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Article

Role of the Nuclear Receptors HNF4α, PPARα, and LXRs in the TNFα-Mediated Inhibition of Human Apolipoprotein A-I Gene Expression in HepG2 Cells[†]

Denis A. Mogilenko,*[,‡,§] Ella B. Dizhe,[‡] Vladimir S. Shavva,[§] Ivan A. Lapikov,[‡,§] Sergey V. Orlov,*[,‡,§] and Andrey P. Perevozchikov*[,‡,§]

[‡]*Department of Biochemistry, Institute of Experimental Medicine, Russian Academy of Medical Sciences, 197376 St. Petersburg, Russia and* [§]*Department of Embryology, St. Petersburg State University, 199034 St. Petersburg, Russia*

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ABSTRACT: The expression of the apolipoprotein A-I gene (apoA-I) in hepatocytes is repressed by pro-inflammatory cytokines such as IL-1β and TNFα. In this work, we have demonstrated that treatment of HepG2 human hepatoma cells with chemical inhibitors for JNK, p38 protein kinases, and NFκB transcription factor abolishes the TNFα-mediated inhibition of human apoA-I gene expression in HepG2 cells. In addition, we have shown that TNFα decreases also the rate of secretion of apoA-I protein by HepG2 cells, and this effect depends on JNK and p38, but not on NFκB and MEK1/2 signaling pathways. The inhibitory effect of TNFα has been found to be mediated by the hepatic enhancer of the apoA-I gene. The decrease in the level of human apoA-I gene expression under the impact of TNFα appears to be partly mediated by the inhibition of HNF4α and PPARα gene expression. Treatment of HepG2 cells with PPARα antagonist (MK886) or LXR agonist (TO901317) abolishes the TNFα-mediated decrease in the level of apoA-I gene expression. PPARα agonist (WY-14643) abolishes the negative effect of TNFα on apoA-I gene expression in the case of simultaneous inhibition of MEK1/2, although neither inhibition of MEK1/2 nor addition of WY-14643 leads to the blocking of the TNFα-mediated decrease in the level of apoA-I gene expression individually. The ligand-dependent regulation of apoA-I gene expression by PPARα appears to be affected by the TNFα-mediated activation of MEK1/2 kinases, probably through PPARα phosphorylation. Treatment of HepG2 cells with PPARα and LXR synthetic agonists also blocks the inhibition of apoA-I protein secretion in HepG2 cells under the impact of TNFα. A chromatin immunoprecipitation assay demonstrates that TNFα leads to a 2-fold decrease in the level of PPARα binding with the apoA-I gene hepatic enhancer. At the same time, the level of LXRβ binding with the apoA-I gene hepatic enhancer is increased 3-fold under the impact of TNFα. These results suggest that nuclear receptors HNF4α, PPARα, and LXRs are involved in the TNFα-mediated downregulation of human apoA-I gene expression and apoA-I protein secretion in HepG2 cells.

Apolipoprotein A-I (apoA-I)[1] is the main structural and functional protein component of human high-density lipoproteins (HDLs). Synthesis of apoA-I protein generally takes place in the liver and small intestine of the adult human (*1*). In most cases, the level of HDLs in serum is positively correlated with the level of expression and secretion of apoA-I by hepatocytes. A high concentration of apoA-I protein in serum protects against atherosclerosis (*2*, *3*). The antiatherogenic properties of apoA-I are associated with the participation of apoA-I in processes of reverse cholesterol transport from peripheral tissues to liver (*4*), and with anti-inflammatory (*5*−*8*), antioxidant (*9*−*11*), and antithrombotic (*12*) properties of apoA-I. Anti-inflammatory activities of apoA-I are supposedly realized through specific cell signaling processes (*13*). Production of pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα) and interleukin 1β (IL-1β), by monocytes and macrophages after contact-mediated activation of these cells by T-lymphocytes was found to be inhibited by apoA-I (*7*). ApoA-I is considered to play the role of a constitutive anti-inflammatory factor, and diminution of the apoA-I concentration in serum during acute inflammation can lead to the development of a chronic inflammatory process (*14*). A decreasing level of apoA-I in HDL structure during inflammation is associated with inhibition of apoA-I synthesis in liver and with displacement of apoA-I in HDL by serum amyloid A (SAA) (*15*, *16*). Pro-inflammatory cytokines (IL-1β and TNFα) decrease the rates of expression and secretion of apoA-I in human hepatocytes (*17*). In vivo and in vitro studies suggest that IL-6 and TNFα inhibit apoA-I gene expression in hepatocytes and decrease the apoA-I protein level in serum during acute inflammation in the pig (*18*). TNFα was found to inhibit rat apoA-I gene transcription in HepG2 cells by acting through a hepatic enhancer of the gene (*19*, *20*), but little is known about the specific transcription factors involved in the process.

Regulation of human apoA-I gene expression at the transcriptional level is mediated by the 5′-regulatory region of the gene. High levels of human apoA-I gene transcription in hepatocytes are controlled by the gene's minimal promoter (positions −41 to +1) and hepatic enhancer (positions −222 to −110). The later

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*To whom correspondence should be addressed: 197376, Acad. Pavlov st., 12, St. Petersburg, Russia. Telephone: 7-812-346-0644. Fax: 7-812-234-0310. E-mail: denis@iem.sp.ru (D.A.M.), serge@iem.sp.ru (S.V.O.), or app@iem.sp.ru (A.P.P.).

[1]Abbreviations: HDLs, high-density lipoproteins; apoA-I, apolipoprotein A-I; TNFα, tumor necrosis factor α; TSP, transcription start point; HE, hepatic enhancer; HRE, hormone responsive element.

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regulatory region is important for increasing the level of apoA-I gene expression in hepatocytes (*21*, *22*). The apoA-I gene hepatic enhancer contains three regulatory regions: A (positions −214 to −192), B (positions −169 to −146), and C (positions −134 to −119). Regions A and C of the apoA-I gene hepatic enhancer contain well-known sites for binding of transcription factors belonging to the nuclear receptor superfamily. Because of their ability to interact with several orphan and ligand-dependent nuclear receptors, the A and C sites are called hormone responsive elements (HREs). The positive regulators of apoA-I gene expression interacting with the HREs include HNF4α (*23*, *24*), PPARα (*25*), and RXRα (*26*, *27*). Nuclear receptors LXRs (*28*) and ARP-1 (*23*) were found to act as apoA-I gene expression repressors.

In this study, we have investigated the regulation of human apoA-I gene expression in the HepG2 human hepatoma cell line treated with TNFα. We have demonstrated that JNK, p38, and MEK1/2 kinases and NFκB transcription factor are involved in the TNFα-mediated inhibition of human apoA-I gene transcription in HepG2 cells. Nuclear receptors HNF4α, PPARα, and LXRs directly regulate human apoA-I gene expression in HepG2 cells treated with TNFα.

MATERIALS AND METHODS

Chemical Inhibitors and Synthetic Ligands. MAP kinase inhibitors and NFκB inhibitor were purchased from Biomol: SB203580 (p38 inhibitor) (catalog number EI-286), SP600125 (JNK1/2/3 inhibitor) (catalog number EI-305), U0126 (MEK1/2 inhibitor) (catalog number EI-282), and QNZ (NFκB inhibitor) (catalog number EI-352). Src kinase inhibitor was purchased from Biomol: PP2 (Srk-kinase inhibitor) (catalog number EI-297). PPARα ligands were purchased from Sigma: WY-14643 (catalog number C7081) and MK-886 (catalog number M2692). LXR ligand was purchased from Biomol: TO901317 (catalog number GR-232). Human recombinant TNFα was purchased from Sigma (catalog number T0157).

