased formation of $PGE_2$ [23] and $PGI_2$ [19–21,23]. In addition, 20-HETE has been shown to activate nuclear factor-κB (NF-κB) signaling and induce expression of cellular adhesion molecules and cytokines, thereby promoting inflammation [24,25]. CYP4A- and CYP4F-derived 20-HETE is also involved in LPS-induced acute systemic inflammation as a proinflammatory mediator [26,27]. In addition, it has been reported that NO inhibits renal CYP ω-hydroxylase activity and the production of 20-HETE [28,29]. Moreover, a NO-induced fall in the endogenous production of 20-HETE has also been found to contribute to the cGMP-independent vasodilator effects of NO in renal and cerebral microcirculations [28,30].

Because of the divergent effects of the NO and eicosanoids in the regulation of vascular tone and inflammation, changes in the functional balance between the production of these mediators might contribute to the pathogenesis and progression of inflammatory diseases, such as sepsis and septic shock. Our previous studies with the use of 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, and a competitive antagonist of vasoconstrictor effects

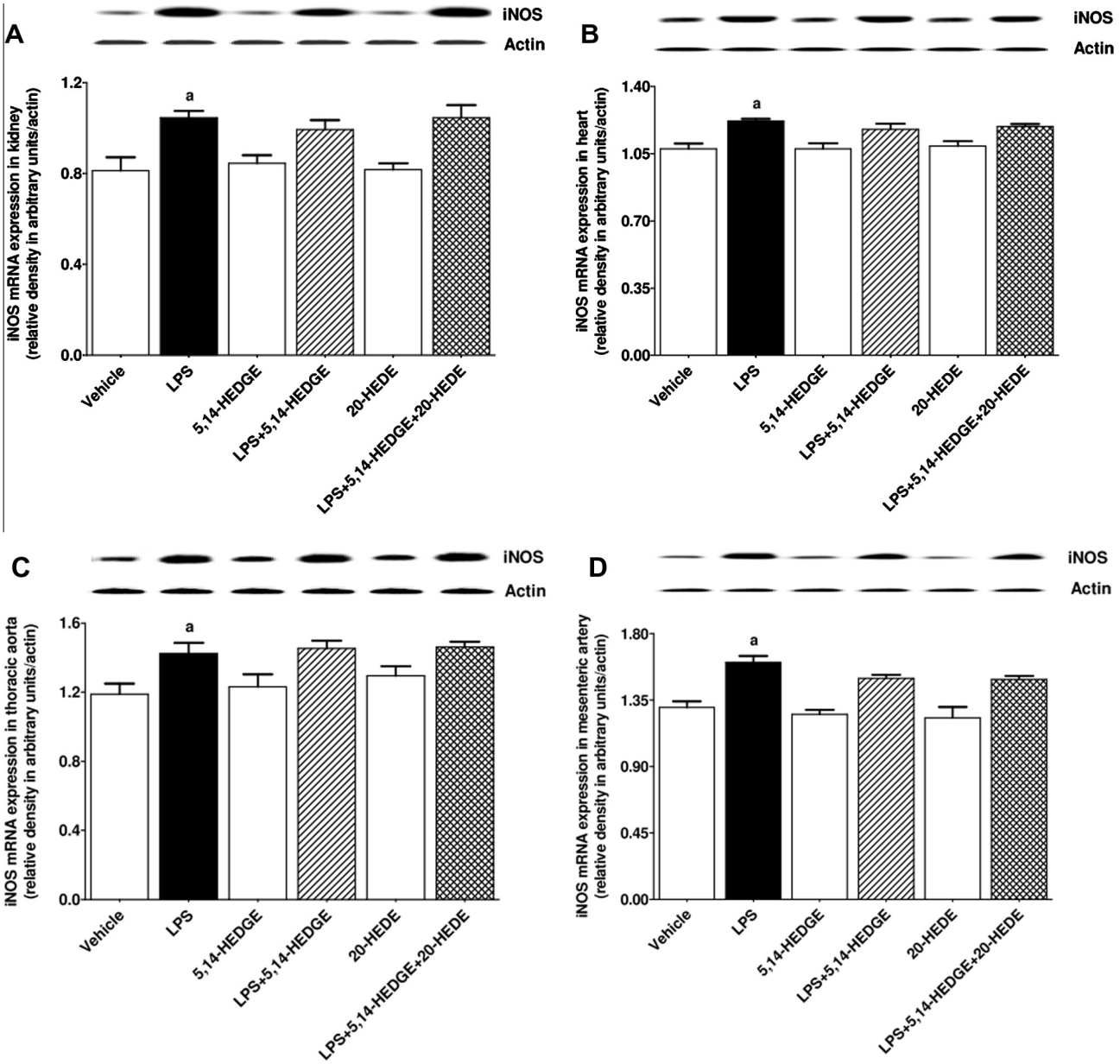


**Fig. 2.** Effects of 5,14-HEDGE and 20-HEDE on changes in iNOS mRNA expression in (A) kidney, (B) heart, (C) thoracic aorta, and (D) superior mesenteric artery measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) and/or 20-HEDE (30 mg/kg, s.c.) were given 1 h after administration of saline or LPS. iNOS mRNA expression in tissue homogenates was measured by RT-PCR. Data are expressed as means ± S.E.M of 4 animals. [a]Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$).

of 20-HETE, 20-hydroxyeicosa-6(*Z*),15(*Z*)-dienoic acid, 20-HEDE, in cardiovascular and/or renal tissues of rats suggested that increased expression and/or activity of soluble epoxide hydrolase (sEH) and MEK1/ERK1/2/IκB kinase (IKK) β/inhibitor of κB (IκB)-α/NF-κB pathway as well as formation of NO and vasodilator prostanoids (i.e., $PGI_2$ and $PGE_2$) by iNOS and COX-2, respectively, peroxynitrite by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, and proinflammatory cytokines (i.e., tumor necrosis factor [TNF]-α and interleukin [IL-8]) associated with decreased CYP2C23 expression and production of anti-inflammatory mediators (i.e., epoxyeicosatrienoic acids; EETs) contributes to hypotension, tachycardia, inflammation, and mortality in a rodent model of septic shock [31–35]. Therefore, the present study was conducted to determine whether decreased expression and activity of iNOS, sGC, PKG, COX-2, $gp91^{phox}$, and $p47^{phox}$, and peroxynitrite production associated with increased expression of COX-1 and CYP4A1 and formation of 20-HETE in renal and cardiovascular tissues of rats contributes to the effect of 5,14-HEDGE to prevent vasodilation, hypotension, tachycardia, and inflammation in LPS-treated rats. The results of this study have been presented in abstract form [33,34].

## Materials and methods

### *Endotoxic shock model*

Experiments were performed on Wistar rats (male; 250–330 g; $n = 72$) (Research Center of Experimental Animals, Mersin

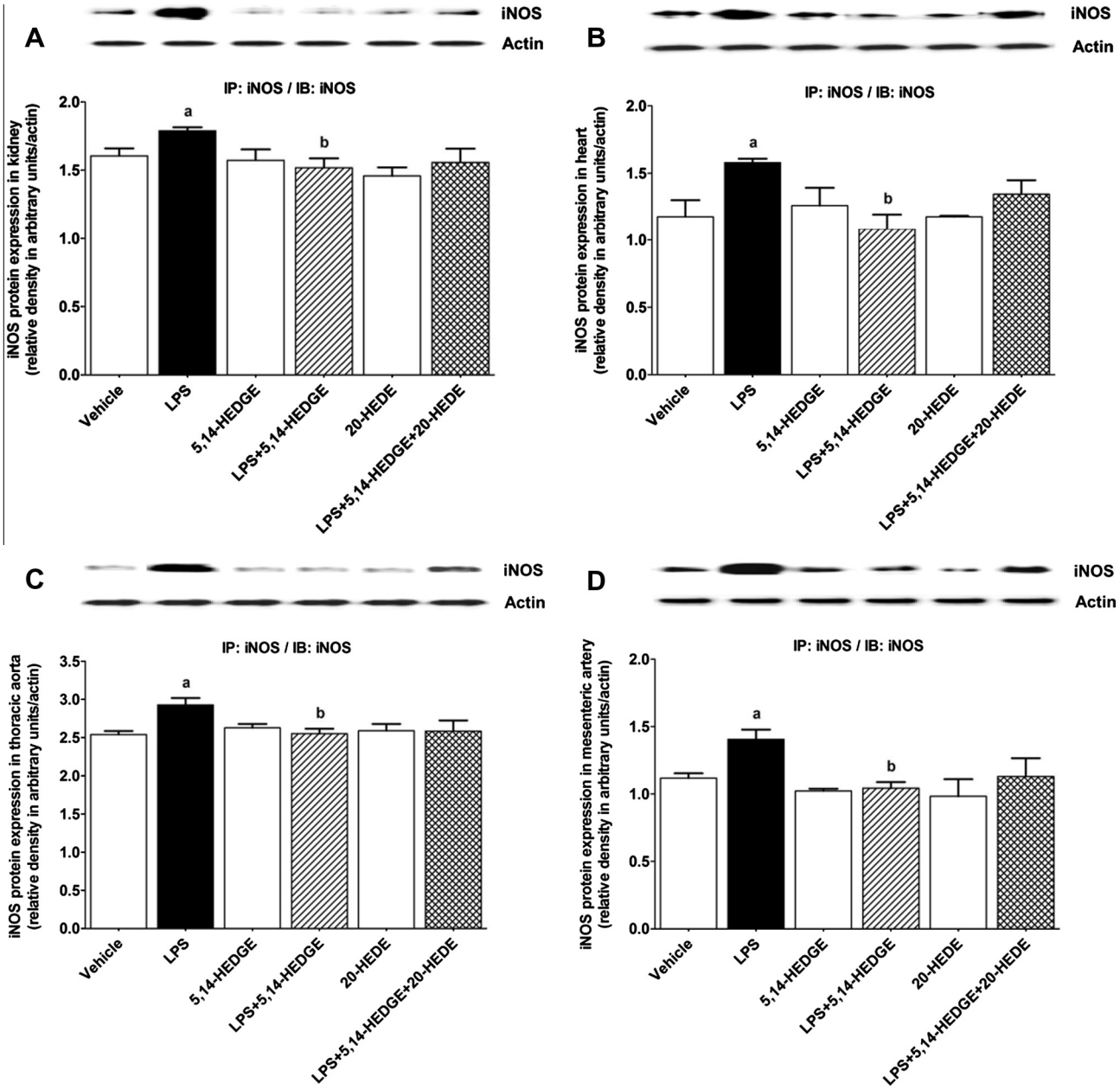


**Fig. 3.** Effects of 5,14-HEDGE and 20-HEDE on changes in iNOS protein expression in (A) kidney, (B) heart, (C) thoracic aorta, and (D) superior mesenteric artery measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) and/or 20-HEDE (30 mg/kg, s.c.) were given 1 h after administration of saline or LPS. Immunoprecipitation was performed with anti-iNOS antibody and immunoprecipitated fractions were incubated with anti-iNOS antibody in immunoblotting studies. Data are expressed as means ± S.E.M of 4 animals. [a]Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). [b]Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$).

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 and mice as previously described by Tunctan et al. [36,37]. Rats were randomly divided into saline ($n = 12$), LPS ($n = 12$), 5,14-HEDGE ($n = 12$), LPS + 5,14-HEDGE ($n = 12$), 20-HEDE ($n = 12$), and LPS + 5,14-HEDGE + 20-HEDE ($n = 12$) groups [32]. In the saline, 5,14-HEDGE, and 20-HEDE groups, animals received saline (4 ml/kg, i.p.) at time 0. Animals in the LPS, LPS + 5, 14-HEDGE, and LPS + 5,14-HEDGE + 20-HEDE groups were treated with LPS (*Escherichia coli* LPS, O111:B4; Sigma Chemical Co., St. Louis, MO, USA) (10 mg/kg, i.p.; sublethal dose) at time 0. In the 5,14-HEDGE, LPS + 5,14-HEDGE, and LPS + 5,14-HEDGE + 20-HEDE groups, animals were treated with a stable synthetic analog of 20-HETE, 5,14-HEDGE (30 mg/kg, s.c.) [32] and/or a competitive antagonist of vasoconstrictor effects of 20-HETE, 20-HEDE (30 mg/kg, s.c.) [32]1 h after injection of saline or LPS, respectively. 5,14-HEDGE and 20-HEDE were synthesized in the Department of Biochemistry University of Texas Southwestern Medical Center, Dallas, Texas, US. Mean arterial pressure (MAP) and heart rate (HR) of the rats were measured using a tail-cuff device (MAY

