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Cationic peptide mR18L with lipid lowering properties inhibits LPS-induced systemic and liver inflammation in rats

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ABSTRACT

The cationic single domain peptide mR18L has demonstrated lipid-lowering and anti-atherogenic properties in different dyslipidemic mouse models. Lipopolysaccharide (LPS)-mediated inflammation is considered as one of the potential triggers for atherosclerosis. Here, we evaluated anti-inflammatory effects of mR18L peptide against LPS-mediated inflammation. First, we tested the efficacy and tolerance of 1, 2.5 and 5 mg/kg mR18L in normolipidemic rats stimulated with 5 mg/kg LPS. LPS and then mR18L were injected in different intraperitoneal regions. By 2 h post LPS, mR18L inhibited LPS-mediated plasma TNF-α elevation at all doses, with the effect being stronger for 2.5 mg/kg ($P < 0.05$ vs. 1 mg/kg, non-significant vs. 5 mg/kg). In a similar model, 2.5 mg/kg mR18L reduced LPS-mediated inflammation in the liver, as assessed by microscopic examination of liver sections and measurements of iNOS expression in the liver tissue. In plasma, 2.5 mg/kg mR18L decreased levels of TNF-α and IL-6, decreased endotoxin activity and enhanced HDL binding to LPS. In another similar experiment, mR18L administered 1 h post LPS, prevented elevation of plasma triglycerides by 6 h post LPS and increased plasma activity of anti-oxidant enzyme paraoxonase 1, along with noted trends in reducing plasma levels of endotoxin and IL-6. Surface plasmon resonance study revealed that mR18L readily binds LPS. We conclude that mR18L exerts anti-endotoxin activity at least in part due to direct LPS-binding and LPS-neutralizing effects. We suggest that anti-endotoxin activity of mR18L is an important anti-inflammatory property, which may increase anti-atherogenic potential of this promising orally active lipid-lowering peptide.

1. Introduction

Inflammation and atherosclerosis are closely related processes [1,2]. Recent experimental studies [3–5] and clinical observations [6–8] suggest that endotoxemia can play a role in the pathogenesis of atherosclerosis. Endotoxin, or lipopolysaccharide (LPS), is a component of bacterial cell wall, and represents a major virulence factor of gram-negative bacteria, which are a common cause of human bloodstream infections [9]. Humans are persistently exposed to LPS mainly through food consumption. Endotoxin levels in blood increase significantly in humans [10] and animals [11] on Western-style diet. Animals on high saturated fat and cholesterol diets demonstrate an enhanced inflammatory response to endotoxin [12]. Chronic metabolically-mediated endotoxemia, and therefore systemic inflammation, is an important pathogenic factor in the development of metabolic syndrome and atherosclerosis [10,11,13]. The liver is the major target of the detrimental effects of endotoxemia and systemic inflammation [14,15]. Because liver is central to the regulation of cholesterol levels in the body, liver inflammation may be involved in the pathogenesis of atherosclerosis [16]. Lowering LPS-mediated inflammation could be a potent strategy to control metabolic diseases and atherosclerosis. Therefore, a specific anti-endotoxin activity would be an important property for an effective anti-atherogenic agent, in addition to its anti-inflammatory and lipid lowering properties.

We have designed several peptides that demonstrate strong anti-atherogenic effects in different animal models [17–20]. One of these peptides, 4F, mimics anti-inflammatory properties of human apoA-I; however 4F has no effect on plasma cholesterol [19]. Several studies indicate that 4F effectively inhibits LPS-mediated inflammatory response *in vitro* and systemic and liver inflammation *in vivo* [21–23]. Another anti-atherogenic peptide Ac-hE18A-NH_2 with lipid-lowering and anti-inflammatory

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properties was designed by covalently linking 141–150 residues from the receptor-binding region of apolipoprotein E (apoE) to the amphipathic α-helical peptide 18A [24,25]. Recent results suggest that Ac-hE18A-NH_2 is a potent inhibitor of LPS-mediated inflammatory response in THP-1 macrophages and HUVEC cells [26]. In apoE-null mice, Ac-hE18A-NH_2 was more effective in inhibiting atherosclerosis than 4F, perhaps due to its cholesterol reducing properties [19]. However, Ac-hE18A-NH_2 is active only when intravenously administered, which limits its potential clinical use.

