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Molecular Mechanism Underlying the Suppression of Lipid Oxidation during Endotoxemia

Urmila Maitra, Samantha Baglin, Neeraj Singh, and Liwu Li

Laboratory of innate immunity and inflammation, Department of Biology, Virginia Tech, Blacksburg, VA 24061

Abstract

Although both inflammatory and metabolic complications occur during sepsis and endotoxemia, relatively few studies have examined the molecular mechanism underlying LPS-modulated metabolic changes during sepsis. In this report, we have demonstrated that LPS suppresses free fatty acid (FFA) oxidation, and consequently contributes to elevated plasma levels of FFA and triglyceride (TG). Furthermore, this process depends upon the interleukin-1 receptor associated kinase 1 (IRAK-1), one of the key TLR4 intracellular signaling kinases. IRAK-1^{-/-} mice fail to exhibit the dramatic rise in plasma FFA and TG levels compared to wild type (WT) mice following lethal LPS injection. Mechanistically, we demonstrated that LPS suppresses FFA oxidation through decreasing the expression levels of key FFA oxidative genes including CPT-1 and MCAD in both liver and kidney tissues of WT but not IRAK-1^{-/-} mice. The expression of CPT-1 and MCAD is controlled by nuclear receptors and co-receptors including PPAR α and PGC-1 α . We observed that LPS selectively suppresses the levels of PPAR α and PGC-1 α in tissues from WT, but not IRAK-1^{-/-} mice. Consequently, IRAK-1^{-/-} mice have a higher survival rate following a lethal dose of LPS. Our current study reveals a novel role for IRAK-1 in the metabolic alterations induced by LPS.

Keywords

Lipopolysaccharide; fatty acid oxidation; innate immunity; regulation; metabolism

INTRODUCTION

Sepsis is a complex yet acute syndrome with both inflammatory and metabolic complications. Disseminated bacterial infection releases the endotoxin lipopolysaccharide (LPS) into circulation, which subsequently triggers a cytokine storm responsible for inflammatory tissue damages (Cohen, 2002; Sriskandan and Altmann, 2008). In addition, there is a dramatic rise in the plasma levels of free fatty acids (FFA) and triglyceride (TG) due to enhanced lipolysis and decreased FFA oxidation in tissues including liver, kidney, heart, and skeletal muscle (Johnson et al., 2005; Khovidhunkit et al., 2004; Rosato et al., 1997; Wang and Evans, 1997; Wolfe and Martini, 2000; Zager et al., 2005). Since the oxidation of FFA, rather than glucose, is the most efficient supplier of energy to vital organs, such a metabolic change significantly

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*Address correspondence to: Liwu Li, Life Science 1 Building, Washington Street, Department of Biology, Virginia Tech, Blacksburg, VA 24061. Tel: 540-231-1433; Fax: 540-231-4043; lwli@vt.edu.

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decreases the energy supply to vital organs (Carre and Singer, 2008; Lind and Lithell, 1994). Collectively, elevated inflammation and reduced energy supply lead to multi-organ failure and death.

Although the molecular signaling processes leading to the induction of inflammatory mediators by LPS is relatively well studied, the mechanism contributing to reduced FFA oxidation is not well understood (Khovidhunkit et al., 2004). Recent studies have revealed that LPS treatment reduces the expression levels of key FFA oxidative enzymes such as carnitine palmitoyltransferase-1 (CPT-1) and medium chain acyl-CoA dehydrogenase (MCAD) in liver, skeletal muscle and kidney tissues (Feingold et al., 2008). The expression of CPT-1 and MCAD is under the control of nuclear receptors including PPAR α and PGC-1 α (Beigneux et al., 2000; Finck and Kelly, 2006; Kim et al., 2007; Kliewer et al., 2001; Schoonjans et al., 1996). Correspondingly, recent studies also reveal that LPS suppresses the levels of PPAR α and PGC-1 α in various tissues both *in vivo* and *in vitro* (Feingold et al., 2008; Wang et al., 2005).

