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n-3 PUFA attenuate lipopolysaccharide-induced down-regulation of toll-like receptor 4 expression in porcine adipose tissue but does not alter the expression of other immune modulators

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Abstract

The objective of this study was to test the hypothesis that the inflammatory response to lipopolysaccharide (LPS) in vivo is accompanied by down-regulation of toll-like receptor (TLR) 4 in adipose tissue, and a source of protected n-3 polyunsaturated fatty acid (PUFA) attenuates this response. Seventy-two castrated male pigs were individually fed either a control (CONT) diet, or the CONT diet containing 1.87% (LF) or 7.50% (HF) protected n-3 PUFA on a weight basis for 7 weeks. Adipose and muscle tissue biopsy samples were taken at Weeks 1, 2, 3, 4 and 7 to assess gene expression and/or confirm tissue enrichment with eicosapentaenoic acid and docosahexaenoic acid and reflected the n-3 PUFA contained in the diet. The LPS challenge was performed at week 7 and consisted of sequential injections of 10 and 2.5 μg LPS per kilogram of body weight 23 h apart. The LPS challenge resulted in a marked down-regulation ($P=.004$) of TLR4 at the protein level in the adipose tissue of challenged vs. control pigs, but LF and HF clearly blocked this response at the mRNA level. Although LF and HF also attenuated ($P<.001$) the LPS-induced acute febrile response and lowered ($P<.002$) serum concentrations of tumour necrosis factor α. Cyclooxygenase 2 and 12-lipoxygenase were readily expressed in porcine adipose tissue, but there was no effect of LF, HF or LPS on expression levels of these inflammatory mediators, or that of TNF and interleukin 6, at the conclusion of the challenge period. These findings indicate that adipose tissue responds to LPS administration in vivo by reducing TLR4 mRNA and protein abundance and that the anti-inflammaotry effects of n-3 PUFA do not include down-regulation of TLR4 in adipose tissue.

Keywords: Pig; n-3 Polyunsaturated fatty acids; Lipopolysaccharide; Inflammation; Toll-like receptor 4

1. Introduction

Historically, proinflammatory cytokines have been viewed predominantly as products of dedicated immunocytes, such as macrophages and lymphocytes. However, the adipocyte is emerging rapidly as an endocrine and immune cell that produces cytokines and other molecules that modulate metabolic and immunological pathways, centrally and in peripheral tissues. Furthermore, at least some of the toll-like receptors (TLRs), which are critical determinants of pathogen recognition [1], are expressed in adipose tissue [2]. Recent findings indicate that the adipocyte mounts a typical innate immune response when cultured with the lipopolysaccharide (LPS) [2,3]. Furthermore, we have also determined that the nuclear factor kappa B (NFκB) transcription factor, a major determinant of inflammatory gene expression, is activated by LPS in primary porcine adipocytes and that this induction is coupled to the production of interleukin (IL) 6 and tumour necrosis factor (TNF) α [3].

Inflammation in adipose tissue is a marked comorbidity of obesity and is intimately linked with the onset and progression of insulin resistance [4,5]. In both human and animal studies, the biological effects of n-3 polyunsaturated fatty acid (PUFA) encompass attenuated inflammatory responses [6] and improved immune status [7,8]. It is also intriguing that enhanced insulin sensitivity and improvements in the hyperglycaemia of diabetes are also associated with n-3 PUFA [9] and may relate directly to immune

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modulation by these fatty acids. In vitro experiments show clearly that n-3 PUFA disrupt the activation of NFκB by LPS in a murine monocytic cell line [10]. Moreover, n-3 PUFA [i.e., docosahexenoic acid (DHA)] modulate TLR4 signaling in vitro in RAW 264.7 macrophages, 293T cells [11] and human monocytes and dendritic cells [12]. In fact, experiments performed with dominant negative TLR4 constructs in cultured immunocytes indicate direct interactions between fatty acids and the TLR4 receptor [12]. It is thus intriguing that immunocytes exposed to LPS for prolonged periods become refractory to the ligand, at least in part, through down-regulation of receptor expression and loss of membrane receptor protein [13]. Currently, there is a paucity of data addressing the potential for LPS or n-3 PUFA to regulate key immune modulators in adipose tissue in vivo. Therefore, the primary objective of this study was to determine whether LPS administration or dietary n-3 PUFA altered the expression of TLR4 or other inflammatory genes in adipose tissue.

2. Methods

2.1. Animals and experimental treatments

All procedures outlined in this experiment were approved by the JBS United Animal Care and Use Committee. Seventy-two crossbred (Large White×Landrace) male castrate pigs (initial weight 42±5 kg) were placed in individual pens, blocked according to initial live weight, and allocated to one of three dietary treatments, in a randomised block design. The diets consisted of a standard pig finisher diet containing 0% (CONT, 30 pigs), 1.87% (LF, 21 pigs) or 7.5% (HF, 21 pigs) n-3 PUFA in the form of a protected fish oil (JBS United, Sheridan, IN, USA). Based on extensive intake data (data not shown), it was estimated that the pigs would receive approximately <1, 5 or 20 g/day of n-3 PUFA from the diet (Table 1). Pigs were fed ad libitum and had free access to water at all times.

