Inhibition of inflammatory signaling pathways in 3T3-L1 adipocytes by apolipoprotein A-I

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ABSTRACT: Activation of inflammatory signaling pathways links obesity with metabolic disorders. TLR4-mediated activation of MAPKs and NF-κB are 2 such pathways implicated in obesity-induced inflammation. Apolipoprotein A-I (apoA-I) exerts anti-inflammatory effects on adipocytes by effluxing cholesterol from the cells via the ATP binding cassette transporter A1 (ABCA1). It is not known if these effects involve inhibition of inflammatory signaling pathways by apoA-I. This study asks if apoA-I inhibits activation of MAPKs and NF-κB in mouse 3T3-L1 adipocytes and whether this inhibition is ABCA1-dependent. Incubation of differentiated 3T3-L1 adipocytes with apoA-I decreased cell surface expression of TLR4 by 16 ± 2% and synthesis of the TLR4 adaptor protein, myeloid differentiation primary response 88, by 24 ± 4% in an ABCA1-dependent manner. ApoA-I also inhibited down-stream activation of MAPKs, such as ERK, p38MAPK, and JNK, as well as expression of proinflammatory adipokines in bacterial LPS-stimulated 3T3-L1 adipocytes in an ABCA1-dependent manner. ApoA-I, by contrast, suppressed nuclear localization of the p65 subunit of NF-κB by 30 ± 3% in LPS-stimulated 3T3-L1 adipocytes in an ABCA1-independent manner. In conclusion, apoA-I inhibits TLR4-mediated inflammatory signaling pathways in adipocytes by preventing MAPK and NF-κB activation.—Sultana, A., Cochran, B. J., Tabet, F., Patel, M., Torres, L. C., Barter, P. J., Rye, K.-A. Inhibition of inflammatory signaling pathways in 3T3-L1 adipocytes by apolipoprotein A-I. FASEB J. 30, 000–000 (2016). www.fasebj.org

KEY WORDS: ABCA1 · MAPK · NF-κB

Adipose tissue inflammation and dysfunction are major factors associated with obesity and its comorbidities, such as insulin resistance, type 2 diabetes, and cardiovascular diseases (1). One of the key events in obesity is macrophage infiltration into adipose tissue and adipokine dysregulation (2), which is characterized by increased secretion of proinflammatory adipokines such as monocyte-chemoattractant protein-1 (MCP-1), IL-6, TNF-α, IL-1β, and resistin (3–6). The plasma level of the anti-inflammatory adipokine, adiponectin, is also reduced in people who are obese (7).

An emerging link between obesity and its associated pathologies is activation of MAPK-mediated signaling pathways in adipocytes (8–10). MAPKs regulate diverse cellular functions, including inflammation and insulin signaling. Three major MAPKs involved in obesity-induced inflammation (11–13) and insulin resistance (14) are ERK, p38MAPK, and JNK. The trans-membrane protein, TLR4, is a known activator of these MAPKs (15, 16).

TLRs play an important role in innate immune signaling in response to microbial infection by utilizing adaptor proteins to activate signal transduction pathways. TLR4 is the main receptor for bacterial LPS, and its activation is associated with lipid-induced inflammation in adipose tissue (17) and insulin resistance in adipocytes (18). When adipocytes are challenged with LPS, TLR4 recruits the adaptor protein myeloid differentiation primary response 88 (MyD88) (19). This results in downstream activation of NF-κB (18) and initiates transcription of proinflammatory genes, such as MCP-1 and IL-6 (15, 17, 20).

A low level of circulating HDL cholesterol is another hallmark feature of obesity (21). Both HDLs and the main HDL apolipoprotein, apolipoprotein A-I (apoA-I), have well documented anti-inflammatory properties (22, 23). For example, apoA-I attenuates TLR4 signal transduction pathways in endothelial cells (24, 25) and inhibits the activation of NF-κB in endothelial cells, monocytes, and macrophages (25–27). Beneficial effects of apoA-I and...
HDLs on adipose tissue were first reported by Van Linthout et al. (28), who established that apoA-I regulates adipose tissue metabolism by decreasing circulating levels of triglycerides and free fatty acids and increasing the level of adiponectin in apoA-I transgenic mice. ApoA-I and HDLs have more recently been shown to inhibit inflammatory signaling in adipocytes by disrupting and removing cholesterol from lipid rafts. These effects were dependent on the ATP binding cassette transporter A1 (ABCA1), ATP binding cassette transporter GL, and scavenger receptor class B type 1 (SR-B1) (29).

Based on these observations, the current study asks if apoA-I can also inhibit inflammation in 3T3-L1 adipocytes by suppressing TLR4-mediated inflammatory signaling pathways. Our findings identify molecular mechanisms by which apoA-I prevents inflammatory responses in adipocytes.