To create an orally active peptide with both cholesterol reducing and anti-inflammatory properties, the sequence of previously characterized cationic amphipathic helical class L (lytic) peptide, 18L [27] was modified by incorporating aromatic amino acids at the center of the nonpolar face and by substituting Lys residues with Arg (Supplementary Fig. 1). The resulting peptide, mR18L had substantially reduced lytic properties and was able to enhance uptake of atherogenic lipoproteins by hepatocytes *in vitro* [20]. Both oral and intravenous administration of mR18L decreased plasma cholesterol levels, mainly VLDL, and inhibited atherosclerotic lesion formation in mouse models [20,28]. Here, we sought to test the potency of mR18L against LPS-induced inflammation and look at the possible mechanisms. We hypothesize that the cationic amphipathic peptide mR18L can readily interact with negatively-charged LPS, thus inhibiting LPS pathogenic effects.

In the present work, we evaluated the anti-endotoxin activity of mR18L in endotoxemic rats. We found that mR18L effectively inhibits systemic and liver inflammation in endotoxemic rats, likely due to a direct interaction with LPS and neutralization of LPS activity.

2. Materials and methods

2.1. Synthesis of peptide

mR18L (Ac-G-F-R-R-F-L-G-S-W-A-R-I-Y-R-A-F-V-G-NH_2) was synthesized by the solid phase peptide synthesis method using fluorenylmethyloxycarbonyl amino acids and suitable protected amino acids as described previously [20]. Peptide purity was ascertained by mass spectral analysis and analytical high-performance liquid chromatography (HPLC). Peptide concentration was determined by monitoring the absorbance of tyrosine and tryptophan residues at 280 nm.

2.2. In vivo experiments with LPS-mediated inflammation

All procedures involving animals were conducted in accordance with the guidelines of the Care and Use of Laboratory Animals and the National Institutes of Health and approved by the IACUC of UAB. Adult male Sprague–Dawley rats, weighing 322 ± 8 g (Charles Rivers, Wilmington, MA), were used throughout the study. Prior to experiments, the rats were acclimatized for one week in a 12 h light/dark cycle with free access to food and water. In the first experiment, nonfasted rats underwent injection of LPS (*Escherichia coli* 026:B6, Sigma–Aldrich, 5 mg/kg, intraperitoneally [i.p.]) and one minute post LPS, saline (1 ml) or mR18L at increasing doses of 1, 2.5 and 5 mg/kg was administered i.p. at a site other than the LPS injection. Blood was collected at 0 h, 2 h, 6 h, and 24 h post LPS injection. Effects of different doses of mR18L on plasma levels of cholesterol and TNF-α were measured. In the next experiments, we chose to use 2.5 mg/kg mR18L, administered either one minutes post LPS (protocol 1) or 1 h post LPS (protocol 2). In protocol 1, the animals were euthanized at 2 h after LPS. Liver tissue and plasma, isolated from the blood by centrifuging, were frozen for later analysis. In protocol 2, blood was collected at 0 h (baseline) and 6 h post LPS injection and animals were euthanized at 24 h. Plasma levels of total cholesterol and triglycerides were measured by commercial kits (Thermo Fisher Scientific Inc.). Plasma enzyme paraoxonase 1 (PON1) activity was determined by adding rat plasma to buffer containing paraoxon (Sigma–Aldrich), and measuring the rate of release of 4-nitrophenol at 405 nm as previously published [21,22]. The plasma concentration of TNF-α, IL-6, and IL-10 was quantified by using commercial ELISA kits (BD Biosciences).

