Infection decreases fatty acid oxidation and nuclear hormone receptors in the diaphragm

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Abstract **Respiratory failure is a major cause of mortality during septic shock and is due in part to decreased ventilatory muscle contraction. Ventilatory muscles have high energy demands; fatty acid (FA) oxidation is an important source of ATP. FA oxidation is regulated by nuclear hormone receptors; studies have shown that the expression of these receptors is decreased in liver, heart, and kidney during sepsis. Here, we demonstrate that lipopolysaccharide (LPS) decreases FA oxidation and the expression of lipoprotein lipase (LPL), FA transport protein 1 (FATP-1), CD36, carnitine palmitoyltransferase beta, medium chain acyl-CoA dehydrogenase (MCAD), and acyl-CoA synthetase, key proteins required for FA uptake and oxidation, in the diaphragm. LPS also decreased mRNA levels of PPARα and β/δ, RXRα, β, and γ, thyroid hormone receptor α and β, and estrogen related receptor alpha (ERRα) and their coactivators PGC-1α, PGC-1β, SRC1, SRC2, Lipin 1, and CBP. Zymosan resulted in similar changes in the diaphragm. Finally, in PPARα deficient mice, baseline CPT-1β and FATP-1 levels were markedly decreased and were not further reduced by LPS suggesting that a decrease in the PPARα signaling pathway plays an important role in inducing some of these changes. The decrease in FA oxidation in the diaphragm may be detrimental, leading to decreased diaphragm contraction and an increased risk of respiratory failure during sepsis.**—Feingold, K. R., A. Moser, S. M. Patzek, J. K. Shigenaga, and C. Grunfeld. **Infection decreases fatty acid oxidation and nuclear hormone receptors in the diaphragm.** ***J. Lipid Res.*** **2009.** 50: **2055–2063.**

Supplementary key words acute phase • thyroid hormone receptor • carnitine palmitoyltransferase I • fatty acid transport proteins

Respiratory failure is a major cause of morbidity and mortality in patients with septic shock (1, 2). This respiratory insufficiency is usually attributed to lung injury, but there is increasing evidence that decreased ventilatory muscle contraction contributes significantly to this respiratory failure (3, 4). Friman (5) reported that in humans, the maximal force that a muscle can produce and the endurance capacity of muscle decreases during infections. Moreover, animal models of infection also showed a decrease in the force that the ventilatory muscles can generate, leading to hypercapneic respiratory failure (3, 4, 6). Additionally, endotoxin (lipopolysaccharide, LPS) administration, a model of gram-negative sepsis, also impairs ventilatory muscle contractility (7–9). Similarly, treatment with tumor necrosis factor-alpha (TNF-α), an important cytokine that mediates many of the effects of sepsis and LPS administration, has been shown to also decrease diaphragmatic pressure and contraction (10). The administration of TNF-α antibodies partially blocked the deleterious effects of LPS on diaphragmatic contractility, suggesting that the effects of sepsis and LPS are mediated by cytokines (9).

The mechanisms accounting for the ventilatory muscle failure during sepsis are likely to be multifactorial (3, 4). In some circumstances, decreased systemic blood pressure could result in insufficient muscle blood flow resulting in a reduction in the delivery of oxygen and nutrients required for normal muscle function. However, studies have shown that blood flow to the ventilatory muscles increases during sepsis, which should compensate for the

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Abbreviations: ACC, acetyl CoA carboxylase; ACS, acyl-CoA synthetase; AGPAT, 1-acyl-glycerol-3-phosphate acyltransferase; Atp5g1, ATP synthase, H^+ transporting, mitochondrial FO complex, subunit c; CBP, CREB binding protein; Cox 5a, cytochrome c oxidase, subunit 5a; CPT-1β, carnitine palmitoyltransferase beta; ERRα, estrogen-related receptor alpha; FATP-1, FA transport protein 1; GPAT, glycerol-3-phosphate acyltransferase; HK1, hexose kinase 1; HK2, hexose kinase 2; Idh3a, isocitrate dehyrogenase 3 (NAD^+) alpha; LPS, lipopolysaccharide; MCAD, medium chain acyl-CoA dehydrogenase; Nduf58, NADH dehydrogenase (ubiquinone) Fe-S protein 8; PDK4, pyruvate dehydrogenase kinase isoenzyme 4; PGC-1, peroxisome proliferator-activated receptor gamma coactivator-1; SAA, serum amyloid A; SRC1, steroid receptor coactivator-1; TNF-α, tumor necrosis factor-alpha; TRAP, thyroid receptor-associated protein.

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increased metabolic demands of muscle (11). Muscle depends on both glucose and free FAs as an energy source required for muscle contraction and, because of its high energy demands, the diaphragm is particularly dependent on FA oxidation, which, compared with the oxidation of glucose, can produce greater amounts of ATP (12, 13).

FA oxidation in muscle and other tissues is regulated by activation of nuclear hormone receptors, particularly PPARs, thyroid hormone receptors (TRs), and estrogen-related receptor α (ERRα) (14–20). These nuclear hormone receptors form obligate heterodimers with RXR, allowing for the activation of gene transcription. In recent studies, we have shown that the expression of RXR, PPARα, TRα and β, and ERRα decrease in liver, kidney, and heart following the administration of LPS, a model of gram-negative bacterial infections, and zymosan, a model for fungal infections (21–26). Moreover, our studies have also demonstrated that in models of sepsis there is a reduction in the expression of crucial nuclear hormone receptor co-activators, such as peroxisome proliferator-activated receptor γ coactivator-1 α and β (PGC-1 α and β), that are required for the nuclear hormone mediated increases in gene transcription (23, 24, 26). In addition, the target genes of PPARα, TR, and ERRα in liver, kidney, and heart that are involved in FA metabolism are also markedly reduced. These data suggest that the decrease in these nuclear hormone receptors and their coactivators plays an important role in regulating FA metabolism during infection. In fact, studies have shown that, during sepsis, there is a decrease in FA oxidation in the liver, kidney, and heart (24, 27–32).

