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Inhibition of Apolipoprotein A-I Expression by TNF-Alpha in HepG2 Cells: Requirement for c-*jun*

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ABSTRACT

Tumor necrosis factor alpha (TNF α) signals in part through the mitogen activated protein (MAP) kinase c-*jun*-N-terminal kinase (JNK). Activation of JNK has been shown to promote insulin resistance and dyslipidemia, including reductions in plasma high-density lipoprotein (HDL) and apolipoprotein A-I (apo A-I). To examine how TNF α-mediated JNK activation inhibits hepatic apo A-I production, the effects of c-*jun* activation on apo A-I gene expression were examined in HepG2 cells. Apo A-I gene expression and promoter activity were measured by Northern and Western blotting and transient transfection. Transient transfection and siRNA were used to specifically over-express or knockout c-*jun*, c-*jun*-N-terminal kinase-1 and -2 (JNK1 and JNK2, respectively) and mitogen-activated protein kinase-4 (MKK4). TNF α-treatment of HepG2 cells induced rapid phosphorylation of c-*jun* on serine 63. In cells treated with phorbol-12-myristate-13-acetate (PMA), apo A-I gene promoter activity was inhibited and apo A-I mRNA content and apo A-I protein secretion decreased. Likewise, over-expression of JNK1 and JNK2 inhibited apo A-I promoter activity. Over-expression of constitutively active MKK4, an upstream protein kinase that directly activates JNK, also inhibited apo A-I promoter activity, while over-expression of a dominant-negative MKK4 de-repressed apo A-I promoter activity in TNF α-treated cells. Inhibition of c-*jun* synthesis using siRNA but not a control siRNA prevented TNF α-mediated inhibition of apo A-I. These results suggest that the MKK4/JNK/c-*jun* signaling pathway mediates TNF α-dependent inhibition of apo A-I synthesis. J. Cell. Biochem. 115: 253–260, 2014. © 2013 Wiley Periodicals, Inc.

KEY WORDS: PRO-INFLAMMATORY CYTOKINES; APOLIPOPROTEIN A-I; HIGH-DENSITY LIPOPROTEIN-CHOLESTEROL; c-*jun*; TRANSCRIPTIONAL REGULATION; CARDIOVASCULAR DISEASE

Dyslipidemia and inflammation are important risk factors for cardiovascular disease (CVD). Pro-inflammatory cytokines promote dyslipidemia by modulating expression of genes involved in cholesterol and triglyceride metabolism and transport [Hikita et al., 2000; Zhao et al., 2002; Murphy et al., 2006], including lowering plasma high-density lipoprotein (HDL) levels. Previous studies have shown that tumor necrosis factor α (TNF α) inhibits expression and/or activity of several nuclear receptors such as the retinoid-X-receptor (RXR) [Sugawara et al., 1998], farnesoid X receptor (FXR) [Kim et al., 2003], thyroid hormone receptor (TR) [Wolf et al., 1994], and peroxisome proliferator activated receptor α and γ (PPARα and PPARγ) [Beier et al., 1994; Zhang et al., 1996]. TNF α and other pro-inflammatory cytokines induce insulin resistance, altering triglyceride and lipid metabolism [Kroder et al., 1996].

Apolipoprotein A-I (apo A-I) is the primary protein component of HDL and promotes many of the anti-atherosclerotic properties of HDL, including reverse-cholesterol transport. Apo A-I gene expression is repressed by TNF α at the level of transcription [Haas

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et al., 2003; Beers et al., 2006], and is dependent on mitogen-activated protein kinases including extracellular signal-regulated kinase 1/2 (ERK1/2), and c-*jun*-N-terminal kinase-1 (JNK1) [Beers et al., 2006].

Stimulation of the TNF α receptor activates mitogen-activated protein kinase kinase 4 (MKK4) which in turn phosphorylates and activates JNK1 [Deacon and Blank, 1997; Fleming et al., 2000]. JNK1 is a stress- and cytokine-inducible kinase that phosphorylates the N-terminal portion of c-*jun*, resulting in activation/repression of c-*jun*-dependent genes. A member of the basic region-leucine zipper family of transcription factors, c-*jun* binds to a c-*fos*-subunit forming the dimeric transcriptional regulator activator protein 1 (AP1) [Abate and Curran, 1990; Busch and Sassone-Corsi, 1990]. Both inhibitory and stimulatory serine/threonine phosphorylation sites regulate c-*jun* activity [Turjanski et al., 2007]. Diacylglycerol- and calcium-sensitive protein kinase C family members (which are activated by phorbol-12-myristate-13-acetate [PMA]) [McDonnell et al., 1990; Whitmarsh and Davis, 1996] and mitogen activated protein (MAP) kinases of the JNK family phosphorylate serine 63 in the N-terminus of c-*jun* [Coso et al., 1995; Minden et al., 1995]. Once activated, c-*jun* forms a homodimer or a heterodimer with c-*fos* and binds AP1-response elements within gene promoters [Abate and Curran, 1990; Busch and Sassone-Corsi, 1990]. Activated c-*jun* also interacts with several steroid hormone nuclear receptors and other select transcription factors inhibiting their activity by trans-repression [Beato, 1991; Lian et al., 1991].

While previous studies suggested that TNF α-mediated inhibition of apo A-I gene expression requires JNK [Beers et al., 2006], it was not determined if the JNK substrate c-*jun* is essential for the response. Therefore, we assessed whether or not c-*jun* directly suppresses apo A-I gene expression by assessing whether or not changes in MKK4, JNK, and c-*jun* expression and/or activity impact apo A-I gene expression.

MATERIALS AND METHODS

MATERIALS

Recombinant human TNF α was from R&D Systems (Minneapolis, MN). Acetyl-coenzyme A and PMA were from Sigma–Aldrich Chemical Company (St. Louis, MO), and lipofectamine was from Invitrogen (Gaithersburg, MD). The ^{14}C-chloramphenicol and [α-^{32}P]-dCTP were from Perkin Elmer-New England Nuclear (Boston, MA). Tissue culture media and fetal calf serum were purchased from BioWittaker (Walkersville, MD). Antibodies to c-*jun* (#9165) and phospho-c-*jun* (serine 63) (#9261) were obtained from Cell Signaling Technology (Danvers, MA), and antibodies to human apo A-I (#178422) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (#MAB374) were obtained from EMD Millipore (Billerica, MA). The anti-albumin antibody 109-4133 was purchased from Rockland (Gilbertsville, PA). All other reagents were from Sigma–Aldrich Chemical Company or Fisher Scientific (Pittsburgh, PA).