2.3. Measurements of mR18L binding to LPS using surface plasmon resonance (SPR)

Binding of LPS to mR18L was assessed using a Biacore 2000 system (BiaCore AB, Piscataway, NJ). mR18L was immobilized on a CM5 sensor chip activated per the manufacturer's protocol with *N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminoisopropyl) carbodimide, and another chip surface was immobilized as a blank. LPS (*E.Coli* 026:B6) was diluted in HEPES buffered saline. 30 μl of LPS (0, 0.05, 0.1, 0.2, 0.4 mg/ml, in duplicates) flowed over the CM5 chip at a rate of 20 μl/min. The interaction was monitored as the changes of SPR response for 2.5 min at 25 °C. The same buffer was then introduced onto the sensor chip without LPS to start the dissociation. Surface regeneration was done by washing the chip with 10 mM glycine, pH 2.5. Bovine serum albumin (BSA, 0.1 mg/ml) was used as a negative control. The dissociation constant (KD) was determined as K_d/K_a by evaluation software.

2.4. Measurements of mR18L effects on endotoxin activity

Endotoxin activity of LPS in aqueous solutions and in rat plasma in the absence and presence of mR18L was measured by limulus amoebocyte lysate (LAL) assay using kinetic-colorimetric test (Endochrome-K, Charles River) in accordance with manufacturer protocol.

2.5. Measurements of mR18L effects on lipoprotein distribution of LPS in rat plasma

Plasma isolated from normal rats was incubated with Bodipy-LPS (4 μg/ml, *E. Coli* 055:B5, Molecular Probes) with or without mR18L (20 μg/ml) for 2 h in the dark and then fractionated by size exclusion chromatography using a Bio-Logic Fast Protein Liquid Chromatography (FPLC) system (Bio Rad, two tandem connected Superose 6 and SuperDex 200 columns, 0.4 ml/ml, 0.5 ml fraction volume). Protein profiles of fractionated lipoproteins were identified by measuring their absorbance at 280 nm. For cholesterol profiles, FPLC fractions were mixed with cholesterol reagent (Thermo Fisher Scientific Inc.), and absorbance at 505 nm was measured. The presence of Bodipy-LPS in eluents was examined by measuring fluorescent emission at 530 nm. Measurements were performed with 2% SDS, which induces complete Bodipy-LPS disaggregation and enhances emission at 530 nm [29].

2.6. Measurements of lipoprotein distribution of mR18L in rat plasma

Normal rat plasma was incubated with mR18L (100 μg/ml) for 2 h at 37° and then fractionated by size exclusion chromatography as described above. VLDL, LDL, and HDL fractions were pooled, delipidated using chloroform:methanol (2:1, v/v) and the aqueous phase, after concentration, was subjected to reverse phase HPLC analysis using an analytical C18 column and Beckman-Coulter HPLC system. A standard solution of mR18L was analyzed under identical conditions to identify the peptide in the pooled lipoprotein fractions.

2.7. Histological analysis

Liver tissues were fixed in 10% neutral buffered formalin, embedded in paraffin blocks, sectioned at 5 μm and stained with hematoxylin and eosin for morphological analysis. Sections ($n = 3$ in control groups, and $n = 4$ in LPS groups) were analyzed using standard histological techniques and the tissue inflammation/damage was subjectively assessed by a blinded experienced pathologist. Neutrophil counts were performed based on the segmented morphology in high power fields (40×) in 30 measurements from 10 different randomly selected alveolar or portal areas, respectively. Liver microscopy was performed using an Olympus BX51 microscope equipped with a Retiga 1300 camera (Q imaging).

