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Effects of 5,14-HEDGE, a 20-HETE mimetic, on lipopolysaccharide-induced changes in MyD88/TAK1/IKK β /I κ B- α /NF- κ B pathway and circulating miR-150, miR-223, and miR-297 levels in a rat model of septic shock

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Abstract

Objectives—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-

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HEDGE), which mimics the effects of endogenously produced 20-HETE, prevents vascular hyporeactivity, hypotension, tachycardia, inflammation, and mortality in a rodent model of septic shock. The present study was performed to determine whether decreased renal and cardiovascular expression and activity of myeloid differentiation factor 88 (MyD88)/transforming growth factor-activated kinase 1 (TAK1)/inhibitor of κ B (I κ B) kinase β (IKK β)/I κ B- α /nuclear factor- κ B (NF- κ B) pathway and reduced circulating microRNA (miR)-150, miR-223, and miR-297 expression levels participate in the protective effect of 5,14-HEDGE against hypotension, tachycardia, and inflammation 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 sacrificed 4 h after LPS challenge and blood, kidney, heart, thoracic aorta, and superior mesenteric artery were collected for measurement of the protein expression.

Results—LPS-induced fall in blood pressure and rise in heart rate were associated with increased MyD88 expression and phosphorylation of TAK1 and I κ B- α in cytosolic fractions of the tissues. LPS also caused an increase in both unphosphorylated and phosphorylated NF- κ B p65 proteins in the cytosolic and nuclear fractions as well as nuclear translocation of NF- κ B p65. In addition, serum miR-150, miR-223, and miR-297 expression levels were increased in LPS-treated rats. These effects of LPS were prevented by 5,14-HEDGE.

Conclusions—These results suggest that downregulation of MyD88/TAK1/IKK β /I κ B- α /NF- κ B pathway as well as decreased circulating miR-150, miR-223, and miR-297 expression levels participate in the protective effect of 5,14-HEDGE against hypotension, tachycardia, and inflammation in the rat model of septic shock.

Keywords

Endotoxin; MyD88/TAK1/IKK β /I κ B- α /NF- κ B pathway; 20-HETE; miRNA; Septic shock

1. 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 [1–3]. In the vasculature, 20-HETE causes vasoconstriction in several vascular beds, including renal, aortic, mesenteric, and coronary arteries [4–8]. Activation of protein kinases, such as mitogen-activated protein kinase (MAPK), MAPK kinase (MEK), and extracellular signal-regulated kinase (ERK) which contribute to the regulation of vascular tone, has been shown to mediate the vasoconstrictor effect of 20-HETE [9–11]. As opposed to its vasoconstrictor effect, 20-HETE has also been reported to produce vasodilation in the vasculature including renal and coronary arteries [12–14]. These vasodilatory responses of 20-HETE have been attributed to nitric oxide (NO) release [15], conversion of 20-HETE to 20-OH-PGE₂ and 20-OH-PGF₂ α by cyclooxygenase (COX) [6, 12, 16], and increased formation of PGE₂ [16] and 2 [12–14, 16]. In addition, 20-HETE has been shown to activate MEK1/ERK1/2/nuclear factor- κ B (NF- κ B) signaling and induce expression of cellular adhesion molecules and cytokines, thereby promoting inflammation [17, 18]. CYP4A- and CYP4F-derived 20-

HETE has been reported to be involved in lipopolysaccharide (LPS)-induced acute systemic inflammation as a proinflammatory mediator [19, 20].

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 [21–23]. In the myeloid differentiation factor 88 (MyD88)-dependent (canonical, classical) pathway, following the recognition of LPS, TLR4 undergoes oligomerization and recruits its downstream adaptors through interactions with Toll-interleukin (IL)-1 receptor domain containing adaptor protein (TIRAP) which in turn induces IL-1 receptor-associated kinase (IRAK) 1/4 and tumor necrosis factor (TNF) receptor-associated factor (TRAF) 6 leading to activation of transforming growth factor-activated kinase 1 (TAK1). Then, TAK1 activates downstream inhibitor of κ B (I κ B) kinase (IKK) and MAPK pathways leading to phosphorylation and degradation of I κ B proteins (e.g., I κ B- α , I κ B- β , I κ B- γ , and I κ B- ζ) by IKKs (e.g., IKK α [IKK1], IKK β [IKK2], and IKK γ [IKK3]) and subsequent nuclear translocation of NF- κ B, and induction of another transcription factor, activating 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, which result in activation of proinflammatory mediator production [21, 23, 24]. It has been reported that inhibition of the NF- κ B pathway improves septic disturbances, restores systemic hypotension, 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 [22, 24]. Therefore, activation of NF- κ B pathway plays a key role in the pathophysiology of septic shock, and the consequences of sepsis inducing tissue injury and multiple organ failure can be prevented by the development of targeted cell-specific approaches inhibiting NF- κ B activation.

