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Insulin Induces Human Acyl-Coenzyme A: Cholesterol Acyltransferase1 Gene Expression Via MAP Kinases and CCAAT/Enhancer-Binding Protein α

Jing Ge,[1] Wei Zhai,[2] Bei Cheng,[1*] Ping He,[1] Benling Qi,[1] Han Lu,[1] Yongli Zeng,[1] and Xin Chen[1]

[1]*Department of Gerontology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430022, China*

[2]*Department of Thoracic Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430022, China*

ABSTRACT

Insulin resistance characterized by hyperinsulinemia is associated with increased risk of atherosclerosis. Acyl-coenzyme A: cholesterol acyltransferase (ACAT) is an intracellular enzyme involved in cellular cholesterol homeostasis and in atherosclerotic foam cell formation. To investigate the relationship between hyperinsulinemia and atherosclerosis, we investigated whether insulin induced *ACAT1* gene expression and found that insulin up-regulated *ACAT1* mRNA, protein and enzyme activity in human THP-1 cells and THP-1–derived macrophages. Moreover, luciferase assays revealed that insulin enhanced the *ACAT1* gene P1 promoter activity but not the P7 promoter. To explore the molecular mechanisms involved, deletion analysis of the human *ACAT1* P1 promoter revealed an insulin response element (IRE) upstream of the P1 promoter (from −603 to −580), EMSA experiments demonstrated that CCAAT/enhancer binding protein α(C/EBPα) bound to the P1 promoter IRE. Insulin-induced *ACAT1* upregulation was blocked by the presence of PD98059 (an inhibitor of extracellular signal-regulated kinase, ERK) and SB203580 (an inhibitor of p38 mitogen-activated protein kinase, p38MAPK) but not by Wortmannin (an inhibitor of phosphatidylinositol 3-kinase, PI3K) or U73122 (an inhibitor of phospholipase C-γ, PLCγ). These studies demonstrate that insulin promotes *ACAT1* gene expression at the transcriptional level. The molecular mechanism of insulin action is mediated via interaction of the functional IRE upstream of the *ACAT1* P1 promoter with C/EBPα and is MAPK-dependent. J. Cell. Biochem. 114: 2188–2198, 2013. © 2013 Wiley Periodicals, Inc.

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The atherosclerosis-related disorders coronary heart disease and stroke constitute the most common cause of mortality and have become a pre-eminent health problem worldwide. Diabetes mellitus is a strong risk factor in all manifestations of atherosclerotic vascular diseases. The molecular mechanism of atherosclerotic development in diabetic patients, however, has not been fully elucidated. Several prospective studies in nondiabetic and diabetic patients have shown an association between hyperinsulinemia resulting from insulin resistance and atherosclerotic disorders, such as cardiovascular disease [Pyörälä et al., 1987], which is one of the major causes of death in diabetics [American Diabetes Association, 1989]. Much evidence has indicated that high-insulin conditions cause atherosclerosis in patients with insulin resistance [Arcaro et al., 2002; Bokemark et al., 2002]. It remains unclear how atherosclerosis develops in patients with hyperinsulinemia.

A critical event in the early stages of atherosclerosis is the focal accumulation of lipid-laden foam cells, largely derived from macrophages, with subsequent fatty streak formation [Ross, 1999]. The bulk of the lipid in foam cells is in the form of cholesteryl esters (CEs), whose synthesis is controlled by acyl-coenzyme A: cholesterol acyltransferases (ACATs), a family of intracellular enzymes responsible for catalyzing the intracellular formation of CEs from cholesterol and long-chain fatty acyl-coenzyme A [Tabas, 1995; Chang et al., 1997]. Two ACAT genes have been identified in mammals

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*Correspondence to: Dr. Bei Cheng, Department of Gerontology, Union Hospital, Jie Fang Avenue 1277#, Wuhan, China, 430022. E-mail: chengbei81@hotmail.com

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(*ACAT1* and *ACAT2*), and *ACAT1* performs the majority of ACAT activity in macrophages [Lee et al., 1998; Joyce et al., 1999] for storing CEs as lipid droplets to protect cells from the toxicity of free intracellular cholesterol [Warner et al., 1995]. The expression and activity of ACAT are increased in human monocytes during differentiation and foam cell formation [Wang et al., 1996], and ACAT has been shown to play a crucial role in the accumulation of CE in foam cells [Tabas, 1995]. Lisa et al. [O'Rourke et al., 2002] found that insulin stimulates *ACAT1* activity in the murine macrophage cell line J774.2, which likely facilitates increased CE synthesis. The regulation mechanism of ACAT, however, in the presence of insulin still needs to be clarified.

Human ACAT1 gene is located in two different chromosomes (1and 7), with each chromosome containing a distinct promoter(P1 and P7) [Li et al., 1999]. Northern analyses have revealed the presence of four ACAT1 mRNAs (7.0, 4.3, 3.6, and 2.8-knt), present in almost all of the human tissues and cells examined. These messages share the same coding sequence. The 2.8- and 3.6-knt messages, comprising more than 70–80% of the total ACAT1 mRNAs, are produced from the P1 promoter [Li et al., 1999]. The 4.3-knt message contains the optional long 5′-UTR that is composed of exons Xa and Xb, and is produced by a novel RNA recombination event that take place between the two discontinuous RNAs transcribes by the P1 promoter and P7 promoter. In the present work we showed that insulin increased *ACAT1* mRNA, protein and enzyme activity in human THP-1 cells and THP-1–derived macrophages. To explore the regulatory mechanism of insulin action on the *ACAT1* gene in monocyte-macrophages, we used THP-1 cells to perform transient transfection experiments and found that insulin enhanced the activity of the human *ACAT1* P1 promoter. Further more, we found that insulin up-regulated *ACAT1* expression at the transcriptional level, which required MAPK-dependent signaling and the putative insulin-responsive element (IRE) in the human P1 promoter between nucleotides -603 and -580, which bound to C/EBP-α.

MATERIALS AND METHODS

REAGENTS

RPMI 1640, Dulbecco's Modified Eagle's Medium, and fetal calf serum (FBS) were obtained from Invitrogen. The Dual-Luciferase Reporter Assay System was from Promega (Madison, WI). Rabbit anti-ACAT-1 (H-125, catalog number sc-20951, 200 μg/ml) polyclonal antibody and anti-C/EBPα (catalog number sc-9314, 200 μg/ml) polyclonal antibody was from Santa Cruz Biotechnology. [1-^{14}C] Oleoyl-CoA was from NEN. Insulin, fatty acid–free bovine serum albumin, cholesterol, cholesteryl oleate, oleoyl-coenzyme A, and Triton WR-1339 were from Sigma. PD98059 and SB203580 was from Promega. Wortmannin was from Fluka Biochemika (Buchs, Switzerland). U73122 was from Cayman Chemical Company (Ann Arbor, MI).