Plasmids. pCMVL, the expression vector for bacterial reporter gene lacZ under the control of the early human cytomegalovirus gene promoter (CMV), has been described previously (*29*). pCMVHNF4, the expression vector of human transcription factor HNF4α, was a generous gift of Dr. Fukamizu (University of Tsukuba, Tsukuba, Japan). pCMVHNF4D, the expression vector of the human HNF4α dominant-negative mutant, was kindly provided by T. Leff (Wayne State University School of Medicine, Detroit, MI). pAPOA-I(−2498/+173)-Luc, pAPOA-I(−2498/+72)-Luc, pAPOA-I(−256/+173)-Luc, and pAPOA-I(−256/+72)-Luc, the plasmids containing the firefly luciferase reporter gene under control of deletion variants of the 5′-regulatory region of the human apoA-I gene (positions −2498 to +173, positions −2498 to +72, positions −256 to +173, and positions −256 to +72, respectively) related to the transcription start point (TSP) of the human apoA-I gene, have been described previously (*30*).

Cell Culture, Transfection, β-Galactosidase, and Luciferase Assays. HepG2 cells were cultivated in DMEM containing 10% fetal calf serum (FCS) and 5% CO_2 at 37 °C. For TNFα administration, cells were seeded on 30 mm culture dishes at a density of 1×10^4 cells/cm^2 and cultivated for 24 h. The cultivation medium was replaced with a fresh one without FCS, and cells were additionally incubated for 24 h before TNFα administration (50 ng/mL). After a 24 h incubation with TNFα, cells were washed three times with sodium phosphate buffer (PBS) (pH 7.5), harvested, and used for RNA isolation and luciferase assays. The cultivation medium was used for ELISA experiments. In the experiments with kinase inhibitors and PPARα or LXR ligands, they had been added 1 h before TNFα. For transfection experiments, HepG2 cells were seeded on 30 mm culture dishes at a density of 1×10^4 cells/cm^2 and grown to a subconfluent layer. The calcium phosphate transfection procedure was performed as described elsewhere (*31*). Seven micrograms of DNA per dish was used in all experiments. The pCMVL plasmid was used to control for the transfection efficiency. The β-galactosidase assay was performed following the standard protocols, using *o*-nitrophenyl β-D-galactopyranoside as a substrate. Relative β-galactosidase activity was calculated as the D_{420} optical density per milligram of total protein of cell lysates per hour. Activity of luciferase was measured on a 20/20^n luminometer (Turner BioSystems) by using a Luciferase Assay System (Promega, catalog number E4030) in accordance with the manufacturer's guidelines. The luciferase activity is shown as a relative light activity (RLA) which corresponds to the percentage of light counts per minute per milligram of total protein of cell lysates relative to the control cells (RLA = 100% in control cells). The protein concentration in cell lysates was measured with the Bradford assay.

Reverse Transcription. Total cellular RNA was isolated from cultivated cells with RNA STAT-60 reagent (Tel-Test) in accordance with the manufacturer's guidelines. After digestion with RNase-free DNase I (Roche Applied Science) (30 min at 37 °C, stopping the reaction by addition of EDTA to a final concentration of 2 mM, and 15 min at 70 °C for DNase inactivation), the concentration of total RNA and RNA purity were determined using an Avaspec-2048 spectrophotometer (Avantes). The ratio of optical densities at 260 and 280 nm was greater than 2.0, whereas the ratio of optical densities at 260 and 230 nm was greater than 1.7. Ribosomal RNA band integrity was confirmed by electrophoresis with a 1% agarose gel. RNA (2 μg) was subjected to reverse transcription, using a dT-16 primer (Invitrogen) and reverse transcriptase (Promega) to generate first-strand cDNA (15 min at 70 °C with dT-16 primer, 1 min on ice, 60 min at 42 °C in reaction mix that contained 0.5 mM dNTPs, 0.3 mM $MgCl_2$, 75 mM KCl, 10 mM DTT, and 8 units/μL reverse transcriptase, and 15 min at 70 °C for reverse transcriptase inactivation).

Real-Time Polymerase Chain Reaction (PCR). Real-time PCRs were performed using the ANK-32 nucleic acid analyzer (Syntol). The instrument determines relative abundances of mRNA by using real-time fluorescence detection of dual-labeled (TaqMan) probes, which are complementary to the PCR amplicon, or the Syber Green technique. All primers and dual-labeled probes were designed with Primer3 (http://primer3.sourceforge.net). The following sets of primers and probes were used: GAPDH (GenBank accession number NM_002046.3) (5-gapdh-rt, AAGGGCATCCTGGGCTAC; 3-gapdh-rt, GTGGAGGAGTGGGTGTCG; h-gapdh-rt, CY5-TGAGCACCAGGTGGTCTCCTCTGAC-RTQ2), apoA-I (GenBank accession number NM_000039.1) (5-cpaponew-rt, CCTTGGGAAAACAGCTAAACC; 3-cpaponew-rt, CAGCTTGCTGAAGGTGGAG; h-cpaponew-rt, FAM-AGCTCCTTGACAACTGGGACAGCGT-BHQ1), LXRα (GenBank accession number NM_001130102.1) (5-lxra, TCACCTTCCTCAAGGATTTCA; 3-lxra, TCGAAGATGGGGTTGATGA; h-lxra, ROX-TAACCGGGAAGACTTTGCCAAAGCA-RTQ2), LXRβ (GenBank accession number

NM_007121.4) (5-lxrb, CAGCAAGGACGACTTCCA; 3-lxrb, CCGCGAGAACTCGAAGAT; h-lxrb, R6G-AGGCCTG-CAGGTGGAGTTCATCAAC-RTQ1), PPARα (GenBank accession number NM_005036.4) (5-ppara-rt, TCACAAGTGC-CTTTCTGTCG; 3-ppara-rt, TCTTGGCATTCGTCCAAAA; h-ppara, ROX-GGATGTCACACAACGCGATTCG-RTQ2), and HNF4α (GenBank accession number NM_175914.3) (5-hnf4a, ATGAGCCGGGTGTCCATA; 3-hnf4a, ACTGGC-GGTCGTTGATGT). All sets of TaqMan primers and probes were designed in a way so they are not able to amplify the genomic DNA templates (one of primers located at the junction of two exons in the case of each pair of primers). The negative (no-reverse transcriptase) as well as the no-template control reactions were conducted to verify the absence of DNA template contamination and probe hybridization with genomic DNA for each real-time PCR. To optimize multiplex real-time PCRs, the conditions that provide the fastest Ct values were selected for each primer−probe set separately and in combination in the case of multiplexing. It was also ascertained during multiplex optimizing that using all primer−probe sets in the multiplexing approach does not influence the efficiency of PCR and the Ct value in comparison with using each primer−probe set alone. The following conditions for real-time PCR were used: 95 °C for 300 s followed by 40 cycles at 95 °C for 25 s and 60 °C for 45 s in the case of TaqMan and 95 °C for 300 s followed by 40 cycles at 95 °C for 30 s, 60 °C for 20 s, and 72 °C for 30 s in the case of Syber Green. TaqMan or Syber Green kits (Syntol, catalog numbers R-412 and R-402) were used. The PCR mix (25 μL) contained 0.25 mM dNTPs, 2.5 mM $MgCl_2$, 0.05 unit/μL Taq polymerase (Syntol), each primer at 0.25 pmol/μL, and each probe at 0.125 pmol/μL, and the reaction was performed in 200 μL tubes (Axygen, catalog number PCR-02D-A). The relative abundances of apoA-I, LXRα, LXRβ, and PPARα mRNAs were assessed by GAPDH detection in the same reaction. The number of cycles (Ct value) required to reach a threshold level of fluorescence that is ~10 standard deviations (of fluctuations in background fluorescence) above the mean background fluorescence was determined for each PCR and primer set by using ANK32 automated software (Syntol). The following Ct values were used: 16 for GAPDH, 15 for apoA-I, 28 for LXRα, 25 for LXRβ, 21 for PPARα, and 22 for HNF4α. The relative amount of mRNA (as a percentage for the control sample) was calculated by relation $(2^{Ct_{control}-Ct_{sample}}) \times 100$.

