Glucose Regulates The Expression Of The Apolipoprotein A5 Gene

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Running title: *APOA5* gene regulation by glucose

Abbreviations: *APOA5*, the human apolipoprotein A5 gene; *apoa5*, the rodent apolipoprotein A5 gene; apoA-V, the human protein; PI3K, the phosphatidylinositol 3-kinase; PP1/PP2A, the protein phosphatases 1/2A; USF, the upstream stimulatory factor; SREBP-1c, the sterol response element binding protein-1c

ABSTRACT

The apolipoprotein A5 gene (*APOA5)* is a key player in determining triglyceride concentrations in humans and mice. Since diabetes is often associated with hypertriglyceridemia, this study explores whether *APOA5* gene expression is regulated by alteration in glucose homeostasis and the related pathways. D-glucose activates *APOA5* gene expression in a time- and dose-dependent manner in hepatocytes, and the glycolytic pathway involved was determined using D-glucose analogs and metabolites. Together, transient transfections, electrophoretic mobility shift assays and chromatin immunoprecipitation assays show that this regulation occurs at the transcriptional level through an increase of USF1/2 binding to an E-box in the *APOA5* promoter. We show that this phenomenon is not due to an increase of mRNA or protein expression levels of USF. Using protein phosphatases 1 and 2A inhibitor, we demonstrate that D-glucose regulates *APOA5* gene *via* a dephosphorylation mechanism, thereby resulting in an enhanced USF1/2-promoter binding. Last, subsequent suppressions of USF1/2 and phosphatases mRNA through siRNA gene silencing abolished the regulation. We demonstrate that *APOA5* gene is up regulated by D-glucose and USF through phosphatase activation. These findings may provide a new cross talk between glucose and lipid metabolism.

INTRODUCTION

Apolipoproteins play a central role in lipid homeostasis and are intimately associated with the cardiovascular disease risk [1; 2]. Recently, the gene coding for a new apolipoprotein family member, *APOA5*, was identified to be an important determinant of plasma triglyceride levels in both humans and mice [3; 4; 5]. Data from mice overexpressing or lacking *APOA5* provide direct evidence that apolipoprotein A5 is inversely correlated with plasma triglyceride levels. In humans, polymorphisms across the *APOA5* locus have been associated with elevated plasma TG concentrations [3; 6], familial combined hypertriglyceridemia [7] and increased risk of cardiovascular disease [8; 9]. Mechanism underlying the hypotriglyceridemic effect of apoA-V protein was evaluated. ApoA-V appears to activate LPL-mediated VLDL-TG hydrolysis, thus accelerates VLDL clearance, and reduces plasma TG by inhibiting hepatic VLDL-TG production [10; 11; 12]. Taken altogether, these data suggest that *APOA5* could be a strong modulator of triglyceride levels. Therefore, studying the regulation of its gene expression is of significant clinical importance for treatment of dyslipidemia. Little is known regarding the *APOA5* gene regulation, although previous studies showed that different transcription factors implicated in triglyceride metabolism regulate the *APOA5* gene. Indeed, PPARα, FXR and RORα [13; 14; 15; 16] induce the *APOA5* gene at the transcriptional level. Moreover, we published that the LXR ligand T0901317 down-regulates human *APOA5* gene expression through an indirect mechanism involving SREBP-1c [17]. Last, we reported that insulin inhibits the *APOA5* gene expression through the activation of the phosphatidylinositol 3-kinase (PI3K) signalling pathway [18]. This work showed for the first time the phosphorylation-dependent binding of USF1/2 to a functional E-box element within the *APOA5* promoter. Interestingly, in agreement with our study, Prieur et al. [19] have reported that the thyroid hormone regulates the hypotriglyceridemic gene *APOA5* through a synergistic activation with USF. Their report combinated with ours raises the possibility of a cross talk between insulin and thyroid signals on the same element in *APOA5*.

The leading cause of death for diabetes is cardiovascular diseases [20; 21]. Diabetes is a highly complex pathology inducing perturbation in glucose homeostasis, and hypertriglyceridemia is often associated with this metabolic disease. Glucose has been implicated as an independent signal in homeostasis of carbohydrate and fatty acids for activating the synthesis of glycolytic and lipogenic enzymes. In liver, adipocytes and β-cells, glucose is metabolized to generate an intracellular signal that allows for transcriptional regulation of metabolic genes. The metabolic pathway involved in the glucose-mediated gene regulation is variable, and glucose analogs or metabolites could mimic the glucose response [22; 23; 24; 25]. Recent studies have identified transcription factors such as USF [26; 27], SREBP1-c [28; 29], COUP-TFII [30], and ChREBP [31; 32; 33], as mediators of glucose-induced gene regulation. However, the mechanism by which glucose regulates the transcriptional activity of these factors is still unclear.

Because we have recently demonstrated the specific implication of USF1/2 in *APOA5* gene regulation by insulin [18], and a forced overexpression of these transcription factors resulted in an important increase of the *APOA5* transactivation, we have explored the role of USF1/2 in this gene regulation after a D-glucose treatment, and the related signalling pathway. Our results indicate that USF1/2 bind directly to the promoter of the *APOA5* gene in a glucose-dependent manner and are absolutely required for its induction by D-glucose. USF1/2 are therefore potentially involved in the regulation of genes during alteration in glucose homeostasis and metabolic disorders, and could contribute to the development of modulation in triglycerides levels.

RESULTS

D-glucose increases *APOA5* gene expression in a time- and dose-dependent manner in human hepatoma cells and primary rat hepatocytes.