CELL CULTURE

THP-1 cells were cultured in RPMI 1640 medium with 10% heat-inactivated FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin under a humidified atmosphere of 5% CO2 and 95% air at 37°C. To trigger differentiation into macrophage-like cells, THP-1 cells were seeded at a density of 2×10^6 cells per 60-mm dish and cultured in the presence of 160 nmol/L PMA for 48 h.

EXTRACTION OF TOTAL RNA AND PRODUCTION OF cDNA

Total RNA was isolated from THP-1 cells using TRI Reagent (Molecular Research Centre, Inc.) following the manufacturer's protocol. cDNA was generated from 1 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Promega) and oligo(dT)$_{15}$ primers (Invitrogen) using protocols recommended by the manufacturers.

SEMI-QUANTITATIVE PCR ANALYSIS

All PCR reactions were performed in a total volume of 25 μl containing 1× *Taq* polymerase buffer, 1.5 mmol/L $MgCl_2$, 200 μmol/L deoxynucleotides, 200 nmol/L each primer, 1 U *Taq* polymerase, and 20 ng cDNA. *GAPDH* expression was assessed to verify that equal amounts of cDNA were added to each PCR. The sets of primers used were 5′-AAAGGAGTCCCTAGAG-3′ and 5′-GGATGAGAACTCTTGC-3′ for human *ACAT1* P1 product (hACAT-1 cDNA K1 1486–2043, amplifying a 558-bp fragment), 5′-GCACGGGTTAAGATCT-3′ and 5′-ACCCACCATTATCTAA-3′ for human *ACAT1* P7 product (hACAT-1 cDNA K1 982–1670, amplifying a 689-bp fragment) [Li et al., 1999; Yang et al., 2001] and 5′-TGATGACATCAAGAAGGTGGTGAAG-3′ and 5′-TCCTTGGAGGCCATGTGGGCCAT-3′ for *GAPDH* (amplifying a 240-bp fragment). Equal volumes of PCR mixtures were resolved in 2% agarose gels and visualized by ethidium bromide staining.

QUANTITATIVE PCR ANALYSIS

SYBR Green I real-time PCR was used to quantitate mRNA expression of ACAT1 and the housekeeping gene GAPDH in THP-1 cells. All PCR reactions were performed using the LightCycler System (Roche Diagnostics, Switzerland) in a total volume of 20 μl containing 1× Taq polymerase buffer, 4 mmol/L MgCl2, 200 μmol/L deoxynucleotides, 200 nmol/L each primer, 0.2 μl of 100× SYBR Green I, 1 U Taq polymerase and 1 μL cDNA. The thermal cycling conditions were as follows: denaturation (94°C for 2 min), amplification and quantification repeated 40× (5 s at 94°C for denaturation, 5 s at respective annealing temperature (Table I) and 10 s at 72°C for elongation and then 1 s at respective fluorescence measurement temperature (Table I). The number of PCR cycles to reach the fluorescence threshold was the

TABLE I. Sequence of the Primers Used in the Amplification of ACAT1 and GAPDH

Gene	Primer sequence (5′--3′)	Annealing (°C)	Measurement temperature(°C)
ACAT1	F: 5′-GCAAAGGAGTCCCTAGAGACA-3′ R:5′-TCATCAAAGTGACTGCCAACTTC-3′	55	80
GAPDH	F: 5′-GAAGGTGAAGGTCGGAGTC-3′ R:5′-GAAGATGGTGATGGGATTTC-3′	60	79

cycle threshold (Ct). The Ct value for each sample was proportional to the log of the initial amount of input cDNA. In the present study, we used the $2^{-\Delta\Delta CT}$ methods to analyze the relative changes in gene expression from real-time quantitative PCR experiments [Livak and Schmittgen, 2001]. Data are expressed as means ± SD.

DNA ISOLATION, PCR, AND REPORTER PLASMIDS

Genomic DNA was purified from THP-1 cells using proteinase K and phenol [Joseph and David, 2001]. The fragments containing the *ACAT1* P1 promoter (−603 to +65) and P7 promoter (−612 to +150) were generated by PCR from genomic DNA. The primers were designed by analyzing the sequence of human *ACAT1* P1 and P7 promoters (GenBank accession AF143319 and AF143320, respectively) [Li et al., 1999]: 5′-ggggtaccGGGCCCTTACATATTACTTAGTT-3′ and 5′-ccgctcgagGTCGACTCCGGGAAGCTCTCCT-3′ for P1 (including a 6-bp *Kpn*I/*Xho*I linker); and 5′-gaagatctGAGCTCCACCTCCTGTCAAAT-3′ and 5′-cccaagcttCCTAGGGTCTGACTTTTGAAAAGGG-3′ for P7 (including a 6-bp *Bgl*II/*Hind*III linker). The PCR products were then digested with *Kpn*I/*Xho*I or *Bgl*II/*Hind*III, respectively, and subcloned into the multiple cloning site of the Firefly luciferase vector pGL3-Enhancer (Promega) to obtain pGL3E-P1 or pGL3E-P7, respectively. The pGL3E-P1 plasmid was used to generate deletions of the *ACAT1* P1 promoter with varying 5′ ends and an identical 3′ end at +65 by PCR. The primer sequences are summarized in Table II, all included a 6-bp KpnI/XhoI linker. The PCR products were then digested with *Kpn*I and *Xho*I and subcloned into an empty pGL3-Enhancer vector to generate the 5′-deletion constructs of the P1 promoter. The 5′-deletion constructs of the P1 promoter represent bases −547 to +65 (−547 Luc), −498 to +65 (−498 Luc), −428 to +65 (−428 Luc), −363 to +65 (−363 Luc), −324 to +65 (−324 Luc), −256 to +65 (−256 Luc), −188 to +65 (−188 Luc) and −125 to +65 (−125 Luc). All these constructs were then identified by restriction enzyme digestion mapping and DNA sequencing.

DELETIONS OF THE C/EBPα, SRF AND HNF-3 BINDING SITES IN THE *ACAT1* P1 PROMOTER IN THE pGL3-ENHANCER PLASMID

According to sequence information indicating the presence of transcription factor binding sites upstream of the P1 promoter, we focused on putative C/EBPα, serum response factor (SRF) and hepatocyte nuclear factor-3 (HNF-3) binding sites to create two different kinds of *ACAT1* P1 promoter deletions, which were separately cloned into Firefly luciferase vector pGL3-Enhancer, producing constructs including bases −579 to +65 or −566 to +65. Deletions were achieved by PCR using the corresponding set of primers that included a 6-bp *Kpn*I/*Xho*I linker. The primer sequences are summarized in Table III. The PCR products were then digested with *Kpn*I and *Xho*I and subcloned into an empty pGL3-Enhancer vector to generate the 5′-deleted *ACAT1* P1 promoter/Luc constructs.