MicroRNAs (miRNAs) are endogenous noncoding small (approximately 20–23 nucleotides) ribonucleic acids (RNAs) that posttranscriptionally regulate protein-coding gene expression by either binding to the 3'-untranslated region of target messenger RNAs (mRNAs) to repress translation or induce mRNA cleavage or induce gene activation [25–27]. miRNAs have been implicated in a variety of processes such as cell proliferation, differentiation, migration, and invasion, immune response, inflammation, apoptosis, angiogenesis, and metastasis [28–31]. In recent years, the diagnostic and prognostic value of circulating miRNAs as biomarkers have been proved for several diseases, including different types of malignancies, acute myocardial infarction, congestive heart failure, oxidative liver injury, type 2 diabetes mellitus, stroke, infectious diseases, and pregnancy [32, 33]. Recent studies have also shown that serum miR-146a and miR-223 expression levels are significantly reduced in septic patients compared with systemic inflammatory response syndrome (SIRS) patients, and healthy controls suggesting serum miR-146a and miR-223 expression levels may serve as new biomarkers for sepsis with high specificity and sensitivity [34]. Decreased levels expression of miR-150 in both leukocytes and plasma have also been shown to be correlated with the aggressiveness of sepsis and can be used as a marker of early sepsis [35]. More recently, it has been demonstrated that serum miR-297 expression levels are more closely associated with survival from sepsis, whereas expression levels of miR-574-5p are

associated with death from sepsis [36]. Furthermore, Schmidt *et al.* [37] reported that miR-146b, miR-150, miR-342, and let-7g were downregulated in peripheral blood leukocytes isolated from LPS-treated healthy volunteers, while miR-143 were upregulated. Despite the importance of the changes in circulating miR-150, miR-223, and miR-297 expression levels in patients with sepsis and septic shock [34–36], there are few studies investigating changes in miR-150 and miR-223 expression in rat serum [38], hepatic stellate cells [39], cardiomyocytes [40], and rat colon mucosa [41], while circulating or tissue miR-297 levels have not been evaluated in rats. Moreover, to the best of our knowledge, there has been no previous attempt to examine involvement of miR-150, miR-223, and miR-297 in rat models of 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 of 20-HETE, 20-hydroxyeicosa-6(Z),15(Z)-dienoic acid, suggested that (1) increased expression and/or activity of MEK1/ERK1/2/IKK β /I κ B- α /NF- κ B/inducible nitric oxide synthase (iNOS)/soluble guanylyl cyclase (sGC)/protein kinase G (PKG) pathway, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, COX-2, and soluble epoxide hydrolase (sEH), (2) overproduction of proinflammatory cytokines (i.e., TNF- α and IL-8), NO, peroxynitrite, and vasodilator prostanoids (i.e., PGI₂ and PGE₂), and (3) decreased CYP4A1 and CYP2C23 expression associated with vasoconstrictor (i.e., 20-HETE) and antiinflammatory mediator formation (i.e., epoxyeicosatrienoic acids; EETs) contribute to hypotension, tachycardia, inflammation, and mortality in a rodent model of septic shock [42–45]. As a continuation of our previous studies, the present study was conducted to determine whether decreased renal and cardiovascular expression and activity of MyD88/TAK1/IKK β /I κ B- α /NF- κ B pathway and circulating miR-150, miR-223, and miR-297 expression levels participate in the protective effect of 5,14-HEDGE against hypotension, tachycardia, and inflammation in LPS-treated rats.

2. Materials and methods

2.1. Endotoxic shock model

Experiments were performed on Wistar rats (male; 200 to 300 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 and mice as previously described by Tunctan *et al.* [46]. Rats were randomly divided into saline (n = 6), LPS (n = 6), 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, 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.; sublethal 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, s.c.) [42–45] 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, US. 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 in the experiments. Rats were euthanized 4 h after the administration of saline or LPS, and samples of blood, kidney, heart, thoracic aorta, and superior mesenteric artery were collected from all animals. Sera were obtained from blood samples by centrifugation at 23,910 x g for 15 min at 4° C and stored at –80° C for measurement of miR-150, miR-223, and miR-297 expression levels as described below. 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 MyD88, TAK1, phosphorylated TAK1, IκB-α, phosphorylated IκB-α, NF-κB, phosphorylated NF-κB, and 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.

