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Atorvastatin might inhibit insulin resistance induced by insulin through the triglyceride-lowering role of Apolipoprotein AV

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Abstract. – OBJECTIVE: We aimed to evaluate the effect of atorvastatin on apolipoprotein AV (ApoAV) in HepG2 cells of insulin resistance (IR), and further explore its mechanism.

MATERIALS AND METHODS: Firstly, a model of IR in HepG2 cells was established by insulin, and then treated with various concentrations of atorvastatin (0, 10, 100 and 500 nM) for 12 h and 24 h, respectively. Detection of glucose concentration was performed by Glucose Oxidase kit. Subsequently, Enzyme-linked immunosorbent assay (ELISA) kits were used to measure the concentrations of triglyceride (TG), high density lipoprotein (HDL), low density lipoprotein (LDL) and very low density lipoprotein (VLDL). The mRNA levels of ApoAV and ApoAV-related genes, including glucose transporter 1 (Glut1), Glut2, peroxisome proliferator activated receptor α (PPARα), and liver X receptor α (LXRα) were detected by qRT-PCR.

RESULTS: We successfully established IR model in HepG2 cells by 10-6 nM insulin. Subsequently, we found that the glucose extraction rate and mRNA level of ApoAV significantly reduced in HepG2 cells of IR ($p < 0.05$); however, atorvastatin increased the glucose extraction rate and ApoAV mRNA level. Furthermore, atorvastatin inhibited the concentration of TG in HepG2 cells of IR ($p < 0.05$); however, atorvastatin had no effect on HDL, LDL and VLDL. Also, atorvastatin could increase the mRNA levels of Glut2 but not Glut1, PPARα, and LXRα.

CONCLUSIONS: Our study indicated that atorvastatin might inhibit IR induced by insulin through the TG-lowering role of ApoAV. Furthermore, Glut2 might be involved in the effect of atorvastatin on ApoAV in HepG2 cells of IR.

Key Words:
Atorvastatin, Insulin resistance, Triglyceride, Apolipoprotein AV.

Introduction

Insulin resistance (IR), a phenomenon with normal dose of insulin resulting in deficient effect on glucose metabolism, is associated with various clinical syndromes, including type 2 diabetes, cardiovascular disease, essential hypertension, polycystic ovary syndrome, nonalcoholic fatty liver disease (NAFLD), certain forms of cancer and sleep apnea[1]. It is well known that hepatic IR is universally complicated by NAFLD through influencing lipid metabolism, which also increases the risk of type 2 diabetes[2]. Startlingly, the prevalence of NAFLD reaches as high as 60% and also is on the rise in urban areas in East and South Asian communities[3,4]. Thus, it is essential to study the underlying molecular mechanism of hepatic IR on lipid metabolism.

Statins, namely hydroxymethyl glutaric acyl coenzyme A reductase inhibitors, has a beneficial role in improvement of endothelial dysfunction, antioxidant effects, stabilization of the atherosclerotic plaque and anti-inflammatory responses[5]. Tang et al[6] has suggested that atorvastatin combined with valsartan or benazepril have a protective effect on cardiorenal syndrome. Fang et al[7] has shown that atorvastatin has an anti-atherosclerotic role by inhibiting synthesis of cholesterol. More recently, atorvastatin have been proved to have a lipid-lowering effect[8,9]. Atorvastatin not only can up-regulate the expression of low density lipoprotein (LDL) receptors by inhibiting hepatocyte cholesterol levels, but also can reduce the triglyceride (TG) levels and modify the composition of lipoprotein in a non-atherogenic manner[9]. Importantly, insulin action can be influenced by elevated plasma TG concentrations[10], which prompted that atorvastatin may be associated with IR. Previous study has demonstrated that atorvastatin can improve IR by inhibiting inflammatory response in adipose tissue of obese mice[11]. In addition, atorvastatin has a protect effect on hypertensive and hypercholesterolemic patients with IR through regulating the fibrinolytic balance[12]. However, few studies have investigated the lipid-lowering effects of atorvastatin on IR.