Based on the above observations, we have hypothesized that the ventilatory muscle failure that occurs in humans and animal models of sepsis may in part be due to abnormalities in FA oxidation that occur secondary to decreased expression in muscle of both nuclear hormone receptors and coactivators that regulate FA oxidation. A decrease in FA oxidation would result in decreased ATP formation leading to reduced muscle contractility. In the present article, we demonstrate that LPS and zymosan treatment decreases the expression of nuclear hormone receptors and coactivators in the diaphragm, a key respiratory muscle. In association with the decrease in nuclear hormone receptors and coactivators, FA oxidation and the expression of key enzymes required for FA oxidation are decreased.

MATERIALS AND METHODS

Materials

LPS (*Escherichia coli* 55:B5) was obtained from Difco and freshly diluted to the desired concentration in pyrogen-free 0.9% saline. Zymosan A and Tri-Reagent was obtained from Sigma. (1-^{14}C)-oleic acid (51.0 mCi/mmol) and (^{14}C [U])-palmitic acid (850 mCi/mmole) were purchased from PerkinElmer Life Sciences. iScript™cDNA Synthesis Kit was purchased from Bio-Rad, Hercules, CA. LightCycler 480 SYBR Green I Master was purchased from Roche Diagnostics.

Animals

Eight-week-old female C57BL/6 mice, PPARα deficient mice, and their wild-type controls, 129S1/SVIMJ mice, were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were maintained in a barrier room with a normal 12 h light-cycle and were provided with Harlan standard rodent chow and water ad libitum. For a typical experiment, mice were injected intraperitoneally with LPS (5 mg/kg) in saline, zymosan A in saline (80 mg/kg body weight), or with saline alone. The doses of LPS and zymosan used in this study have significant effects on triglyceride and cholesterol metabolism, but are not lethal. The half-maximally lethal dose (LD_{50}) for LPS in rodents is ∼30–50 mg/kg body weight. Food was withdrawn immediately after the injection of LPS, zymosan, or saline because LPS and zymosan induce anorexia in rodents. At 16 h, mice were euthanized with an overdose of halothane and diaphragms were excised, rapidly frozen, and stored at −80°C. All experiments were performed according to protocols approved by the Animal Studies Subcommittee of the San Francisco Veterans Affairs Medical Center.

FA oxidation and triglyceride synthesis

Fresh diaphragms were removed from control and 16 h LPS-treated animals, quickly weighed, and placed in cold 0.9% saline solution. 0.5 mm slices were then made with a McIlwain tissue slicer and 50 mg portions were placed in center well 25 ml Erlenmeyer flasks. Each flask contained 2 ml Krebs-Ringer phosphate buffer (pH 7.4), 1μCi 0.4 mM ^{14}C-oleic acid or ^{14}C-palmitic acid, and 0.15 mM BSA (FA free). Blank samples contained no tissue. Flasks were then sealed with septum caps and incubated in a shaking water bath for 2 h at 37°C. After the 2 h incubation, 0.9 ml 1N NaOH was added to each center well through the stopped cap and 1 ml H_2SO_4 to the incubation mix, also through the stopper cap. Flasks were allowed to incubate an additional 15 min, after which time flasks were removed from the water bath, caps removed, and 450 μl aliquots of NaOH (containing trapped $^{14}CO_2$) were removed from center wells and added to counting vials containing 12 ml of ScintiSafe 30% LSC-counting cocktail from Fisher Scientific. Incorporation of FA into triglyceride levels and cholesterol ester were measured after tissues were homogenized and lipids extracted by the Folch method. Extracted lipids were then run on a SIL G plate and eluted with hexane-diethyl ether-acetic acid (85:15:2) and visualized in an iodine tank. Triglyceride and cholesterol ester bands identified by comparing to triglyceride and cholesterol ester standards on the same plate were cut out and counted in ICN BetaMax scintillation fluid in a Beckman LS 6500 scintillation counter. In separate experiments, the protein levels in diaphragm from control and LPS-treated mice were determined by the Bradford method (Bio-Rad Laboratories, Inc.). Control protein was 71.9 μg/mg wet weight ± 6.1 versus 69.8 μg/mg wet weight ± 3.4 in LPS-treated animals (not significant).

Isolation of RNA and quantitative real-time PCR

Total RNA from mouse diaphragm was isolated from 100 mg of snap frozen tissue using Tri-Reagent. First strand cDNA was synthesized from 1 μg of total RNA with the iScript™cDNA Synthesis Kit, Bio-Rad). The real-time PCR contained in a final volume of 20 μl, 20 ng of reversed transcribed total RNA, 450 nM forward and reverse primers, and 10 μl of 2 × LightCycler 480 SYBR Green I Master. PCR was carried out in 96-well plates using Mx3000P™ Real-time PCR System (Stratagene, La Jolla, CA). The relative amount of all mRNAs was calculated using the comparative C_T method. 36B4 mRNA was used as the invariant control for all experiments. Quantitative PCR primers are listed in **Table 1**.

TABLE 1. Primer sequences used for quantitativePCR and NCBI gene accession numbers

