Atorvastatin ameliorates endothelium-specific insulin resistance induced by high glucose combined with high insulin

OU YANG[1], JINLIANG LI[2], HAIYAN CHEN[1], JIE LI[1] and JIAN KONG[1]

[1]Department of Cadre Ward, First Hospital, Jilin University, Changchun, Jilin 130021; [2]Department of Cardiology, Heilongjiang Provincial Hospital, Harbin, Heilongjiang 150001, P.R. China

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Abstract. The aim of the present study was to establish an endothelial cell model of endothelium-specific insulin resistance to evaluate the effect of atorvastatin on insulin resistance-associated endothelial dysfunction and to identify the potential pathway responsible for its action. Cultured human umbilical vein endothelial cells (HUVECs) were pretreated with different concentrations of glucose with, or without, 10^{-5} M insulin for 24 h, following which the cells were treated with atorvastatin. The tyrosine phosphorylation of insulin receptor (IR) and insulin receptor substrate-1 (IRS-1), the production of nitric oxide (NO), the activity and phosphorylation level of endothelial NO synthase (eNOS) on serine1177, and the mRNA levels of endothelin-1 (ET-1) were assessed during the experimental procedure. Treatment of the HUVECs with 30 mM glucose and 10^{-5} M insulin for 24 h impaired insulin signaling, with reductions in the tyrosine phosphorylation of IR and protein expression of IRS-1 by almost 75 and 65%, respectively. This, in turn, decreased the activity and phosphorylation of eNOS on serine1177, and reduced the production of NO by almost 80%. By contrast, the mRNA levels of ET-1 were upregulated. All these changes were ameliorated by atorvastatin. Taken together, these results demonstrated that high concentrations of glucose and insulin impaired insulin signaling leading to endothelial dysfunction, and that atorvastatin ameliorated these changes, acting primarily through the phosphatidylinositol 3-kinase/Akt/eNOS signaling pathway.

Correspondence to: Professor Jian Kong or Professor Jie Li, Department of Cadre Ward, First Hospital, Jilin University, 71 Xinmin Avenue, Changchun, Jilin 130021, P.R. China
E-mail: kongjian-2005@163.com
E-mail: yabianwanghai@163.com

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Introduction

Cardiovascular disease, particularly, coronary artery disease, is a leading contributor to mortality rates worldwide. Compelling evidence suggests that endothelial dysfunction, which results from the reduction of nitric oxide (NO) and the impairment of NO bioavailability, is a key early event in the pathogenesis of atherosclerosis and an important predictor of cardiovascular disease (1-4).

Insulin resistance, the early stage of type 2 diabetes, is not only accompanied by glucose and lipid metabolism disorder, but is also associated with endothelial dysfunction (5). A number of studies have suggested that insulin resistance is one of the independent risk factors for the progression of cardiovascular diseases (6,7).

The reciprocal association between insulin resistance and endothelial dysfunction in the development of diabetes and cardiovascular disease has been suggested in animal studies (8,9). Notably, studies have suggested that the impairment of insulin signaling results in the reduced production of NO in an animal model (10,11). In addition, animal and clinical studies have demonstrated that endothelial cells exhibit dysfunction in response to high glucose concentrations (12,13), or prolonged modest hyperinsulinemia (14,15). However, *in vivo*, several complicated factors, including individual differences in experimental and insulin receptor sensitivity, affect endothelial dysfunction; thus a reproducible and highly stable endothelial cell model of endothelium-specific insulin resistance is essential to examine the specific insulin signaling associated with endothelial dysfunction.

Atorvastatin, a vessel protective drug, have been confirmed to reduce cardiovascular events in patients by protecting endothelial function, in addition to decreasing levels of cholesterol (16). This drug has pleiotropic effects, including the upregulation and activation of endothelial NO synthase (eNOS), which in turn causes an increase in the production of NO, and reduces oxidative stress and vascular inflammation (17,18). Phosphatidylinositol 3-kinase (PI3K) is one of the most well-characterized downstream effectors of insulin receptor substrate-1 (IRS-1) proteins (19). *In vivo* studies have suggested that atorvastatin improves endothelial function via the phosphatidylinositol 3-kinase (PI3K)/Akt/eNOS pathway (20-22).