**MATERIALS AND METHODS**

**Isolation of apoA-I**

HDLs were isolated from pooled samples of normal human plasma (Healthscope Pathology, Adelaide, South Australia) by sequential ultracentrifugation (1.063 < density < 1.21 g/ml) as described previously (30). The isolated HDLs were delipidated and apoA-I was purified by chromatography on a Q Sepharose Fast Flow column attached to an Äkta-FPLC system (GE Healthcare, Chalfont St. Giles, United Kingdom) (31, 32). The purified apoA-I was lyophilized, reconstituted in 3 mM guanidine hydrochloride/10 mM Tris/0.01% (w/v) EDTA-Na2 (pH 8.2) and dialyzed against endotoxin-free PBS (pH7.4) before use (33).

**apoA-I assay**

ApoA-I concentrations were quantified immunoturbidimetrically using an AU480 autoanalyzer (Beckman Coulter, Brea, CA, USA) as described previously (34) using a goat anti-human apoA-I antibody (Calbiochem, San Diego, CA, USA), purified apoA-I standards (0–500 µg/ml), and a pooled sample of normal human plasma as a quality control.

**Cell culture and treatments**

Mouse preadipocyte 3T3-L1 cells (American Type Culture Collection, Manassas, VA, USA), were cultured and differentiated according to the supplier’s protocol. Mature adipocyte formation and lipid accumulation in the cells was achieved by d 7, as observed by phase contrast microscopy. All treatments were completed by d 8 of differentiation.

To determine the effects of apoA-I on MCP-1 and IL-6 expression, differentiated 3T3-L1 adipocytes were incubated for 16 h with PBS or apoA-I (final concentration as indicated in figure legends) in serum-free high-glucose DMEM. The cells were rinsed (×3) with warm PBS then incubated for 6 h at 37°C with LPS (Escherichia coli, serotype 055:B5, final concentration 100 ng/ml; Sigma-Aldrich, St. Louis, MO, USA) or PBS (control) in serum-free, high-glucose DMEM. MCP-1 and IL-6 mRNA levels and secretion were quantified by qPCR and ELISA, respectively.

For MAPK and signal transducer and activator of transcription 3 (STAT3) signaling experiments, differentiated 3T3-L1 cells were preincubated for 16 h with PBS or apoA-I (final concentration 1 mg/ml) in serum-free high-glucose DMEM, rinsed with PBS (×3), then incubated for up to 60 min with LPS or PBS (control) in serum-free high-glucose DMEM. Phosphorylation of MAPKs and STAT3 was quantified in whole cell lysates by Western blotting. NF-κB activation was determined by Western blotting for the p65 subunit in nuclear and cytoplasmic extracts. Cell lysates and medium were assayed for apoA-I as described above.

To determine if the ability of apoA-I to reduce MCP-1 and IL-6 expression was dependent on MAPK activation, differentiated adipocytes were preincubated for 30 min in serum-free DMEM with selective inhibitors of ERK1/2 (U0126), p38MAPK (SB203580), and JNK1/2 (SP600125) (final concentration 10 µM for all; Cell Signaling, Danvers, MA, USA), then incubated for 6 h with LPS. For analysis of TLR4 and MyD88 expression, differentiated adipocytes were incubated for 16 h with PBS or apoA-I in serum-free high-glucose DMEM. Cell surface TLR4 levels were determined by flow cytometry and TLR4 and MyD88 mRNA levels were quantified by qPCR.

TLR4 and caveolin-1 protein expression in isolated lipid rafts was determined by incubating 3T3-L1 adipocytes in 6-well plates for 16 h with PBS or with apoA-I in serum-free high-glucose DMEM in 6-well plates. TLR4 and caveolin-1 protein expression was assessed by Western blotting.

**Transfection**

Transfections were carried out as described (35), with slight modifications. At d 4 after the initiation of differentiation, 3T3-L1 adipocytes (4.4 × 10^5 cells/well) were transfected at 37°C for 24 h with ABCA1 siRNA (50 nM, On-Targetplus Smartpool), SR-B1 siRNA (100 nM, On-Targetplus Smartpool), or scrambled siRNA (control) (On-Targetplus Nontargeting Control Pool, Thermo Scientific, Lafayette, CO, USA), using the lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA). After transfection, the cells were cultured for a further 3 d. ABCA1 and SR-B1 gene knockdown was confirmed by Western blotting of whole cell lysates. mRNA levels were quantified by qPCR, using β-actin as a control.

**Cell viability**

Cell viability was determined using trypan blue. Differentiated 3T3-L1 cells were washed in PBS, trypsinized, and centrifuged. The cell suspension (20 × 10^4 cells/ml) was added to 20 µl of a trypan blue solution and cells were counted in a hemocytometer. Cell viability was expressed as a percentage of the trypan blue positive cells.

**qPCR**

Total RNA was extracted from 3T3-L1 adipocytes using the TRI reagent (Sigma-Aldrich). RNA concentrations were normalized to 200–300 ng/µl using the SYBR Green I assay (Invitrogen) and reverse transcribed using iScript/IQ SYBR Green Supermix in a BioRad iQ5 thermocycler. Relative changes in mRNA levels were determined by the ΔΔC_t method using β-actin as the control. Primer sequences are shown in Table 1.