2.2. Sample and data collection

At Weeks 1, 2, 3, 4 and 7, adipose and muscle tissue samples were collected by surgical biopsy from three pigs from each dietary treatment to determine the fatty acid profile. Biopsies were taken using a biopsy punch following anesthesia with an im injection (0.04 ml/kg) of a 1:1 mixture of Xylazine-HCl (Lloyd Laboratories, Shenandoah, IA, USA) and Telazol-HCl (Fort Dodge Animal Health, Fort Dodge, IA, USA). The biopsy was taken 5 cm caudal to the last rib and approximately 5 cm off the dorsal midline. The tissue was snap-frozen in liquid nitrogen, the wound closed with nylon suture material and a topical analgesic was applied. The pigs also received a single injection of an antibiotic. Sutures were removed within 3 weeks. No pig was biopsied more than once throughout the 7 weeks.

Table 1
Ingredient composition of the diets (%) as fed

Ingredient	Diet		
	Control	LF	HF
Corn — yellow, shelled	66.40	64.39	58.35
48% Soybean meal	31.10	31.23	31.65
Custom 40-L*	2.50	2.50	2.50
Protected fish oil	0.00	1.87	7.50

* Custom 40-L vitamin, mineral, and phytase premix (JBS United, Inc., Sheridan, IN).

At 7 weeks of feeding, 18 control pigs and nine pigs from each of the LF and HF diets, which had not been biopsied previously, were used for the LPS challenge portion of the experiment. The challenge treatments consisted of CONT−LPS, CONT+LPS, LF+LPS and HF+LPS. Pigs weighing 96±8 kg where given either an initial (i.e., 0 h) intramuscular injection of LPS [10 μg/kg body weight (BW)] from *Escherichia coli* serotype 055:B5 (Sigma–Aldrich, St Louis, MO, USA) or a no-LPS control injection of sterile saline in the lower abdominal region. At 23 h, the pigs received a second injection of either saline or a lower level of LPS (2.5 μg/kg BW). The LPS was dissolved in sterile 0.9% (wt/vol) NaCl solution so that an injection of 0.1 ml/kg of BW would achieve the desired quantity of LPS. At approximately 36 h, adipose and muscle biopsies were taken from three pigs per treatment as previously described.

Pigs were restrained by snaring and blood samples (10 ml, jugular venipuncture), and rectal temperatures were obtained from all pigs in the challenge experiment at 0, 2, 6, 12, 25 and 31 h after the initial injection. Blood samples were placed on ice pending transport back to the laboratory. Serum was separated by centrifugation (2000*g* for 15 min at 4°C) and stored at −80°C until analysed.

2.3. Serum analysis

Serum concentrations of IL10 and TNF-α were measured at 0, 2, 25 and 36 h post LPS challenge using porcine-linked ELISA kits (R&D Systems, Minneapolis, MN, USA and Pierce Endogen, Rockford, IL, USA, respectively).

2.4. Fatty acid analysis

Lipids from muscle and adipose tissue samples were extracted by the method of Lepage and Roy [14] with minor modifications. Briefly, 0.5 g of tissue was homogenised in 2.5 ml 4:1 methanol/hexane, and 200 μl of 3.7 mmol heptadecanoic acid per liter of methanol was added to each sample as an internal standard. Fatty acid methyl esters (FAMES) were analysed by gas chromatography (model 6890; Hewlett–Packard, Palo Alto, CA, USA) fitted with a Omegawax 320 (30 m×0.32 mm internal diameter, 0.25 μm) capillary column (Sigma–Aldrich). The injector and detector temperatures were 250°C, and the oven temperature was at 200°C.

2.5. RNA extraction and real-time polymerase chain reaction

Total RNA was recovered from adipose tissue using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Residual DNA was

Table 2
Primers used for qPCR analysis

Gene	Primer	Oligonucleotide sequence (5′–3′)	Annealing temperature (°C)	Cycles
TLR4	S	GAATATTTTTCTAACCTGCCCAACCTGGAG	60	40
	AS	CCAGCCAGACCTTGAATACAAGTTTTCATTACATC		
TNFα	S	ACTCGGAACCTCATGGACAG	55	42
	AS	AGGGGTGAGTCAGTGTGACC		
IL6	S	TACTGGCAGAAAACAACCTG	55	40
	AS	GTACTAATCTGCACAGCCTC		
IL10	S	ATGGGCGACTTGTTGCTGAC	60	40
	AS	CACAGGGCAGAAATTGATGACA		
COX2	S	ATAAGTGTGACTGCACCCGAAC	60	40
	AS	GGTGGGCTATCAATCAGATGTG		
12LO	S	TCCTGAAACCAGACACGACA	60	40
	AS	AAACCCTGACGCCAAATACC		
AMPKα2	S	CGACGTGGAGCTGTACTGCTT	65	40
	AS	CATAGGTCAGGCAGAACTTGC		
RPL32	S	TGGAAGAGACGTTGTGAGCAA	55	40
	AS	CGGAAGTTTCTGGTACACAATGTAA		