IRAK-1 is a key intracellular signaling component downstream of TLR4, an LPS receptor (Gottipati et al., 2008; Huang et al., 2004; Li, 2004). A series of studies have revealed that IRAK-1 positively contributes to the activation of NF κ B, STAT1/3, and IRF5/7 (Huang et al., 2004; Oganessian et al., 2006; Uematsu et al., 2005). Consequently, IRAK-1 mediates LPS-induced expression of pro-inflammatory mediators (Deng et al., 2003; Swantek et al., 2000). Additionally, IRAK-1 has been linked to the pathogenesis of sepsis (Arcaroli et al., 2006), in that a genetic variant of the human IRAK-1 gene is associated with an elevated mortality rate in sepsis patients. Despite the prominent role IRAK-1 within the TLR4 signaling pathway, its involvement in LPS-mediated suppression of FFA oxidation has never been defined.

In the current study, we examined the contribution of IRAK-1 to LPS-mediated suppression of FFA oxidation *in vivo* and *in vitro* using IRAK-1^{-/-} mice and cells. Furthermore, we studied the effect of LPS on the expression profile of FFA oxidative enzymes in wild type and IRAK-1^{-/-} cells and tissues. Mechanistically, we analyzed the levels of key nuclear receptors such as PPAR α and PGC-1 α involved in the expression of FFA oxidative genes.

MATERIALS AND METHODS

Reagents

LPS (*E. Coli* O111:B4) was obtained from Sigma. The antibodies against PGC1 α , β -actin and PPAR α were purchased from Santa Cruz Biotechnology. The primer sets were obtained from IDT.

Mice

Wild-type C57BL/6 mice were obtained from the Charles River Laboratory. IRAK1^{-/-} mice with C57BL/6 background were kindly provided by Dr. James Thomas from the University of Texas Southwestern Medical School. All mice were housed and bred at Derring Hall Animal Facility in compliance with approved Animal Care and Use Committee protocols at Virginia Polytechnic Institute and State University. Wild type and IRAK-1^{-/-} mice of matched gender and age were injected with LPS (*E. Coli* O111:B4, Sigma) (25mg/kg body weight) or PBS intraperitoneally. Total blood was drawn 16 hours after the injection and plasma was collected for downstream analysis. Liver and kidney tissues were harvested and used for described assays.

Mouse survival study

WT and IRAK-1^{-/-} mice (n=14 per genotype) were injected with LPS (25mg/kg body weight) or PBS intraperitoneally. Injected mice were provided with drinking water only, and closely

monitored at 2 hr intervals. Survival and mortality were recorded for a period lasting for 50 hrs.

Western blot analysis

Isolation of whole cell lysates was performed using the T-PER Tissue protein extraction reagent (Thermo Scientific) according to the manufacturer's protocol. Briefly, the tissue samples (kidney and liver) were weighed and homogenized using the T-PER reagent containing protease inhibitors. The samples were centrifuged at $10,000 \times g$ for 5 min to pellet tissue debris. The supernatant was collected and stored at $-80^{\circ}C$ for downstream analysis. Western blotting analysis of the protein samples were performed as described previously. Immunoblots were developed using the Amersham ECL Plus chemiluminescent detection system (GE Healthcare). The intensities of the bands were quantified using the Fujifilm Multi Gauge software, and then normalized against β -actin levels.

Real time RT-PCR

Total RNA was prepared from small sections of mouse liver and kidney (50–100 μ g) using TRIzol (Invitrogen) according to the manufacturer's protocol. Reverse transcription was carried out using the High-Capacity cDNA Reverse transcription kit (Applied Biosystems) and subsequent real-time RT-PCR analyses were performed using the SYBR green supermix on an IQ5 thermocycler (Biorad). The relative levels of transcripts were calculated using the comparative $\Delta\Delta C_t$ method after normalizing with *GAPDH* levels as the internal control.