ChIP Assay. Chromatin immunoprecipitation (ChIP) was performed as described previously (*32*) with slight modifications. Briefly, cells were cross-linked with 1% formaldehyde prepared on PBS for 10 min. After reactions had been quenched with 125 mM glycine, cells were lysed at 4 °C for 10 min. The lysates were sonicated with a UZDN1 sonic disintegrator (Nasosenergomash) (four times for 20 s at the current 0.3 A and sound frequency of 44 kHz) to an average length of chromatin of approximately 200−300 bp. Murine monoclonal antibodies against PPARα (Abcam, catalog number ab2779) and rabbit polyclonal antibodies against LXRβ (Abcam, catalog number ab56237) were used. Purified DNA of the immunoprecipitates and of input DNA was analyzed by real-time PCR using fluorescence detection of dual-labeled (TaqMan) probes, which are complementary to the PCR amplicon as described earlier. The primer and probe set (left primer, 5′-GCTTGCTGTT-TGCCCACT-3′; right primer, 5′-GGTCCTGGCAATGTG-GAA-3′; dual-labeled probe, 5′-FAM-CCCAGGGACA-GAGCTGATCCTTG-BHQ1-3′) amplifies an 82 bp region within the human apoA-I gene hepatic enhancer (positions −175 to −94 vs the canonical TSP). Results were normalized, and the relative level of PPARα or LXRβ bound to apoA-I hepatic enhancer has been calculated as a part of the PPARα or LXRβ binding in the control probe. The human IgG fraction was used instead of PPARα or LXRβ antibodies as a negative control.

ELISA. Human apoA-I in cultural medium was detected by the sandwich enzyme-linked immunosorbent assay (ELISA) with rabbit polyclonal antibodies to human apoA-I and secondary goat antibodies to rabbit IgG conjugated with horseradish peroxidase. Diaminobenzidine was used as a chromogenic substrate. Polyclonal antibodies to human apo A-I were obtained as described previously (*33*).

Statistical Analysis. Results are presented as the mean ± standard error of the mean. The statistical analyses of differences between compared groups were performed using a nonpaired *t*-test or Dannet's criterion for multiple comparisons. Differences were considered statistically significant at the $p < 0.05$ level. All statistical analyses were performed using Statistica version 5.0 (StatSoft).

RESULTS

NFκB and MAP Kinases Are Involved in the TNFα-Mediated Inhibition of apoA-I Gene Expression and apoA-I Protein Secretion in HepG2 Cells. TNFα was found to inhibit the expression of the apoA-I gene in HepG2 cells (*17*, *19*). To investigate the mechanism of the TNFα-mediated inhibition of endogenous apoA-I gene expression in HepG2 cells, we have used chemical inhibitors for NFκB transcription factor and JNK, p38, and MEK1/2 protein kinases. Real-time PCR analysis demonstrates that TNFα decreases the level of apoA-I gene expression by 30 ± 3.7% against the control level in HepG2 cells 24 h after administration (Figure 1a). Inhibition of NFκB, JNK, and p38 leads to abolishment of the TNFα-mediated inhibition of apoA-I gene expression in HepG2 cells. Inhibition of MEK1/2 increases the level of apoA-I gene expression but does not affect the inhibition of apoA-I gene expression under the impact of TNFα (Figure 1a).

We have investigated the roles of NFκB and MAP kinases in the process of the TNFα-mediated inhibition of apoA-I protein secretion in HepG2 cells. TNFα decreases the level of apoA-I secretion by HepG2 cells by 36.6 ± 2.7% 24 h after administration. Treatment of HepG2 cells with MEK1/2 inhibitor provokes an increase in the rate of apoA-I secretion, whereas inhibition of JNK, p38, and NFκB decreases the rate of apoA-I secretion by HepG2 cells. The TNFα-mediated inhibition of apoA-I secretion by HepG2 cells is blocked by addition of JNK or p38 inhibitors, but not MEK1/2 or NFκB inhibitors (Figure 1b).

TNFα-Dependent Inhibition of Human ApoA-I Gene Expression Is Mediated by the apoA-I Gene Hepatic Enhancer. As described previously using the 5′-regulatory region of the rat apoA-I gene, TNFα inhibits transcription of the gene by acting through the hepatic enhancer of the rat apoA-I gene in HepG2 cells (*19*, *20*). To determine the region of the human apoA-I gene promoter that is responsible for the TNFα-mediated decrease in the level of the gene expression, we have used plasmids containing the firefly luciferase reporter gene under the control of several deletions of the 5′-regulatory region of the human apoA-I gene (Figure 2a). TNFα inhibits the activities of all plasmids including plasmid pAPOA-I(−256/+72)-Luc containing the fragment of the 5′-regulatory region

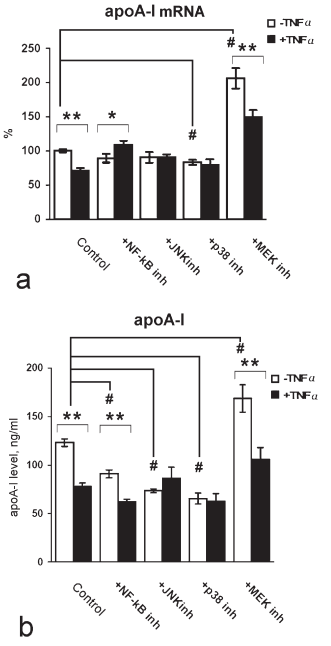

FIGURE 1: Regulation of endogenous apoA-I expression and secretion under the impact of TNFα in HepG2 cells. Role of MAP kinase cascades and transcription factor NFκB. (a) Real-time PCR. The *Y*-axis values correspond to the relative level of gene expression (100% in control HepG2 cells). (b) Analysis of apoA-I protein in cultivation medium after treatment of HepG2 cells with TNFα (ELISA): control, HepG2 cells without inhibitors; p38 inh, SB203580 (p38 inhibitor) (25 μM); JNK inh, SP600125 (JNK1/2/3 inhibitor) (10 μM); MEK inh, U0126 (MEK1/2 inhibitor) (10 μM); NFκB inh, QNZ (NFκB inhibitor) (10 nM). The *Y*-axis values correspond to the apoA-I protein level (nanograms per milliliter) in cultivation medium of HepG2 cells. HepG2 cells were administered by TNFα (50 ng/mL) for 24 h. The inhibitors had been added 1 h before TNFα. White columns represent data for untreated cells, and black columns represent data for cells treated with TNFα. Values are presented as means ± the standard error of the mean of four independent experiments. The statistical analyses of differences between compared groups (with or without TNFα) were performed using a nonpaired Student's *t*-test (*$p < 0.05$; **$p < 0.01$) and Dannet's criterion (#$p < 0.05$).

at base pairs −256 to +72 of the human apoA-I gene related to TSP which contains only minimal promoter and hepatic enhancer of apoA-I gene and firefly luciferase reporter gene (Figure 2a).

The results obtained in the experiments with the endogenous apoA-I gene in HepG2 cells have been confirmed in the transfection experiments using the pAPOA-I(−2497/+173)-Luc plasmid containing the fragment of 5′-regulatory region at base pairs −2497 to +173 of the human apoA-I gene related to TSP and the firefly luciferase reporter gene. The TNFα-mediated inhibition of the activity of the plasmid is abolished with a treatment of transfected HepG2 cells by NFκB or JNK inhibitors, but not by p38 or MEK1/2 inhibitors (Figure 2b). As opposed to the endogenous apoA-I gene, inhibition of p38 does not lead to the blocking of the TNFα-mediated inhibition of the activity of a plasmid containing the fragment of the 5′-regulatory region of the human apoA-I gene. Therefore, p38-mediated inhibition of apoA-I gene expression under the impact of TNFα appears to involve sites for transcription factors outside the 5′-regulatory region (positions −2487 to +173) of the human apoA-I gene.

