Inflammatory Signaling Pathways Regulating ApoE Gene Expression in Macrophages*

Anca V. Gafencu1,2, Marius R. Robciuc3, Elena Fuior1, Vassilis I. Zannis4,5, Dimitris Kardassis3, and Maya Simionescu3

From the 1Institute of Cellular Biology and Pathology Nicolae Simionescu, 8 B. P. Hasdeu Street, P. O. B. 35-14, Sect. 5, Bucharest 050568, Romania, the 2University of Crete Medical School, Heraklion, Crete GB-71110 Greece, and the 3Boston University School of Medicine, Boston, Massachusetts 02718

The atheroprotective role of apolipoprotein E (apoE) is well established. During inflammation, expression of apoE in macrophages is reduced leading to enhanced atheromatous plaque development. In the present study, we investigated the signaling pathways involved in the repression of apoE gene expression in response to lipopolysaccharide (LPS) treatment, a condition that mimics the inflammatory stress, in mouse macrophages RAW 264.7. We identified Tpl-2 and MEKK1 as the kinases that are primarily responsible for the down-regulation of apoE promoter activity by LPS. Using a dominant negative form of IκB, we established that Tpl-2 and MEKK1 signaling pathways converge to NF-κB acting on the apoE core promoter —55/+73. In addition to NF-κB activation, LPS also activated c-Jun via its phosphorylation by JNK. The activity of the apoE promoter was repressed by c-Jun, whereas small interference RNA-mediated inhibition of endogenous c-Jun expression reversed the inhibitory effect of Tpl-2 on the apoE promoter. Transfection experiments and DNA binding assays showed that the binding site for c-Jun is in the —55/+73 region of the apoE promoter. Finally, we showed that LPS inhibited apoE gene expression via activation of the Tpl-2/MEK/ERK pathway acting on a different apoE promoter region. In summary, LPS represses apoE gene expression in macrophages via signaling pathways that involve the upstream kinases Tpl-2 and MEKK1, the intermediate mitogen-activated protein kinases ERK and JNK, and the downstream transcription factors AP-1 and NF-κB that inhibit the apoE promoter activity via distinct regions.

Human apolipoprotein E (apoE),2 a glycoprotein of 35 kDa, plays a key role in lipid metabolism, as a major component of various plasma lipoprotein classes, including chylomicrons, very low density lipoproteins, and large high density lipoproteins (1). As a ligand for the low density lipoprotein receptor family, apoE scavenges lipoprotein remnants and mediates clearance of apoE-rich large high density lipoprotein particles from the serum, a significant determinant of the redistribution of cholesterol and triglycerides between the peripheral tissues and the liver.

ApoE is mainly synthesized by the liver, kidney, lung, spleen, skin, brain, and various cells such as macrophages (2). ApoE production is of particular significance at sites endangered by the uptake of lipoproteins, such as the atheromatous plaques. The protective role of apoE in atherosclerosis was recognized in apoE knockout mice, which is a well established animal model for atherogenesis (3). At the level of atheromas, macrophages are the primary providers of apoE. This was elegantly demonstrated in transgenic mice expressing apoE only in macrophages, which were protected from atherosclerosis, even though the plasma levels of apoE were exceedingly low and the animals were hypercholesterolemic (4). Consistently, transgenic mice with normal levels of plasma apoE and apoE-null macrophages were more susceptible to atherosclerosis (5). ApoE secretion by macrophages within the atherosclerotic plaque facilitates efflux of cholesterol to exogenous acceptors (such as high density lipoprotein), assisting reversal of cholesterol transport to the liver.

The human apoE gene is 3.6 kb in length and is located on chromosome 19, at the 5'-end of the apoE/apoCI/apoCIV/apoCII gene cluster (6). Transcriptional regulation of apoE requires the aggregation of several transcription factors on the proximal promoter. Several positive and negative elements have been detected on the apoE promoter (7), including the AP-2 and Zic1/Zic2 sites and the non-canonical E-box, which are important regulatory elements within the proximal apoE promoter in astrocytes (8–10). Transcription factors that drive the apoE expression in macrophages are not fully identified. Currently, there is a general agreement that atherogenesis entails both a lipid disorder and an inflammatory process. The prominent role of inflammation in atherosclerosis (11) requires further elucidation of the molecular mechanisms at the level of the atherosclerotic plaque, which may lead to novel or more complex anti-inflammatory therapeutic targets. The aim of this study was to elucidate the regulatory mechanisms that control apoE promoter activity in macrophages in response to inflammatory signals. To mimic the inflammatory state, we used lipopolysaccharide (LPS), which activates numerous signal
transduction cascades, including the NF-κB-dependent production of inflammatory cytokines (12). We report here that LPS represses apoE gene expression in macrophages. The mechanism involves Toll-like receptor-4-mediated activation of the upstream kinases Tpl-2 and MEKK1, activation and nuclear translocation of the NF-κB, and AP-1 transcription factors that act directly on the apoE promoter.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction and modification enzymes (T4 ligase, DNA polymerase, and calf intestinal alkaline phosphatase) were purchased from Invitrogen or Promega (Madison, WI). Dulbecco’s modified Eagle’s medium and fetal calf serum were from Invitrogen, Titan™ One Tube RT-PCR System was from Roche Applied Science, ECL Western blotting kit was from Pierce, Luciferase assay system was from Promega, and all other reagents were from Sigma. The primers were obtained from Invitrogen; siRNA for human c-Jun and all antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