To determine whether D-glucose can modulate *APOA5* gene expression, human hepatoma HepG2 and HuH7 cells were treated with D-glucose under several different conditions as described in Figure 1. The *APOA5* mRNA levels were increased in a time-dependent manner to reach a maximum of 2.5-fold after 48h treatment with 25mM D-glucose (Fig. 1A). Moreover, this up-regulation was found to be D-glucose dose-dependent (Fig. 1B) with a maximal stimulatory effect observed with 25mM of D-glucose. Results were confirmed in primary rat hepatocytes treated with 25mM of D-glucose for 24h and 48h (Fig. 1C). Thus, human and rat *APOA5* gene expression were significantly increased after treatment with 25mM of D-glucose for 48h.

Identification of the glycolytic pathway involved in the regulation of *APOA5* gene expression.

To explore the metabolic pathway involved in the D-glucose response, HuH7 cells were treated with several glucose analogs and metabolites (Fig. 2). Compounds were added for 48h in a culture medium without D-glucose. L-Glucose, a non-metabolizable sugar, did not influence the expression of *APOA5* gene, indicating that intracellular processing of D-glucose is needed to promote *APOA5* induction. To analyse whether the metabolites of the glycolytic pathway stimulate *APOA5* expression, Lactate, Pyruvate, and Glycerol were tested. None of these molecules increases *APOA5* mRNA levels, thus indicating that the induction of *APOA5* is mediated by a glucose-metabolite generated during glycolysis and upstream to triose phosphate pathway. To examine whether the pentose pathway is implicated in the D-glucose-dependent induction of *APOA5*, we treated cells with xylitol, a precursor of xylulose-5-phosphate, an intermediate of the non-oxidative branch of the pentose phosphate pathway. Xylitol was unsuccessful in restoring *APOA5* gene induction. To verify whether the hexosamine pathway is implicated in this up-regulation, cells were treated with azaserine, an inhibitor of glutamine/fructose-6-phosphate amidotransferase, the rate-limiting enzyme in the conversion of glucose to glucosamine. The induction of *APOA5* expression by 25mM D-glucose was not inhibited by azaserine, suggesting that the hexosamine pathway is not implicated. Last, to evaluate whether analogs could mediate the D-glucose *APOA5* up-regulation, we tested D-fructose and D-mannose and we observed that both sugars restored the D-glucose dependent-induction of *APOA5*. Fructose is phosphorylated to fructose-1-P (F-1-P) and this metabolite split into glyceraldehyde (GAD) and dihydroxyacetone phosphate (DHAP). Both sugars give glyceraldehydes-3-P (G3P). Moreover, fructose can be phosphorylated into fructose-6-P (F-6-P) and then metabolized to reach G3P. In the glycolysis pathway, D-glucose is phosphorylated to glucose-6-phosphate (G-6-P) by glucokinase and a specific isomerase change G-6-P to F-6-P. Because D-mannose enters glycolysis at the F-6-P step and pyruvate does not mimic the D-glucose response, we conclude that the regulation of *APOA5* occurs through a metabolite during the glycolysis between G3P and pyruvate. A last treatment was done with D-mannitol as control to demonstrate that this not an osmotic effect. D-mannitol, which is not transported into cells, did not mediate the D-glucose effect.

D-glucose regulates *APOA5* gene at the transcriptional level and requires a functional E-box in the promoter.

We performed transient transfections of HepG2 cells to determine if the induction of *APOA5* by D-glucose occurs at the transcriptional level. We observed that 25 mM of D-glucose increases the promoter activity in a significant manner after 48h (Fig. 3A). We controlled

experiences using transient transfections with the *L-PK* glucose-responsive promoter. To underline the presence of a response element in the promoter, 5'-deletion analyses of the human *APOA5* promoter were performed (Fig. 3B). We showed that the promoter activity was increased by 25mM D-glucose when cells were transfected with both –304/+63 and –146/+63 fragments. However, D-glucose has no effects when cells were transfected with the –61/+63 fragment. Moreover, when cells were transfected with a reporter plasmid driven by the *APOA5* promoter fragment –146/+63 in which E-box was mutated (5'-CCCGCG3-3'), *APOA5* gene was not regulated by D-glucose at the transcriptional level. These results confirm the presence, the functionality and the requirement of the E-box element 5'-CACGTG-3' located at –76/-81 in the promoter.

D-glucose increases the USF1/2-DNA complex in the human *APOA5* promoter.
Previously, we have demonstrated in our laboratory the binding of USF1 and USF2 [18] and SREBP-1c [17] to the *APOA5* promoter. Thus, we investigated whether these transcription factors are involved in *APOA5* regulation by D-glucose. We showed with EMSA that treatment of cells with D-glucose for 48h increased the binding of nucleoproteins to the *APOA5* E-box (Fig. 4A). Towards this goal, we tested the ability of the specific antibodies anti-USF1, anti-USF2, or anti-SREBP-1c to disrupt the binding from the nucleoproteins. Only anti-USF1 and anti-USF2 antibodies, but not anti-SREBP-1c, were able to disrupt the binding. Specificity of USF was confirmed by using *in vitro* transcribed and translated human protein. Moreover, we found that the mutated E-box-containing probe failed to bind USF. To characterize the specific binding of USF to the promoter *in vitro* and *in vivo* in cells after 48h D-glucose treatment, we performed respectively biotinylated oligoprecipitation assays using nuclear extracts followed by western blot analyses of USF1 (Fig. 4B) and ChIP assays followed by PCR amplification of *APOA5* gene (Fig. 4C). *In vitro* and *in vivo* association between USF1 and h*APOA5* promoter was evaluated after three independent experiments, and results demonstrate a two-fold increase after D-glucose treatment.