In the present study, an endothelial cell model of endothelium-specific insulin resistance was established, which was induced by high concentrations of glucose in combination with high concentrations of insulin. Subsequently, the cell model was treated with atorvastatin to evaluate its effect on insulin resistance-associated endothelial dysfunction and to identify the potential pathway responsible for its action.

Materials and methods

Cell culture. Human umbilical vein endothelial cells (HUVECs) (23), provided by the Department of Pathology, Jilin University (Jilin, China), were purchases from Cobioer Biosciences (Nanjing, China). The cells were maintained at 37˚C in humidified air (5% CO_2) and cultured in serum-free Dulbecco's modified Eagle's medium (DMEM; GE Healthcare Life Science HyClone Laboratories, Logan, UT, USA) with 5 mM D-glucose (GE Healthcare Life Science HyClone Laboratories), 4 mM L-glutamine, and 20% fetal bovine serum (GE Healthcare Life Science HyClone Laboratories).

Treatment of HUVECs with high glucose and high insulin concentrations to impair insulin signaling. HUVECs (10^5/ml) were incubated in culture medium containing 5, 15 or 30 mM D-glucose. The cells were maintained at 37˚C in humidified air (5% CO_2) with or without 10^{-5} M insulin for 24 h. The medium was then discarded, the cells were washed twice with phosphate-buffered saline (PBS), and were then incubated for 6 h following the addition of 10^{-5} M insulin at 37˚C with 5% CO_2.

Treatment with atorvastatin. HUVECs (10^6/ml) cultured in normal culture medium (Nor vec) and HUVECs pretreated with 30 mM glucose and 10^{-5} M insulin for 24 h at 37˚C with 5% CO_2, defined as insulin-resistant HUVECs (IR vec), were treated with varying concentrations of atorvastatin (0, 10^{-6}, 10^{-5} and 10^{-4} M) for 24 h or were treated with 10^{-4} M atorvastatin for 0, 3, 6, 9, 12, 15, 18, 21 and 24 h at 37˚C with 5% CO_2.

Treatment with LY29004, an inhibitor of PI3K. The Nor vec and IR vec cells (10^6/ml) were treated with PBS (control), 10^{-4} M atorvastatin only, 25 μM LY29004 (Merck Millipore, Darmstadt, Germany) alone or with a combination of 10^{-4} M atorvastatin and 25 μM LY29004 for 24 h at 37˚C with 5% CO_2.

Western blot analysis. The HUVECs were pretreated, as described above, in 10 cm cell dishes. Cell lysates were prepared by washing twice in cold PBS followed by lysis buffer, containing 50 mM Tris-HCl (pH 7.4), 10 mM EDTA, 5 mM EGTA, 0.5% NP40, 1% Triton X-100 and protease inhibitor (Roche, Mannheim, Germany) and sonication. The cell lysate was centrifuged at 14,000 x g for 10 min at 4˚C, and the supernatant was discarded. Lysis buffer (150-200 μl) was added to each Eppendorf tube. Protein concentration was measured using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Inc. Waltham, MA, USA). Protein samples (40-60 μg) were mixed with SDS-PAGE loading dye, boiled and loaded onto an SDS-PAGE gel (4-20%; Roche). Following transfer of the proteins onto PVDF membranes (Roche), the proteins were blotted with anti-IR (cat. no. sc-57344), anti-phosphorylated (phosphor)-IR (tyrosine; cat. no. sc-17200), anti-IRS-1 (cat. no. SC-8038) and anti-phosphor-IRS-1 (tyrosine; cat. no. sc-17194) antibodies from Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and anti-phosphor-eNOS (serine1177) and total eNOS antibody (cat. no. orb6756) from BD Transduction Laboratories (San Diego, CA, USA) in Tris-buffered saline with Tween, containing 50 mM Tris (pH 7.5), 0.15 M NaCl and 0.05% Tween-20, with 5% fat free milk for 24 h at 4˚C. Following incubation with antibodies, the membranes were washed and then reacted with horseradish-peroxidase-conjugated IgG (Santa Cruz Biotechnology, Inc.) for 1 h at 4˚C. The membranes were then treated with ECL reagent (GE Healthcare, Piscataway, NJ, USA) and exposed to X-ray film. β-actin was used as the protein loading control. Each experiment contained three replicates and was repeated three times.