2.8. SDS polyacrylamide gel electrophoreisis (SDS–PAGE); immunobloting analysis

Frozen (at −80 °C) liver tissue (30 mg) was powdered, then thawed and homogenized on ice in T-PER tissue lysis buffer (Pierce) supplemented with 2 mm phenylmethylsulphonyl fluoride, 2 mM EDTA, 0.25% Nonidet P-40, a protease and phosphatase inhibitor cocktail (EMD Chemicals). Lysates (30 μg protein, $n = 2$ in control groups, and $n = 6$ in LPS groups) were separated on a 7.5% bisacrylamide gel by SDS–PAGE, transferred to a PVDF membrane, and probed with antibodies to NOS2 (Santa Cruz) and α-Tubulin for loading control (Cell Signaling) diluted in 5% BSA, TBS.T-0.1% Tween-20 for 1 h at room temperature. Membranes were then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz), and proteins of interest were visualized on the film using Enhanced Chemiluminescent Reagent (Amersham). The film was optically scanned and iNOS and α-Tubulin bands were analyzed with Image-J Software (NIH, Bethesda, MD).

2.9. Statistical analysis

All results, unless otherwise specified, are reported as the mean ± SEM. Statistical analysis was performed using GraphPad Prism V.4.0.1 (GraphPad Software Inc.). Differences between multiple groups were assessed by one-way ANOVA with post-hoc Bonferroni's Multiple Comparison test. Differences between two groups were assessed by either unpaired (LPS vs. mR18L at 6 h) or paired (6 h vs. baseline) t-test. A value of $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Effects of mR18L on plasma cytokine levels and liver inflammation in LPS-treated rats

Initially, we assessed the efficacy and tolerance of 1, 2.5, and 5 mg/kg mR18L in the rats stimulated by 5 mg/kg LPS (i.p.). All rats demonstrated tolerance to mR18L administered one minute post LPS i.p. Compared to the effect of LPS alone, none of the mR18L doses decreased plasma cholesterol in normolipidemic rats with baseline plasma cholesterol level of 91.4 ± 5.3 mg/dL (Fig. 1A). At the same time, mR18L significantly reduced LPS-mediated plasma elevation of TNF-α starting at the lowest tested dose of 1 mg/kg (Fig. 1B). The effect of 2.5 mg/kg mR18L on TNF-α levels was stronger than the effect of 1 mg/kg mR18L ($P < 0.05$) and did not differ from the effect of 5 mg/ml mR18L, therefore we chose a dose of 2.5 mg/kg mR18L for subsequent experiments.

We then studied effects of 2.5 mg/kg mR18L on LPS-mediated inflammation in depth. When administered alone, mR18L had no effects on normal levels of TNF-α, IL-6, and IL-10 in rat plasma. However, the peptide significantly reduced LPS-mediated elevation of pro-inflammatory cytokines, TNF-α and IL-6, without significantly reducing anti-inflammatory cytokine IL-10 (Fig. 1C–E). Endotoxemia and systemic inflammation results in liver inflammation and injury [14,15]. Therefore, liver tissues from different treatment groups were stained with H&E and analyzed for tissue inflammation (Fig. 2A). H&E staining and microscopic examination of liver sections from saline and mR18L-treated animals showed normal morphological and histological features without signs of inflammation or tissue damage. In contrast, liver sections from LPS groups showed tissue inflammation, which was less in rats treated with LPS and mR18L than in rats treated with LPS alone. This observation was supported by counting of infiltrating neutrophils in the liver tissue as depicted in Fig. 2B.

Oxidative stress is an important mechanism of LPS-induced liver damage [30,31]. In the liver, LPS triggers the production of reactive oxygen species, including superoxide [32]. LPS also stimulates iNOS activity in the liver and vascular cells, which leads to increased production of nitric-oxide [33]. In addition to systemic hypotension and poor perfusion, large amounts of nitric-oxide produce direct hepatotoxic effects [34]. Moreover, nitric-oxide and superoxide react spontaneously to form the more toxic oxidant peroxynitrite [31]. Inhibition of iNOS has been shown to reduce LPS-induced oxidative stress and liver injury in rats [31]. Therefore, we measured the relative change of iNOS expression in the liver tissue in our experiment. As presented in Fig. 2C (see also Supplementary Fig. 2), LPS alone increased iNOS expression in the rat liver 2 h post LPS injection. When rats received both LPS and mR18L simultaneously, the LPS-mediated iNOS elevation was reduced ($P < 0.05$ vs. LPS alone, Fig. 2C).