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Apolipoprotein AV (ApoAV), a novel identified apolipoprotein, plays a vital role in the regulation of TG metabolism in human and animal studies[13,14]. The human *APOA5* gene was originally identified in the *ApoA1-ApoC3-ApoA4* gene cluster and located on chromosome 11q23[15,16]. O'Brien et al[17] have shown that ApoAV is mainly expressed in liver and distributes in high density lipoprotein (HDL), very low density lipoprotein (VLDL), and chylomicrons. Recently, ApoAV is proved to be regulated by various nuclear transcription factors related to lipid metabolism, including peroxisome proliferator activated receptor α (PPARα)[18], liver X receptor α (LXRα)[19], hepatocyte nuclear factor-4 α (HNF4α)[20], and farnesoid X receptor[21]. In addition, insulin and glucose are also involved in the regulation of ApoAV, suggesting the role of ApoAV in diabetes[22,23]. Also, a decreased expression of ApoAV is found in IR-related hypertriglyceridemia in obesity[24]. Thus, we speculated that lipid metabolism disorder might be a cause of these IR syndromes and ApoAV might be closely associated with hepatic IR.

In this study, we induced a model of IR in HepG2 cells by insulin, and then interfered IR by atorvastatin. Subsequently, the expressions of ApoAV and ApoAV-related genes, including glucose transporter 1 (Glut1), Glut2, PPARα, LXRα, as well as the concentrations of TG, HDL, LDL and VLDL were detected. We aimed to evaluate the effect of atorvastatin on HepG2 cells of IR and ApoAV, and further explore its mechanism.

Materials and Methods

Ethics Statement

All studies have been approved by The Ethics Committee of The Second Xiangya Hospital, Central South University and performed in accordance with the ethical standards.

Cell Culture

Human HCC cell line, HepG2, was purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were maintained in Dulbecco's Modified Eagle Media (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA). Cultures were maintained at 37°C in a saturation humidity containing 5% CO_2.

Cell Treatment

In order to establish a model of IR, HepG2 cells were treated with various concentrations of insulin (0, 10^{-7}, 5×10^{-7}, and 10^{-6} nM) (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. The appropriate concentration of insulin was selected by detecting the glucose concentration and used for the subsequent experiments.

Next, HepG2 cells were treated with the optimum concentration of insulin for 24 h in order to induce IR, and then treated with various concentrations of atorvastatin (0, 10, 100 and 500 nM) (Sigma-Aldrich, St. Louis, MO, USA) for 12 h and 24 h, respectively. Cell culture supernatant was collected for the detection of glucose concentration.

Detection of Glucose Concentration

Glucose concentration of cell culture supernatant was detected by Glucose kit (Applygen Technologies Inc., Beijing, China) according to the manufacturer's instructions. Briefly, 5 μL cell culture supernatant was incubated with 195 μL reaction buffer in a 96-well plate for 30 min at room temperature. The absorbance was read at 550 nm using a microplate reader (Thermo, Mass, USA). All determinations were carried out in triplicate. Glucose extraction rate was calculated according to the following formula: glucose extraction rate = (concentration of blank well – concentration of cell with no insulin) / (concentration of blank well – concentration of cell with insulin) × 100%.

Detection of TG, HDL, LDL and VLDL Concentrations

After treatment, the cells were crushed by ultrasonication and then supernatant was collected by centrifugation (10,000 g for 6 min). Subsequently, the concentrations of TG, HDL, LDL and VLDL were detected using Enzyme-Linked Immunosorbent Assay (ELISA) commercial kits (Enzyme-linked Biological Technology Co., LTD., Shanghai, China) following the manufacturer's instruction.