2.2. miRNA isolation and quantitation

Total RNA including miRNAs was isolated from 200 µl of serum samples by miRNeasy Serum/Plasma Kit (Qiagen, Valencia, CA, USA) and quantitative real-time polymerase chain reaction (qRT-PCR) was performed with miScript II RT Kit (Qiagen) and miScript SYBR Green PCR Kit (Qiagen) using specific primers for miR-150, miR-223, and miR-297 (Qiagen) following the manufacturer's instructions. qRT-PCR was performed using TaqMan probes on ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, USA) according to the protocol provided by the manufacturer. The relative quantities of miRNA were determined using comparative threshold cycle (CT) method and normalized against Hs_RNU6B_2 (small nuclear RNA; snRNA) (Qiagen) as an endogenous control.

2.3. Immunoblotting

Immunoblotting for MyD88, TAK1, phosphorylated TAK1, IκB-α, phosphorylated IκB-α, NF-κB p65, phosphorylated NF-κB p65, and actin proteins were performed according to the method described previously [42, 44, 45]. Briefly, tissue homogenates (75 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) MyD88: MyD88 (F-19) monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA); (2) TAK1: TAK1 (D94D7) rabbit monoclonal antibody (Cell Signalling Technology, Danvers, MA, USA); (3) phosphorylated TAK1: phospho-TAK1 (Ser⁴¹²) antibody (Cell Signalling); (4) IκB-α: IκB-α mouse monoclonal IgG (Santa Cruz); (5) phosphorylated IκB-α: p-IκB-α (Ser³²) goat IgG (Santa Cruz); (6) NF-κB: NF-κB p65 mouse monoclonal IgG (Santa Cruz); and (7) phosphorylated NF-κB: p-NF-κB p65 (Ser⁵³⁶) mouse monoclonal IgG (Santa Cruz). The membranes were then incubated with goat anti-rabbit IgG-horseradish peroxidase (Amersham Life Sciences, Cleveland, OH, USA) for

TAK1 and phosphorylated TAK1, and sheep anti-mouse IgG-horseradish peroxidase (Amersham) for MyD88, I κ B- α , phosphorylated I κ B- α , NF- κ B p65, and phosphorylated NF- κ B p65 in 0.1% BSA (1:1,000) at room temperature for 1 h. The blots were developed with enhanced chemiluminescence (ECL Plus Western Blotting Detection Reagent) (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-actin antibody [2Q1055] (Abcam, Cambridge, MA, USA) (1:500 in 5% BSA), monoclonal anti- α -sarcomeric actin (Sigma Chemical Co., St. Louis, MO, USA) (1:500 in 5% BSA), or monoclonal anti- β -actin (Sigma) (1:500 in 5% BSA) as a loading control followed by incubation with sheep anti-mouse IgG-horseradish peroxidase (Amersham) (1:1,000 in 0.1% BSA). Relative densities of immunoreactive bands for MyD88, TAK1, phosphorylated TAK1, I κ B- α , phosphorylated I κ B- α , NF- κ B p65, phosphorylated NF- κ B p65 proteins in each sample were normalized to the density of corresponding bands for actin, α -sarcomeric actin, and β -actin for kidney, heart, and vascular tissues, respectively.

2.4. Statistical analysis

Data are expressed as means $\pm$ S.E.M. Data were analysed 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.

3. Results

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

LPS caused a gradual fall in MAP and an increase in HR over the 4 h course of the experiment ($p < 0.05$) (Fig. 1A). The change in MAP and HR reached a maximum 4 h after the administration of LPS. MAP fell by 35 mmHg and HR rose by 75 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 ($p > 0.05$).

3.2. Effect of 5,14-HEDGE on LPS-induced increase in circulating miR-150, miR-223, and miR-297 expression levels

To determine the effect of 5,14-HEDGE on LPS-induced changes in circulating miR-150, miR-223, and miR-297, miR-150, miR-223, and miR-297 expression levels were measured in the serum samples of endotoxemic rats. LPS increased serum expression levels of miR-150 (Fig. 2A), miR-223 (Fig. 2B), and miR-297 (Fig. 2C) (Fig. 1A) ($p < 0.05$). The increase in miR-150, miR-223, and miR-297 expression levels was not observed in the sera of rats treated with LPS and 5,14-HEDGE ($p < 0.05$) (Fig. 2). 5,14-HEDGE had no effect on basal miR-150, miR-223, and miR-297 expression levels in vehicle-treated rats ($p > 0.05$) (Fig. 2).