TRANSFECTION AND LUCIFERASE ASSAY

A series of luciferase reporter (Luc) constructs containing the *ACAT1* P1 or P7 promoter were transfected into THP-1 cells using the Diethylaminoethyl dextran (DEAE-dextran) method [Yang et al., 2001]. Briefly, after washing twice with PBS, 1×10^6 cells were transfected with 0.5 μg of *ACAT1* promoter/Luc plasmids and 10 ng Renilla Luciferase Report Vector (pRL-TK) as internal control in 200 μl of STBE (25 mmol/L Tris–HCl, pH 7.4, 5 mmol/L KCl, 0.7 mmol/L $CaCl_2$, 137 mmol/L NaCl, 0.6 mmol/L Na_2HPO_4, 0.5 mmol/L $MgCl_2$) containing 60 μg of DEAE-dextran. The cells were incubated for 20 min at 37°C, washed once with RPMI 1640

TABLE II. Sequence of the Primers Used in the Amplification of the Deletion Mutants of the ACAT1 P1 Promoter

Position(bp)	Primer sequence (5′→3′)	Annealing(°C)
−547 to +65	F: 5′-ATGATCAGgtaccCAGGTTTTTTCCCCTTATC-3′ R: 5′-GATCActcgagGTCGACTCCGGGAAGCTCTCC-3′	52
−498 to +65	F: 5′-ATGATCAGgtaccTTCAAACGGTAAGGAATC-3′ R: 5′-GATCActcgagGTCGACTCCGGGAAGCTCTCC-3′	52
−428 to +65	F: 5′-ATGATCAGgtaccCTGGCTAGTTCTACG-3′ R: 5′-GATCActcgagGTCGACTCCGGGAAGCTCTCC-3′	52
−363 to +65	F: 5′- ATGATCAGgtaccGGCTTCTTCCAGTCG-3′ R: 5′-GATCActcgagGTCGACTCCGGGAAGCTCTCC-3′	52
−324 to +65	F: 5′-ATGATCAGgtaccGCTCCATGCTACCACGC-3′ R: 5′- GATCActcgagGTCGACTCCGGGAAGCTCTCC-3′	52
−256 to +65	F: 5′-ATGATCAGgtaccACATTCTACTGCTGGGGTG -3′ R: 5′- GATCActcgagGTCGACTCCGGGAAGCTCTCC-3′	52
−188 to +65	F: 5′- ATGATCAGgtaccAGCTTCCTTGGCAAGGTTGCC-3′ R: 5′- GATCActcgagGTCGACTCCGGGAAGCTCTCC-3′	52
−125 to +65	F: 5′- ATGATCAGgtaccGGGAAGGGGCGGGGAGG-3′ R: 5′-GATCActcgagGTCGACTCCGGGAAGCTCTCC-3′	52

TABLE III. Sequence of the Primers Used in the Amplification of the Deletion Mutants of the ACAT1 P1 Promoter

Position (bp)	Primer sequence (5′–3′)	Annealing(°C)
−579 to +65	F: 5′-ATGATCAGgtaccTTCCATTATTTGGTGTG-3′ R: 5′-GATCActcgagGTCGACTCCGGGAAGCTCTCC-3′	52
−566 to +65	F: 5′-ATGATCAGgtaccTGTGTTTTATTATTTGG-3′ R: 5′-GATCActcgagGTCGACTCCGGGAAGCTCTCC-3′	52

without FBS, resuspended in 500 μl of fresh RPMI 1640 with 10% FBS, and plated in a 24-well plate. All cells were transfected with pGL3-Enhancer and pGL3-Control, which served as negative and positive controls, respectively. Each transfection reaction was performed in triplicate. After incubation for 7 h, cells were treated with or without insulin (100 nM). Forty hours later, the cells were harvested and assayed using the Dual-Luciferase Reporter Assay System in a Lumat LB9507 luminometer (EG&G Bertold, Freiburg, Germany). Results were obtained from three different transfection experiments after normalization for the internal control of TK activity. Experimental variations are indicated as mean ± SD.

CELL EXTRACTS AND WESTERN BLOT ANALYSIS

After washing three times with ice-cold PBS, cells were harvested in a lysis buffer consisting of 10 mmol/L HEPES, pH 7.8, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.1 mmol/L EDTA, 1 mmol/L dithiothreitol, 0.5 mmol/L PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin and 0.5 g/L NP40. The protein samples were then subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Rabbit polyclonal anti–ACAT-1 (1:1,000, Santa Cruz Biotechnology, CA) and anti–β-actin (Santa Cruz Biotechnology, CA) antibodies were used. Horseradish peroxidase–conjugated goat anti-rabbit antibody (Pierce, Rockford, IL) was used as the secondary antibody. Signals were visualized using ECL detection reagent (Pierce). Scanning densitometry was performed using the Gel Doc XR imaging System and Quantity One V4.6 software (Bio-Rad).

ACAT ACTIVITY ASSAY

Treated with or without insulin for 24 h, the proteins of THP-1 cells were extracted according to the method of Yang et al. [2001]. The ACAT activity assay was performed according to the method of Billheimer et al. [1981] with some modifications. The standard assay in a final volume of 200 μl contained 100 μg of protein, 1 mg of fatty acid–free bovine serum albumin in 0.1 M potassium phosphate buffer (pH 7.4), and 100 μM [1-^{14}C] oleoyl-CoA. Where designated, exogenous cholesterol (20 μg) was added as an aqueous dispersion in Triton WR-1339 (600 μg). All components except oleoyl-CoA were preincubated for 15 min; the reaction was initiated by adding [1-^{14}C] oleoyl-CoA and incubated for 10 min. The reaction was stopped by adding 4 ml of chloroform:methanol (2:1, v/v). After separation into two layers by adding 0.8 ml of water, the chloroform layer was removed, and the lipids were separated by thin-layer chromatography using hexane:diethyl ether:acetic acid (85:15:0.5, v/v). The spots corresponding to CEs were scraped out and placed directly into scintillation vials for counting using MicroBeta1450 Liquid Scintillation and Luminescence Counters (WALLAC, PE). Cholesteryl oleate (10 μg) was added as an internal standard.

ELECTROPHORETIC MOBILITY SHIFT ASSAYS (EMSAs)

Nuclear extracts were prepared from the THP-1 cells treated with or without insulin (100 nM) using NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Pierce) following the manufacturer's protocol. A DIG Gel Shift kit (Roche, Mannheim, Germany) was used for EMSA assays. The oligonucleotide probe (5′- GGGCCCTTACATATTACTTAGTTG-3′) containing the C/EBPα binding motif was end-labeled with DIG-ddUTP. A mutant oligonucleotide with the sequence 5′-GGGCCCTTACATCGGCAGGCTGTG-3′ was also prepared(mutated region is underlined).