Quantitative Real-Time PCR (qRT-PCR)

The total RNA was extracted using 800 μL Trizol reagent (Invitrogen, USA) according to manufacturer's instruction. High-quality RNA was reversely transcribed into complementary DNA (cDNA) with a Reverse Transcription Kit (DRR036A, TaKaRa). Primers for *ApoAV*, *Glut1*, *Glut2*, *PPARα*, *LXRα* and glyceraldehyde-3-

phosphate dehydrogenase (GAPDH) are shown in Table I. PCR amplification was performed with SYBR® Premix Ex Taq™ (Applied Biosystems, Foster City, CA, USA). The PCR program was: 95°C for 3 min, 40 cycles of 95°C for 10 s and 59°C for 20 s, using the ABI Stepone plus Real-time PCR system (Applied Biosystems, Foster City, CA, USA). Relative quantification and calculations were done with the comparative threshold (*C*t) cycle method ($2^{-\Delta\Delta}$*C*t).

Statistical Analysis

Statistical analysis was performed by SPSS 12.0 statistical analysis software (SPSS Inc., Chicago, IL, USA). Data were expressed as the mean ± SD and analyzed by one-way analysis of variance. A value of $p < 0.05$ was considered significant.

Results

Atorvastatin Increased glucose Extraction Rate in HepG2 Cells of IR

The glucose extraction rate in cells with 10^{-6} nM insulin significantly lower than that in cells with other doses of insulin ($p < 0.05$), suggesting that a model of IR in HepG2 cells was successfully established by 10^{-6} nM insulin (Figure 1A). Noteworthily, after treatment for 12 h and 24 h, atorvastatin could increase the glucose extraction rate in HepG2 cells of IR ($p < 0.05$) (Figure 1B).

Table I. Primer sequences for specific genes.

Gene	Primer sequences (5′-3′)
ApoAV	Forward: TGGGCTCTGGCTCTTCTTT Reverse: GCTCCACCCTGCCTTTGTC
Glut1	Forward: ACAGGCTCAAAGAGGTTATG Reverse: TGGGTGGAGTTAATGGAGTAG
Glut2	Forward: AATTGCTCCAACCGCTCTCA Reverse: CTAATAAGAATGCCCGTGACGAT
LXRα	Forward: TTGCTAAACAGCTACCCGGCT Reverse: ATCACCTCGATCGCAGAGGT
PPARα	Forward: CAGGCTATCATTACGGAGTC Reverse: CTGGCATTTGTTTCTGTTCT
GAPDH	Forward: TGACAACTTTGGTATCGTGGAAGG Reverse: AGGCAGGGATGATGTTCTGGAGAG

ApoAV: Apolipoprotein AV; Glut1: glucose transporter 1; LXRα: liver X receptor; PPARα: peroxisome proliferator activated receptor α; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Effect of Atorvastatin on ApoAV in HepG2 Cells of IR

qRT-PCR analysis showed that the mRNA level of *ApoAV* was significantly inhibited in HepG2 cells of IR compared with HepG2 cells without treatment ($p < 0.05$) (Figure 2). However, after treatment with different dose of atorvastatin for 12 h and 24 h, the mRNA level of *ApoAV* was increased compared with HepG2 cells of IR ($p < 0.05$) (Figure 2).

Atorvastatin Inhibited the Concentrations of TG But Not HDL, LDL and VLDL in HepG2 cells of IR

ELISA analysis showed that the concentration of TG was increased in HepG2 cells of IR compared with HepG2 cells without treatment ($p < 0.05$) (Figure 3A). However, after treatment with different doses of atorvastatin for 12 h and 24 h, TG concentration remarkably reduced compared with that in HepG2 cells of IR ($p < 0.05$) (Figure 3A). Also, insulin could increase the concentration of VLDL at 12 h but not 24 h ($p < 0.05$), while the concentration of VLDL was not significantly changed after treatment with atorvastatin in HepG2 cells of IR (Figure 3B). In addition, insulin and atorvastatin had no obvious effect on HDL (Figure 3C) or LDL (Figure 3D).