36B4	GCGACCTGGAAGTCCAACTAC ATCTGCTGCATCTGCTTGG	X15267
ACC 1	CTGTGCCTGGGATCTTTTGTGTC CCAGGAACACTCCCAGCATGG	NM_133360.2
ACC 2	CCCAGCCGAGTTTGTCACT GGCGATGAGCACCTTCTCTA	NM_133904
ACOX1	GGTGGTATGGTGTCGTACTTGA GAATCTTGGGGAGTTTATCTGC	NM_015729
ACSL1	CAGGCAAAGCATGTCTTCAA TCCCAGATTTTTGGCTTGTC	NM_007981.3
AGPAT 2	AGCGGACAGAAGAAACTGGA TGAAGTAGACACCCCCAAGG	XM_130130
AtP5g1	AGTTGGTGTGGCTGGATCA GCTGCTTGAGAGATGGGTTC	NM007506
CBP	TGGAGTGAACCCCCAGTTAG TTGCTTGCTCTCGTCTCTGA	NM_001025432.1
CD36	GATGTGGAACCCATAACTGGAT CCCAGTCTCATTTAGCCACAGTA	NM_007643
Cox5a	GGGTCACACGAGACAGATGA GGAACCAGATCATAGCCAACA	NM_007747
CPT1β	CCCATGTGCTCCTACCAGAT CCTTGAAGAAGCGACCTTTG	NM_009948
DGAT 1	GGCCCAAGGTAGAAGAGGAC GATCAGCATCACCACACACC	NM_010046.2
DGAT 2	AGTGGCAATGCTATCATCACGT AAGGAATAAGTGGGAACCAGATCA	NM_026384
ERRα	CCTTCCCTGCTGGACCTC CGACACCAGAGCGTTCACT	NM_007953.2
FAS	GCTGCGGAAACTTCAGGAAAT AGAGACGTGTCACTCCTGGACTT	AF_127033
FATP 1	TGCTTTGGTTTCTGGGACTT GCTCTAGCCGAACACGAATC	NM_011977
GLUT1	GGATCTCTCTGGAGCACAGG TCCTCCTGGACTTCACTGCT	NM_011400
GLUT4	ACTCTTGCCACACAGGCTCT CCTTGCCCTGTCAGGTATGT	NM_009204
GPAT 1	CAACACCATCCCCGACATC GTGACCTTCGATTATGCGATCA	NM_008149
HK 1	TCTCCAGAATCATGCACCA GATCCTGCTCTTAGGCGTTC	NM_010438
HK 2	AACCGAACAAGCTGGTGTAC TGCACACATCTATAGGTGGC	NM_013820
Idh3a	CCTCCTGCTTAGTGCTGTGA CGTTGCCTCCCAGATCTTT	NM_029573
IL-1β	AGGCAGGCAGTATCACTCATTGT GGAAGGTCCACGGGAAAGA	NM_008361
LIPIN 1	CGCCAAAGAATAACCTGGAA TGAAGACTCGCTGTGAATGG	NM_172950.2
LPL	GCTGGTGGGAAATGATGTG TGGACGTTGTCTAGGGGGTA	NM_008509.2
LXRα	AGGAGTGTCGACTTCGCAAA CTCTTCTTGCCGCTTCAGTTT	NM_013839.1
LXRβ	AAGGACTTCACCTACAGCAAGGA GAACTCGAAGATGGGATTGATGA	NM_009473
MCAD	ATGCCCTGGATAGGAAGACA CATAGCCTCCGAAAATCTGC	NM_007382
Ndufs8	TGGCGGCAACGTACAAGTAT CCTCGGATGAGTTCTGTCCA	NM_144870
PDK4	CCGCTGTCCATGAAGCA GCAGAAAAGCAAAGGACGTT	NM_13743.2
PPARα	CCTGAACATCGAGTGTCGAATAT GTTCTTCTTCTGAATCTTGCAGCT	NM_011144
PPARβ/δ	CCACGAGTTCTTGCGAAGTC AACTTGGGCTCAATGATGTCA	NM_011145
PPARγ	CCACCAACTTCGGAATCA TTTGTGGATCCGGCAGTTA	NM_005037
PGC1α	TAGGCCCAGGTACGACAGC GCTCTTTGCGGTATTCATCC	NM_008904
PGC1β	CAAGCTCTGACGCTCTGAAGG TTGGGGAGCAGGCTTTCAC	NM_133249
RXRα	TGCCCATCCCTCAGGAA TGTTGTCTCGGCAGGTGTAG	XM_123763
RXRβ	CAAACGGCTCTGTGCAATCT AGCCCTCGCAGCTGTAAAC	NM_011306
RXRγ	GCCACCCTGGAGGCCTATA AGCAGAAGCTTGGCAAACCT	NM_009107
SAA	TTCTGCTCCCTGCTCCTG GTAATTGGGGTCTTTGCCACT	NM_178060
SRC1	TGGGTACCAGTCACCAGACA GAATGTTTGCGTTTCCACCT	NM_010881
SRC2	ACAGAACCAGCCAAACCAAC TGGTTGAGGATTTCCCTCTG	NM_008678
TNFα	CGGAGTCCGGGCAGGT GCTGGGTAGAGAATGGATGAACA	NM_013693.2
TRAP	CCTTCTTTCTCCGCAGTCAC GGAAGAGCAGCGTAAAATCG	NM_013634.2
TRα	GGATGGAATTGAAGTGAATGGAA CCGTTCTTTCTTTTTCGCTTTC	NM_178060
TRβ	AACCAGTGCCAGGAATGTC CCTCTTCTCACGGTTCTCCTC	NM_009380

Western blotting

Total protein extracts were obtained from diaphragm homogenized in RIPA buffer. Crude homogenates were centrifuged at 14,000 rpm for 30 min. The resulting supernatants were used for Western analysis. Protein concentration was determined using a Pierce BCA protein assay kit. Protein extracts (100μg) separated by SDS-PAGE transferred onto a PVDF membrane. Immunoblotting was performed by using rabbit anti-PPARα at 1:200 (101710, Cayman Chemicals) or rabbit anti-RXRα at 1:1000 (sc-553, Santa Cruz Biotechnology) and was detected using anti-rabbit IgG conjugated to horseradish peroxidase at a dilution of 1:50000. Immune complexes were visualized using SuperSignal Substrate (Pierce Biotechnology). Immuno-reactive bands obtained by autoradiography were quantified using the GS-710 Calibrated Imaging Densitometer (Bio-Rad Laboratories).

Statistical analysis

Data are expressed as the mean ± SE of experiments from four to five animals. The difference between two experimental groups was analyzed using the Student's *t*-test. In Fig. 6, a one way ANOVA with a Tukey-Kramer test was used to compare multiple groups to the control group.