**ELISA**

The concentrations of MCP-1, IL-6, adiponectin, resistin, IL-1β, and IFN-γ in cell culture supernatants were determined by
ELISA (R&D Systems, Minneapolis, MN, USA) using an ELISA plate reader (Fluostar Omega; BMG Labtech, Ortenberg, Germany). All results were normalized to total protein using the bicinchoninic acid assay (Thermo Scientific, Waltham, MA, USA).

Western Blotting

3T3-L1 adipocytes were washed with ice-cold PBS and lysed in RIPA buffer containing 50 mM Tris base (pH 7.4), 150 mM NaCl, 1% (v/v) NP-40, 1% (w/v) deoxycholate, 1 mM EDTA-Na2, 0.1% (w/v) SDS, the phosphatase inhibitor Na3VO4 (1:500) and protease inhibitors (1:100). Nuclear and cytoplasmic extracts were isolated using the NucBuster protein extraction kit (Merck Millipore, Darmstadt, Germany). Whole cell, nuclear, and cytoplasmic lysates (30–60 µg) were subjected to SDS-PAGE, transferred to nitrocellulose membranes (Invitrogen) and incubated overnight, at 4°C, with monoclonal antibodies: rabbit anti-mouse phospho-ERK1/2 (catalog no. 4370, 1:1000), ERK1/2 (catalog no. 4695, 1:1000), phospho-p38 MAPK (#4511, 1:500), p38 MAPK (catalog no. 8690, 1:1000), JNK1/2 (catalog no. 9258, 1:1000), STAT3 (catalog no. 9145, 1:1000), and pSTAT3 (catalog no. 12640, 1:1000) from Cell Signaling; rat anti-mouse ABCA1 (catalog no. NB400-164, 1:1000), and pSTAT3 (catalog no. 9145, 1:1000), and pSTAT3 (catalog no. 12640, 1:1000) from Cell Signaling; rat anti-mouse SR-B1 (#GTX61567, 1:200) from Genetex (Irvine, CA, USA); rabbit anti-mouse NF-κB p65 (catalog no. sc-8008, 1:200) from Santa-Cruz Biotechnology (Santa Cruz, CA, USA); rabbit anti-mouse TLR4 (catalog no. ab22048, 1:500), mouse anti-Caveolin-1 (#ab17052, 1:250) from Abcam, mouse anti-phospho-STAT3 (catalog no. ab96879, Abcam). The cells were then washed (×3) with binding buffer, and incubated for 30 min at 4°C with a TLR4 antibody (10 µg/1 × 10^6 cells, catalog no. ab22048, Abcam) or an isotype control antibody (mouse IgG2b [PLP219], catalog no. ab91366, Abcam). The cells were then washed (×3) with binding buffer, and incubated for 30 min at 4°C with DyLight 488 goat anti-mouse IgG (1:50/1 × 10^6 cells, catalog no. ab96879; Abcam). TLR4 cell surface expression was analyzed using a FACSVersus flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). Data were analyzed with FlowJo software (Ashland, OR, USA). Typically, acquisition of 10,000 cells was analyzed based on forward vs. side scatter gating.

Nondetergent isolation of lipid rafts

Lipid rafts were isolated as previously described (36). Confluent 3T3-L1 adipocytes were washed (×3) with ice-cold PBS. Na2CO3 (333 µl of a 500 mM solution) was added to each well. The cells (2 ml total) were transferred to a prechilled Dounce (glass-glass) homogenizer and disrupted (20 strokes on ice). The cell homogenates were then transferred to a prechilled 50 ml conical tube and homogenized (3 × 10 s) with a polytron handheld homogenizer (VWR International, Tingalpa, QLD, Australia). The samples were further homogenized (3 × 20 s with a 60 s rest between homogenization) using an ultrasonic cell disruptor. The cell homogenates (2 ml) were then mixed with 90% sucrose/[2-(N-morpholino)ethanesulfonic acid]-buffered saline (MBS, 2 ml). Sucrose (35% (w/v) in MBS/Na2CO3 (4 ml) was layered on top of the sample/90% sucrose/MBS layer. A 5% (w/v) sucrose solution in MBS/Na2CO3 (4 ml) was layered on top of the 35% sucrose /MBS/Na2CO3 layer. The samples were centrifuged at 4°C for 38 h at 39,000 rpm in an SW41Ti rotor (Beckman Coulter, Brea, CA, USA) and 12 × 1 ml fractions were collected. Fractions 1 and 2, 9 and 10, 11 and 12 were pooled. As protein concentration does not provide an accurate baseline parameter for comparison across fractions, equal sample volume loading (39 µl) was used for Western blotting. This loading method accounts for the presence of lipid rafts.

