Inhibition of Apolipoprotein AI Gene Expression by Tumor Necrosis Factor α: Roles for MEK/ERK and JNK Signaling

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ABSTRACT: Plasma high-density lipoprotein and apolipoprotein AI (apoAI) levels are suppressed by tumor necrosis factor α. To determine the molecular mechanisms responsible for the effect of TNF α on the apoAI promoter activity, HepG2 cells were exposed to both genetic and pharmacological modulators of TNF α-mediated signaling in the presence or absence of TNF α. Exogenous ERK1 and ERK2 expression suppressed basal apoAI promoter activity; however, only ERK2 enhanced the ability of TNF α to suppress apoAI promoter activity. Exogenous expression of all three MEK isoforms (MEK1, MEK2A, and MEK2E) suppressed basal apoAI promoter activity and further aggravated TNF α-related apoAI promoter activity inhibition. Treatment with SB202190 (p38 MAP kinase inhibitor) alone significantly increased apoAI promoter activity; however, in the presence of TNF α, apoAI promoter activity was suppressed to an extent similar to that in cells not treated with SB202190. ApoAI promoter activity increased in cells treated with the specific JNK inhibitor SP600125, but unlike SB202190 treatment, the level of TNF α-related apoAI promoter inhibition was reduced by 50%. Similarly, the level of TNF α-related apoAI promoter inhibition was reduced in cells transfected with JNK1 siRNA. Finally, treatment of cells with the NF-κB inhibitors BAY and SN-50 or overexpression of NF-κB subunits p50 and p65 had no effect on the ability of TNF α to repress apoAI promoter activity. These results suggest that TNF α suppresses apoAI promoter activity through both the MEK/ERK and JNK pathways but is not mediated by either p38 MAP kinase activity or NF-κB activation.

Apolipoprotein AI (apoAI)[1] is the primary protein component of the cholesterol-transporting high-density lipoprotein (HDL) particle. HDL is believed to participate in the process of reverse cholesterol transport (RCT), by which cholesterol in the periphery is transported to the liver where it is converted to bile acids for elimination (*1*, *2*). Through RCT or by other mechanisms (*3*−*5*), HDL levels are inversely related to the risk of developing atherosclerosis and cardiovascular disease (*6*−*8*). Conditions associated with either low plasma HDL or apoAI levels, including diabetes, obesity, and the metabolic syndrome, include an increased risk for the rapid progression of atherosclerosis (*9*−*11*). Several mechanisms may account for the decrease in plasma HDL levels, including changes in cholesterol ester transfer protein (CETP) or lecithin-cholesterol acyltransferase (LCAT) activity, leading to HDL remodeling or improved turnover accompanied by decreased rates of synthesis.

Hepatic apoAI expression accounts for the majority of the apoAI protein present in plasma. Cytokines such as TNF α and IL-1β have been shown to repress apoAI gene expression at the transcriptional level (*12*−*14*). This may partially explain the association between inflammatory states and low plasma HDL or apoAI levels as reported in patients with rheumatoid arthritis (*15*) and during acute infections (*16*). The cytokine-mediated reduction in apoAI promoter activity requires the presence of a previously characterized region of the promoter, namely site A (*12*). This element, located between nucleotides −214 and −195 (relative to the transcriptional start site, +1), has been shown to bind several transcriptional factors involved in either activating or repressing apoAI gene expression (*17*−*24*). The precise molecular pathways by which TNF α interacts with site A are not known. Although deletion and mutagenesis of site A prevented repression of apoAI promoter activity in the presence of TNF α, no changes in site A binding were observed in nuclear protein extracts prepared from TNF α-treated cells (*12*).