USF1 and USF2 mRNA and protein levels are not regulated by D-glucose treatment.
To study the effect of D-glucose on USF1 and USF2 gene expression, we evaluated USF1 and USF2 mRNA levels by quantitative RT-PCR after cell treatment with 5mM or 25mM D-glucose for 48h (Fig. 5A). We conclude that D-glucose does not regulate the expression of the USF1 and USF2 gene. Then, to verify the role of D-glucose on the USF1 and USF2 protein expression, we performed western blot analyses using specific anti-USF1 or anti-USF2 antibodies, and nuclear extracts from cell treated with 5mM or 25mM D-glucose for 48h (Fig. 5B). Quantifications of USF1 or USF2 are expressed in figure 5C as the USF1 or USF2 to β-actin protein ratio. In both immunoblots, no significant differences for protein expression appeared after D-glucose treatment. Thus, D-glucose does not regulate USF1 and USF2 mRNA and protein levels

Effect of okadaic acid on the D-glucose-mediated activation of *APOA5* gene.
Since D-glucose treatment did not influence the expression of USF, we investigated whether a phosphorylation/dephosphorylation mechanism is involved in the D-glucose signalling pathway (Fig. 6). Thus, we tested okadaic acid, a potent inhibitor of type 1 and 2A protein phosphatases (PP1/PP2A) the most abundant phosphatases present in the liver known to be implicated in the glucose effect. The *APOA5* gene expression was evaluated after D-glucose treatment in the presence of different concentrations of phosphatases inhibitor. Okadaic acid has no inhibitory effects in the lower concentration (0.1 nM), but suppress in part the *APOA5* gene regulation by D-glucose when it is used at 1 nM. At this concentration, only the PP2A phosphatase is inhibited. We conclude that D-glucose needs the PP2A activation to regulate the

expression of *APOA5* gene. Used at 10 nM the okadaic acid completely inhibits both PP1 and PP2A protein phosphatases resulting in the suppression of the D-glucose-mediated activation of *APOA5* mRNA levels. We show here that okadaic acid inhibits in a dose-dependent manner the D-glucose-mediated increase of *APOA5* gene expression. Thus, to increase the *APOA5* gene expression, D-glucose involved a dephosphorylation mechanism *via* the PP1/PP2A signalling pathway

Effect of USF1, USF2, PP1 and PP2A siRNA on *APOA5* gene expression.

To further examine whether transcription factors and protein phosphatases could mediate the D-glucose effect on *APOA5* gene regulation, we attempted to suppress the USF1, USF2, PP1 and PP2A gene products through RNA interference. With quantitative RT-PCR we demonstrated that mRNA levels for the target genes were significantly decreased (50%) after transfection with siRNA (Fig. 7A). Thus, HuH7 cells were transfected with siRNA to finally inhibit the expression of the respective mRNA (Fig. 7B). First, we controlled that *APOA5* mRNA levels were significantly increased after D-glucose treatment when cells were transfected with nonspecific control siRNA. Next, we showed that *APOA5* mRNA levels were not increased after transfection of cells with USF1 siRNA or USF2 siRNA. In conclusion, both transcription factors are required for *APOA5* gene regulation by D-glucose. Last, we used PP1 siRNA or PP2A siRNA to demonstrate the implication of these protein phosphatases in *APOA5* gene regulation by D-glucose. In both cases, *APOA5* mRNA levels were not increased after D-glucose treatment. Taken together, these results showed that D-glucose induced *APOA5* gene regulation *via* USF1/2 transcription factors and this regulation occurs through the PP1/PP2A protein phosphatases signalling pathway.

DISCUSSION

APOA5 is a major determinant of triglyceride homeostasis. This, coupled with the fact that diabetes-associated hypertriglyceridemia may in part be due to the hypertriglyceridemic *APOC3* gene overexpression [34], led us to explore the potential regulation of the recently identified *APOA5* gene by D-glucose. In the present study, we demonstrate that 25mM D-glucose increases *APOA5* gene expression in a time- and dose-dependent *in vitro* and our results suggest that a glucose-metabolite generated during glycolysis between G3P and triose phosphate could regulate the expression of *APOA5* gene. Since we found that insulin and the LXR ligand T0901317 down-regulate *APOA5* gene expression through USF phosphorylation and SREBP1-c activation respectively [17; 18], we studied here the specificity of the transcription factor. We clearly demonstrated that SREBP-1c is not involved and the novelty rests in the fact that the D-glucose treatment highly increases the USF1/2 binding to the *APOA5* promoter. After treatment, expressions of USF1/2 mRNA and protein levels were not modulated by D-glucose. Thus, in order to explain how D-glucose acts and increases the binding of these nucleoproteins, we investigated whether a post-translational modification of USF1/2 is involved in cell. Previously, we demonstrated that insulin down-regulates *APOA5* gene through the PI3K signalling pathway [18]. Since an activation of PI3K inhibits *APOA5* gene transcription, it follows that activation of phosphatases could stimulate *APOA5* gene transcription. Furthermore, we showed that only the non-phosphorylated form of USF could bind the *APOA5* E-box. Taken together, these results suggested that the association of USF1/2 to the promoter is mediated *via* a phosphorylation/dephosphorylation mechanism.