RESULTS

Effect of LPS treatment on FA utilization by the diaphragm

Our initial experiments examined, in C57BL/6 mice, the effect of treatment with LPS, a TLR4 activator and a model of gram-negative infections, on the metabolism of FAs (oxidation and triglyceride synthesis) in the diaphragm, a key respiratory muscle. As shown in **Fig. 1A**, the production of $^{14}CO_2$ from FAs (1-^{14}C oleic acid) and (^{14}C [U]palmitic acid) was decreased by approximately 40% in diaphragms obtained from LPS-treated animals compared with controls, indicating a significant decrease in FA oxidation. In contrast, there was an approximate 20% increase in the incorporation of 1-^{14}C oleic acid or ^{14}C palmitic acid into triglycerides in the diaphragms of animals treated with LPS (Fig. 1B). The incorporation of FAs into cholesterol esters was similar in controls and LPS-treated animals (data not shown). These results indicate that FA oxidation in the diaphragm is reduced by LPS treatment,

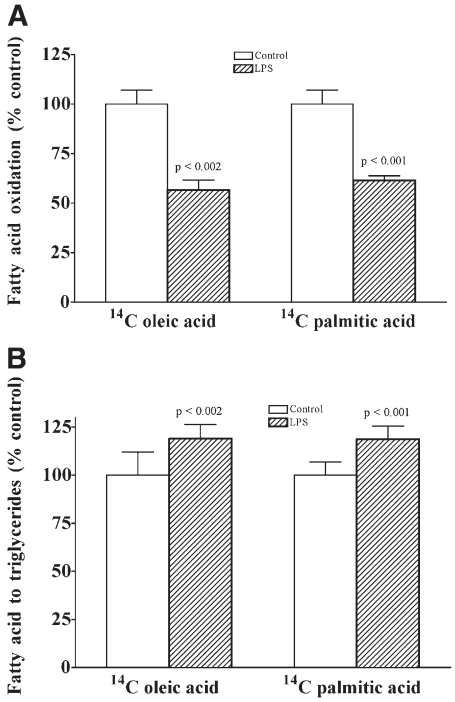

Fig. 1. Effect of LPS treatment on fatty acid oxidation and incorporation into triglycerides. Female C57BL/6 mice were injected with either 5 mg/kg LPS or vehicle (0.9% sterile saline). After 16 h, the animals were euthanized (N = 5), the diaphragms removed, and the following performed. A: FA oxidation was measured in the diaphragms as described in detail in the Methods section. Data (means ± SE) are expressed as percent control. B: Incorporation of FAs into triglycerides was measured in the diaphragms as described in detail in the Methods section. Data (means ± SE) are expressed as percent control.

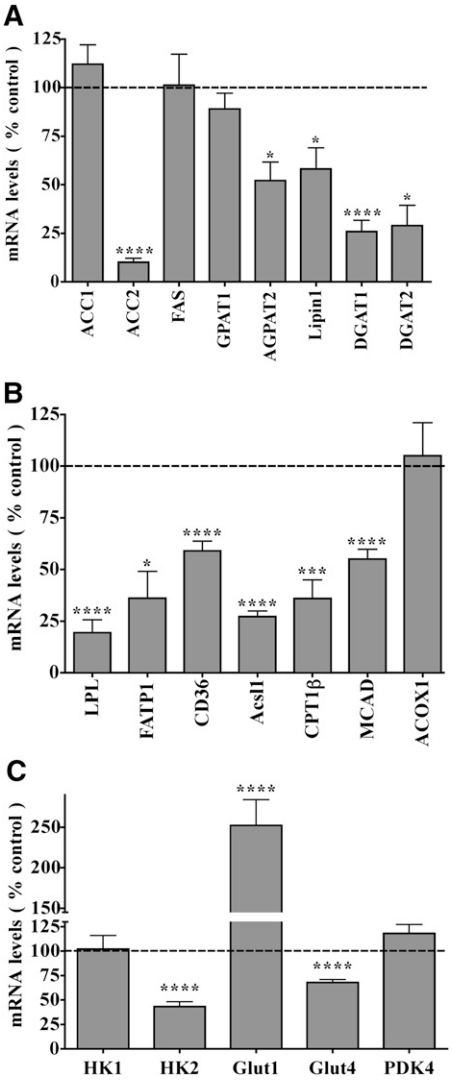

Fig. 2. Effect of LPS treatment on gene expression in mouse diaphragm. Female C57BL/6 mice were injected with either 5 mg/kg LPS or vehicle (0.9% sterile saline). After 16 h, total RNA was isolated from diaphragms, cDNA was synthesized with reverse transcriptase, and quantitative real-time PCR performed as described in Materials and Methods section. N = 4–5 per group. A: mRNA levels of genes that synthesize FA and triglycerides. The data are presented as the mean ± SEM. Data are expressed as a percentage of controls. *, $P < 0.02$; ****, $P < 0.001$. CT values for control animals are ACC1 = 25, ACC2 = 25, FAS = 26, GPAT1= 28, AGPAT 2 = 30, Lipin 1 = 22, DGAT 1 = 33, and DGAT 2 = 29. B: mRNA levels of genes that mediate FA uptake, transport, and oxidation. The data are presented as the mean ± SEM. Data are expressed as a percentage of controls. *, $P < 0.02$; ***, $P < 0.005$; ****, $P < 0.001$. CT values for control animals are LPL = 24, FATP-1 = 23, CD36 = 18, CPT-1β = 21, MCAD = 18, Acsl1 = 19, and ACOX1 = 22. C: mRNA levels of genes involved in glucose metabolism. The data are presented as the mean ± SEM. Data are expressed as a percentage of controls. ****, $P < 0.001$. CT values for control animals are HK1 = 28, HK2 = 24, Glut1 = 26, Glut4 = 22, PDK4 = 18.

whereas the incorporation of FAs into triglycerides is increased.