Binding of a ligand to the TNF α receptor activates several signal transduction pathways. These include NF-κB (*25*, *26*) and c-*jun* (*27*, *28*) activation as well as signaling through the MEK/ERK, p38 MAP kinase, and *jun*-N-terminal kinase (JNK) pathways (*29*−*31*), regulating expression of stress-responsive genes (*32*, *33*). Some of these biochemical

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[1] Abbreviations: apoAI, apolipoprotein AI; CAT, chloramphenicol acetyltransferase; CETP, cholesterol ester transfer protein; ERK, extracellular signal-regulated kinase; HDL, high-density lipoprotein; IL-1β, interleukin-1β; LCAT, lecithin-cholesterol acyltransferase; MAP kinase, mitogen-activated protein kinase; MEK1, MAP kinase kinase, also known as MKK (microtubule-associated ERK kinase); JNK, c-*jun* N-terminal kinase; NFκB, nuclear factor κB; RCT, reverse cholesterol transport; siRNA, small interfering RNA; TNF α, tumor necrosis factor α; PPAR α, peroxisome proliferator-activated receptor α.

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pathways have been shown to modulate apoAI gene expression. For example, lipopolysaccharide-induced NF-κB has been demonstrated to suppress apoAI promoter activity by inhibiting peroxisome proliferator-activated receptor α (PPAR α) activity and binding to site A (*34*). MAP kinases have been shown to be required for activation of the apoAI gene by growth factors such as epidermal growth factor (*35*). A potential role for JNK in regulating apoAI gene expression has not been previously reported.

To determine which signal transduction pathways are necessary for repression of the apoAI gene by TNF α, we used both genetic and pharmacologic approaches. These studies demonstrate that repression of apoAI promoter activity by TNF α requires the MEK/ERK and JNK signaling pathways.

MATERIALS AND METHODS

Materials. Recombinant human TNF α was purchased from R&D Systems (Minneapolis, MN). Acetyl-coenzyme A was from Sigma-Aldrich (St. Louis, MO), and Lipofectamine was purchased from Life Technologies, Inc. (Gaithersburg, MD). The radionuclide [^{14}C]chloramphenicol was from New England Nuclear (Boston, MA). The JNK inhibitor SP600125 (SP) and the p38 MAP kinase inhibitor SB202190 (SB) were purchased from Calbiochem (La Jolla, CA), while the NF-κB inhibitors SN50 cell permeable inhibitory peptide and BAY 11-7085 were purchased from BIOMOL (Plymouth Meeting, PA). Tissue culture media and fetal calf serum were purchased from BioWittaker (Walkersville, MD). All other reagents were from Sigma-Aldrich or Fisher Scientific (Pittsburgh, PA).

Cell Culture. HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and penicillin and streptomycin (100 units/mL and 100 μg/mL, respectively). Cells were maintained in a humidified environment at 37 °C and 5% CO_2. Cell viability was monitored with the trypan blue exclusion method. The viability was greater than 95% in all experiments.

Plasmids and Transient Transfection Analysis. The plasmid pAI.474.CAT contains the apoAI promoter region from base pair −474 to 7 (relative to the transcriptional start site) that contains the TNF α-responsive site A element and is attached to a heterologous reporter gene, namely the bacterial chloramphenicol acetyltransferase (CAT) gene (*12*). Expression constructs for MEK1, MEK2A, and MEK2E were kindly provided by D. J. Templeton (University of Virginia Medical School, Charlottesville, VA). The ERK1/2 expression plasmids as well as their kinase-defective mutants were kindly provided by M. Cobb (The University of Texas Southwestern Medical Center, Dallas, TX). The kinase-defective ERK1/2 mutant proteins contain a lysine-to-arginine change at the ATP binding site near the kinase catalytic domain, rendering it inactive. Expression constructs for NF-κB subunits p65 (pCMV4/p65) and p50 (pCMV4/p50) were kindly provided by D. Ballard (Vanderbilt University, Nashville, TN). To inhibit JNK1 with siRNA, HepG2 cells were transfected with either 1 μg of siRNA to human JNK1 (sc-29380, Santa Cruz Biotechnology, Santa Cruz, CA) or 1 μg of control scrambled siRNA that will not degrade any cellular mRNA (src-37007). Each plasmid was transfected into HepG2 cells as indicated in each figure using Lipofectamine. The plasmid pCMV.SPORT-β-gal (Life Technologies, Inc.) expresses β-galactosidase under the control of the cytomegalovirus immediate-early promoter which was included to control for transfection efficiency. After 24 h, the culture medium was replaced with serum-free medium containing either vehicle (0.1% bovine serum albumin in PBS) or 30 ng/mL TNF α. After an additional 24 h, the cells were collected and assayed for CAT (*36*) and β-galactosidase (*37*) activity.

