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Angiopoietin like protein 4 expression is decreased in activated macrophages

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ABSTRACT

Angiopoietin like protein 4 (ANGPTL4) inhibits lipoprotein lipase (LPL) activity. Previous studies have shown that Toll-like Receptor (TLR) activation increases serum levels of ANGPTL4 and expression of ANGPTL4 in liver, heart, muscle, and adipose tissue in mice. ANGPTL4 is expressed in macrophages and is induced by inflammatory saturated fatty acids. The absence of ANGPTL4 leads to the increased uptake of pro-inflammatory saturated fatty acids by macrophages in the mesentery lymph nodes due to the failure of ANGPTL4 to inhibit LPL activity, resulting in peritonitis, intestinal fibrosis, weight loss, and death. Here we determined the effect of TLR activation on the expression of macrophage ANGPTL4. LPS treatment resulted in a 70% decrease in ANGPTL4 expression in mouse spleen, a tissue enriched in macrophages. In mouse peritoneal macrophages, LPS treatment also markedly decreased ANGPTL4 expression. In RAW cells, a macrophage cell line, LPS, zymosan, poly I:C, and imiquimod all inhibited ANGPTL4 expression. In contrast, neither TNF, IL-1, nor IL-6 altered ANGPTL4 expression. Finally, in cholesterol loaded macrophages, LPS treatment still decreased ANGPTL4 expression. Thus, while in most tissues ANGPTL4 expression is stimulated by inflammatory stimuli, in macrophages TLR activators inhibit ANGPTL4 expression, which could lead to a variety of down-stream effects important in host defense and wound repair.

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1. Introduction

Angiopoietin like protein 4 (ANGPTL4) is a member of a family of proteins that have a similar structure consisting of a fibrinogen/angiopoietin like domain, an N-terminal signal sequence, a unique region of variable length, and a coiled-coil domain [1–4]. ANGPTL4 is primarily expressed in white adipose tissue with more modest expression in liver, heart, and skeletal muscle [1–4]. ANGPTL4 has been shown to inhibit lipoprotein lipase (LPL) activity by disrupting enzyme dimerization [5]. Over-expression of ANGPTL4 results in hypertriglyceridemia due to a delay in the clearance of triglyceride rich lipoproteins secondary to inhibition of LPL activity [1–4]. Conversely, deletion of ANGPTL4 results in a marked decrease in serum triglyceride levels associated with increased LPL activity [1–4]. ANGPTL4 also stimulates adipose tissue lipolysis [6]. Expression of ANGPTL4 is stimulated by hypoxia, an effect mediated by HIF-1α, and by activation of PPARs [7–10].

Studies in our laboratory have shown that the administration of LPS, an activator of TLR4 and a model of Gram negative bacterial infections, stimulates ANGPTL4 expression in liver, heart, muscle, and adipose tissue [11]. Moreover, serum levels of ANGPTL4 were markedly increased following LPS treatment [11]. Zymosan, an activator of TLR2 and a model of fungal infections, also increased ANGPTL4 mRNA levels in heart, muscle, and adipose tissue and increased ANGPTL4 serum levels [11]. Thus, ANGPTL4 is a positive acute phase protein and the increase induced by inflammatory stimuli could contribute to the hypertriglyceridemia that characteristically occurs during infection and inflammation by inhibiting LPL activity [12].

Recently, studies have shown that mice deficient in ANGPTL4 develop peritonitis, ascites, intestinal fibrosis, weight loss, and death between 15 and 25 weeks of age when fed a diet high in saturated fat [13]. ANGPTL4 is expressed in macrophages and the absence of ANGPTL4 leads to the marked uptake of pro-inflammatory saturated fatty acids by macrophages in the mesentery lymph nodes due to the failure of ANGPTL4 to inhibit LPL activity [13]. The increased LPL activity results in the breakdown of triglycerides carried in chylomicrons allowing for the enhanced uptake of free fatty acids by macrophages, which is toxic to the macrophages [13]. Thus, ANGPTL4 plays an important role in protecting macrophages in mesenteric lymph nodes from the toxic effects of dietary saturated fat. Given the recent recognition of the importance of the expression of ANGPTL4 in macrophages, the purpose of the present study was to determine the effect of inflammation induced by TLR activation on the expression of ANGPTL4 in macrophages.