3.3. Effect of 5,14-HEDGE on LPS-induced increase in MyD88 expression in endotoxemic rats

To examine the effect of 5,14-HEDGE on LPS-induced changes in MyD88 expression, MyD88 protein expression was measured in the cytosolic fractions of kidney, heart, thoracic aorta, and superior mesenteric artery of endotoxemic rats. LPS increased protein expression of MyD88 in the kidney (Fig. 3A), heart (Fig. 3B), thoracic aorta (Fig. 3C), and superior mesenteric artery (Fig. 3D) ($p < 0.05$). The increase in MyD88 protein expression was prevented in the tissues of rats treated with 5,14-HEDGE ($p < 0.05$). 5,14-HEDGE had no effect on basal MyD88 protein expression in vehicle-treated rats ($p > 0.05$) (Fig. 3).

3.4. Effect of 5,14-HEDGE on LPS-induced increase in TAK1 expression and phosphorylation in endotoxemic rats

To demonstrate the effect of 5,14-HEDGE on LPS-induced changes in TAK1 expression and phosphorylation at Ser⁴¹², unphosphorylated and phosphorylated TAK1 protein expression were measured in the cytosolic fractions of kidney, heart, thoracic aorta, and superior mesenteric artery of endotoxemic rats. LPS treatment increased phosphorylated ($p < 0.05$), but not unphosphorylated ($p > 0.05$), protein expression of TAK1 in the kidney (Fig. 4A), heart (Fig. 4B), thoracic aorta (Fig. 4C), and superior mesenteric artery (Fig. 4D). The increase in TAK1 phosphorylation caused by LPS was prevented in the renal and cardiovascular tissues of rats treated with 5,14-HEDGE ($p < 0.05$) (Fig. 4). 5,14-HEDGE had no effect on basal unphosphorylated and phosphorylated TAK1 protein expression in vehicle-treated rats ($p > 0.05$) (Fig. 4).

3.5. Effect of 5,14-HEDGE on LPS-induced increase in I κ B- α expression and phosphorylation in endotoxemic rats

To demonstrate the effect of 5,14-HEDGE on LPS-induced changes in I κ B- α expression and phosphorylation at Ser³², unphosphorylated and phosphorylated I κ B- α protein expression were measured in the cytosolic fractions of kidney, heart, thoracic aorta, and superior mesenteric artery of endotoxemic rats. LPS treatment increased phosphorylated protein expression of I κ B- α in the kidney (Fig. 5A), heart (Fig. 5B), thoracic aorta (Fig. 5C), and superior mesenteric artery (Fig. 5D) while unphosphorylated I κ B- α protein expression was decreased in the renal and cardiovascular tissues ($p < 0.05$). The changes in unphosphorylated and phosphorylated I κ B- α protein expression caused by LPS were prevented in the tissues of rats treated with 5,14-HEDGE ($p < 0.05$) (Fig. 5). 5,14-HEDGE had no effect on basal unphosphorylated and phosphorylated I κ B- α protein expression in vehicle-treated rats ($p > 0.05$) (Fig. 5).

3.6. Effect of 5,14-HEDGE on LPS-induced increase in NF- κ B p65 expression and phosphorylation

To demonstrate the effect of 5,14-HEDGE on LPS-induced changes in NF- κ B p65 expression and phosphorylation of the subunit p65 at Ser⁵³⁶, unphosphorylated and phosphorylated NF- κ B p65 protein expression were measured in the cytosolic and nuclear fractions of kidney, heart, thoracic aorta, and superior mesenteric artery of endotoxemic rats. LPS increased unphosphorylated and phosphorylated NF- κ B p65 protein expression in both

fractions of kidney (Fig. 6A), heart (Fig. 6B), thoracic aorta (Fig. 6C), and superior mesenteric artery (Fig. 6D) ($p < 0.05$). The increase in both unphosphorylated and phosphorylated NF- κ B p65 expression produced by LPS was prevented in the renal and cardiovascular tissues of rats treated with 5,14-HEDGE ($p < 0.05$) (Fig. 6). 5,14-HEDGE had no effect on basal unphosphorylated and phosphorylated NF- κ B p65 protein expression in vehicle-treated rats ($p > 0.05$) (Fig. 6).