**Flow cytometry**

3T3-L1 adipocytes were washed with PBS and incubated at 37°C with Accutase (Sigma-Aldrich) until the cells detached from the wells. The cells were gently resuspended in binding buffer containing 0.98% (w/v) HBSS, 20 mM HEPES, 1 mM CaCl2, 1 mM MgCl2, and 0.1% (w/v) bovine serum albumin and incubated for 30 min at 4°C with a TLR4 antibody (10 µg/1 × 10^6 cells, catalog no. ab22048, Abcam) or an isotype control antibody (mouse IgG2b [PLP219], catalog no. ab91366, Abcam). The cells were then washed (×3) with binding buffer, and incubated for 30 min at 4°C with DyLight 488 goat anti-mouse IgG (1:50/1 × 10^6 cells, catalog no. ab96879; Abcam). TLR4 cell surface expression was analyzed using a FACSVersus flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). Data were analyzed with FlowJo software (Ashland, OR, USA). Typically, acquisition of 10,000 cells was analyzed based on forward vs. side scatter gating.

**TABLE 1. Sequences of primers used for qPCR**

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<tr>
<th>Primer</th>
<th>Sequence, 5′–3′</th>
<th>Accession number</th>
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<tbody>
<tr>
<td>MCP-1</td>
<td>CGGAACCAATGGATCGAA</td>
<td>NM_011333.3</td>
</tr>
<tr>
<td>IL-6</td>
<td>TGTTGAAAAGTGGATGTC</td>
<td>NM_031168.1</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>CAGTGTTGATCCAGACCAA</td>
<td>NM_009605.4</td>
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<tr>
<td>Resistin</td>
<td>TGCAAGGTATGCAAGGAAA</td>
<td>NM_022984.4</td>
</tr>
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<td>ABCA1</td>
<td>GCHTTGUGAGTGTATAACAATGTTG</td>
<td>NM_013454.3</td>
</tr>
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<td>SR-B1</td>
<td>CAGTGTTGATCCAGACCAA</td>
<td>NM_001205083.1</td>
</tr>
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<td>TLR4</td>
<td>TGCAAGGTATGCAAGGAAA</td>
<td>NM_021297.2</td>
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<td>MyD88</td>
<td>GCHTTGUGAGTGTATAACAATGTTG</td>
<td>NM_010851.2</td>
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<tr>
<td>β-actin</td>
<td>ATGGTACAGACCAAGGAGG</td>
<td>NM_007393.3</td>
</tr>
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**MCP-1** (253 bp, NM_011333.3)

**IL-6** (262 bp, NM_031168.1)

**Adiponectin** (256 bp, NM_009605.4)

**Resistin** (250 bp, NM_022984.4)

**ABCA1** (306 bp, NM_013454.3)

**SR-B1** (257 bp, NM_001205083.1)

**TLR4** (250 bp, NM_021297.2)

**MyD88** (250 bp, NM_010851.2)

**β-actin** (237 bp, NM_007393.3)
for differential separation of proteins into specific fractions following density gradient ultracentrifugation. The samples were loaded onto 4–12% SDS polyacrylamide gels and run at 125 V for 1.5 h, electrophoretically transferred to nitrocellulose membranes, blotted for TLR4, then stripped and blotted for caveolin-1.

Statistical analysis

All data are presented as mean ± SEM. Results were analyzed using the Student’s t test (unpaired, 2-tailed) or 1-way ANOVA with Dunnett’s post hoc test or 2-way ANOVA with Tukey’s post hoc test, where appropriate. All analyses were performed using GraphPad Prism software Version 4.03 (GraphPad Software, Inc., La Jolla, CA, USA). A value of P < 0.05 was considered significant.

RESULTS

apoA-I inhibits LPS-induced proinflammatory adipokine expression in 3T3-L1 adipocytes

Because LPS increases MCP-1 and IL-6 production in adipocytes (15), we first asked if apoA-I inhibits the LPS-induced secretion of these adipokines in 3T3-L1 cells. Preincubation of 3T3-L1 adipocytes with increasing concentrations of apoA-I progressively inhibited LPS-mediated MCP-1 secretion (Fig. 1A). MCP-1 levels in the medium decreased from 2526 ± 387 pg/mg in 3T3-L1 cells, which were incubated with LPS alone, to 1204 ± 177 pg/mg protein when the cells were preincubated with apoA-I at a final concentration of 1 mg/ml prior to stimulation with LPS (P < 0.01). Preincubation with apoA-I also reduced LPS-induced IL-6 secretion from 3T3-L1 cells in a concentration-dependent manner (Fig. 1B). The concentration of IL-6 in the medium decreased from 187 ± 17 pg/mg, when the cells were incubated with LPS alone, to 144 ± 2 pg/mg protein (P < 0.05) when the cells were preincubated with apoA-I (final concentration 0.05 mg/ml), and decreased further to 73 ± 5 pg/mg cell protein (P < 0.0001) at a final apoA-I concentration of 1 mg/ml. Consistent with these findings, preincubation with apoA-I (final concentration 1 mg/ml) also reduced MCP-1 and IL-6 mRNA levels by 40 ± 5-fold (Fig. 1C) and 5 ± 0.3-fold (Fig. 1D), respectively, in LPS-stimulated 3T3-L1 cells (P < 0.0001 for both). A potential explanation for a partial apoA-I-mediated reduction in IL-6 and MCP-1 secretion, despite their respective mRNA levels being reduced to baseline, is that a proportion of the secreted cytokines may have originated in intracellular stores that are not targeted by apoA-I. It is also possible that LPS affected adipokine production post-transcriptionally by mechanisms that are not apoA-I-dependent. ApoA-I was not detected in the cell lysates or cell culture medium, indicating that the aforementioned results were not due to the binding of LPS to apoA-I, or to the presence of residual apoA-I in the culture medium of the LPS-stimulated cells.