The DNA probe was prepared using a DIG gel shift kit (Roche) according to the manufacturer's protocol. The DNA binding and electrophoresis were performed as described previously [Qian et al., 2010]. The labeled DNA fragments were further purified to remove the redundant DIG-ddUTP and salts. For the binding reaction, different amounts of the nuclear extracts in 20 μl binding buffer were incubated for 20 min at 25°C with 4 μl of binding Buffer, 1 μg poly (dI-dC), 0.1 μg poly L-lysine, and 0.8 μg labeled oligonucleotide. For a competition assay, a 300-fold molar excess of cold probe was added to the reaction. To perform supershift assay, 1 μl of anti-C/EBPα antibody (sc-9314) was added to the reaction. Bovine serum albumin (BSA) was used as a nonspecific protein binding control.

The DNA–protein complexes formed in the mixture were resolved in 7% nondenaturing PAGE in in a TBE buffer (22.5 mM Tris, 22.5 mM boric acid, and 0.5 mM EDTA, pH 8.3) and blotted onto a nylon membrane. Probes were immobilized by baking, washed and hybridized with anti-DIG-AP Fab fragments for 30 min. Then the membrane was washed in washing solution for 30 min and moved to detection buffer. The detection buffer was supplemented with 100 μg/ml disodium 3-(4-methoxyspiro{1,2-dioethane-3,2′-(5′-chloro) tricyclo [3.3.1.13,7) decane}-4-yl] phenylphosphate (CSPD), and this CSPD working solution was applied to the membrane. The membrane was incubated for 5 min at 25°C and further at 37°C for 10 min, after which it was exposed to an X-ray film for 30 min.

STATISTICAL ANALYSIS

Results presented are the mean ± SD from at least three independent experiments performed in duplicate. Statistical analysis was performed using one-way ANOVA or the two independent sample *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

INSULIN UP-REGULATES HUMAN *ACAT1* GENE EXPRESSION AND ACTIVITY IN THP-1 CELLS AND THP-1–DERIVED MACROPHAGES

In this study, we treated THP-1 cells and THP-1–derived macrophages for 24 h with insulin, and then examined *ACAT1* at the levels of mRNA, protein, and enzyme activity. Real-time quantitative RT-PCR analysis showed that the level of the *ACAT1* mRNA in treated THP-1 cells and THP-1–derived macrophages increased in a dose-dependent manner (Fig. 1A). Consistent with these results, western blot and enzyme activity assays also showed that the *ACAT1* protein level (Fig. 1B) and enzyme activity (Fig. 1C) were enhanced in a dose-dependent manner. These results suggest that the insulin-mediated enhancement of *ACAT1* gene expression and enzyme activity might act principally at the transcriptional level.

INSULIN ENHANCES *ACAT1* P1 PROMOTER ACTIVITY IN THP-1 CELLS

To investigate the functional responses of *ACAT1* P1 and P7 promoters to insulin, we tested the activity of *ACAT1* P1 and P7 promoters in THP-1 cells using luciferase reporter activity assays. The

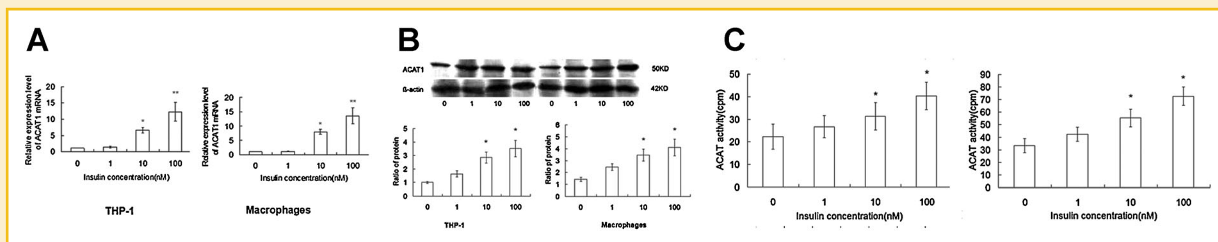

Fig. 1. Effect of insulin on human ACAT1 expression and activity in THP-1 cells and THP-1–derived macrophages. A: Total RNAs were prepared from THP-1 cells and THP-1–derived macrophages treated for 24 h without or with insulin (1–100 nM). *ACAT1* mRNA was quantitated by SYBR Green I real-time PCR (normalized to *GAPDH*). B and C, THP-1 cells and THP-1–derived macrophages were incubated for 24 h in RPMI 1640 media without or with insulin (1–100 nM). Cell extracts were prepared, and immunoblotting and enzyme activity assay were conducted as described under "Experimental Procedures." The data represent the mean ± SD of three independent experiments. One-way ANOVA was performed to determine statistical significance. * and ** indicate differences of $P < 0.05$ and $P < 0.01$, respectively, compared with untreated insulin group.

results (Fig. 2A) show that insulin had no detectable effect on the P7 promoter, but insulin enhanced the luciferase expression driven by the P1 promoter. Treated with or without insulin for 24 h, total RNAs of THP-1 cells were extracted for RT-PCR. As shown in Figure 2B, the *ACAT1* P1 promoter transcript level increased in insulin-treated THP-1 cells in comparison with untreated cells. In contrast, the level of the *ACAT1* P7 promoter transcript was not significantly altered. We concluded that insulin up-regulated *ACAT-1* gene expression by activating the P1 promoter, so next we analyzed the structure of the P1 promoter.

DELETION ANALYSIS OF HUMAN *ACAT1* P1 PROMOTER

The *ACAT1* P1 promoter is contiguous with the coding sequence and spans from −603 to +65 of the *ACAT1* genomic sequence [Li et al., 1999]. By sequence analysis (Fig. 3A), neither a typical TATA box nor a typical CAAT box was found. Four copies of a typical GC box (Sp1) were found in this region, however. In the P1 promoter sequence, other potential binding sites for various transcription factors existed, including C/EBPα, SRF, HNF-3, winged helix protein, c-Myb, GATA binding factor, nuclear factor-1 (NF-1), cyclic AMP response element binding protein, and nuclear factor-kappaB.