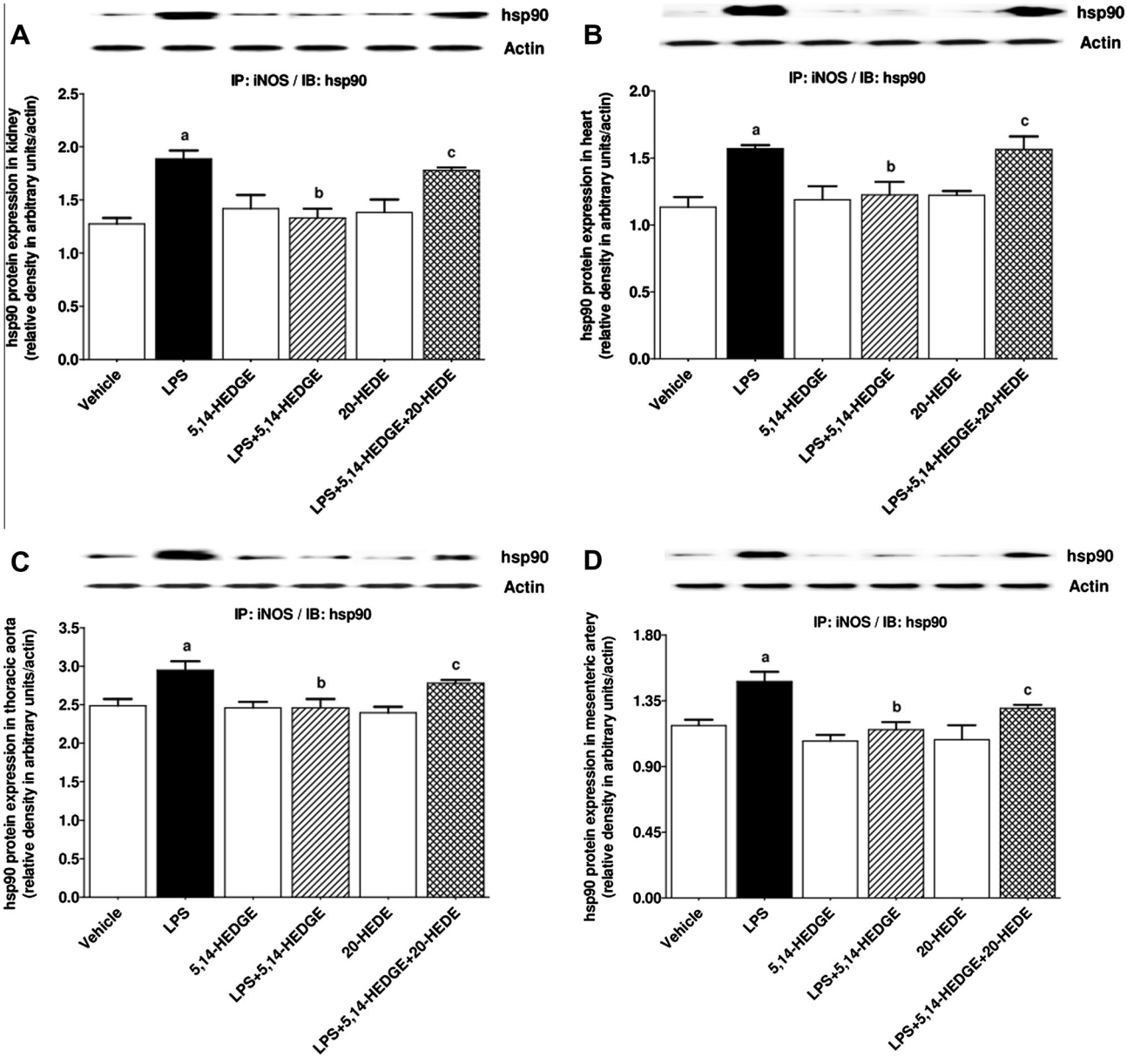


**Fig. 4.** Effects of 5,14-HEDGE and 20-HEDE on changes in iNOS-hsp90 complex formation in (A) kidney, (B) heart, (C) thoracic aorta, and (D) superior mesenteric artery measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) and/or 20-HEDE (30 mg/kg, s.c.) were given 1 h after administration of saline or LPS. Immunoprecipitation was performed with anti-iNOS antibody and immunoprecipitated fractions were incubated with anti-hsp90 antibody in immunoblotting studies. Data are expressed as means ± S.E.M of 4 animals. [a]Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). [b]Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$). [c]Significant difference from the corresponding value seen in the rats treated with LPS and 5,14-HEDGE ($p < 0.05$).

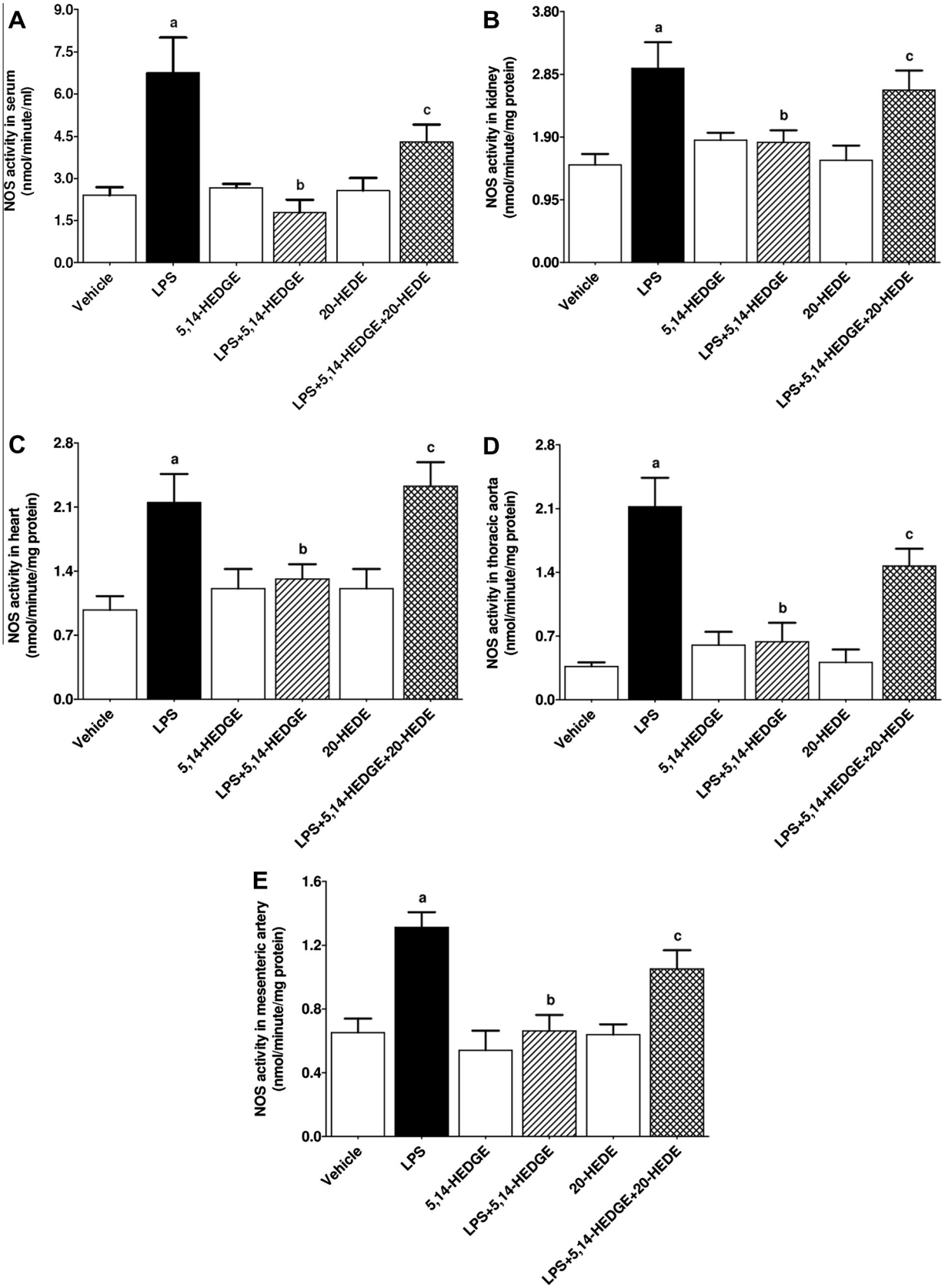


**Fig. 5.** Effects of 5,14-HEDGE and 20-HEDE on changes in NOS activity in (A) serum, (B) kidney, (C) heart, (D) thoracic aorta, (E) superior mesenteric artery measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) and/or 20-HEDE (30 mg/kg, s.c.) were given 1 h after administration of saline or LPS. NOS activity in sera and tissue homogenates was measured by the rate of L-arginine to nitrite/nitrate conversion using the Ultrasensitive Colorimetric Assay for Nitric Oxide Synthase ELISA Kit following the manufacturer's instructions. Data are expressed as means ± S.E.M of 4–6 animals. [a]Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). [b]Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$). [c]Significant difference from the corresponding value seen in the rats treated with LPS and 5,14-HEDGE ($p < 0.05$).

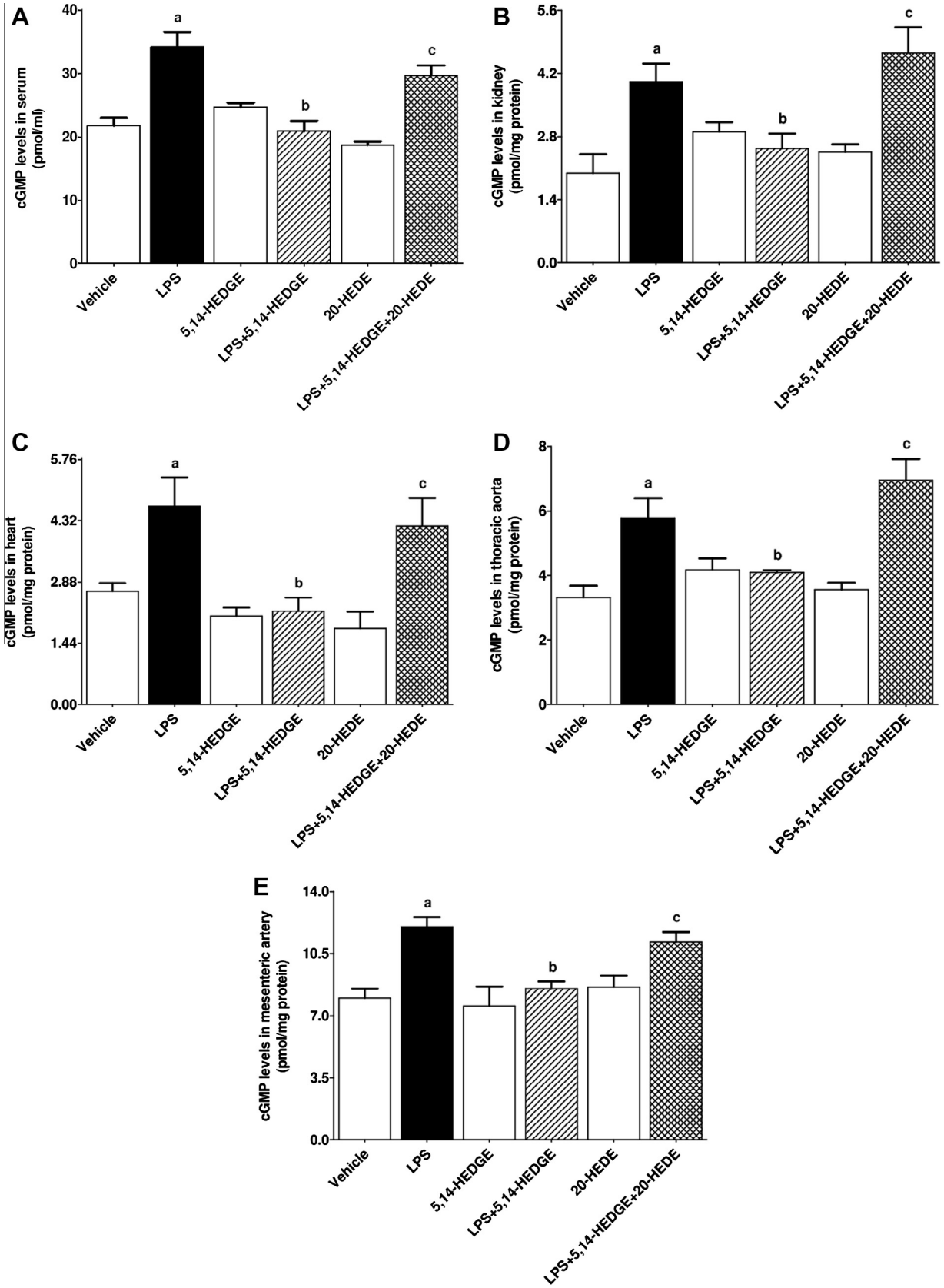


**Fig. 6.** Effects of 5,14-HEDGE and 20-HEDE on changes in cGMP levels in (A) serum, (B) kidney, (C) heart, (D) thoracic aorta, and (E) superior mesenteric artery measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) and/or 20-HEDE (30 mg/kg, s.c.) were given 1 h after administration of saline or LPS. cGMP levels in sera and tissue homogenates were measured by the Cyclic GMP EIA Kit following the manufacturer's instructions. Data are expressed as means ± S.E.M of 4–6 animals. [a]Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). [b]Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$). [c]Significant difference from the corresponding value seen in the rats treated with LPS and 5,14-HEDGE ($p < 0.05$).

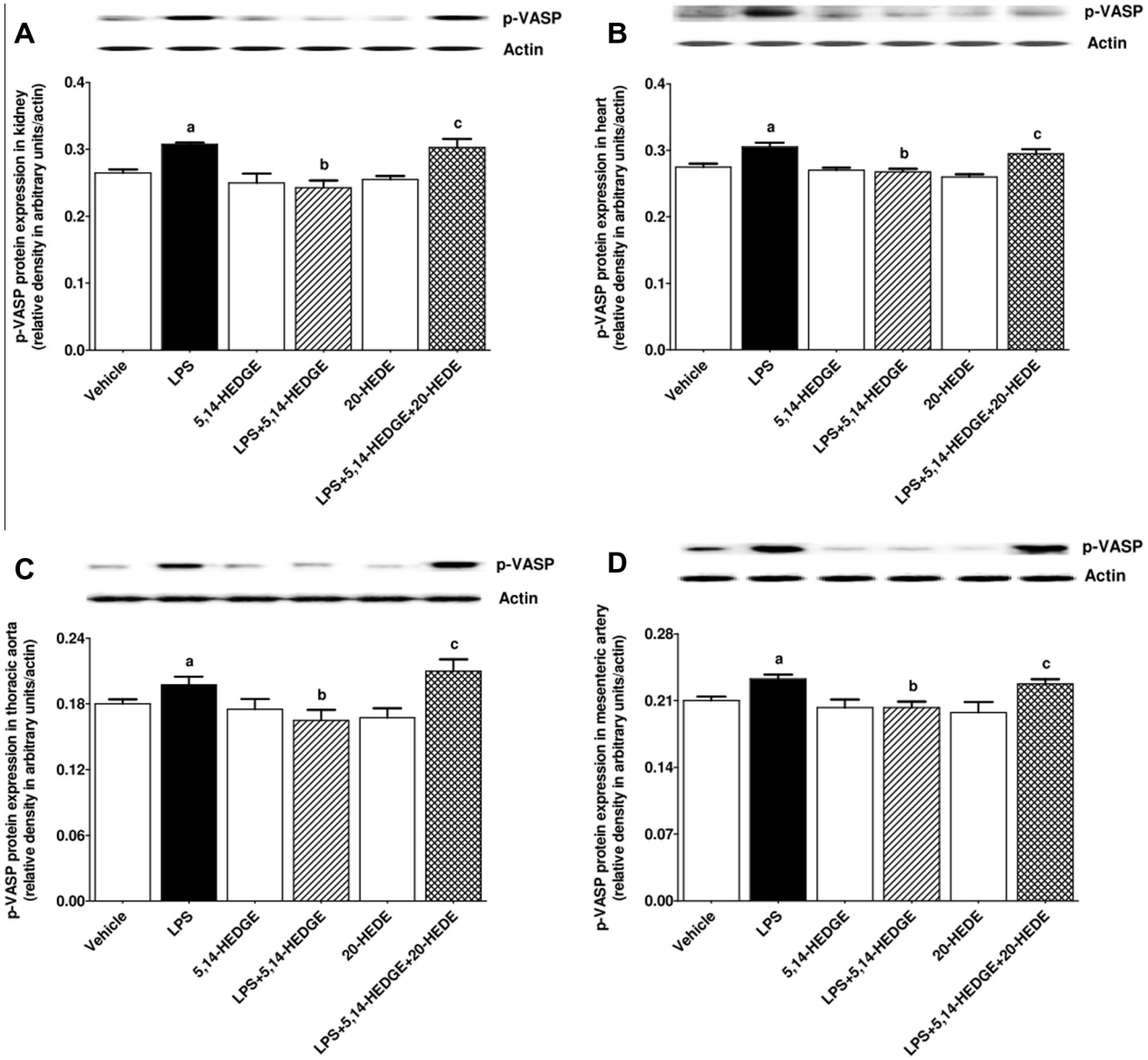


**Fig. 7.** Effects of 5,14-HEDGE and 20-HEDE on changes in p-VASP ($Ser^{239}$) protein expression in (A) kidney, (B) heart, (C) thoracic aorta, and (D) superior mesenteric artery measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) and/or 20-HEDE (30 mg/kg, s.c.) were given 1 h after administration of saline or LPS. p-VASP ($Ser^{239}$) protein levels in tissue homogenates were measured by immunoblotting. Data are expressed as means ± S.E.M of 4 animals. [a]Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). [b]Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$). [c]Significant difference from the corresponding value seen in the rats treated with LPS and 5,14-HEDGE ($p < 0.05$).

