Phosphorylation-dependent down-regulation of apolipoprotein A5 by insulin

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Abbreviations: APOA5, the human gene; *apoa5*, the rodent gene; ApoAV, the human protein; PI3K, the phosphatidylinositol 3-kinase; USF, the upstream stimulatory factor.

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ABSTRACT

The apolipoprotein A5 (*APOA5)* gene has been shown to be important in lowering plasma triglyceride levels. Since several studies have shown that hyperinsulinemia is associated with hypertriglyceridemia, we sought to determine whether *APOA5* gene is regulated by insulin. We show here that cell and mouse treatments with insulin down-regulated *APOA5* expression in a dose-dependent manner. Furthermore, we determined that insulin decreases *APOA5* promoter activity and subsequent deletion analyses revealed an E-box-containing fragment. We showed that Upstream Stimulatory Factors, USF1/USF2, bind to the identified E-box in the *APOA5* promoter. Moreover, in cotransfection studies, USF1 stimulates *APOA5* promoter activity. The treatment with insulin reduces the binding of USF1/USF2 to *APOA5* promoter. The inhibition of PI3K pathway with wortmannin abolished the insulin's effect on *APOA5* gene transcription. Using oligoprecipitation method of USF from nuclear extracts, we demontrated that phosphorylated USF1 failed to bind to *APOA5* promoter. This indicates that the *APOA5* gene transrepression by insulin involves a phosphorylation of USF through PI3K, that modulate their binding to *APOA5* promoter and results in *APOA5* down-regulation. The effect of exogenous hyperinsulinemia in healthy men shows a decrease of the plasma ApoAV level. These data suggest a potential mechanism involving *APOA5* gene in hypertriglyceridemia associated with hyperinsulinemia.

INTRODUCTION

Several epidemiological studies have established that, in addition to elevated cholesterol level in LDL and reduced cholesterol level in HDL, hypertriglyceridemia is an independent risk factor for coronary heart diseases (8, 15). In addition, hypertriglyceridemia is often associated with the metabolic syndrome that characterizes diabetes and obesity (14, 20). Type 2 diabetes is frequently linked to hyperglycemia, hyperinsulinemia and hypertriglyceridemia, and the leading cause of death for individuals with diabetes is cardiovascular diseases (19).

Insulin plays a major role in the regulation of carbohydrate and lipid metabolism in liver, adipose tissue and muscle. Hepatic fatty acid oxidation, lipogenesis and glycerolipid synthesis are subject to regulation by insulin (24). Insulin controls the lipids synthesis from glucose in liver and adipose tissue where they are utilized, and controls the exportation of fatty acids into lipoproteins from the liver to the extrahepatic organs in which they are utilized or stored. First, insulin increases apolipoprotein A1 (*APOA1*), the major apolipoprotein component of HDL which has an intrinsic antiatherogenic properties (23, 29). In addition, insulin decreases apolipoprotein C3 (*APOC3*), which is positively associated with hypertriglyceridemia (5, 17). Insulin also increases apolipoprotein A4 (*APOA4*), which play a role in the prevention of atherosclerosis through the reverse cholesterol transport from peripheral tissues to the liver (10, 12, 31).

The apolipoprotein A5 gene (*APOA5*) was identified through comparative sequence analysis of genomic DNA sequences and has been shown to be important in determining plasma triglyceride levels in mice and humans (25). This gene is mainly expressed in the liver and resides in HDL and VLDL lipoprotein particles (25, 35). It has been demonstrated that mice expressing a human *APOA5* transgene showed a decrease in plasma triglyceride concentration to one-third those in control mice. Conversely, knockout mice lacking *APOA5*

had four times as much plasma triglycerides as controls. Moreover, adenoviral overexpression of *APOA5* reduces serum levels of triglycerides and cholesterol in mice (36). Furthermore, strong genetic associations have been described between polymorphisms the human *APOA5* gene and triglyceride concentrations(1, 2, 11, 21, 22, 27, 30, 33). Finally, PPARα agonists are known to have hypotriglyceridemic effect, and several recent studies have shown that the *APOA5* gene is highly up-regulated by PPARα and FXR (26, 37).

In the current study, we investigated the regulation of *APOA5* gene expression by insulin both *in vitro* and *in vivo* models. We showed that insulin negatively regulates *APOA5* gene expression at the transcriptional level in a dose-dependent manner. We elucidated the mechanism of this down-regulation which involves a phosphorylation pathway. Moreover, the human ApoAV protein level is down-regulated after insulin infusion.

EXPERIMENTAL PROCEDURES

Cloning and construction of recombinant plasmids

Human *APOA5* promoter fragments (-304/+63, -146/+63 and -61/+63) were amplified by PCR using an *APOA5* genomic BAC clone as template and cloned in the pGL3 luciferase vector. The followed forward oligonucleotide 5'-TCT GTT GGT GGG CCA GC C AG-3', 5'-GGT GCC AGG GAA AGG GCA GG-3', 5'-CAA TTG GTG CCA GAG GCT CAG-3' and the reverse oligonucleotide 5'-AAT GCC CTC CCT TAG GAC TGT GAC-3' primers were used for the PCR reaction.

Cell culture

The human hepatoma cell line HepG2 was maintained in Dulbecco's modified Eagle's minimal (DMEM) medium containing 4.5 g/L of glucose, supplemented with 10% fetal calf serum (FCS), 1% glutamin, 1% penicillin-stretomycin, 1% sodium pyruvate and 1% non-essential amino acids (Invitrogen). The culture was maintained at 37°C in a humidified atmosphere contained 5% CO_2. Medium was changed every other day. For treatments, cell culture medium was changed for 18 h in order to deprive the cells of glucose. The treatment was done in fresh medium identical to this used for the weaning and added with different insulin concentrations during 24 h.

For experiment using wortmannin the same volume of vehicle (DMSO) was used as control. Wortmannin 200 nM was added to culture medium 30 min before adding of insulin.

Primary rat hepatocytes

Rat hepatocytes were isolated by collagenase perfusion from livers of Wistar rats as described previously (34). Hepatocytes (cell viability higher than 85% by trypan blue exclusion test) were cultured as a monolayer in serum-free William's E medium supplemented with 2 mM glutamin, 25 µg/mL gentamicin, 100 nM dexamethasone and 0.1% fatty acid free-BSA at 37°C in a humidified atmosphere containing 5% CO_2. After a 6 h

incubation, medium was changed to glucose-free DMEM medium supplemented with 2 mM glutamin, 25 µg/mL gentamicin, 100 nM dexamethasone, 10 mM lactate and 5.5 mM glucose. After overnight culture, cells were incubated for the indicated time in fresh culture medium supplemented with 2 mM glutamine, 25 µg/mL gentamicine and 100 nM dexamethasone.

Primary human hepatocytes

Human hepatocytes were isolated from the liver of a 21 years old woman. Hepatocytes were cultured in William's E medium supplemented with 10% FCS, 0.1% BSA, 10 nM T3 and 1 µM dexamethasone at 37°C in a humidified atmosphere containing 5% CO_2. After 6 h of incubation, medium was changed to FCS free medium supplemented with 0.1% BSA, 10 nM T3 and 1 µM dexamethasone for 18 h. Next, cells were incubated for 16 h in a culture medium deprived of glucose and FCS, supplemented with 100 nM dexamethasone and 10 nM T3. Finally, cells were treated in the same fresh medium with different concentrations of insulin as described in the "*Results*" section.

RNA extraction and real-time PCR analysis

Total RNA from HepG2 cells or from mouse liver were extracted using RNeasy kit (Qiagen). mRNA quantification analysis was performed after a reverse transcription and a real-time PCR using the MX 4000 apparatus (Stratagene).