Nuclear Receptors HNF4α, PPARα, and LXRs Regulate the Expression and Secretion of apoA-I in HepG2 Cells under the Impact of TNFα. Nuclear receptors HNF4α (*23*, *24*), PPARα (*25*), and LXRs (*28*) were found to play an important role in the process of regulation of apoA-I gene expression by interacting with HREs within the apoA-I gene hepatic enhancer. To determine the role of HNF4α in the TNFα-mediated inhibition of human apoA-I gene expression, HepG2 cells were cotransfected with plasmid pAPOA-I(−2497/+173)-Luc and expression vectors of human transcription factor HNF4α or human HNF4α dominant-negative mutant (Figure 3a). TNFα represses the activity of the plasmid containing the 5′-regulatory region of the human apoA-I gene 4.9 ± 1.4-fold over the control level in HepG2 cells. The level of TNFα-mediated inhibition of the activity of the 5′-regulatory region of the apoA-I gene has not been reduced in the case of HNF4α overexpression (6.2 ± 0.4-fold over the control level) and has been increased under the HNF4α dominant-negative mutant overexpression (9.7 ± 0.3-fold over the control level) (Figure 3a). Inhibition of NFκB or JNK leads to the statistically significant decrease in the level of TNFα-mediated repression of plasmid pAPOA-I(−2497/+173)-Luc activity under HNF4α overexpression [levels of repression are 1.7 ± 0.3-fold (NFκB inhibition) and 1.4 ± 0.4-fold (JNK inhibition) over the control level in HepG2 cells] (Figure 3b). Alternatively, inhibition of p38 increases the level of TNFα-mediated repression of plasmid pAPOA-I(−2497/+173)-Luc activity under HNF4α overexpression (level of repression is 9.5 ± 1.2-fold over the control level in HepG2 cells) (Figure 3b).

To determine the role of PPARα and LXR in the TNFα-mediated inhibition of human apoA-I gene expression, we used the synthetic ligand approach. According to previously published data, synthetic PPARα agonist WY-14643 increases the level of endogenous apoA-I gene expression and protein secretion in primary monkey hepatocytes (*34*) and HepG2 cells (*35*), whereas synthetic PPARα antagonist MK886 (*35*) or LXR agonist TO901317 (*28*) decreases the level of apoA-I gene expression in HepG2 cells. Treatment of HepG2 cells with MK886 or TO901317 abolishes the TNFα-mediated effect on the apoA-I gene expression in HepG2 cells (Figure 4a).

The previous investigations have shown that the synthetic PPARα ligands such as WY-14643 and nafenopin can regulate expression of several genes in a PPARα-independent manner by activating Src, p38, and MEK1/2-Erk1/2 signaling pathways in rat hepatocytes and HepG2 cells (*36*, *37*). Since p38 and MEK1/2-Erk1/2 signaling pathways are involved in regulation of human apoA-I gene expression, we have verified whether regulation of apoA-I gene expression in the presence of WY-14643 is a PPARα-dependent or PPARα-independent process. Thereto, HepG2 cells were treated with WY-14643 and p38, MEK1/2, and Src inhibitors (Figure 4b). Treatment of the HepG2 cells with Src, p38, or MEK1/2 inhibitors in combination with WY-14643 does not abolish the activation of endogenous apoA-I gene transcription in HepG2 cells under the impact of WY-14643 alone (Figure 4a,b). These results suggest that PPARα agonist WY-14643 regulates apoA-I gene expression in a PPARα-dependent manner. Simultaneous treatment of HepG2 cells with WY-14643 and p38 inhibitors increases the level of apoA-I gene transcription almost 3-fold over the control level and abolishes TNFα-mediated inhibition of human apoA-I gene expression in HepG2 cells (Figure 4b). Interestingly, simultaneous treatment of the HepG2 cells with WY-14643 and MEK1/2 inhibitor abolishes the TNFα-mediated inhibition of human apoA-I gene expression (Figure 4b), whereas neither WY-14643 nor MEK1/2 inhibition blocks the TNFα-mediated decrease in the level of human apoA-I gene expression by itself (Figures 1a and 4a). Simultaneous