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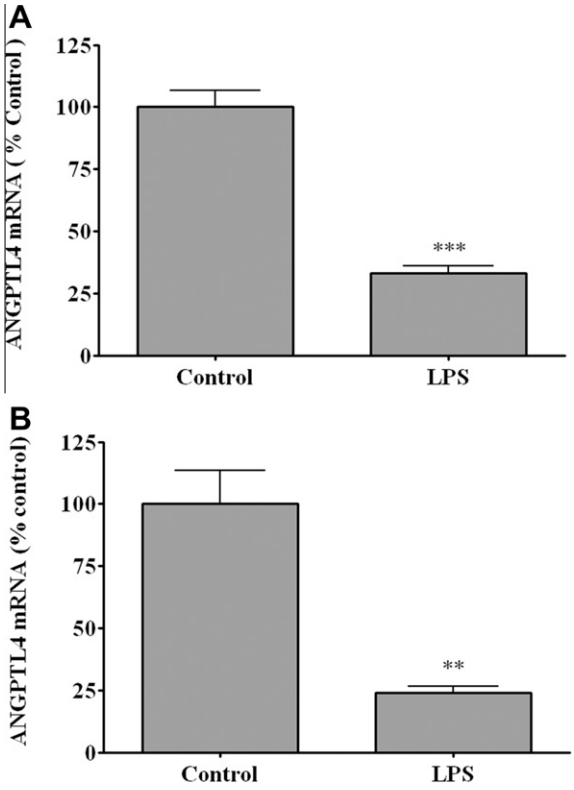

Fig. 1. Effect of LPS on ANGPLT4 expression. For mRNA quantification, total RNA was isolated, cDNA was synthesized with reverse transcriptase, and quantitative real-time PCR performed as described in Section 2. (A) Mice were injected with 100 μg LPS and euthanized at 16 h. Spleen was excised and total RNA was isolated; ANGPLT4 mRNA levels were measured. (B) Mouse peritoneal macrophages were treated with 100 ng/ml LPS for 16 h. RNA was isolated and ANGPTL4 mRNA measured. Data are presented as percent change of control (mean ± SEM). $N = 3–4$ per group. $^{**}p < 0.01$, $^{***}p < 0.001$.

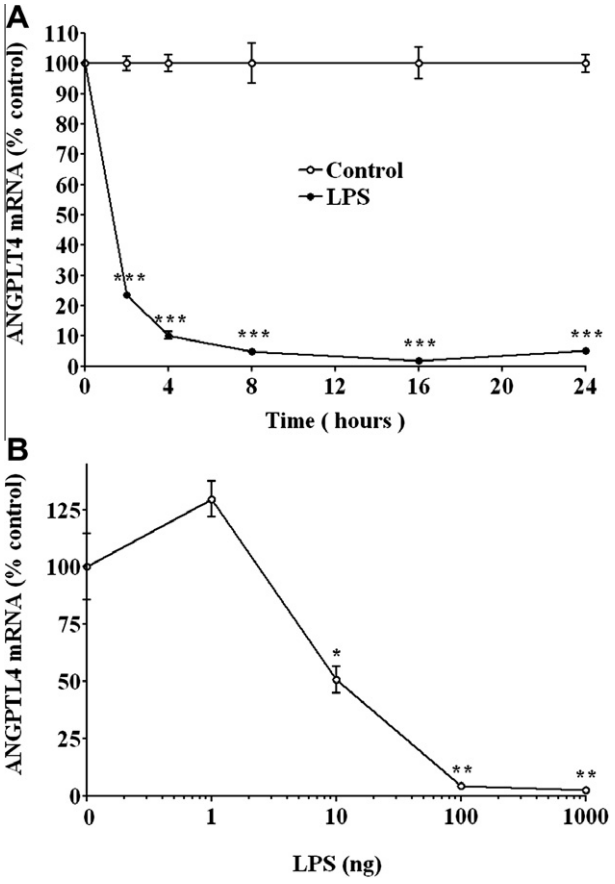

Fig. 2. Effect of LPS on ANGPLT4 expression in RAW cells. Cells were treated with LPS at indicated doses and times. For mRNA quantification, total RNA was isolated, cDNA was synthesized with reverse transcriptase, and quantitative real-time PCR performed as described in Section 2. (A) Cells were treated with 100 ng/ml LPS, at indicated times total RNA was isolated and ANGPTL4 mRNA levels were measured. (B) Cells were treated for 16 h with indicated doses of LPS. Total RNA was isolated and ANGPTL4 mRNA levels were measured. Data are presented as percent change of control (mean ± SEM). $N = 3–4$ per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