To examine the functional regions of the P1 promoter involved in modulation of basal *ACAT1* expression in THP-1 cells and to identify the IRE of the P1 promoter, sequential 5′-deletion analysis of the promoter fragment was carried out. A series of 5′-deletion fragments, with 5′ ends ranging from −603 to −125 and 3′ ends at +65, was fused to the luciferase reporter gene of pGL3-Enhancer. We transiently transfected these plasmids into THP-1 cells and measured luciferase activities. The luciferase activities observed with different deletions were compared to that of construct −603 LUC. As shown in

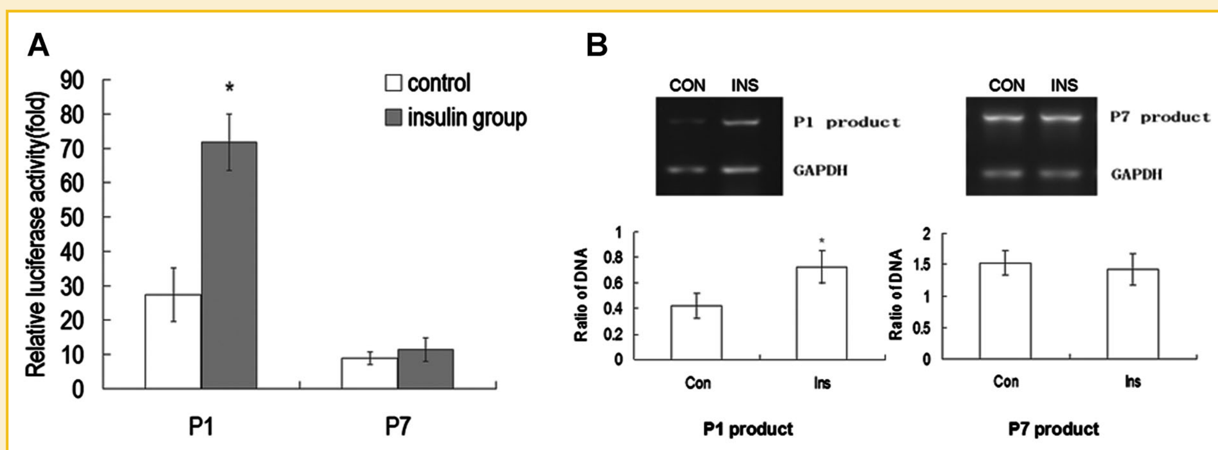

Fig. 2. Effect of insulin on *ACAT1* P1 and P7 promoter activity in THP-1 cells. A: The Luc constructs containing the *ACAT1* P1 or P7 promoter ligated to the luciferase reporter vector (pGL3-Enhancer) were transfected into THP-1 cells. Seven hours after transfection, cells were treated with or without insulin (100 nM). The luciferase activity was determined by the Dual-Luciferase Reporter Assay System in lysates of THP-1 cells 40 h later and normalized to TK activity. Values represent the means from triplicate determinations. Afterward, the luciferase activity was determined as described under "Experimental Procedures." B, Semi-quantitative RT-PCR results (normalized to *GAPDH*) show the expression level of *ACAT-1* P1 and P7 promoter–driven transcripts (designated as P1 and P7 products) in THP-1 cells treated for 24 h with or without 100 nM insulin. The ratio of DNA concentrations (shown in the bottom panels) was determined using the Gel Doc XR imaging System and Quantity One V4.6 software (Bio-Rad). The data represent the mean ± SD of three independent experiments. Two independent sample t-test was performed to determine statistical significance. * indicates differences of $P < 0.05$, compared with untreated insulin group.

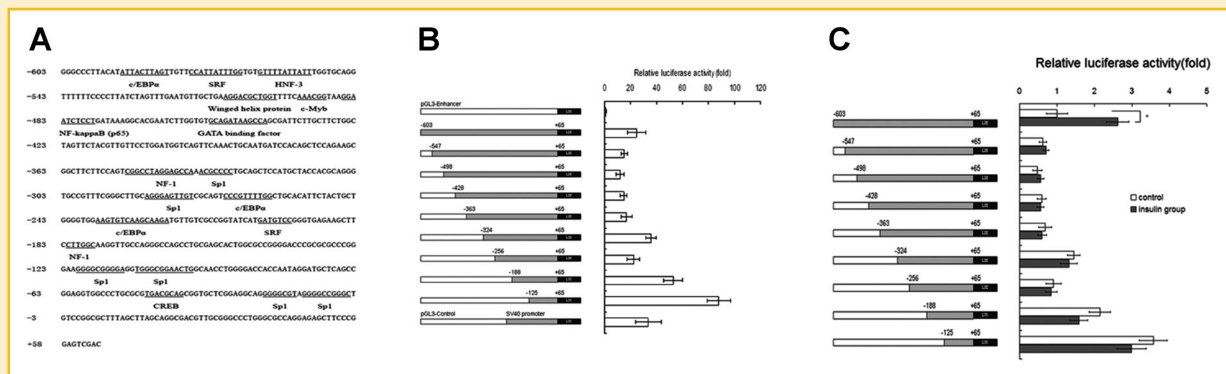

Fig. 3. Deletion analysis of human *ACAT1* P1 promoter (A), Nucleotide sequence analysis of human ACAT1 P1 promoter. B, ACAT1 P1 promoters with varying 5′ ends and an identical 3′ end (+65) were fused to the luciferase reporter gene in pGL3-Enhancer. The constructs were transfected into THP-1 cells, along with pRL-TK to normalize for transfection efficiency. Cells were also transfected with pGL3-Enhancer and pGL3-Control, which served as negative and positive controls, respectively. The cells were harvested for activity assays 48 h after transfection. Luciferase activity is expressed as fold increase over promoterless reporter plasmid pGL3-Enhancer. C, The Luc constructs containing serial 5′ deletions of the ACAT1 P1 promoter were transfected into THP-1 cells. Seven hours after transfection, cells were treated with or without insulin (100 nM). Luciferase activity was determined in lysates of THP-1 cells 40 h later and normalized to TK activity. All data are expressed as luciferase activities relative to the value from the construction −603 to +65 in untreated cells, which was defined as 1. Results presented are the mean ± SD from at least three independent experiments performed in duplicate. Two independent sample *t*-test was performed to determine statistical significance. *Indicates differences of $P < 0.05$ compared to −603 LUC.

Figure 3B, the luciferase activities of plasmids −188 LUC and −125 LUC were higher than that of −603 LUC when transfected into THP-1 cells, and the maximal transcriptional activity was located within the 159 bp from −125 to +65, suggesting that the core sequence of the *ACAT1* P1 promoter was located in the region from −125 to +65. Multiple GC boxes, which are likely to be Spl binding sites, are present within this region (Fig. 3A).

To delineate promoter elements that may be responsive to insulin, the luciferase plasmids containing a serial 5′ deletions of the *ACAT1* P1 promoter were transfected into THP-1 cells, treated with or without insulin (100 nM). As shown in Figure 3C, insulin stimulated luciferase expression 2.6-fold when the plasmid containing the P1 promoter (−603 to +65) was transiently transfected into THP-1 cells. Insulin did not stimulate luciferase expression, however, when plasmids containing progressive 5′ deletions from position −547 to −125 of the P1 promoter were used. In other words, deletion of the region −603 to −547 reduced insulin-stimulated luciferase expression significantly. These results indicate that at least part of the cis-acting DNA sequences that mediate insulin response in THP-1 cells may be located in the region between −603 and −548 upstream of the *ACAT1* P1 promoter.

