Adipocyte Fatty Acid–Binding Protein Expression and Lipid Accumulation Are Increased During Activation of Murine Macrophages by Toll-Like Receptor Agonists

Mahmood R. Kazemi, Carol M. McDonald, Judy K. Shigenaga, Carl Grunfeld, Kenneth R. Feingold

Objective—Toll-like receptors (TLRs) recognize pathogens and mediate signaling pathways important for host defense. Recent studies implicate TLR polymorphisms in atherosclerosis risk in humans. Adipocyte fatty acid–binding protein (aP2) is present in macrophages and has an important role in atherosclerotic plaque development. We investigated aP2 expression in RAW 264.7 cells treated with lipopolysaccharide (LPS) and other TLR agonists and assessed lipid accumulation in these activated murine macrophages.

Methods and Results—Stimulation with LPS, a TLR4 ligand, resulted in a 56-fold increase in aP2 mRNA expression, and zymosan, a TLR2 ligand, induced an ∼1500-fold increase. Polynosine: polycytidylic acid (poly I:C), a TLR3 ligand, led to a 9-fold increase. Levels of aP2 protein were significantly increased in LPS or zymosan-treated macrophages compared with control or poly I:C–treated cells. In addition, the cholesteryl ester content of LPS or zymosan-treated macrophages was ∼5-fold greater in the presence of low-density lipoprotein, and triglyceride content was ∼2-fold greater in the absence of exogenous lipid than control or poly I:C–treated cells.

Conclusions—Expression of macrophage aP2 is induced on TLR activation and parallels increases in cholesteryl ester and triglyceride levels. These results provide a molecular link between the known roles of TLR and aP2 in foam cell formation. (Arterioscler Thromb Vasc Biol. 2005;25:1220-1224.)

Key Words: atherosclerosis ■ foam cell ■ macrophage ■ toll-like receptor ■ aP2

Atherosclerosis is increasingly recognized as an inflammatory disorder.1 Epidemiologic studies have reported an elevated risk of atherosclerosis with a number of infectious agents, including Chlamydia pneumoniae and cytomegalovirus.2,3 Local immune responses to these pathogens initiate inflammatory cascades that can result in atheroma formation.

Macrophages are central mediators of innate immune responses. Like other antigen-presenting cells, macrophages detect pathogen-associated molecular patterns by means of toll-like receptors (TLRs).4,5 At least 10 different TLRs have been identified to date, and each appears to recognize molecules of different microbial origin. For example, TLR4 mediates the cellular response to bacterial lipopolysaccharide (LPS), whereas TLR2 recognizes the fungal cell wall constituent zymosan, and TLR3 binds the double-stranded RNA viral analog polynosine: polycytidylic acid (poly I:C).6–9 The importance of these receptors to arterial inflammation is highlighted by the decreased atherosclerosis risk in humans with a TLR4 polymorphism that attenuates receptor signaling.10 There has also been recent interest in potential endogenous ligands of the TLRs, such as heat shock proteins and the extra domain A of fibronectin.11,12

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Activation of macrophages by TLR ligands such as LPS has been shown previously to increase low-density lipoprotein (LDL) uptake and cholesterol content, leading to foam cell formation.13,14 In addition, our laboratory has reported an increase in intracellular triglyceride with LPS stimulation of murine macrophages in the absence of exogenous lipid.14 In vivo studies have also documented an increase in atherosclerotic lesion size with LPS administration.15

During the course of lipid accumulation, fatty acids present in macrophages associate with cytoplasmic fatty acid–binding proteins (FABPs) for intracellular transport.16 The adipocyte FABP (aP2; also known as FABP4) is a marker of terminal adipocyte differentiation and is under transcriptional regulation by fatty acids in these cells.17,18 Monocytes have been shown to express aP2 after phorbol myristate acetate stimulation of macrophage differentiation.19 Macrophages also show an increase in aP2 expression when treated with dexamethasone or oxidized LDL to induce foam cell formation.20–22 In vivo, the role of aP2 in atherosclerosis has been studied in knockout mouse models. Cholesteryl ester accumulation is
reduced in lipid-loaded macrophages from aP2−/− mice compared with wild-type.19 When fed a Western diet, apolipoprotein E−/− (apoE−/−) aP2−/− mice show significant reductions in atherosclerotic lesion size compared with apoE−/− mice.23 Bone marrow transplants have further demonstrated that this reduction is mediated by the absence of macrophage aP2 expression.24 Given the important role of aP2 in atherosclerosis and the increase in macrophage lipid content with LPS stimulation and foam cell formation, we hypothesized that LPS and other TLR ligands would increase the expression of aP2 in murine macrophages. We also postulated that this TLR-mediated enhancement of aP2 expression would be paralleled by an increase in the lipid content of these cells.