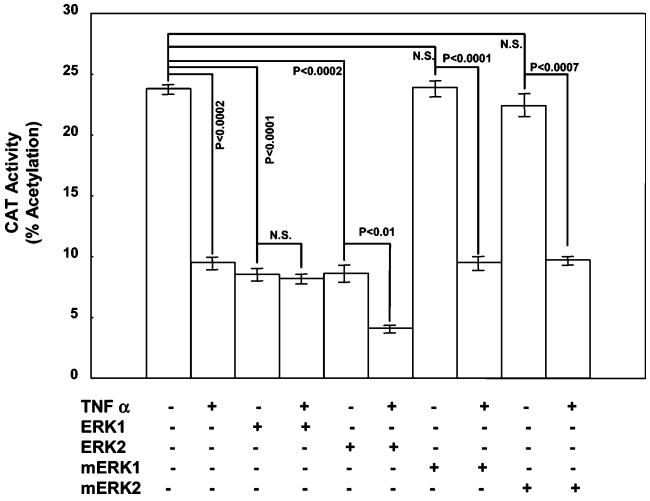

FIGURE 1: Effect of Erk1/2 overexpression on apoAI promoter activity in the presence and absence of TNF α. CAT activity decreased in cells transfected with both ERK1 and ERK2 expression constructs, but not in the presence of kinase-defective ERKs (mERK1 and mERK2). Only ERK2 expression enhanced the ability of TNF α to suppress apoAI promoter activity. *p* values are shown. NS stands for not significant. $n = 6$.

Statistics and Data Analysis. All results are expressed as means ± the standard error of the mean. A Student's *t*-test for independent variables was used to assess the significance using the statistical package Statistica for Windows (Statsoft Inc., Tulsa, OK). Significance was defined as a two-tailed *p* of <0.05.

RESULTS

Effect of Exogenous ERK1/2 Expression on TNF α-Mediated Repression of ApoAI Promoter Activity. Overexpression of ERK1 or ERK2 alone suppressed apoAI promoter activity 62.4% (from 23.8 ± 0.6 to 8.67 ± 0.8% acetylation, $p < 0.0001$) or 63.9% (from 23.8 ± 0.6 to 8.60 ± 1.0% acetylation, $p < 0.0002$), respectively (Figure 1). This was similar to the 60.1% decrease in apoAI promoter activity in cells exposed to TNF α relative to that in vehicle-treated cells (from 23.8 ± 0.6 to 9.5 ± 0.8% acetylation, $p < 0.0002$). When ERK1-transfected cells were treated with TNF α, apoAI promoter activity did not change (8.67 ± 0.8% acetylation in solvent-treated cells vs 8.15 ± 0.5% acetylation in TNF α-treated cells). However, when ERK2-transfected cells were treated with TNF α, apoAI promoter activity decreased 52.0% (from 8.60 ± 1.0 to 4.13 ± 0.2% acetylation, $p < 0.012$). These results suggest that ERK2 overexpression, but not ERK1, can potentiate the effect of TNF α on apoAI promoter activity.