LOCALIZATION OF INSULIN RESPONSE SEQUENCES IN HUMAN *ACAT1* PROMOTER

To predict the insulin-activated transcription factor binding sites between −603 and −548 upstream of the *ACAT1* P1 promoter, we searched for sequences similar to known transcription factor binding sequences. The search itself revealed multiple potential sequences, including a C/EBPα binding site in the −591 to −582 region, a SRF binding site in the −577 to −567 region and a HNF-3 binding site in the −563 to −553 region of the P1 promoter (Fig. 3A). To determine whether C/EBPα, SRF and HNF-3 are functionally important for inducing *ACAT1* transcription by insulin, we generated deletions of these potential elements, which resulted in P1 promoter sequences that included bases −579 to +65, −566 to +65 and −547 to +65, respectively. As shown in Figure 4, insulin did not stimulate luciferase expression when plasmids with the deletions were transiently transfected into THP-1 cells. As the putative C/EBPα site was the farthest upstream and all deletions lacked this sequence, these results indicate that deletion of the putative C/EBPα binding sequence (−591 to −582) completely eliminated insulin-stimulated luciferase expression. This suggests that an IRE was located in the region between −603 and −580 (5′ GGGCCCTTACATATTACTTAGTTG 3′) upstream of the human *ACAT1* gene P1 promoter, which could contain a C/EBPα binding site.

INSULIN-STIMULATED HUMAN *ACAT1* GENE EXPRESSION AND ENZYMATIC ACTIVITY ARE DEPENDENT ON MAP KINASES

As shown in Figure 5A, real-time quantitative RT-PCR analysis showed that insulin increased the level of *ACAT1* mRNA in THP-1 cells. This effect of insulin was almost completely inhibited by the ERK inhibitor PD98059 and partly inhibited by the p38MAPK inhibitor SB203580, but not by the PI3K inhibitor Wortmannin or the PLCγ inhibitor U73122. To confirm these results, we used western blotting (Fig. 5B*)* and enzyme activity assays (Fig. 5C), which also showed that PD98059 almost completely, SB203580 partly blocked the effect of insulin-mediated enhancement of *ACAT1* protein level and enzyme activity. In contrast, Wortmannin and U73122 had no effect. These results indicate that the insulin induction of *ACAT1* gene expression and enzymatic activity was mediated specifically by MAPK.

Next we sought to determine whether insulin activation of the *ACAT1* gene promoters involved the same insulin-signaling intermediates. As shown in Figure 5D, insulin-stimulated *ACAT1* P1 promoter activity was completely inhibited by addition of PD98059, partly inhibited by SB203580, but not by Wortmannin

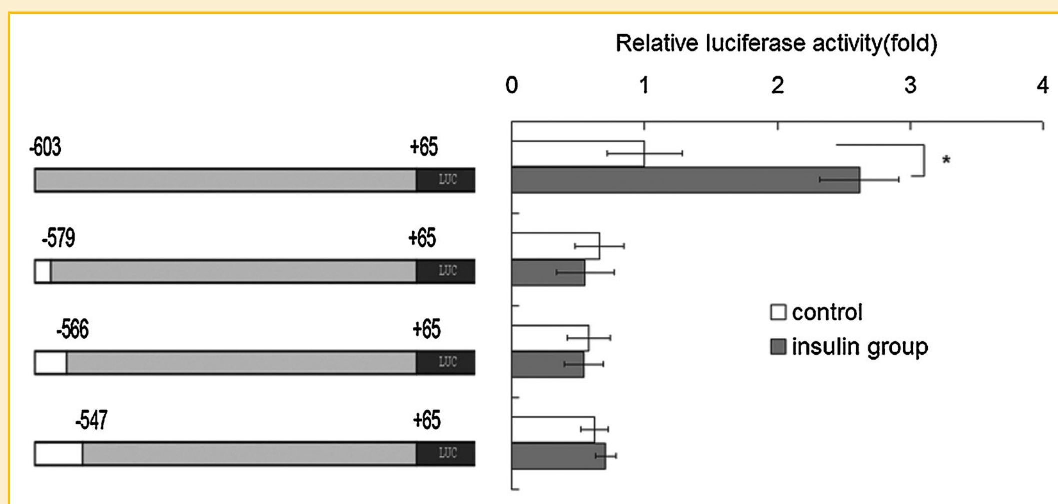

Fig. 4. Localization of insulin regulatory sequences in the human *ACAT1* gene. The deletion constructs ligated to the luciferase reporter vector (pGL3-Enhancer) were transfected into THP-1 cells. Seven hours after transfection, cells were treated with or without insulin (100 nM). Luciferase activity was determined in lysates of THP-1 cells 40 h later and normalized to TK activity. All data are expressed as luciferase activities relative to the value from the construction −603 to +65 in untreated cells, which was defined as 1. Results presented are the mean ± SD from at least three independent experiments performed in duplicate. Two independent sample *t*-test was performed to determine statistical significance. *Indicates differences of $P < 0.05$ compared to −603 LUC.

or U73122, consistent with the results above. Similarly, insulin increased the level of the *ACAT1* P1 promoter transcript, an effect that was also prevented by PD98059 and SB203580 (Fig. 5E). We concluded, therefore, that insulin stimulates the activity of the *ACAT1* P1 promoter and enhances *ACAT1* gene expression through a MAPK-dependent pathway.

HUMAN *ACAT1* GENE P1 PROMOTER WAS MEDIATED BY THE C/EBPα BOUNDING TO THE FUNCTIONAL IRE

To verify the binding of C/EBPα to this potential IRE, electrophoresis mobility shift assays (EMSA) were carried out by using a labeled probe relevant to the −603 to −580 bp region of human ACAT1 gene P1 promoter. As shown in Figure 6, the specific DNA-protein bands were observed with the nuclear extracts from insulin-treated THP-1 cells (lanes 2), by comparing to those of adding 300-fold excess of the unlabeled cold probe (lanes 4). The nuclear extracts failed to bind to mutant labeled probe (lanes 5), and the probe containing the mutated C/EBP sequences could not deplete the binding of C/EBPα to the labeled wild type probe (lanes 6). A supershift was observed in the presence of C/EBPα antibody, indicating the specificity of this interaction for C/EBPα (lanes 7). Taken together, nucleotide sequence analysis, luciferase reporter assays, and EMSA showed that the activity of human ACAT1 gene P1 promoter might be mediated by the C/EBPα bounding to the functional IRE at the −603 to −580 bp of human ACAT1 gene P1 promoter region, indicating that C/EBPα was functionally important for human ACAT1 gene expression.