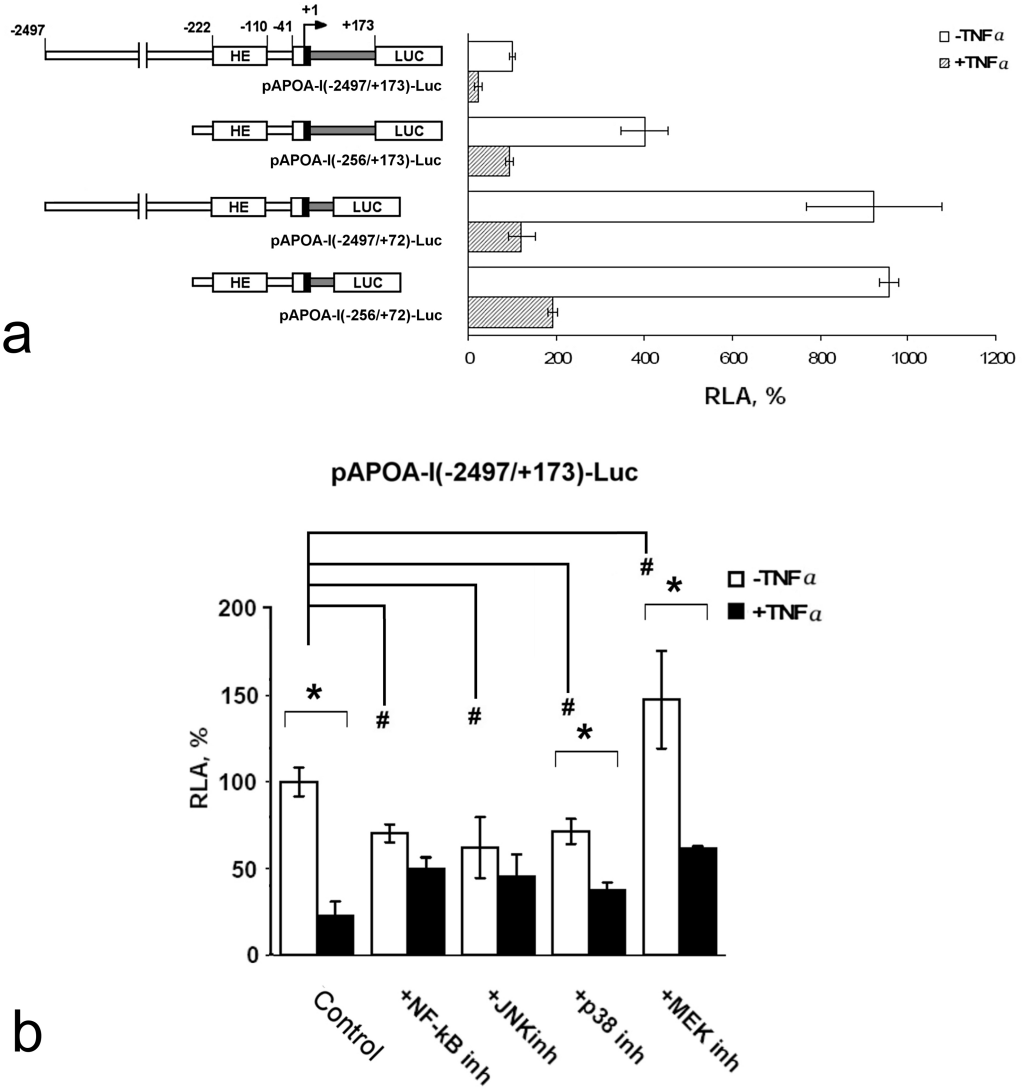

FIGURE 2: Effect of TNFα on the 5′-regulatory region of the human apoA-I gene (luciferase assay). (a) Activity of plasmids containing deletion variants of the 5′-regulatory region of the human apoA-I gene in HepG2 cells: LUC, firefly luciferase gene; HE, human apoA-I gene hepatic enhancer; black rectangle, human apoA-I gene I exon; arrow, human apoA-I gene TSP. Numbers indicate coordinates related to human apoA-I gene TSP (+1). (b) Role of MAP kinase cascades and transcription factor NFκB in the TNFα-mediated downregulation of pAPOA-I(−2498/+173)-Luc in HepG2 cells: control, HepG2 cells without inhibitors; NFκB inh, QNZ (NFκB inhibitor) (10 nM); JNK inh, SP600125 (JNK1/2/3 inhibitor) (10 μM); p38 inh, SB203580 (p38 inhibitor) (25 μM); MEK inh, U0126 (MEK1/2 inhibitor) (10 μM). Cells were transfected by plasmids (7 μg per 30 mm dish). Plasmid pCMVL (2 μg) was added to all probes as an internal control (see Materials and Methods). One day after transfection, HepG2 cells were treated with TNFα (50 ng/mL) for 24 h. The inhibitors had been added 1 h before TNFα. White columns represent data for untreated cells, and black columns represent data for the cells treated with TNFα. RLA is the relative luciferase activity. Values are presented as means ± the standard error of the mean of five independent experiments. The statistical analyses of differences between compared groups (with or without TNFα) were performed using a nonpaired Student's *t*-test (*$p < 0.05$) and Dannet's criterion (#$p < 0.05$).

treatment of the HepG2 cells with p38 and MEK1/2 inhibitors leads to the superadditive activation of apoA-I gene expression as compared with the sum of the individual inhibitor effects but does not block the TNFα-mediated inhibition of human apoA-I gene expression (Figure 4b; see also Figure 1a). Treatment of HepG2 cells with PP2, the Src inhibitor, and simultaneous treatment of HepG2 cells with PP2 and WY-14643 increase the level of apoA-I gene expression but do not abolish the TNFα-mediated inhibition of human apoA-I gene expression (Figure 4b).

Synthetic PPARα ligands (WY-14643 and MK886) or the LXR ligand (TO901317) decreases the rate of secretion of apoA-I protein and abolishes the TNFα-mediated inhibition of apoA-I secretion by HepG2 cells (Figure 4c).

The transfection experiments suggest that WY-14643 increases but MK886 or TO901317 decreases the activities of plasmids pAPOA-I(−2497/+173)-Luc and pAPOA-I(−256/+72)-Luc in HepG2 cells (Figure 4d,e). Treatment of HepG2 cells with MK886 or TO901317 abolishes the TNFα-mediated repression of plasmid pAPOA-I(−2497/+173)-Luc and pAPOA-I(−256/+72)-Luc activity in HepG2 cells (Figure 4e).

TNFα Decreases the Level of Expression of HNF4α, PPARα, LXRα, and LXRβ Genes in HepG2 Cells. The activity of apoA-I gene transcription was found to correlate with the level of HNF4α gene expression (*38*). We have studied the influence of TNFα on HNF4α gene expression in HepG2 cells. Real-time PCR analysis demonstratets that TNFα decreases the level of HNF4α gene expression by 29 ± 3.4% against the control level in HepG2 cells 24 h after administration. Inhibition of NFκB or JNK but not p38 or MEK1/2 abolishes the TNFα-mediated repression of HNF4α gene transcription (Figure 5a).

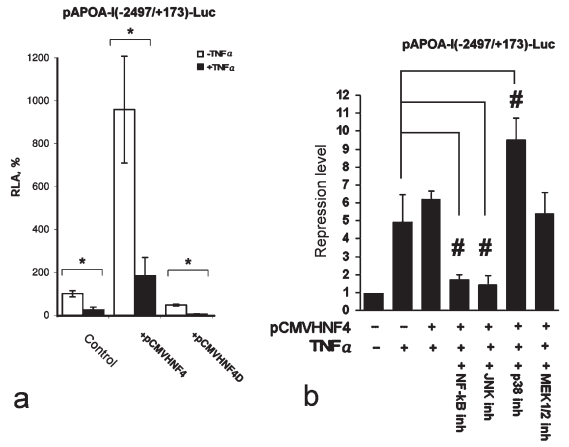

FIGURE 3: Role of HNF4α in the TNFα-mediated inhibition of human apoA-I gene expression (luciferase assay). (a) Effect of overexpression of HNF4α or HNF4α dominant-negative mutant on TNFα-mediated regulation of human apoA-I. HepG2 cells were cotransfected with plasmids pAPOA-I(−2498/+173)-Luc (7 μg per 30 mm dish) and pCMVHNF4 (0.5 μg), the expression vector of human transcription factor HNF4α, or pCMVHNF4D (0.5 μg), the expression vector of human HNF4α dominant-negative mutant. pCMVL (2 μg) was added to all probes as an internal control (see Materials and Methods). White columns represent data for untreated cells, and black columns represent data for the cells treated with TNFα. RLA is the relative luciferase activity. (b) Repression levels of pAPOA-I(−2498/+173)-Luc activity under the impact of TNFα in the case of pCMVHNF4 cotransfection (the repression level indicates how many times the plasmid activity decreases compared with the control level): NFκB inh, QNZ (NFκB inhibitor) (10 nM); JNK inh, SP600125 (JNK1/2/3 inhibitor) (10 μM); p38 inh, SB203580 (p38 inhibitor) (25 μM); MEK inh, U0126 (MEK1/2 inhibitor) (10 μM). HepG2 cells were cotransfected with pAPOA-I(−2498/+173)-Luc (7 μg per 30 mm dish) and pCMVHNF4 (0.5 μg). pCMVL (2 μg) was added to all probes as an internal control (see Materials and Methods). One day after transfection, HepG2 cells were incubated with TNFα (50 ng/mL) for 24 h. The inhibitors had been added 1 h before TNFα. Values are presented as means ± the standard error of the mean of five independent experiments. The statistical analyses of differences between compared groups were performed using a nonpaired Student's *t*-test (*$p < 0.05$; **$p < 0.01$) and Dannet's criterion (#$p < 0.05$).

TNFα also decreases the level of PPARα gene expression by 18 ± 0.8%, that of LXRα gene expression by 40 ± 1.8%, and that of LXRβ gene expression by 17 ± 4.7% against the control level in HepG2 cells 24 h after administration (Figure 5b−d). To identify the signaling pathways involved in the regulation of the gene expression, we have used the chemical inhibitors for MAP kinases and NFκB. The TNFα-dependent decrease in the level of PPARα gene expression was found to be mediated by NFκB and JNK signaling pathways. The TNFα-mediated decrease in the level of LXRβ gene expression is associated with NFκB, whereas the inhibition of LXRα gene expression is mediated by NFκB, JNK, and p38 (Figure 5b−d). The level of PPARα gene expression increases 2.3 ± 0.1-fold over the control level in HepG2 cells after inhibition of MEK1/2 (Figure 5b). Interestingly, simultaneous treatment of HepG2 cells with TNFα and MEK1/2 inhibitor leads to a 3.3 ± 0.3-fold increase in the level of LXRβ gene expression over the control level, whereas treatment of HepG2 cells with MEK1/2 inhibitor alone stimulates expression of the gene only 1.6 ± 0.1-fold over the control level (Figure 5c).