APOA5 mRNA was quantified and normalized with the *36B4* gene which code for the human acidic ribosomal phosphoprotein. Specific primers used for the amplifications are as follow: h*APOA5* forward : 5'-ACG CAC GCA TCC AGC AGA AC-3'; h*APOA5* reverse : 5'-TCG GAG AGC ATC TGG GGG TC-3'; r*APOA5* forward : 5'-GCC TGG GAA GGA GCC TCC TCG GC-3'; r*APOA5* reverse :5'-GCT CCA TCA GCT CGA CCG TGT AGG G-3'; m*APOA5* forward : 5'-CTC TGT CCC ACA AAC TCA CAC G-3'; m*APOA5* reverse : 5'-AGG TAG GTG TCA TGC CGA AAA G-3' ; *36B4* forward : 5'-CAT GCT CAA CAT CTC CCC CTT CTC C-3'; *36B4* reverse : 5'-GGG AAG GTG TAA TCC GTC TCC ACA G-3'.

The PCR amplifications were performed with the Brilliant Quantitative PCR Core Reagent Kit (Stratagene).

Treatment of mice with insulin

Female C57/BL6 mice weighing between 20-25 g were purchased from Charles River Laboratories. Mice were maintained on standard rodent chow diet. Three groups of six mice were studied. After 4 h of fasting, mice were then injected intraperitoneally with 0.5 U/Kg or 1 U/Kg of insulin, or vehicle as control. 6 h after the injection of insulin, the livers were excised and snap-frozen in liquid nitrogen for total RNA extraction. Mouse *apoa5* mRNA was quantified and normalized with the *36B4* gene.

Transient transfection assays

Transfection studies were carried out with human hepatoma HepG2 cells plated in 24-well plates. Culture medium was changed to fresh medium in order to deprive the cells of glucose for 18 hours. Transfections studies were performed at 50-60% confluence by the calcium phosphate co-precipitation procedure as described previously (38). Cells were transfected with 0.3 µg of reporter vector, which contains the –304/+63 human *APOA5* promoter fragment. Simultaneously, 30 ng of CMV-β-galactosidase expression vector as a control were transfected in cells for transfection efficiency. For the deletion analysis, cells were transfected with different constructions of the human *APOA5* promoter. Three luciferase constructs were used for this experiment:-304/+63, -146/+63 and –61/+63. After incubation for 2 h, cells were washed with PBS solution and incubated for 24 h with different concentrations of insulin. Cells were lysed and the cytosolic fraction was collected. Luciferase activity was measured and normalized according to β-galactosidase activity.

For the co-transfection experiments with USF1, 30 ng of this expression vector were added for the precipitation.

Preparation of nuclear extracts and electrophoretic mobility shift assays.

HepG2 cells were treated with 10 nM of insulin for 24 h, washed and resuspended in DMEM medium containing 10% FCS and 10% DMSO, frozen in liquid nitrogen and conserved at –80°C. To prepare nuclear extracts, cells were centrifuged for 5 min at 1000 rpm and resuspended in 5 mL of buffer A (15 mM Tris-HCl, pH8, 15 mM NaCl, 60 mM KCl, 0.5 mM EDTA, 1 mM PMSF), centrifuged for 5 min at 1000 rpm and at 4°C and resuspended in 200 µL of buffer A with 0.05% Triton X100 and centrifuged for 10 min at 1200 rpm. The pellet was washed with 5 mL of buffer A 0.05% Triton X100 and then with 5 mL of buffer A. The nuclei were incubated in 50 µL of buffer A supplemented with 360 mM of KCl at 4°C for 30 min and centrifuged for 5 min at 13000 rpm. The concentration of proteins in the supernatant was determined.

To study the insulin's regulatory region in the *APOA5* gene, a synthetic double-stranded oligonucleotide from the human *APOA5* gene was used (5'-CTT TTG AAC TTC CAC GTG GTA TTT ACT CAG-3'). A 23-bp double-stranded oligonucleotide containing the E-box consensus for the binding of USFs (5'-CAC CCG GTC ACG TGG CCT ACA CC-3') was used as a control probe. Double-stranded oligonucleotide were T4-polynucleotide kinase end-labeled using [γ-^{32}P]ATP and purified using the QIAquick Nucleotide Removal Kit (Qiagen). 2.5 µg of nuclear extracts were preincubated in a total volume of 20 µL for 30 min on ice with 2.5 µg of poly (dIdC) and 1 µg of herring sperm DNA in a binding buffer. For supershift experiments, 1 µg of anti-USF1 or anti-USF2 antibodies (Santa Cruz Biotechnology) were added in this binding reaction. 1 µL of end-labeled double-stranded oligonucleotide was added before a second incubation for 30 min on ice. Then, DNA-protein complexes were separated by electrophoresis on 5% polyacrylamide gel in 0.25X TBE buffer. The gel was dried and exposed to a film with an intensifying screen.

Oligoprecipitation assay

For this experiment, 5'-biotin labelled oligonucleotides corresponding to *APOA5* E-box used in EMSA were employed. 2 µL of double-stranded biotin-labeled oligonucleotides were incubated with 10 µg of nuclear extracts from HepG2 cells in EMSA mix for at least 1 h at 4°C. After 3 wash steps with EMSA binding buffer, 20 µL streptavidin-sepharose HP beads (Amersham Bioscience) were added in the oligoprotein complex for 1 h at 4°C. Then, samples were centrifuged, washed and boiled in order to remove protein from the probe. Proteins were separated on 10% SDS-PAGE and incubated with antibodies according to manufacturer's instructions (Santa Cruz Biotechnology).

Insulin clamp study in humans

Twelve healthy men participated in the study. All subjects underwent a history and physical examination and laboratory tests for exclusion of hepatic, renal, thyroid and hematological abnormalities. None of the subjects used any medication. The purpose, nature and potential risks of the studies were explained to the subjects before their written consent was obtained. The study protocol was approved by the ethical committee of the Helsinki University Central Hospital. All subjects were admitted at 7.30 am after an overnight (12 h) fast to the metabolic ward of the Department of Medicine, University of Helsinki. An indwelling cannula was inserted in an antecubital vein for infusions. A second cannula was inserted retrogradely into a heated hand vein to obtain arterialized venous blood for blood samplings. The study design allowed each subject to serve as their own controls. At 0 minute, infusion of saline or insulin was started. The euglycemic clamp study was performed as previously described (9). Insulin (Actrapid Human, Novo Nordisk, Copenhagen, Denmark) was infused in a primed continuous manner at a rate of 1 mU/Kg/min for 8 h. Normoglycemia was maintained by adjusting the rate of a 20% glucose infusion based on plasma glucose measurements which were performed at 5 minutes intervals.

Measurement of ApoAV protein level in human plasma

An enzyme-linked immunosorbent sandwich assay was used to measure ApoAV in sera. A pool of two monoclonal anti-human ApoAV antibodies solution, raised in mice by using recombinant protein, was used at 10 µg/mL in PBS 0.1 M, pH 7.2 to coat the wells of the microtiter plates at room temperature overnight. The wells were washed twice with PBS 0.1 M. The remaining sites for protein binding were saturated with 3% BSA/PBS for 1 h at 37°C. The wells were washed twice with PBS. 90 µL of the antigen solution was added to the wells. For quantitation, a pool of human plasma was calibrated and titrated using ApoAV recombinant protein as a primary standard. Then, the pool of human plasma was used for the calibration curve. The antigen solution is incubated for 2 h at room temperature. The wells were washed with PBS. The horseradish peroxidase labeled second anti-ApoAV polyclonal antibody, produced in rabbit using synthetic peptide, was diluted in the blocking buffer and added to the wells. After incubation of 2 hours at 37°C, the plates were washed with PBS. Prior to developing the enzyme label, 30 mg of o-phenylenediamine (ODP) was dissolved in 20 mL 0.1 M citrate/phosphate buffer and 20 µL of 30% H_2O_2. Then 100 µL of the enzyme substrate solution were added to each micotiter well. After incubation of 30 min, the reaction was terminated by adding HCl l M and the absorbance at 492 nm was measured using a microplate photometer (Dynex Technologies).