DISCUSSION

In some cholesterol-loaded cells, cholesterol esterification is increased without changes in ACAT1 mRNA or protein expression levels, but with an increase of ACAT activity [Rea et al., 1996]. The main mode of sterol-specific regulation of ACAT1 has been identified at the post-translational level, involving allosteric regulation by its substrate cholesterol [Chang et al., 1997]. ACAT1, however, could also be regulated at the transcriptional level. Yang et al. showed that a glucocorticoid response element in the human *ACAT1* P1 promoter responsible for the dexamethasone-induced elevation of *ACAT1* gene expression could be functionally bound with glucocorticoid receptor [Yang et al., 2004]. In human THP-1 monocytic cells, the combination of IFN-γ and all-trans retinoic acid enhanced *ACAT1* P1 promoter activity in a synergistic manner [Yang et al., 2001]. Other factors associated with atherosclerosis up-regulate *ACAT1* gene expression in human monocyte-macrophages, such as 1,25-dihydroxyvitamin D (3) or 9-cis-retinoic acid, transforming growth factor-β1, serotonin, salusin-beta and leptin [Maung et al., 2001; Hori et al., 2004; Suguro et al., 2006; Watanabe et al., 2008; Hongo et al., 2009]. However, incretins and hydrogen sulfide prevent the development of atherosclerosis through downregulation of *ACAT1* gene expression [Nagashima et al., 2011; Zhao et al., 2011]. In the present work, we showed that insulin enhanced human ACAT1 enzyme activity and expression at both the mRNA and protein levels in THP-1 cells and THP-1-derived macrophages. Further analysis showed that insulin up-regulated *ACAT1* gene expression by stimulating the activity of the *ACAT1* P1 promoter. These results suggest that insulin could up-regulate *ACAT1* expression and enzyme activity in human monocyte-macrophages at the transcriptional level by activating the P1 promoter.

Macrophages play important roles in the development of atherosclerosis. Some reports have demonstrated that insulin has a direct effect on macrophages. Insulin increases the expression of the type 2 scavenger receptor CD36 on human macrophage foam cell formation, and decreases the expressions of hormone-sensitive lipase

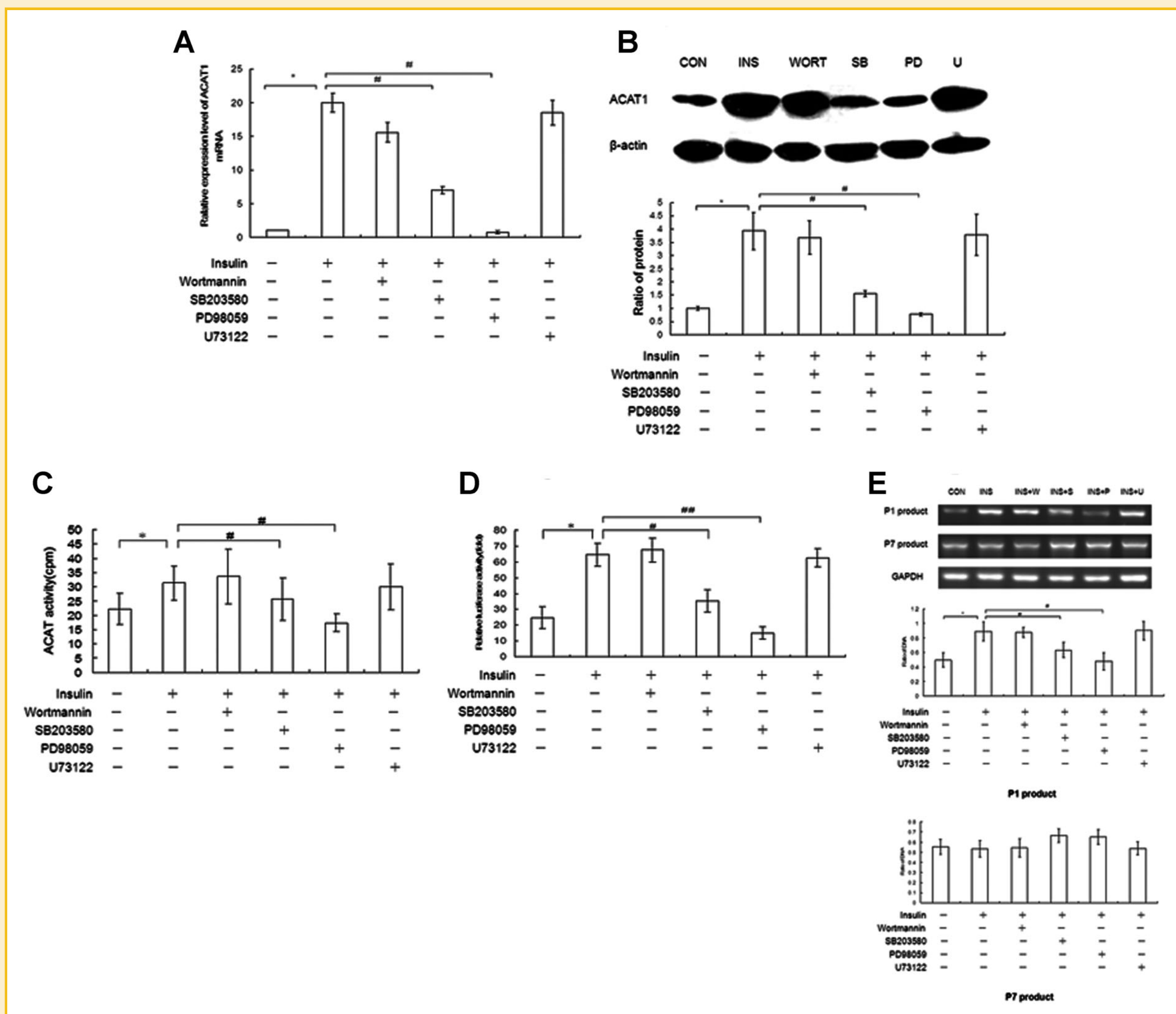

Fig. 5. Insulin-stimulated *ACAT1* expression requires MAP kinases. (A, B, C and E): THP-1 cells were treated with the specific inhibitor of PI3K (Wortmannin, 200 nM), p38MAPK (SB203580, 10 μM),ERK (PD98059, 50 mM), or PLCγ (U73122, 10 μM) for 30 min prior to the addition of insulin or buffer alone. Cell extracts were prepared 24 h later. A, *ACAT1* mRNA was quantitated by SYBR Green I real-time PCR (normalized to *GAPDH*). B and C, Immunoblotting and enzyme activity assays were conducted as described under "Experimental Procedures." The data are expressed as relative protein and ACAT activity using the values from untreated THP-1 cells. E, Semi-quantitative RT-PCR (normalized to *GAPDH*) for the *ACAT1* P1 and P7 promoter-driven transcripts (designated as P1 and P7 products). D: The Luc construct containing the *ACAT1* P1 promoter ligated to pGL3-Enhancer was transfected into THP-1 cells. Seven hours after transfection, cells were treated with the specific inhibitor of PI3K (Wortmannin, 200 nM), p38MAPK (SB203580, 10 μM),ERK (PD98059, 50 mM), or PLCγ (U73122, 10 μM) for 30 min prior to the addition of insulin or buffer alone. The luciferase activity was determined in lysates of THP-1 cells 40 h later and normalized to TK activity. Values represent the means from triplicate determinations. One-way ANOVA was performed to determine statistical significance. *Indicates differences of $P < 0.05$ compared with control, # and ## indicates differences of $P < 0.05$ and $P < 0.01$ compared with insulin group.