**Plasmid Constructions**—The apoE proximal promoter (−500/+73) was cloned in the pGL3 basic vector (Promega) that contains as a reporter the promoterless luciferase (luc) gene. Briefly, the primer sequence −500/+302 was amplified by PCR using the following primers: F, 5′-TTTGGTACC-GCTGGTCTCACAAGGTCATGGATTGC-3′; R, 5′-TTTAGGTTAACTCGTGACTGGGAT-3′. After amplification, the DNA fragments were digested with KpnI and SacI and cloned in the pGL3 vector and the plasmid containing the luciferase reporter gene were isolated with Dynabeads M-280 streptavidin (Invitrogen). The primers were obtained from Invitrogen; siRNA for human c-Jun and all antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

**Transient Transfections**—Transient transfections were performed by the Ca3(PO4)2 coprecipitation method (13). Transiently transfected RAW 264.7 macrophages were treated for 18 h with 1 μg/ml LPS, alone or together with inhibitors (10 μM U0126, 10 μM cyclosporine, 10 nM PMA). Forty hours after transfection, the cells were harvested and subsequently lysed in the buffer included in the luciferase assay kit (Promega) followed by a freeze-thaw cycle and centrifugation at 13,000 rpm for 5 min. Luciferase assays were performed using the luciferase assay kit from Promega (as indicated by the manufacturer). The activity of β-galactosidase was determined using o-nitrophenyl galactopyranoside.

**DNA Pulldown Assay**—DNA fragments of the apoE promoter were biotinylated by PCR, using the corresponding apoE-luc plasmids as templates and the primers described for cloning except that the reverse primer (R + 73) was biotinylated at the 5′-end. In other experiments, biotinylated forward primer RV3 primer (5′-CTAGCAAATAGGCTGTCCC, Promega), and R + 73 unbiotinylated were employed. In this case, the amplified product contained a fragment of 58 bp corresponding to the pGL3-basic vector. Biotinylated DNA was coupled to the Dynabeads M-280 streptavidin according to the manufacturer’s instructions. Briefly, biotinylated DNA was incubated with Dynabeads M-280 streptavidin for 15 min at room temperature in B & W buffer (5 mM Tris-HCl, pH 7.5, with 0.5 mM EDTA and 1 mM NaCl) in a slight excess of DNA. After coupling, the beads were washed twice with B & W buffer to remove the unbound DNA and once with the binding buffer (see below). When RV3 primer was used, after immobilization of the DNA on Dynabeads, half of the total amount of the beads was treated with KpnI, to remove the (−55/+73) apoE fragment; the beads containing the 58 bp from pGL3 vector were used as negative control for the DNA binding assay. Nuclear extracts were purified as previously described (16); these extracts were incubated with the biotinylated DNA immobilized on Dynabeads for 90 min at 4 °C in binding buffer (10 mM Hepes, pH 7.9, with 50 mM KCl, 2 mM MgCl2, 4 mM spermidine, 0.1 mg/ml bovine serum albumin, 20 μM zinc acetate, 10% glycerol, and 0.05% Nonidet P-40). The complexes formed were isolated with Dynabeads M-280 streptavidin, washed with binding buffer, and then subjected to SDS-PAGE.
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**RESULTS**

**LPS Induces ApoE Gene Expression in Monocytes and Inhibits ApoE Gene Expression in Macrophages**—To determine the effect of bacterial LPS on the expression of apoE in monocytes and macrophages, we employed two monocytic cell lines (U937 and THP-1), a macrophage cell line (RAW 264.7), and thioglycollate-elicited MPMs. The cells were incubated with 1 μg/ml LPS for 18 h, and apoE expression was determined by RT-PCR. The results showed a differential modulation of apoE by LPS in monocytes and macrophages. As illustrated in Fig. 1A, apoE is expressed in both monocytic cell lines only in the presence of LPS. By contrast, this treatment decreased the basal levels of apoE mRNA in macrophage cell line by 30%, and in mouse peritoneal macrophages by 48%. Semiquantitative evaluation of the products obtained by RT-PCR showed a dose-dependent repression of apoE in RAW264.7 cells by LPS treatment (for 18 h). As compared with the control, the repression induced by LPS was statistically significant (p < 0.035, Fig. 1B).