S, sense primer; AS, antisense primer.

removed with Turbo DNase (Ambion, Houston, TX, USA). Thereafter, 2 μg of DNA-free RNA was reverse-transcribed using the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). The quantitative polymerase chain reaction (qPCR) conditions and sequences encoding the genes for porcine RPL32 (housekeeper), TLR4, TNF-α, IL6, IL10, cyclo-oxygenase-2 (COX2), 12-lipoxygenase (12LO) and adenosine monophosphate-activated protein kinase (AMPK) are shown in Table 2. Thermal cycler conditions for PCR were 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, annealing temperature (Table 3) for 30 s and 72°C for 30 s. The PCR products were cloned into pGEMT vector (Promega, Madison, WI, USA) and sequenced for transcript verification. Real-time reactions were carried out in an iCycler using the IQ SYBR Green Supermix kit (BioRad, Hercules, CA, USA). Transcript abundance was calculated by regression against the standard curves, which were generated in the same reaction by serial dilution of the respective plasmids. Expression was normalized against the RPL32 housekeeper and expressed as the log of the starting quantity.

Table 3
Dietary fatty acid profile (g/100g)

Fatty acid		Diet		
		CONT	LF	HF
Capric acid	12:0	1.39	1.67	1.27
Myristic acid	14:0	0.00	0.78	2.54
	14:1	0.00	0.00	0.00
	15:0	0.00	0.00	0.00
Palmitic acid	16:0	15.24	15.50	16.66
	16:1	0.00	1.11	3.50
	17:1	0.00	0.00	0.00
Stearic acid	18:0	2.81	2.85	3.07
	18:1	22.11	21.03	18.49
Linoleic	18:2n-6	56.88	52.74	42.59
γ-Linolenic acid	18:3n-6	0.00	0.00	0.00
α-Linolenic acid	18:3n-3	1.58	3.08	2.85
	20:1	0.00	0.00	0.61
	20:2	0.00	0.00	0.00
Arachidonic acid	20:4n-6	0.00	0.00	0.00
EPA	20:5n-3	0.00	1.19	4.04
Docosapentaenoic acid	22:5n-3	0.00	0.00	0.70
DHA	22:6n-3	0.00	1.07	3.68
	Total	100.00	100.00	100.00
	Saturated	19.44	19.79	23.54
	n-3	1.58	5.33	11.27
	n-6	58.46	52.74	42.59
	n-6/n-3	36.1	9.9	3.8

2.6. Western blotting

Whole frozen adipose samples (0.5 g) were homogenised on ice in 700 μl buffer A [50 mM Tris-HCl (pH 7.5), 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM DTT, 0.1 mM Phenylmethylsulfonyl fluoride, 10% glycerol] containing 1% Triton X-100, 5 μM aprotinin, leupeptin and pepstatin. The lysates were centrifuged at 6000*g* for 20 min at 4°C to remove insoluble material. Thereafter, the supernatant extracts were collected and protein concentration determined using the BCA reagent (Pierce, Rockford, IL, USA). The extracts were frozen at −80°C until the western blot analyses were performed.

To measure TLR4 protein expression, 50 μg of total whole cell protein extract were separated by sodium docecyl sulfate–polyacrylamide gel electrophoresis (12% resolving gel), transferred to a nitrocellulose membrane and probed (1:500 dilution) with a rabbit polyclonal anti-TLR4 antibody (sc-10741; Santa Cruz Biotechnology, CA, USA) overnight. The membranes were then probed with a goat antirabbit IgG–horseradish peroxidase conjugate (1:20,000) (Pierce) at for 1 h at room temperature. Blots were developed using the SuperSignal West Pico Chemiluminescent Substrate system (Pierce) and imaged onto microfilm for image analysis and densitometry. Signal intensity was quantified using Quantity One 1-D analysis software (Bio-Rad, Hercules, CA, USA).