There is controversy about the involvement and the action of USF as transcription factor mediated in the glucose responsiveness. Findings suggest that USF regulation is complex, with possible cell-specific and species-specific regulation of USF proteins by glucose [35]. For example, although Kahn [26] claims that the binding of USF proteins to the GlRE is not modulated by nutritional conditions, others suggested that glucose and insulin regulate the USF protein-DNA interactions [36; 37; 38]. Moreover, the role of USF in the regulation of glucose-stimulated *L-PK* expression in hepatocytes and in insulin-secreting cells is unclear [39; 40; 41; 42]. Two groups showed contradictory results of the *L-PK* reporter enzyme activity using transient transfection of hepatocytes with USF and dominant-negative USF (DN-USF) [27; 31]. However, the expression levels of USF and DN-USF were not examined in either study [33]. To assess the role of USF1/2 in the regulation of *APOA5* gene expression, we used an inactivation strategy. Suppression of USF1 or USF2 by siRNA assays indicated that both transcription factors are linked and act in the heterodimer form to increase *APOA5* gene. Indeed, results suggested that when USF1 or USF2 is not synthesised, no regulation was observed. This could be explained, in part, by the fact that in physiological conditions, mutually USF1 or USF2 alone were not able to bind the *APOA5* promoter. Moreover, the major USF species present in most tissues and cell lines is the heterodimer of USF1 and USF2 [35]. In the same way, results obtained after suppression of PP1 or PP2A suggested that the *APOA5* gene is regulated *via* a dependent role of both protein phosphatases. Findings may prove that USF1 and USF2 could be dephosphrorylated and involved in the D-glucose regulation only when both PP1 and PP2A are present in cell. Taken together, we could hypothesize a potential activation of the protein phosphatases signalling pathway, which may induce the nuclear dephosphorylation of USF1/2 and consequently increase their binding to the promoter, resulting in the transactivation of *APOA5* gene expression.

Moreover, it has been well documented that glucose activates the nuclear translocation of an other transcription factor distinct from USF, named ChREBP, and suggested by Uyeda *et al.* [43; 44] as the regulator of the glucose responsiveness of the *L-PK* expression. Like us, authors proposed that ChREBP activity was regulated acutely in response to high glucose by a series of

phosphorylation/dephosphorylation reactions in hepatocytes, with two phosphorylation sites, the first located near the nuclear localization signal and the second near the DNA binding ability [24; 43]. So, it could be interesting to determine the phosphorylation sites present in USF1/2 transcription factors, that are involved in insulin and glucose response, to give more insights into the regulation of *APOA5* gene. Dentin et al. [45] demonstrated using siRNA that ChREBP is implicated in glucose signalling and regulation of *L-PK*, *FAS* or *ACC* gene expression in hepatocytes, but others published that this transcription factor does not act alone to regulate these genes. Indeed, Stoeckman et al. [46] demonstrated that ChREBP dimerize with Mlx (Max-like protein X) and this heterodimer is functional in regulating the expression of glucose-responsive genes. Then, Ma et al. [47] confirmed that ChREBP and Mlx dimerize and function together as the principal mediator of glucose-induced gene expression in the liver. Moreover, authors proposed the consensus ChoRE for ChREBP, consisting of two E-box motifs with the sequence 5'-CACGTG-3' separated by five base pairs. The ChREBP-Mlx heterodimer is unable to bind the E-box motif located at –76/-81 in the *APOA5* promoter whose the analysis shows the 5'-CACGTG$_5$TACTCA-3' sequence. Thus, this is not a correct ChoRE for ChREBP and this reinforces the specific implication of USF as transcription factor in the *APOA5* gene regulation by glucose.

In conclusion, we show here that D-glucose, through a metabolite generated at the beginning of glycolysis, activates the PP1/PP2A signalling pathway and increases the regulation of *APOA5* gene in cells. This activation of phosphatases mediates the dephosphorylation of USF1/2 transcription factors, and thus, their specific binding to the E-box element in the *APOA5* promoter, driving to the *APOA5* transactivation. These important new data strengthen the relation between *APOA5* and alteration in glucose homeostasis, metabolic disorder and modulation in triglyceride levels observed in the physiopathology of diabetes.

MATERIALS AND METHODS

Cell Culture and Transfection Assays.

Hepatoma HepG2 and HuH7 cells were grown in Dulbecco's modified Eagles's medium supplemented with 10% foetal calf serum, streptomycin/penicillin, sodium pyruvate, glutamine and nonessential amino acids (Invitrogene) at 37°C in a humidified 5% CO2 atmosphere. The medium was changed every 48h. Primary rat hepatocytes were isolated and cultured as described [18]. Before treatment cells were deprived for D-glucose during 18h. Human *APOA5* promoter fragments (residues -304/+63, -146/+63 and -61/+63) or *L-PK* glucose-responsive promoter were cloned in the pGL3 luciferase vector. Site-directed mutagenesis (Stratagene) for the E-box 5'-CACGTG- 3' located in the –146/+63 promoter fragment was done to obtained the mutated E-box 5'-C**C**CG**C**G-3' (mutated bases are indicated in bold). Transient transfection assays were performed using jetPEI cationic polymer transfection reagent (Q-biogene) and luciferase activity was measured by using the Mithras LB 940 plate reader (Berthold Technologies). Transfection efficiency was monitored by co-transfecting 300 ng of cytomegalovirus –driven β–galactosidase expression plasmid. For Okadaic acid treatment, inhibitor was added to culture media 1h prior to D-glucose to abolish the protein phosphatases PP1 and PP2A signalling pathway. The same volume (less than 0.1% of the total volume of the culture media) of DMSO was used as control. To determine the metabolic pathway involved in the D-glucose regulation, cells were treated in the same conditions with glucose analogs and metabolites as indicated in Figure 2. Independent experiments were done at least three times in triplicate. The data were expressed as mean ± SD. Statistic significance were assessed with the Student's *t* test.

RNA Extraction and Quantification.