RESULTS

To determine whether insulin can modulate *APOA5* gene expression in human HepG2 cell line and in both primary human and rat hepatocytes, we analyzed *APOA5* mRNA levels after a 24 h treatment with insulin 10 nM. We observed a down regulation of *APOA5* gene expression in all cellular models (Fig. 1A). In HepG2 cells and human primary hepatocytes at 10 nM of insulin, *APOA5* gene expression is reduced by 46% and 60% respectively after 24 h of treatment (Fig. 1A). In rat primary hepatocytes, at 10 nM of insulin the *apoA5* gene expression is dramatically decreased by 90%. These data demonstrate that insulin decreases both human and rat *APOA5* mRNA levels in culture. We demonstrated that in rat primary hepatocytes the down-regulation of *apoa5* by insulin is dose-dependent (Fig. 1B). In this experiment, we also analyzed the expression of *apoc3* gene and confirmed its down-regulation by insulin (Fig. 1C) as that has been reported before (4, 16).

To investigate the regulation of *apoa5* by insulin *in vivo*, C57/BL6 mice were injected with 0.5 or 1 U/Kg of insulin. 6 hours later, the mice were sacrificed, RNA was extracted from liver, and *apoa5* gene expression was analyzed by quantitative PCR. A significant dose-dependent decrease of *apoa5* mRNA levels was observed (Fig. 2). In mice treated with 1 U/Kg of insulin, *apoa5* gene expression was decreased by 53% versus controls.

To determine if the decreased *APOA5* mRNA levels by insulin occurs at the transcriptional level, we analyzed the promoter activity in transiently transfected HepG2 cells with the –304/+63 fragment of the human *APOA5* promoter. We observed that insulin down-regulated the human promoter activity with a nearly 40% reduction at the highest concentration of insulin (Fig. 3). This result supports that the regulation of human *APOA5* by insulin occurs at the transcriptional level.

Deletion analyses were performed by transient transfections of HepG2 with three different constructs of the human *APOA5* promoter (Fig. 4). After 24 h of insulin treatment, the

promoter activity with the transfected -304/+63 and -146/+63 fragments were decreased. The effect of insulin is abolished when cells were transfected with the -61/+63 fragment. These results suggest the presence of a responsive element to insulin in the human *APOA5* promoter spanning the differential region from –146 to -61. This response element is hypothesized to be responsible for the transrepression of *APOA5* gene expression.

The insulin's regulatory region in the *APOA5* promoter contains a sequence 5'-CAC GTG-3' strongly homologous with the consensus E-box. The nuclear extracts from insulin treated HepG2 cells and controls were tested in electrophoretic mobility shift assay using an *APOA5* probe containing the E-box. The results showed a decrease of the binding with insulin treated extracts (Fig. 5A). Since the ubiquitinous Upstream Stimulatory Factors (USFs) were initially identified by their ability to bind to the E-box, we investigated whether USFs are involved in the *APOA5* gene regulation. For this, we tested whether the band observed in the gel shift assay with the nuclear extract is affected by addition of specific anti-USF antibodies. As shown in Figure 5A, the anti-USF1 or anti-USF2 antibodies disrupted the band and resulted in a supershift. These data strongly support that USF1 and USF2 are involved in the regulation of the *APOA5* gene at the transcriptional level. These data were confirmed by using the translated protein USF1 *in vitro* which binds to the *APOA5* E-box. When the *APOA5* E-box is mutated, the binding with either USF1 or nuclear extracts is abolished (Fig.5B).

To determine whether the binding of USF1 to *APOA5* promoter is functional, we analyzed the promoter activity in transiently co-transfected HepG2 cells with the –304/+63 fragment of the human *APOA5* promoter and USF1. We observed that USF1 clearly increased the promoter activity by 8.5-fold (Fig. 6). This increase by USF1 is affected when insulin is added.

To study the signaling pathway mediating the down-regulation of the *APOA5* gene expression by insulin, HepG2 cells were treated with wortmannin (200nM) , an inhibitor of the PI 3-kinase pathway, followed by the addition of insulin (10nM) after 30 minutes. The

290 results showed that the down-regulation of the *APOA5* gene by insulin (10 nM) was totally
291 abolished in the presence of wortmannin (Fig. 7). These data suggest that the inhibitory effect
292 of insulin on *APOA5* gene expression involves a phosphorylation mechanism via the PI3K
293 signaling pathway.

294 To investigate the phosphorylation state of USF that binds to the *APOA5* promoter,
295 nuclear extracts were isolated from HepG2 cells and incubated with the oligonucleotide probe
296 containing the *APOA5* E-box. After incubation with streptavidin-sepharose beads, the
297 oligoprotein complex and the supernatant were analysed by western blot using anti-USF1 and
298 anti-phosphothreonine or anti-phosphoserine antibodies. The results demonstrated that a large
299 amount of USF1 binds the *APOA5* E-box (Fig. 8, left lane) and a small amount of USF1,
300 which did not bind to *APOA5* E-box, was detected in the supernatant (Fig. 8, right lane). The
301 analyses with anti-phosphoserine (data not shown) and anti-phosphothreonine do not
302 recognize the USF1 which binds to the *APOA5* E-box (Fig. 8, left lane) but the USF1 in the
303 supernatant was detected with anti-phosphthreonine (Fig. 8, right lane). These data indicate
304 clearly that in HepG2 cells a large quantity of USF1 is not phosphorylated, this is consistent
305 with what has been reported before (13) and that the phosphorylated form of USF1 does not
306 bind to *APOA5* E-box.

307 An insulin clamp study in humans was carried out to analyze the effect of insulin on
308 human ApoAV levels *in vivo* (Fig. 9). In this case protein levels were examined with a human
309 ApoAV antibody since liver RNA was not easily collectable. For this study, twelve healthy
310 men were recruited and insulin was infused in a continuous manner at a rate of 1 mU/Kg/min
311 over 8 hours. The glycemia was maintained at normal level by adjusting the rate with a 20%
312 glucose infusion. This experimentally induced hyperinsulinemia significantly reduced serum
313 ApoAV protein level at 3 h 30 and 8 h by 52 and 72%, respectively.

DISCUSSION

Interest in this topic stems from the clinical observations showing that hyperinsulinemia and insulin resistance are associated with hypertriglyceridemic state and the fact that the *APOA5* gene is negatively correlated with triglyceride levels. We examined the regulation of *APOA5* gene expression by insulin and found a significant decrease. These studies used an *in vitro* model for further investigation of the molecular mechanism involved in this regulation, and were further supported by our observations *in vivo* in humans and mice.

HepG2 cells were chosen for the study because they express the *APOA5* gene as well as the insulin receptor. When HepG2 cells were exposed to high insulin concentrations (10 nM), it reduced significantly endogenous human *APOA5* mRNA. This *APOA5* gene down-regulation was confirmed in human and rat primary hepatocytes and also in mice models. Insulin has previously been shown to regulate the expression of several genes including some apolipoproteins, such as apolipoprotein A1 (*APOA1),* apolipoprotein A4 (*APOA4)* and apolipoprotein C3 (*APOC3)*. We confirmed that rat *apoc3* gene expression is nearly 4-fold decreased in our experiments (4, 12). It is well established that the plasma concentration of ApoCIII is positively correlated with levels of plasma triglycerides (18, 28) and its decrease by insulin could not explain the cause of hypertriglyceridemia associated with the hyperinsulinemia.

This study shows that the repression of *APOA5* gene regulation by insulin occurs at the transcriptional level. The promoter activity of human *APOA5* in transfected HepG2 cells is decreased in a dose-dependent manner. Deletion analyses of the promoter and the transcriptional activity studies allowed the identification of a 85 bp DNA fragment likely to contain an element responsive to insulin.