Atorvastatin Influenced the mRNA Level of Glut2 But Not LXRα, Glut1 and PPARα in HepG2 Cells of IR

Compared with HepG2 cells without treatment, the mRNA level of *Glut2* was prominently decreased in HepG2 cells of IR ($p < 0.05$); however, which was increased as the increasing dose of atorvastatin at 12 h but not 24 h in HepG2 cells of IR ($p < 0.05$) (Figure 4A). In addition, insulin could inhibit the mRNA level of *LXRα* ($p < 0.05$), while atorvastatin had no effect on *LXRα* in HepG2 cells of IR (Figure 4B). Nonetheless, insulin and atorvastatin did not influence the mRNA levels of *Glut1* (Figure 4C) or *PPARα* (Figure 4D) in HepG2 cells.

Discussion

It had been acknowledged that high dose of insulin could induce IR in HepG2 cells[25,26]. Consistent with this, we also demonstrated that 10^{-6} nM insulin caused IR. Previous study had shown that IR could contribute to the occurrence of cardiovascular disease and type 2 diabetes[27]. Note-

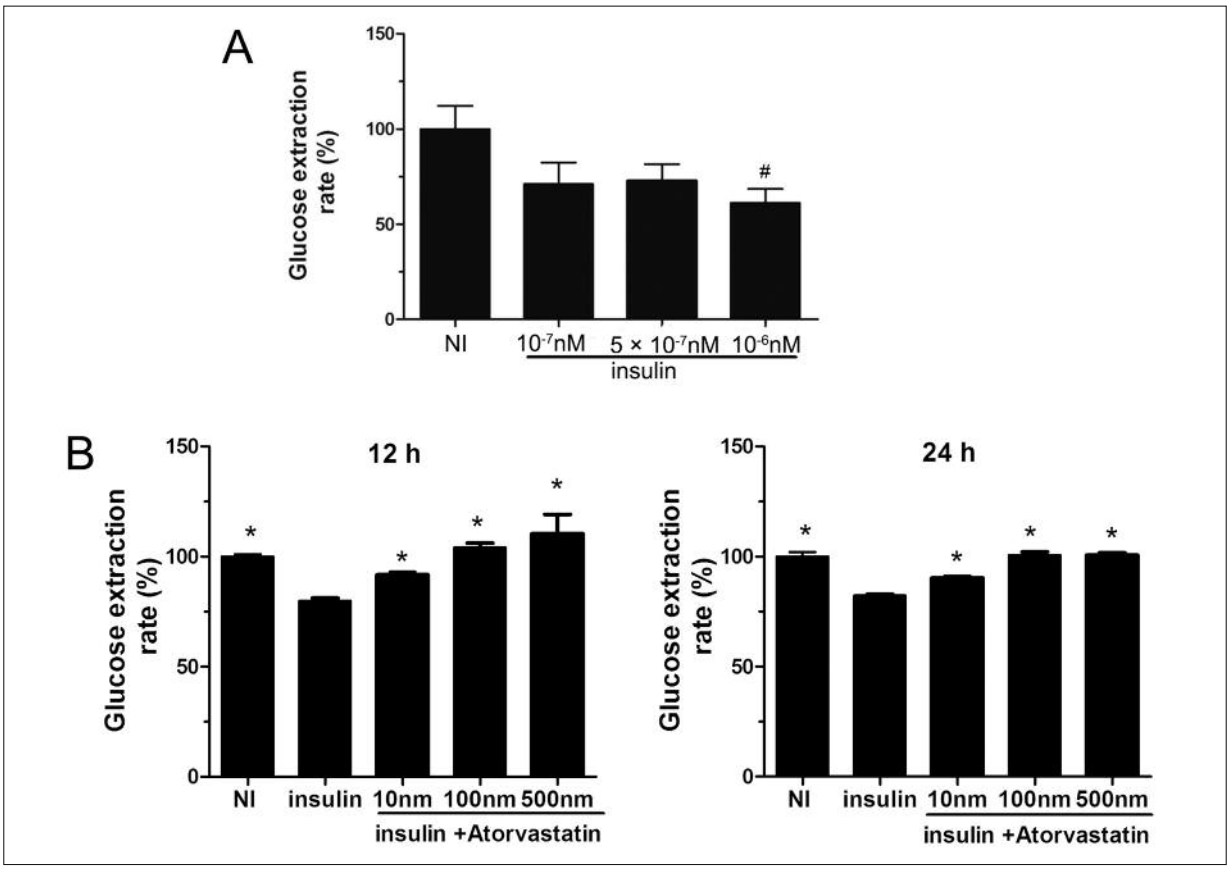

Figure 1. Effect of atorvastatin on glucose extraction rate in HepG2 cells of IR. ***A,*** A model of IR in HepG2 cells was successfully established by 10-6 nM insulin. ***B,*** After treatment for 12 h and 24 h, atorvastatin increased the glucose extraction rate in a dose-dependent manner in HepG2 cells of IR. NI: cell without treatment. $^{\#}p < 0.05$ versus NI group, $^{*}p < 0.05$ versus insulin group.

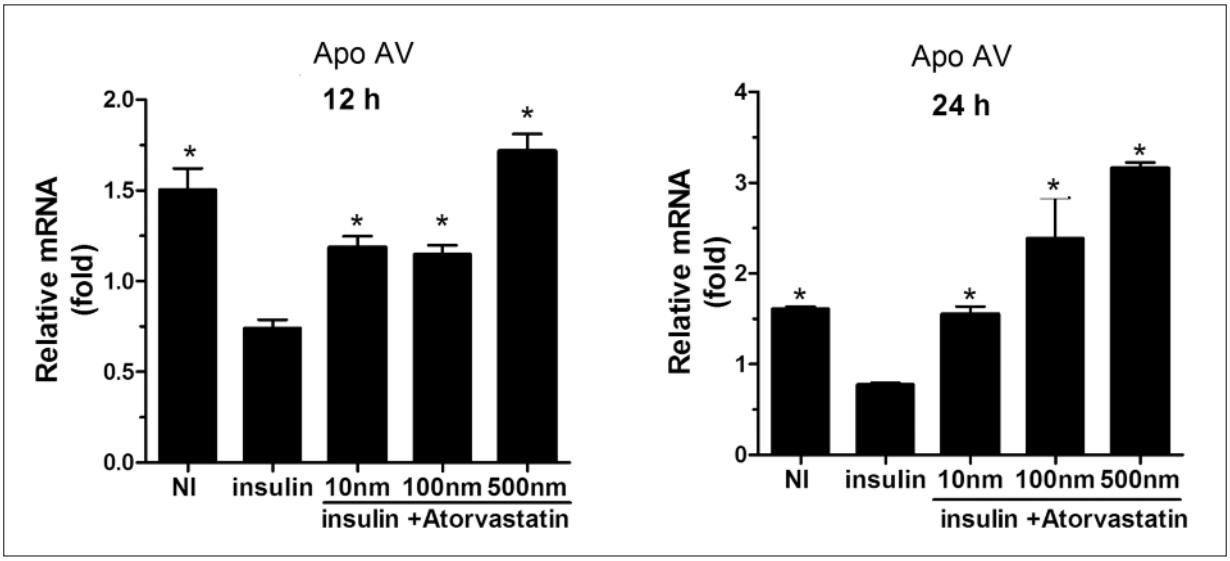

Figure 2. Effect of atorvastatin on Apo AV in HepG2 cells of IR. qRT-PCR analysis showed that compared with NI group, the mRNA level of ApoAV was significantly inhibited in insulin group; however, atorvastatin increased ApoAV mRNA level after treatment for 12 h and 24 h . $^{*}p < 0.05$ versus insulin group.