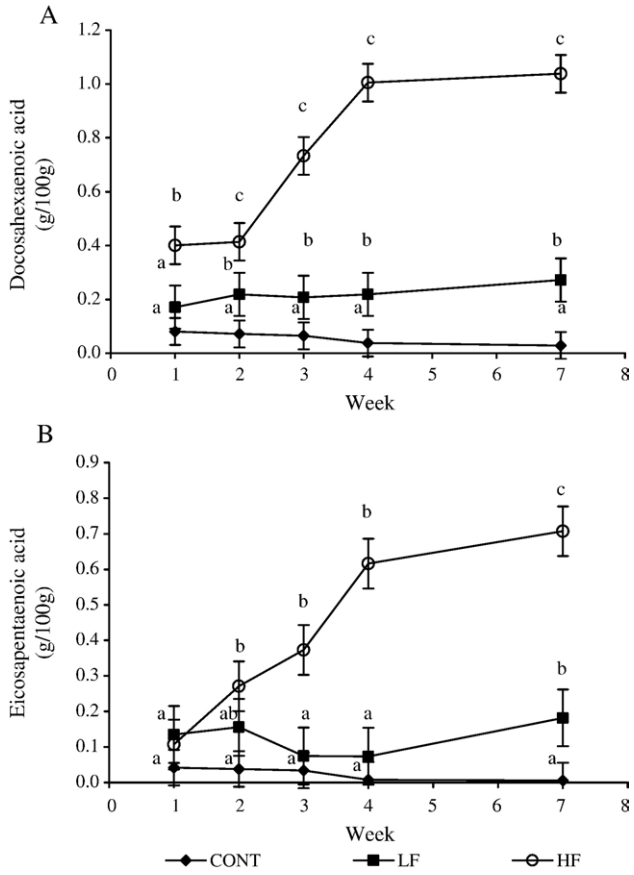

Fig. 1. Subcutaneous adipose tissue enrichment of (A) docosahexaenoic acid and (B) eicosapentaenoic acid fatty acid in the finisher pigs fed 0 (CONT), 1.87% (LF) or 7.5% (HF) n-3 PUFA for 7 weeks. Letters a, b and c represent significant differences within week ($P<.05$) ($n=3$ pigs per treatment per week).

2.7. Statistical analysis

All data were analysed using the PROC MIXED procedure of SAS (Version 9.1; SAS Institute, Cary, NC, USA). Blocks were formed based on initial BW where there was no significant block effect. Least-squares means are presented, and differences are considered significant at $P<.05$ and tending towards significance at $P<.10$.

3. Results

3.1. Diet analysis and tissue fatty acid enrichment

All diets contained between 1.6 and 11.3 g per 100 g of total n-3 PUFA (Table 3). As expected, the control diet only contained the n-3 PUFA, α-linolenic acid (1.6 g/100 g total fatty acids). While the DHA and eicosapentaenoic acid (EPA) percentages were evenly match in both the LF and HF diets and these were not detected in the CONT diet, this resulted in an increase in the total n-3 PUFA content by 3.8% (LF) and 9.7% (HF) from the CONT (Table 3). This in turn, greatly altered the n-6:n-3 PUFA ratios between the CONT, LF and HF diets (36, 10 and 4, respectively) (Table 3). Measured feed intake among the treatments prior to the LPS challenge were not significantly different (2.5 kg/d, $P>.10$). Measured n-3 PUFA intake for EPA in the CONT, LF and HF treatments was 0.0, 1.1 and 3.7 g/d, respectively. Similarly, DHA intake was calculated to be 0.0, 1.2 and 4.0 g/d, respectively. Therefore, the total n-3 PUFA intake was increased in the LF- (5.3 g/d) and HF-fed (11.3 g/d) pigs, compared to the CONT (1.6 g/d), which primarily resulted from the EPA and DHA inclusion from the protected fish oil. The sequential changes in DHA and EPA content of subcutaneous adipose tissue over the 7-week period are shown in Fig. 1. The magnitude of adipose n-3 enrichment was greater in pigs fed the HF diet, and the extent of DHA and EPA incorporation was similar within-diet. The adipose