(HSL), neutral cholesteryl ester hydrolase (nCEH), ATP-binding cassette transporter A1 (ABCA1) and G1 (ABCG1), suggesting that insulin increases the accumulation of CE in macrophages and accelerates atherosclerotic lesion development [O'Rourke et al., 2002; Sporstøl et al., 2007; Yamashita et al., 2010; Meilin et al., 2011; Park et al., 2012]. Insulin binds to and activates high-affinity receptors located in the plasma membrane. The signal transduction pathways activated from these receptors include three major mediators: Ras/MAPK (including ERK and p38MAPK), PI3K and PLCγ. Stimulation of any of these pathways can eventually lead to the activation/inhibition of target genes, either by transcriptional or post-transcriptional mechanisms. In the present work, we found that the induction of ACAT1 mRNA, protein and enzyme activity by insulin was almost completely inhibited by the ERK inhibitor PD98059 and partly inhibited by the p38MAPK inhibitor SB203580. Insulin-stimulated ACAT1 P1 promoter activity and P1 promoter transcript level were also prevented by PD98059 and SB203580. We have previously reported that both SB203580 and PD98059 reduces ACAT1 mRNA and protein expression in macrophages [Xin et al., 2009], which are consistent with our present results. This implied that insulin signals through MAPK to stimulate the ACAT1 P1 promoter and increase ACAT1 gene expression. These

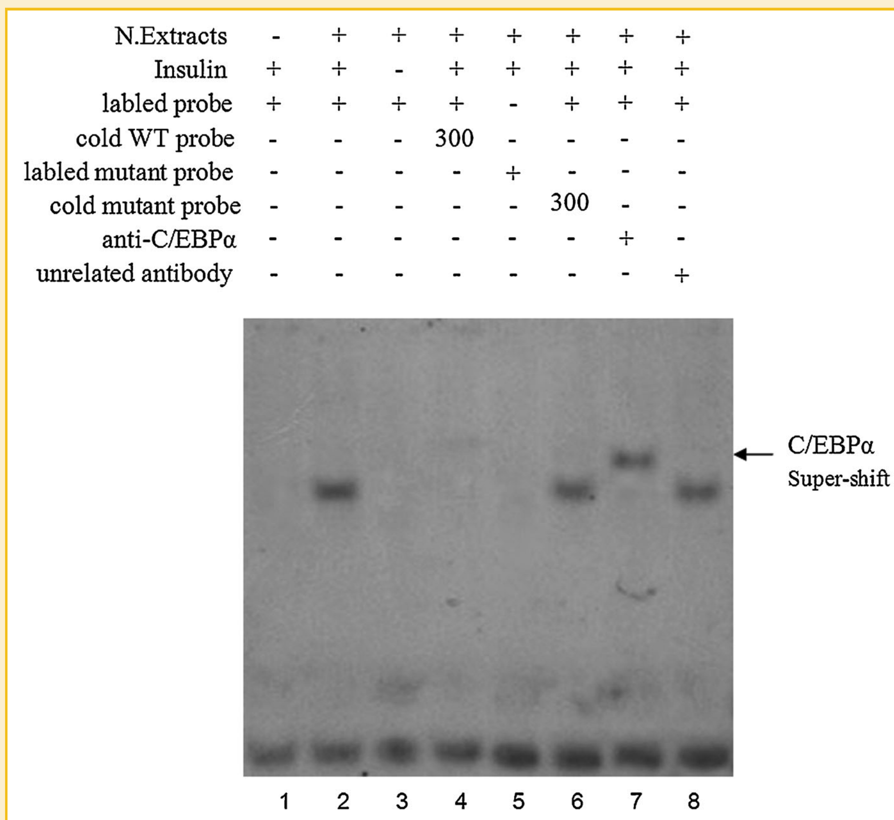

Fig. 6. Binding of C/EBPα to the IRE of the human ACAT1 gene P1 promoter. Lane 1,labled Probe; Lane 2 and 3, binding reaction between the 0.1 pmol of labeled wide type probe and 1 ul of the nuclear extract obtained from THP-1 cells treated with or without insulin(100 nM); Lane 4, competition by adding 300-fold molar excess of the relevant cold unlabed probe to the binding reaction described as lane 2; Lane 5, binding reaction between the 0.1 pmol of labeled mutant type probe and 1 ul of the nuclear extract; Lane 6, competition by adding 300-fold molar excess of the relevant cold unlabed mutant probe to the binding reaction described as lane 2; Lane 7, supershift reaction by adding 1 ul of anti-C/EBPα to binding reaction described 2; Lane 8, competition by adding BSA to the binding reaction described as lane 2. Similar observations were obtained from two other experiments.

data implicate ERK and p38MAPK may be involved in the insulin-mediated regulation of ACAT1 expression.

Insulin induces transcriptional changes of more than 100 genes [O′Brien and Granner, 1996]. The stimulatory and inhibitory effects of insulin on gene transcription are mediated through various cis-acting elements collectively referred to as insulin response elements or sequences (IREs/IRSs). IREs have been identified in the promoters of different genes, and it is now accepted that there is not a consensus IRE. The trans-acting factors that activate these IREs are largely unknown. C/EBPs, HNF-3, SRF, NF-1, AP-1, forkhead box proteins and sterol response element binding proteins [Treisman, 1995; Karin et al., 1997; Scassa et al., 2004; Cagen et al., 2005; Yeagley and Quinn, 2005; Fan et al., 2007; Jackson et al., 2007] have been implicated in gene regulation by insulin, although this action is restricted to a few genes. In the present work, deletion analysis of the *ACAT1* P1 promoter revealed that the IRE was located in the region from −603 to −548. By sequence analysis, a number of potential transcription factor binding sites, including C/EBPα, SRF and HNF-3, were located in the upstream region of the P1 promoter between −603 and −548. Further experiments showed that an IRE was located specifically in the region between −603 and −580 (5′ GGGCCCTTACATATTACT-TAGTTG 3′), which could be a C/EBPα binding site. To verify the binding of C/EBPα to this potential IRE, EMSA showed that the activity of human ACAT1 gene P1 promoter might be mediated by the C/EBPα bounding to the functional IRE at the −603 to −580 bp of human ACAT1 gene P1 promoter region.

In sum, our findings demonstrate that a novel effect of insulin on *ACAT1* gene expression in THP-1 cells is transcriptionally mediated. The insulin effect is mediated, at least in part, via interaction between the functional IRE located in the −603 to −580 region of the *ACAT1* P1 promoter and C/EBPα, and is MAPK dependent. These findings could explain why foam cell formation is particularly accelerated in insulin-resistant individuals and why death from atherosclerotic heart disease correlates closely with blood insulin levels, and this could lead to new treatment strategies.

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