Insulin regulates many genes at the transcriptional level, but the molecular mechanism responsible for this regulation is hampered by the fact that there is no consensus insulin

response element that can account for the regulation of all insulin-responsive genes (16). The analysis of the 85 bp candidate insulin responsive fragment allowed for the selection of 30 bp fragment containing a putative E-box which could potentially explain *APOA5's* responsiveness to insulin. This 30 bp fragment was used in electrophoretic mobility shift assay of nuclear extracts from HepG2 cells treated or not with insulin. The nucleoproteins binding to the probe from cells treated in hyperinsulinemic conditions were quantitatively less important than those from control cells. The 30 bp element responsive to insulin of the *APOA5* promoter contains an E-box identical to the consensus motif. Results obtained with electrophoretic mobility supershift assay clearly show a binding of Upstream Stimulatory Factors, USF1 and USF2, to the E-box present in the *APOA5* promoter. An other known insulin's action on the Fatty Acid Synthase (FAS) gene functionally requires USF binding to the E-box (4, 32, 40). USF proteins, USF1 and USF2, are members of basic helix-loop-helix leucine-zipper family of transcription factors able to interact as homo- and/or heterodimers on E-box of 5'-CANNTG-3' sequence. The binding of the heterodimer USF1/USF2 is partially inhibited when cells were treated with 10 nM of insulin. Indeed, in normal conditions, USF1 and USF2 appear to bind as heterodimers to the E-box motif and allow the basal regulation of the *APOA5* gene. The transfection assay showed that USF1 increased significantly the *APOA5* promoter activity and this increase is affected when insulin is added. The repression of the *APOA5* gene by insulin may occur via an exclusion of the binding of transcriptional factors USF1 and USF2 to DNA. We addressed the question how insulin's action may inhibit the binding of USF1/USF2 to *APOA5* promoter. Many metabolic actions of insulin are mediated through pathways which include the activation of phosphatidylinositol 3-kinase (PI3K) and, consequently, the activation of its downstream target, the protein kinase B/Akt (PKB/Akt) (7, 39). We showed that the repression of the *APOA5* gene by insulin is completely abolished in the presence of wortmannin, an inhibitor of PI3K. Indeed, when the PI3K pathway is blocked, the regulation of *APOA5* gene remains unchanged at its basal level.

These data taken together with the binding decrease of USF1/USF2 to *APOA5* promoter provide evidence that phosphorylation/dephosphorylation could modulate the ability of USF to bind DNA and in fact their transactivation function. The oligoprecipitation of USF from HepG2 nuclear extracts followed by immunoblot analyses with anti-USF1 and antiphosphothreonine showed that USF1 which binds E-box *APOA5* is not phosphorylated and exists in a large amount in HepG2 cells, however, only a small amount of USF1 is phosphorylated on threonines and this phosphoprotein failed to bind to E-box *APOA5*. By contrast, it has been demonstrated that the phosphorylated form of USF1 *in vitro* bound DNA preferentially to transactivate certain genes (6, 13).

Our data suggest a potential mechanism where insulin stimulates the PI3K pathway inducing the phosphorylation and binding exclusion to *APOA5* promoter of the transcriptional activator USF and thereby resulting in the down-regulation of *APOA5* gene expression (Fig. 10). The insulin treatment could induce a phosphorylation of certain amino acids in the helix-loop-helix DNA binding domain of USF, thereby inhibiting its binding to *APOA5* promoter. It has been reported that the insulin stimulation induces translocation, phosphorylation and activation of two isoforms of PKC, PKC-beta2 and PKC-zeta (3) and that these effects are PI3K-dependents. The phosphorylation site prediction for the helix-loop-helix DNA binding domain of USF1 by PKC shows two potential threonines, T195 and T234, and two potential serines, S194 and S233. The phosphorylation of these sites could probably alter the binding of USF1 to DNA.

We assessed also the effect of the insulin in healthy men using an euglycemic hyperinsulinemic clamp study. The data showed a decrease of plasma ApoAV protein level in a short time following treatment. The ApoAV enhances the triglyceride rich particles catabolism (F.G. Schaap, P.J. Voshol, P.C Rensen, H.N. van der Vliet, R.A. Chamuleau, N.M. Maeda, L.M. Havekes, A.K. Groen and K.W. van Dijk, Abstr. American Heart Association, abstr. 1215, 2003 and I. Grosskopf, N. Baroukh, S-J. Lee, E.M. Rubin, L.A.

Pennacchio and A.D. Cooper, Abstr. American Heart Association, abstr. 1217, 2003). In our study, the triglyceride levels were not yet affected within this short time but the early decrease of ApoAV could initiate a delay of the catabolism of the triglyceride rich particles resuling in their accumulation in the plasma and therefore, exhibiting hypertriglyceridemia.

In summary, our *in vitro* and *in vivo* studies demonstrated that insulin decreases *APOA5* gene expression in human and rodents. This down-regulation occurs at the transcriptional level and is mediated by the PI3K signaling pathway. Binding study allowed us to underline the importance of USFs as transcriptional factors required for the regulation of the *APOA5* gene through a phosphorylation/dephosphorylation mechanism. Moreover, we have shown that insulin decreases ApoAV protein level in humans after insulin administration.

These results suggest that the down-regulation of *APOA5* gene by insulin could contribute to the development of hypertriglyceridemia associated with hyperinsulinemia.

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FIGURE LEGENDS

Figure 1. Effects of insulin on *APOA5* gene expression in HepG2 cells, human primary hepatocytes, and rat primary hepatocytes (A) and dose-dependent down-regulation of *apoa5* (B) and *apoc3* (C) gene expression by insulin.

After 24 h of treatment of different cells with 10 nM of insulin, total RNA was isolated and subjected to a quantitative PCR using *36B4* as normalizing gene (Fig. 1A). The amplification was obtained with species specific primers. Fig 1B and 1C shown insulin's down-regulation of respectively *apoa5* and *apoc3* gene expression after a dose-dependent treatment of rat primary hepatocytes. Fold decrease of *APOA5* gene expression are shown (black bars) versus control (white bar) (means ± SD, n=3). Statistically significant differences between groups versus control were obtained with a student t-test and are indicated by *asterisks* (*:0.01<p<0.05, **:0.0001<p<0.01, and ***:p<0.0001).

Figure 2. Effects of insulin on mouse *apoa5* gene expression *in vivo* in C57/BL6 mice.

Mice were injected intraperitoneally respectively with 0.5 U/Kg or 1 U/Kg of insulin (black bars), or vehicle as control (white bars). Total RNA from liver was extracted 6 h after injection and subjected to a quantitative PCR using *36B4* as normalizing gene. Fold decrease are shown (means ± SD, n=6). Statistically significant differences between groups versus control were obtained with a student t-test and are indicated by *asterisks* (*:0.01<p<0.05, **:0.0001<p<0.01, and ***:p<0.0001).

Figure 3. Effect of insulin on the human *APOA5* promoter activity.

HepG2 cells were transfected with empty reporter plasmid (striped bars) or the -304/+63 human *APOA5* promoter fragment reporter plasmid (full bars). After 2 h of transfection, cells were treated with different concentrations of insulin (0, 10, 100 or 1000 nM). Cells were lysed after 24 h of treatment and the luciferase activity was measured. Results were expressed in RLU (means ± SD, n=3). Experiments were repeated at least 3 times. Statistically significant

differences between groups versus control were obtained with a student t-test and are indicated by *asterisks* (*:0.01<p<0.05, **:0.0001<p<0.01, and ***:p<0.0001).

Figure 4. Deletion of the human *APOA5* promoter and effects of insulin on the promoter activity.

Schematic illustration of the reporter constructs used in the transfection studies. Boxed and bold sequence represents the E-box. HepG2 cells were transfected with different human *APOA5* promoter fragments reporter plasmids, and treated with the maximal concentration of insulin (1 µM). After 24 h of treatment, the luciferase activity was measured. White bar represents the control without insulin and black bars represent the promoter activity after a treatment with insulin 1 µM. Fold induction are shown (means ± SD, n=3). Experiments were repeated at least 3 times.

Figure 5. Electrophoretic mobility shift assays of nuclear extracts from HepG2 cells.