Overexpression of the kinase-defective ERK1/2 isoforms mERK1 and mERK2 had no effect on apoAI promoter

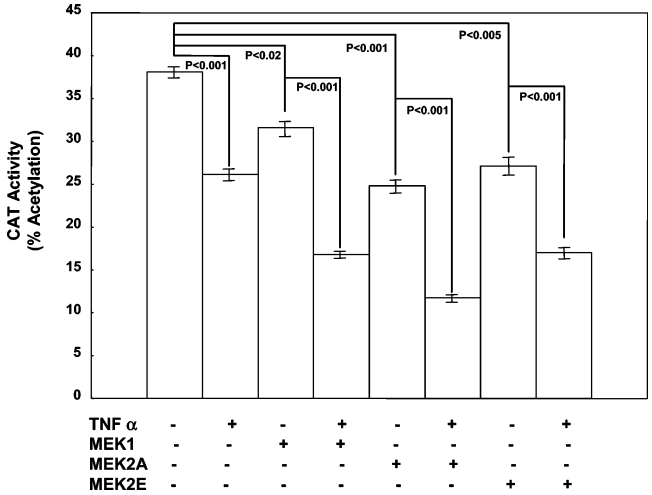

FIGURE 2: Effect of MEK overexpression on apoAI promoter activity in the presence and absence of TNF α. CAT activity decreased in cells transfected with either MEK1, MEK2A, or MEK2E expression construct in vehicle-treated cells, and in the presence of TNF α. CAT activity was further reduced by overexpression of each MEK. *p* values are shown. NS stands for not significant. $n = 6$.

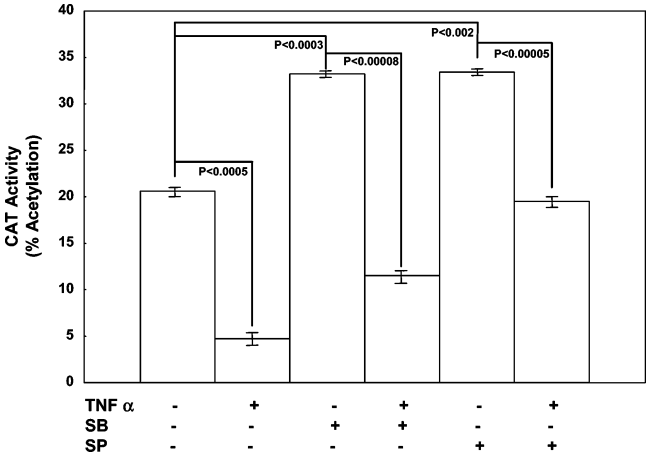

FIGURE 3: Effect of p38 MAP kinase and JNK inhibitors, SB202190 (SB) and SP600125 (SP), on TNF α-mediated repression of apoAI promoter activity. Treatment with SB alone significantly increased apoAI promoter activity; however, in the presence of TNF α, apoAI promoter activity was suppressed to an extent similar to that in cells not treated with SB. ApoAI promoter activity increased in cells treated with the specific JNK inhibitor SP, but unlike that with the SB treatment, the level of TNF α-related apoAI promoter inhibition was reduced by 50%. *p* values are shown. NS stands for not significant. $n = 6$.

activity compared to control cells (23.8 ± 0.6% acetylation in control cells vs 23.9 ± 0.8 and 22.4 ± 1.3% acetylation in cells transfected with mERK1 and mERK2, respectively) (Figure 1). In cells treated with TNF α, apoAI promoter activity was suppressed in the presence of mERK1 and mERK2 to an extent similar to that observed in control cells (9.5 ± 0.8% acetylation in TNF α-treated cells vs 9.5 ± 0.5 and 9.7 ± 0.3% acetylation in cells transfected with mERK1 and mERK2, respectively).