Preincubation of 3T3-L1 cells with apoA-I, followed by stimulation with LPS, had no effect on adiponectin and resistin secretion or mRNA levels (Supplemental Fig. 1). IL-1β and IFN-γ were not secreted from 3T3-L1 adipocytes, irrespective of whether the cells were incubated in the presence or absence of LPS (not shown).

apoA-I inhibits the LPS-stimulated expression of MCP-1 and IL-6 in 3T3-L1 adipocytes in an ABCA1-dependent manner

Because apoA-I is known to inhibit inflammatory processes in adipocytes in an ABCA1-dependent manner (29), we next determined if the inhibition of LPS-induced MCP-1 and IL-6 synthesis and secretion in 3T3-L1 cells by apoA-I was dependent on ABCA1 or SR-B1.

3T3-L1 cells were transiently transfected with scrambled siRNA, ABCA1 siRNA, or SR-B1 siRNA. ABCA1 mRNA and protein levels were decreased by 51 ± 3% (Fig. 2A; P < 0.0001) and 75 ± 8% (Fig. 2B; P < 0.005), respectively, in the ABCA1 siRNA-transfected cells. Trypan blue exclusion confirmed that siRNA transfection did not compromise cell viability (Supplemental Fig. 2).

When 3T3-L1 cells were transfected with scrambled siRNA and preincubated with apoA-I, the LPS-induced secretion of MCP-1 into the medium decreased from 1660 ± 160 to 934 ± 128 pg/mg protein (Fig. 2C, open bars; P < 0.05), while the LPS-mediated secretion of IL-6 into the medium reduced from 326 ± 26 to 170 ± 20 pg/mg protein (Fig. 2D, open bars; P < 0.05). However, when the cells were transfected with ABCA1 siRNA, preincubation with apoA-I had no effect on MCP-1 secretion (Fig. 2C, closed bars) or on IL-6 secretion (Fig. 2D, closed bars).

Preincubation with apoA-I reduced MCP-1 mRNA levels in the scrambled siRNA-transfected cells by 1.5 ± 0.03-fold (Fig. 2E, open bars; P < 0.01) and decreased IL-6 mRNA levels by 1.5 ± 0.05-fold (Fig. 2F, open bars; P < 0.01). By contrast, apoA-I had no effect on MCP-1 mRNA levels (Fig. 2E, closed bars) and increased IL-6 mRNA levels by 1.6 ± 0.2-fold (Fig. 2F, closed bars; P < 0.01) in the ABCA1 siRNA-transfected cells.

SR-B1 mRNA and protein levels were reduced by 49 ± 2% (Fig. 3A, P < 0.0001) and 44 ± 6% (Fig. 3B, P < 0.05), respectively, in the SR-B1 siRNA-transfected cells. Unlike ABCA1 siRNA-transfected cells, transfection of LPS-stimulated 3T3-L1 cells with SR-B1 siRNA did not prevent apoA-I from inhibiting MCP-1 secretion (Fig. 3C, closed bars), IL-6 secretion (Fig. 3D, closed bars), MCP-1 mRNA levels (Fig. 3E, closed bars) and IL-6 mRNA levels (Fig. 3F, closed bars) in LPS-stimulated 3T3-L1 adipocytes transfected with SR-B1 siRNA. When considered together, these results indicate that the inhibition of LPS-induced inflammatory adipokine synthesis and secretion by apoA-I is dependent on ABCA1, but not on SR-B1.

apoA-I inhibits the LPS-induced activation of MAPKs and nuclear translocation of the NF-κB p65 subunit in 3T3-L1 adipocytes

To establish whether the increased MCP-1 and IL-6 mRNA levels and protein secretion that were observed in LPS-stimulated 3T3-L1 cells in Fig. 1 were
regulated by MAPKs, 3T3-L1 cells were incubated with specific inhibitors to ERK1/2 (U0126), p38MAPK (SB203580), and JNK1/2 (SP600125) prior to stimulation with LPS. The results show that MCP-1 protein secretion (Supplemental Fig. 3A) and mRNA levels (Supplemental Fig. 3C) were regulated by ERK1/2 and JNK1/2, but not p38MAPK, whereas IL-6 protein (Supplemental Fig. 3B) and mRNA levels (Supplemental Fig. 3D) were regulated by ERK1/2 and p38MAPK. These findings are consistent with previous studies showing that the LPS-induced generation of inflammatory adipokines in adipocytes involves the activation of MAPKs (11, 16, 37).