Each reaction (20 µL) contained 2.5 µg of nuclear extracts from HepG2 cells control or insulin 10 nM treated, 1 X gel shift reaction buffer, 2.5 µg of poly(dI-dC), 1 µg of herring sperm DNA and 1 µL of [γ-^{32}P]ATP-labeled probe containing the *APOA5* E-box. For supershift assay, 1 µg of anti-USF1 or 1 µg of anti-USF2 antibodies was added in the reaction mix. After incubations on ice, samples were applied to a 5% nondenaturing polyacrylamide gel. The gel was dried and exposed to a film with an intensifying screen.

Figure 6. Effect of insulin on the human *APOA5* promoter activity co-transfected with USF1

HepG2 cells were co-transfected with the -304/+63 human *APOA5* promoter fragment reporter plasmid and the USF1 expression plasmid. After 2 h of transfection, cells were treated with 1 µM of insulin. Cells were lysed after 24 h of treatment and the luciferase activity was measured. Results were expressed in fold induction (means ± SD, n=3). Experiments were repeated at least 3 times.

Figure 7. Effect of wortmannin on down-regulation of *APOA5* gene expression by insulin.

HepG2 cells were incubated with 10 nM of insulin (black bar) or with wortmannin 200 nM 30 min before adding 10 nM of insulin (gray bar). The same volume of vehicle was used as control (white bar). After 24 h of treatment, total RNA was isolated from the cells and subjected to a quantitative PCR using *36B4* as normalizing gene. Fold induction of *APOA5* gene expression are shown (means ± SD, n=3). Experiments were repeated at least 3 times. Statistically significant differences between groups versus control were obtained with a student t-test and are indicated by *asterisks* (*:0.01<p<0.05, **:0.0001<p<0.01, and ***:p<0.0001).

Figure 8. **Phosphorylation state of bound USF to E-box *APOA5***.

Western blot of oligoprecipitated USF1 (left lane) from nuclear extracts and the oligoprecipitation's supernatant (right lane). Experiment was performed using 10 µg of nuclear extracts from HepG2 cells. Immunoblot analyses were done using anti-USF1 or anti-phosphothreonin as primary antibody.

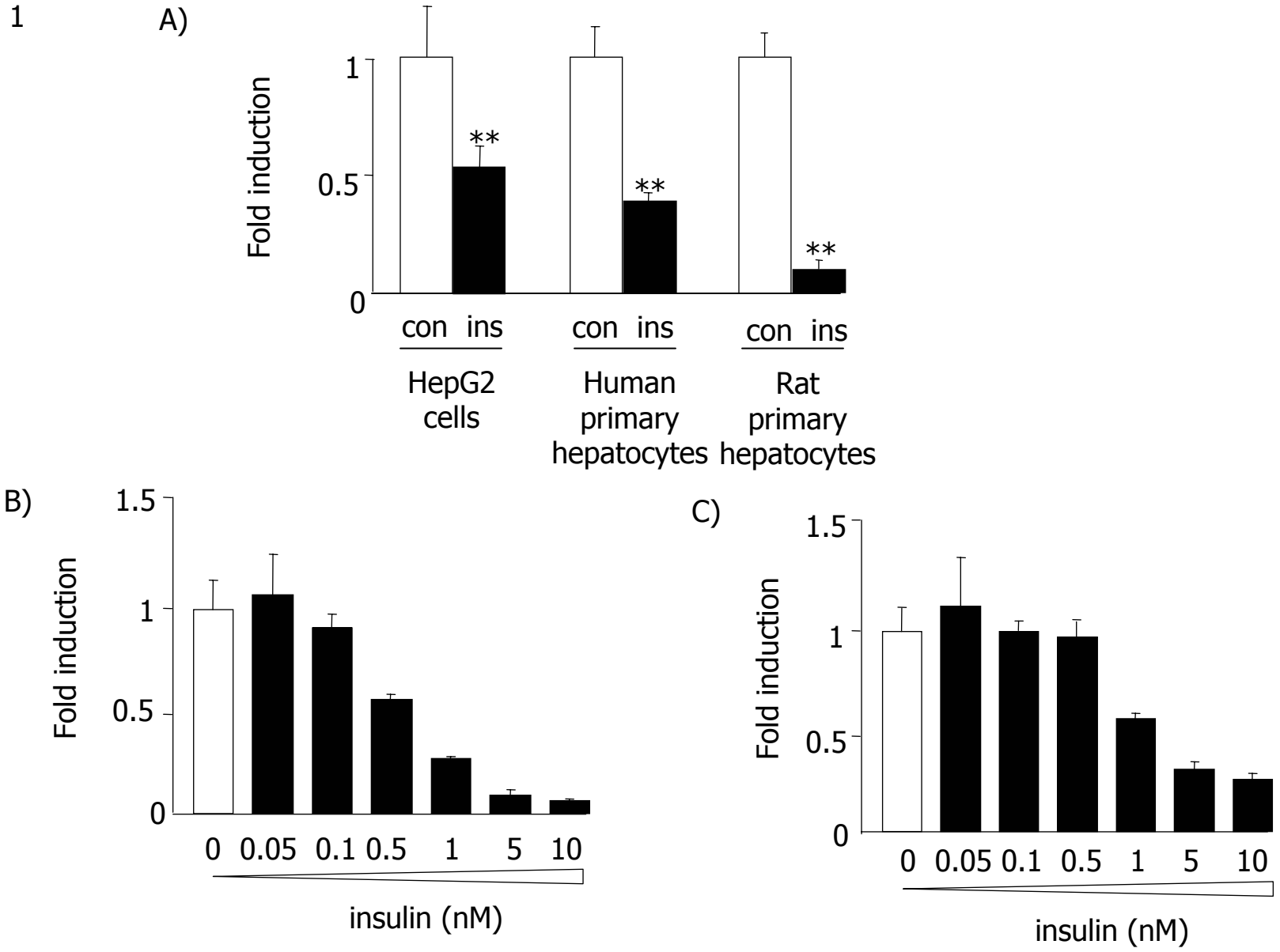
Figure 9. Effects of insulin on the plasma ApoAV protein level in humans.

Plasma ApoAV protein level was measured in humans after infusion of insulin (full line) or saline (dotted line). Insulin was infused at a rate of 1 mU/Kg/min for 8 h. Blood samplings of each subjects was collected at different times. The first sample was collected 30 min before the beginning of insulin or saline infusion, the second sample was collected just after the start of infusion. The third and the fourth samples were collected 3 h 30 and 8 h respectively after the infusion. Results were done in means ± SD, n=12. Statistically significant differences between groups versus control were obtained with a student t-test and are indicated by *asterisks* (*:0.01<p<0.05, **:0.0001<p<0.01, and ***:p<0.0001).

Figure 10. A proposed model for the roles of USF1 and USF2 in the regulation of *APOA5* gene transcription.

Under insulin, the PI3K pathway is stimulated inducing a phosphorylation of USF. The phosphorylated complexes lose their ability to bind *APOA5* promoter and consequently their transactivation function.