2. Materials and methods

2.1. Materials

LPS from *Escherichia coli* strain O55:B5 was purchased from Difco (Detroit, MI) and diluted in sterile normal saline to the desired concentration. Dulbecco's Modified Eagle's Medium (DMEM) was from Fisher Scientific (Pittsburgh, PA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Human serum albumin (HSA) was obtained from Bayer (Elkhart, IN). Tri Reagent and concanavalin A were from Sigma (St. Louis, MO). Zymosan, polyinosine–polycytidylic acid (poly-I:C), and imiquimod were from InvivoGen (San Diego, CA). Mouse TNF α, IL-1β, and IL-6 were purchased from R&D Systems (Minneapolis, MN). Acetylated low density lipoprotein (AcLDL) was from Intracel (Frederick, MD).

2.2. Animal experiments

Female C57BL/6 mice (8–12 weeks of age, ~20 g) were obtained from Charles River Laboratories (Wilmington MA). The animals were maintained in a normal-light-cycle room and were fed Tekland 8656 rodent chow (Harlan Laboratories, Indianapolis, Indiana) and water ad libitum. Animals were injected with either saline or LPS (100 μg/mouse ip) and food was removed from both control and treated animals following injection. At 16 h after injection, mice were rapidly euthanized with an overdose of halothane and the spleen was removed and snap frozen in liquid nitrogen, placed in storage tubes in dry-ice bath until the end of experiment, and then stored at −80 °C until RNA extraction. All experimental protocols were approved by the Animal Studies Subcommittee of the San Francisco Veterans Affairs Medical Center.

2.3. Cell culture

RAW 264.7 cells, a murine macrophage cell line, were from American Type Culture Collection (Manassas, VA). Cells were grown in DMEM supplemented with 10% FBS and incubated at 37 °C in 5% CO_2. When confluent, cells were washed with serum free medium once and then treated in medium supplemented with 2.5% human serum albumin for indicated times (2–24 h) prior to RNA isolation. For lipid loading, cells were co-incubated with LPS at 100 ng/ml and acetylated LDL (AcLDL) at 100 μg/ml for 16 h. Cells were treated with LPS at the doses and for the times indicated in the text and figure legends. Cells were treated with zymosan at 500 μg/ml, poly I:C at 50 μg/ml, or imiquimod at 100 μg/ml for 16 h. For treatment with cytokines, cells were treated with TNFα, IL-1β, or IL-6 at 10 ng/ml for 16 h.

2.4. Mouse peritoneal macrophage culture

Peritoneal macrophages were harvested from C57BL/6 mice 3 days after the ip injection of 40 μg of concanavalin A in 0.5 ml of phosphate-buffered saline and then cultured as described previously by Tang et al. [14]. Cells were plated in 12-well plates in

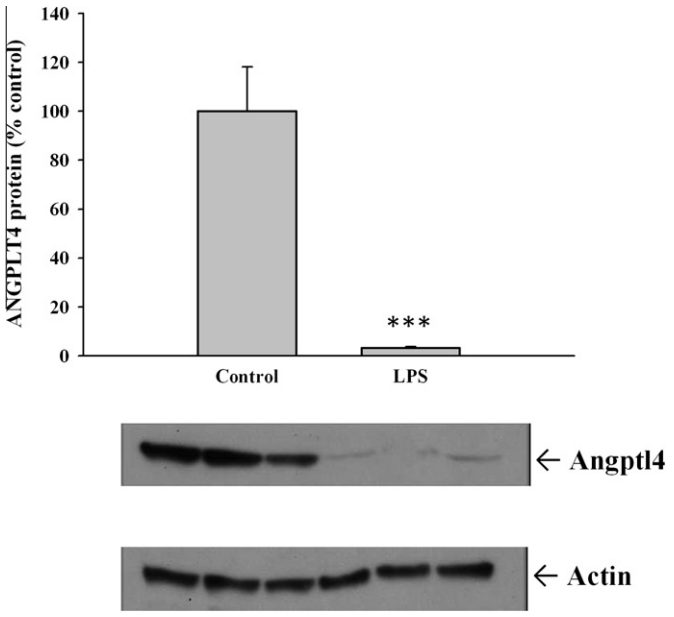

Fig. 3. Effect of LPS on ANGPTL4 protein in RAW cells. Cells were treated with 100 ng/ml LPS for 16 h, protein was extracted and ANGPTL4 Western blot was performed as described in Section 2. Blot was washed and re-probed with actin antibody. Data are presented as percent change of control (mean ± SEM). $N = 3$ per group. $^{***}p < 0.001$.