3.2. Effects of mR18L on LPS

To explain the mechanisms of reduction of LPS-mediated effects in rats by mR18L, we hypothesize that the positively-charged amphipathic peptide mR18L can readily interact with negatively-charged LPS molecule, thus inhibiting LPS pathogenic effects. To test this hypothesis, we first evaluated binding characteristics of mR18L to LPS using surface plasmon resonance (Fig. 3A). The peptide readily bound LPS. The binding was fast and relatively strong with association (ka) and dissociation (kd) constants being 6.72E+4 1/Ms. and 0.003 1/s, respectively, and with steady-state affinity (equilibrium dissociation constant, KD) being 4.51E-7 M. Due to a direct binding to LPS, mR18L could inhibit LPS pathogenic activity by limiting LPS interaction with plasma components in rats. To demonstrate such an inhibitory effect, we measured endotoxin activity in rat plasma using LAL assay. The peptide significantly reduced endotoxin activity in LPS-treated rats ($P < 0.05$ vs. LPS alone, Fig. 3B). It has been also shown that LPS is mainly associated in plasma with HDL due to its highest binding capacity and then transferred to LDL and VLDL [35]. LPS binding to LDL and LVDL is considered pro-inflammatory and atherogenic [2,36,37]. In an *in vitro* experiment, we found that mR18L inhibits association of LPS with VLDL in rat plasma while increasing the association of LPS with HDL (Fig. 3C). We did not measure the distribution of mR18L between lipoprotein fractions in this experiment, however we suggest that mR18L-LPS complexes might associate with HDL in rat plasma as has been shown for apoA-I mimetic peptide L-4F [22]. In a separate *in vitro* experiment, we incubated mR18L in rat plasma and then searched for mR18L in plasma lipoproteins using HPLC. mR18L was detected only in HDL fractions. In rats, HDL is the predominant lipoprotein and that in part explains association of mR18L with HDL in rat plasma.