Total RNA was isolated from cells using NucleoSpin RNA II kit (Macherey-Nagel). Quantification analysis was performed after a reverse transcription by using random hexamere primers with the Omniscript Reverse transcriptase kit (Qiagen) followed by a real-time PCR using the MX 4000 apparatus (Stratagene). *APOA5* mRNA was quantified and normalized with the *36B4* gene, which codes for the human acidic ribosomal phosphoprotein. Specific primers used for the amplifications are as follow: human *APOA5* forward: 5'-ACG CAC GCA TCC AGC AGA AC-3'; human *APOA5* reverse: 5'-TCG GAG AGC ATC TGG GGG TC-3'; rat *apoa5* forward: 5'-GCC TGG GAA GGA GCC TCC TCG GC-3'; rat *apoa5* reverse: 5'-GCT CCA TCA GCT CGA CCG TGT AGG G-3'; *36B4* forward: 5'-CAT GCT CAA CAT CTC CCC CTT CTC C-3'; and *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 as recommended by the manufacturer (Stratagene). Independent experiments were done at least three times in triplicate. Quantification of mRNA levels was expressed relative to control as means ± standard deviation (SD). After siRNA experiments, quantification of mRNA levels was done for each gene by real-time PCR with the specific oligonucleotides, and normalized with the *36B4* gene.

Electrophoretic Mobility Shift Assay (EMSA), Oligonucleotide Precipitation Assay, Chromatin Immunoprecipitation (ChIP) Assay and Western Blot Analysis.

To detect the association of nuclear proteins with human *APOA5* promoter *in vitro* and *in vivo*, EMSA, biotinylated oligonucleotide precipitation and ChIP assays were conducted as previously described [18]. Each binding experiment was done at least three times using independent nuclear extracts. Three samples per condition were pooled to amplify the signal for precipitation assays.

Small Interfering RNAs (SiRNAs).

Pre-designed small interfering RNA targeting USF1, USF2, PP1, and PP2A mRNA were provided by Ambion. Cells were plated at a density of 250 000 cells/well into 6-well plates and were transfected with 20 nM of siRNA for 8h using the siPORT Amine transfection agent as recommended by the manufacturer (Ambion). After 48h treatment with 5 mM or 25 mM D-glucose, mRNA extraction and analysis were performed as described under "RNA extraction and Quantification".

Statistical Analysis.
Statistical significance was analysed by using the Student *t* test. All values are reported as mean ±SD. Values with asterisks were considered significant.
(*:0.01<p<0.05, **:0.0001<p<0.01, and ***:p<0.0001).

ACKNOWLEDGMENTS

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Figure 1

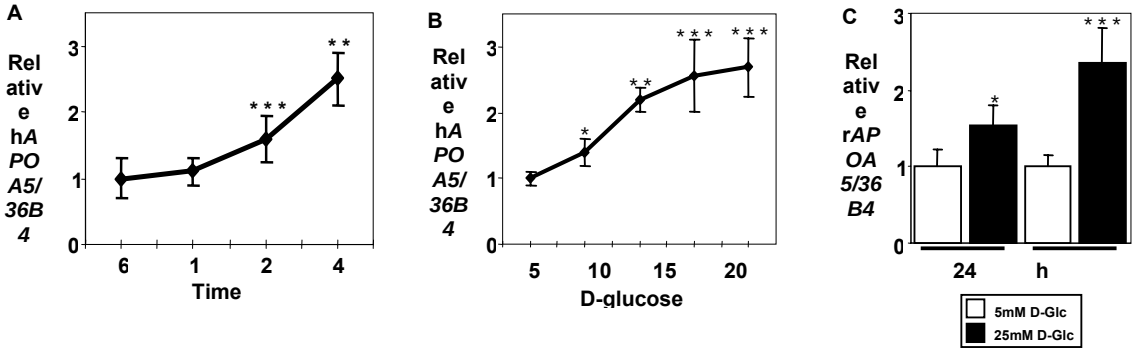


Figure 1. D-glucose increases *APOA5* gene expression in a time- and dose-dependent manner in human hepatoma cells and in primary rat hepatocytes.
HepG2 and HuH7 were cultured A) for different times with D-glucose 25mM, and B) for 48h with different concentrations of D-glucose. Results are expressed in fold induction of *APOA5* gene expression relative to control obtained with cells treatment with D-glucose 5mM. C) Primary rat hepatocytes were cultured for 24h and 48h with D-glucose 25mM. Fold induction of *apoa5* gene expression after D-glucose treatment is shown (black bars) versus control (white bars). For each experiment, results are representative of three independent treatments (means ± SD, n=3) (*:0.01<p<0.05, **:0.0001<p<0.01, and ***:p<0.0001).