**Inhibition of ApoE Gene Expression by LPS Involves Kinases Tpl-2 and MEKK1**—To investigate the mechanism of apoE gene repression by LPS, the apoE proximal promoter followed by Western blotting, using anti-p50 or anti-c-Jun antibodies. The blots were developed using an ECL kit from Pierce. Immuno blotting—RAW 264.7 cells treated with LPS for various time intervals were washed with phosphate-buffered saline, harvested, and solubilized in Laemmli buffer. The samples were subjected to SDS-PAGE and transferred onto nitrocellulose, and the blots were tested with the following antibodies: anti-ERK, anti-phospho-ERK, anti-phospho-SAPK, anti-c-Jun, anti-phospho c-Jun, and anti-p50. In other experiments, LPS-treated cells were subjected to hypo-osmotic lysis using 7 mM phosphate buffer containing protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride and 1 mM benzamidine). The cell homogenate was passed 10 times through a syringe needle (24-gauge) and then centrifuged 10 min at 750 × g. The pellet containing the nuclear fraction was washed twice with phosphate-buffered saline and solubilized in Laemmli buffer. The proteins from the supernatant, considered the cytoplasmic fraction, were precipitated with trichloroacetic acid (12% final concentration) and solubilized in Laemmli buffer. The protein concentration was determined using Amido Black (17). From each sample, aliquots of 30 μg of protein were subjected to Western blot using the polyclonal antibodies anti-p50 and anti-c-Jun, followed by the secondary horseradish peroxidase-conjugated antibodies, which were revealed using an ECL kit (Pierce). The bands were quantified by densitometry using the Total Lab software (Amersham Biosciences).

Statistics—Values in the text are means ± S.E. One-way analysis of variance was performed using OriginPro 7.5. For p values <0.05, the population means are statistically different.
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NF-κB Is Involved in LPS-induced ApoE Down-regulation and Is a Common Downstream Target of Tpl-2 and MEKK1—Next, we examined the involvement of the NF-κB pathway in the LPS-induced inhibition of apoE promoter activity in macrophages. For this purpose, we determined the nuclear and cytosolic distribution of the p50 subunit of NF-κB under LPS treatment. The Western blot data showed that the p50 precursor (p105) was present in the cytoplasmic fraction of untreated macrophages. After 3 h of exposure to LPS, the p50 subunit was translocated to the nucleus, where it remained during the subsequent 18 h of exposure to LPS. However, after 18 h of LPS exposure, p105 (p50 precursor) reappeared in the cytosolic fraction (Fig. 3A). Similar to the p50 subunit, tracking of the p65 subunit of NF-κB showed nuclear translocation after 3 h of LPS treatment (data not shown).

Next, we evaluated whether NF-κB is involved in LPS-induced suppression of apoE promoter activity. Macrophages were transiently transfected with the (−500/+73) apoE promoter, along with Tpl-2 or MEKK1 in the presence or in the absence of a dominant negative form of IκB (IκBΔN), and exposed to the LPS challenge. As illustrated in Fig. 3B, the inhibitory effect of LPS on the (−500/+73) apoE promoter activity (columns 1 and 2) was reversed by the overexpression of IκBΔN (column 3). Moreover, inhibition of apoE promoter activity by Tpl-2 and MEKK1 (columns 4 and 6, respectively), was completely reversed by IκBΔN overexpression (columns 5 and 7, respectively).

The region of the apoE promoter that responds to NF-κB activation was investigated by transient transactivation assays. Macrophages were transfected with the three fragments of the apoE promoter and either wild-type IKKβ kinase or a p50/65 hybrid. This hybrid is composed of the Rel homology domain of p50 and the transactivation domain of p65. The hybrid is a constitutively active protein that simulates the active p50/65 heterodimer of NF-κB. Overexpression of either IKKβ kinase or the p50/65 hybrid abolished the activity of all apoE promoter fragments (Fig. 3C), showing that the binding site for NF-κB is contained in the smallest apoE promoter fragment (−55/+73).

To validate the activation of the NF-κB pathway by the plasmids employed here, HEK293 cells were cotransfected with a vector containing three binding sites for NF-κB in sequence with the luciferase reporter gene (NF-κB)3-luc. In transfected cells, overexpression of Tpl-2, MEKK1, and IκBΔN activated the (NF-κB)3 promoter to various degrees (Fig. 3D). By contrast, overexpression of MEKK1 did not affect the activity of the (NF-κB)3 promoter. These data demonstrate that NF-κB directly inhibits the apoE promoter and that the inhibitory effect of Tpl-2 and MEKK1 on the apoE promoter is mediated by their interference with the NF-κB pathway.