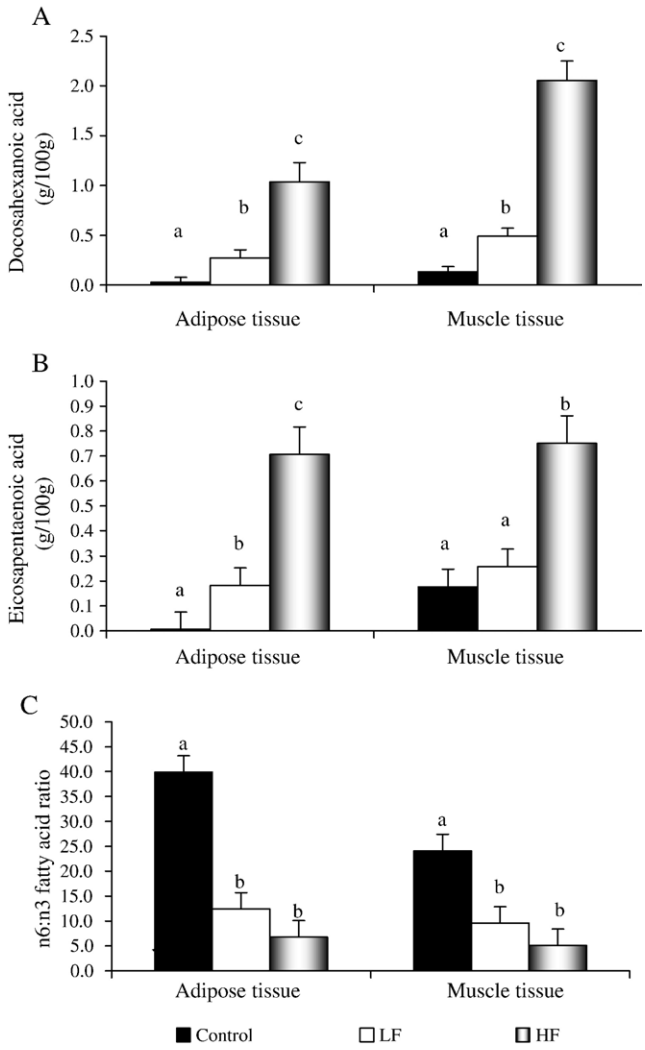

Fig. 2. Adipose and muscle fatty acid analysis from finisher pigs fed n-3 PUFA for 7 weeks at 0% (CONT), 1.87% (LF) or 7.5% (HF). (A) docosahexaenoic acid g/100 g of the total fatty acid content. (B) Eicosapentaenoic acid (g/100 g). (C) The n-6:n-3 fatty acid ratio in muscle and adipose tissue. Tissue samples were collected by biopsy from 3 pigs per treatment, and results represent the means±S.E. Different letters (a-c) within tissue represent significant differences ($P<.05$).

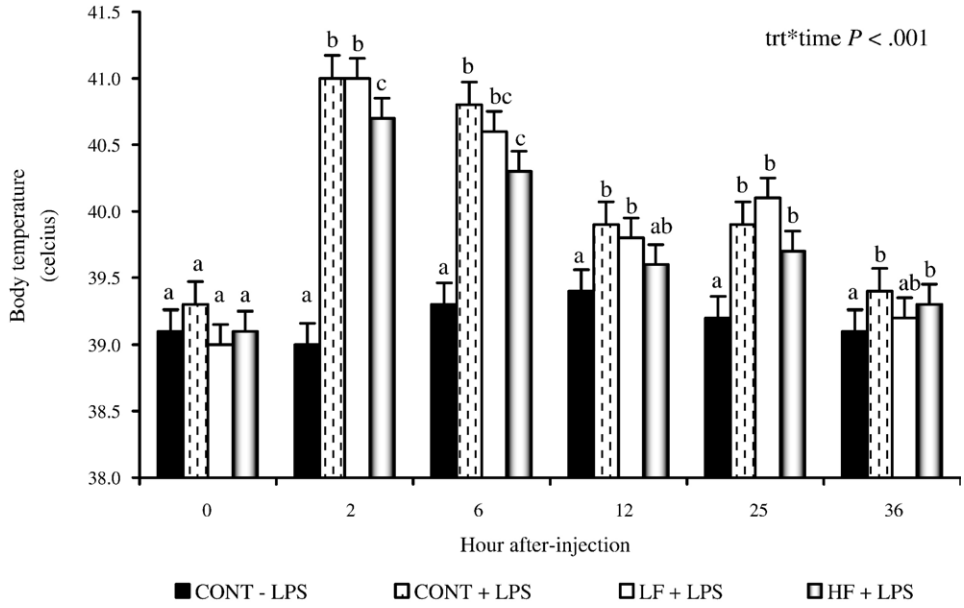

Fig. 3. Core body temperatures of finisher pigs fed n-3 PUFA for 7 weeks at 0% (CONT), 1.87% (LF), or 7.5% (HF) and challenged with 10 and 2.5 μg/kg BW LPS or saline at 0 and 23 h. Rectal temperatures were taken at 0, 2, 6, 25 and 36 h post initial challenge. Data represent means of $n=9$ pigs per treatment±S.E.M. Different letters (a-c) within time point represent significant differences ($P<.05$).

tissue sample collected from the muscle biopsy site (off the midline, caudal to the last rib) showed a similar diet-dependent pattern of n-3 PUFA enrichment at 7 weeks (Fig. 2).

As with adipose tissue, there was marked incorporation of n-3 PUFA into skeletal muscle tissue (Fig. 2) of pigs fed the HF diet for 7 weeks. However, the EPA content of muscle from pigs fed the LF diet was not different than that of control animals.

3.2. Clinical signs of inflammation

Within 1 h of the LPS injection, pigs became visibly depressed, and dyspnoeic and several pigs vomited. Over the Week 7 challenge period, feed intake was 6% higher in CONT+LPS pigs, compared with CONT-LPS, LF+LPS and HF+LPS (3.06 vs. 2.87 vs. 2.83 vs. 2.70 kg/day, respectively).