Figure 2

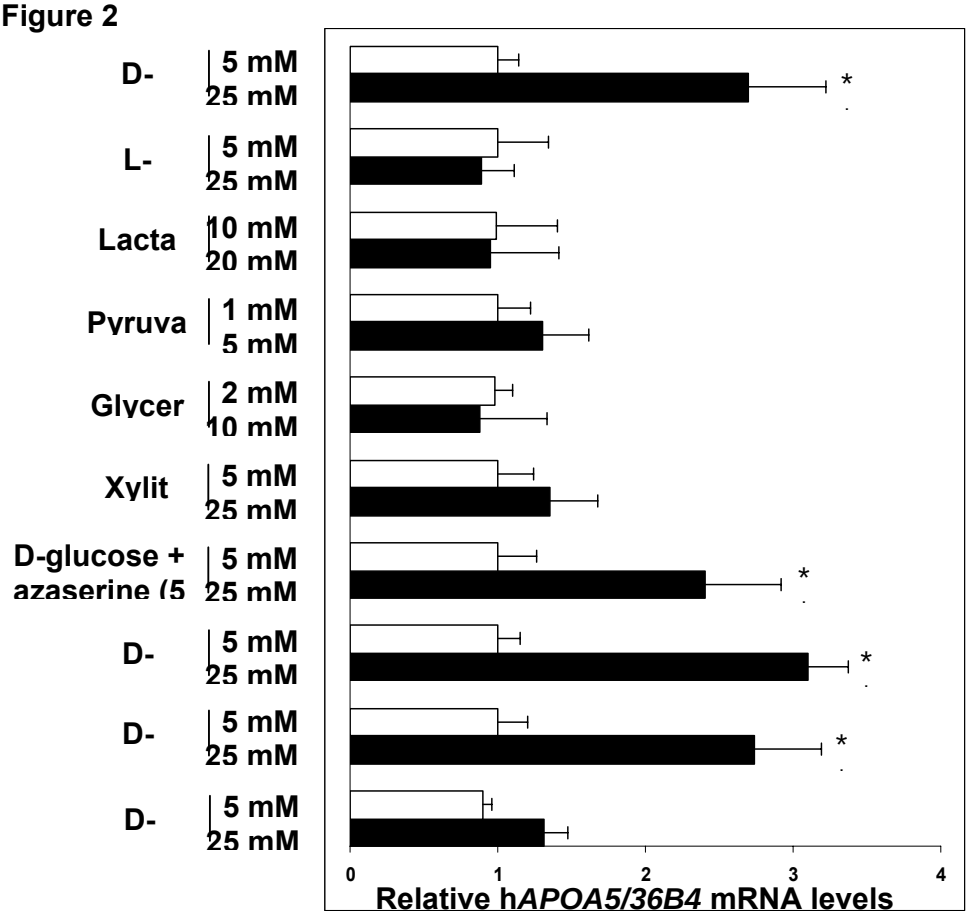


Figure 2. Effect of D-glucose analogs and metabolites on *APOA5* gene expression.
APOA5 mRNA levels were measured in HuH7 cells cultured for 48 h in medium containing different chemicals as indicated. Fold induction of *APOA5* gene expression after treatment is shown (black bars) versus control (white bars). Data are expressed as means ± SD, n=3. (***:p<0.0001).

Figure 3

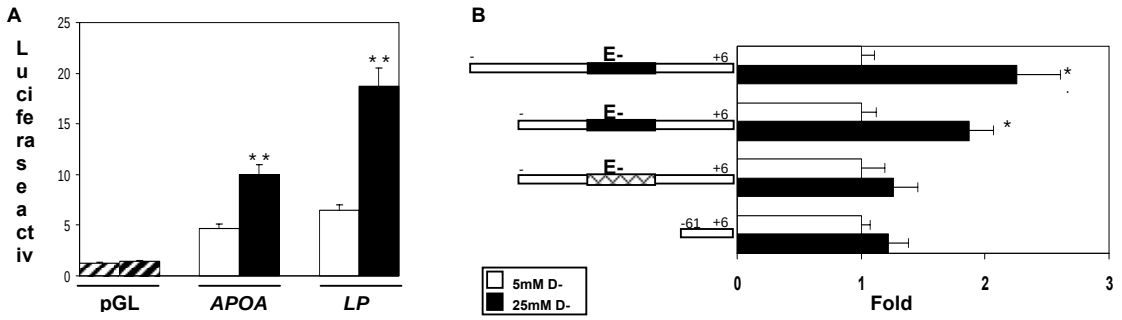


Figure 3. D-glucose regulates the *APOA5* gene expression at the transcriptional level through an E-box element in the promoter.

A) HepG2 cells were transient transfected with luciferase reporter plasmid containing the –304/+63 human *APOA5* promoter fragment or the *L-PK* glucose responsive promoter promoter (full bars) or the control vector pGL3 (stripped bars). Cells were treated with different concentrations of D-glucose for 48 h and luciferase activity was measured. B) HepG2 cells were transfected with luciferase reporter constructs containing a series of 5'-deletions of the *APOA5* promoter, with or without the wild-type E-box 5'-CACGTG-3' (black element) or the mutated E-box 5'-CCCGCG-3' (hatched element). Luciferase activity was measured and normalized according to β-galactosidase activity as described under "Experimental procedures". Results are expressed as mean ± SD, n=3. Independent assays were repeated at least three times and the representative result is shown (**:0.0001<p<0.01, and ***:p<0.0001).