AP-1 Transcription Factors Mediate the ApoE Promoter Down-regulation by Tpl-2 in Macrophages Exposed to LPS—We next evaluated the role of AP-1 transcription factors in apoE gene regulation in response to LPS treatment. For this purpose, macrophages were homogenized after incubation with 1 μg/ml LPS for variable periods of time and probed using specific antibodies in Western blot experiments. Up-regulated expression of c-Jun was noticed after a brief incubation (15 min) of the macrophages with LPS, and peak amounts were found after 60 min of incubation (Fig. 4A). Phosphorylated c-Jun was consistently increased as early as 15 min following LPS addition and was decreased after prolonged incubation of the macrophages with LPS (Fig. 4A). Phosphorylation of c-Jun implied early activation of Jun N-terminal kinase (JNK), as observed by immunoblotting (data not shown). Activation of c-Jun depends on its translocation to the nucleus. In our experiments, an increase in the nuclear to cytosolic partition was directly related to the duration of macrophage incubation with LPS (Fig. 4A). Thus, in LPS-treated macrophages, c-Jun is up-regulated, phosphorylated, and translocates to the nucleus.

Next, we investigated whether Tpl-2 and MEKK1 are responsible for c-Jun up-regulation and phosphorylation by LPS. HEK293 cells were transfected with Tpl-2 or MEKK1, and c-Jun expression and phosphorylation were evaluated by immunoblotting. c-Jun expression was quantified by normalization against β-galactosidase, cotransfected as an internal reference. Both Tpl-2 and MEKK1 induced c-Jun expression (Fig. 4B, lanes 2 and 4, respectively) as compared with the control cells (Fig. 4B, lane 1). c-Jun induction by Tpl-2 and MEKK1 was decreased by ~50% after c-Jun silencing using siRNA (Fig. 4B, lanes 3 and 5, respectively). Moreover, we detected the phos-
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phosphorylated form of c-Jun in cells overexpressing Tpl-2 and MEKK1 (Fig. 4B, lanes 7 and 8, respectively), as compared with the control (Fig. 4B, lane 6).

To determine whether c-Jun is involved in the down-regulation of apoE promoter activity by Tpl-2 and MEKK1, cells were transiently transfected with an expression vector for Tpl-2 or MEKK1, the (-500/+73) apoE-luc plasmid and a siRNA specific for c-Jun. Partial inhibition of c-Jun expression by siRNA ameliorated the inhibitory effect of Tpl-2 and MEKK1 on the apoE promoter (Fig. 4C). The same results were obtained in RAW macrophages (not shown).

The inverse relationship between the amount of total or phosphorylated c-Jun and the apoE promoter activity strongly suggested that c-Jun is a central mediator of apoE promoter regulation by Tpl-2 and MEKK1. Consistent with the data related to NF-κB, the site of c-Jun-mediated inhibition was identified in the shortest (-55/+73) apoE promoter fragment (Fig. 4D).

To validate the binding of c-Jun to the (-55/+73) apoE promoter fragment, we performed DNA pulldown experiments. In these assays, the apoE promoter fragments -445/+73 and -55/+73 were amplified by PCR using biotinylated primers and were incubated with nuclear extract of LPS-treated RAW 264.7 macrophages. These experiments showed that both -445/+73 and -55/+73 apoE promoter fragments bind c-Jun (Fig. 4E, lanes 1 and 3, respectively). Competition experiments, in which two times excess of unbiotinylated DNA was added (under the same experimental conditions), revealed the specificity of the binding (Fig. 4E, lanes 2 and 4). Control experiments in which untreated RAW 264.7 cells were used under

FIGURE 3. LPS-induced engagement of NF-κB pathway in RAW macrophages decreases apoE expression. A, assayment by Western blot of the presence of p50 subunit or its precursor (p105) in the cytoplasmic (C) and the nuclear fraction (N) obtained from macrophages exposed for various time intervals to 1 μg/ml LPS. B, RAW macrophages were transiently cotransfected with 3 μg of the (-500/+73) apoE-luc plasmid along with 1 μg of cytomegalovirus β-galactosidase plasmid and 1.5 μg of vectors expressing Tpl-2 wild-type or MEKK1 in the presence or absence of a dominant negative form of IkB (IkBDN), and treated with 1 μg/ml LPS for 18 h or left untreated, as indicated. 40 h following the transfection, the cells were harvested and the luciferase activity was determined in the cell lysate. The mean values (±S.E.) from at least two independent transfections performed in duplicate are presented in the form of a bar graph. Note that IkBDN reverts the negative effects of LPS or of Tpl-2 and MEKK1 on apoE promoter activity. The difference in the activity of apoE promoter under LPS treatment or Tpl-2 and MEKK1 overexpression in the absence or in the presence of IkBDN overexpression was statistically significant (p < 0.05). C, RAW macrophages were transiently cotransfected with 2 μg of the apoE proximal promoter (-500/+73) apoE-luc or the deletion mutants (-100/+73) apoE-luc and (-55/+73) apoE-luc in the absence or in the presence of expression vectors for IKKβ and for the p65/p50 hybrid of NF-κB (2 μg). The activity of the apoE promoter fragments was normalized to the β-galactosidase activity. Note that all apoE promoter fragments tested were inhibited by IKKβ and IKKβ/p65 (p < 0.015 for IKKβ and p < 0.03 for p65/50). D, activation of the NF-κB pathway in HEK293 cells by Tpl-2, MEKK1, and IKKβ. HEK293 cells were transfected with a plasmid containing three binding sites for NF-κB (NFκB3-luc, 2 μg) in the absence or in the presence of expression vectors for Tpl-2, MEKK1, and IKKβ (2 μg). The normalized luciferase activity is shown in the form of a bar graph. Note that Tpl-2 as well as MEKK1 and IKKβ (p < 0.01), but not MEK1 (p > 0.8) increase the activity of the promoter containing three NF-κB binding sites.
similar experimental conditions gave negative results (data not shown). In addition, another strategy was used to confirm the binding of c-Jun to the $-55/+73$ fragment of apoE promoter.
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the reverse apoE +73 primer. This DNA fragment bound c-Jun present in LPS-treated nuclear extracts (Fig. 4E, lane 5), whereas no specific binding was detectable when the apoE promoter fragment was removed by Kpnl digestion (Fig. 4E, lane 6).