The capacity of apoA-I to inhibit MAPK phosphorylation in LPS-stimulated 3T3-L1 adipocytes was next investigated. Incubation for 10 and 20 min with LPS increased ERK1/2 phosphorylation by 480 ± 46% (P < 0.0001) and 211 ± 38% (P < 0.01), respectively (Fig. 4A, closed bars). Preincubation of the cells with apoA-I abrogated the increase in ERK1/2 phosphorylation by 66 ± 6% (P < 0.0001) at 10 min and by 70 ± 10% at 20 min (P < 0.005) (Fig. 4A, striped bars). Similarly, p38MAPK phosphorylation was increased by 80 ± 33% and 83 ± 11% (P < 0.05 for both) when 3T3-L1 cells were incubated with LPS for 10 and 20 min, respectively (Fig. 4B, closed bars), and preincubation with apoA-I decreased p38MAPK phosphorylation by 60 ± 9% (P < 0.005) at 10 min and by 60 ± 12% at 20 min (P < 0.01) (Fig. 4B, striped bars). Incubation for 10 and 20 min with LPS increased JNK1/2 phosphorylation by 60 ± 15% (P < 0.01) and 49 ± 14% (P < 0.05), respectively (Fig. 4C, closed bars). This effect was suppressed when the cells were preincubated with apoA-I, with JNK1/2 phosphorylation returning to baseline at 10 min (P < 0.01) and 20 min (P < 0.05) (Fig. 4C, striped bars). The STAT3 signal transduction pathway was not activated when 3T3-L1 cells were preincubated with apoA-I prior to activation with LPS (result not shown).

Importantly, ERK1/2, p38MAPK or JNK1/2 phosphorylation and NF-κB p65 subunit levels in the nucleus were not increased when the cells were preincubated with apoA-I alone.

As NF-κB is a key regulator of MCP-1 and IL-6 expression in adipocytes (11), the effect of apoA-I on NF-κB activation in 3T3-L1 adipocytes was also investigated. Incubation of 3T3-L1 cells with LPS for 10 min increased nuclear levels of the NF-κB p65 subunit by 27 ± 9% (Fig. 4D, closed bar; P < 0.05). By contrast, nuclear translocation of the NF-κB p65 subunit was decreased by 29 ± 3% in 3T3-L1 cells that had been preincubated with apoA-I (Fig. 4D, striped bar; P < 0.01).

Figure 1. ApoA-I inhibits LPS-stimulated MCP-1 and IL-6 synthesis and secretion in 3T3-L1 adipocytes. 3T3-L1 cells were incubated for 16 h in the presence (striped bars) or absence of apoA-I (open and closed bars) at the indicated concentrations. The apoA-I was removed and the cells were incubated for an additional 6 h with (closed bar) or without LPS (open bar, final concentration 100 ng/mL). Secretion of MCP-1 (A) and IL-6 (B) into the cell culture medium was measured by ELISA. MCP-1 (C) and IL-6 (D) mRNA levels were quantified by qPCR. Values represent means ± SEM of 3 independent experiments. *P < 0.05, **P < 0.01, ****P < 0.0001 vs. incubation with LPS.
apoA-I inhibits MAPK activation in 3T3-L1 adipocytes in an ABCA1-dependent manner

Preincubation of scrambled siRNA-transfected cells with apoA-I decreased the LPS-induced phosphorylation of ERK1/2 by 53 ± 6% (Fig. 5A, striped bar; P < 0.05), and p38MAPK and JNK1/2 phosphorylation were reduced by 52 ± 6% (Fig. 5B, striped bar; P < 0.05) and 59 ± 6% (Fig. 5C, striped bar; P < 0.005), respectively. Preincubation with apoA-I did not inhibit the LPS-induced phosphorylation of ERK1/2 (Fig. 5A, striped bar), p38MAPK (Fig. 5B, striped bar), or JNK1/2 (Fig. 5C, striped bar) in 3T3-L1 cells that were transfected with ABCA1 siRNA. When 3T3-L1 cells that had been transfected with scrambled siRNA or ABCA1 siRNA, were preincubated with apoA-I, the LPS-mediated increase in nuclear NF-κB p65 subunit protein levels was attenuated by 47 ± 22% (P < 0.05) and 30 ± 3% (P < 0.05) (Fig. 5D, striped bars), respectively.