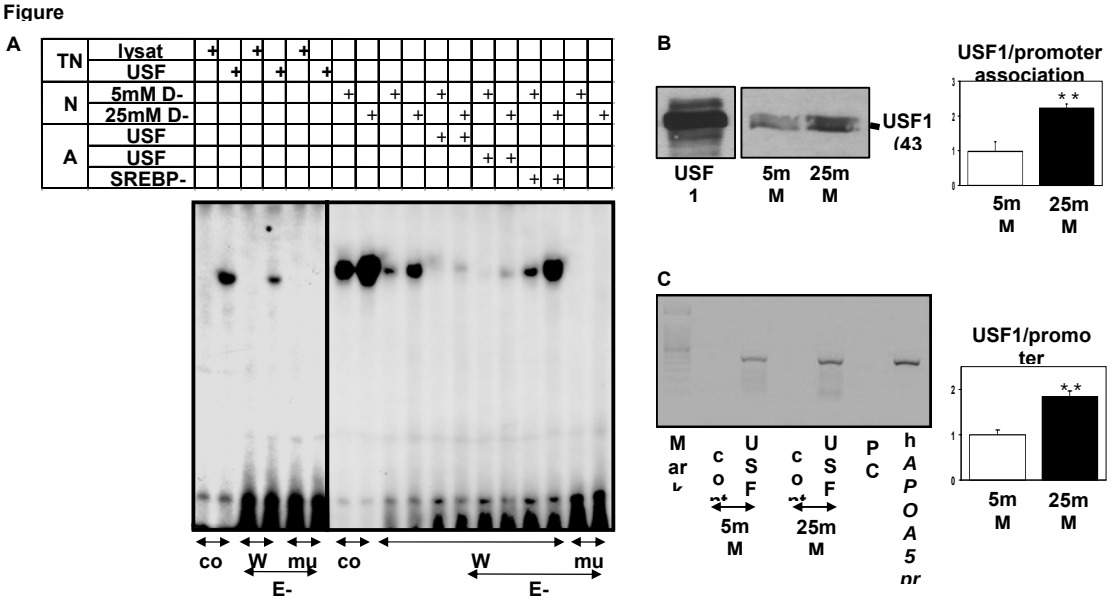


Figure 4. USF1 and USF2 specifically bind *in vitro* and *in vivo* the E-box element in the *APOA5* promoter.

A) EMSA were performed at least three times using independent nuclear extracts (NE) from HepG2 cells treated during 48h with 5mM D-glucose or 25mM D-glucose and with *in vitro* transcribed-translated (TNT) human USF1 protein or unprogrammed reticulocyte lysate. Three probes were used for experiments: an USF consensus E-box as the control (con) probe, a wild type (WT) *APOA5* E-box containing probe, and a mutated (mut) *APOA5* E-box containing probe. Antibodies (Ab) against USF1, USF2 or SREBP-1c were added to check the specificity of the nucleoproteins. B) Immunoblot of the transcribed-translated human USF1 protein and *APOA5* bound-USF1 from nuclear extracts. Quantification of the USF1/promoter association after three independent biotinylated oligoprecipitation assays (**:0.0001<p<0.01). C) Formaldehyde-cross-linked chromatin isolated from cells was immunoprecipitated with USF1 antibody. *In vivo* USF1/promoter association was evaluated by PCR. Negative (PCR-) and positive controls were obtained after PCR amplification with no sample and plasmid containing the –304/+63 *APOA5* promoter fragment respectively. ChIP assays were repeated independently three times and multiple samples were pooled in each experiment (**:0.0001<p<0.01).