Figure 1


A)
Fold induction
1
0.5
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con ins
con ins
con ins
HepG2 cells
Human primary hepatocytes
Rat primary hepatocytes
B)
Fold induction
1.5
1
0.5
0
0 0.05 0.1 0.5 1 5 10
insulin (nM)
C)
Fold induction
1.5
1
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0
0 0.05 0.1 0.5 1 5 10
insulin (nM)

Figure 2

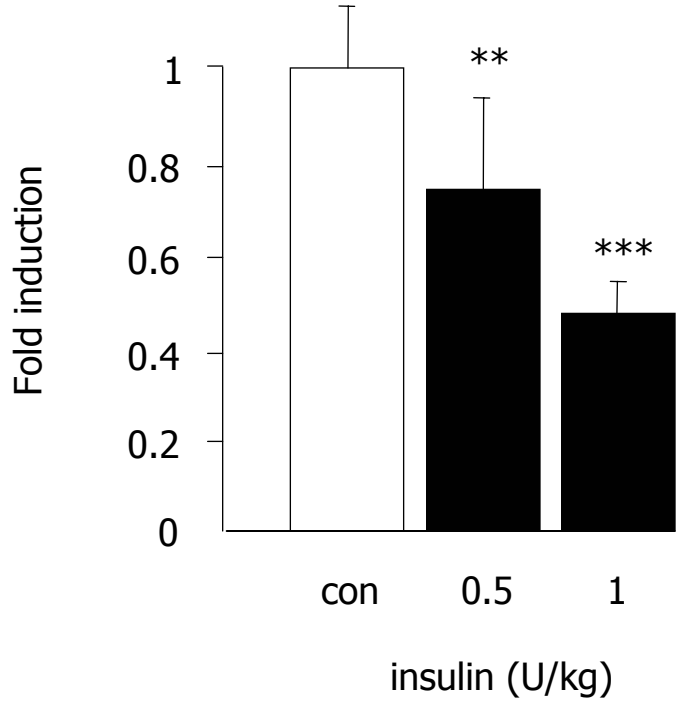


Figure 3

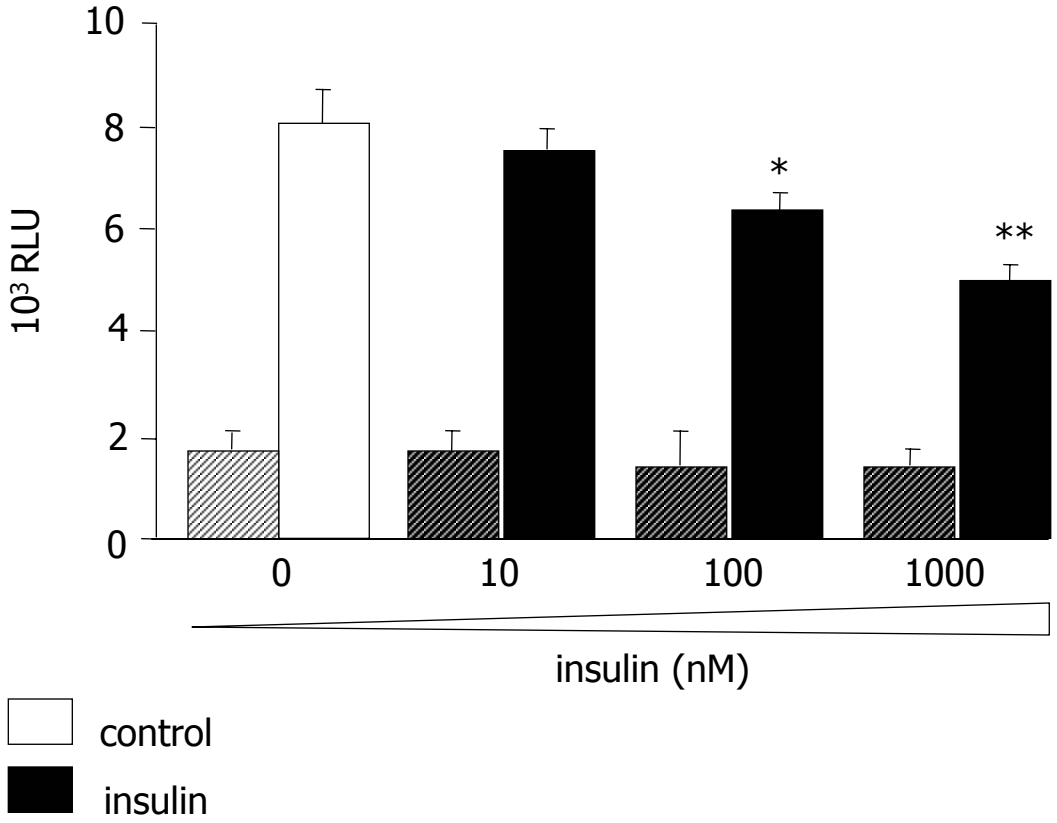


Figure 4

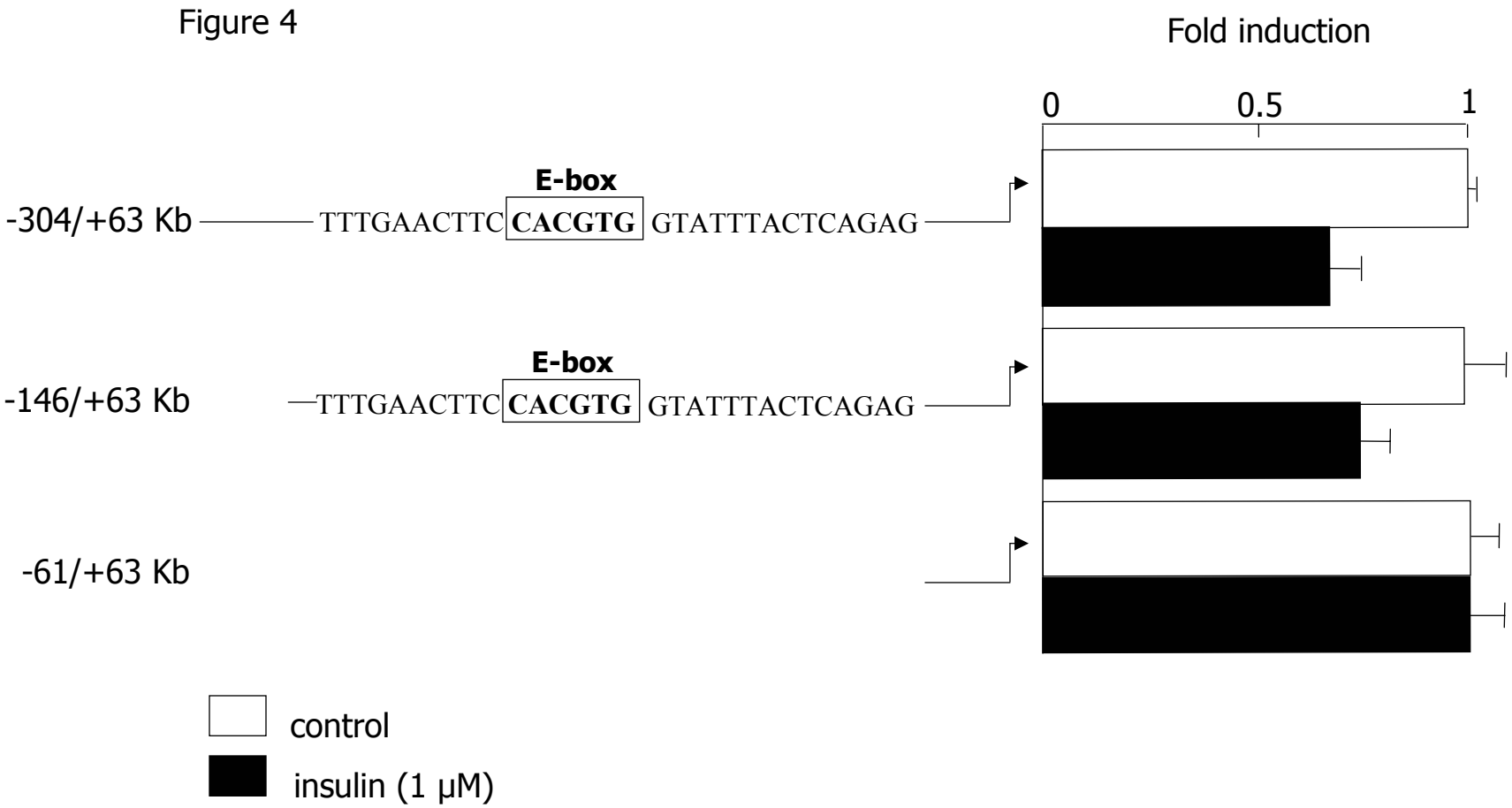

Fold induction
0
0.5
1
E-box
-304/+63 Kb
TTTGAACTTC CACGTG GTATTTACTCAGAG
E-box
-146/+63 Kb
TTTGAACTTC CACGTG GTATTTACTCAGAG
-61/+63 Kb
control
insulin (1 μM)