Taken together, these results demonstrate that apoA-I suppresses the activation of MAPKs in 3T3-L1 cells in an ABCA1-dependent manner and that apoA-I inhibits NF-κB activation in adipocytes by a mechanism that is ABCA1-independent.

apoA-I reduces cell surface TLR4 expression and MyD88 mRNA levels in 3T3-L1 adipocytes in an ABCA1-dependent manner

LPS is the main ligand of TLR4 and uses the adaptor protein, MyD88, to activate MAPKs and NF-κB and induce inflammation in adipocytes (19, 38). Incubation of 3T3-L1 adipocytes with apoA-I did not affect TLR4 mRNA or total TLR4 protein levels (Figs. 6A, B, respectively). However, incubation with apoA-I decreased cell surface TLR4 protein levels by 26 ± 3% (Fig. 6C; P < 0.0001). This reduction in cell surface TLR4 protein levels did not reflect depletion of TLR4 from lipid rafts (Supplemental Fig. 4). ApoA-I also reduced cell surface TLR4 protein levels by 16 ± 2% in 3T3-L1 cells that had been transfected with scrambled siRNA (Fig. 6D; P < 0.005). Incubation with apoA-I, by contrast, did not alter cell surface TLR4 protein levels in 3T3-L1 cells transfected with ABCA1 siRNA.

ApoA-I decreased MyD88 mRNA levels by 18 ± 3% in nontransfected 3T3-L1 cells (Fig. 6E; P < 0.01) and by 24 ± 4% in 3T3-L1 cells transfected with scrambled siRNA (Fig. 6F; P < 0.01). Once again, this effect of apoA-I was lost in 3T3-L1 cells that were transfected with ABCA1 siRNA (Fig. 6F). Taken together, these results suggest that apoA-I suppresses cell surface expression of TLR4 and MyD88 synthesis in an ABCA1-dependent manner.

DISCUSSION

Chronic inflammation in adipose tissue is closely associated with metabolic disorders such as obesity, insulin resistance, and type 2 diabetes. In this regard, activation of inflammatory signaling pathways has emerged as an...
important link between such disorders. TLR4 plays a key role in the transduction of inflammatory signals and has recently been associated with insulin resistance in adipocytes (18). The current study establishes for the first time that apoA-I inhibits the TLR4-mediated activation of MAPKs, NF-κB, and downstream inflammatory signal transduction pathways in cultured adipocytes. Moreover, the inhibition of MAPKs, but not NF-κB, was shown to be ABCA1-dependent.

This mechanism of action of apoA-I is initiated by an ABCA1-dependent reduction in cell surface TLR4 expression and MyD88 expression (Fig. 7i). ApoA-I also inhibits the TLR4/MyD88-mediated activation of ERK, p38MAPK, and JNK (Fig. 7ii) and the synthesis and secretion of MCP-1 and IL-6, in an ABCA1-dependent manner (Fig. 7iii). The TLR4/MyD88-stimulated nuclear translocation of the p65 subunit of NF-κB is, by contrast, inhibited by apoA-I independent of ABCA1 (Fig. 7iv). The finding that apoA-I inhibits proinflammatory adipokine expression has important therapeutic implications as both MCP-1 and IL-6 are associated with adipose tissue inflammation (39–41). MCP-1 induces infiltration of macrophages into adipose tissue, which exacerbates adipose tissue inflammation and impairs whole body insulin sensitivity and glucose homeostasis (40). Adipocytes from insulin-resistant humans have increased expression of IL-6, which also inhibits the expression of the insulin-sensitizing adipokine, adiponectin (42–44). Additionally, IL-6 induces insulin resistance in 3T3-L1 adipocytes (41).

ApoA-I is the primary acceptor of cholesterol that is exported from cells that express ABCA1. The results showing that apoA-I inhibits LPS-induced MCP-1 and IL-6 expression in 3T3-L1 cells in an ABCA1-dependent manner indicate that the anti-inflammatory effects of apoA-I in adipocytes are likely to be related to the cholesterol content of cell membranes. This was recently found to be the case by Umemoto et al. (29), who reported that apoA-I inhibits the palmitate-induced synthesis of MCP-1 in 3T3-L1 adipocytes in an ABCA1-dependent manner. Similar effects were observed when cholesterol was removed from 3T3-L1 adipocytes by incubation with methyl-β-cyclodextrin, and incubation of 3T3-L1 adipocytes with cholesterol-loaded cyclodextrin increased MCP-1 synthesis. Overall, these results are consistent with cholesterol efflux being a requirement for the anti-inflammatory effects of apoA-I in adipocytes.

The apoA-I concentrations that were used to inhibit MCP-1 synthesis in LPS-stimulated 3T3-L1 adipocytes in the present study were higher than what has been reported previously for palmitate-stimulated 3T3-L1 adipocytes (29). This difference reflects the fact that LPS is more bioactive than palmitate. Treatment of 3T3-L1
adipocytes for 24 h with 2 ng/ml LPS increases MCP-1 synthesis and secretion, whereas incubation for 24 h with 500 μM of palmitate had no effect (20). Additionally, free fatty acids do not induce TLR signaling or proinflammatory cytokine production (45, 46). These findings show that a higher apoA-I concentration is required to inhibit an LPS-mediated increase in MCP-1 mRNA levels in 3T3-L1 cells relative to palmitate-stimulated 3T3-L1 cells.