Analysis of total NO concentrations. Total NO concentrations were determined using a nitrate reduction assay, in which the summation of NO_2^- and NO_3^- (24) were measured using a commercially available NO kit (Nanjing Jiancheng Biological Products Co., Ltd., Nianjing, China), according to the manufacturer protocols. This assay was used to quantify the dose effects, as measured by the absorbance at 550 nm. The following equation was used: NO (μM) = (A - A0) / (As - A0) * Cs * P; where A=sample absorbance, A0=control absorbance As=standard absorbance, Cs=standard concentration (100 μM), P=dilution factor. Each experiment contained six replicates and was repeated three times.

Reverse transcription-quantitative polymerase chain reaction analysis (RT-qPCR) of the mRNA levels of ET-1. Following the 24 h incubation period, total RNA was extracted from the HUVECs using TRIzol reagent (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's protocol. Total RNA (2 μg) was subjected to random-primed reverse transcription using SuperScript-2 reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). qPCR was performed in triplicate using an Applied Biosystem 7900 HT system (Applied Biosystems Life Technologies, Foster City, CA, USA) with 5 ng of cDNA, 1 μM of each primer pair and SYBR Green PCR master mix (Roche). RT-qPCR was performed with the following primers: ET-1, forward 5'-AGAGTGTGTCTACTTCTGCCA-3' and reverse CTG CCGTCTAGAA-3'; GAPDH, forward 5'-GGACCTGAC CTGCCGTCTAGAA-3' and reverse 5'-GGTGTCGCTGTT GAAGTCAGAG-3'. Relative mRNA levels were normalized to GAPDH and quantified using the $2^{-\Delta\Delta Cq}$ method (25). Each experiment contained three replicates and was repeated three times.

Assessment of eNOS activity. Cell lysates were prepared, as described for the western blot analysis. The cell lysates were centrifuged at 14,000 x g for 10 min and the supernatant was discarded from the pelleted cells. The activity of eNOS was detected by measuring the conversion of [^{3}H] l-arginine to [^{3}H] l-citrulline using an eNOS assay kit. (Cayman Chemical