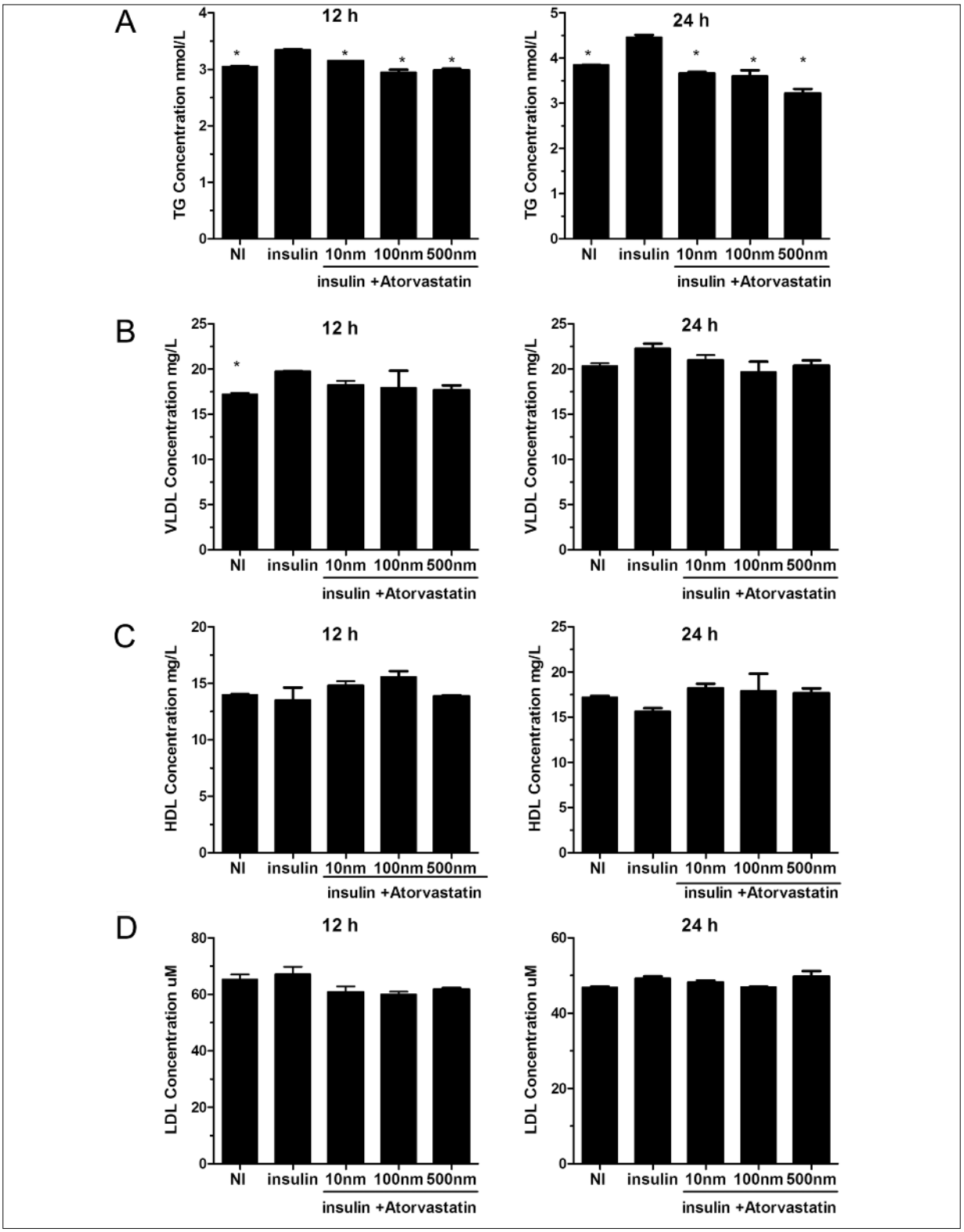

Figure 3. Effect of atorvastatin on TG, HDL, LDL and VLDL in HepG2 cells of IR. ***A,*** Compared with NI group, the concentration of TG increased in insulin group; however, TG concentration remarkably reduced after treatment with different doses of atorvastatin for 12 h and 24 h. ***B,*** Compared with NI group, the concentration of VLDL increased in insulin group at 12 h but not 24 h, while atorvastatin did not influence VLDL concentration. ***C, D,*** Insulin and atorvastatin had no effect on HDL and LDL. *$p < 0.05$ versus insulin group.