Free fatty acid (FFA) and triglyceride (TG) Assay

The levels of free fatty acids were measured in the plasma using the BioVision Free fatty acid colorimetric quantification kit according to the manufacturer's recommendation. Briefly, 7 μ l of sample was measured against a standard of varying concentrations of palmitic acid (provided by the kit) and the O.D. was measured at 570 nm in a 96-well microplate reader (Molecular Devices). Plasma triglyceride levels were quantified using the Wako triglyceride colorimetric assay kit using 2 μ l sample per well and a triglyceride standard provided by the kit. The samples were incubated for 5 min at $37^{\circ}C$ and measured on a microplate reader at 600 nm absorbance. Quantification was based on a standard curve derived by linear dilution of the standards included in the respective kits. The levels of both free fatty acid and triglyceride samples were calculated using the slope of the standard curve and the concentration was expressed as nmol/ μ l.

Statistical analyses

The results are expressed as means $\pm$ standard deviations (SD). Statistical significance was determined using the unpaired 2-tailed Student's *t*-test. *P*-values less than 0.05 were considered statistically significant.

RESULTS

IRAK-1^{-/-} mice have a higher survival rate following lethal LPS challenge

To determine the effect of IRAK-1 in the host response to disseminated endotoxin challenge, we injected either PBS or a lethal dose of LPS (25mg/kg body weight) intraperitoneally into WT and IRAK1^{-/-} mice. The mice were monitored on an hourly basis and their survival times were recorded. As shown in Fig. 1, LPS injection caused significant mortality in the WT mice, with 90% of the mice succumbing over the 50 hour time period. In contrast the mortality rate of IRAK-1^{-/-} was significantly lower, with only a 50% mortality rate during the same observation period. The differences in mortality between the WT and IRAK-1^{-/-} groups were compared using the log rank test, which showed a statistically significant difference ($p=0.018$)

at 50 h endpoint. This is consistent with a previous study demonstrating a higher survival rate in IRAK-1^{-/-} mice following lethal LPS challenge (Swantek et al., 2000).

Lethal LPS injection causes dramatic elevation of plasma FFA and TG levels in WT, but not IRAK-1^{-/-} mice

Elevated plasma levels of FFA and TG are hallmarks of endotoxemia and sepsis, due to elevated lipolysis and decreased FFA oxidation and utilization in vital organs and tissues (Khovidhunkit et al., 2004; Wolfe and Martini, 2000; Zager et al., 2005). Since IRAK-1^{-/-} mice display significantly higher survival rate following lethal LPS injection, we subsequently examined the plasma levels of FFA and TG in WT and IRAK-1^{-/-} mice. As shown in Fig. 2, 16 hrs post LPS injection, the plasma levels of FFA and TG were significantly higher in WT mice, changing from 0.2±0.052 to 0.5±0.091 for FFA and from 0.4±0.086 to 0.8±0.19 for TG. On the contrary, LPS injection failed to significantly alter the plasma levels of FFA and TG in IRAK-1^{-/-} mice.

LPS treatment decreases the expression of lipid oxidative genes in WT but not in IRAK1^{-/-} mice

To further elucidate the mechanism underlying IRAK-1 mediated lipid metabolism, we examined the expression levels of several key FFA oxidative genes in WT and IRAK1^{-/-} mice following lethal LPS injection. CPT-1 is necessary for transporting FFA across the mitochondrial membrane for subsequent breakdown and generation of energy in the mitochondria. MCAD is a key enzyme responsible for the first step of β -oxidation and breakdown of FFA carbon chains. As shown in Fig. 3A and B, lethal LPS challenge led to a significant decrease in the expression levels of CPT1 α and MCAD in WT liver tissues. Likewise, LPS injection also caused significant decrease in the levels of CPT1 β and MCAD in the kidney tissues from WT mice (Fig. 3C and D). In contrast, LPS failed to alter the levels of CPT-1 or MCAD in the liver and kidney tissues harvested from IRAK-1^{-/-} mice.