MEK1/2 Kinases Are Involved in the Repression of the ApoE Promoter Activity by Tpl-2 in Macrophages—We next determined the involvement of other kinases in apoE gene repression by LPS. We found that the repression in apoE gene expression by LPS in cultured MPMs (to 57 ± 4.6% relative to the control) was reversed by the specific MEK1 inhibitor U0126, as determined by RT-PCR. Furthermore, the MEK1 inhibitor counterbalanced very effectively the inhibition of apoE promoter activity by LPS in transiently transfected RAW 264.7 macrophages (Fig. 5B). The data represent the average of the intensity of the bands corresponding to the apoE gene obtained by RT-PCR normalized to the expression levels of GAPDH, using MPM isolated from three mice.

To test whether MEK1 is a downstream target of Tpl-2 or MEKK1 in macrophages and mediates their negative effect on apoE promoter activity, RAW 264.7 cells were cotransfected with the plasmid (−500/+73) apoE-luc and the expression vectors for Tpl-2 (Fig. 5C) or MEKK1 (Fig. 5D) in the absence or presence of the inhibitor U0126. The results showed that the MEK1/2 inhibitor can only partially reverse the Tpl-2 repressive effect on the apoE promoter (Fig. 5C), whereas the MEKK1 inhibitory activity on the apoE promoter was not affected by U0126 (Fig. 5D). The same results were obtained using a different MEK1 inhibitor, PD98059 (data not shown).

Another downstream target known to be engaged in MEKK1 signaling (through SAPK/ERK kinase) is p38 (20). Cotransfection experiments in which an expression vector for p38 was used showed that overexpression of p38 did not influence the apoE promoter activity; moreover, the p38 inhibitor, SB203580, used showed that overexpression of p38 did not influence the promoter activity by LPS in transiently transfected RAW 264.7 macrophages (Fig. 5B). The data represent the average of the intensity of the bands corresponding to the apoE gene obtained by RT-PCR normalized to the expression levels of GAPDH, using MPM isolated from three mice.

To test whether MEK1 is a downstream target of Tpl-2 or MEKK1 in macrophages and mediates their negative effect on apoE promoter activity, RAW 264.7 cells were cotransfected with the plasmid (−500/+73) apoE-luc and the expression vectors for Tpl-2 (Fig. 5C) or MEKK1 (Fig. 5D) in the absence or presence of the inhibitor U0126. The results showed that the MEK1/2 inhibitor can only partially reverse the Tpl-2 repressive effect on the apoE promoter (Fig. 5C), whereas the MEKK1 inhibitory activity on the apoE promoter was not affected by U0126 (Fig. 5D). The same results were obtained using a different MEK1 inhibitor, PD98059 (data not shown).

In other experiments, we tested whether LPS can activate ERK1/2, a known downstream target of MEK1/2. For this purpose, RAW 264.7 macrophages were treated with 1 μg/ml LPS for different time intervals and were tested for the ERK1/2 phosphorylation by immunoblotting using antibodies that recognize phosphorylated ERK. The results showed that ERK1/2 phosphorylation is an early response to the LPS treatment, and both isoforms, p44 and p42, were phosphorylated after 10 min of LPS exposure of the cells (Fig. 5E, lane 2), whereas the control (untreated) cells expressed only a very faint amount of phospho-p42 (Fig. 5E, lane 1). Extended LPS treatment did not lead to the sustained activation of ERK1/2; at 1 h of LPS treatment the amount of phospho-ERK1 and -2 significantly decreased (Fig. 5E, lane 3). To determine whether ERK1/2 is phosphorylated by MEK1/2, the RAW cells were treated with LPS for 10 min, in the presence or absence of the MEK1/2 inhibitor U0126. The results showed that ERK phosphorylation was dependent on MEK1/2, because the MEK inhibitor blocked the ERK phosphorylation by LPS (Fig. 5E, lane 4).