3.7. Effect of 5,14-HEDGE on LPS-induced increase in nuclear translocation of NF- κ B 65

To investigate the effect of 5,14-HEDGE on LPS-induced changes in nuclear translocation of NF- κ B p65, the ratios of nuclear over cytosolic unphosphorylated and phosphorylated NF- κ B p65 proteins were calculated in the kidney, heart, thoracic aorta, and superior mesenteric artery of endotoxemic rats. LPS increased calculated nuclear/cytosolic unphosphorylated as well as phosphorylated NF- κ B p65 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 both unphosphorylated and phosphorylated nuclear/cytosolic NF- κ B p65 ratios produced by LPS was prevented in the renal and cardiovascular tissues of rats treated with 5,14-HEDGE ($p < 0.05$) (Fig. 7). 5,14-HEDGE had no effect on basal nuclear/cytosolic ratios of unphosphorylated and phosphorylated NF- κ B p65 in vehicle-treated rats ($p > 0.05$) (Fig. 7).

4. Discussion

The results of the present study indicate that increased expression and/or activity of MyD88/TAK1/IKK β /I κ B- α pathway which results in nuclear translocation of both unphosphorylated and phosphorylated forms of NF- κ B p65 as well as enhanced serum miR-150, miR-223, and miR-297 expression levels 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 decreased expression and activity of the MyD88/TAK1/IKK β /I κ B- α /NF- κ B pathway as well as circulating miR-150, miR-223, and miR-297 expression levels leading to vasodilatory and inflammatory mediator production in the rat model of septic shock.

NF- κ B has been implicated in the regulation of multiple biological phenomena and disease states, including inflammation and septic shock. Activation of CD14/MD-2/TLR4/TIRAP/IRAK1/4/MyD88/TAK1/IKK β /I κ B- α pathway in response to LPS has been reported to act as an important regulator of NF- κ B p65 activation and increased expression of NF- κ B p65-dependent proinflammatory genes (e.g., iNOS, COX-2, acute phase proteins, and proinflammatory cytokines) [22, 24]. On the other hand, it has been demonstrated that NF- κ B subunits p65 (RelA; encoded by the RELA gene) and p50 (generated from its precursor, p105/NF- κ B1, encoded by the NFKB1 gene) have critical inhibitory functions during the systemic response to LPS and raise the possibility that these functions could be essential in preventing mortality associated with SIRS [46]. NF- κ B proteins can be phosphorylated in the cytoplasm or nucleus by such kinases as glycogen synthase kinase 3 β , NF- κ B-activator-binding kinase 1, protein kinase A, mitogen- and stress-activated protein kinase-1, MAP3K NF- κ B-inducing kinase, Tpl2, protein kinase C (PKC)- θ , PI3K, Akt, p38 MAPK, protein tyrosine kinase, PKC- δ , Rho-kinase 2, MEK3, and receptor tyrosine kinases [22, 24]. In the