LPS treatment reduces the protein levels of PPAR α and PGC1 α in WT but not in IRAK1^{-/-} mice

Since the expression of CPT-1 and MCAD is known to be controlled by PPAR α (Kliwer et al., 2001; Schoonjans et al., 1996), we then examined the effect of LPS on the levels of PPAR in WT and IRAK-1^{-/-} mice. As shown in Fig. 4, LPS injection significantly reduced the levels of PPAR α in both liver and kidney tissues from WT mice (an 80% reduction in the liver and a 70% reduction in the kidney compared to the tissues from PBS injected control mice). In contrast, the PPAR α levels were not altered by LPS in either liver or kidney tissues harvested from IRAK-1^{-/-} mice.

In addition to PPAR α , several co-activators such as PGC-1 α also play a critical role in mediating the expression of genes responsible for FFA oxidation (Finck and Kelly, 2006). Earlier studies have indicated that LPS treatment also reduces the expression levels of PGC1 α (Feingold et al., 2008; Kim et al., 2007). Therefore, we examined the levels of PGC1 α in the liver extracts of WT and IRAK1^{-/-} mice after LPS administration. Similarly, the protein levels of PGC1 α were significantly reduced in the liver tissues of WT mice following LPS injection (an 85% reduction compared to the PBS injected control mice). Instead, the levels of PGC-1 α remained steady in liver tissues collected from IRAK1^{-/-} mice treated with either PBS or LPS.

DISCUSSION

We have demonstrated that IRAK-1 plays a critical role in LPS-modulated FFA oxidation during endotoxemia. IRAK-1^{-/-} mice do not exhibit the dramatic alteration in plasma levels

of FFA and TG, and have improved survival rates following a lethal LPS challenge. Mechanistically, IRAK-1 participates in LPS-mediated suppression of key FFA oxidative genes including CPT1 and MCAD, via suppressing the transcription factors PPAR α and PGC-1 α .

Our finding confirms and extends previous studies demonstrating the suppressive effect of LPS on FFA oxidation (Feingold et al., 2008; Khovidhunkit et al., 2004). The reduced expression of key FFA oxidative genes due to the LPS challenge is most likely responsible for this effect (Feingold et al., 2008). LPS exerts its pleiotropic effects through TLR4 and multiple downstream intracellular adaptor molecules as well as effector kinases (Su, 2005). Given the fact that multiple pathways diverge downstream of the LPS receptor TLR4, it is likely that selected intracellular molecules may be specifically involved in suppressing the expression of FFA oxidative genes. Our current study is the first to provide solid evidence that defines IRAK-1 as a key intracellular signaling molecule involved in the suppression of FFA oxidative genes.

Mechanistically, our data reveals that IRAK-1 is required for LPS-mediated suppression of nuclear receptors (PPAR α and PGC-1 α), necessary for the active expression of CPT-1 and MCAD (Figure 5). However, the means by which IRAK-1 related downstream signaling processes lead to reduced levels of PPAR α and PGC-1 α remains unknown. Several potential possibilities for the reduced levels of these nuclear receptors, including reduced transcription and/or translation, and elevated protein degradation, may be involved (Blanquart et al., 2003; Blanquart et al., 2004). In particular, ubiquitin-mediated degradation of PPAR has been previously reported (Blanquart et al., 2002). Moreover, IRAK-1 and its associated molecules such as TRAF6 and Tollip are known to be involved in protein ubiquitination and degradation (Brissoni et al., 2006; Conze et al., 2008; Didierlaurent et al., 2006). Further biochemical analyses using cultured cell lines are warranted to test whether LPS may trigger degradation of PPAR α and/or PGC-1 α via a pathway involving IRAK-1.