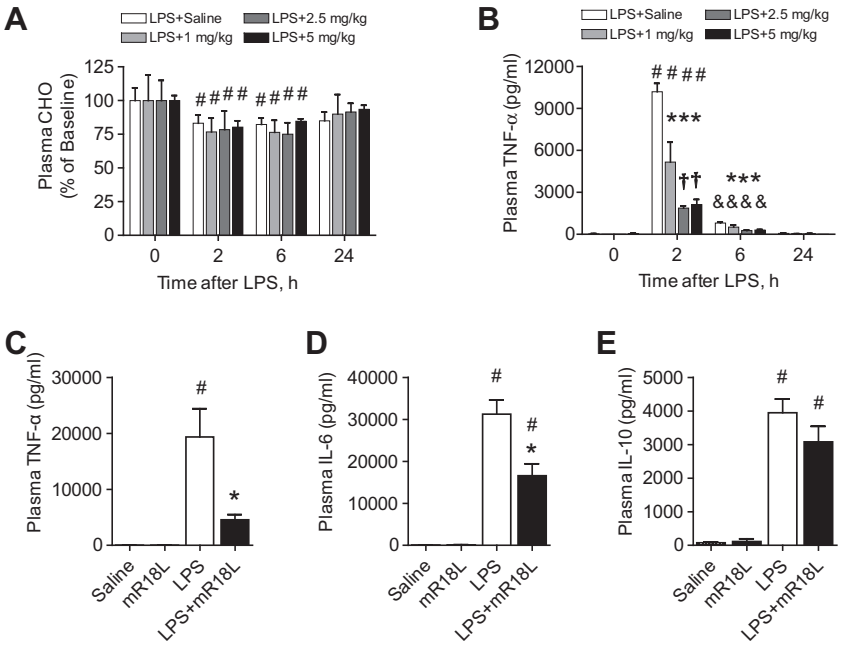

Fig. 1. mR18L inhibits inflammatory response in plasma of endotoxemic rats. (A and B) Dose- and time-dependent changes of plasma cholesterol (A) and IL-6 (B) levels in normolipidemic rats treated with 5 mg/kg LPS and 0, 1, 2.5, and 5 mg/kg mR18L (n = 3 rats/group). (C–E) Plasma levels of TNF-α (C), IL-6 (D), and IL-10 (E) at 2 h post 5 mg/kg LPS and 2.5 mg/kg mR18L (n = 5 rats/control groups and 5–7 rats/LPS groups). $^{\#}P < 0.05$ vs. Baseline or control groups, $^{*}P < 0.05$ vs. corresponding LPS, $^{\dagger}P < 0.05$ vs. 1 mg/kg, $^{\&}P < 0.05$ vs. 2 h.

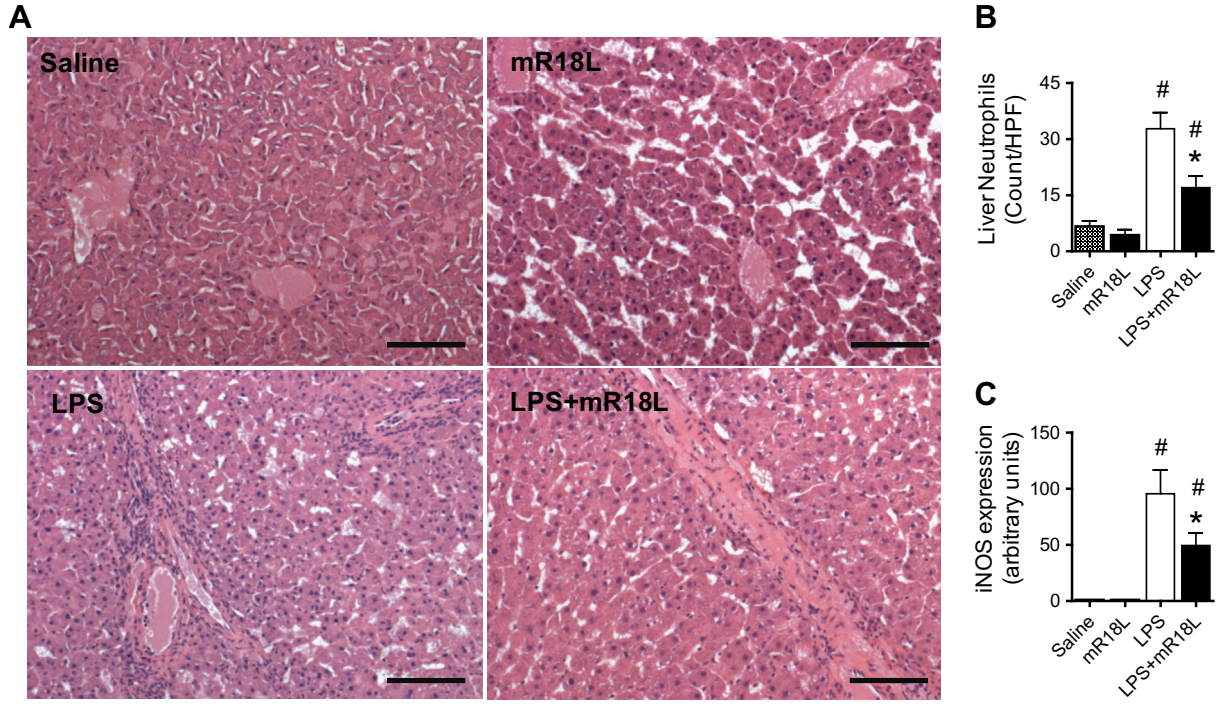

Fig. 2. mR18L inhibits liver inflammation in endotoxemic rats. (A) Liver sections, stained with H&E. Liver sections from saline and mR18L-treated rats showed no signs of inflammation. Liver sections from rats treated with LPS demonstrated significant infiltration of inflammatory cells, whereas liver sections from rats treated with LPS and mR18L had reduced infiltration of inflammatory cells. Scale bars, 100 μm. (B) Neutrophil counts in liver tissue (n = 3/control groups and n = 4/LPS groups). (C) Changes in iNOS levels in liver tissue measured by immunoblotting (see also Supplementary Fig. 2). $^{\#}P < 0.05$ vs. control groups, $^{*}P < 0.05$ vs. LPS.