CELL CULTURE

HepG2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) 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. In most experiments, cells were treated with the indicated compounds in serum-free medium (SFM), consisting of DMEM and penicillin and streptomycin as indicated above. Cell viability was monitored by trypan blue exclusion [Jauregui et al., 1981] and was >95% in all experiments.

MEASUREMENT OF APO A-I mRNA AND PROTEIN ACCUMULATION

HepG2 cells were treated with 50 nM PMA or an equivalent amount of the solvent dimethylsulfoxide (DMSO) in SFM for 24-h. Apo A-I and albumin levels were measured in the conditioned medium by Western blot using 5-μg protein/lane as described [Towbin et al., 1979], and the signal was quantified with a scanning densitometer (Molecular Dynamics, Sunnyvale, CA).

Total RNA was isolated from PMA-treated cells as described [Chomczynski and Sacchi, 1987], fractionated by electrophoresis on a 1% formaldehyde-agarose gel (15-μg/lane) and transferred to a nylon hybridization membrane. Apo A-I and GAPDH probes were labeled with ^{32}P [Feinberg and Vogelstein, 1983], and hybridized to the membrane in Rapid Hyb (GE Healthcare, Piscataway, NJ), washed under high-stringency conditions (0.1× standard saline citrate, 0.1% sodium dodecylsulfate (SDS), 65°C), and exposed to Hyperfilm MP autoradiography film (GE Healthcare). Hybridization signals were quantified with a scanning densitometer (Molecular Dynamics).

PLASMIDS AND TRANSIENT TRANSFECTION ANALYSIS

The plasmid pAI.474.CAT, containing the rat apo AI promoter region between −474 and +7 bp and the TNF-responsive region site A (−214 to −195 bp) [Haas et al., 2003], was used to assess transcription of the apo A-I gene. Expression constructs for c-*jun* (SRα3-c-*jun*), JNK1 (pGEX-KG-JNK1), and the AP1-responsive human collagenase promoter-containing reporter plasmid (phu.collase-73/+63-CAT) were kindly provided by Dr. Michael Karin (The University of California—San Diego, San Diego, CA). The JNK2 expression construct pCNDA3-Flag-JNK2 was kindly provided by Dr. Roger Davis (University of Massachusetts Medical School, Boston, MA). The plasmid pEBG-MKK4/SEK1E → D, kindly provided by Dr. John Kyriakis (Tufts University of Medicine, Boston, MA) expresses a constitutively active (CA) MAP kinase kinase 4 (MKK4). The plasmid pSRα-MEKK(KM)Dead, kindly provided by Dr. Francois-Xavier Claret, (MD Anderson Cancer Center, Houston, TX) expresses a dominant-negative (DN) MKK4. HepG2 cells were transfected as indicated in each figure using Lipofectamine. The plasmid pCMV. SPORT-β-gal (Invitrogen), expressing β-galactosidase under the control of the cytomegalovirus immediate-early promoter, was used to control for transfection efficiency. After 24-h, the culture media was replaced with media containing either vehicle (0.1% bovine serum albumin in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.47 mM KH_2PO_4, pH 7.4 (PBS)), 30 ng/ml TNFα, and PMA dissolved in DMSO (50 nM). After 24-h, the cells were collected and assayed for chloramphenicol acetyltransferse (CAT) [Gorman et al., 1982] and β-galactosidase activity [Herbomel et al., 1984].

INHIBITION OF C-*JUN* EXPRESSION BY siRNA-MEDIATED SILENCING

To determine if silencing of c-*jun* effects apo A-I gene expression, HepG2 cells in 25-cm^2 flasks were transfected with both control and

c-*jun*-specific siRNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and 72-h later, c-*jun* levels were measured by Western blot. Briefly, lipofectamine-based transfection was performed by scaling up the reaction twofold and 7.5- and 10-μl of the c-*jun* and 10-μl of the control siRNA were added to the mixtures. After 4 h of incubation with the cells in SFM, 2× DMEM containing 20% FBS was added to the cells which were returned to the incubator overnight. After 24-h, the cells were fed fresh growth medium containing 10% FBS and the cells were placed in the incubator for 48-h. To prepare cell extracts, the cells were washed three times with ice-cold PBS and the cells were lysed in a solution containing 1% SDS, 50 mM tris(hydroxymethyl) aminomethane–hydrochloride (Tris–Cl) pH 8.0, 12.5% glycerol and 5% 2-mercaptoethanol. Protein concentration was measured using the Bradford dye-binding assay and 100 μg of protein was loaded per lane on a 10% SDS–polyacrylamide gel for Western blot analysis. After electrophoresis, the samples were transferred to nitrocellulose as described above and subjected to immunoblotting with the anti-c-*jun* antiserum (1:1,000) followed by incubation with a goat-anti-rabbit IgG-horse radish peroxidase (HRP) secondary antibody (1:5,000). After ECL, the membrane was stripped in 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris–Cl, pH 6.7 at 50°C and subjected to Western blot with an anti-GAPDH antibody (1:500). The optical density of each band was measured by densitometry and is expressed in arbitrary units (A.U.).

MEASUREMENT OF C-*JUN* ACTIVATION

Activation of c-*jun* by TNF α was measured by Western blotting with an antibody specific to c-*jun* phosphorylated on serine 63. HepG2 cells in 25-cm^2 flasks were treated with 30 nM TNF α in SFM for 0, 1, 3, 6, and 24 h. After washing the cells in ice-cold PBS, the cells were lysed by addition of 300 μl of 12.5% glycerol, 50 mM Tris–Cl, pH 8.0, 5% 2-mercaptoethanol, and 1% SDS. The protein content was measured with the Bradford protein assay using bovine serum albumin as the standard [Bradford, 1976]. One hundred microgrms of protein from each sample was fractionated by electrophoresis on a 10% SDS–polyacrylamide gel, transferred to nitrocellulose, and after blocking in 5% non-fat dry milk in PBS-Tween, the anti-phospho-c-*jun* (ser 63) antibody (1:1,000) was added. After ECL, the blots were stripped for 30 min in 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris–Cl, pH 6.7 at 50°C. After two washes room-temperature 10-min washes in PBS-Tween, the blots were blocked in 5% non-fat dry milk in PBS-Tween and incubated for 24 h with the anti-c-*jun* antiserum (1:1,000) for 24 h and the optical density of each band was measured by densitometry.

STATISTICS AND DATA ANALYSIS

The mean ± SEM are presented. Statistical significance between means was analyzed using Student's *t*-test for independent variables and Statistica for Windows (Statsoft, Inc., Tulsa, OK). Significance was defined as a two-tailed $P < 0.05$.