Transient transfection experiments were employed to search for the region in the apoE promoter that responds to ERK1/2 activation. The results illustrated in Fig. 5F showed that the overexpression of the ERK2 inhibited the activity of the (−500/ +73) apoE promoter in RAW 264.7 cells, and this inhibition was drastically decreased by deletion of the region between −500 and −100. Thus, the region of the apoE promoter that responds to ERK2 activation is the −500 to −100.

DISCUSSION

ApoE has important functions in lipoprotein clearance and cholesterol redistribution, which counteracts atheroma formation. This protein is supplied to the atherosclerotic plaques primarily by macrophages locally differentiated. Accordingly, reduction or absence of macrophage synthesis of apoE promotes the development of atheromas. Within the atherosclerotic plaques, macrophages are often submitted to the inflammatory stress. In this study, we aimed to determine the molecular mechanisms of apoE gene suppression in macrophages under inflammatory stress mimicked by LPS treatment.

Previous studies have revealed that LPS has variable effects on apoE gene expression in different cell types. For example, in CaCo-2 epithelial cells, LPS represses apoE gene expression, and apical secretion in a dose- and time-dependent manner (21). LPS induces apoE gene expression in monocytes but represses gene expression in macrophages (Fig. 1). Finally, apoE gene expression is induced by LPS in astrocytes (22). Thus, it seems that apoE gene regulation by LPS depends on the cellular context.

It has been reported previously that murine bone marrow-derived monocytic precursors do not synthesize apoE and that apoE gene expression is up-regulated during macrophage maturation (23). In agreement with these findings, we show here that the monocytic lines THP-1 and U937 did not express apoE under basal conditions (Fig. 1B). In sharp contrast, thioglycolate-induced peritoneal macrophages synthesized and secreted apoE (Fig. 1B), and this expression was repressed by LPS confirming previous reports of reduced apoE gene expression caused by bacterial endotoxins and other inflammatory agents (24). Modulation of apoE gene expression by LPS was similar in both peritoneal macrophages and in RAW 264.7 cells, thus we focused on the RAW 264.7 cell line.

Through binding to the Toll-like receptor 4, LPS activates intracellular signaling cascades that culminate in the expression of pro-inflammatory proteins (25). For example, in macrophages, LPS is known to up-regulate the pro-inflammatory protein COX-2 (27). Here we demonstrate that Tpl-2 is involved in down-regulation of apoE, an anti-inflammatory factor. Thus, LPS may alter the balance between the pro- and anti-inflammatory molecules, leading to the aggravation of the atherogenic process.

The Toll-like receptor 4 signaling pathway involves activation of the Tpl-2 proto-oncogene (26), a member of the mitogen-activated protein kinase (MAPK) family. The serine/threonine kinase Tpl-2 (also known as Cot in humans) is a member of the MAPK kinase kinase (MAP3K) family, which regulates oncogenic and inflammatory pathways (18, 19). Transcription factors activated by Tpl-2 include c-Jun, NF-κB, and NF-AT (27).

3 A. V. Gafencu, unpublished data.
We also found that MEKK1, another downstream target of Toll-like receptor 4 activation (28), mimicked the modulation of the apoE gene expression by LPS in macrophages. As schematically illustrated in Fig. 6, the two kinases, Tpl-2 and MEKK1, are intercalated in the signaling pathway of Toll-like receptor 4 that suppresses apoE gene expression. Our data revealed that NF-κB is a downstream target not only of Tpl-2, but also of MEKK1. This was demonstrated by using a domi-
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FIGURE 6. Schematic model of apoE gene regulation by LPS in macrophages. ApoE down-regulation induced by LPS in macrophages is mediated by two upstream kinases (Tpl-2 and MEKK1), leading to the activation of intermediate kinases (IKKβ, JNK, MEK, and ERK) that lead to the activation of NF-κB and AP-1 complexes and possibly other factors acting on different regions of the apoE promoter.

nant negative form of IκB (that does not detach from NF-κB), which abolished the effect of LPS and its downstream transcription factors Tpl-2 and MEKK1 (Fig. 3, B and D). In silico analysis revealed a potential NF-κB binding site at −43 of the apoE promoter (CONSITE-TF selection). The location of this NF-κB binding site close to the site of recruitment of the preinitiation complex suggested to us that this transcription factor may sterically inhibit the aggregation of certain components of the basal transcription apparatus. Thus, instead of increasing the promoter activity by positive interactions, the activation of these transcription factors inhibited promoter activity and downregulated gene expression. The surprising repressive effect of NF-κB on the apoE promoter corroborates a recent report showing that NF-κB mediates suppression of the cyplal1 promoter by LPS (29), and the inhibition of the APOC3 promoter by tumor necrosis factor-α (30).

TRANSFAC® analysis predicted NF-AT binding sites at the −297 and −313 of the apoE proximal promoter (31). We recently observed translocation of NF-AT to the nucleus after brief exposure to LPS (not shown). However, neither a dominant negative form of NF-AT nor cyclosporin reversed the inhibitory activity of Tpl-2 on the apoE promoter (data not shown). These data suggested that NF-AT is not involved in the apoE repression by LPS.