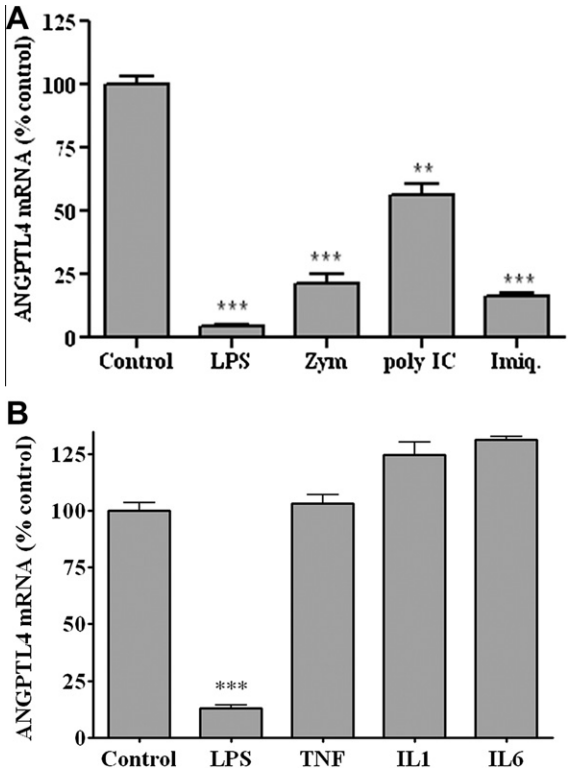

Fig. 4. Effect of macrophage activation on ANGPTL4 expression in RAW cells. Cells were treated with indicated activators for 16 h. For mRNA quantification, total RNA was isolated, cDNA was synthesized with reverse transcriptase, and quantitative real-time PCR performed as described in Section 2. (A) RAW cells were treated with LPS (100 ng/ml), Zym (500 μg/ml), poly I:C (50 μg/ml), or imiquimod (100 μg/ml) and ANGPTL4 mRNA levels were measured. (B) RAW cells were treated with LPS (100 ng/ml), TNFα, IL-1β, or IL-6 at 10 ng/ml and ANGPTL4 mRNA levels were measured. Data are presented as percent change of control (mean ± SEM). $N = 3$ per group. $^{**}p < 0.01$, $^{***}p < 0.001$.

DMEM containing 10% FBS and 20% L-cell culture medium and allowed to adhere to wells for 1 h. Cells were washed with serum free medium and then treated in DMEM supplemented with 2.5% human serum albumin with LPS (100 ng/ml) for 16 h.

2.5. RNA isolation and quantitative PCR

Total RNA was isolated using Tri Reagent. First strand cDNA was synthesized from 1 μg of total RNA with the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The real-time PCR contained 20 ng of reversed transcribed total RNA, 450 nM forward and reverse primers, and 10 μl of 2× LightCycler 480 SYBR Green IMaster in a final volume of 20 μl in 96-well plates using Mx3000PTM real-time PCR System (Stratagene, La Jolla, CA). Quantification was performed by the comparative Ct method with 36B4 used for normalization. The following primers were used: ANGPTL4, forward: 5′-CACCAATGTTTCCCCCAAT-3′; ANGPTL4, reverse: 5′-AAGATACC CTTTTTACGCTCCTG-3′; 36B4, forward: 5′-GCGACCTGGAAGTC CAACTAC-3′, reverse: 5′-ATCTGCTGCATCTGCTTGG-3′.