RESULTS

THE EFFECT OF PMA ON APO A-I mRNA AND PROTEIN EXPRESSION

To determine if PMA represses apo A-I gene expression, HepG2 cells were treated with 50 nM PMA and apo A-I mRNA and protein levels were measured by Northern blot and Western blot, respectively (Fig. 1). When HepG2 cells were treated with 50 nM PMA for 24 h (Fig. 1A, quantified in Fig. 1C), apo A-I mRNA levels decreased 65.3% (from 585 ± 16 A.U. (mean ± SEM) to 203 ± 1.6 A.U.; $P < 0.0001$). Apo A-I protein accumulation in the conditioned medium (Fig. 1B, quantified in Fig. 1C) decreased 44.9% in response to PMA treatment (from 1272 ± 23 to 701 ± 12 A.U.; $P < 0.0003$l; Fig. 1). GAPDH mRNA levels (Fig. 1A) and albumin expression (Fig. 1B) did not change, indicating that the effect of PMA was specific. Treatment with PMA had no effect on cell viability, as assessed by trypan blue staining (data not shown).

THE EFFECT OF PMA, TNF α, AND EXOGENOUS C-*JUN* EXPRESSION ON APO A-I GENE TRANSCRIPTION

To determine if changes in apo A-I mRNA and protein levels in response to PMA treatment are due to changes in gene transcription, HepG2 cells were transfected with the reporter plasmid pAI.474.CAT and after 24 h, treated with 50 nM PMA. After 24 h (Fig. 2A), PMA suppressed apo A-I promoter activity 83.8% (from 31.5 ± 1.1% acetylation in control cells to 5.1 ± 0.7% acetylation in PMA-treated cells; $P < 0.0004$). In HepG2 cells treated with 30 ng/ml TNF α for 24 h, apo A-I promoter activity declined 41.0% (from 31.5 ± 1.1% acetylation in control cells to 18.6 ± 0.7% acetylation in TNF α-treated cells; $P < 0.0006$).

To determine if exogenous c-*jun* expression inhibits apo A-I promoter activity, HepG2 cells were transfected with the plasmid SRα3-c-*jun*, containing the c-*jun* cDNA driven by the SV-40 virus

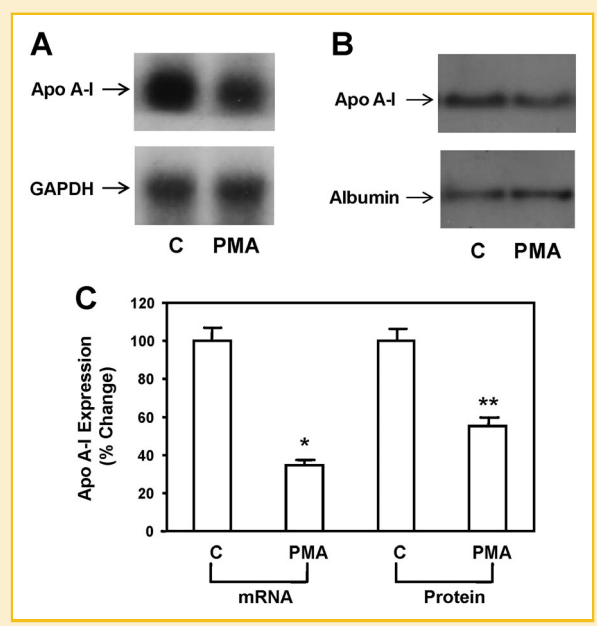

Fig. 1. The effect of PMA on Apo A-I protein and mRNA levels. HepG2 cells were treated with 50 nM PMA for 24-h and apo A-I and GAPDH mRNA levels (A) and apo A-I and albumin levels in the conditioned medium (B) were measured by Northern and Western blot, respectively (C). PMA suppressed both apo A-I mRNA and protein levels. N = 6, *treated versus control, $P < 0.0001$; **treated versus control, $P < 0.0003$.

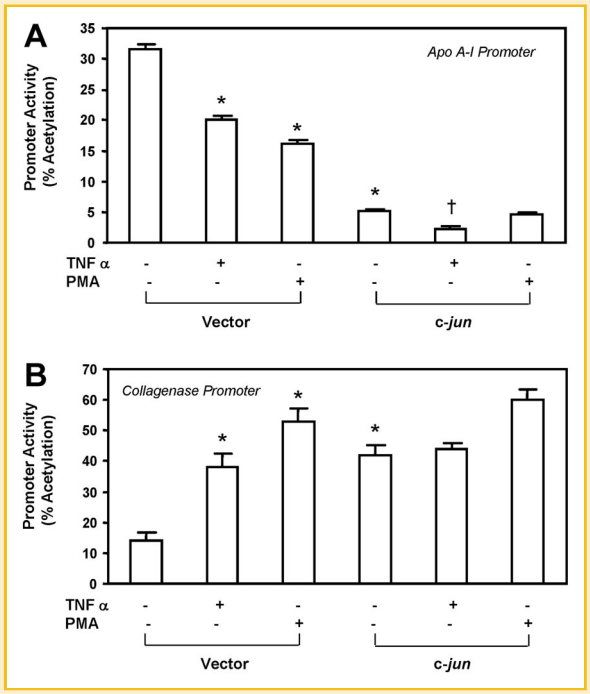

Fig. 2. The effect of exogenous c-*jun* expression on apo A-I promoter activity. A: HepG2 cells were transfected with the apo A-I reporter plasmid in the presence (c-*jun*) or absence (vector) of a c-*jun* expression plasmid, and after 24-h, treated with 30 ng/ml TNF α or 50 nM PMA. Apo A-I promoter activity is presented as percent acetylation. TNF α, PMA, and exogenous c-*jun* inhibited apo A-I promoter activity. N = 6. *Treated versus control, $P < 0.005$; †c-*jun* transfected versus c-*jun*-transfected treated with TNF α, $P < 0.001$. B: HepG2 cells were transfected with the c-*jun*-responsive human collagenase promoter reporter plasmid with (c-*jun*) or without (vector) the c-*jun* expression plasmid and treated with 30 ng/ml TNF α or 50 nM PMA for 24-h. Collagensase promoter activity is presented as percent acetylation. PMA, TNF α, and exogenous c-*jun* stimulated collagenase promoter activity. N = 6. *Treated versus control, $P < 0.001$.

constitutive promoter, and after 24 h treated with TNF α or PMA (Fig. 2A). Transfection with c-*jun* alone suppressed basal apo A-I promoter activity 83.3% (from 31.5 ± 1.1% acetylation to 5.3 ± 0.6% acetylation; $P < 0.005$). In the presence of exogenous c-*jun*, addition of TNF α and PMA suppressed apo A-I promoter activity from 5.3 ± 0.6% acetylation to 2.2 ± 0.8% and 4.7 ± 0.1% acetylation, respectively ($P < 0.01$ and N.S., respectively). These results suggest that exogenous c-*jun* expression alone suppresses apo A-I promoter activity.