AP-1 is another transcription factor identified in this study to be involved in the apoE gene suppression by LPS. A recent study demonstrated that JNK-interacting protein 3 was involved in LPS-mediated JNK activation (32). We have shown that c-Jun was activated following LPS treatment and bound to the apoE promoter in the −55/+73 region (Fig. 4). The involvement of c-Jun on apoE gene transcription is further supported by recent findings showing that overexpression of a dominant negative c-Jun in mice via adenovirus-mediated gene transfer significantly elevated apoE mRNA levels in the liver as well as the plasma apoE levels (33). In addition to the known positive role of c-Jun on gene activation, recent data revealed that AP-1 transcription factors participate in down-regulation responses. For example, tumor necrosis factor-α suppresses the human Cu/Zn superoxide dismutase 1 promoter via the JNK/AP-1 signaling pathway (34). Interaction of c-Jun homodimers and heterodimers with other AP-1 partners bound to regulatory elements of the apoCIII promoter could mediate repression of apoCIII promoter activity (35). Franklin et al. (36) revealed that the C terminus of the c-Jun protein associates directly with TBP (TATA box-binding protein) and TFIIIB in vitro, independently of c-Jun phosphorylation at the N terminus. In our system, c-Jun recognized the apoE core promoter and inhibited its activity. This may suggest that binding of c-Jun to the proximal site of the apoE promoter may be stabilized by TBP, which interacts with an adjacent region.

An alternative mechanism of promoter inhibition by c-Jun is via inhibitory interactions with other transcription factors that bind to the same regulatory region. In this scenario, c-Jun could either bind directly to the DNA and inhibit the transcriptional activity of an adjacently bound factor or bind directly to the transcription factor without a requirement for direct DNA binding. In support of the latter scenario, we have observed the translocation of NF-AT to the nucleus beginning with 3-h post LPS addition.

In precursors of B cells and dendritic cells, NF-κB orchestrates the early effects of LPS signaling and controls subordinated activation of AP-1 that mounts the appropriate biological responses (39). In macrophages, we have detected activation of AP-1 complexes as an early response to LPS (44). By contrast, a late response to LPS treatment implicated NF-κB translocation to the nucleus beginning with 3-h post LPS addition. Our experimental data revealed that NF-κB and AP-1 bind the apoE promoter adjacent to the TATA box suggesting the concerted action of these two transcription factors in the repression of apoE gene expression in macrophages. AP-1 and NF-κB may interfere with the functions of transcription factors that bind to the apoE promoter in the region −55/+73. This is in agreement with previous data revealing that the region, including nucleotides −54 to −45, is required for maximum transcriptional activity of the apoE promoter (40).

We also show here that activation of ERK by phosphorylation mediates inhibition of apoE gene expression by LPS (Fig. 5). In addition, LPS activation of the MEK-ERK1/2 pathway in human monocytes induce Elk-1 phosphorylation and Egr-1 expression that up-regulates tissue factor and tumor necrosis factor-α (41).
factor-α expression (41). These factors may also be involved in the apoE gene regulation in macrophages. Because the ERK-responsive region of the apoE promoter is distinct from the NF-κB and AP-1 sites, one can postulate that the repressive mechanisms of these transcription factors are different.

In conclusion, our results show that LPS down-regulates the expression of apoE in macrophages at the level of transcription and inhibits the activity of the proximal apoE promoter. This effect is mediated by at least two upstream kinases,Tpl-2 and MEKK1, and subsequent activation of kinases IKKβ, JNK, MEK1/2, and ERK1/2, which activate NF-κB and AP-1 complexes (Fig. 6). These data imply that stress-associated factors might aggravate the development of atheromas, the signaling proteins Tpl-2, MEKK1, NF-κB, and c-Jun could be effective molecular targets for therapies aiming at the prevention of atheroma formation in human patients.