Effect of LPS treatment on genes that regulate FA metabolism in the diaphragm

We then examined the mechanism for the increase in FA incorporation into triglycerides in C57BL/6 mice. The expression of the key enzymes that synthesize triglycerides was not increased in the diaphragm by LPS administration (**Fig. 2A**). Indeed, the mRNA levels of 1-acyl-glycerol-3-phosphate acyltransferase 2 (AGPAT2), Lipin 1, diacylglycerol acyltransferase 1 and 2 (DGAT 1and 2) were all decreased in LPS-treated animals, whereas mRNA levels for glycerol-3-phosphate acyltransferase 1 (GPAT1) were not altered. The absence of an increase in the expression of the enzymes that synthesize triglycerides suggests that the increase in FA incorporation into triglycerides is substrate driven, likely due to a decrease in FA oxidation.

The decrease in FA oxidation following LPS treatment may result from the reduced expression of several proteins required for FA oxidation, including the genes that mediate triglyceride breakdown and FA uptake, esterification, transport, and oxidation in the diaphragm. As shown in Fig. 2B, 16 h after LPS treatment, mRNA levels of LPL, an enzyme required for the breakdown of triglyceride in circulating lipoproteins to FAs was decreased by 81%. In addition, the mRNA levels of FA transport protein 1 (FATP1) and CD36, two important FA transporters expressed in muscle, were decreased by 64% and 41%, respectively, in the diaphragm following LPS treatment. LPS treatment also dramatically decreased expression of

acyl-CoA synthetase (ACS), an enzyme responsible for converting FAs to fatty acyl-CoA, by 73%. Moreover, mRNA levels for the key enzyme that transports FA moieties across the mitochondrial membranes in muscle, carnitine palmitoyltransferase 1 β (CPT-1β), was decreased by 64%. Lastly, the expression of medium-chain acyl CoA dehydrogenase (MCAD), an enzyme that mediates FA oxidation, was decreased by 45% in diaphragms from LPS-treated animals. In contrast, mRNA levels of ACOX1, an enzyme that is involved in peroxisomal FA oxidation, was not altered by LPS treatment. These data clearly indicate that the expression of multiple genes that play important roles in FA oxidation are downregulated in the diaphragm following LPS administration. Although a decrease in the expression of any one of these genes could decrease FA oxidation, it is likely that the LPS-induced reduction of FA oxidation activity is the result of a coordinate downregulation of several genes that mediate multiple steps along the FA oxidation pathway. In contrast to the effects of LPS on the genes involved in FA oxidation, the mRNA levels of genes involved in FA synthesis, acetyl CoA carboxylase 1 (ACC1) and FAS, were not altered in the diaphragm following LPS treatment (Fig. 2A). However, the mRNA levels of acetyl CoA carboxylase 2 (ACC2) were markedly decreased.

We also examined the effect of LPS treatment on genes involved in glucose metabolism. We found that the mRNA levels of hexokinase 1 (HK1) were unchanged whereas the expression of hexokinase 2 (HK2) and Glut 4 was decreased by 43% and 34% respectively (Fig. 2C). In contrast, the expression of Glut 1 was increased 2.5-fold in the diaphragms of LPS-treated animals (Fig. 2C). The expression of pyruvate dehydrogenase kinase, isoenzyme 4 (PDK4) was also unchanged with LPS treatment.

Effect of LPS treatment on nuclear hormone receptor expression in the diaphragm

Nuclear hormone receptors play a key role in regulating FA metabolism in muscle and other tissues. Therefore, we next determined the effect of LPS administration on the expression of a number of nuclear hormone receptors that are well recognized to regulate FA metabolism in C57BL/6 mice. As shown in **Fig. 3A**, mRNA levels of PPARα and PPARβ/δ, which regulate FA oxidation, were reduced by 71% and 31%, respectively. Western blotting revealed that PPARα protein levels were decreased by 48% following LPS treatment (Fig. 3B). In contrast, mRNA levels of PPARγ were not significantly altered by LPS treatment. The expression of all three RXRs was also decreased in the diaphragms of LPS-treated animals (RXRα 56%, RXRβ 41%, and RXRγ 76% decrease). Western blotting demonstrated that RXRα protein levels were decreased by 53% after LPS administration (Fig. 3C). Both TRα and β mRNA levels were also markedly decreased in the diaphragms of animals treated with LPS. Additionally, LPS treatment decreased ERRα expression by 50% in the diaphragm. Finally, LXRβ expression was not altered by LPS treatment whereas LXRα was only slightly decreased. These data indicate that LPS treatment specifically