canonical pathway, IKK β is known to be the most important kinase in the IKK signalosome which consists of IKK α , IKK β , and IKK γ (also known as NF- κ B essential modulator) to activate NF- κ B in response to LPS [47]. Besides activation itself by a transphosphorylation mechanism [48], IKK β has been shown to catalyze not only phosphorylation of I κ B- α at Ser^{32/36} [49], but also NF- κ B p65 at Ser⁵³⁶ in both the cytosol and nucleus to regulate NF- κ B-dependent and -independent gene expression [50–53]. On the other hand, Chew *et al.* [54] reported that WIP1, a Ser/Thr PP2C family of phosphatases, act as a negative regulator of NF- κ B activation. NF- κ B activation is also tightly regulated mainly through its subcellular localization. In resting cells, NF- κ B proteins are kept in the cytoplasm in association with inhibitory I κ B proteins including I κ B- α , I κ B- β , and I κ B- ϵ among which I κ B- α is the most abundant [55]. I κ Bs inhibit NF- κ B activation through three mechanisms: (i) sequestering NF- κ B dimers in the cytoplasm, (ii) facilitating dissociation of complementary deoxyribonucleic acid (DNA)-bound NF- κ B dimers from their DNA binding sites, and (iii) exporting NF- κ B dimers from the nucleus [22]. In addition, although I κ B- α /NF- κ B complexes are localized mainly in cytosol in the presence of I κ B- α , I κ B- α and NF- κ B molecules as well as I κ B- α /NF- κ B complexes have been reported to shuttle between cytoplasm and nucleus [56–58]. Formation of I κ B- α /NF- κ B complex has been shown to mask the nuclear localization sequences on p65, but not on p50, resulting in a partial blockage of nuclear entry of NF- κ B dimers [59]. Some of the I κ B- α /NF- κ B complexes are also able to enter the nucleus due to the unmasked nuclear localization sequences on p50. On the other hand, those I κ B- α /NF- κ B complexes entering the nucleus are actively transported back to cytoplasm by means of the nuclear export sequences on I κ B- α [55]. Furthermore, it has been reported that exposure to LPS in *ex vivo* or *in vitro* conditions results in increased nuclear NF- κ B p65 protein levels associated with decreased amounts in cytosol, suggesting increased nuclear translocation of NF- κ B p65 during endotoxemia [60–64]. Moreover, Ishizuka *et al.* [18] demonstrated that 20-HETE activates the MEK1/ERK1/2/NF- κ B pathway and increases nuclear translocation of NF- κ B p65 leading to expression of proinflammatory mediators in human endothelial cells. In bovine endothelial cells, Cheng *et al.* [17] 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- κ B activity through IKK β /I κ B- α pathway. In the present study, we measured changes in the expression and/or activity of MyD88, TAK1, IKK β , I κ B- α , and NF- κ B p65 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 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 increases in MyD88, TAK1, phosphorylated TAK1 (Ser⁴¹²), and phosphorylated I κ B- α (Ser³²) protein expression, (2) decreases in I κ B- α protein levels in cytosolic fractions, and (3) elevated amounts of both unphosphorylated and phosphorylated NF- κ B p65 (Ser⁵³⁶) protein expression in the cytosolic and nuclear fractions of kidney, heart, thoracic aorta, and superior mesenteric artery of rats associated with a rise in serum miR-150, miR-223, and miR-297 levels. 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 A₂ and cause release of arachidonic acid from

tissue lipids which result in increased prostaglandin synthesis and 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 [3]. Indeed, the results of the present study together with our previous observations [42–45] suggest that 5,14-HEDGE prevents LPS-induced vascular hyporeactivity, hypotension, tachycardia, and inflammatory response in endotoxemic rats and mortality in mice by (1) increasing CYP4A1 and CYP2C23 expression together with production of vasoactive eicosanoids (e.g., 20-HETE) and antiinflammatory mediators (e.g., EETs), (2) suppressing of both MyD88/TAK1/IKK β /I κ B- α /NF- κ B and MyD88/TAK1/MEK1/ERK1/2/AP-1 pathways, (3) inhibiting iNOS, sGC, PKG, COX-2, and gp91^{phox} expression/activity associated with superoxide formation, and (4) reducing production of proinflammatory cytokines (e.g., TNF- α and IL-8). In the present study, 5,14-HEDGE also prevented the increase in both calculated nuclear/cytosolic unphosphorylated and phosphorylated NF- κ B p65 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 NF- κ B p65 as well as phosphorylation of NF- κ B p65 in the nucleus through IKK β /I κ B- α pathway during endotoxemia. However, additional experiments need to be conducted to demonstrate the validity of this hypothesis. Further characterization of the molecular mechanisms of the effects of 5,14-HEDGE on the molecules (e.g., importin- α 3 and Crm1) responsible for nuclear import and export of I κ B- α and/or NF- κ B p65 as well as phosphorylation in the nucleus through IKK β /I κ B- α will provide the framework for extension of this work into understanding the role of the MyD88/TAK1/IKK β /I κ B- α /NF- κ B pathway in the protective effect of 5,14-HEDGE against hypotension, tachycardia, and inflammation in the rat model of septic shock.