**Methods**

**Materials**

LPS from *Escherichia coli* strain O55:B5 was purchased from Difco and diluted in sterile normal saline to the desired concentration. DME was purchased from Fisher Scientific. FCS was purchased from Gemini Bioproducts, and human serum albumin was obtained from ZLB Bioplasma. Intralipid, a 10% emulsion of soybean oil, was obtained from Fresenius Kabi Clayton. Human LDL was purchased from ZLB Bioplasma. Intralipid, a 10% emulsion of soybean oil, was obtained from Fresenius Kabi Clayton. Human LDL was purchased from ZLB Bioplasma. Intralipid, a 10% emulsion of soybean oil, was obtained from Fresenius Kabi Clayton. Human LDL was purchased from ZLB Bioplasma. Intralipid, a 10% emulsion of soybean oil, was obtained from Fresenius Kabi Clayton. Human LDL was purchased from ZLB Bioplasma. Intralipid, a 10% emulsion of soybean oil, was obtained from Fresenius Kabi Clayton. Human LDL was purchased from ZLB Bioplasma.

**Cell Culture**

RAW 264.7, a murine macrophage cell line, was obtained from American Type Culture Collection. Cells were grown in 75-cm² flasks in DME supplemented with 10% FCS and incubated at 37°C in 5% CO₂. Confluent flasks were trypsinized and used to seed 100-mm dishes or 6-well plates for experiments. When confluent, cells were washed with serum-free DME once and then treated in DME with 2.5% human serum albumin. Cells were treated for 4 to 16 hours before RNA isolation and 16 hours before protein isolation or lipid assay.

**RNA Isolation and Northern Blot Analysis**

Total RNA was isolated from 100-mm dishes using Tri Reagent. A total of 30 μg of total RNA was denatured and electrophoresed on 7% agarose-formaldehyde gels. The uniformity of sample loading was verified by UV visualization of the ethidium-bromide–stained gel before electrotransfer to Nytran membrane. The cDNA probe for aP2 was a gift from Dr Bruce Spiegelman (Harvard Medical School, Boston, Mass). 32P-labeled cDNA was prepared using the random priming method (Amersham Biosciences). mRNA levels were quantified by means of the Personal FX phosphorimager (Bio-Rad).

**Protein Isolation**

Cells grown in 100-mm dishes were washed twice with ice-cold PBS and scraped into 2 mL per dish of chilled PBS with protease inhibitor cocktail added. Cells were pelleted at 1000 rpm for 5 minutes at 4°C, and pellets were resuspended in cell lysis buffer containing 1% Triton X-100, 0.5% deoxycholate, 2 mM/L sodium vanadate, and 100 mM/L sodium fluoride with protease inhibitor cocktail added. After incubation on ice for 30 minutes, the suspension was centrifuged at 10000 rpm for 30 minutes at 4°C. The supernatant was collected and assayed for protein concentration by the Bradford method (Bio-Rad).

**Western Blot Analysis**

SDS-PAGE was performed on 100-μg aliquots of protein under reducing conditions. Samples were electrophoresed on a 10% to 20% gradient gel using a minigel apparatus (Bio-Rad). Proteins were electrotransferred to a polyvinylidene fluoride membrane (Amersham), followed by blocking with 5% nonfat dry milk in PBS. After washing with PBS containing 0.1% Tween 20, the membrane was incubated with goat anti-mouse aP2 primary antibody (1:100 dilution) in 1% milk for 1 hour at room temperature. After repeat washing, the membrane was incubated with the appropriate secondary antibody and signal was detected with ECL Plus (Amersham).