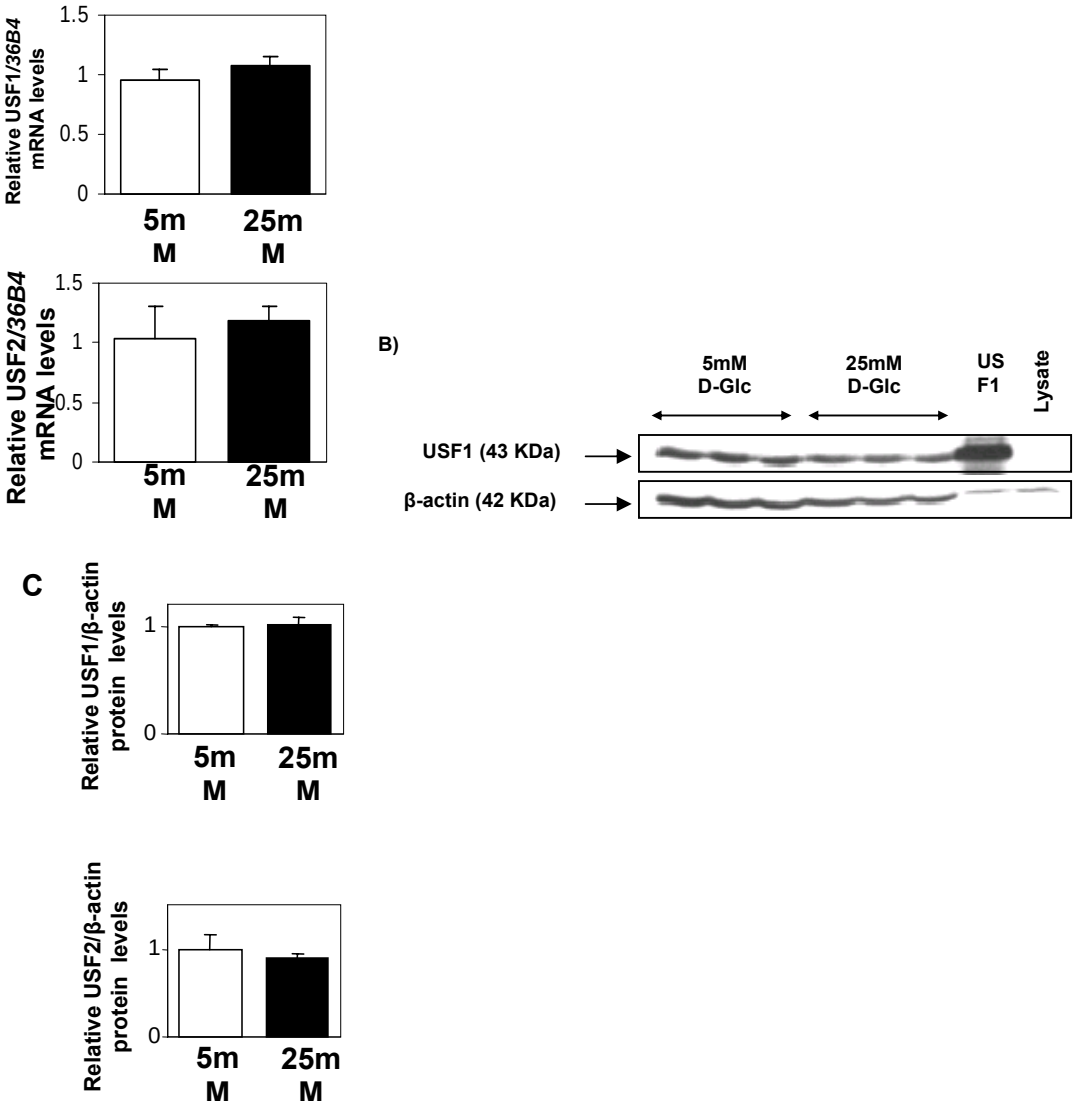


Figure 5. Effect of D-glucose on USF1 and USF2 mRNA and protein levels.
A) USF1 and USF2 mRNA levels were quantified by RT-PCR and normalized with the *36B4* gene after HuH7 treatment with 5mM or 25mM D-glucose for 48h. B) Western blot analyses of USF1 and USF2 protein levels using nuclear extracts from cells treated with 5mM or 25mM D-glucose for 48h. In both immunoblots, *in vitro* transcribed-translated human USF1 protein or unprogrammed reticulocyte lysate were used as controls to verify the specificity of antibodies. C) Quantification of USF1 or USF2 expressed as the USF1 or USF2 to β-actin protein ratio.

Figure

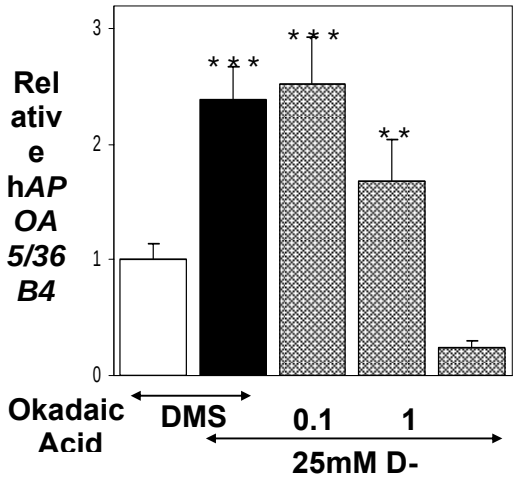
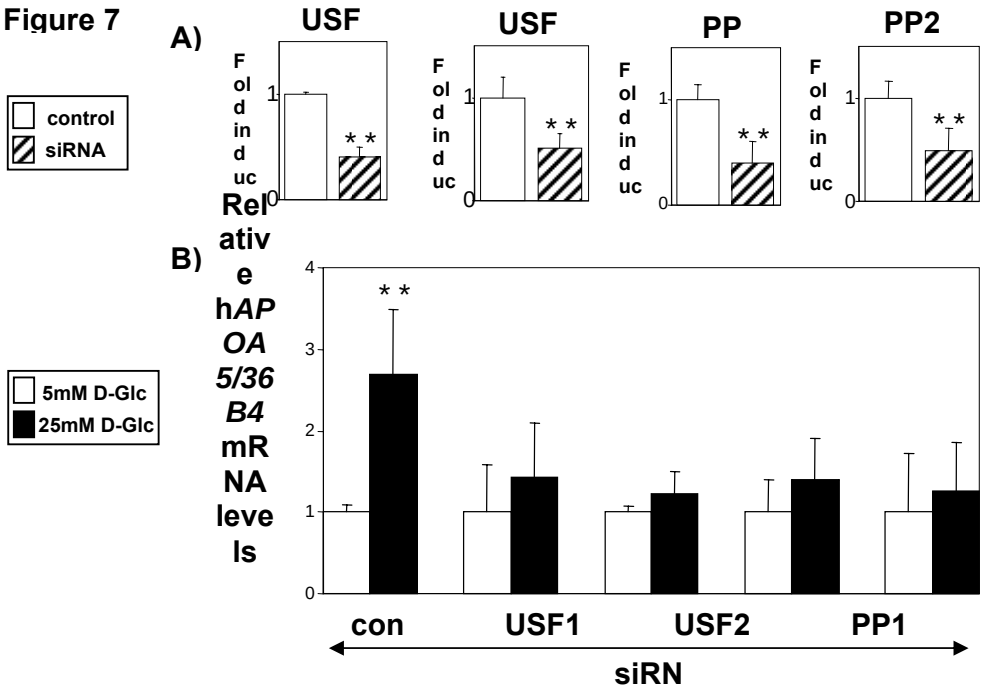


Figure 6. D-glucose activates the PP1/PP2A protein phosphatases signalling pathway.
The phosphatases inhibitor okadaic acid was added to HuH7 cells for 1h before D-glucose treatment. The same volume of vehicle (DMSO) was used as the negative control. Fold induction of *APOA5* gene expression are shown (means ± SD, n=3). Experiments were repeated at least three times and the representative result is shown (**:0.0001<p<0.01, and ***:p<0.0001).

Figure 7



Figure

7. Effect of USF1, USF2, PP1 and PP2A siRNA on *APOA5* gene expression.
A) Expression of target genes after siRNA transfections was evaluated by quantification of mRNA levels with RT-PCR. B) HuH7 cells were transfected for 8h with specific siRNAs or nonspecific control siRNA (con) and subsequently treated with 5mM D-glucose or 25mM D-glucose for further 48h. Experiments were repeated at least three times and *APOA5* mRNA levels were analysed by quantitative RT-PCR. Values are expressed as mean ± SD. Results are representative of three independent assays. (**:0.0001<p<0.01, and ***:p<0.0001).

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