In recent years, it has been demonstrated that serum miR-223 expression levels are significantly reduced in septic patients compared with SIRS patients, and healthy controls suggesting serum miR-223 expression levels may serve as a new biomarker for sepsis with high specificity and sensitivity [34]. Vasilescu *et al.* [34] reported that decreased expression levels of miR-150 in both leukocytes and plasma are correlated with the aggressiveness of sepsis. In a recent study, it has been demonstrated that serum miR-297 expression levels are more closely associated with survival from sepsis [35]. Moreover, it has been reported that miR-150 was downregulated in peripheral blood leukocytes isolated from LPS-treated healthy volunteers [36]. On the other hand, to the best of our knowledge, there is no information about the involvement of miR-150, miR-223, and miR-297 during rat endotoxemia. In the present study, serum miR-150, miR-223, and miR-297 expression levels were increased at 4 h post-LPS administration. The increase in serum miR-150, miR-223, and miR-297 expression levels was prevented in endotoxemic rats treated with 5,14-HEDGE. Increasing evidence suggest that NF- κ B, as a key molecule in the pathogenesis of many inflammatory diseases such as sepsis and septic shock, regulates biogenesis of several miRNAs some being inhibitory and others activating by increasing (e.g., miR-9, miR-21, miR-125a, miR-132, miR-146a, miR-147b, miR-155, and miR-301a) or decreasing (e.g., let-7i) their expression. It has also been reported that miRNAs can play roles as both positive and negative feedback regulators of NF- κ B signalling network. For example,

miR-9, miR-21, miR-146a, and miR-155 inhibit NF- κ B signalling pathway, while miR-301a induces NF- κ B activity [29–31, 65]. In contrast to previous reports [34, 35, 37], our findings suggest that miR-150 and miR-223 expression are increased during endotoxemia. On the other hand, our results are in agreement with previous studies showing that relative serum miR-297 expression levels of surviving and nonsurviving sepsis patients are significantly higher in nonsurvivors than in survivors [36]. Thus, the discrepancy between previous studies and our results could be attributed to differences in humans and animals as well as type of infection, and time points for measurement of miRNA expression levels which might reflect the differences in response to LPS treatment. Collectively, these data demonstrate that 5,14-HEDGE prevents the increase in expression and activity of the MyD88/TAK1/IKK β /I κ B- α /NF- κ B pathway as well as enhanced expression levels of circulating miR-150, miR-223, and miR-297 through this pathway leading to changes in vasoactive and proinflammatory mediator production which results in hypotension, tachycardia, inflammation, and mortality in the rodent model of septic shock. It is also possible that increased expression levels of circulating miR-150, miR-223, and miR-297 might activate this pathway during endotoxemia. However, additional experiments in the renal and cardiovascular tissues of endotoxemic rats treated with NF- κ B inhibitors or anti-miR oligonucleotides against miR-150, miR-223, and miR-297 need to be conducted to demonstrate the validity of this hypothesis.

In conclusion, the present study 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, causes increased MyD88, TAK1, phosphorylated TAK1, and phosphorylated I κ B- α protein expression and diminished I κ B- α protein levels in cytosolic fractions as well as raised NF- κ B p65 and phosphorylated NF- κ B p65 protein expression in cytosolic and nuclear fractions of renal and cardiovascular tissues as well as enhanced serum expression levels of miR-150, miR-223, and miR-297 leading to hypotension, tachycardia, inflammation, and mortality in the rodent model of septic shock (Fig. 8). 5,14-HEDGE, a 20-HETE mimetic, prevents the effects of endotoxin on the increase in MyD88, TAK1, phosphorylated TAK1, and phosphorylated I κ B- α protein expression associated with decreased I κ B- α protein levels in cytosolic fractions, increase in NF- κ B p65 and phosphorylated NF- κ B p65 protein expression in cytosolic and nuclear fractions which results in nuclear translocation of both NF- κ B p65 and phosphorylated NF- κ B p65 as well as a rise in miR-150, miR-223, and miR-297 serum expression levels. Therefore, it can be concluded that decreased expression and nuclear translocation of both phosphorylated and unphosphorylated forms of NF- κ B p65 in a MyD88/TAK1/IKK β /I κ B- α pathway-dependent manner and downregulated circulating miR-150, miR-223, and miR-297 expression levels participate in the protective effect of 5,14-HEDGE against vascular hyporeactivity, hypotension, tachycardia, and mortality in the rat model of septic shock. Moreover, it is likely that on the one hand both the MyD88/TAK1/IKK β /I κ B- α /NF- κ B pathway and miR-150, miR-223, and miR-297 upregulated through this pathway might cause changes in gene expression of certain enzymes (i.e., iNOS, COX-2, and CYP4A1) that are responsible for vasoactive and proinflammatory mediator formation; on the other hand, enhanced biogenesis of miR-150, miR-223, and miR-297 in response to LPS might cause increased expression and activity of this pathway which results in gene expression during endotoxemia. Hence, we speculate that 5,14-