LPS increased rectal temperatures over the 36-h challenge period. The HF diet attenuated the LPS-induced increase in rectal temperature ($P<.001$, Fig. 3) at 2 and 6 h post LPS injection. The LF diet did not alter the febrile response.

LPS endotoxin administration resulted in a significant treatment by time interaction in serum TNF-α concentrations ($P=.002$, Fig. 4A). Peak concentrations occurred 2 h post injection in CONT+LPS group (501 pg/ml). Serum TNF concentrations in the LF+LPS and HF+LPS groups were similar to the CONT-LPS (501 vs. 258 vs. 194 vs. 131 pg/ml, respectively; $P<.05$) (Fig. 4A). There were no treatment differences detected at 25 or 36 h post challenge, at which time serum TNF-α concentrations had returned to prechallenge (0 h) concentrations.

Serum levels of the anti-inflammatory cytokine IL10 was not influenced by diet or tended to be unchanged at 0,

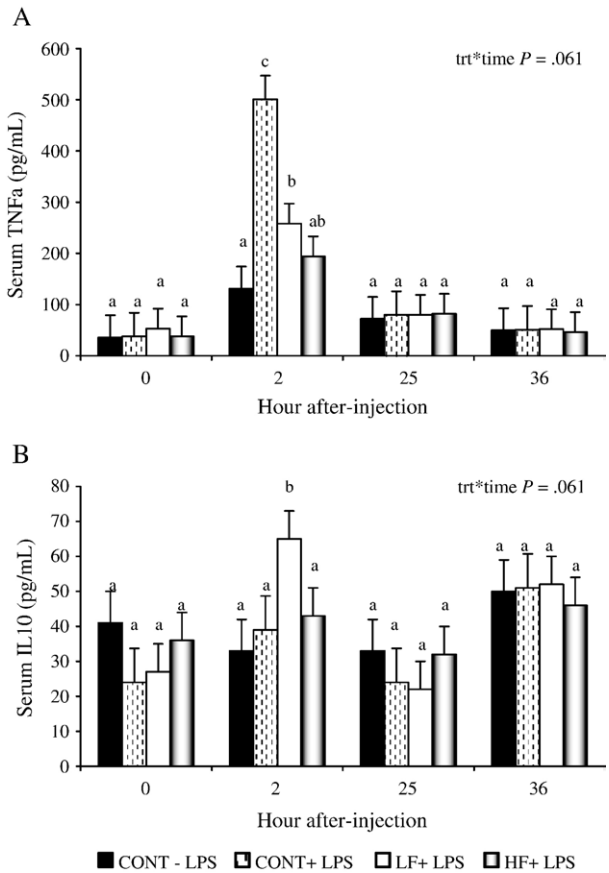

Fig. 4. Serum cytokine concentrations. (A) TNF-α. (B) Interleukin 10. Pigs were challenged with 10 and 2.5 μg/kg BW LPS or saline at 0 and 23 h and blood samples taken at 0, 2, 25 and 36 h post initial challenge. Data represents means of $n=9$ pigs per treatment±S.E.M. Different letters (a-c) within time point represent significant differences ($P<.05$).

25 and 36 h post LPS challenge by dietary treatment (P=.06, Fig. 4B). However, LF+LPS serum IL10 concentrations were up to 46% higher at 2 h post challenge, compared with the either CONT or HF treatments. IL10 peaked at 65 pg/ml for the LF+LPS, compared with the CONT-LPS, CONT+LPS and HF+LPS (33, 39 and 43 pg/ml, respectively, P<.05) (Fig. 4B).

3.3. The effects of dietary n-3 PUFA and LPS on gene and TLR4 protein expression

The expression levels of several cytokines within adipose tissue were measured by qPCR. Differences in adipose gene expression after 7 weeks of dietary intervention following LPS challenge are shown in Table 4. There was no change in expression levels of IL6 or IL10 as a result of LPS or LF and HF treatments (P>.05, Table 4). Polyunsaturated fatty acids have been shown to compete with n-6 PUFA for 12LO and COX2 enzymes and alter eicosonoid metabolism and prostaglandin production. Neither LPS nor LF and HF altered the expression of these two genes. Interestingly, TNFα gene expression was not detected in pig adipose tissue in this experiment using 40 cycles on the qPCR protocol.

Compared with the CONT+LPS pigs, the energy-sensing status gene, AMPK, was 41% lower in adipose expression than in HF+LPS pigs (1.36 vs. 0.80 log starting units, P<.05) (Table 4). However, LF+LPS and CONT-LPS were unchanged, compared with either the HF+LPS or CONT+LPS pig adipose AMPK expression (Table 4).