3.3. Effects of mR18L administered 1 h post LPS

Since an important property of an anti-inflammatory and anti-atherogenic drug would be its ability to reduce inflammation, we tested anti-endotoxin and anti-inflammatory effects of mR18L when administered 1 h after LPS injection. By 1 h post LPS, LPS initiates an inflammatory cascade with rapid increase in plasma TNF-α [38], a major mediator of LPS effects [39]. In this experiment, while not affecting plasma cholesterol, mR18L reduced plasma endotoxin activity by 30% and plasma levels of IL-6 by 57% in rat plasma at 6 h post LPS (Fig. 4A–C). mR18L also reduced plasma levels of anti-inflammatory IL-10 by 38% (Fig. 4D), however the

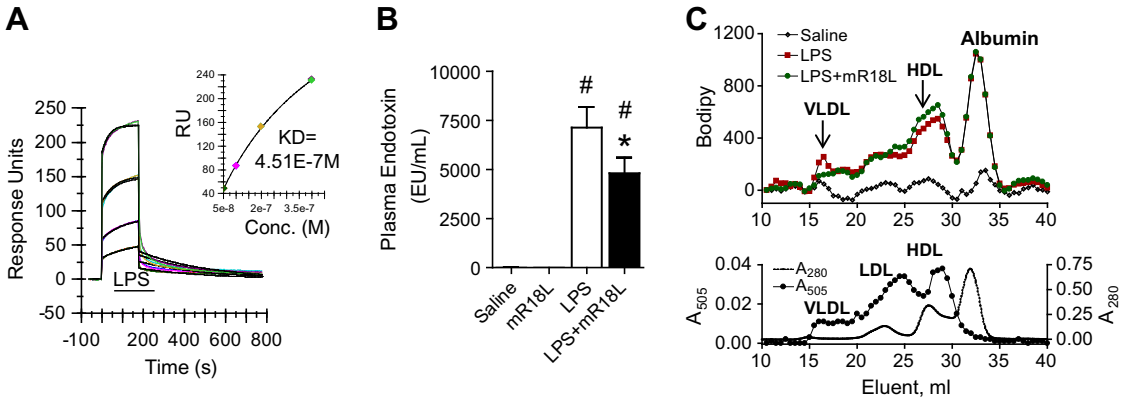

Fig. 3. Interaction of mR18L with LPS and plasma lipoproteins. (A) mR18L has high binding affinity to LPS as measured using surface plasmon resonance technique. mR18L was immobilized on a CM5 chip, and LPS (0, 0.05, 0.1, 0.2, 0.4 mg/ml, in duplicates) flowed over the samples. Binding was measured by observing the change in the SPR angle of mR18L. KD was calculated for LPS micelles with molecular weight of 1000 kDa. (B) mR18L inhibits endotoxin activity in rat plasma at 2 h post 5 mg/kg LPS and 2.5 mg/kg mR18L (n = 5–7/group). Endotoxin activity was measured by LAL assay. (C) mR18L inhibits association of LPS with VLDL and increases association of LPS with HDL. Plasma isolated from normal rats was incubated with Bodipy-LPS (4 μg/ml) with or without mR18L (20 μg/ml) for 2 h and fractionated by gel filtration. Bodipy fluorescence was measured at 530 nm (top panel). Plasma lipoproteins were identified by their specific cholesterol and protein profiles, measured at 505 nm and 280 nm, respectively (bottom panel). VLDL = very low density lipoprotein; LDL = low density lipoprotein; HDL = high density lipoprotein.