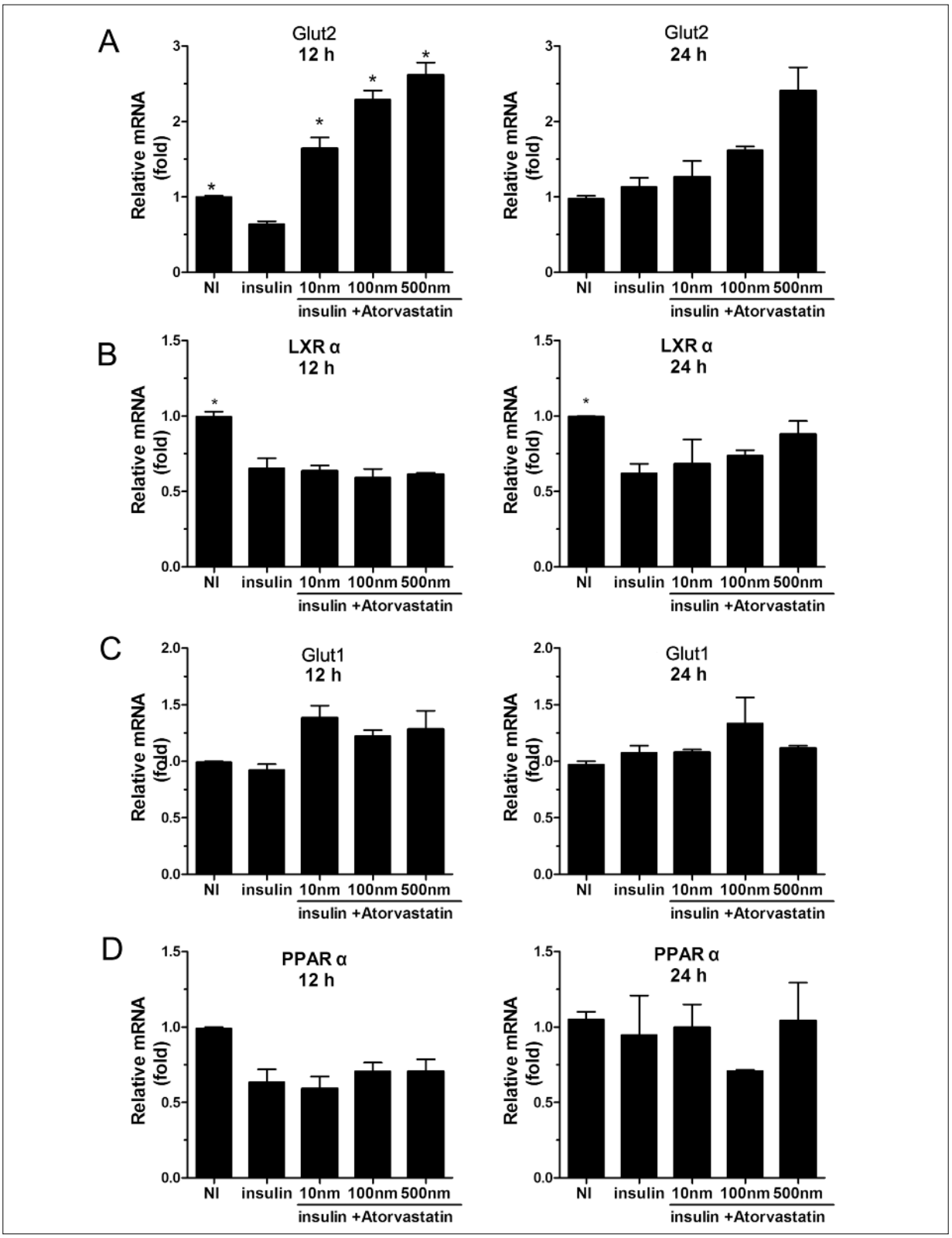

Figure 4. Effect of atorvastatin on ApoAV-related gene in HepG2 cells of IR. ***A,*** The mRNA level of Glut2 was prominently decreased in insulin group compared with NI group; however, atorvastatin increased Glut2 mRNA level after treatment for 12 h but not 24 h. ***B,*** Insulin inhibited LXRα mRNA level, while atorvastatin had no effect on LXRα in HepG2 cells of IR. ***C, D,*** Insulin and atorvastatin did not influence the mRNA level of Glut1 or PPARα in HepG2 cells. *$p < 0.05$ versus insulin group.