The induction of the immune cascade and NFκB by LPS requires TLR4. CONT-LPS and both the LF and HF treatments, in conjunction with LPS, increased TLR4 gene expression by 57%, compared with the CONT+LPS (2.79, 2.87, 2.94 and 1.87 log starting units, respectively, P<.05) (Table 4). Furthermore, to identify whether the TLR4 receptor itself was influencing the attenuation of the immune response due to LPS, the protein expression of this receptor was examined in adipose tissue (Fig. 5). All LPS treatments significantly (P<.05) reduced TLR4 protein expression in adipose tissue post LPS challenge, compared to the CONT-LPS. The magnitude of the reduction was maximal (approximately 90%) between the control tissue samples. Importantly, the HF diet tended to attenuate this reduction, compared with the CONT+LPS (344 vs. 32 arbitrary units, P<.10) (Fig. 5).

Table 4
The effects of dietary n-3 PUFA gene expression (log starting quantity) in pig subcutaneous adipose tissue

Gene	Treatment				S.E.M.
	CONT − LPS	CONT + LPS	LF + LPS	HF + LPS	
TNFα	ND	ND	ND	ND	–
IL6	0.82	1.17	1.01	1.07	0.33
IL10	2.57	2.56	2.50	2.58	0.06
COX2	1.76	2.09	1.98	1.87	0.22
12LO	1.25	1.72	1.62	1.34	0.28
TLR4	2.79^{b}	1.87^{a}	2.87^{b}	2.94^{b}	0.38
AMPKα2	0.89^{ab}	1.36^{b}	0.90^{ab}	0.80^{a}	0.20

Pigs were challenged sequentially with 10 and 2.5 μg/kg BW LPS or saline at 0 and 23 h, and tissue biopsies taken at 36 h. Values are given in log starting quantity and represent means of 3 pigs/treatment.
ND, not detected.
Means without a common superscript letter (a, b) are different (P<.05).

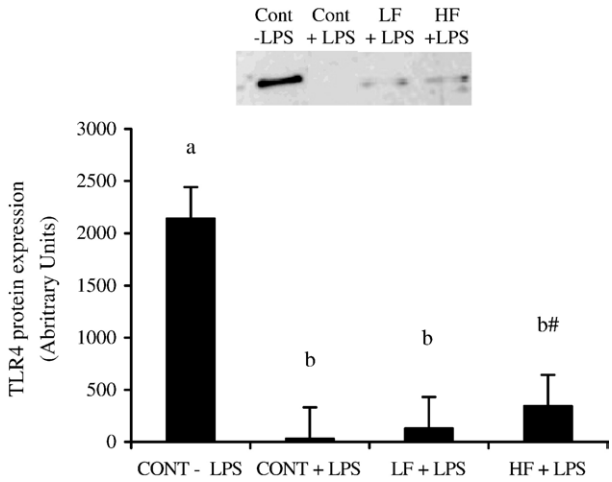

Fig. 5. Effects of dietary n-3 PUFA on TLR4 protein expression in subcutaneous adipose tissue of pigs at 36 h post LPS challenge. Pigs were challenged sequentially with either saline (CONT-LPS) or 10 and 2.5 μg/kg BW LPS at 0 and 23 h. Adipose tissue biopsies were collected 36 h after the initial injection. Data represent means of 3 pigs per treatment±S.E. Different letters (a, b) within tissue represent significant differences (P<.05). #Tendency (P<.10) from the CONT+LPS.

4. Discussion

The data presented herein support several novel and significant concepts relative to our hypotheses. Results from serial tissue biopsies clearly demonstrate that enrichment of n-3 PUFA, DHA and EPA in porcine adipose tissue and skeletal muscle is rapidly accomplished via dietary modifications. This is consistent with previous reports [15,16] in which the fatty acid content of porcine adipose tissue was altered by diet. However, in one 4-week study, the latter group showed that EPA and DHA content in adipose tissue tended to plateau earlier, as fish oil levels were increased in the diets of pigs [17]. However, based on our findings, enrichment can be improved, perhaps due to the protected source of n-3 PUFA we used and the longer feeding period. Collectively, these data support the novel concept of decreasing inflammation in the adipose tissue by manipulating the dietary fatty acid profile.