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 in the experiments. Rats were euthanized 4 h after the administration of saline or LPS, and blood sample, kidney, heart, thoracic aorta, and superior mesenteric artery were collected from all animals. Detailed method about preparation of serum and tissue samples is reported in the Supplementary material.

*Messenger ribonucleic acid (mRNA) isolation and reverse transcription-polymerase chain reaction (RT-PCR)*

Complementary deoxyribonucleic acids (cDNAs) for iNOS, COX-1, COX-2, CYP4A1, β-actin, α-skeletal actin, and α-smooth muscle actin were synthesized followed by mRNA isolation from the frozen tissue powders as given in detail in the Supplementary material.

*Immunoblotting*

Immunoblotting for iNOS, heat shock protein (hsp90), phosphorylated VASP (p-VASP), COX-1, COX-2, CYP4A1, $gp91^{phox}$, $p47^{phox}$, nitrotyrosine, β-actin, α-sarcomeric actin, and α-smooth muscle actin proteins were performed according to the method reported in the Supplementary material.

*Measurement of NOS, COX, sGC, and CYP hydroxylase activities and nitrotyrosine levels*

NOS, COX, and sGC activities and nitrotyrosine levels in the sera and tissue samples were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions in Ultrasensitive Colorimetric Assay for Nitric Oxide Synthase (Oxford Biomedical Research Inc., Oxford, MI, USA), Cyclic GMP Enzyme Immunoassay Kit (Cayman Chemical, Ann Arbor, MI, USA),

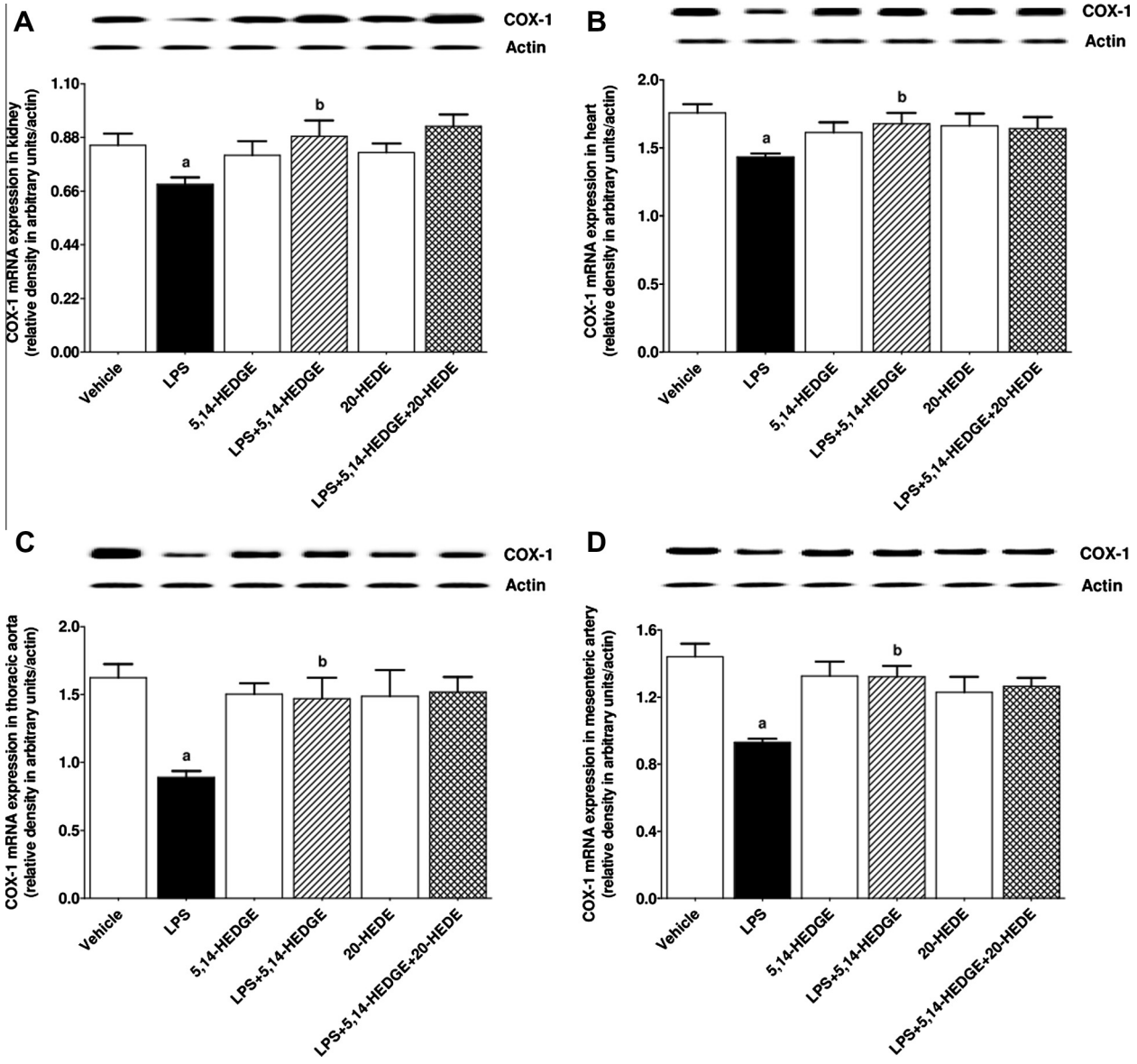


**Fig. 8.** Effects of 5,14-HEDGE and 20-HEDE on changes in COX-1 mRNA expression in (A) kidney, (B) heart, (C) thoracic aorta, and (D) superior mesenteric artery measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) and/or 20-HEDE (30 mg/kg, s.c.) were given 1 h after administration of saline or LPS. COX-1 mRNA expression in tissue homogenates was measured by RT-PCR. Data are expressed as means ± S.E.M of 4 animals. [a]Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). [b]Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$).

6-keto-$PGF_{1\alpha}$ ELISA Kit (Cayman Chemical Co., Ann Arbor, MI, USA), $PGE_2$ ELISA Kit (Cayman Chemical Co., Ann Arbor, MI, USA), and nitrotyrosine ELISA Kit (Northwest Life Science Specialities, LSS, Vancouver, WA, USA), respectively. As an index for CYP hydroxylase activity, the capacity of renal microsomes to produce 20-HETE from ($^{14}$C)-AA was determined as described in the Supplementary material.

*Statistical analysis*

Data are expressed as means ± S.E.M. Data were analyzed by one-way ANOVA followed by Student–Newman–Keuls test for multiple comparisons, Kruskal–Wallis test followed by Dunns 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 28 mmHg (Fig. 1A) and HR rose by 47 bpm (Fig. 1B) 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 (Fig. 1A) and the

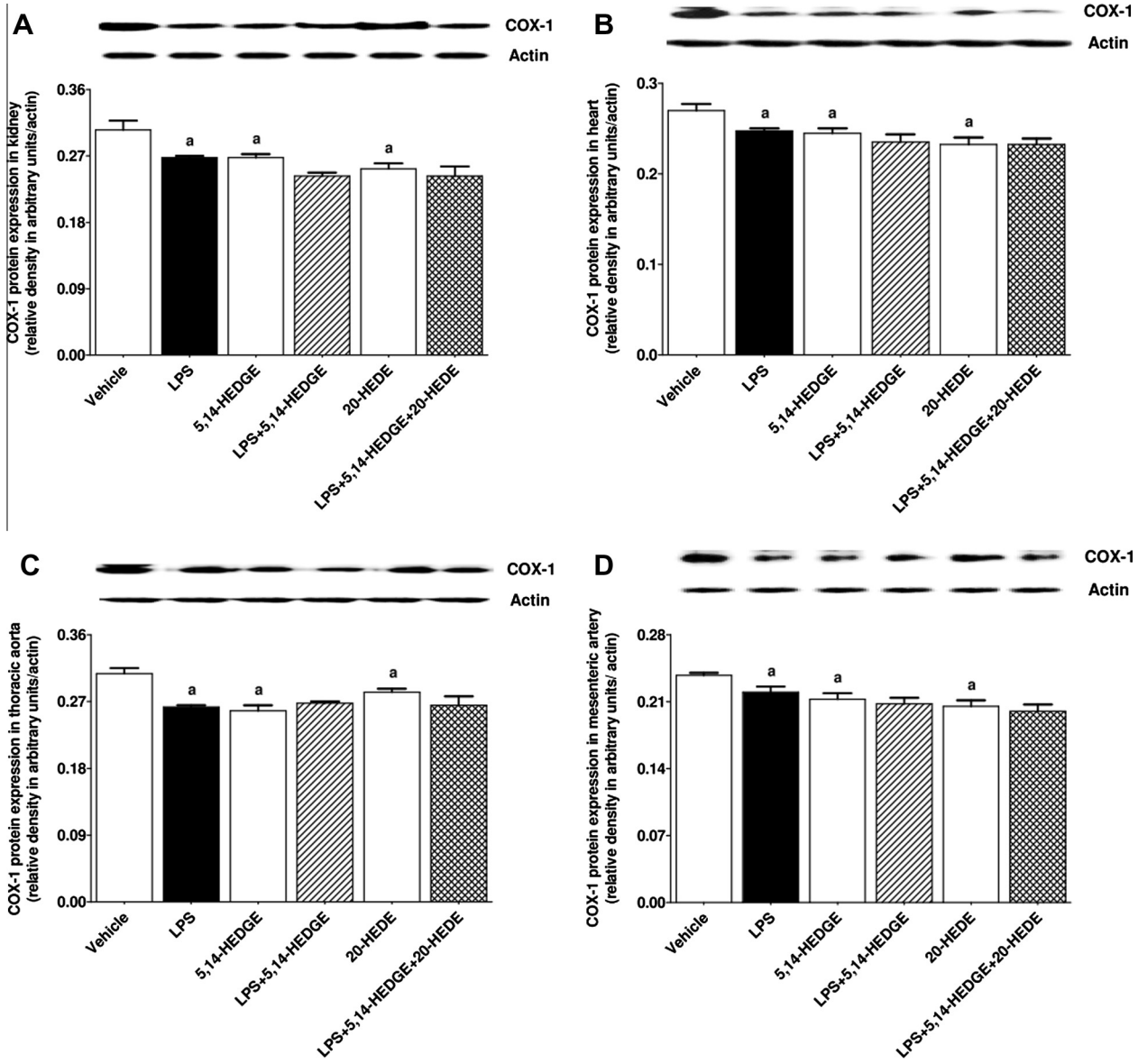


**Fig. 9.** Effects of 5,14-HEDGE and 20-HEDE on changes in COX-1 protein expression in (A) kidney, (B) heart, (C) thoracic aorta, and (D) superior mesenteric artery measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) and/or 20-HEDE (30 mg/kg, s.c.) were given 1 h after administration of saline or LPS. COX-1 protein levels in tissue homogenates were measured by immunoblotting. Data are expressed as means ± S.E.M of 4 animals. [a]Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$).

increase in HR (Fig. 1B) in rats given LPS ($p < 0.05$). A competitive antagonist of the vasoconstrictor effects of 20-HETE, 20-HEDE, reversed the ability of 5,14-HEDGE to oppose the effects of endotoxin on MAP (Fig. 1A) and HR (Fig. 1B) ($p < 0.05$). 5,14-HEDGE or 20-HEDE had no effect on MAP (Fig. 1A) or HR (Fig. 1B) in rats treated with vehicle ($p > 0.05$).

*Effect of 5,14-HEDGE on LPS-induced increase in iNOS expression, iNOS-hsp90 complex formation, and NOS activity*

To determine the effect of 5,14-HEDGE on LPS-induced changes in iNOS expression and activity, expression of iNOS mRNA and protein as well as iNOS-hsp90 complex formation and NOS activity (as indexes for iNOS activity) was measured in the serum, kidney, heart, thoracic aorta, and/or superior mesenteric artery of endotoxemic rats. LPS increased expression of iNOS mRNA (Fig. 2) and protein (Fig. 3) as well as iNOS-hsp90 complex formation (Fig. 4) in the kidney, heart, thoracic aorta, and superior mesenteric artery, and NOS activity in the sera and renal and cardiovascular tissues (Fig. 5) ($p < 0.05$). 5,14-HEDGE prevented the increase in iNOS protein (Fig. 3), but not mRNA (Fig. 2), expression, iNOS-hsp90 complex formation (Fig. 4), and NOS activity (Fig. 5) in the sera and/or tissues of rats caused by LPS ($p < 0.05$). 20-HEDE reversed the effect of 5,14-HEDGE on iNOS-hsp90 complex formation (Fig. 4) and NOS activity (Fig. 5), but not mRNA (Fig. 2) and protein (Fig. 3) expression, in LPS-treated rats ($p < 0.05$). 5,14-HEDGE or 20-HEDE had no effect on the basal iNOS expression (Fig. 3), iNOS-hsp90 complex formation (Fig. 4), and NOS activity (Fig. 5) in vehicle-treated rats ($p > 0.05$).