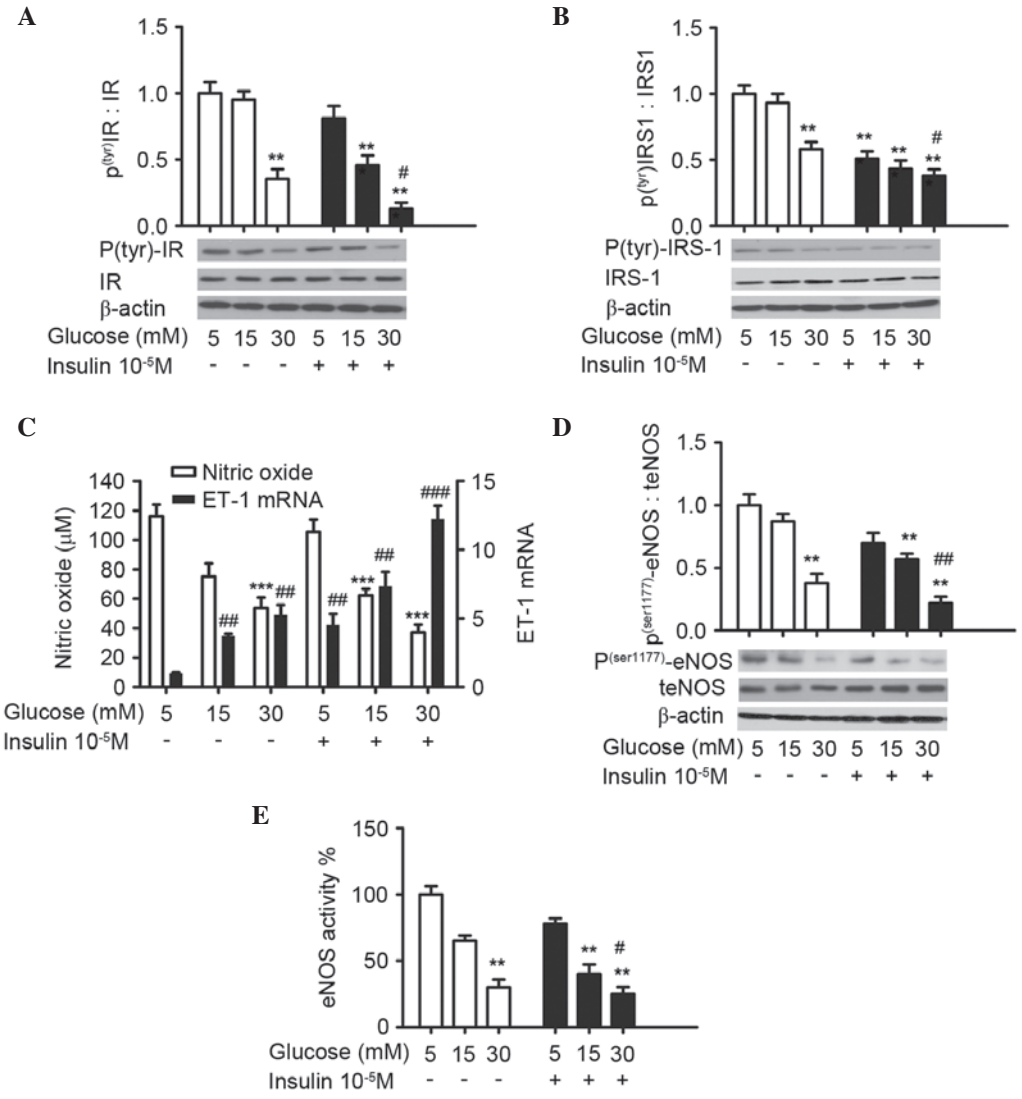

Figure 1. High concentrations of glucose and insulin reduce the protein expression levels of phosphor$^{(tyr)}$-IR and phosphor$^{(tyr)}$-IRS-1, production of NO, protein expression of phosphor $^{(ser1177)}$ -eNOS and activity of eNOS, and increase the mRNA expression of ET-1 in HUVECs. The HUVECs were treated with indicated concentrations of glucose in the absence (open bars) or presence (filled bars) of insulin (10^{-5} M) for 24 h. Western blot analysis of (A) phosphor $^{(tyr)}$ IR and (B) phosphor $^{(tyr)}$ IRS-1. (C) Nitrate reduction assay of NO concentrations and reverse transcription-quantitative polymerase chain reaction analysis of ET-1 mRNA. ***P<0.001 vs. media containing 5 mM glucose only. The values for ET-1 mRNA in 5 mM glucose without insulin are defined as 1 ($^{##}$P<0.01 and $^{###}$P<0.001 vs. 1). (D) Western blot analysis of the protein expression levels of phosphor $^{(ser1177)}$ eNOS and total eNOS. (E) Assessment of eNOS activity. (A, B, D and E) **P<0.01 vs. media containing 5 mM glucose only; $^{#}$P<0.05, $^{##}$P<0.01 vs. media containing 30 mM glucose only. The data are presented as the mean ± standard deviation of three independent experiments. HUVECs, human umbilical vein endothelial cells; phosphor/P, phosphorylated; t, total; IR, insulin receptor; IRS-1, insulin receptor substrate-1; NO, nitric oxide; eNOS, endothelial NO synthase.

Co., Ann Arbor, MI, USA), according to the manufacturer's protocol. The results are expressed as a percentage of the control. Each experiment contained three replicates and was repeated three times.

MTS cell proliferation assays to assess the toxicity of atorvastatin. The cells were seeded in 96-well plates (1,000 cells/well) and were treated with vehicle or with increasing doses of atorvastatin (10^{-6}, 10^{-5} and 10^{-4} M) for 24 and 48 h at 37˚C with 5% CO_2. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (Promega, Madison, WI, USA; 20 μl/well) was added for 2 h on the indicated days. Cell proliferation was measured, according to the manufacturer's protocol. Each experiment contained six replicates and was repeated three times.