We show herein, and for the first time in any species, that the TLR4 receptor is markedly down-regulated at both the mRNA and protein level in the adipose tissue of pigs challenged with LPS vs. nonchallenged controls. We have shown previously [3] that adipocytes exposed to LPS in vitro undergo a somewhat transient pattern of NFκB activation. Although this is partly attributable to sequential depletion and replenishment of the cytosol with the inhibitor

protein IkB-α inhibitor of NFκB, it is also likely that the TLR4 receptor itself is depleted to protect against prolonged or excessive stimulation. It is also important to note that, in lean pigs, similar to those used for this study, the abundance of monocytes or macrophages in the adipose tissue is negligible (unpublished data). Consequently, we think it is likely that the reduction in TLR4 mRNA and protein most likely reflects changes in the adipocyte itself, rather than in other cell types residing in the adipose tissue, which might express TLR4. It is intriguing that the n-3 PUFA source negated the LPS-induced reduction in TLR4 mRNA abundance, and western blot analyses, although not statistically significant, suggested that the HF diet might have sustained a marginally higher level of receptor protein. This seemingly contradictory result argues for a proinflammatory effect of the n-3 PUFA, yet it is important to note that the recognized anti-inflammatory effects of the n-3 PUFA may instead require direct interactions with the TLR4 receptor [12]. Thus, while expression at the mRNA and protein level may be greater in the HF group, the signaling potential for the TLR4 receptor may be attenuated. It should also be noted that others have shown direct and indirect modulation of TLR signaling by members of the Suppressors of Cytokine Signaling (SOCS) family [18,19], and it is quite possible that the regulation of TLR4 by n-3 PUFA relate to an induction of SOCS.

LPS evokes a rapid febrile response, causes anorexia, and increases proinflammatory cytokine and cortisol concentrations in blood [20–22]. The notion that n-3 PUFA attenuates the immune response to LPS is also well established [7,23,24]. Our data confirm previous reports in which dietary provision of these fatty acids attenuated LPS challenge-induced febrile responses. In the current study, the protective effect of the n-3 PUFA, particularly the HF diet, on rectal temperature was readily apparent following the first LPS injection. The second LPS injection in our regimen produced a lower febrile response overall, and the protective effect of the HF diet was diminished. LPS challenge-induced changes in serum TNF-α concentrations reflect a similar pattern to the febrile response in that the strong acute response to the initial LPS challenge was markedly attenuated by either the LF or HF diet.

It is intriguing that there was a clear increase in IL10, a major anti-inflammatory cytokine, in the serum of challenged pigs fed the LF diet. Dietary conjugated linoleic acid has been shown to attenuate the production and gene expression of proinflammatory cytokines in weaned pigs challenged with LPS and to increase the IL10 in the plasma and mRNA expression in spleen and thymus tissue [25]. Furthermore, IL10 is known to be secreted by white adipose tissue explants and is up-regulated by LPS and TNFα, both in vitro and in subcutaneous and visceral adipose tissue in obese humans and rodents [26]. Given this, it is intriguing that, in the current study, there was a clear increase in IL10 in the serum of LPS-challenged pigs fed the LF diet. However, there was no effect of LPS or diet on IL10 expression in adipose samples collected at the conclusion of the challenge period. Thus, while it is possible that adipose tissue contributed to the transient increase in serum IL10, additional studies with adipose sampling on a more acute timeline will be necessary to resolve this question. We also acknowledge that it is not readily apparent as to why the stimulatory response of the n-3 PUFA was limited to the LF vs. HF diets.

The oxygenases, COX2 and 12LO, are key determinants of fatty acid metabolism and eicosanoid production and, as such, are central to integrated immune modulation. Although COX2 expression in murine adipose tissue has already been documented through transcript profiling strategies [27], we have confirmed this in the pig, and established that 12LO is readily expressed. Although dietary fish oil has been reported to substantially increase 3-series prostoglandin production (10–50-fold) [28], neither COX2 nor 12LO transcript levels were influenced by LPS or the n-3 PUFA source. However, we cannot rule out the possibility that acute but transient changes in expression occurred with LPS administration, as is possible for the other transcripts quantified.

Finally, AMPK is a heterotrimeric protein with two different catalytic α, two β and three γ regulatory subunits, with isoforms encoded by distinct genes (α1, α2; β1, β2; γ1, γ2, γ3) that act as sensors of cellular energy status [29]. However, AMPK is a not only a sensor of cellular energy status, but Lihn et al. [30] reported that the synthetic AMPK agonist 5-aminoimidazole-4-carboxamide ribonucleoside attenuated the release of TNF and IL6 from human adipose tissue. It is interesting that LPS challenge tended to increase expression of the AMPKα2 subunit, whereas the HF diet blunted this response. Given the potential anti-inflammatory activity of the AMPK, the tendency for up-regulation by LPS seems consistent with a self-limiting inflammatory response as a means of protecting against an overzealous immune response. Albeit, it is clear that the n-3 PUFA blunted this up-regulation, perhaps by altered TLR4 signalling. Additional studies are ongoing to reconcile these findings.

In conclusion, we have shown clearly that dietary n-3 PUFA modify the fatty acid profile of adipose tissue. We have also shown, for the first time, that LPS administration causes down-regulation of TLR4 at the mRNA and protein level in adipose tissue and that dietary n-3 PUFA blocks this response, albeit less apparent at the protein level. These findings indicate that diet may be an effective means of influencing the inflammatory status of adipose tissue, particularly for those pathways influenced by TLR4 signaling.

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