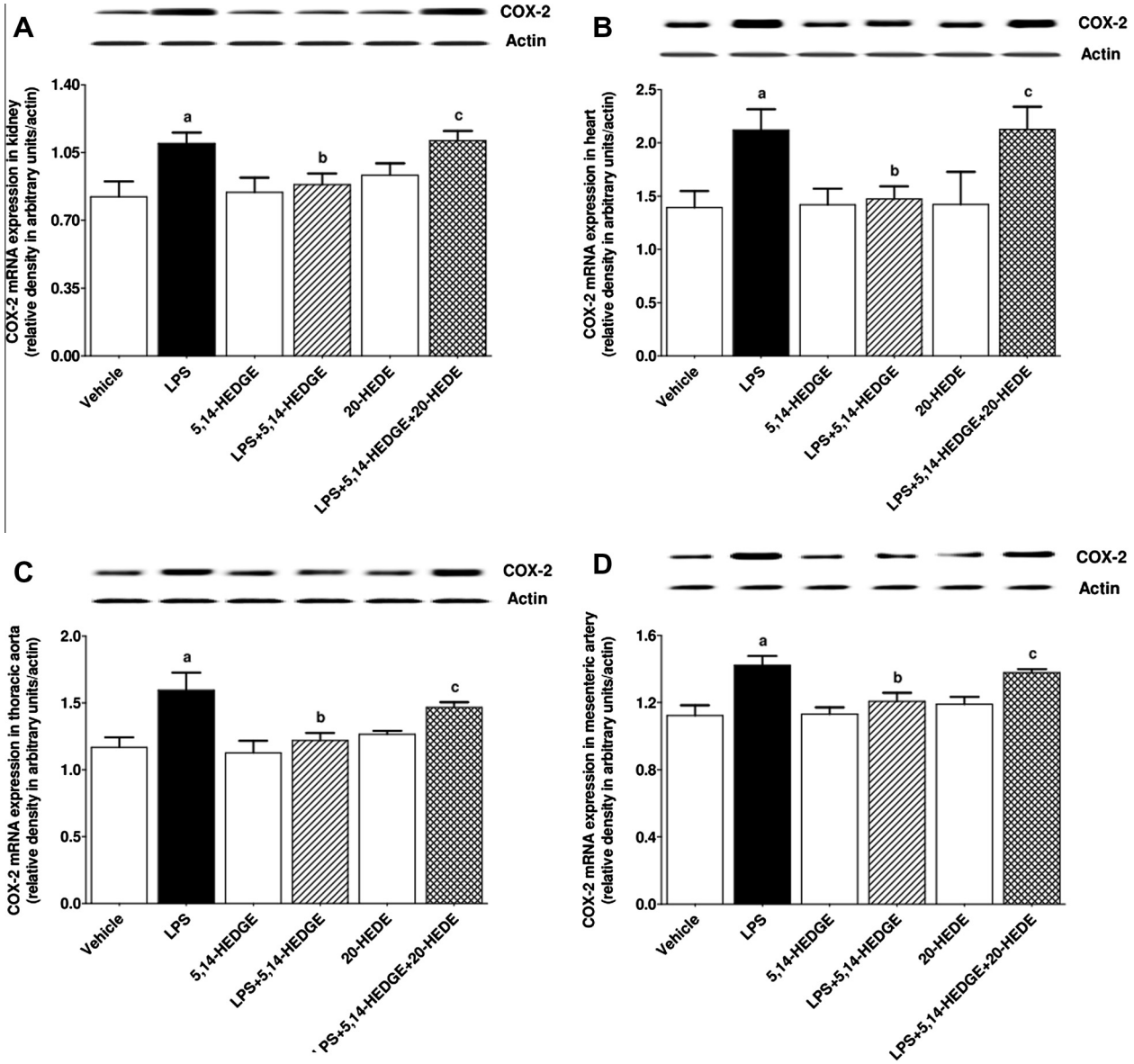


**Fig. 10.** Effects of 5,14-HEDGE and 20-HEDE on changes in COX-2 mRNA expression in (A) kidney, (B) heart, (C) thoracic aorta, and (D) superior mesenteric artery measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) and/or 20-HEDE (30 mg/kg, s.c.) were given 1 h after administration of saline or LPS. COX-2 mRNA expression in tissue homogenates was measured by RT-PCR. Data are expressed as means ± S.E.M of 4 animals. [a]Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). [b]Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$). [c]Significant difference from the corresponding value seen in the rats treated with LPS and 5,14-HEDGE ($p < 0.05$).

*Effect of 5,14-HEDGE on LPS-induced increase in sGC activity*

To determine the effect of 5,14-HEDGE on LPS-induced vascular hyporeactivity, cGMP levels (as an index for sGC activity) were measured in the serum, kidney, heart, thoracic aorta, and superior mesenteric artery of endotoxemic rats. LPS increased cGMP levels in the serum (Fig. 6A), kidney (Fig. 6B), heart (Fig. 6C), thoracic aorta (Fig. 6D), and superior mesenteric artery (Fig. 6E) ($p < 0.05$). The increase in cGMP levels in the sera (Fig. 6A) and tissues (Fig. 6B–E) of rats caused by LPS was prevented by treatment with 5,14-HEDGE ($p < 0.05$). 20-HEDE reversed the effect of 5,14-HEDGE on cGMP levels in LPS-treated rats ($p < 0.05$) (Fig. 6). 5,14-HEDGE or 20-HEDE had no effect on the basal cGMP levels in vehicle-treated rats ($p > 0.05$) (Fig. 6).

*Effect of 5,14-HEDGE on LPS-induced increase in PKG activity*

To determine the effect of 5,14-HEDGE on LPS-induced vascular hyporeactivity, p-VASP protein expression (as an index for PKG activity) was measured in the kidney, heart, thoracic aorta, and superior mesenteric artery of endotoxemic rats. LPS increased p-VASP protein expression in the kidney (Fig. 7A), heart (Fig. 7B), thoracic aorta (Fig. 7C), and superior mesenteric artery (Fig. 7D) ($p < 0.05$); this increase was prevented by 5,14-HEDGE ($p < 0.05$) (Fig. 7). 20-HEDE reversed the effect of 5,14-HEDGE on p-VASP protein expression in LPS-treated rats ($p < 0.05$) (Fig. 7). 5,14-HEDGE or 20-HEDE had no effect on the basal p-VASP protein expression in vehicle-treated rats ($p > 0.05$) (Fig. 7).

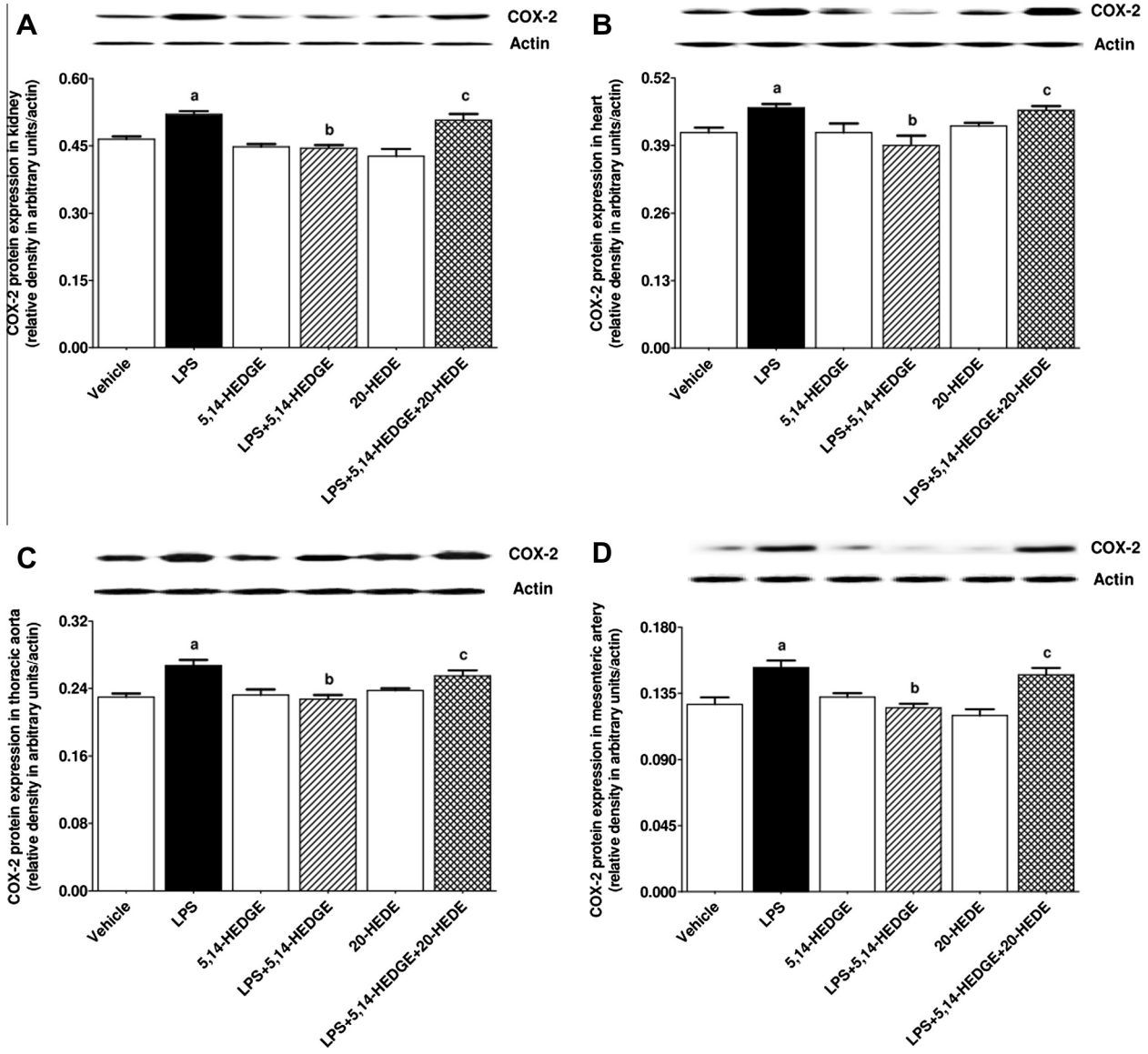


**Fig. 11.** Effects of 5,14-HEDGE and 20-HEDE on changes in COX-2 protein expression in (A) kidney, (B) heart, (C) thoracic aorta, and (D) superior mesenteric artery measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) and/or 20-HEDE (30 mg/kg, s.c.) were given 1 h after administration of saline or LPS. COX-2 protein levels in tissue homogenates were measured by immunoblotting. Data are expressed as means ± S.E.M of 4 animals. [a]Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). [b]Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$). [c]Significant difference from the corresponding value seen in the rats treated with LPS and 5,14-HEDGE ($p < 0.05$).

*Effect of 5,14-HEDGE on LPS-induced changes in COX-1 and COX-2 expression, and COX activity*

To determine the effect of 5,14-HEDGE on LPS-induced changes in COX-1 and COX-2 expression and COX activity, expression of COX-1 and COX-2 mRNA as well as 6-keto-$PGF_{1\alpha}$ and $PGE_2$ levels (as indexes for COX activity) was measured in the serum, kidney, heart, thoracic aorta, and/or superior mesenteric artery of endotoxemic rats. LPS decreased expression of COX-1 mRNA (Fig. 8) and protein (Fig. 9) in the kidney, heart, thoracic aorta, and superior mesenteric artery while expression of COX-2 mRNA (Fig. 10) and protein (Fig. 11) was increased in the renal and cardiovascular tissues ($p < 0.05$). The LPS-induced increase in COX-2 expression (Figs. 10 and 11) was associated with an increase in 6-keto-$PGF_{1\alpha}$ (Fig. 12) and $PGE_2$ (Fig. 13) levels in the kidney, heart, thoracic aorta, and superior mesenteric artey ($p < 0.05$). The decrease in COX-1 mRNA (Fig. 8), but not protein (Fig. 9), expression, the increase in COX-2 expression (Fig. 10, Fig. 11) and prostanoid levels (Figs. 12 and 13) in the tissues of rats produced by LPS was prevented by 5,14-HEDGE ($p < 0.05$). 20-HEDE reversed the effect of 5,14-HEDGE on COX-2 expression (Figs. 10 and 11) and prostanoid levels (Figs. 12 and 13), but not COX-1 expression (Figs. 8 and 9) in LPS-treated rats ($p < 0.05$). 5,14-HEDGE or 20-HEDE had no effect on the basal COX-1 mRNA (Fig. 8) and COX-2 expression (Figs. 10 and 11) ($p > 0.05$), however, COX-1 protein expression (Fig. 9) and prostanoid levels (Figs. 12 and 13) were decreased in the tissues of 5,14-HEDGE- or 20-HEDE-treated control rats ($p < 0.05$).

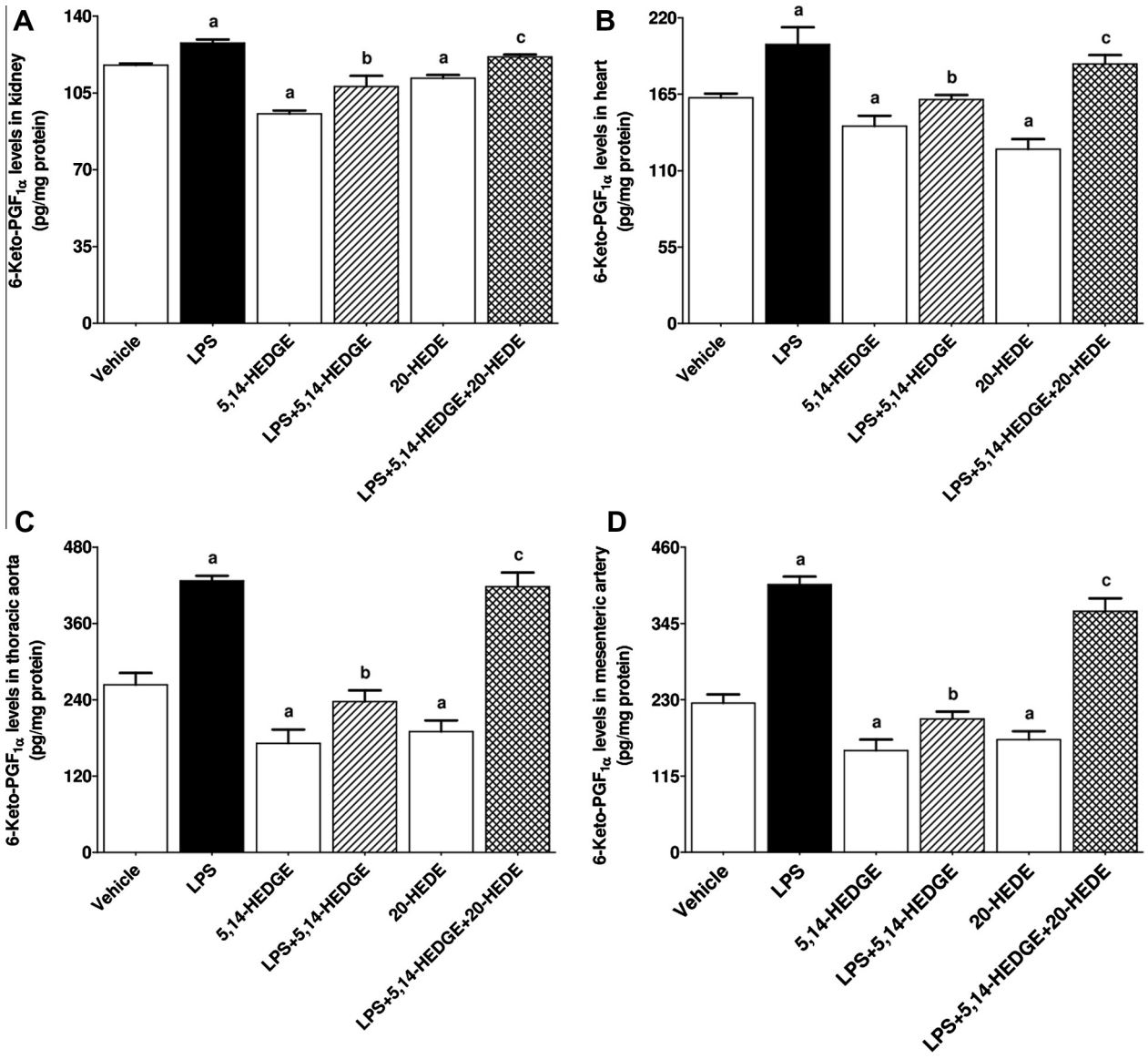


**Fig. 12.** Effects of 5,14-HEDGE and 20-HEDE on changes in 6-keto-$PGF_{1\alpha}$ levels in (A) kidney, (B) heart, (C) thoracic aorta, and (D) superior mesenteric artery measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) and/or 20-HEDE (30 mg/kg, s.c.) were given 1 h after administration of saline or LPS. 6-keto-$PGF_{1\alpha}$ levels in tissue homogenates were measured by the 6-Keto-$PGF_{1\alpha}$ ELISA Kit following the manufacturer's instructions. Data are expressed as means ± S.E.M of 4 animals. [a]Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). [b]Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$). [c]Significant difference from the corresponding value seen in the rats treated with LPS and 5,14-HEDGE ($p < 0.05$).