**Lipid Assays**

Cells grown in 6-well plates were washed twice with ice-cold PBS and scraped into 100 μL of doubly distilled water. The resulting suspension was kept on ice and sonicated for 15 seconds with a Branson Sonifier 350. Aliquots of 10 μL were assayed separately for total cholesterol and free cholesterol using a kit from Wako. A 20-μL aliquot of each sample was assayed for total triglyceride and corrected for free glycerol using a kit from Sigma. Protein concentration was determined by the Bradford method.

**Statistics**

Data are presented as mean±SEM. Student’s t test was used for comparisons between groups. A P value <0.05 was considered significant.

**Results**

LPS stimulation of RAW cells results in a dose-dependent increase in aP2 mRNA levels (Figure 1A). There is a substantial increase even at 1 ng/mL (8-fold greater than control), demonstrating the sensitivity of the response to LPS. Half-maximal response is at ~10 ng/mL, and maximal increase is at 100 ng/mL (56-fold greater than control), with no appreciable further increase at 1000 ng/mL. Levels of aP2 mRNA rise within 8 hours after RAW cells are treated with LPS (Figure 1B). No significant induction is apparent at 4 hours (Figure 1B), and there is no further increase at 24 hours (data not shown).

Because aP2 is an FABP involved in the intracellular transport of lipid, it is possible that the magnitude of the response to LPS stimulation may be modulated by extracellular or intracellular lipid concentration. RAW cells treated with a triglyceride emulsion (Intralipid) showed no increase in aP2 expression (Figure 2A), despite having a 5-fold increase in triglyceride content compared with control (Figure 2B). When RAW cells are treated with LPS and Intralipid, cells showed a substantial increase in aP2 expression from baseline (36-fold increase over Intralipid alone). However, this increase is not greater than with LPS alone (Figure 2A), even with higher triglyceride levels (Figure 2B), indicating that TLR4 activation, per se, provides a specific signal for regulation of aP2 gene expression in the macrophage, inde-
pendent of an increase in lipid content. This is further supported by the rise in aP2 with LPS stimulation despite the lack of cholesteryl ester accumulation in the absence of exogenous LDL (data not shown).

We next studied macrophage activation by 2 other TLR ligands: zymosan as a model for fungal infection, and poly I:C as a model for viral infection (Figure 3). At doses comparable to that used for LPS stimulation, zymosan increases aP2 mRNA levels \( \approx 1500 \)-fold (Figure 3, inset; note logarithmic scale), whereas poly I:C shows an \( \approx 9 \)-fold increase. Activation of each TLR results in a different magnitude of change in aP2 expression, with a particularly robust response after TLR2 activation. This is especially notable in light of the unique microbial origins for each class of TLR agonist.

A significant increase in macrophage aP2 protein is also apparent on stimulation with LPS or zymosan but not poly I:C (Figure 4). Therefore, the enhanced expression of aP2 mRNA leads to an increase in protein level. Because baseline expression of aP2 protein in macrophages is quite low and difficult to detect, the strong signal found on Western blotting of LPS or zymosan-treated cell lysates suggests a significant change in the cellular program for lipid metabolism. Given the low level of expression in control cells, we cannot rule out a small increase induced by poly I:C.

Consistent with the change in aP2, a considerable accumulation of triglyceride is seen in RAW cells treated with LPS or zymosan in the absence of serum or exogenous lipid when compared with control or poly I:C–treated cells (Figure 5A). Approximately twice the triglyceride content is found in the LPS or zymosan-treated cells. Notably, cells treated with Intralipid and LPS also show an \( \approx 2.2 \)-fold increase in triglyceride content compared with Intralipid treatment alone (Figure 2B). This suggests a specific effect of TLR signaling on macrophage triglyceride content.

No significant change is seen in macrophage cholesteryl ester content with LPS, zymosan, or poly I:C in the absence of exogenous LDL (data not shown), consistent with previ-
Discussion

Macrophages play an essential role in the development of atherosclerotic plaque by accumulating and storing lipid particles in the arterial wall. Activation of macrophages by TLR ligands has been shown to induce foam cell formation in murine macrophages by our laboratory and others. We now extend these findings to show LPS-mediated increases in mRNA and protein levels of aP2, an FABP found in macrophages that has been shown recently to have an important role in atherosclerosis. This intracellular transport protein is present at extremely low levels in macrophages at baseline, and its expression is dramatically increased on exposure to LPS and zymosan. Notably, this change in gene expression is paralleled by a substantial increase in intracellular cholesteryl ester and triglyceride levels consistent with foam cell formation but is not regulated by increased lipid concentration alone.