To determine if inhibition of apo A-I gene expression by PMA and TNF α is specific, the effects of PMA, TNF α, and c-*jun* over-expression on the c-*jun*-inducible collagenase promoter [Mitchell and Cheung, 1991] was examined. TNF α and PMA increased collagenase promoter activity 2.7- and 3.8-fold, respectively ($P < 0.001$ and $P < 0.001$, respectively; Fig. 2B). In the presence of exogenous c-*jun*, collagenase promoter activity increased 3.0-fold ($P < 0.001$). In c-*jun*-expressing cells treated with TNF α and PMA, promoter activity changed 1.0- (N.S.) and 1.4-fold (N.S.), respectively relative to c-*jun* expressing cells alone. These results indicate that inhibition of apo A-I gene expression by PMA, TNF α, and transfected c-*jun* is specific. Treatment with PMA, TNF α, or co-transfection with SRα3-c-*jun* had no effect on the CMV promoter-driven β-galactosidase reporter gene (data not shown).

THE EFFECT OF C-*JUN* KNOCKDOWN ON REPRESSION OF APO A-I PROMOTER ACTIVITY BY TNF α

To determine if silencing c-*jun* expression prevents TNF α from inhibiting apo A-I promoter activity, c-*jun* levels were knocked down using siRNA. Transfection of c-*jun* siRNA (Fig. 3A, quantified in Fig. 3B) inhibited c-*jun* expression 49.1% and 80.2% (from 597 ± 40 A.U. in cells transfected with the control siRNA to 304 ± 61 and 118 ± 40 A.U. in cells transfected with 7.5 and 10 μg of c-*jun* siRNA; $P < 0.002$ and <0.0001, respectively).

To determine if c-*jun* knockdown prevents TNF α-mediated inhibition of apo A-I promoter activity, HepG2 cells were transfected with 10 μg of the control siRNA or 10 μg of the c-*jun* siRNA and the apo A-I reporter plasmid pAI.474.CAT. After 24 h, the cells were treated with 30 ng/ml TNF α, and 24 h later, apo A-I promoter activity was measured (Fig. 3C). In cells transfected with the control siRNA, TNF α inhibited apo A-I promoter activity 49.4% (from 25.8 ± 0.9% acetylation in untreated cells to 13.0 ± 1.6% acetylation in cells treated with TNF α; $P < 0.0007$). However, in cells transfected with the c-*jun* siRNA, TNF α inhibited apo A-I promoter activity only 14.2% (from 27.4 ± 1.5% acetylation in untreated cells to 24.5 ± 0.8% acetylation in cells treated with TNF α; $P < 0.01$). Apo A-I promoter activity in cells transfected with the control siRNA and treated with TNF α (13.0 ± 1.6% acetylation) was significantly reduced compared to apo A-I promoter activity in cells transfected with the c-*jun* siRNA and treated with TNF α (24.5 ± 0.8% acetylation; $P < 0.001$). These results suggest that inhibition of apo A-I promoter activity by TNF α requires c-*jun*.

THE EFFECT OF TNF α ON C-*JUN* PHOSPHORYLATION ON SERINE 63

To determine if TNF α treatment activates c-*jun* activity, HepG2 cells were treated with 30 ng/ml TNF α for 1, 2, 3, 6, and 24 h and c-*jun* phosphorylation on serine 63 was measured by Western blotting with a phospho-specific anti-serum (Fig. 4A, quantified in Fig. 4B). TNF α treatment induced maximal c-*jun* phosphorylation by 6 h which returned to control levels after 24 h. Total c-*jun* levels did not change with TNF α treatment during the 24 h time course while apo A-I protein levels declined only after 24 h of treatment (Fig. 4A).

THE EFFECT OF JNK OVER-EXPRESSION ON APO A-I PROMOTER ACTIVITY

Exogenous JNK1 (Fig. 5) suppressed apo A-I promoter activity 60.0% (from 33.4 ± 1.6% acetylation in control cells to 13.3 ± 0.3% acetylation in JNK1 expressing cells; $P < 0.0002$). Exogenous JNK2 suppressed apo A-I promoter activity 79.3% (from 33.4 ± 1.6% acetylation in control cells to 6.89 ± 0.1% acetylation in JNK2 expressing cells; $P < 0.0001$). These result suggest that both JNK1 and JNK2 are equally efficacious at inhibiting apo A-I gene expression.

THE EFFECT OF DN AND CA MKK4 ON APO A-I PROMOTER ACTIVITY

To examine the effect of DN MKK4 on apo A-I gene expression, HepG2 cells were transfected with pAI.474.CAT and pCMV.SPORT.