*Effect of 5,14-HEDGE on LPS-induced decrease in CYP4A1 expression and CYP hydroxylase activity*

To determine the effect of 5,14-HEDGE on LPS-induced decrease in CYP4A1 expression and CYP hydroxylase activity during endotoxemia, expression of CYP4A1 mRNA and protein as well as the capacity of renal microsomes to produce 20-HETE from ($^{14}$C)AA were measured. LPS decreased expression of CYP4A1 mRNA (Fig. 14) and protein (Fig. 15) as well as 20-HETE formation in renal microsomes ($p < 0.05$) (Fig. 16); these effects of LPS were prevented by 5,14-HEDGE ($p < 0.05$) (Figs. 14–16). 20-HEDE reversed the effect of 5,14-HEDGE on CYP4A1 protein (Fig. 15), but not mRNA expression (Fig. 14), and 20-HETE formation in LPS-treated rats ($p < 0.05$). 5,14-HEDGE or 20-HEDE had no effect on the basal CYP4A1 expression (Figs. 14 and 15) and 20-HETE formation (Fig. 16) in vehicle-treated rats ($p > 0.05$).

*Effect of 5,14-HEDGE on LPS-induced increase in gp91$^{phox}$ protein expression*

To determine the effect of 5,14-HEDGE on LPS-induced increase in gp91$^{phox}$ (NOX2; a superoxide producing NOX enzyme), protein expression of gp91$^{phox}$ and p47$^{phox}$ (organizer subunit of gp91$^{phox}$) was measured in the kidney, heart, thoracic aorta, and superior mesenteric artery of endotoxemic rats. LPS increased expression of gp91$^{phox}$ (Fig. 17) and p47$^{phox}$ (Fig. 18) in the kidney, heart, thoracic aorta, and superior mesenteric artery ($p < 0.05$); these increases were prevented by treatment with 5,14-HEDGE ($p < 0.05$) (Figs. 17 and 18). 20-HEDE reversed the effect of 5,14-HEDGE on expression of gp91$^{phox}$ (Fig. 17) and p47$^{phox}$ (Fig. 18) in LPS-treated rats ($p < 0.05$). 5,14-HEDGE or 20-HEDE had no effect on the basal expression of gp91$^{phox}$ and p47$^{phox}$ in vehicle-treated rats ($p > 0.05$) (Figs. 17 and 18).

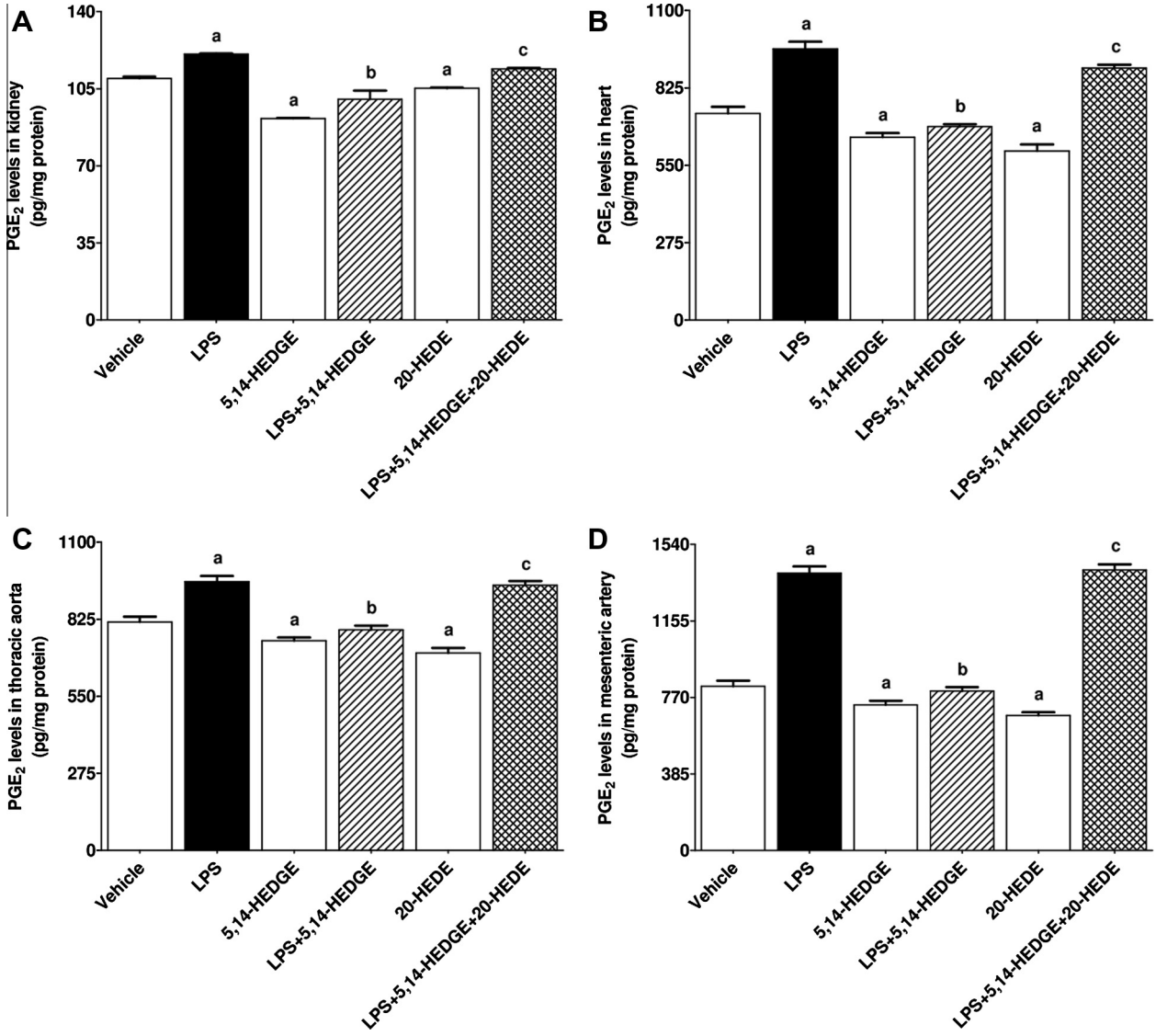


**Fig. 13.** Effects of 5,14-HEDGE and 20-HEDE on changes in $PGE_2$ levels in (A) kidney, (B) heart, (C) thoracic aorta, and (D) superior mesenteric artery measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) and/or 20-HEDE (30 mg/kg, s.c.) were given 1 h after administration of saline or LPS. $PGE_2$ levels in tissue homogenates were measured by the $PGE_2$ ELISA Kit following the manufacturer's instructions. Data are expressed as means ± S.E.M of 4 animals. [a]Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). [b]Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$). [c]Significant difference from the corresponding value seen in the rats treated with LPS and 5,14-HEDGE ($p < 0.05$).

*Effect of 5,14-HEDGE on LPS-induced increase in peroxynitrite formation*

To determine the effect of 5,14-HEDGE on LPS-induced increase in peroxynitrite formation, nitrotyrosine (a stable end-product of peroxynitrite) levels were measured in the serum, kidney, heart, thoracic aorta, and superior mesenteric artery of endotoxemic rats. LPS increased nitrotyrosine protein expression in the kidney (Fig. 19A), heart (Fig. 19B), thoracic aorta (Fig. 19C), and superior mesenteric artery (Fig. 19D) ($p < 0.05$). LPS-induced increase in nitrotyrosine expression was also associated with enhanced nitrotyrosine levels in the serum (Fig. 20A), kidney (Fig. 20B), heart (Fig. 20C), thoracic aorta (Fig. 20D), and superior mesenteric artery (Fig. 20E) ($p < 0.05$). The increase in nitrotyrosine levels in the sera and tissues of rats caused by LPS was prevented by 5,14-HEDGE ($p < 0.05$) (Figs. 19 and 20). 20-HEDE reversed the effect of 5,14-HEDGE on nitrotyrosine levels in LPS-treated rats ($p < 0.05$) (Figs. 19 and 20). 5,14-HEDGE or 20-HEDE had no effect on the basal nitrotyrosine levels in vehicle-treated rats ($p > 0.05$) (Figs. 19 and 20).

## Discussion

The results of the present study indicate that increased expression and/or activity of iNOS/sGC/PKG pathway, COX-2, $gp91^{phox}$, and peroxynitrite production associated with decreased CYP4A1 expression and 20-HETE formation participate in the decrease in vascular reactivity, 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 vascular hiporeactivity, hypotension, tachycardia, and inflammation which may be due to increased CYP4A1 expression and 20-HETE formation associated with decreased expression and/or activity of the iNOS/sGC/PKG

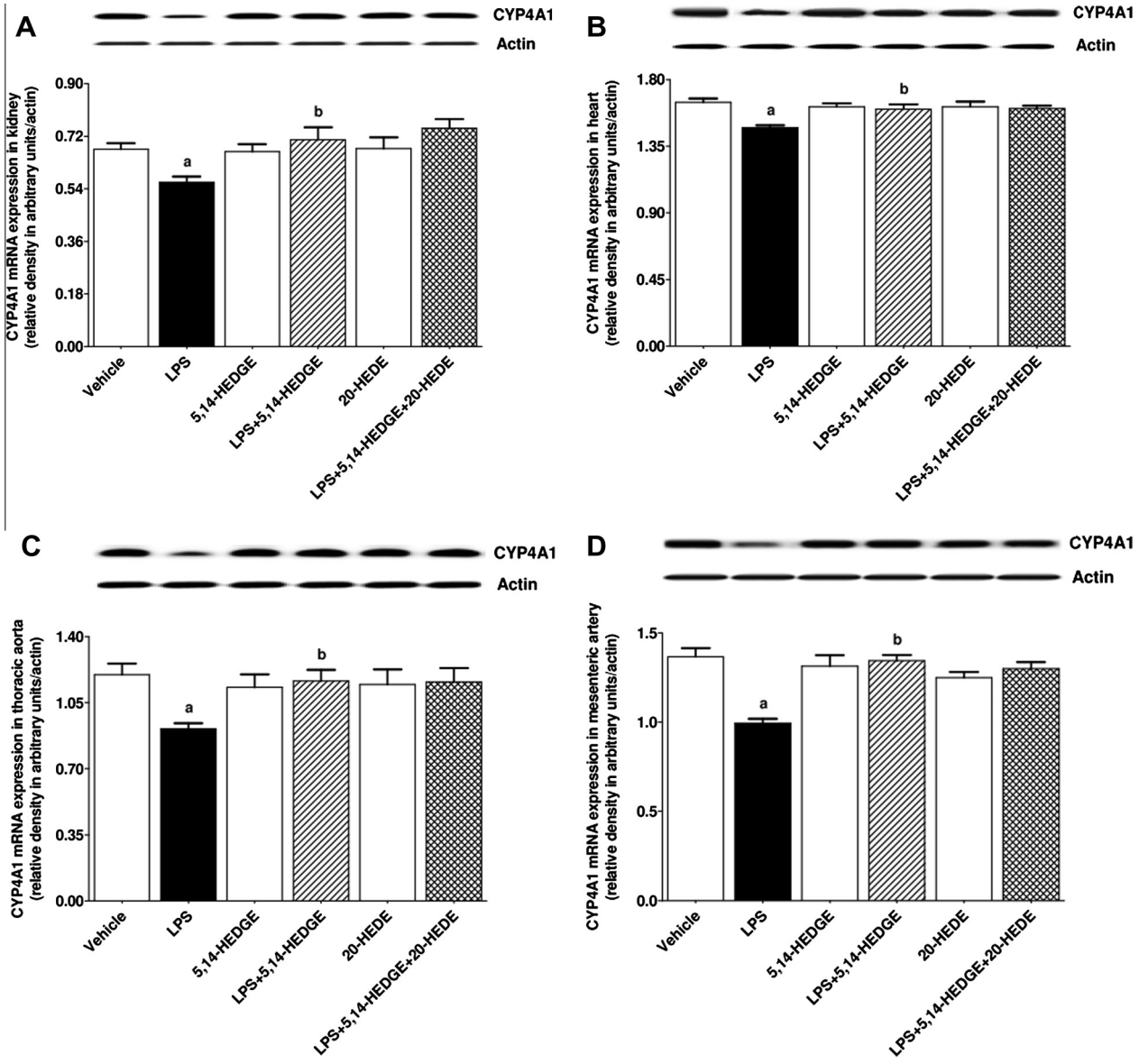


**Fig. 14.** Effects of 5,14-HEDGE and 20-HEDE on changes in CYP4A1 mRNA expression in (A) kidney, (B) heart, (C) thoracic aorta, and (D) superior mesenteric artery measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) and/or 20-HEDE (30 mg/kg, s.c.) were given 1 h after administration of saline or LPS. CYP4A1 mRNA expression in tissue homogenates was measured by RT-PCR. Data are expressed as means ± S.E.M of 4 animals. [a]Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). [b]Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$).

pathway, COX-2, and gp91$^{phox}$ leading to vasodilatory and inflammatory mediator production in the rat model of septic shock.