TNFα Decreases the Level of PPARα and Increases the Level of LXRβ Binding with the Human apoA-I Gene Hepatic Enhancer. To study the binding of PPARα and LXRβ nuclear receptors to the hepatic enhancer of the human apoA-I gene, we used the ChIP assay. It was shown that treatment of HepG2 cells with TNFα leads to redistribution of nuclear receptors occupying the apoA-I hepatic enhancer 24 h after TNFα administration. In particular, treatment of the HepG2 cells with TNFα leads to a decrease in the level of PPARα binding with the apoA-I gene hepatic enhancer 2-fold over the control level (Figure 6a). In contrast, the level of LXRβ binding with the apoA-I gene hepatic enhancer is increased 3-fold over the control level in HepG2 cells after TNFα administration (Figure 6b).

DISCUSSION

Currently, atherosclerosis is thought to be not only a disorder of lipid transport but also a chronic inflammatory process (*39*). Pro-inflammatory cytokine TNFα is one of the key factors taking part in the inflammatory processes during development of atherogenic lesion of blood vessels (*40*). In addition, TNFα decreases the level of expression and secretion of apoA-I in human hepatocytes (*17*, *19*) and also leads to decreases in both the content of apoA-I protein in HDL and the level of HDL in serum (*15*, *16*). It was shown earlier by transfection experiments that TNFα inhibits transcription of the rat apoA-I gene in HepG2 cells by acting through the hepatic enhancer of the gene (*19*, *20*), but little is known about the molecular mechanisms and the specific transcription factors involved in this process. In this work, we have confirmed that TNFα leads to a significant decrease in the level of endogenous apoA-I gene expression and apoA-I protein secretion in HepG2 cells (Figure 1a,b) and that the inhibitory effect of TNFα is mediated by the hepatic enhancer of the human apoA-I gene (Figure 2a).

We have shown that NFκB, JNK, and p38 are involved in the TNFα-mediated decrease in the level of endogenous apoA-I gene expression in HepG2 cells, while MEK1/2 appears not to be involved in this process (Figure 1a). Interestingly, p38 is involved in the TNFα-mediated inhibition of endogenous apoA-I gene expression, but not in the TNFα-mediated repression of the plasmid pAPOA-I(−2497/+173)-Luc activity in HepG2 cells (Figure 2b). These results suggest the p38 signaling pathway takes part in the TNFα-dependent inhibition of apoA-I gene expression through sites or regions that are not included within the 5′-regulatory region of the gene. Differences in the influence of NFκB and JNK signaling pathways on endogenous apoA-I gene expression and the 5′-regulatory region of the human apoA-I gene (Figures 1a and 2b) may be explained by the involvement of sites that are located outside of the human apoA-I gene 5′-regulatory region. One of the regions important for control of apoA-I gene expression in hepatocytes is a common cluster enhancer for apoA-I, apoA-IV, and apoCIII genes located within the 5′-regulatory region of the apoCIII gene (positions −792 to −592 relative to apoCIII gene TSP) (*41*). Our data suggest the p38-dependent signaling pathway may be essential for the TNFα-mediated inhibition of apoA-I gene expression, and it probably acts through the cluster enhancer for apoA-I, apoC-III, and apoA-IV genes.

There are previously published data obtained from transfection experiments on HepG2 cells demonstrating the role of MEK/ERK and NFκB signaling pathways in the TNFα-mediated inhibition of rat apoA-I gene expression (*20*). It was shown that inhibition of the NFκB pathway leads to a decrease and inhibition of p38 and JNK pathways to an increase in the

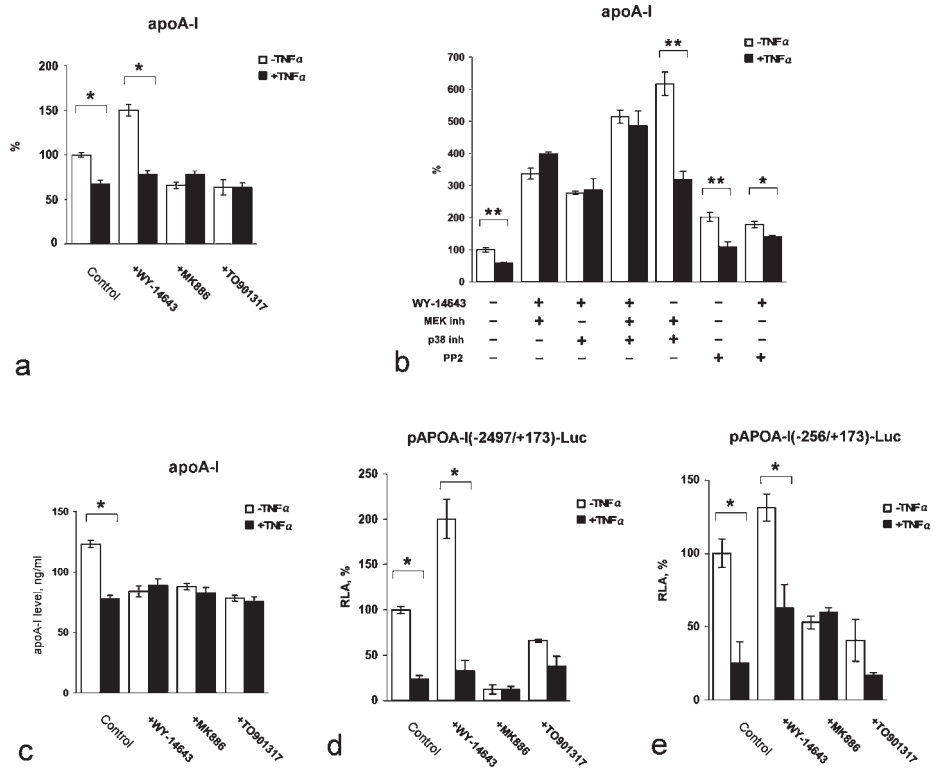

FIGURE 4: Role of PPARα and LXRs in the TNFα-mediated inhibition of human apoA-I gene expression in HepG2 cells. (a) Endogenous apoA-I gene expression under the impact of TNFα and PPARα or LXR synthetic ligands in HepG2 cells, via real-time PCR: control, HepG2 cells without nuclear receptor ligands; WY-14643, an agonist of PPARα (10 μM); MK886, an antagonist of PPARα (10 μM); TO901317, an agonist of LXRs (5 μM). HepG2 cells were treated with TNFα (50 ng/mL) for 24 h. The nuclear receptor ligands had been added 1 h before TNFα; the *Y*-axis values correspond to the relative level of gene expression (100% in control HepG2 cells). (b) Endogenous apoA-I gene expression under the impact of TNFα, WY-14643, and kinase inhibitors in HepG2 cells, via real-time PCR: WY-14643, an agonist of PPARα (10 μM); MEK inh, U0126 (MEK1/2 inhibitor) (10 μM); p38 inh, SB203580 (p38 inhibitor) (25 μM); PP2, PP2 (Src-kinase inhibitor) (10 μM). HepG2 cells were treated with TNFα (50 ng/mL) for 24 h. The WY-14643 and kinase inhibitors had been added 1 h before TNFα; the *Y*-axis values correspond to the relative level of gene expression (100% in control HepG2 cells). (c) apoA-I protein secretion under the impact of TNFα and PPARα or LXR synthetic ligands in HepG2 cells, via ELISA: control, HepG2 cells without nuclear receptor ligands; WY-14643, an agonist of PPARα (10 μM); MK886, an antagonist of PPARα (10 μM); TO901317, an agonist of LXRs (5 μM). HepG2 cells were treated with TNFα (50 ng/mL) for 24 h. The nuclear receptor ligands had been added 1 h before TNFα; the *Y*-axis values correspond to apoA-I protein levels (nanograms per milliliter) in cultivation medium of HepG2 cells. (d and e) Effect of TNFα and PPARα or LXR synthetic ligands on the 5′-regulatory region of the human apoA-I gene (luciferase assay): control, HepG2 cells without nuclear receptor ligands; WY-14643, an agonist of PPARα (10 μM); MK886, an antagonist of PPARα (10 μM); TO901317, an agonist of LXRs (5 μM). HepG2 cells were transfected with pAPOA-I(−2498/+173)-Luc (d) (7 μg per 30 mm dish) or pAPOA-I(−256/+173)-Luc (e) (7 μg per 30 mm dish). pCMVL (2 μg) was added to all probes as an internal control (see Materials and Methods). One day after transfection, HepG2 cells were treated with TNFα (50 ng/mL) for 24 h. The nuclear receptor ligands had been added 1 h before TNFα. RLA is the relative luciferase activity. White columns represent data for untreated cells, and black columns represent data for the cells treated with TNFα. Values are presented as means ± the standard error of the mean of five independent experiments. The statistical analyses of differences between compared groups (with or without TNFα) were performed using a nonpaired Student's *t*-test (*$p < 0.05$).