Figure 5

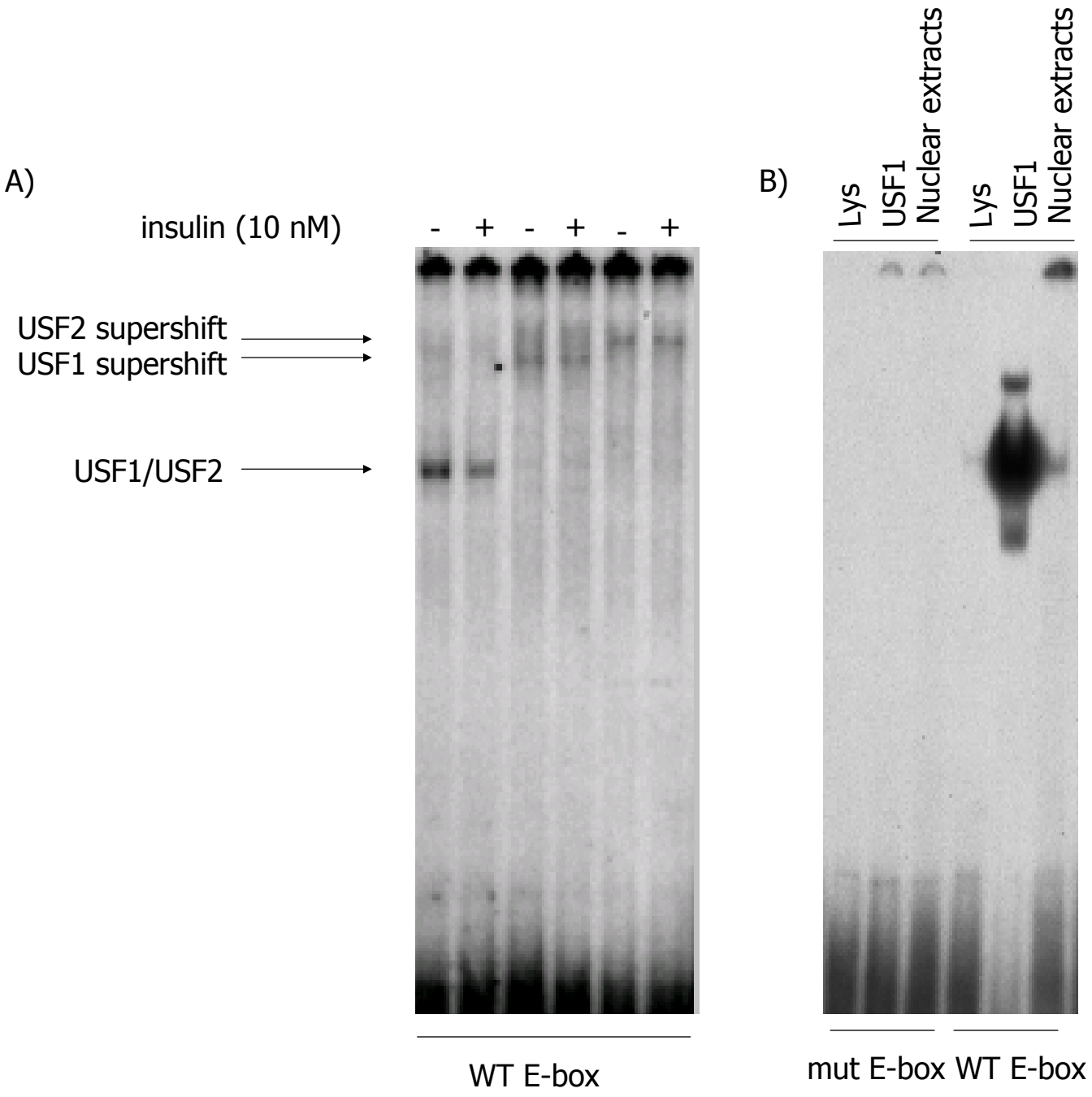

A)
insulin (10 nM)
- + - + - +
USF2 supershift
USF1 supershift
USF1/USF2
WT E-box
B)
Lys
USF1
Nuclear extracts
Lys
USF1
Nuclear extracts
mut E-box
WT E-box