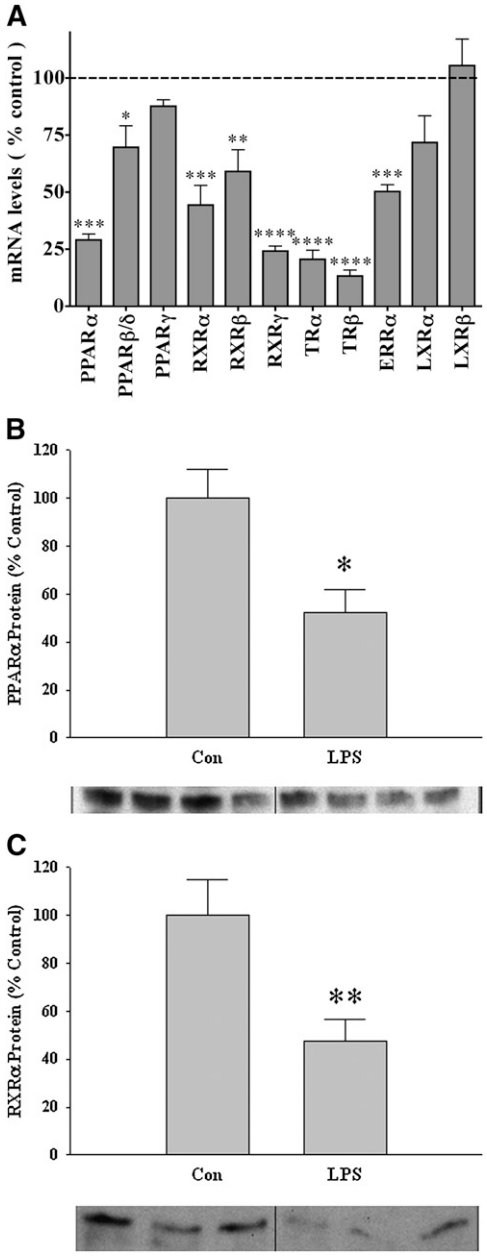

Fig. 3. Effect of LPS treatment on the expression of nuclear hormone receptors. Female C57BL/6 mice were injected with either 5 mg/kg LPS or vehicle (0.9% sterile saline). After 16 h, total RNA was isolated from diaphragms, cDNA was synthesized with reverse transcriptase, and quantitative real-time PCR performed as described in Materials and Methods section. A: mRNA levels of nuclear hormone receptor genes. The data are presented as the mean ± SEM. Data are expressed as a percentage of controls. N = 4–5 per group. *, $P < 0.02$; ** $P < 0.01$, ***, $P < 0.005$; ****, $P < 0.001$. CT values for control animals are PPARα = 25, PPARβ/δ = 25, PPARγ = 25, RXRα = 26, RXRβ = 26, RXRγ = 29, TRα = 24, TRβ = 26, ERRα = 25, LXRα = 25, and LXRβ = 27. B: Protein extract was prepared from mouse diaphragm and PPARα protein levels were measured by Western blot as described in Materials and Methods section. C: Protein extract was prepared from mouse diaphragm and RXRα protein levels were measured by Western blot as described in Materials and Methods section. The data are presented as the mean ± SEM. Data are expressed as a percentage of controls. N = 6–7 per group. *, $P < 0.02$; **, $P < 0.01$. Autorads shown are from representative blots. GAPDH protein levels were unchanged by LPS treatment.

decreases the expression of a number of nuclear hormone receptors that are important regulators of FA oxidation.

Effect of LPS treatment on nuclear hormone receptor coactivators in the diaphragm

Recent studies by our laboratory have shown that LPS treatment not only decreases the expression of key nuclear hormone receptors that regulate lipid metabolism in liver, heart, and kidney, but LPS also decreases the expression of coactivators that are required for the increase in gene transcription that occurs with activation of these nuclear hormone receptors. As shown in **Fig. 4A**, the mRNA levels of PGC-1α and β, steroid receptor coactivator-1 and 2 (SRC 1 and 2), lipin 1, and CREB binding protein (CBP) are all decreased in the diaphragms of animals treated with LPS. In contrast, mRNA levels of thyroid receptor-associated protein (TRAP) are not altered in the diaphragms of LPS-treated animals.

Because PGC-1α is well known to regulate mitochondria formation and function, we next determined the effect of LPS treatment on the expression of a number of mitochondria proteins in the diaphragm. As shown in Fig. 4C, mRNA levels of ATP synthase, H^+ transporting mitochondrial FO complex, subunit c (atp5g1), cytochrome c oxidase, subunit 5a (Cox5a), isocitrate dehyrogenase 3 (NAD^+) α (Idh3a), and NADH dehydrogenase (ubiquinone) Fe-S protein 8 (Ndufs8) are all decreased in the diaphragm of LPS-treated animals. These results indicate that infection decreases the expression of not only nuclear hormone receptors in the diaphragm but also key coactivators. Moreover, there is a decrease in the expression of mitochondrial proteins, which could contribute to the decrease in FA oxidation.

Although the expression of many genes is decreased in the diaphragm following LPS treatment, there are several genes that increase. Specifically, TNF-α mRNA levels were increased 1.8-fold, IL-1β mRNA levels were increased 2.2-fold, and serum amyloid A levels were increased 75-fold in the diaphragm 16 h after LPS treatment (data not shown).

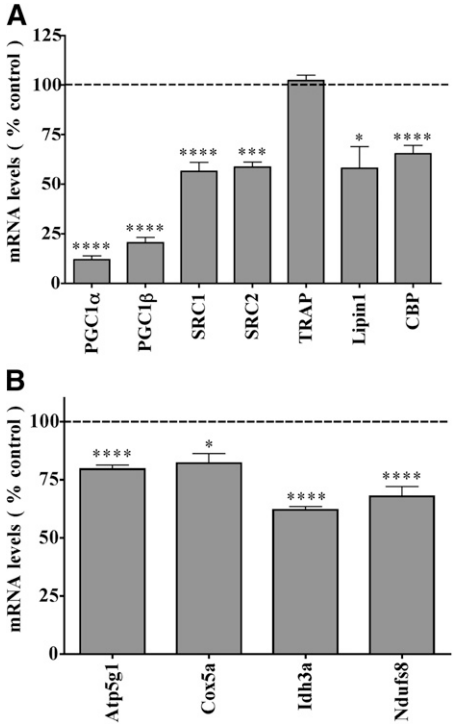

Fig. 4. Effect of LPS treatment on coactivators and mitochondrial genes. Female C57BL/6 mice were injected with either 5 mg/kg LPS or vehicle (0.9% sterile saline). After 16 h, total RNA was isolated from diaphragms, cDNA was synthesized with reverse transcriptase, and quantitative real-time PCR performed as described in Materials and Methods section. N = 4–5 per group. A: mRNA levels of nuclear hormone receptor coactivators are measured. The data are presented as the mean ± SEM. Data are expressed as a percentage of controls. *, $P < 0.02$; ***, $P < 0.005$; ****, $P < 0.001$. CT values for control animals are PGC-1α = 23, PGC-1β = 26, SRC1 = 25, SRC2 = 25, TRAP = 25, Lipin 1 = 22, and CBP = 22. B: mRNA levels of mitochondrial genes are measured. The data are presented as the mean ± SEM. Data are expressed as a percentage of controls. *, $P < 0.02$; ****, $P < 0.001$. CT values for control animals are Atp5g1 = 18, Cox5a = 18, Idh3a = 20, and Ndufs8 = 21.

