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
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 purchased from Sigma. Poly I:C was purchased from InvivoGen. \(-\alpha\)-P\( [\text{dCTP} \) (3000 Ci/mmol) was purchased from Perkin-Elmer Life Sciences. Goat anti-mouse \( aP2 \) primary antibody (1:100 dilution) was purchased from BD Biosciences. QPCR was performed with the MX3000P (Stratagene) using 2X SYBR Green Biosciences. Quantitative Polymerase Chain Reaction (QPCR) was performed by the comparative CT method, with 36B4 used for normalization.

**Cell Culture**

RAW 264.7, a murine macrophage cell line, was obtained from American Type Culture Collection. Cells were grown in 75-cm\(^2 \) flasks in DME supplemented with 10% FCS and incubated at 37°C in 5% CO\(_2\). 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 5% CO\(_2\). Confluent flasks were trypsinized and used to seed 100-mm dishes using Tri Reagent. A total of 3 \( \mu \)g of total RNA from RAW 264.7 cells was reverse transcribed to cDNA using a commercially available kit (BD Biosciences). Quantitative polymerase chain reaction (QPCR) was performed with the MX3000P (Stratagene) using 2X SYBR Green Biosciences. QPCR was performed by the comparative CT method, with 36B4 used for normalization.

**RNA Isolation and Northern Blot Analysis**

Total RNA was isolated from 100-mm dishes using Tri Reagent. A total of 3 \( \mu \)g of total RNA was denatured and electrophoresed on 1% 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 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 mmol/L sodium vanadate, and 100 mmol/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-\( \mu \)g 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 \( \mu \)L of doubly distilled water. The resulting suspension was kept on ice and sonicated for 15 seconds with a Branson Sonifier 350. Aliquots of 10 \( \mu \)L were assayed separately for total cholesterol and free cholesterol using a kit from Wako. A 20-\( \mu \)L 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\( \pm \)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 ∼1500-fold (Figure 3, inset; note logarithmic scale), whereas poly I:C shows an ∼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 ∼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-
synthesis and secretion on exposure to low doses of LPS.25 
early studies by Werb et al demonstrated reductions in apoE 
genes thought to have key roles in atherosclerosis. One of the 
concentration alone.

in intracellular cholesteryl ester and triglyceride levels consistent 
this finding to show LPS-mediated increases in mRNA and 
macrophages by our laboratory and others.13,14 We now extend 
ligands has been shown to induce foam cell formation in murine 
Inflammation and lipid metabolism in RAW cells such that LXR agonists inhibit 
expression of genes that mediate the inflammatory response in 
LPS-treated cells.29 This hypothesis provides an intriguing 
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.20,30,31 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.32 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.33

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.34,35 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.36

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.37 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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