LPS has been shown to regulate a number of macrophage genes thought to have key roles in atherosclerosis. One of the early studies by Werb et al demonstrated reductions in apoE synthesis and secretion on exposure to low doses of LPS. Decreases in 2 important high-density lipoprotein (HDL) receptors, scavenger receptor B1 and ATP-binding cassette A1 (ABCA1), are also regulated in an LPS dose-dependent fashion. These changes contribute to decreased cholesterol efflux from macrophages and enhanced foam cell formation. Of note, induction of gene expression with LPS stimulation in macrophage models, such as that shown for aP2 here, has been reported less frequently in the literature than suppression of macrophage genes.

One proposed mechanism for the decrease in cholesterol efflux on LPS stimulation is the inhibition of liver X receptor (LXR) transcriptional activity in macrophages activated by TLR agonists. Castrillo et al have shown that this inhibition in RAW cells leads to decreases in the expression of LXR target genes, such as ABCA1. However, LPS also downregulates ABCA1 by a non-LXR–dependent mechanism in J774 murine macrophages. Nevertheless, a reciprocal relationship may exist between the regulation of inflammation and lipid metabolism in RAW cells such that LXR agonists inhibit expression of genes that mediate the inflammatory response in LPS-treated cells. This hypothesis provides an intriguing explanation for the cholesterol accumulation seen in this study with TLR activation under specific conditions.

The response of macrophage aP2 to LPS stimulation is exquisitely sensitive to LPS dose and occurs rapidly. Effects can be seen with as little as 1 ng/mL of LPS and as early as 8 hours after treatment. For comparison, the LD₅₀ of LPS in mice is ~15 mg/kg or ~300 μg for a 20-g mouse. Previous studies have shown that expression of the aP2 gene is enhanced in macrophage cell lines treated with low levels of oxidized LDL, HDL, or peroxisome proliferator-activated receptor-γ agonists. Induction of aP2 mRNA and protein expression under these conditions has a similar time course to that shown for LPS here. During infection, the presence of higher concentrations of LPS would be expected to lead to significant increases in the aP2 expression of participating macrophages. In fact, recent studies of periodontitis in human subjects have shown a correlation between the area of inflammation in the oral cavity and macrophage cytokine production and LDL cholesteryl ester uptake. These proatherogenic changes are thought to be mediated by increases in serum LPS during periodontal infection and have been postulated to augment the risk of vascular disease in affected individuals.

Another novel finding here is that ligands for other TLRs also induce macrophage aP2 expression. Zymosan, a TLR2 ligand, and LPS, a TLR4 ligand, dramatically increase aP2 mRNA and protein levels, whereas poly I:C, a TLR3 ligand, shows much smaller increases. These data suggest that foam cell formation is part of an immune program activated by the TLR-mediated recognition of molecules associated with potentially harmful pathogens. However, the class of TLR activated is key to the changes seen in the regulation of lipid metabolism. In fact, it has been proposed that C pneumoniae, a microorganism associated with increased atherosclerosis risk, may stimulate cytokine production by means of non-LPS components recognized by TLR2, the same TLR that mediates the potent response to zymosan.

Although there is a 5-fold increase in cholesteryl ester and 2-fold increase in triglyceride content of cells treated with either LPS or zymosan, neither accumulate to a significant extent in poly I:C–stimulated cells compared with control. These results parallel the enhanced expression of aP2 with LPS or zymosan treatment. Given the significant role of aP2 in atheroma formation that has been shown in knockout mouse models, this parallel increase is highly suggestive of a key role for aP2 in macrophage lipid accumulation. The increase in aP2 expression likely assists with the transport of...
fatty acids needed for esterification of cholesterol and accumulation of triglyceride. The data with Intralipid demonstrate that it is the TLR ligand that induces aP2 expression, not the increase in lipid concentration.

Thus, aP2 is induced during the innate immune response of macrophages in which stored lipid likely plays a role in host defense. Notably, the growth of *C. pneumoniae* in lipid-loaded macrophages is inhibited. This metabolic shift may serve as an effective method for neutralization of intracellular pathogens in the short term. However, chronic activation of macrophage TLRs can lead to plaque formation and clinical atherosclerosis.

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**References**


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