2.6. Western blots

Macrophage cell lysates were subjected to SDS–PAGE (10% gel) with pre-stained protein markers. Proteins on the gel were transferred onto polyvinylidene difluoride membranes, the membranes were incubated with primary antibody for ANGPTL4 (1:250 dilution, Invitrogen 40-9800), or antibody for actin (1:200 dilution, Santa Cruz sc-1616), and then with peroxidase labeled anti-rabbit IgG antibody (1:50,000 dilution, Amersham Biosciences). The immuno-reactive bands were detected using the SuperSignal West Dura Extended Duration Substrate Kit (Pierce, Rockford, IL) and quantified with the Bio-Rad Multi-Imaging System.

2.7. Statistics

Data are presented as mean ± SEM. The Student's *t*-test was used for comparisons between groups. A p-value <0.05 was considered significant. When multiple samples were compared one way ANOVA was used to determine statistical significance.

3. Results and discussion

Our initial experiment determined the effect of LPS treatment on the expression of ANGPTL4 in the spleen, a tissue enriched in macrophages. As shown in Fig. 1A, LPS treatment results in an approximately 70% reduction in ANGPTL4 mRNA levels in the spleen. This is in contrast to our previous results where LPS treatment increased the expression of ANGPTL4 in heart, muscle, and adipose tissue [11]. We next determined the effect of LPS treatment on ANGPTL4 expression in isolated peritoneal macrophages. As shown in Fig. 1B, LPS treatment results in an approximately 75% decrease in ANGPTL4 mRNA levels in isolated peritoneal macrophages.

We then determined the effect of LPS on the expression of ANGPTL4 in RAW cells, a murine macrophage cell line. As shown in Fig. 2A, LPS treatment results in a rapid decrease in ANGPTL4 expression with a 90% decrease in ANGPTL4 mRNA levels at 4 h. This effect is sustained for at least 24 h with over a 90% decrease in ANGPTL4 expression at 24 h. Fig. 2B shows the dose response effect of LPS on ANGPTL4 mRNA levels in RAW cells. The maximal effect is seen at 100 ng/ml with a ½ maximal response at 10 ng/ml. This LPS induced decrease in ANGPTL4 mRNA levels is accompanied by a 96% decrease in ANGPTL4 protein levels (Fig. 3). These results clearly indicate that LPS treatment of macrophages results in marked decrease in ANGPTL4 expression.

While LPS binds with TLR4 to activate macrophages, there are other ligands that bind to other TLRs and activate macrophages [15]. As shown in Fig. 4A, treatment of RAW cells with zymosan, a fungal product that binds to TLR2 [15], also results in a decrease in ANGPTL4 expression. Similarly, poly I:C, which binds to TLR3 and is a model of viral infections [15], decreases ANGPTL4 expression (Fig. 4A). Lastly, imiquimod, a TLR7 ligand [15], similarly decreases ANGPTL4 expression in RAW cells (Fig. 4A). Thus, activators of a variety of different TLRs all suppress the expression of ANGPTL4. In contrast, cytokines that activate macrophages, including TNF, IL-1, and IL-6, do not affect ANGPTL4 expression (Fig. 4B) indicating that the method of macrophage activation is crucial.

The increased accumulation of cholesterol in macrophages is characteristic of atherosclerosis [16]. We next determined if TLR activators would inhibit ANGPTL4 expression in macrophages incubated with acetylated LDL, which leads to increased cholesterol accumulation in macrophages [16]. Under these conditions, LPS treatment still decreased ANGPTL4 expression in macrophages (AcLDL − Control = 100% ± 3.9, AcLDL − LPS = 3.7% ± 0.01), indicating that TLR activation decreases ANGPTL4 expression even in cholesterol enriched macrophages.

The decrease in ANGPTL4 expression in macrophages would increase the activity of LPL in the environment surrounding macrophages, which would allow increased uptake of fatty acids that is thought to play a role in host defense. Previous studies have suggested that during infection and inflammation, fatty acids are mobilized from storage tissues and directed to macrophages. However, it is important to recognize that ANGPTL4 has other functions. First, studies have suggested that ANGPTL4 regulates angiogenesis [8,17,18]. Second, ANGPTL4 binds to vitronectin and fibronectin and delays their proteolytic degradation by metalloproteinases, which could affect wound healing [19]. Third, ANGPTL4 can interact with specific integrins (β1 and β5), which can regulate cell migration [20]. Thus, the inhibition of ANGPTL4 expression in macrophages by TLR activators could lead to a variety of downstream effects important in host defense and wound repair.

Acknowledgments

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