Effect of Exogenous MEK on TNF α-Mediated Repression of ApoAI Promoter Activity. Activation of the ERK1/2 kinases in the MAP kinase signaling cascade is dependent on the upstream MEK kinases. Since TNF α-mediated inhibition of apoAI promoter activity required primarily ERK2 activity, roles for the MEK isoforms MEK1, MEK2A, and MEK2E in modulating the effect of TNF α on apoAI promoter activity were examined. Overexpression of each MEK isoform (Figure 2) inhibited apoAI promoter activity in the absence of TNF α [from 38.1 ± 1.0% acetylation in control cells to 31.6 ± 1.6, 24.5 ± 1.4, and 27.1 ± 1.7% acetylation in cells transfected with MEK1 ($p < 0.02$), MEK2A ($p < 0.001$), and MEK2E ($p < 0.005$), respectively]. However, in cells treated with TNF α, there was an even greater reduction in apoAI promoter activity with all three MEK family members [from 26.1 ± 0.9% acetylation in TNF α-treated cells receiving empty vector to 16.9 ± 0.4, 11.7 ± 0.4, and 17.0 ± 0.7% acetylation in TNFα-treated cells expressing exogenous MEK1 ($p < 0.001$), MEK2A ($p < 0.001$), and MEK2E ($p < 0.001$), respectively].

Effect of p38 MAP Kinase and c-jun-N-Terminal Kinase Inhibitors on Repression of the ApoAI Gene by TNF α. In cells treated with 500 nM SB (p38 MAP kinase inhibitor) or 200 nM SP (JNK inhibitor), apoAI promoter activity was induced 1.65- or 1.63-fold, respectively, from 20.1 ± 0.9% acetylation in control cells to 33.2 ± 0.7 and 33.5 ± 0.4% acetylation in cells treated with SB ($p < 0.0003$) and SP ($p < 0.002$), respectively (Figure 3). In the presence of TNF α, apoAI promoter activity decreased 77.0% (from 20.6 ± 0.9 to 4.7 ± 1.2% acetylation, $p < 0.0005$), while in the presence of SB, TNF α suppressed apoAI promoter activity 65.2% (from 33.2 ± 0.7 to 11.5 ± 1.2% acetylation, $p < 0.0001$). However, in the presence of SP, apoAI promoter activity was suppressed only 41.2% (from 33.5 ± 0.3 to 19.5 ± 0.6% acetylation, $p < 0.00005$). These results suggest that TNF α does not inhibit apoAI promoter activity through a p38 MAP kinase-dependent pathway; however, JNK activity is at least partially necessary for repression by TNF α.

Effect of JNK1 siRNA on ApoAI Promoter Activity. To determine if JNK1 is involved in suppressing apoAI promoter activity by TNF α, HepG2 cells were transfected with the apoAI reporter plasmid pAI.474.CAT with either a JNK1-specific or scrambled nonspecific siRNA and then treated with TNF α (Figure 4). TNF α repressed apoAI promoter activity 72.1% (from 35.5 ± 0.7 to 9.9 ± 1.1% acetylation, $p < 0.0003$). Similarly, in cells transfected with the scrambled siRNA, TNF α repressed apoAI promoter activity 71.2% (from 35.8 ± 0.9 to 9.9 ± 0.6% acetylation, $p < 0.0001$). However, in cells transfected with the JNK1 siRNA, TNF α repressed CAT activity only 34.9% (from 25.8 ± 0.5 to 16.8 ± 0.5% acetylation, $p < 0.0002$).

Effect of NF-κB Inhibition or Overexpression on Repression of ApoAI Promoter Activity by TNF α. In control cells, addition of BAY alone suppressed basal apoAI promoter activity 34.7% (from 32.5 ± 1.0 to 21.2 ± 0.4% acetylation, $p < 0.0005$), while in TNF α-treated cells, addition of BAY suppressed apoAI promoter activity 88.2% (from 21.2 ± 0.4 to 2.5 ± 0.6% acetylation, $p < 0.00002$) (Figure 5A). SN50 had no effect on apoAI promoter activity in vehicle-treated cells, but in TNF α-treated cells, SN50 decreased apoAI promoter activity 80.3%, from 31.4 ± 1.2 to 6.2 ± 0.9% acetylation ($p < 0.00002$). Since this degree of inhibition was similar to that observed with TNF α treatment (82.5%, from 32.5 ± 1.0 to 5.7 ± 0.4% acetylation, $p < 0.00002$), we conclude that neither NF-κB inhibitor was capable of potentiating or preventing TNF α-mediated repression.