Effect of zymosan on gene expression in the diaphragm

We next determined whether treatments that mimic other infections also affect gene expression in the diaphragm of C57BL/6 mice. Zymosan activates TLR 2 and the administration of zymosan is a model of fungal infections. As shown in **Fig. 5**, zymosan induces a marked decrease in the expression of the same nuclear hormone receptors, coactivators, and proteins that play a key role in FA metabolism. The changes induced by zymosan treatment are very similar to those observed after LPS administration and suggest that these changes are not unique to LPS, but rather are also induced by other infections.

Effect of LPS treatment on mRNA levels in PPARα deficient mice

To determine the role of PPARα in mediating these changes in the diaphragm, we next determined the effect of LPS treatment in PPAR deficient mice (knockout mice). As described above for C57/Bl6 mice, LPS treatment of

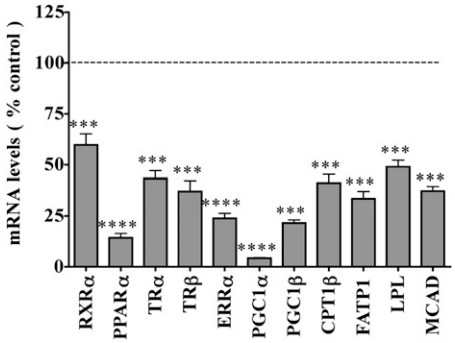

Fig. 5. Effect of zymosan treatment on gene expression in the diaphragm. Female C57BL/6 mice were injected with either 80 mg/kg zymosan or vehicle (0.9% sterile saline). After 16 h, total RNA was isolated from diaphragms, cDNA was synthesized with reverse transcriptase, and quantitative real-time PCR performed as described in Materials and Methods section. The data are presented as the mean ± SEM. Data are expressed as a percentage of controls. N = 4–5 per group. ***, $P < 0.005$; **** $P < 0.001$.

the wild-type control mice (1291/SVIMJ) also induced a marked decrease in the expression of TRα and β, ERRα, PGC-1α and β, CPT-1β, FATP1, and MCAD in the diaphragm (**Fig. 6**). In PPARα deficient mice, basal mRNA levels of TRα and β, ERRα, and PGC-1α and were not markedly altered in the diaphragm and LPS treatment resulted in a marked reduction in their expression similar to that observed in wild-type mice (Fig. 6). However, CPT-1β and FATP1 mRNA levels were markedly decreased in the basal state in PPAR deficient mice, indicating that PPARα plays a key role in regulating the basal expression of these genes in the diaphragm (Fig. 6). Moreover, LPS administration did not result in a significant decrease in the mRNA levels of CPT-1β or FATP1 in the diaphragms of PPARα deficient mice, suggesting that the LPS-induced decrease in the PPARα signaling pathway plays a key role in the downregulation of the expression of these genes during infection. With regard to MCAD, the basal level of expression is reduced by approximately 40% in PPARα deficient mice (not statistically significant compared with wild-type mice) and LPS treatment results in a further reduction in mRNA levels (Fig. 6; not statistically significant compared with PPARα knock-out control), suggesting that whereas PPARα signaling may play a role in the regulation of MCAD, other factors are also involved. It is well known that MCAD is regulated by ERRα (33) and, as shown in Fig. 6, LPS treatment also results in a decrease in the expression of ERRα.

DISCUSSION

The present study demonstrates that infection decreases FA oxidation in the diaphragm, a muscle that plays an important role in respiration. This decrease in FA oxidation is accompanied by a decrease in the expression of a large number of genes that play key roles in the uptake and oxidation of FAs by muscle cells. Specifically, LPL, FATP1, CD36, ACS, CPT-1β, and MCAD mRNA levels are all decreased in the diaphragm in two different models of infection (LPS or zymosan administration), indicating that there is a coordinate downregulation of a large number of proteins that mediate various steps in the FA oxidation pathway. In addition, a number of mitochondrial proteins, including Atp5g1, Cox5a, Idh3a, and Ndufs8, are also downregulated by infection and likely also contribute to the decrease in FA oxidation. Whereas FA oxidation is decreased, the incorporation of FAs into triglycerides is somewhat increased in the diaphragm. This increase appears to be substrate driven as expression of the enzymes that catalyze the incorporation of FAs into triglycerides is decreased rather than increased. Thus, in the diaphragm, similar to the liver, heart, and kidney, infection induces a decrease in FA oxidation with an increase in FA incorporation into triglycerides (24, 27–32).

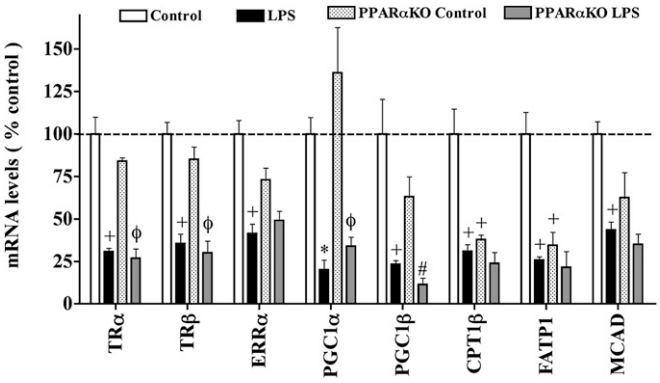

Fig. 6. Effect of LPS treatment in PPARα deficient mice. Female 129S1/SVIMJ mice or PPARα knock-out mice were injected with either 5 mg/kg LPS or vehicle (0.9% sterile saline). After 16 h, total RNA was isolated from diaphragms, cDNA was synthesized with reverse transcriptase, and quantitative real-time PCR performed as described in Materials and Methods section. The data are presented as the mean ± SEM. Data are expressed as a percentage of controls. N = 5 per group. *, $P < 0.05$ versus WT control; +, $P < 0.01$ versus WT control;#, $P < 0.05$ versus KO control; φ, $P < 0.01$ versus knock-out control.