There are several reports suggesting a direct link between AA metabolites and reactive nitrogen species (e.g., NO and peroxynitrite) under physiological and pathophysiological conditions [1,6,8–10]. The constitutive isoforms of COX and NOS enzymes play an important role in the regulation of several physiological states. On the other hand, under inflammatory conditions such as endotoxic shock, the inducible isoforms of these enzymes as well as the NOX family of reactive oxygen species-generating NADPH oxidases are expressed in a variety of cells and tissues resulting in the production of large amounts of prostanoids and NO. In the present study, we measured changes in the expression and/or activity of iNOS, sGC, PKG, COX-1, COX-2, CYP4A1, and gp91$^{phox}$ (NOX2) and p47$^{phox}$, organizer subunit of gp91$^{phox}$, as well as peroxynitrite formation 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. The effects of LPS on the expression and/or activity of iNOS, sGC, PKG, COX-1, COX-2, CYP4A1, gp91$^{phox}$, and p47$^{phox}$ as well as peroxynitrite formation, except iNOS mRNA and COX-1 protein expression, were also prevented by 5,14-HEDGE given 1 h after LPS. One might argue that vasodilator mediators (e.g., NO and prostanoids) and decreased levels of 20-HETE generated from an increased expression of iNOS and COX-2 following activation of the MEK1/ERK1/2/IKKβ/IκB-α/NF-κB pathway, and decreased expression of CYP4A1, respectively, contribute to the sustained decrease in blood pressure.

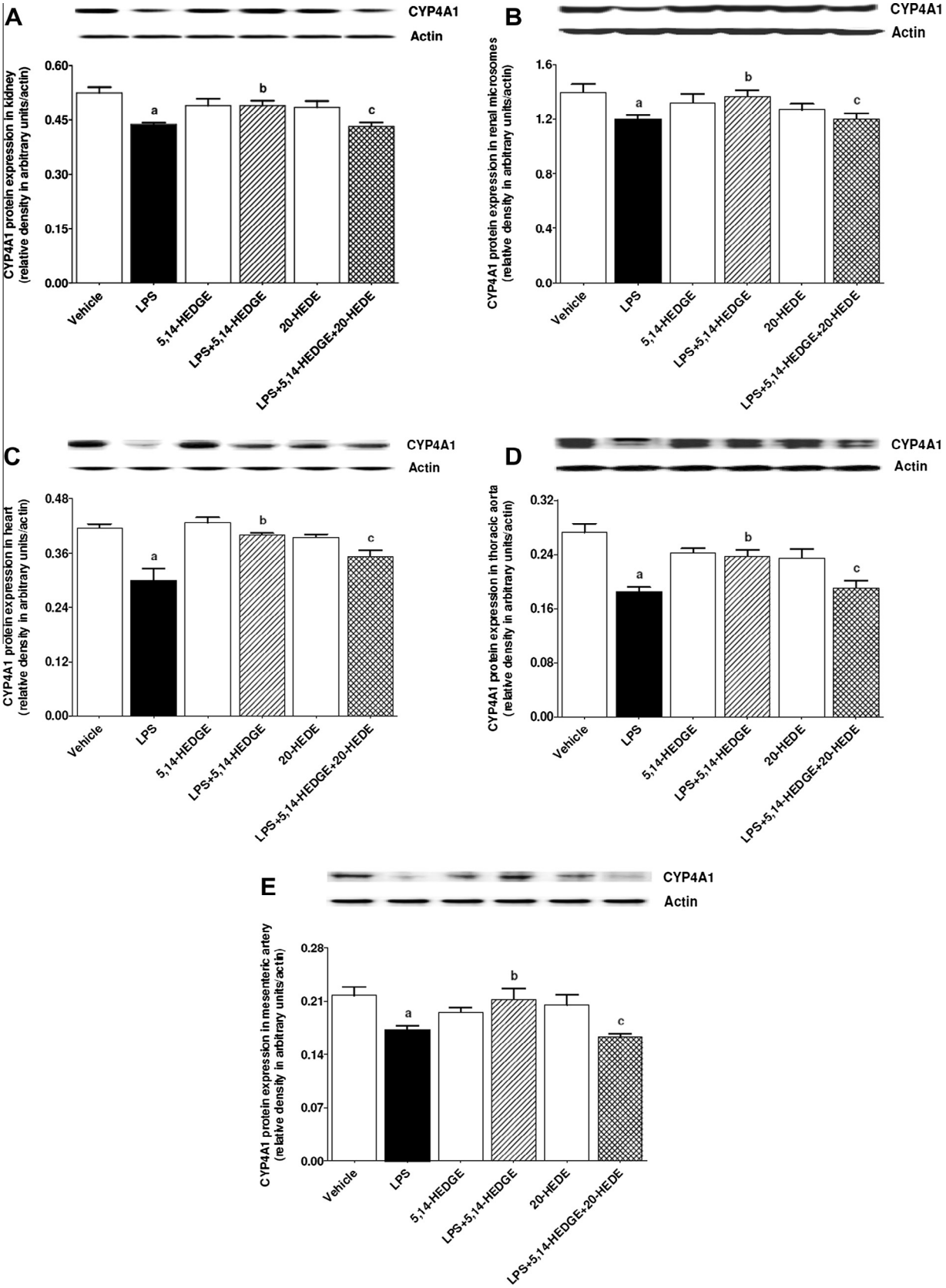


**Fig. 15.** Effects of 5,14-HEDGE and 20-HEDE on changes in CYP4A1 protein expression in (A) kidney, (B) renal microsomes, (C) heart, (D) thoracic aorta, and (E) superior mesenteric artery measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) and/or 20-HEDE (30 mg/kg, s.c.) were given 1 h after administration of saline or LPS. CYP4A1 protein levels in tissue homogenates and renal microsomes were measured by immunoblotting. Data are expressed as means ± S.E.M of 4 animals. [a]Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). [b]Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$). [c]Significant difference from the corresponding value seen in the rats treated with LPS and 5,14-HEDGE ($p < 0.05$).

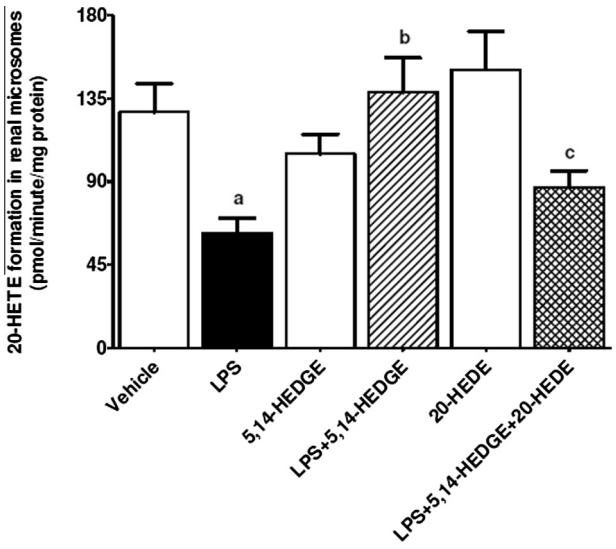


**Fig. 16.** Effects of 5,14-HEDGE and 20-HEDE on changes in 20-HETE formation in renal microsomes measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) and/or 20-HEDE (30 mg/kg, s.c.) were given 1 h after administration of saline or LPS. The capacity of renal microsomes to produce 20-HETE from radiolabeled AA was determined as described in the Supplementary material. Data are expressed as means ± S.E.M of 4 animals. [a]Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). [b]Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$). [c]Significant difference from the corresponding value seen in the rats treated with LPS and 5,14-HEDGE ($p < 0.05$).

It is well established that iNOS and COX-2 expression are induced 1–2 h after LPS administration, and eNOS and COX-1 expression are downregulated [38–40]. It is also known that increased NO production by eNOS is responsible for the fall in blood pressure within 1 h after LPS administration while iNOS-derived NO is responsible for the delayed hypotension and vascular hyporeactivity [40,41]. Compensatory mechanisms, including activation of the renin-angiotensin-aldosteron system, increased sensitivity of baroreceptor reflex mechanisms, and increased production of endothelin-1 and catecholamines, which are known to activate phospholipase $A_2$ and cause release of AA from tissue lipids, and results in prostaglandin synthesis, and also stimulate production of reactive nitrogen (e.g., NO and peroxynitrite) and oxygen (e.g., 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 during the early phase of LPS-induced endotoxemia [38–46]. We have previously demonstrated that selective inhibition of iNOS with phenylene-1,3-bis(ethane-2-isothiourea) dihydrobromide (1,3-PBIT) prevents the fall in MAP, increase in HR, and vascular reactivity to norepinephrine as well as increased sGC and PKG activity associated with systemic and/or aortic NO and nitrotyrosine production 4 h after LPS administration to rats [36,47–49]. Recently, we have reported that an increase in iNOS protein expression is associated with a decrease in eNOS and COX-1 protein expression in the renal, cardiac, and vascular tissues as well as vascular hyporeactivity to norepinephrine in thoracic aorta and superior mesenteric artery 4 h after LPS administration to rats [31,32,50]. These data suggested that overproduction of NO by iNOS associated with an increase in sGC and PKG activity in vascular tissue contributes to the hypotension and vascular hyporeactivity to norepinephrine 4 h after LPS injection to rats. In the present study, administration of LPS to rats caused an increase in mRNA and protein expression of iNOS and COX-2 as well as a decrease in COX-1 and CYP4A1 mRNA and protein expression in the kidney, heart, thoracic aorta, and superior mesenteric artery. Increased NOS activity, iNOS-hsp90 complex formation, protein expression of p-VASP, $gp91^{phox}$, $p47^{phox}$, and nitrotyrosine as well as cGMP, $PGI_2$, $PGE_2$, and nitrotyrosine levels associated with decreased 20-HETE formation were also observed in the renal and cardiovascular tissues of LPS-treated rats. Therefore, the results of the present study, together with above-mentioned observations, suggest that the NO, vasodilator prostanoids, and peroxynitrite could cause a decrease in blood pressure which is followed by increased expression of iNOS, COX-2, and $gp91^{phox}$ and decreased expression of CYP4A and 20-HETE formation, resulting in a sustained fall in blood pressure and tachycardia. Furthermore, a competitive antagonist of vasoconstrictor effects of 20-HETE, 20-HEDE, reversed the effects of 5,14-HEDGE, except iNOS and COX-1 mRNA and protein expression as well as expression of CYP4A1 mRNA. These findings also suggest that 20-HEDE is not only an antagonist of vasoconstrictor effects of 20-HETE, but also have additional effects on the 5,14-HEDGE-induced changes in the expression and/or activity of iNOS, COX-2, CYP4A1, and $gp91^{phox}$ during endotoxemia, for example, it reverses the effects of 5,14-HEDGE on iNOS-hsp90 association and NOS activity without a change in iNOS mRNA and protein expression leading to NO production and activation of sGC/PKG pathway resulting in vascular hyporeactivity and hypotension in LPS-treated rats. In the present study, 5,14-HEDGE and 20-HEDE also caused a decrease in COX-1 protein, but not mRNA, expression as well as serum and tissue levels of 6-keto-$PGF_{1\alpha}$ and $PGE_2$ in vehicle-treated rats. A possible mechanism by which 20-HEDE decreases COX-1 protein expression and systemic and tissue $PGI_2$ and $PGE_2$ production in control rats could be through inhibiting the effects of endogenously produced 20-HETE on COX-1 protein expression and prostanoid production. It is also possible that both 5,14-HEDGE and 20-HEDE might directly inhibit COX-1 protein expression and activity at posttranslational level leading to decreased prostanoid levels. However, additional experiments need to be conducted to demonstrate the validity of this hypothesis.

There are conflicting data regarding the effect of LPS on CYP4A expression and activity. Our previous studies suggested that the endotoxemia-induced increase in NO production primarily via iNOS suppresses renal CYP4A expression and activity, and selective inhibition of iNOS with 1,3-PBIT restores renal CYP4A protein and activity and MAP presumably due to increased production of AA metabolites derived from CYP4A [49,51]. The results of the present study together with our previous findings using 5,14-HEDGE and COX inhibitors [31,33,50,52] demonstrate that LPS causes a decrease in CYP4A1 mRNA and protein expression not only in the kidney, but also in the heart, thoracic aorta, and superior mesenteric artery, together with reduced systemic and tissue levels of 20-HETE. In contrast to our findings, Anwar-Mohamed et al. [26] demonstrated that CYP4A1 mRNA expression was increased in the heart of inflamed animals at 6, 12, and 24 h by 400%, 900%, and 6000%, respectively, after injection of LPS (*E. coli* LPS, O127:B8, 1 mg/kg, i.p.) to Sprague–Dawley rats. Similarly, but with a lower magnitude, renal mRNA expression of CYP4A1 was increased only at 24 h by 100% after injection of LPS (*E. coli* LPS, O127:B8, 1 mg/kg, i.p.) to rats. The LPS-induced changes in the expression of CYP4A1 as well as enhanced expression of TNF-α and IL-6 genes in cardiac and renal tissues were also associated with increased production of 20-HETE in the heart microsomes of inflamed animals by 40% *ex vivo*. The authors suggested that acute inflammation causes alteration in cardiac CYP-mediated AA metabolism in favor of 20-HETE formation. However, Theken et al. [27] reported that 20-HETE formation in the kidney, but not in the heart, decreased 6 or 24 h after injection of LPS (*E. coli* LPS, O111:B4, 1 mg/kg, i.p.) to C57BL/6 mice leading to the conclusion that acute activation of the innate immune response alters CYP expression and eicosanoid metabolism in an isoform-,