Figure 6

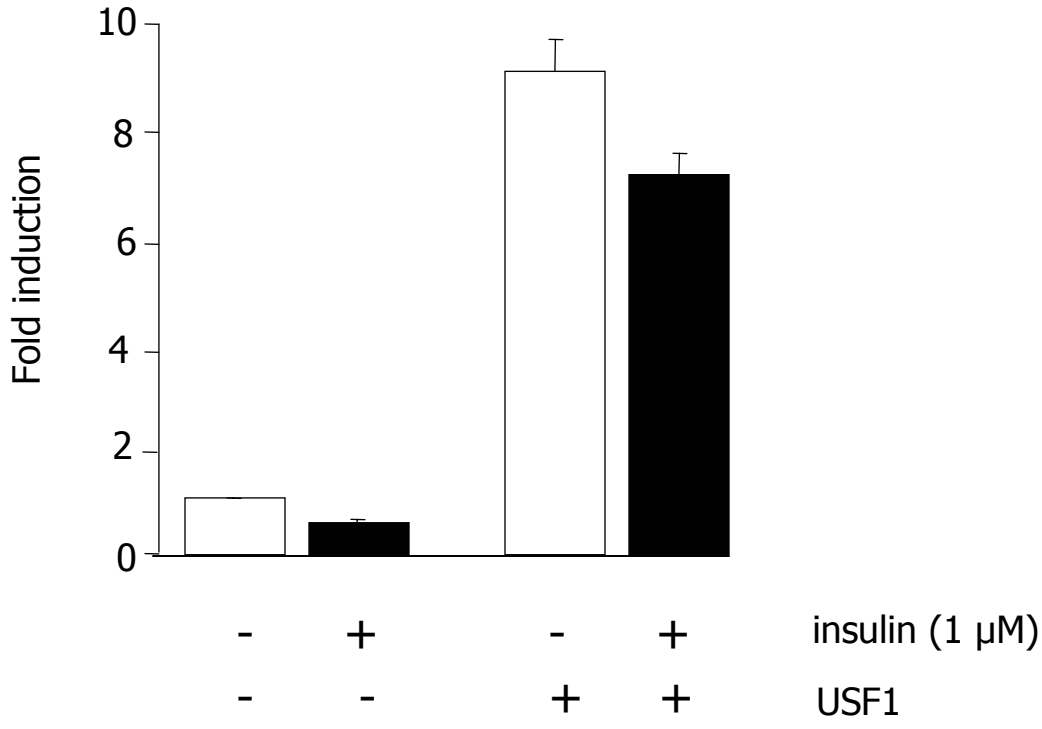


Figure 7

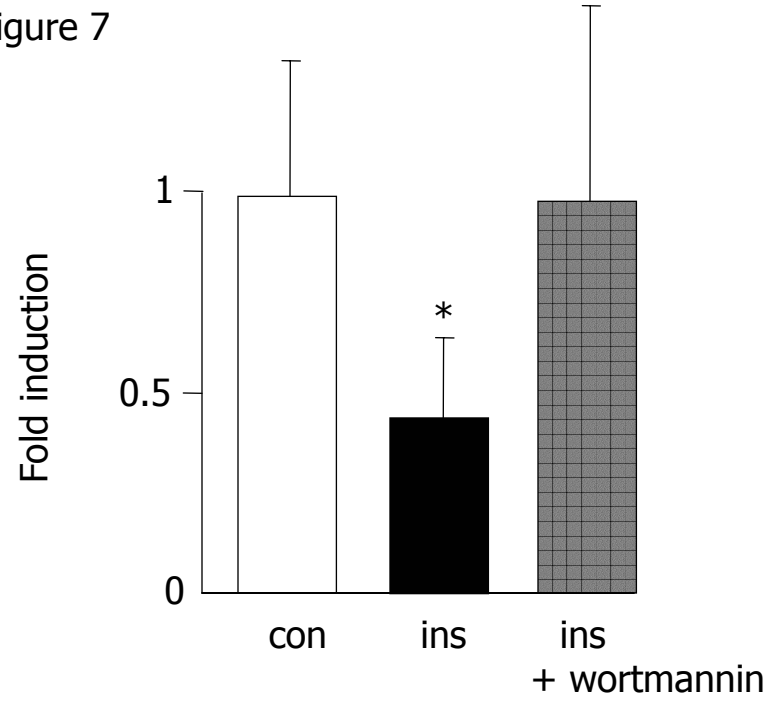


Figure 8

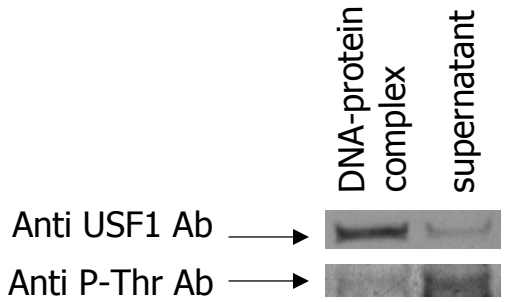


Figure 9

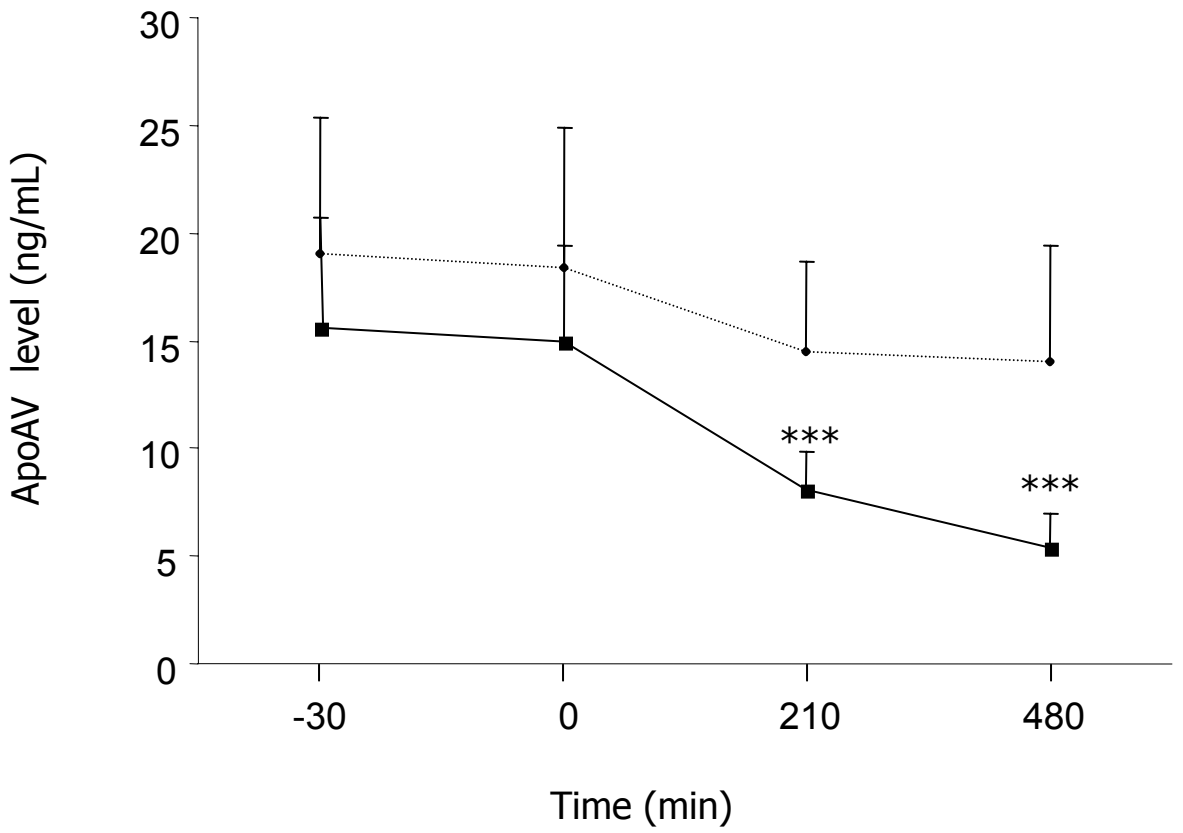
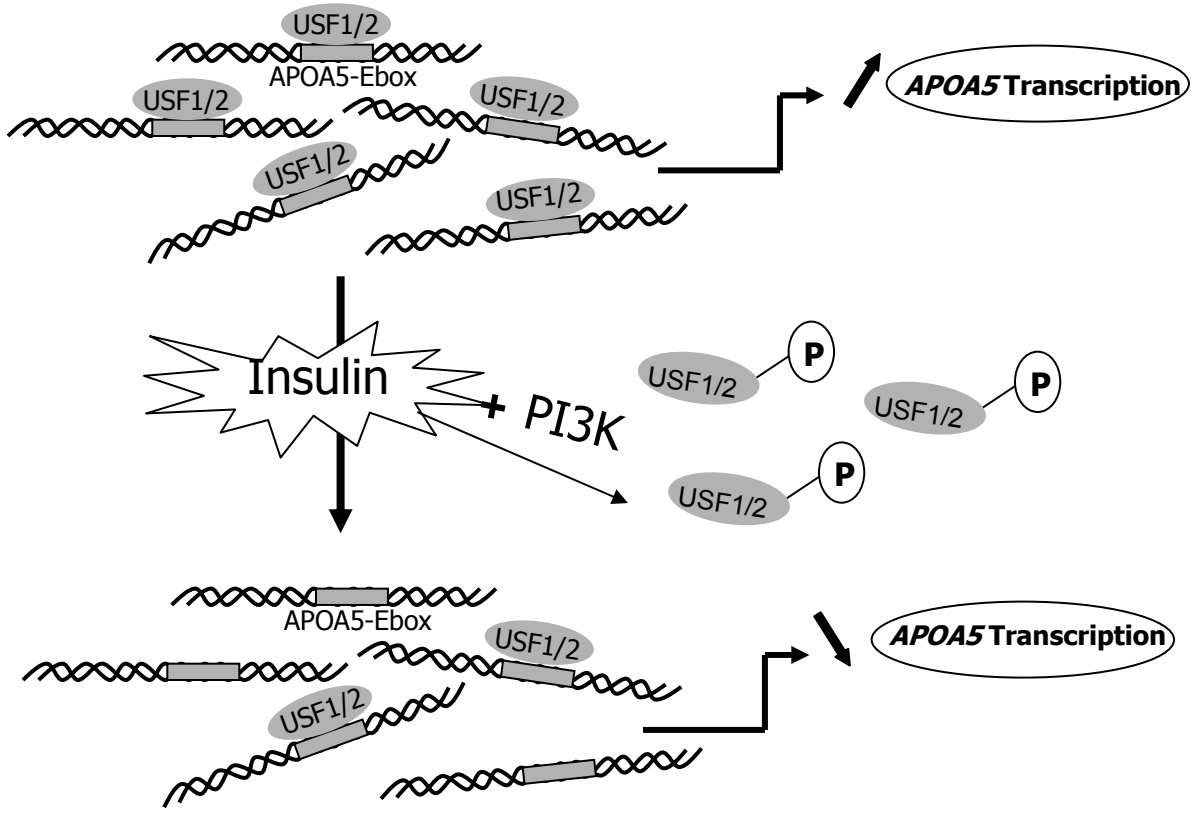


Figure 10


USF1/2
APOA5-Ebox
USF1/2
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USF1/2
USF1/2
APOA5 Transcription
Insulin
+ PI3K
USF1/2
P
USF1/2
P
USF1/2
P
APOA5-Ebox
USF1/2
USF1/2
APOA5 Transcription