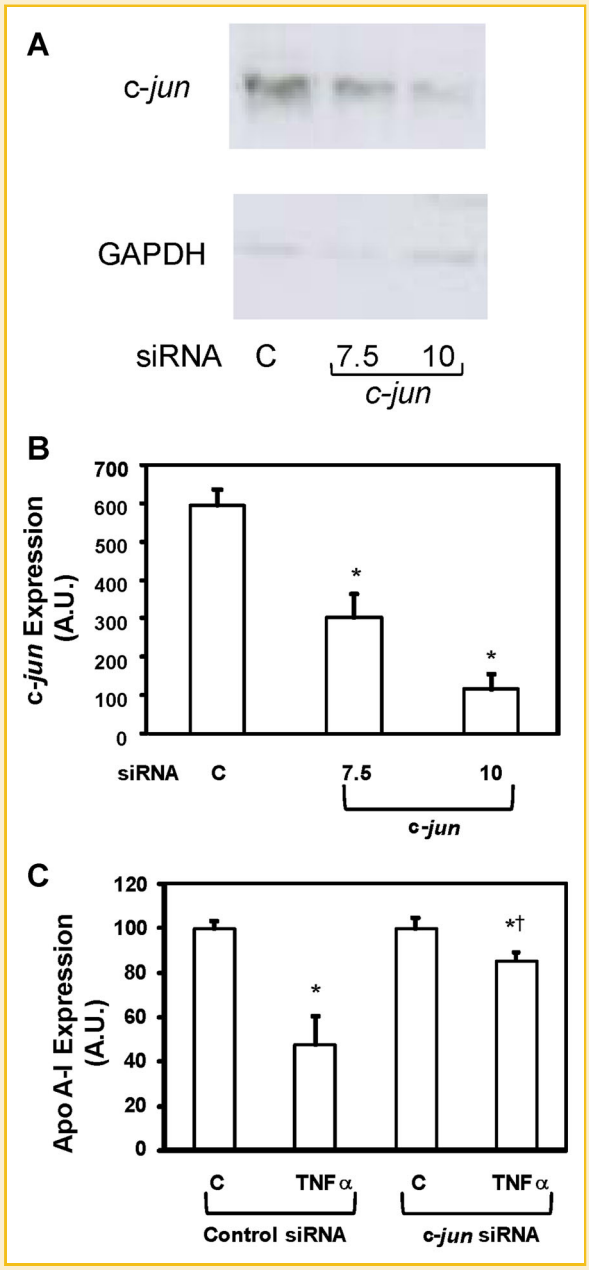

Fig. 3. The effect of c-*jun* inhibition on apo A-I gene expression. HepG2 cells were transfected with either control or c-*jun*-specific siRNA and 48-h later c-*jun* expression was measured by Western blot (A) and quantified (B). N = 6; *control siRNA versus c-*jun* siRNA, $P < 0.002$ and 0.0001 for 7.5 and 10 μg of siRNA, respectively. C: HepG2 cells were transfected with pAI.474.CAT and pCMV.SPORT.β-gal and either control or c-*jun*-specific siRNA and 48-h later treated with 30 ng/ml TNF α or solvent (0.1% BSA in PBS). Reporter gene expression was measured 24-h later. Silencing of c-*jun* expression prevented TNF α from inhibiting apo A-I promoter activity. N = 6; *treated versus control, control siRNA, $P < 0.0007$; treated versus control, c-*jun* siRNA, $P < 0.01$; †control siRNA-transfected cells treated with TNF α versus c-*jun* transfected cells treated with TNF α, $P < 0.001$.

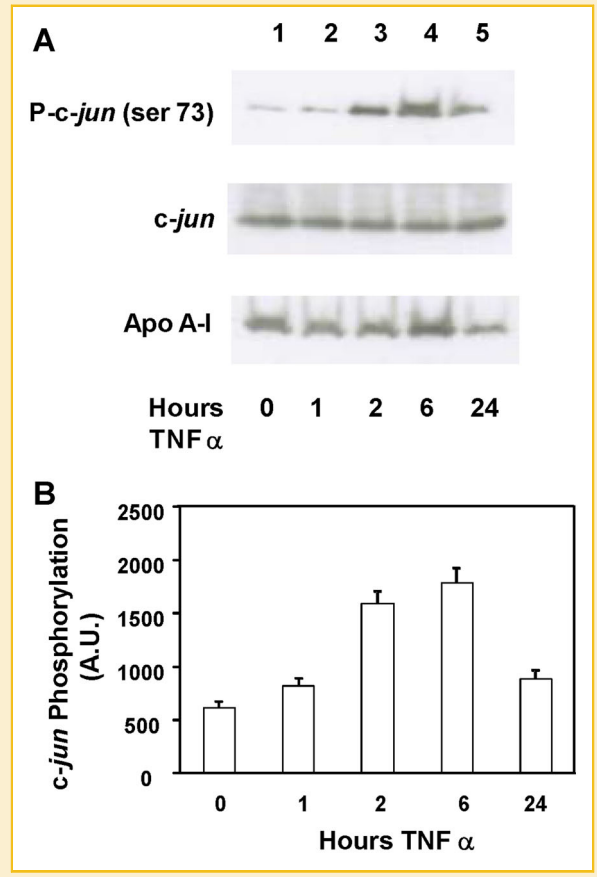

Fig. 4. The effect of TNF α on c-*jun* phosphorylation on serine 63. HepG2 cells were treated with 30 ng/ml TNF α for 1, 2, 3, 6, and 24-h and c-*jun* phosphorylation on serine 63 was measured by Western blotting with a phospho-specific anti-serum (A) and quantified in (B). Expression of c-*jun* did not change, however apo A-I levels in the conditioned medium decreased significantly at 24-h (A).

β-gal with and without the DN MKK4 plasmid pSRα-MEKK(KM)Dead (Fig. 6B). In cells treated with TNF α, apo A-I promoter activity decreased 69.7% (from 9.3 ± 1.1% acetylation in control cells to 2.8 ± 0.4% acetylation in TNF α-treated cells; $P < 0.004$; Fig. 6A). Control and DN MKK4-expressing cells had similar apo A-I promoter activity (9.3 ± 1.1% acetylation and 9.2 ± 1.0% acetylation; N.S.), while apo A-I promoter activity in TNF α-treated cells transfected with the DN MKK4 construct (5.7 ± 0.3% acetylation) was significantly higher than apo A-I promoter activity in TNF α-treated cells without the DN MKK4 plasmid (2.8 ± 0.4% acetylation; $P < 0.004$; Fig. 6A).

To examine the effect of CA MKK4 on apo A-I gene expression, HepG2 cells were transfected with pAI.474.CAT and pCMV.SPORT.β-gal with and without the CA MKK4 plasmid pEBG-MKK4/SEK1E → D (Fig. 6B). TNF α inhibited apo A-I promoter activity 52.3% (from 13.7 ± 0.1% acetylation in control cells vs. 6.5 ± 0.6% acetylation in TNF α -treated cells; $P < 0.001$), while transfection with CA MKK4 inhibited apo A-I promoter activity 26.2% (13.7 ± 0.1% acetylation vs. 10.1 ± 0.1% acetylation in CA MKK4-tranfected cells; $P < 0.007$). In the presence of TNF α, CA MKK4 had no effect on TNF

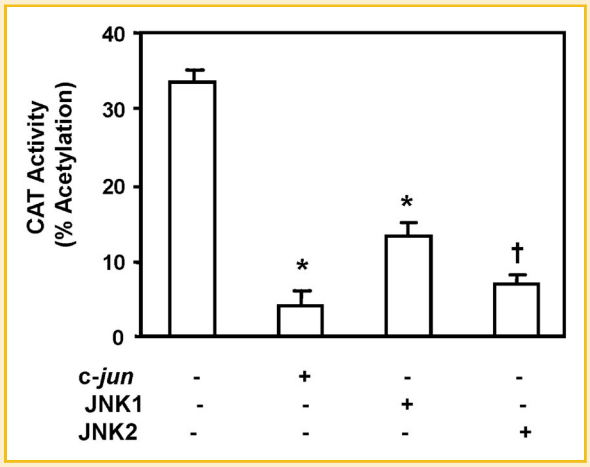

Fig. 5. The effect of exogenous JNK1 and JNK2 expression on apo A-I promoter activity. HepG2 cells were transfected with the apo A-I reporter plasmid with or without expression constructs for c-*jun*, JNK1, and JNK2. After 48-h, reporter enzyme activity was measured. Exogenous JNK1 and JNK2 were equally efficacious as c-*jun* at inhibiting apo A-I promoter activity. N = 6. *c-*jun* and JNK1 expressing cells versus control cells, $P < 0.0002$; †JNK2 expressing cells versus control cells, $P < 0.0001$.