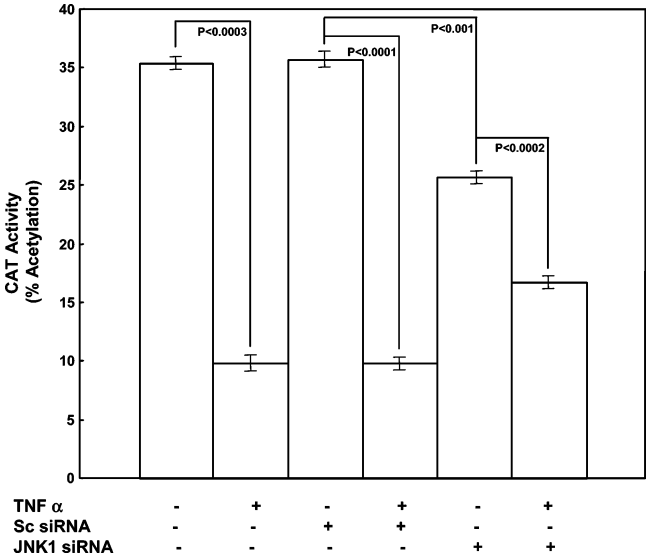

FIGURE 4: Effect of JNK1 siRNA on apoAI promoter activity. HepG2 cells were transfected with pAI.474.CAT and either a scrambled nonspecific siRNA (Sc siRNA) or a siRNA specific for JNK1 (JNK1 siRNA) and after 48 h treated with TNF α. CAT activity was suppressed by TNF α in all cases; however, in cells transfected with the JNK1 siRNA, there was a smaller reduction in CAT activity. *p* values are shown. NS stands for not significant. $n = 6$.

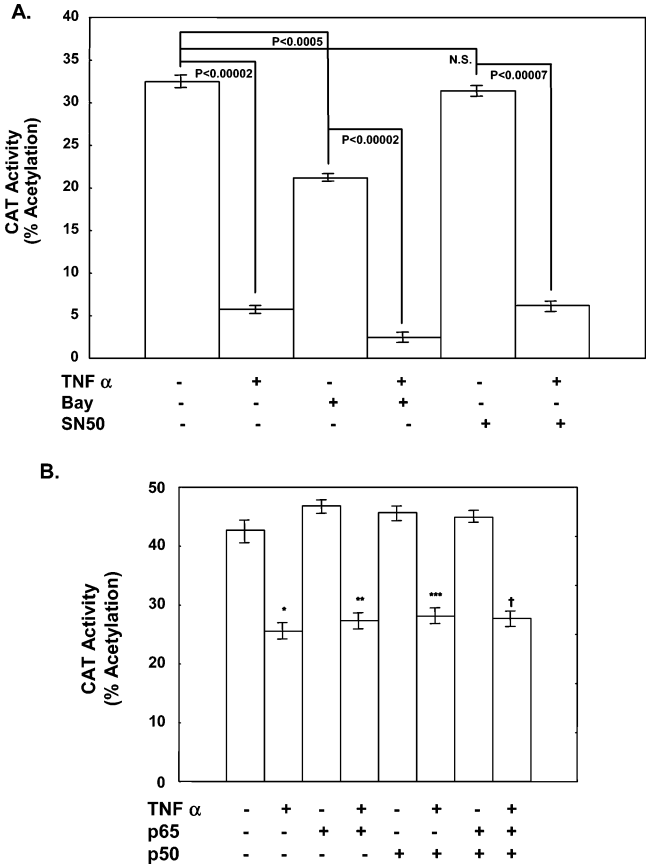

FIGURE 5: Effect of NF-κB on TNF α-mediated repression of apoAI promoter activity. (A) Treatment with NF-κB inhibitor BAY but not SN50 suppressed basal apoAI promoter activity. Neither BAY nor SN50 had any significant effect on the ability of TNF α to repress apoAI promoter activity. (B) Overexpression of NF-κB subunits p50 and p65 alone or in combination had no effect on the ability of TNF α to repress apoAI promoter activity. *p* values are shown. NS stands for not significant. $n = 6$.