HEDGE could have antihypotensive effect due to its 20-HETE mimetic activity and inhibition of vasodilatory mediator formation as well as antiinflammatory effect which could be due to decreased proinflammatory mediator production. In addition, these three miRNAs might be useful biomarkers for patients with septic shock, although additional studies will be needed to confirm this. 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 and also diagnostic and prognostic value of circulating miRNAs for several diseases, 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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Abbreviations

20-HETE	20-Hydroxyeicosatetraenoic acid
5.14-HEDGE	N-(20-hydroxyeicosa-5[Z],14[Z]-dienoyl)glycine
AP	activating protein
BSA	bovine serum albumin
CD	cluster of differentiation
DNA	complementary deoxyribonucleic acid
COX	cyclooxygenase
CT	comperative threshold cycle
CYP	cyclochrome P450
EET	epoxyeicosatrienoic acid
ERK	extracellular signal-regulated kinase
HR	heart rate
IKK	IκB kinase
IκB	inhibitor of κB
IL	interleukin
i.p	intraperitoneally

IL	interleukin
IRAK	IL-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	MAP kinase
MKK	MAPK kinase
miRNA	microRNA
mRNA	messenger RNA
MyD88	myeloid differentiation factor 88
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor- κ B
NIK	NF- κ B-inducing kinase
NO	nitric oxide
PG	prostaglandin
PKC	protein kinase C
PKG	protein kinase
RNA	ribonucleic acid
qRT-PCR	quantitative real time polymerase chain reaction
s.c	subcutaneously
sEH	soluble epoxide hydrolase
sGC	soluble guanylyl cyclase
SIRS	systemic inflammatory response syndrome
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

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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 means $\pm$ S.E.M. of 6 animals. *Significant difference from the corresponding value seen in rats treated with saline (vehicle) ($p < 0.05$). #Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$). §Significant difference from the time 0 h value within a group ($p < 0.05$). + Significant difference from the time 1 h value within a group ($p < 0.05$).

Fig. 2.

Effects of 5,14-HEDGE on changes in serum expression levels of (A) miR-150, (B) miR-223, and (C) miR-297 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. Serum miRNA expression levels were measured by qRT-PCR, determined using comparative CT method, and normalized against Hs_RNU6B_2 as an endogenous control. Data are expressed as means±S.E.M of 4 animals. *Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). #Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$).

Fig. 3.

Effects of 5,14-HEDGE on changes in MyD88 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.) was given 1 h after administration of saline or LPS. MyD88 protein expression in tissue homogenates was measured by immunoblotting. Data are expressed as means±S.E.M of 4 animals. *Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). #Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$).

Fig. 4.

Effects of 5,14-HEDGE on changes in TAK1 protein expression and phosphorylation at Ser⁴¹² 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.) was given 1 h after administration of saline or LPS. TAK1 and p-TAK1 protein levels in tissue homogenates were measured by immunoblotting. Data are expressed as means±S.E.M of 4 animals. *Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). #Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$).

Fig. 5.

Effects of 5,14-HEDGE on changes in IκB-α protein expression and phosphorylation at Ser³² 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.) was given 1 h after administration of saline or LPS. IκB-α and p-IκB-α protein levels in tissue homogenates were measured by immunoblotting. Data are expressed as means±S.E.M of 4 animals. *Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). #Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$).

Fig. 6.

Effects of 5,14-HEDGE on changes in NF-κB p65 protein expression and phosphorylation at Ser⁵³⁶ in cytosolic and nuclear fractions of (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.) was given 1 h after administration of saline or LPS. NF-κB p65 and p-NF-κB p65 protein levels in tissue homogenates were measured by immunoblotting. Data are expressed as means±S.E.M of 4 animals. *Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). #Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$).

Fig. 7.

Effects of 5,14-HEDGE on changes in the calculated ratios of nuclear/cytosolic NF-κB p65 and nuclear/cytosolic p-NF-κB p65 proteins 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.) was given 1 h after administration of saline or LPS. NF-κB p65 and p-NF-κB p65 (Ser⁵³⁶) protein levels in tissue homogenates were measured by immunoblotting. Data are expressed as means±S.E.M of 4 animals. *Significant difference from the corresponding value seen in rats treated with saline ($p < 0.05$). #Significant difference from the corresponding value seen in the rats treated with LPS ($p < 0.05$).

Fig. 8.

Schematic diagram showing the involvement of the MyD88/TAK1/IKKβ/IκB-α/NF-κB pathway and miR-150, miR-223, and miR-297 in endotoxin-induced hypotension, tachycardia, inflammation, and survival based on the results of the current study and our previous findings. (↑), increase; (↓), decrease.