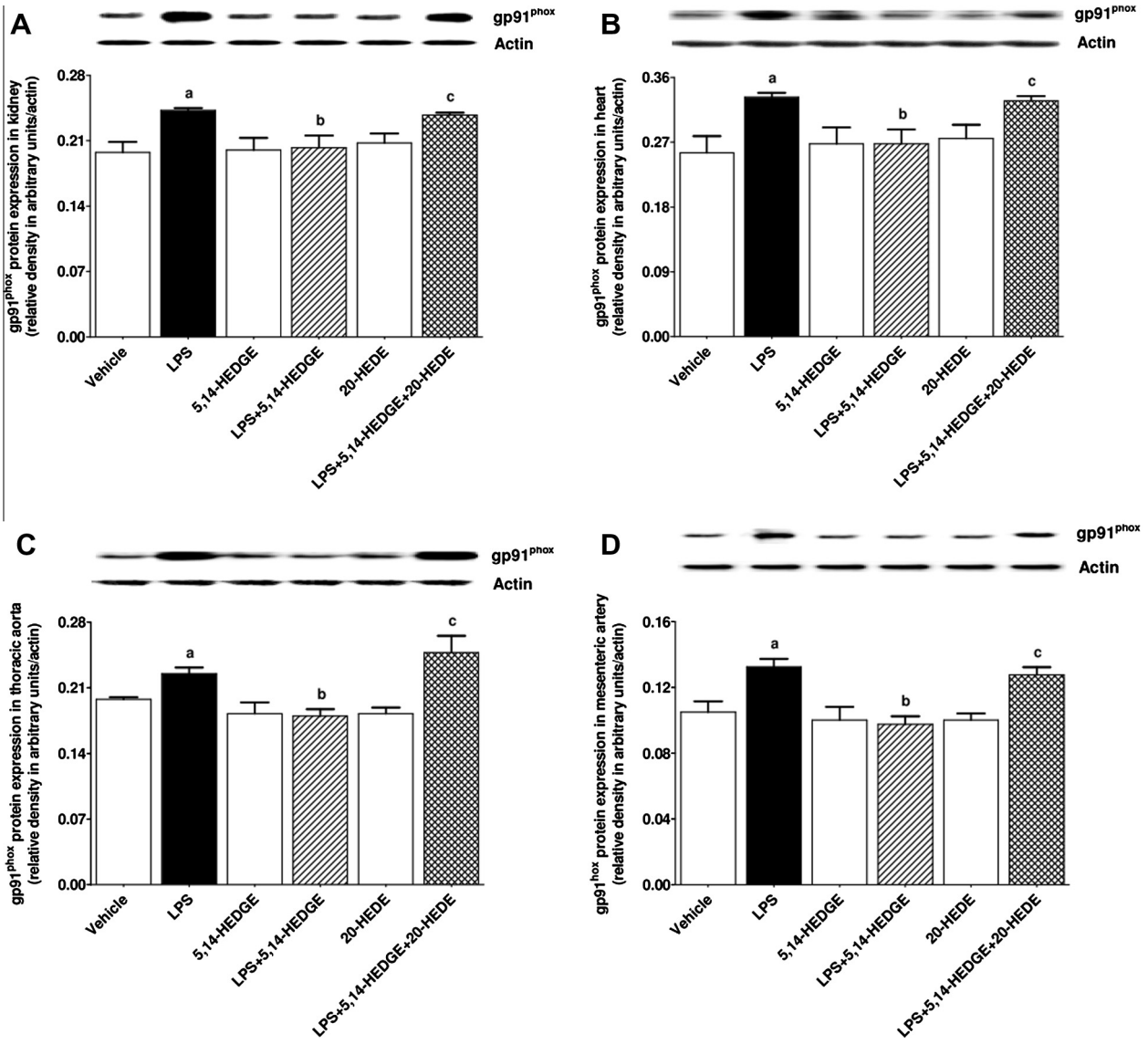


**Fig. 17.** Effects of 5,14-HEDGE and 20-HEDE on changes in $gp91^{phox}$ protein expression in (A) kidney, (B) heart, (C) thoracic aorta, and (D) superior mesenteric artery measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) and/or 20-HEDE (30 mg/kg, s.c.) were given 1 h after administration of saline or LPS. $gp91^{phox}$ protein levels in tissue homogenates were measured by immunoblotting. Data are expressed as means ± S.E.M of 4 animals. [a]Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). [b]Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$). [c]Significant difference from the corresponding value seen in the rats treated with LPS and 5,14-HEDGE ($p < 0.05$).

tissue-, and time-dependent manner. Thus, the contradiction between previously published studies and our results could be attributed to differences in type and strain of animals, dose regimen, strain of LPS, and time points for measurement of enzyme expression and activity which might reflect the differences in the response to LPS treatment.

A chaperone molecule, hsp90, has been identified as a signaling molecule in the activation of all the isoforms of NOS [53]. Hsp90 associates with NOS and facilitates its phosphorylation and, thereby, increases NO production. Vo et al. [54] demonstrated that maximal vascular iNOS expression and its function are achieved via the up-regulation and increased association of eNOS with hsp90, and that in the absence of functional eNOS, the vascular effects of iNOS are delayed during rodent endotoxemia. Yoshida and Xia [55] also reported that hsp90 is an important post-translational modulator of iNOS in iNOS-transfected cells. On the other hand, there are several studies reporting that endotoxin up-regulates iNOS protein, but does not alter basal hsp90 expression in *in vitro* [56] and *in vivo* [57] studies. Recently, Cheng et al. [58] have shown that 20-HETE impairs NO production *in vitro* and its function *in vivo* by inhibiting association of eNOS with hsp90. It has been shown that increased production of 20-HETE in the vasculature is associated with endothelial dysfunction and increased vascular tone which contributes to the development of hypertension in animal models [59]. At least three different pathways have been suggested to play a role in these responses including increased vascular expression of subunits of reduced nicotinamide-adenine dinucleotide phosphate oxidase by 20-HETE, leading to production of superoxide [60], decreased association of eNOS with hsp90 by 20-HETE, leading to diminished formation of NO and increased formation of superoxide [58,61,62], and increased superoxide production directly by 20-HETE in endothelial cells [62]. We have previously demonstrated that 5,14-HEDGE did not prevent the LPS-induced decrease in endothelium-dependent relaxations

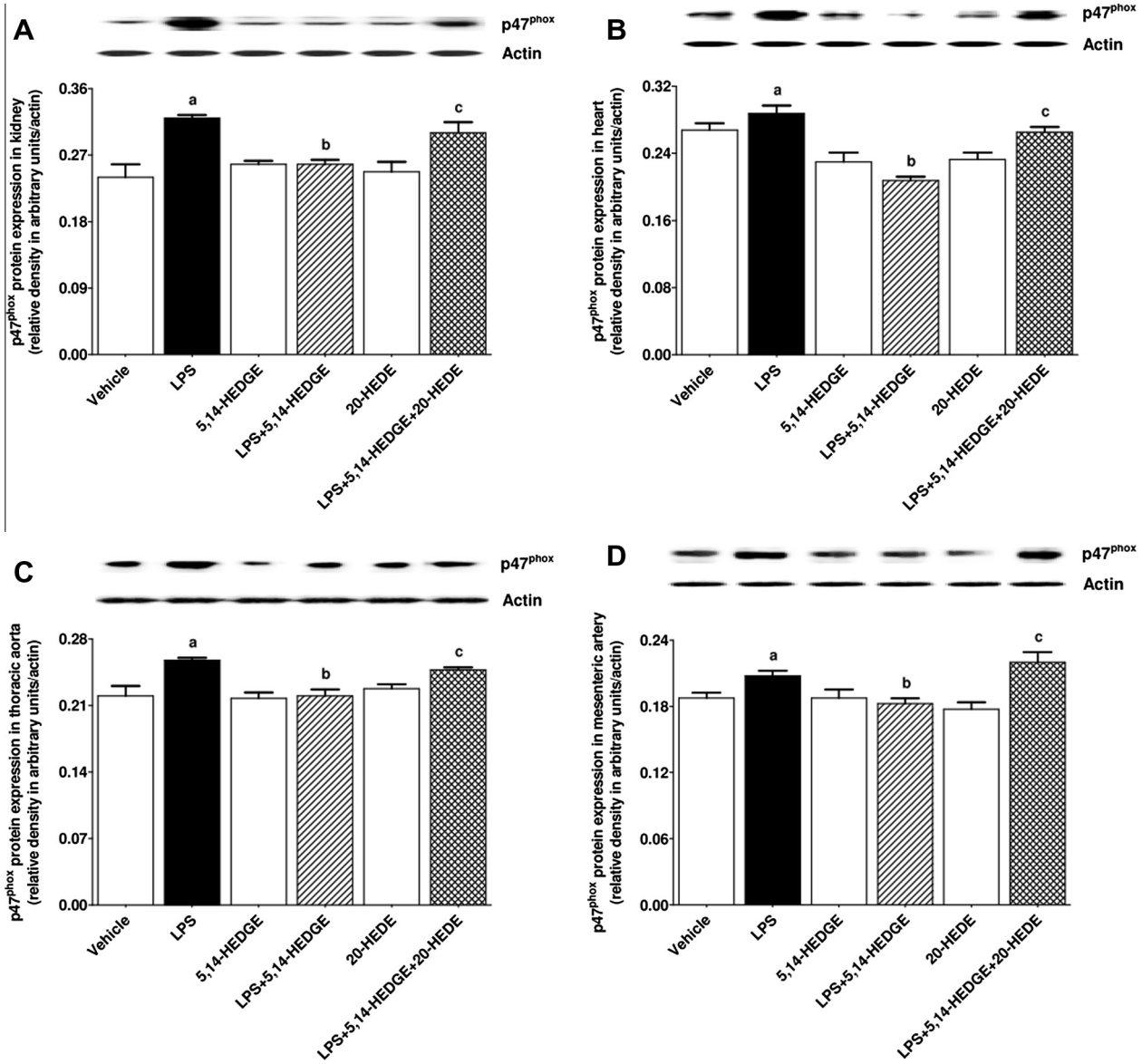


**Fig. 18.** Effects of 5,14-HEDGE and 20-HEDE on changes in $p47^{phox}$ protein expression in (A) kidney, (B) heart, (C) thoracic aorta, and (D) superior mesenteric artery measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) and/or 20-HEDE (30 mg/kg, s.c.) were given 1 h after administration of saline or LPS. $p47^{phox}$ protein levels in tissue homogenates were measured by immunoblotting. Data are expressed as means ± S.E.M of 4 animals. [a]Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). [b]Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$). [c]Significant difference from the corresponding value seen in the rats treated with LPS and 5,14-HEDGE ($p < 0.05$).

induced by acetylcholine in thoracic aorta and superior mesenteric artery, although it reversed the effects of LPS on vascular hyporeactivity to norepinephrine and overproduction of NO in the tissues [32]. More recently, we have demonstrated that the LPS-induced decrease in CYP4A1 protein expression and 20-HETE formation was accompanied with increased iNOS protein expression, decreased expression of eNOS protein, and prostanoid levels without alterations in hsp90 expression in the kidney, heart, thoracic aorta, and/or superior mesenteric artery of endotoxemic rats [31,50]. These effects of LPS, except for eNOS and hsp90 protein expression, were prevented by 5,14-HEDGE. In the present study, 5,14-HEDGE inhibited the iNOS, $gp91^{phox}$, $p47^{phox}$, and nitrotyrosine protein expression, iNOS-hsp90 complex formation, and NOS activity as well as nitrotyrosine levels in the renal and cardiovascular tissues of rats. Therefore, the results of the present study suggest that 20-HETE impairs NO synthesis and its function by inhibiting association of iNOS with hsp90 and superoxide formation.

The results of the present study together with our previous observations [31–37,47–51,63–65] suggest that increased CYP4A1 and CYP2C23 expression together with formation of vasoactive eicosanoids (e.g., 20-HETE) and antiinflammatory mediators (e.g., EETs) associated with suppression of not only MEK1/ERK1/2/IKKβ/IκB-α/NF-κB pathway and production of proinflammatory cytokines (e.g., TNF-α and IL-8), but also iNOS, sGC, PKG, COX-2, $gp91^{phox}$, and superoxide formation participate in the effect of 5,14-HEDGE to prevent LPS-induced vascular hyporeactivity, hypotension, tachycardia, and inflammatory response in endotoxemic rats and mortality in mice. In addition, it seems that transcriptional (e.g., gene expression), translational (e.g., mRNA expression and stability), and posttranslational mechanisms (e.g., protein expression and stability) as well as changes in enzyme activity are involved in the regulation of iNOS, COX-1, COX-2, and CYP4A1 expression by 5,14-HEDGE in the rodent model of septic shock. Although our results demonstrated that

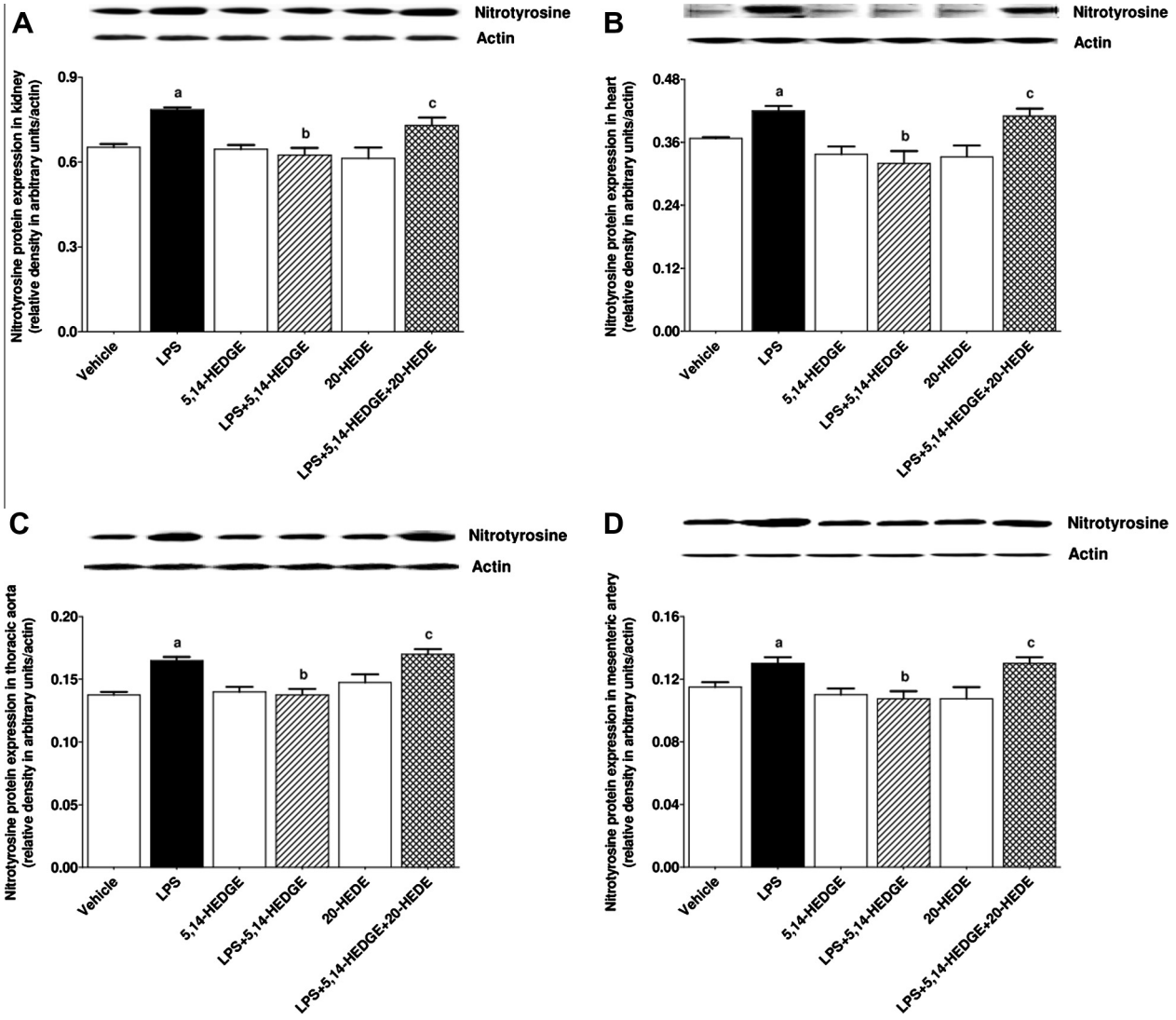


**Fig. 19.** Effects of 5,14-HEDGE and 20-HEDE on changes in nitrotyrosine protein expression in (A) kidney, (B) heart, (C) thoracic aorta, and (D) superior mesenteric artery measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) and/or 20-HEDE (30 mg/kg, s.c.) were given 1 h after administration of saline or LPS. Nitrotyrosine protein levels in tissue homogenates were measured by immunoblotting. Data are expressed as means ± S.E.M of 4 animals. [a]Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). [b]Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$). [c]Significant difference from the corresponding value seen in the rats treated with LPS and 5,14-HEDGE ($p < 0.05$).