Expression of p50, p65, or p50 and p65 had no effect on either basal apoAI promoter activity (42.7 ± 1.9% acetylation in control cells vs 46.8 ± 1.0, 45.7 ± 1.4, and 45.0 ± 1.1% acetylation in cells transfected with p50, p65, and p50 and p65 expression plasmids, respectively, compared to control cells) or the ability of TNF α to suppress apoAI promoter activity (25.5 ± 1.3% acetylation in control cells vs 27.4 ± 1.4, 28.2 ± 1.5, and 27.7 ± 1.6% acetylation in cells transfected with p50, p65, and p50 and p65, respectively) (Figure 5B). In the presence of an empty vector, TNF α suppressed apoAI promoter activity 40.3% (from 42.7 ± 1.9 to 25.5 ± 1.3% acetylation, $p < 0.002$). These studies, as well as those with the NF-κB inhibitors, suggest that repression of apoAI promoter activity by TNF α does not require NF-κB activation.

DISCUSSION

Three MAP kinase pathways (MEK/ERK, p38, and JNK) mediate many of the effects of TNF α on gene expression. Overexpression of ERK1 and ERK2 in HepG2 cells suppressed apoAI promoter activity to an extent similar to that of TNF α treatment (Figure 1). Furthermore, in ERK2-expressing cells, TNF α suppressed apoAI promoter activity to a greater extent than TNF α or ERK2 expression alone. These observations suggest that ERK2 kinase may be implicated in the effects of TNF α the on apoAI promoter.

ERK2 kinase activity requires upstream activation of the MEK kinases in the MAP kinase signaling cascade (*38*−*40*). Since ERK2 is able to further repress apoAI promoter activity in the presence of TNF α, roles for MEK signaling were examined in a similar manner. ApoAI promoter activity was significantly suppressed in cells expressing all isoforms of MEK that have been examined, MEK1, MEK2A, and MEK2E (Figure 2). In the presence of TNF α, cells expressing exogenous MEK had even lower apoAI promoter activity than TNF α-treated cells receiving the empty vector (Figure 2). These findings support the hypothesis that the MEK/ERK signaling cascade is important in negatively regulating apoAI promoter activity by TNF α.

The stress-related JNK and p38 MAP kinase also mediate many of the effects of TNF α. To examine their potential roles in modulating the effects of TNF α on apoAI promoter activity in HepG2 cells, their respective activities were inhibited pharmacologically with SP (*41*) and SB (*42*). By themselves, in the absence of TNF α, both inhibitors increased apoAI promoter activity (Figure 3), suggesting that both of these pathways exert some negative control over the gene under normal culture conditions. Furthermore, while addition of SB to TNF α-treated cells had no significant effect on apoAI promoter activity compared to that of TNF α-treated cells alone, addition of SP was partially effective at relieving the repressive effects of TNF α. This underscores the role of JNK in the effects of TNF α on the apoAI promoter.