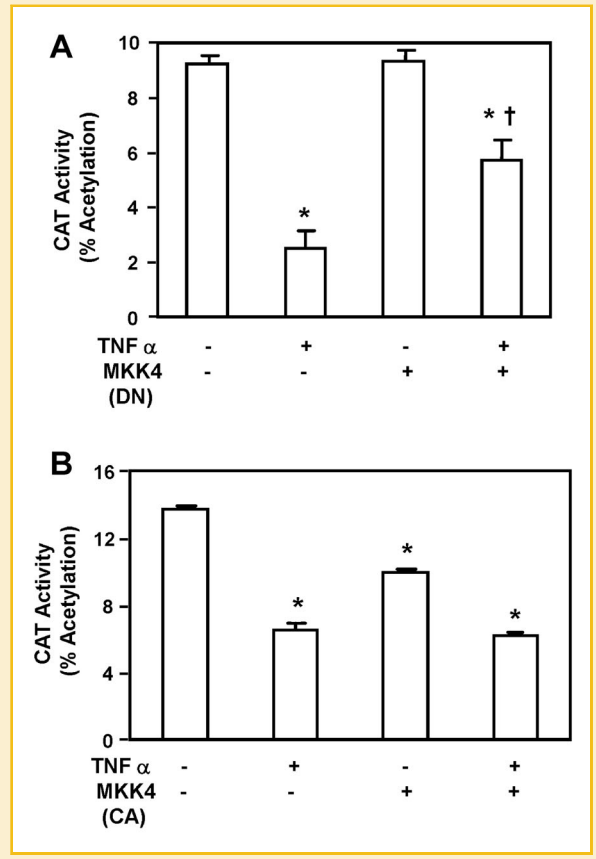

Fig. 6. The effect of dominant-negative and constitutively active MKK4 on TNF α-mediated suppression of apo A-I promoter activity. HepG2 cells were transfected with the apo A-I reporter plasmid pAI.474.CAT with or without expression plasmids for DN MKK4 (A) or CA MKK4 (B). Cells were then either treated with solvent or 30 ng/ml TNF α. Dominant-negative (DN) MKK4 alone had no effect on apo A-I promoter activity, however it did prevent some inhibition of apo A-I promoter activity by TNF α. Constitutively active (CA) MKK4 suppressed apo A-I promoter activity in the absence of TNF α, and addition of TNF α had no further effect on apo A-I promoter activity. N = 6. *Any treated group versus control, $P < 0.01$; †DN MKK4 expressing cells treated with TNF α versus TNF α treated cells, $P < 0.004$.

α-mediated suppression of apo A-I promoter activity (6.5 ± 0.6% acetylation in TNF α-treated cells vs. 6.2 ± 0.3% acetylation in CA MKK4-transfected cells treated with TNF α; N.S.). These results suggest that MKK4 is an intermediate in a signaling pathway linking TNF α receptor activation and repression of the apo A-I gene expression.

DISCUSSION

Pro-inflammatory cytokines contribute significantly to the pathogenesis associated with dyslipidemia and atherosclerosis. PMA treatment suppressed apo A-I mRNA and protein levels (Fig. 1) and promoter activity (Fig. 2) in HepG2 cells, as did expression of exogenous c-*jun* (Fig. 2). Apo A-I promoter inhibition by c-*jun* was selective since PMA-treatment of HepG2 cells transfected with a control human collagenase promoter construct increased reporter gene expression, as did TNF α-treatment and c-*jun* transfection (Fig. 2). Knockdown of c-*jun* expression via siRNA also prevented TNF α-mediated apo A-I gene inhibition (Fig. 3). Treatment of HepG2 cells with TNF α induced c-*jun* phosphorylation on ser 63 (Fig. 4). Exogenous over-expression of JNK1 and JNK2 also suppressed basal apo A-I promoter activity, further implying that these MAP kinase family members participate in a TNF α-dependent signaling pathway that inhibits apo A-I gene expression (Fig. 5). Likewise, exogenous over-expression of a CA upstream regulator of JNK, MKK4, also repressed apo A-I promoter activity (Fig. 6), while over-expression of a dominant-negative MKK4 prevented TNF α-mediated suppression of apo A-I promoter activity (Fig. 6). These results suggest that TNF α-mediated signaling in HepG2 cells suppresses apo A-I gene expression in part through MKK4/JNK/c-*jun* (Fig. 7).

One limitation of our study was our use of HepG2 cells in all of our experiments. We used HepG2 cells in our experiments since these studies are a continuation of previous studies in the same cell line [Haas et al., 2003; Beers et al., 2006]. HepG2 cells were recently shown to express many of the same secretory proteins as primary human hepatocytes. In a shotgun analysis of the secretome of primary human hepatocytes, HepG2 cells, and Hep3B cells [Slany et al., 2010], primary human hepatocytes expressed 72 secreted proteins, 46 of which were plasma proteins. HepG2 cells secreted 139 proteins, 55 of which were plasma proteins. In contrast, Hep3B cells secreted 72 proteins, 24 of which were plasma proteins. In the cytoplasm, 2-D-PAGE analysis demonstrated that primary hepatocytes expressed 104 of 107 hepatocyte-specific proteins, while HepG2 and Hep3B cells expressed 20 and 6 hepatocyte-specific proteins. These results and others by Slany et al. [2010] suggest that HepG2 cells demonstrate more features of hepatocytes and that Hep3B cells are more