This study provides a potential therapeutic target for the development of anti-septic therapies. There is currently no effective drug available to treat sepsis, due to the complex inflammatory and metabolic complications involved in this syndrome. Antibiotics, fluid therapy, and corticosteroids remain the mainstay of sepsis treatment, but these administrations remain supportive at best. Additionally, therapies solely targeting inflammatory cytokines such as TNF α or IL-1 β have all failed clinical testing in the past (Abraham et al., 1998; Abraham et al., 1995; Goode et al., 2006). It is likely that interventions in both inflammatory and metabolic alterations are necessary in the prevention of devastating multi-organ failure that ensues following severe disseminated endotoxemia. Thus, compounds that could potentially inactivate IRAK-1, combined with anti-inflammatory agents, may be useful in treating sepsis.

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Fig. 1. Loss of IRAK-1 protects against LPS-induced endotoxemia

Effect of LPS on the survival rate percentage in WT and IRAK1^{-/-} mice. Fourteen mice of each genotype were injected with either 500 μ g (25mg/kg body weight) of LPS or vehicle (PBS) intraperitoneally. The survival rate (%) was monitored hourly and plotted against time. Differences in survival curves were analyzed with *log-rank* tests. *p=0.018

Fig. 2. Increased plasma FFA and TG levels in WT compared to IRAK1^{-/-} mice in response to LPS

(A) Differential effect of LPS on plasma free fatty acid levels in WT and IRAK1^{-/-} mice. Mice (n=4 per group) were injected with LPS or PBS (control) and the free fatty acid levels in the plasma were analyzed after 16 h. The results are representative of three independent experiments. (B) Differential effect of LPS on plasma TG levels. WT and IRAK1^{-/-} mice (n=4 per group) were injected with either 500 µg LPS or vehicle (PBS) intraperitoneally. The triglyceride levels in the plasma were measured after 16 h and expressed as nmol/µl by multiplying mg/dl by a conversion factor of 0.011. The error bar represents standard deviation (SD).

Fig. 3. Differential effect of LPS on fatty acid oxidation genes in WT and IRAK1^{-/-} mice

Five mice of each genotype were injected with either 500 μ g LPS or vehicle (PBS) intraperitoneally. After 16 h, liver and kidney tissues were isolated and total RNA was prepared using TRIzol reagent. RNA was reverse transcribed and the mRNA levels of multiple genes (A) Liver CPT1 α ; (B) Liver MCAD; (C) Kidney CPT1 β ; (D) Kidney MCAD mRNA levels were analyzed using real-time RT-PCR and plotted after normalization with GAPDH levels as the internal control. Each data point represents the mean $\pm$ SD of five animals. * $P < 0.05$, compared with untreated control, assigned a value of 1. The results are representative of at least three independent experiments.

Fig. 4. Effect of LPS on PPARα and PGC1α protein levels in WT and IRAK1^{-/-} mice
 (A) Mice were injected with either 500 μg LPS (25mg/kg body weight) or vehicle (PBS). After 16 h, the levels of PPARα were detected in the liver by immunoblotting using PPARα specific antibodies. The same blots were probed with β-actin as a measure of internal loading control. (B) The band intensities were quantified using the Fujifilm Multi Gauge software, and then expressed as the average of the percentages compared to the PBS-injected control group. (C) The levels of PPARα in the kidney 16 h after LPS injection in WT and IRAK1^{-/-} mice. Control animals of each genotype were injected with PBS. (D) The band intensities were quantified and expressed as bar graphs showing densitometric analysis. (E) The protein levels of PGC1α were detected in the liver tissues of WT and IRAK1^{-/-} mice (n=4) after LPS administration. (F) The band intensities were quantified and expressed as bar graphs showing densitometric analysis.

Fig. 5. A schematic illustration of LPS-mediated regulation of FFA oxidation

LPS reduces the levels of PPAR α and PGC-1 α via IRAK-1, and subsequently contributes to reduced expression of CPT-1 and MCAD in vital tissues such as liver and kidney. The reduced expression of CPT-1 and MCAD leads to decreased tissue FFA oxidation, and accumulation of FFA in circulation.