These changes in FA metabolism in the diaphragm are likely mediated by alterations in the levels of nuclear hormone receptors and their coactivators that regulate the expression of the enzymes and transport proteins that play an important role in FA oxidation. In the present study, we demonstrate that mRNA levels of PPARα, PPARβ/δ, TRα, TRβ, and ERRα are all decreased in the diaphragm following the administration of LPS or zymosan. Moreover, the expression of all three RXR isoforms, the obligate heterodimer partners required for the activity of these nuclear hormone receptors, is also decreased during infection. Furthermore, many of the coactivators that are required for the nuclear hormone mediated changes in transcription, including PGC-1α and β, SRC 1 and 2, lipin 1, and CBP, also are decreased during infection. Thus, the decrease in the obligate heterodimer partners RXRα, RXRβ, and RXRγ, coupled with a decrease in coactivators, together with a decrease in the levels of the receptors that regulate FA metabolism would be expected to lead to reductions in the enzymes and transport proteins noted above. Just as the decrease in FA oxidation is the result of a coordinate downregulation of several genes that mediate multiple steps along the FA oxidation pathway, the decrease in expression of enzymes and transport proteins is also likely to be due to downregulation at a number of different sites that control gene expression. Finally, the changes in nuclear hormone receptor and coactivator expression in the diaphragm that occur during infection are very similar to those seen in liver, heart, and kidney (21–26).

Lecarpentier et al. (34) have shown that PPARα deficiency impaired the mechanics and energetics of the diaphragm and led to histological abnormalities including a decrease in the number of active myosin cross-bridges. Additionally, a marked increase in entropy production leading to thermodynamic dysfunction was reported, indicating abnormalities in fuel homeostasis (34). In the present manuscript, studies in PPARα deficient mice suggest that the decrease in expression of certain genes during infection is dependent on alterations in the activity of PPARα.

Specifically, in PPARα deficient mice, basal CPT-1β and FATP1 expression is decreased in the diaphragm and LPS administration does not result in a further decrease in either CPT-1β or FATP1. In contrast, MCAD expression is also decreased in the diaphragm of PPARα deficient mice and LPS administration results in a further decrease in MCAD, suggesting that other pathways independent of PPARα can account for the LPS induced downregulation. It is well known that ERRα also regulates MCAD expression and, therefore, it is likely that the decrease in ERRα signaling would also contribute to the decrease in MCAD expression (33). It should be noted that the decrease in PPARα activity that occurs in infection could be due to a decrease in PPARα, a decrease in RXR, or a decrease in coactivators. In previous studies in cultured hepatocytes, we were unable to demonstrate that the change in any single component was able to entirely account for the alterations in gene expression regulated by PPARα transcription (26). Rather, it appears that there are multiple changes that together account for the decreases in PPARα activity.

During infection, decreased FA oxidation is seen in conjunction with decreases in nuclear hormone receptors and coactivators in multiple tissues, including the liver, heart, and kidney, as well as the diaphragm. Most of the changes in protein synthesis and lipid metabolism that occur during infection are beneficial to the organism. One can only speculate on the potential benefits of decreasing FA oxidation in these organs. Infection stimulates an increase in triglyceride-rich lipoproteins, which have been shown to directly bind toxic bacterial products, such as endotoxin and lipoteichoic acid, and thereby reduce their harmful effects (35). Additionally, triglyceride-rich lipoproteins could provide FAs for metabolism by cells of the immune system that play a crucial role in host defense or tissue repair. For example, studies have shown that LPS stimulates the uptake of triglyceride by macrophages (35, 36). Decreased FA oxidation in the liver directs more FAs into triglyceride synthesis in the liver, thereby enhancing VLDL synthesis and secretion. The decrease in the oxidation of FAs by heart, kidney, and diaphragm along with the mobilization of FAs from adipose tissue are likely part of a coordinated systemic metabolic response to make more FAs available to the liver for the production of VLDL. Together, these metabolic changes could facilitate host defense and/or tissue repair.

However, the decrease in FA oxidation in the heart, kidney, and diaphragm could also have detrimental effects. As compared with the oxidation of glucose or lactate, the oxidation of FA produces the most energy per molecule. ATP generated from FA oxidation is an important energy source for metabolically active tissues such as the heart, kidney, and diaphragm. During severe sepsis, multi-organ failure, including renal and heart failure, often occur and one can speculate that the inability to generate energy via FA oxidation might contribute to the development of these abnormalities (2). Similarly, as discussed in detail in the introduction, respiratory failure, which is in part due to decreased ventilatory muscle contraction, is a major cause of morbidity and mortality in patients with overwhelming infections (1, 2). The decrease in FA oxidation in the diaphragm demonstrated in this study could contribute to the ventilatory muscle failure. It should be recognized that ventilatory demand increases during infections because of both an increase in minute ventilation and alterations in airway mechanics (3). Additionally, lung disease, such as acute respiratory distress syndrome, also increases respiratory needs and the alterations in FA oxidation described in this article could prevent an adequate respiratory response to these burdens.

Studies have shown estrogen signaling regulates lipid metabolism in liver and heart. Female PPARα deficient mice are able to survive treatment with etomoxir, which blocks FA oxidation, whereas male PPARα deficient mice have a very high mortality. Treating male PPARα deficient mice with estrogens improves their survival and reduces triglyceride accumulation in the liver and heart (37). It is likely that estrogen signaling increases FA oxidation. In the present study, female mice were evaluated and it is likely that the metabolic abnormalities that occur with infection would be at least as severe or even more severe in male mice.

In summary, the present study demonstrates that infection results in a decrease in FA oxidation in the diaphragm because of the decreased expression of key proteins required for the uptake and oxidation of FAs. This decrease is accounted for by decreases in the transcription factors that regulate FA oxidation and metabolism, RXRs, PPARα, PPARδ, TFα, TRβ, ERRα and their coactivators. Whereas this decrease in FA oxidation may be beneficial by increasing the availability of FAs for liver VLDL production, it may also be detrimental, leading to decreased diaphragm muscle contraction, which contributes to increased risk of respiratory failure.

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