worthily, atorvastatin reduced the risk of cardiovascular disease caused by type 2 diabetes[28]. These results prompted that atorvastatin might be associated with IR. In addition, Paolisso et al[29] also had suggested that atorvastatin had a inhibiting effect on IR in non-insulin dependent diabetic patients. Similarly, our results showed that atorvastatin increased the glucose extraction rate in a dose-dependent manner in HepG2 cells of IR, suggesting that atorvastatin might inhibit IR in HepG2 cells.

Furthermore, we found that IR could inhibit ApoAV mRNA expression in HepG2 cells, while atorvastatin had the opposite effect on ApoAV. In accordance with our results, *in vitro* and *in vivo* studies of Nowak et al[22] had shown that insulin could reduce the expression of ApoAV in humans and rodents. Huang et al[30] had further demonstrated that atorvastatin increased ApoAV level. ApoAV was a critical regulator involved in triglyceride homeostasis[13]. The earlier studies had demonstrated that knockdown of ApoAV led to increased plasma TG in mice, whereas its overexpression resulted in a reduction of TG[13,16]. Interestingly, our results in this study were consistent with these earlier found. Currently, there were three explanations for TG-lowering role of ApoAV, including inhibiting the production and secretion of VLDL[31], promoting TG hydrolysis induced by lipoprotein lipase (LPL)[32], and accelerating hepatic uptake of VLDL[33]. To explore whether atorvastatin affected ApoAV expression by lipoprotein, we detected the concentration of HDL, LDL and VLDL. Unfortunately, our results only found that the concentration of VLDL increased in HepG2 cells of IR at 12 h and atorvastatin did not influence the concentration of HDL, LDL and VLDL. We speculated that atorvastatin might inhibit IR through the TG-lowering role of ApoAV. However, it should be further investigated whether ApoAV inhibit TG level through HDL, LDL and VLDL in HepG2 cells of IR.

Previous study had demonstrated that atorvastatin enhanced ApoAV expression and reduced TG content through up-regulating PPARα expression[30,34]. On the contrary, our results found that atorvastatin did not influence the mRNA level of PPARα in HepG2 cells of IR. Noteworthily, the mRNA level of LXRα reduced in HepG2 cells of IR, while atorvastatin had no effect on LXRα. LXRα ligand T0901317 was found to down-regulate the expression of ApoAV through the activation of sterol response element binding protein 1[19]. Also, Qiu et al[35] had shown that atorvastatin decreased LPL level by inhibiting the activation of LXRα. These results indicated that decreased mRNA level of *ApoAV* induced by IR might be associated with LXRα in HepG2 cells; however, atorvastatin did not influence *ApoAV* mRNA expression by LXRα. In addition, we further found that the mRNA level of *Gult2* but not *Glut1* had the same changing trend with *ApoAV*. Glut proteins were necessary to cellular glucose uptake[36] and glucose concentration could be gradually reduced by the regulation of Glut proteins[37], suggesting the vital role of Glut proteins in IR. Thus, we speculated that Glut2 but not Glut1 might affect the mRNA level of *ApoAV* caused by atorvastatin in HepG2 cells of IR.

Conclusions

Our study indicated that atorvastatin might inhibit IR induced by insulin through the TG-lowering role of ApoAV. Furthermore, Glut2 might be involved in the effect of atorvastatin on ApoAV in HepG2 cells of IR. However, the further study is essential to explore whether other pathways are involved in the TG-lowering role of ApoAV.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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