Since inhibition of JNK activity by SP relieved some of the inhibition of apoAI promoter activity with TNF α, the effect of TNF α on apoAI promoter activity was examined in cells lacking JNK1 following transfection with JNK1 siRNA. ApoAI promoter activity was suppressed 71.2% in cells receiving a nonspecific siRNA but only 34.9% in cells receiving the JNK1 siRNA (Figure 4). This observation

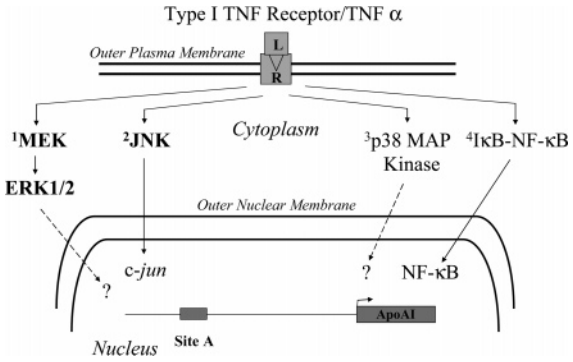

FIGURE 6: Intracellular signaling pathways activated by TNF α. Activation of the type 1 TNF α receptor (R) by TNF α (L) induces the three MAP kinase pathways listed above [(1) MEK/ERK, (2) JNK, and (3) p38 MAP kinase], as well as the nuclear import and regulation of NF-κB-dependent genes. ERK1/2 and p38 MAP kinase activation regulate gene expression through several mechanisms. The JNK pathway modulates AP1-dependent gene expression by activating c-*jun*. TNF α suppresses apoAI promoter activity through both the MEK/ERK and JNK pathways (shown in bold letters), but that is not mediated by either p38 MAP kinase activity or NF-κB activation.

supports a role of JNK in TNF α-mediated suppression of apoAI. It is noteworthy that in cells transfected with siRNA, the basal CAT activity was also reduced relative to that of the control cells receiving the nonspecific mRNA. This reduction in apoAI promoter activity was unexpected since SP, a pharmacologic inhibitor of JNK, actually increased apoAI promoter activity in control cells. This discrepancy may possibly be due to nonspecific effects of the JNK inhibitor SP or the presence of other JNK isoforms that may mediate some of the TNF α response that are not inhibited to similar extents by the siRNA or SP. In these experiments, there was no evidence of nonspecific toxicity as the β-galactosidase activity did not vary significantly with different experimental manipulations.

Expression of the apoAI gene in HepG2 cells treated with lipopolysaccharides is repressed in part due to NF-κB-mediated repression of PPAR α (*34*). Unlike lipopolysaccharides, TNF α-mediated repression of apoAI promoter activity did not require activation of NF-κB since two pharmacologic inhibitors of NF-κB neither potentiated nor suppressed apoAI promoter activity in the presence of TNF α (Figure 5A). In addition, overexpression of NF-κB subunit p50 and/or NF-κB subunit p65 in the presence of TNF α had no effect on apoAI promoter activity (Figure 5B). These results suggest that apoAI gene expression may be repressed by different mechanisms, depending on the type of inflammatory stimulus, either chronic (cytokine-mediated) or acute (lipopolysaccharide-mediated).

The two NF-κB inhibitors used in these studies had different effects on apoAI promoter activity. While Bay treatment repressed basal apoAI promoter activity, SN50 did not significantly alter the promoter activity (Figure 5A). This difference could be related to the differences in the mechanism of NF-κB inhibition. BAY is an irreversible inhibitor of the IκB-α kinase, the enzyme that phosphorylates IκB-α leading to the release of cytoplasmic NF-κB from an inactive complex and subsequent nuclear localization (*43*−*45*). SN50 is a cell permeable peptide that inhibits the translocation of active NF-κB into the nucleus (*46*)

Intracellular signaling pathways activated by TNF α are shown in Figure 6. Activation of the type 1 TNF α receptor induces the three MAP kinase pathways, namely, MEK/ERK, JNK, and p38 MAP kinase, as well as the nuclear import and regulation of NF-κB-dependent genes. ERK1/2 and p38 MAP kinase activation regulate gene expression through several mechanisms. The JNK pathway modulates AP1-dependent gene expression by activating c-*jun*. These studies show that TNF α suppresses apoAI promoter activity through both the MEK/ERK and JNK pathways (shown in bold letters in Figure 6) but is not mediated by either p38 MAP kinase activity or NF-κB activation.

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