activity of the rat apoA-I gene promoter in HepG2 cells (*20*), whereas according to our results, NFκB and JNK do not influence and p38 decreases the level of human endogenous apoA-I gene expression in HepG2 cells (Figure 1a). On the other hand, the MEK-ERK pathway is involved in increasing the level of expression of both rat (*20*) and human apoA-I genes in HepG2 cells (Figures 1a and 2b). The difference between the above-mentioned signaling pathways which take part in the TNFα-mediated inhibition of apoA-I gene expression and the impact of these pathways on the level of apoA-I gene expression in the human and rat can be explained by species-specific regulation of the gene expression mediated by different transcription factors. One of these transcription factors is PPARα which is predominantly bound with site A (positions −214 to −192) within the human apoA-I gene hepatic enhancer (*35*). Site A of the apoA-I hepatic enhancer responsible for PPARα binding in humans is nonfunctional in rodents because of three nucleotide mismatches (*42*). Fibrates, the synthetic PPARα agonists, have different effects on the regulation of murine and human apoA-I gene expression, activating human apoA-I gene expression and inhibiting mouse apoA-I gene expression, which was shown by using human apoA-I gene transgenic mice (*43*). We have demonstrated that the TNFα-mediated inhibition of human apoA-I gene expression involves PPARα, in particular via a decrease in the level of nuclear receptor binding to the human apoA-I hepatic enhancer (Figure 6a). It has been shown earlier that NFκB inhibits the capacity of PPARα to bind to the human apoA-I hepatic enhancer under the impact of bacterial lipopolysaccharide (LPS) which results in a decrease in the level of apoA-I gene transcription (*35*). Our data suggest the TNFα-mediated inhibition of apoA-I gene expression may be realized through the involvement of different signaling pathways and transcription factors in the rat and the human.

Besides inhibition of apoA-I gene expression, TNFα decreases the rate of apoA-I protein secretion in HepG2 cells, and this effect is abolished by inhibition of p38 or JNK MAP-kinase pathways. Interestingly, inhibition of NFκB abolishes the TNFα-mediated decrease in the level of apoA-I gene expression, but not apoA-I

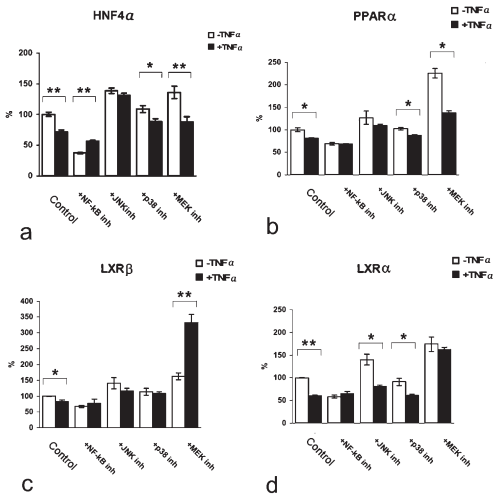

FIGURE 5: Effect of TNFα, NFκB, and MAP kinase inhibitors on HNF4α, PPARα, LXRα, and LXRβ gene expression in HepG2 cells. Expression of HNF4α (a), PPARα (b), LXRβ (c), and LXRα (d) genes in HepG2 cells, via real-time PCR: control, HepG2 cells without inhibitors; NFκB inh, QNZ (NFκB inhibitor) (10 nM); JNK inh, SP600125 (JNK1/2/3 inhibitor) (10 μM); p38 inh, SB203580 (p38 inhibitor) (25 μM); MEK inh, U0126 (MEK1/2 inhibitor) (10 μM). HepG2 cells were incubated with TNFα (50 ng/mL) for 24 h. The inhibitors had been added 1 h before TNFα. The *Y*-axis values correspond to the relative levels of gene expression (100% in control HepG2 cells). White columns represent data for untreated cells, and black columns represent data for the cells treated with TNFα. Values are presented as means ± the standard error of the mean of four independent experiments. The statistical analyses of differences between compared groups (with or without TNFα) were performed using a nonpaired Student's *t*-test (*$p < 0.05$; **$p < 0.01$).

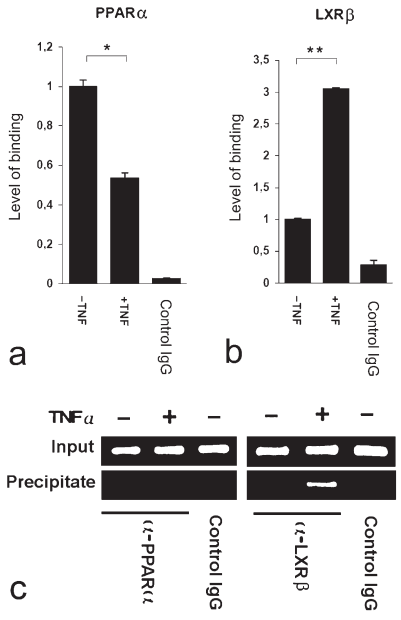

FIGURE 6: Effect of TNFα on PPARα and LXRβ binding with the human apoA-I gene hepatic enhancer in HepG2 cells. Levels of PPARα (a) and LXRβ (b) binding with the human apoA-I gene hepatic enhancer, via real-time PCR calculation of ChIP: level of binding, relative abundance of PPARα or LXRβ bound with the hepatic enhancer of apoA-I (given 1 in the control probe); −TNF, untreated HepG2 cells; +TNF, HepG2 cells treated with TNFα (50 ng/mL) for 24 h; control IgG, chromatin immunoprecipitation with an unspecific human serum IgG fraction (negative control). (c) Agarose gel electrophoresis analysis of real-time PCR products (late logarithmic stage, 32 cycles): α-PPARα and α-LXRβ, antibodies against PPARα and LXRβ, respectively; the size of the PCR band is 82 bp. Chromatin immunoprecipitation was performed using chromatin of HepG2 cells and antibodies against human PPARα and LXRβ as described in Materials and Methods. Values are presented as means ± the standard error of the mean of four independent experiments. The statistical analyses of differences between compared groups (with or without TNFα) were performed using a nonpaired Student's *t*-test (*$p < 0.05$; **$p < 0.01$).

protein secretion in HepG2 cells (Figure 1b). Our results demonstrate that inhibition of NFkB or JNK has no effect on endogenous apoA-I gene expression but decreases the rate of apoA-I protein secretion (Figure 1a,b). All of the used PPARα and LXRs synthetic ligands decrease the rate of apoA-I protein secretion and abolish the TNFα-mediated inhibition of apoA-I protein secretion by HepG2 cells (Figure 4c). Since PPARα, NFkB, and JNK take part in the regulation of various biological processes in hepatocytes, the effects of those signaling pathways involved in the regulation of apoA-I transcription and secretion can be studied by the influence on differential intracellular pathways of apoA-I secretion and/or catabolism regulation. Further investigations will allow us to test this suggestion.