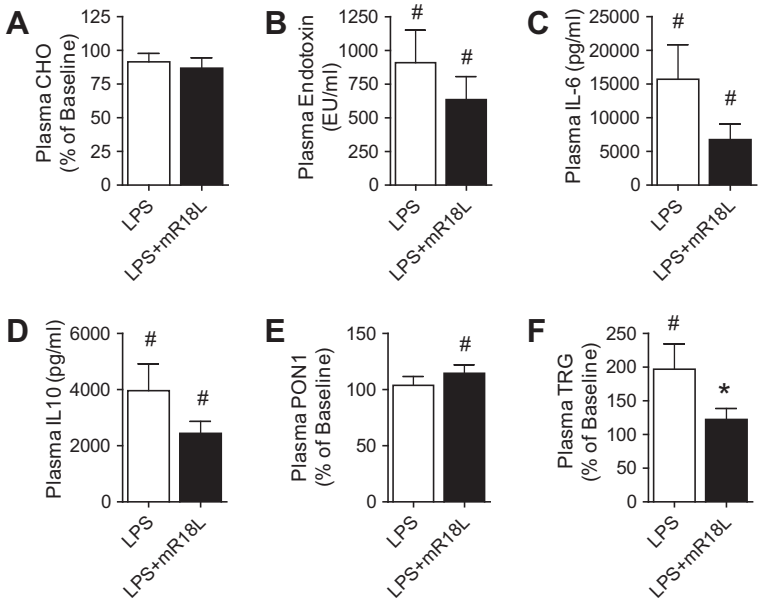

Fig. 4. Effects of mR18L administered 1 h post LPS injection. mR18L (2.5 mg/kg) was injected in rats 1 h post LPS (5 mg/kg) (n = 5 rats/group). Rat plasma was analyzed at 0 h (baseline, BL) and 6 h post LPS. There was no difference in baseline levels between groups. (A) Plasma cholesterol (BL = 115.9 ± 3.4 mg/dL, n = 10). (B) Plasma levels of endotoxin activity measured by LAL assay (BL = low). (C) Plasma IL-6 (BL = 824 ± 119 pg/mL). (D) Plasma IL-10 (BL = 449 ± 115 pg/mL). (E) Plasma PON1 activity (BL = 652 ± 14 U). (F) Plasma triglycerides (TRG, BL = 105.8 ± 7.0 mg/dL). $^{\#}P < 0.05$ vs. BL, $^{*}P < 0.05$ vs. LPS.

IL-10 to IL-6 ratio was more favorable in mR18L-treated rats (increased by 24%, not shown). In addition, mR18L increased the activity of potent plasma antioxidant enzyme PON1 in LPS-treated rats by 15% ($P < 0.05$ vs. baseline, Fig. 4E). PON1 is mainly associated with HDL in plasma [40], which again suggests that mR18L improves HDL function in endotoxemic rats. Finally, mR18L reduced LPS-mediated elevation of plasma triglyceride at 6 h post LPS (Fig. 4F), which might be mediated by the reduction of LPS-induced liver dysfunction.

4. Conclusion

We evaluated anti-endotoxin activity of promising apoE mimetic peptides, mR18L. In endotoxemic rats, mR18L decreased plasma levels of endotoxin and pro-inflammatory cytokines and inhibited LPS-mediated liver inflammation. Anti-inflammatory effects of mR18L were evident even after being administered 1 h post LPS. We suggest that anti-endotoxin activity of mR18L might be due to a direct interaction of mR18L with LPS and HDL. Such anti-endotoxin activity is an important anti-inflammatory property, which may increase anti-atherogenic potential of this promising orally active lipid lowering peptide. Because mR18L does not affect plasma cholesterol in normolipidemic animals, it might be also useful in gram-negative sepsis/inflammation.

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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.bbrc.2013.06.020.

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