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REFERENCES

1. Mahley, R. W. (1988) Science 240, 622–630
2. Zannis, V. I., Kan, H. Y., Kritis, A., Zanni, E. E., and Kardassis, D. (2001) Curr. Opin. Lipidol. 12, 181–207
3. Brown, M. S., and Goldstein, J. L. (1983) Annu. Rev. Biochem. 52, 223–261
4. Bellosta, S., Mahley, R. W., Sanan, D. A., Murata, J., Newland, D. L., Taylor, J. M., and Pitas, R. E. (1995) J. Clin. Invest. 96, 2170–2179
5. Fazio, S., Babaev, V. R., Murray, A. B., Hasty, A. H., Carter, K. J., Gleave, L. A., Atkinson, J. B., and Linton, M. F. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4647–4652
6. Olaisen, B., Teisberg, P., and Gedde-Dahl, T., Jr. (1982) Hum Genet. 62, 233–236
7. Smith, J. D., Melian, A., Leff, T., and Breslow, J. L. (1988) J. Biol. Chem. 263, 8300–8308
8. Garcia, M. A., Vazquez, J., Gimenez, C., Valdivieso, F., and Zafra, F. (1996) J. Neurosci. 16, 7550–7556
9. Salero, E., Gimenez, C., and Zafra, F. (2003) Biochem. J. 370, 979–986
10. Salero, E., Perez-Sen, R., Aruga, J., Gimenez, C., and Zafra, F. (2001) J. Biol. Chem. 276, 1881–1888
11. Libby, P. (2002) Nature 420, 868–874
12. Zhang, G., and Ghosh, S. (2000) J. Endotoxin Res. 6, 453–457
13. Prokova, V., Mosialos, G., and Kardassis, D. (2002) J. Biol. Chem. 277, 9342–9350
14. Kardassis, D., Papakosta, P., Pardali, K., and Moustakas, A. (1999) J. Biol. Chem. 274, 92572–92578
15. Agelaki, S., Tsatsanis, C., Gravanis, A., and Margioris, A. N. (2002) Infect. Immun. 70, 6068–6074
16. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475–1489
17. Sheffield, J. B., Graff, D., and Li, H. P. (1987) Anal. Biochem. 166, 49–54
18. Beinke, S., and Ley, S. C. (2004) Biochem. J. 382, 393–409
19. Tsatsanis, C. (2006) UCSD-Nature Molecule Pages doi:10.1038/mp.a000092.01
20. Ichijo, H. (1999) Oncogene 18, 6087–6093
21. Ripolles Piquer, B., Nazih, H., Neunlist, M., Huvelin, J. M., and Bard, J. M. (2004) J. Cell. Biochem. 91, 786–795
22. Qiao, X., Cummins, D. J., and Paul, S. M. (2001) Eur. J. Neurosci. 14, 474–482
23. Werb, Z., and Chin, J. R. (1983) J. Cell Biol. 97, 1113–1118
24. Werb, Z., and Chin, J. R. (1983) J. Exp. Med. 158, 1272–1293
25. Chow, J. C., Young, D. W., Golenbock, D. T., Christ, W. J., and Gusovsky, F. (1999) J. Biol. Chem. 274, 10689–10692
26. Dumitru, C. D., Ceci, J. D., Tsatsanis, C., Kontoyiannis, D., Stamatakis, K., Lin, J. H., Patriotis, C., Jenkins, N. A., Copeland, N. G., Kollias, G., and Tsichlis, P. N. (2000) Cell 103, 1071–1083
27. Tsatsanis, C., Patriotis, C., and Tsichlis, P. N. (1998) Oncogene 17, 2609–2618
28. O’Reilly, S. M., and Moynagh, P. N. (2003) Biochem. Biophys. Res. Commun. 303, 586–593
29. Ke, S., Rabson, A. B., Germino, J. F., Gallo, M. A., and Tian, Y. (2001) J. Biol. Chem. 276, 39638–39644
30. Nikolaidou-Neokosmidou, V., Zannis, V. I., and Kardassis, D. (2006) Biochem. J. 398, 439–450
31. Quandt, K., Frech, K., Karas, H., Wingender, E., and Werner, T. (1995) Nucleic Acids Res. 23, 4878–4884
32. Matsuguchi, T., Masuda, A., Sugimoto, K., Nagai, Y., and Yoshikai, Y. (2003) EMBO J. 22, 4455–4464
33. Drosatos, K., Sanoudou, D., Kypreos, K. E., Kardassis, D., and Zannis, V. I. (April 24, 2007) J. Biol. Chem. doi:10.1074/jbc.M700986200
34. Afonso, V., Santos, G., Collin, P., Khatib, A. M., Mitrovic, D. R., Lomri, N., Leitman, D. C., and Lomri, A. (2006) Free Radic. Biol. Med. 41, 709–721
35. Hadzopoulos-Cladaras, M., Lavrentiadou, S. N., Zannis, V. I., and Kardassis, D. (1999) Biochemistry 37, 14078–14087
36. Franklin, C. C., McCulloch A. V., and Kraft, A. S. (1995) Biochem. J. 305, 967–974
37. Bilson, A. E., Stevenson, K., Atkinson, S., Kolch, W., and Keith, W. N. (2006) Cancer Res. 66, 1363–1370
38. Paik, Y. K., Chang, D. J., Reardon, C. A., Walker, M. D., Taxman, E., and Taylor, J. M. (1988) J. Biol. Chem. 263, 13340–13349
39. Krappmann, D., Wegener, E., Sunami, Y., Essen, M., Thiel, A., Mordmüller, B., and Scheidereit, C. (2004) Mol. Cell. Biol. 24, 6488–6500
40. Chang, D. J., Paik, Y. K., Leren, T. P., Walker, D. W., Howlett, G. J., and Taylor, J. M. (1990) J. Biol. Chem. 265, 9496–9504
41. Guha, M., O’Connell, M. A., Pawlinski, R., Hellis, A., McGovern, P., Yan, S. F., Stern, D., and Mackman, N. (2001) Blood 98, 1429–1439

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