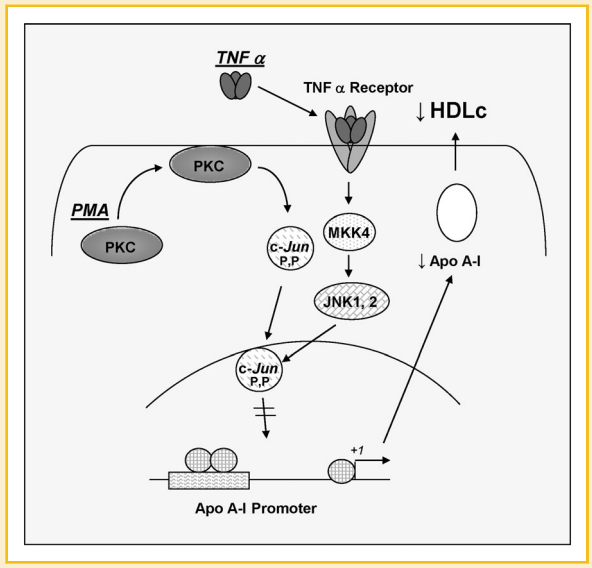

Fig. 7. Model for c-*jun*-mediated repression of apo A-I gene expression. TNF α and PMA induce c-*jun* activity through MKK4/JNK1, 2 and PKC, respectively. Once phosphorylated by PKC or JNK1, 2 (P, P), c-*jun* inhibits apo A-I promoter activity by trans-repression, by directly inhibiting nuclear hormone receptor (PPARα?) expression, or by another mechanism that remains to be discovered. As a result, decreased apo A-I levels reduces hepatic HDL secretion.

characteristic of hepatocytes undergoing the epithelial-to-mesenchymal transition.

PKC β and JNK activation have been demonstrated to induce insulin resistance [Hirosumi et al., 2002; Ragheb et al., 2008; Schmitz-Peiffer and Biden, 2008; Kennedy et al., 2009; Han et al., 2013]. Deletion of JNK1 but not JNK2 prevented mice from gaining weight when fed a high fat diet, and prevent weight gain in genetically obese mice [Hirosumi et al., 2002]. Recently, Han et al. demonstrated that deletion of JNK1 and JNK2 in myeloid cells protected mice from developing insulin resistance [Han et al., 2013]. These mice also had fewer activated macrophage cells infiltrating their adipose tissue [Han et al., 2013]. These results and ours suggest that therapies targeting JNK and PKC may not only prevent impaired glucose tolerance but may also have beneficial effects on lipids.

Several nuclear hormone receptors modulate apo A-I gene expression [Taylor et al., 1996; Claudel et al., 2002; Millar et al., 2009], and most are inhibited by c-*jun*, either by trans-repression or by inhibiting nuclear hormone receptor gene expression. In inflammation models, c-*jun* decreases PPARα, FXR, TR, and RXR mRNA levels, all of which regulate apo A-I. These changes, as well as changes in liver-X-receptor (LXR), sterol regulatory element binding proteins (SREBPs) and carbohydrate-responsive element binding protein, which regulate enzymes involved in glycolytic and lipogenic pathways, may be the molecular basis for many of the pathological metabolic changes associated with obesity, diabetes, and the metabolic syndrome.

The composition of AP-1 is complicated and we are not sure which forms suppress apo A-I gene expression. Our transfection experiments used c-*jun*, however we have not tested other family members including *jun*B and *jun*D. Likewise, we also need to determine if c-*fos* or other *fos* family members are involved. *Fos* family members includes FosB (and its two splice variants FosB2 and deltaFosB2), Fra-1, and Fra-2. Depending on the cell type and expression levels, AP-1 binding activity is composed of c-*jun*, *jun*B, or *jun*D homodimers or heterdimers containing *fos*. Which combination is utilized in a particular situation is cell-type and promoter-dependent. There are few studies on c-fos and CVD. Recently, cardiomyocyte c-fos expression was shown to correlate positively with infarct size in a rat model of acute myocardial infarction [Zhang et al., 2013]. Treatment with metoprolol reduced both infarct size and decreased cardiomyocyte c-fos expression [Zhang et al., 2013] Due to these limitations, we will examine AP-1 composition on the apo A-I gene promoter in vivo in future experiments using chromatin immunoprecipitation assays.

Overall the present study demonstrates that the MKK4/JNK/c-*jun* signaling pathway is important for TNF α-dependent inhibition of apo A-I synthesis and may be an important target for anti-inflammatory agents in the treatment of dyslipidemia.

REFERENCES

Abate C, Curran T. 1990. Encounters with fos and jun on the road to AP-1. Semin Cancer Biol 1:19–26.

Beato M. 1991. Transcriptional control by nuclear receptors. FASEB J 5:2044–2051.

Beers A, Haas MJ, Wong NC, Mooradian AD. 2006. Inhibition of apolipoprotein AI gene expression by tumor necrosis factor alpha: Roles for MEK/ERK and JNK signaling. Biochem 45:2408–2413.

Beier K, Volkl A, Fahimi HD. 1994. TNF-alpha downregulates the peroxisome proliferator activated receptor-alpha and the mRNAs encoding peroxisomal proteins in rat liver. FEBS Lett 412:385–387.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Busch SJ, Sassone-Corsi P. 1990. Dimers, leucine zippers and DNA-binding domains. Trends Genet 6:36–40.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Claudel T, Sturm E, Duez H, Torra IP, Sirvent A, Kosykh V, Fruchart JC, Dallongeville J, Hum DW, Kuipers F, Staels B. 2002. Bile acid-activated nuclear receptor FXR suppresses apolipoprotein A-I transcription via a negative FXR response element. J Clin Invest 109:961–971.

Coso OA, Chiariello M, Yu JC, Teramoto H, Crespo P, Xu N, Miki T, Gutkind JS. 1995. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell 81:1137–1146.

Deacon K, Blank JL. 1997. Characterization of the mitogen-activated protein kinase kinase 4 (MKK4)/c-jun NH2-terminal kinase 1 and MKK3/p38 pathways regulated by MEK kinases 2 and 3. MEK kinase 3 activates MKK3 but does not cause activation of p38 kinase in vivo. J Biol Chem 272:14489–14496.

Feinberg AP, Vogelstein B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13.

Fleming Y, Armstrong CG, Morrice N, Paterson A, Goedert M, Cohen P. 2000. Synergistic activation of stress-activated protein kinase 1/c-jun N-terminal kinase (SAPK1/JNK) isoforms by mitogen-activated protein kinase kinase 4 (MKK4) and MKK7. Biochem J 352:145–154.

Gorman CM, Moffat LF, Howard BH. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol Cell Biol 2:1044–1051.

Haas MJ, Horani M, Mreyoud A, Plummer B, Wong NC, Mooradian AD. 2003. Suppression of apolipoprotein AI gene expression in HepG2 cells by TNF alpha and IL-1beta. Biochim Biophys Acta 1623:120–128.