5,14-HEDGE directly increases CYP4A1 expression at both transcriptional and posttranscriptional levels and 20-HETE production in endotoxemic rats, it is also possible that 5,14-HEDGE might increase COX-1 and CYP4A1 expression and activity indirectly by inhibiting expression of iNOS, COX-2, $gp91^{phox}$, $p47^{phox}$, and nitrotyrosine, and/or decreasing the effects of NO, superoxide, $PGI_2$, $PGE_2$, 20-HETE, cytokines, and/or transcription factors (e.g., NF-κB) on the expression and activity of COX-1 and CYP4A1. However, additional experiments need to be conducted to demonstrate the validity of the proposed hypothesis. Further characterization of the molecular mechanisms of the effects of 5,14-HEDGE on iNOS, COX-2, CYP4A1, and $gp91^{phox}$ enzymes will provide the framework for extension of this work into understanding the role of AA products in the inflammatory response associated with vascular hyporeactivity, hypotension, tachycardia, inflammation, and mortality during endotoxemia.

In conclusion, the present study provides evidence that endotoxin induces expression and/or activity of iNOS/sGC/PKG pathway, COX-2, and $gp91^{phox}$ associated with decreased CYP4A1 expression and 20-HETE formation during endotoxemia which may result in vascular hyporeactivity, hypotension, tachycardia, inflammation, and mortality in the rodent model of septic shock (Fig. 21). Our findings also suggest that 5,14-HEDGE, a 20-HETE mimetic, prevents vascular hyporeactivity, hypotension, tachycardia, inflammation, and mortality presumably due to increased CYP4A1 expression and 20-HETE formation associated with decreased expression and/or activity of iNOS/sGC/PKG pathway, COX-2, and $gp91^{phox}$ leading to decreased vasodilatory and inflammatory 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 [1]. In the light of the important role of 20-HETE in the regulation of renal and cardiovascular hemostasis as well as inflammatory process, further studies with 20-HETE mimetics in experimental models of endotoxemia could provide a novel approach to treat hypotension,

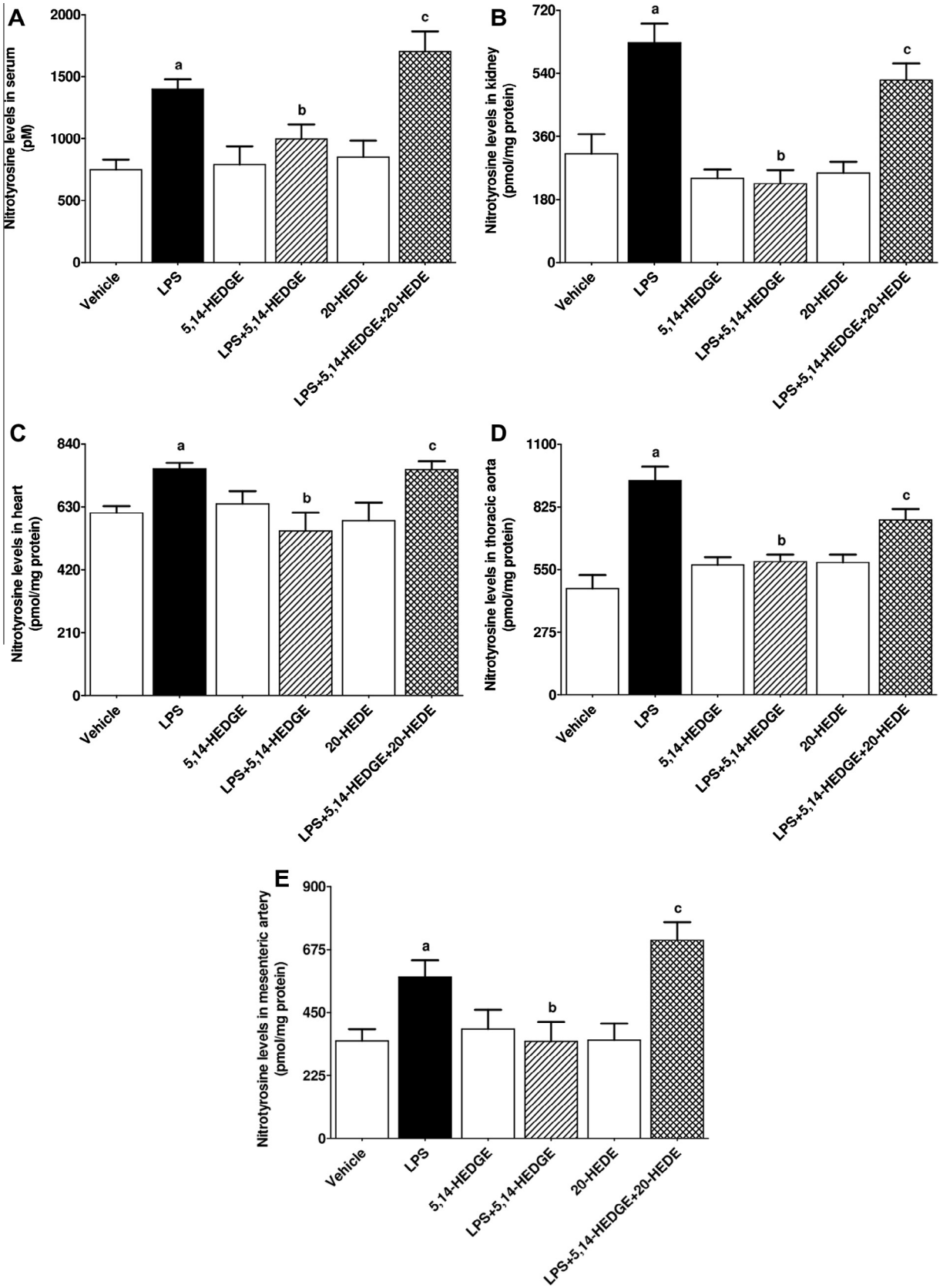


**Fig. 20.** Effects of 5,14-HEDGE and 20-HEDE on changes in nitrotyrosine levels in (A) serum, (B) kidney, (C) heart, (D) thoracic aorta, and (E) superior mesenteric artery measured 4 h after saline (vehicle) (4 ml/kg, i.p.) or LPS (10 mg/kg, i.p.) injection to conscious rats. 5,14-HEDGE (30 mg/kg, s.c.) and/or 20-HEDE (30 mg/kg, s.c.) were given 1 h after administration of saline or LPS. Nitrotyrosine levels in sera and tissue homogenates were measured by the Nitrotyrosine ELISA Kit following the manufacturer's instructions. Data are expressed as means ± S.E.M of 4–8 animals. [a]Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). [b]Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$). [c]Significant difference from the corresponding value seen in the rats treated with LPS and 5,14-HEDGE ($p < 0.05$).

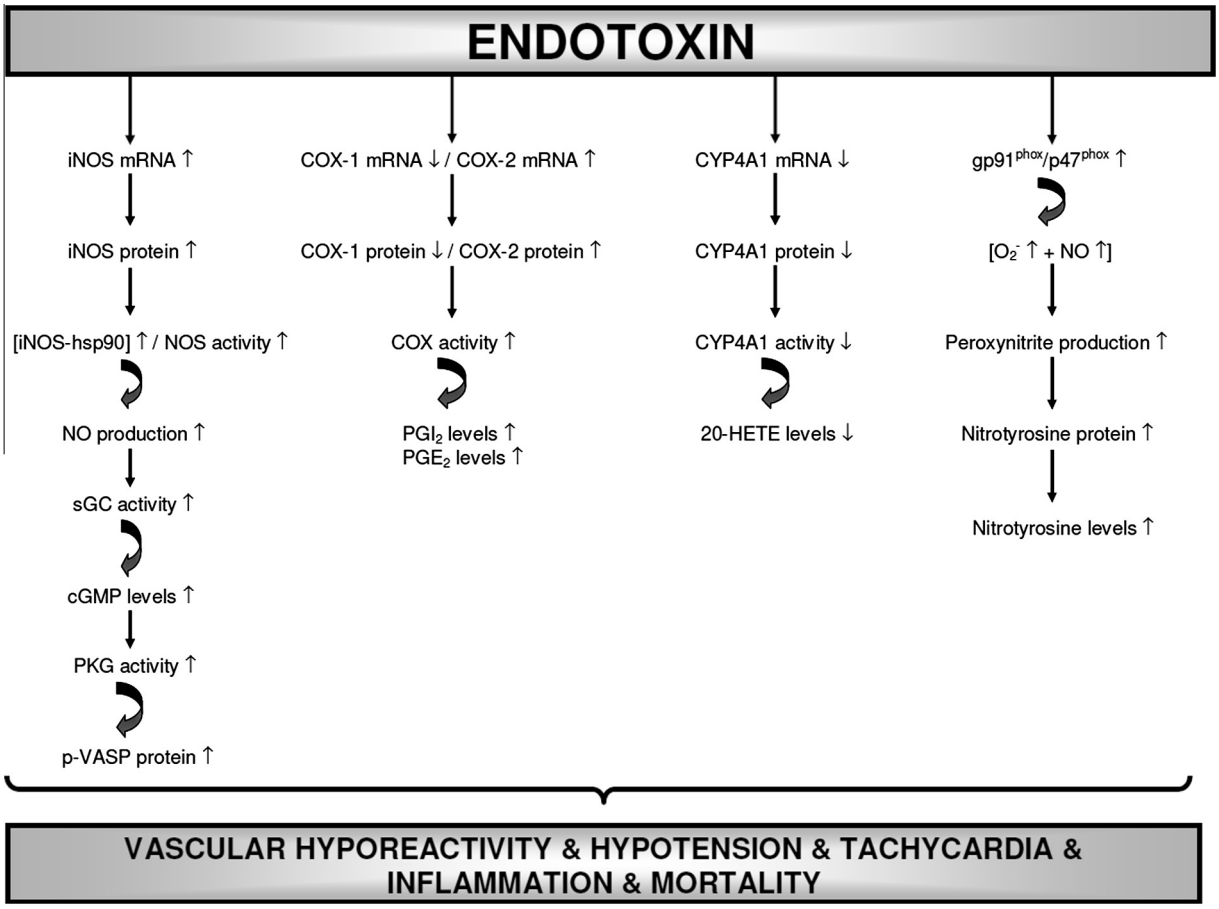


**Fig. 21.** Schematic diagram showing the involvement of iNOS, sGC, PKG, COX-1, COX-2, CYP4A1, and gp91$^{phox}$ in endotoxin-induced vascular hyporeactivity, hypotension, tachycardia, and survival based on the results of the present and our previous findings. Endotoxin, the lipid A part of LPS which is the most potent microbial mediator of the pathogenesis of sepsis and septic shock, increases iNOS and COX-2 mRNA expression, iNOS, p-VASP, COX-2, gp91$^{phox}$ (NOX2; a superoxide generating NOX enzyme), p47$^{phox}$ (NOXO2; organizer subunit of gp91$^{phox}$), and nitrotyrosine protein expression, iNOS-hsp90 complex formation, NOS activity, and levels of cGMP, 6-keto-PGF$_{1\alpha}$, PGE$_2$, and nitrotyrosine associated with decreased COX-1 and CYP4A1 mRNA expression, COX-1 protein expression, and 20-HETE levels in renal and cardiovascular tissues leading to vascular hyporeactivity hypotension, tachycardia, and mortality in the rodent model of septic shock. 5,14-HEDGE, a 20-HETE mimetic, prevents the effects of endotoxin on the increase in expression of COX-1, COX-2, and CYP4A1 mRNA expression, iNOS, p-VASP, COX-2, CYP4A1, gp91$^{phox}$, p47$^{phox}$, and nitrotyrosine protein expression, iNOS-hsp90 complex formation, NOS activity, and levels of cGMP, 6-keto-PGF$_{1\alpha}$, PGE$_2$, 20-HETE, and nitrotyrosine, and thus, restores blood pressure, prevents tachycardia, and improves survival during rodent endotoxemia. It should be noted that a competitive antagonist of vasoconstrictor effects of 20-HETE, 20-HEDE, prevents the effects of 5,14-HEDGE on blood pressure, HR, p-VASP, COX-2, CYP4A1, gp91$^{phox}$, p47$^{phox}$, and nitrotyrosine protein expression, iNOS-hsp90 complex formation, NOS activity, and 6-keto-PGF$_{1\alpha}$, PGE$_2$, 20-HETE, and nitrotyrosine levels in rats treated with LPS. It can be concluded that decreased expression and activity of iNOS, sGC, PKG, COX-2, and gp91$^{phox}$ associated with increased CYP4A1 expression and activity participate in the protective effect of 5,14-HEDGE against vascular hyporeactivity, hypotension, tachycardia, and mortality in the rodent model of septic shock. (↑), increase; (↑) decrease.

tachycardia, inflammation, and mortality which lead to multiple organ failure and death in septic shock.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.niox.2013.05.001.

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