HNF4α plays an important role in the apoA-I gene transcription regulation in hepatocytes (*23*). Inhibition of MAP-kinase pathways was found to increase both HNF4α mRNA and protein levels in HepG2 cells (*44*). The transcription activity of the human apoA-I gene was found to correlate with the level of HNF4α gene expression in HepG2 cells (*38*). Our results suggest that TNFα inhibits human HNF4α gene expression through NFκB and JNK signaling pathways in HepG2 cells (Figure 5a). Since HNF4α binds with HREs as a homodimer, the decrease in the level of HNF4α protein in the cell under the impact of TNFα may dramatically repress apoA-I gene expression. Although overexpression of HNF4α increases the activity of the 5′-region of the human apoA-I gene, it does not affect the TNFα-mediated inhibition of apoA-I gene expression. In addition, blocking of NFκB or JNK leads to a significant decrease, while blocking of p38 leads to an increase in the level of TNFα-mediated inhibition of apoA-I gene expression upon HNF4α overexpression (Figure 3b). Interestingly, JNK-dependent induction of transcription factor c-Jun under the impact of pro-inflammatory cytokine IL-1β in human hepatocytes was found to interfere with HNF4α and PPAR-γ coactivator-1α (PGC-1α) interaction, which leads to inhibition of cholesterol 7α-hydroxylase (CYP7A1) gene expression (*45*). Increasing apoA-I gene transcription activity was also thought to require the cooperation between HNF4α and PGC-1α for achieving the coactivation effect of the transcription factors (*38*). JNK-dependent downregulation of apoA-I gene expression under the impact of TNFα appears to be realized through inhibition of the interaction between HNF4α and PGC-1α. A second possibility is that NFκB can inhibit HNF4α binding with cis-acting elements within promoters of target genes (*46*). Thereby, the TNFα-mediated inhibition of human apoA-I gene expression can involve both NFκB and JNK pathways through via a decrease in the level of HNF4α gene expression and through downregulation of HNF4α binding with HREs within the apoA-I gene hepatic enhancer and/or through reducing of HNF4α coactivation properties (Figure 7a). Nevertheless, our results suggest that decreasing the levels of HNF4α gene expression is not the only trigger of human apoA-I gene expression inhibition under the impact of TNFα in HepG2 cells.

We have demonstrated that the ligand-dependent regulation of apoA-I gene expression by PPARα and LXRs can interfere with

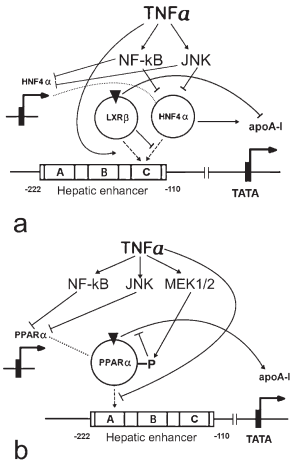

FIGURE 7: Hypothetical scheme illustrating a possible mechanism of the TNFα-mediated inhibition of human apoA-I gene expression in HepG2 cells. Role of HNF4α and LXRs (a) and PPARα (b) in TNFα-mediated inhibition of human apoA-I gene expression in HepG2 cells. Dotted arrow indicates nuclear receptor binding with HREs (the A and C sites) within the human apoA-I gene hepatic enhancer. The black triangle indicates a ligand, and the triangle-derived arrow indicates ligand-dependent activation/repression of apoA-I gene transcription. The gray circle enclosing a P indicates PPARα phosphorylation. For an explanation, see Discussion.

the TNFα-mediated inhibition of apoA-I gene expression in HepG2 cells (Figure 4a,d,e). Those results suggest PPARα and LXRs are involved in the TNFα-mediated regulation of human apoA-I gene expression. Although WY-14643 and nafenopin can act in a PPARα-independent manner (*36*), our data suggest the regulation of apoA-I gene expression by WY-14643 depends on PPARα (Figure 4b). MEK-ERK-dependent phosphorylation of PPARα seems to be involved in the regulation of ligand-dependent coactivation activity of the nuclear receptor (*47*), though an effect of PPARα phosphorylation on the activity of target genes may be both positive (*48*) and negative (*49*). Our results suggest MEK1/2-ERK1/2 activation under the impact of TNFα leads to the PPARα phosphorylation and thereby to a weakening of the PPARα-mediated activation of apoA-I gene expression. Indeed, blocking of the PPARα phosphorylation by addition of MEK1/2 inhibitor seems to restore the PPARα-mediated ligand-dependent regulation of apoA-I gene expression (Figure 7b).

TNFα decreases the level of PPARα gene expression in hepatocytes (*50*), but little is known about signaling pathways involved in the process. Our results suggest the TNFα-dependent inhibition of PPARα gene expression is mediated by NFκB and JNK in HepG2 cells (Figure 5b). Since PPARα increases the level of human apoA-I gene expression in hepatocytes, decreases in the level of PPARα under the impact of TNFα might be involved in the inhibition of apoA-I gene transcription. However, the observed TNFα-mediated decrease in PPARα mRNA levels in HepG2 cells could not be the only cause of apoA-I gene expression inhibition. On the other hand, regulation of gene expression by nuclear receptors appears to be determined not only by the amount of those transcription factors in cells but also by the level of their binding with specific sites within target gene promoters (*51*). We have demonstrated that the TNFα-dependent inhibition of human apoA-I gene expression is mediated by a decrease in the level of positive (PPARα) and an increase in the level of negative (LXRβ) regulators of apoA-I gene transcription binding with the apoA-I gene hepatic enhancer in HepG2 cells. During nuclear translocation of NFκB (p50-p65), the p65 subunit was found to bind PPARα and to inhibit the ability of PPARα to interact with HREs within target gene promoters, blocking PPARα-mediated activation of gene expression (*52*). NFκB plays an important role in the mechanism of the LPS-mediated decrease in the level of apoA-I gene expression through inhibition of the PPARα-mediated activation of apoA-I gene expression (*35*). TNFα appears to inhibit PPARα binding with HREs within the human apoA-I gene hepatic enhancer in a NFκB-dependent manner, and in addition to the TNFα-mediated inhibition of PPARα gene expression, this process is also important for negative regulation of human apoA-I gene expression (Figure 7b). Increasing the level of LXRβ binding with HREs within the human apoA-I gene hepatic enhancer mediates intensification of ligand-dependent repression of apoA-I gene expression by LXRβ under the impact of TNFα. In addition, since LXRs can displace HNF4α from site C (positions −134 to −119) of the human apoA-I gene hepatic enhancer (*28*), a decrease in the level of HNF4α binding with the HREs appears to be involved in inhibition of apoA-I gene expression (Figure 7a).

In conclusion, we have demonstrated the roles of JNK, p38, MEK1/2, and NFκB signaling pathways in the TNFα-mediated inhibition of apoA-I gene expression in HepG2 cells. TNFα-dependent MEK1/2 activation appears to affect the ligand-dependent regulation of the human apoA-I gene by PPARα, probably through PPARα phosphorylation. We have also shown that TNFα decreases the level of PPARα binding with the human apoA-I gene hepatic enhancer while the level of LXRβ binding with this region is increased. These results suggest the substantial complexity of the mechanism of the TNFα-mediated regulation of apoA-I gene expression and apoA-I protein secretion in hepatocytes, with the involvement of nuclear receptors HNF4α, PPARα, and LXRs.

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