Han MS, Jung DY, Morel C, Lakhani SA, Kim JK, Flavell RA, Davis RJ. 2013. JNK expression by macrophages promotes obesity-induced insulin resistance and inflammation. Science 339:218–222.

Herbomel P, Bourachot B, Yaniv M. 1984. Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. Cell 39:653–662.

Hikita M, Bujo H, Yamazaki K, Taira K, Takahashi K, Kobayashi J, Saito Y. 2000. Differential expression of lipoprotein lipase gene in tissues of the rat model with visceral obesity and postprandial hyperlipidemia. Biochem Biophys Res Commun 277:423–429.

Hirosumi J, Tuncman G, Chang L, Görgün CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS. 2002. A central role for JNK in obesity and insulin resistance. Nature 420:333–336.

Jauregui HO, Hayner NT, Driscoll JL, Williams-Holland R, Lipsky MH, Galletti PM. 1981. Trypan blue dye uptake and lactate dehydrogenase in adult rat hepatocytes–freshly isolated cells, cell suspensions, and primary monolayer cultures. In Vitro 17:1100–1110.

Kennedy A, Martinez K, Chuang CC, LaPoint K, McIntosh M. 2009. Saturated fatty acid-mediated inflammation and insulin resistance in adipose tissue: Mechanisms of action and implications. J Nutr 139:1–4.

Kim MS, Shigenaga J, Moser A, Feingold K, Grunfeld C. 2003. Repression of farnesoid X receptor during the acute phase response. J Biol Chem 278:8988–8995.

Kroder G, Bossenmaier B, Kellerer M, Capp E, Stoyanov B, Mühlhöfer A, Berti L, Horikoshi H, Ullrich A, Häring H. 1996. Tumor necrosis factor-alpha- and hyperglycemia-induced insulin resistance. evidence for different mechanisms and different effects on insulin signaling. J Clin Invest 97:1471–1477.

Lian JB, Stein GS, Bortell R, Owen TA. 1991. Phenotype suppression: A postulated molecular mechanism for mediating the relationship of proliferation and differentiation by Fos/Jun interactions at AP-1 sites in steroid responsive promoter elements of tissue-specific genes. J Cell Biochem 45: 9–14.

McDonnell SE, Kerr LD, Matrisian LM. 1990. Epidermal growth factor stimulation of stromelysin mRNA in rat fibroblasts requires induction of proto-oncogenes c-fos and c-jun and activation of protein kinase C. Mol Cell Biol 10:4284–4293.

Minden A, Lin A, Claret FX, Abo A, Karin M. 1995. Selective activation of the JNK signaling cascade and c-jun transcriptional activity by the small GTPases rac and Cdc42Hs. Cell 81:1147–1157.

Millar JS, Duffy D, Gadi R, Bloedon LT, Dunbar RL, Wolfe ML, Movva R, Shah A, Fuki IV, McCoy M, Harris CJ, Wang MD, Howey DC, Rader DJ. 2009. Potent and selective PPAR-alpha agonist LY518674 upregulates both ApoA-I production and catabolism in human subjects with the metabolic syndrome. Arterioscler Thromb Vasc Biol 29:140–146.

Mitchell PG, Cheung HS. 1991. Tumor necrosis factor alpha and epidermal growth factor regulation of collagenase and stromelysin in adult porcine articular chondrocytes. J Cell Physiol 149:132–140.

Murphy N, Grimsditch DC, Parkin S, Vidgeon-Hart MP, Overend P, Groot PH, Graham A, Benson GM. 2006. Hypercholesterolaemia and circulating levels of CXC chemokines in apoE*3 leiden mice. Atherosclerosis 163:69–77.

Ragheb R, Medhat AM, Shanab GM, Seoudi DM, Fantus IG. 2008. Links between enhanced fatty acid flux, protein kinase C and NFkappaB activation, and apoB-lipoprotein production in the fructose-fed hamster model of insulin resistance. Biochem Biophys Res Commun 370:134–139.

Schmitz-Peiffer C, Biden TJ. 2008. Protein kinase C function in muscle, liver, and beta-cells and its therapeutic implications for type 2 diabetes. Diabetes 57:1774–1783.

Slany A, Haudek VJ, Zwickl H, Gundacker NC, Grusch M, Weiss TS, Seir K, Rodgarkia-Dara C, Hellerbrand C, Gerner C. 2010. Cell characterization by proteome profiling applied to primary hepatocytes and hepatocyte cell lines Hep-G2 and Hep-3B. J Proteome Res 9:6–21.

Sugawara A, Uruno A, Nagata T, Taketo MM, Takeuchi K, Ito S. 1998. Characterization of mouse retinoid X receptor (RXR)-beta gene promoter: Negative regulation by tumor necrosis factor (TNF)-alpha. Endocrinol 139: 3030–3033.

Taylor AH, Wishart P, Lawless DE, Raymond J, Wong NC. 1996. Identification of functional positive and negative thyroid hormone-responsive elements in the rat apolipoprotein AI promoter. Biochem 35:8281–8288.

Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354.

Turjanski AG, Vaque JP, Gutkind JS. 2007. MAP kinases and the control of nuclear events. Oncogene 26:3240–3253.

Whitmarsh AJ, Davis RJ. 1996. Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. J Mol Med 74:589–607.

Wolf M, Hansen N, Greten H. 1994. Interleukin 1 beta, tumor necrosis factor-alpha and interleukin 6 decrease nuclear thyroid hormone receptor capacity in a liver cell line. Eur J Endocrinol 131:307–312.

Zhang B, Berger J, Hu E, Szalkowski D, White-Carrington S, Spiegelman BM, Moller DE. 1996. Negative regulation of peroxisome proliferator-activated receptor-gamma gene expression contributes to the antiadipogenic effects of tumor necrosis factor-alpha. Mol Endocrinol 10:1457–1466.

Zhang S, Zhang M, Goldstein S, Li Y, Ge J, He B, Ruiz G. 2013. The effect of c-fos on acute myocardial infarction and the significance of metoprolol intervention in a rat model. Cell Biochem Biophys 65:249–255.

Zhao L, Cuff CA, Moss E, Wille U, Cyrus T, Klein EA, Praticò D, Rader DJ, Hunter CA, Puré E, Funk CD. 2002. Selective interleukin-12 synthesis defect in 12/15-lipoxygenase-deficient macrophages associated with reduced atherosclerosis in a mouse model of familial hypercholesterolemia. J Biol Chem 277:35350–35356.