The current finding showing that apoA-I reduces TLR4 protein levels at the adipocyte surface in an ABCA1-dependent manner is also consistent with reports suggesting that accumulation of unesterified cholesterol in the plasma membrane is associated with increased TLR4 signaling (47, 48). Although a possible mechanism underlying this effect may be an apoA-I-mediated disruption of cholesterol-rich lipid rafts within the plasma membrane (29), we did not detect a reduction in the TLR4

![Figure 4](image-url)

Figure 4. ApoA-I inhibits activation of MAPKs and nuclear translocation of the NF-κB p65 subunit. 3T3-L1 adipocytes were incubated for 16 h in the presence (striped bars) or absence of apoA-I (1 mg/ml), then incubated with (closed bars) or without LPS (open bar, 100 ng/ml) for 10, 20, 30, and 60 min. Cell lysates were Western blotted for phospho-ERK1/2, and ERK1/2 (n = 3) (A), phospho-p38MAPK and p38MAPK (n = 4) (B), and phospho-JNK1/2 and JNK1/2 (n = 5) (C). Nuclear and cytoplasmic cell lysates were Western blotted for the p65 subunit of NF-κB (n = 3) (D). Mean values for the control samples were normalized to 100%. Values represent the means ± SEM. All experiments were carried out in duplicate. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.0001 vs. incubation with LPS.
content of lipid rafts in 3T3-L1 cells following incubation with apoA-I. The current results further indicate that, although incubation with apoA-I significantly decreases 3T3-L1 cell surface TLR4 levels, TLR4 synthesis and total protein levels remained unchanged. This suggests that apoA-I may regulate intracellular TLR4 trafficking, which is supported by the observation that apoA-I decreases the synthesis of MyD88 in 3T3-L1 cells in an ABCA1-dependent manner, thereby suppressing downstream TLR4 signal transduction pathways. Similar observations have been made in other cell types. For example, apoA-I decreases cell surface expression of TLR4 in human microvascular endothelial cells (24) and inhibits MyD88 synthesis in human monocyte-derived macrophages (26). ApoA-I also attenuates palmitate-induced TLR4 recruitment into the lipid rafts in bovine aortic endothelial cells (25), and apoA-I and the apoA-I mimetic peptide, 4F, reduce TLR4 expression and protect against
LPS-stimulated inflammation in human monocyte-derived macrophages (49).

In vitro studies suggest that TLR4 activates MAPKs such as ERK and JNK in 3T3-L1 adipocytes (11, 15) and activates p38MAPK in primary cultures of human adipocytes (16) in response to LPS stimulation. MAPKs have recently been linked to obesity-associated inflammation and insulin resistance as evidenced by reports of ERK1- and JNK1-deficient mice having decreased adiposity and reduced susceptibility to diet-induced obesity and insulin resistance (13, 50). Additionally, oral administration of a p38MAPK inhibitor reduced high-fat diet-induced obesity in mice (51). Mice with diet-induced obesity and genetically obese mice also have high JNK activity in adipose tissue, liver, and muscle (13). Chronic activation of ERK, p38MAPK, and JNK in 3T3-L1 adipocytes by inhibiting the insulin signaling pathway (14). The present study extends these observations by showing for the first time that apoA-I exerts anti-inflammatory effects in 3T3-L1 adipocytes by inhibiting the LPS-mediated activation of all 3 MAPKs.

NF-κB is a master regulator of inflammation. In the basal state, the NF-κB subunits p50 and p65 associate with the NF-κB inhibitor, IκB, in the cytoplasm. In the presence of an inflammatory stimulus, IκB is degraded, and the NF-κB subunits translocate to the nucleus where they increase transcription of inflammatory genes, such as MCP-1 and IL-6 (46, 52, 53). Previous studies have reported that inhibition of NF-κB with salicylates, or targeted disruption of IκB kinase-B, protects against obesity-induced insulin resistance (54, 55). A key finding from the present study is that apoA-I down-regulates the LPS-induced nuclear localization of the NF-κB p65 subunit in 3T3-L1 adipocytes. However, unlike the effects of apoA-I on TLR4, MyD88, and MAPK signaling, inhibition of NF-κB signaling by apoA-I was independent of ABCA1. This is contrary to what has been reported in human coronary artery endothelial cells (56) and human monocyte-derived macrophages (26), where the apoA-I mimetic peptide ETC-642 and full-length apoA-I inhibit nuclear localization of the p65 subunit of NF-κB in an ABCA1-dependent manner. The reason for this discrepancy remains unknown.
In summary, this report provides new insights into the mechanisms by which apoA-I inhibits inflammation in adipocytes. As adipose tissue inflammatory responses are a hallmark feature of obesity and type 2 diabetes and increased cardiovascular risk (57), this study suggests that interventions that increase apoA-I levels may be of potential therapeutic value in these pathologies.

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REFERENCES


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