Statistical analysis. Data are presented as the mean ± standard deviation. Differences between groups were analyzed using a two-tailed unpaired *t*-test (GraphPad InStat; GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

High concentrations of glucose and insulin reduce the protein expression levels of phosphor$^{(tyr)}$-IR and phosphor$^{(tyr)}$-IRS-1, production of NO, protein expression of phosphor$^{(ser1177)}$-eNOS and activity of eNOS, and increase the mRNA level of ET-1 in HUVECs. To establish the effects of high glucose and high insulin concentrations on the function of the HUVECs, the cells were cultured in DMEM containing 5, 10 or 30 mM

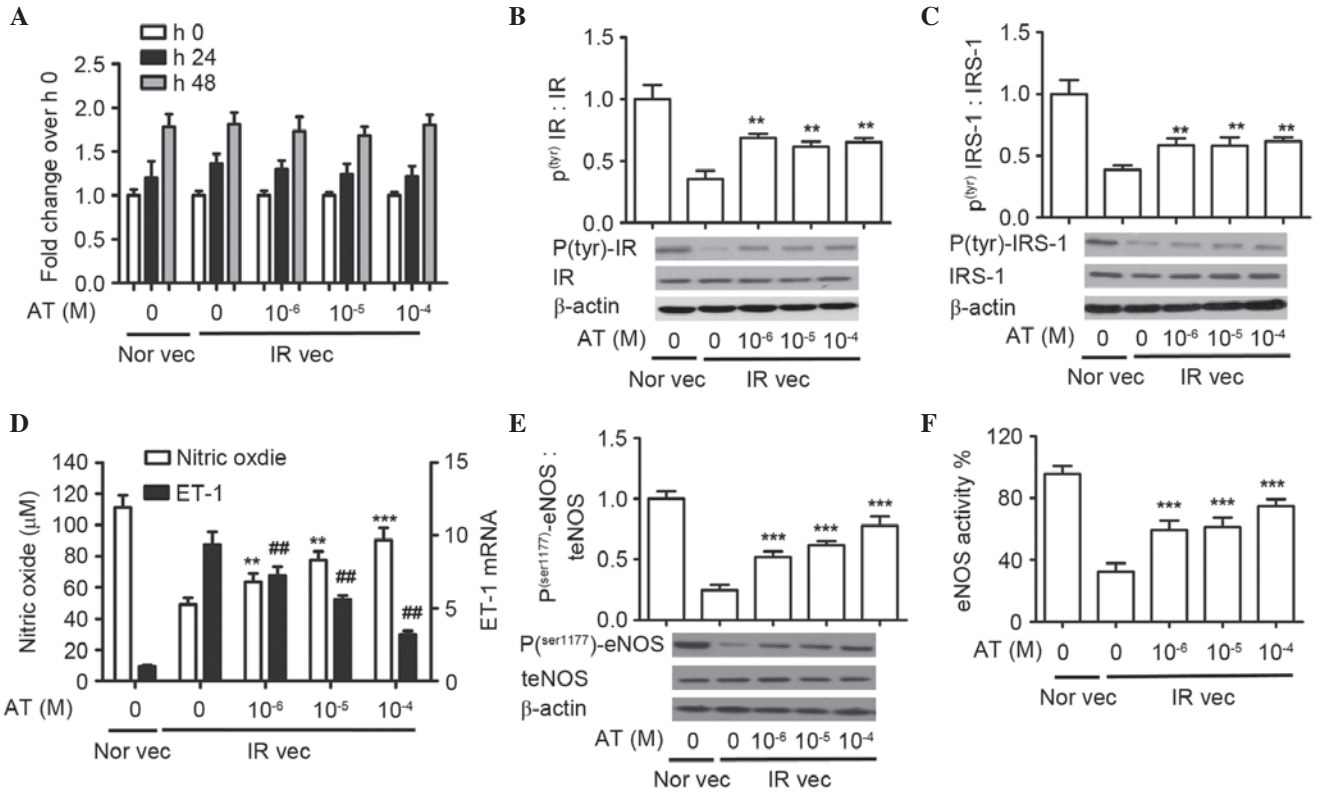

Figure 2. Atorvastatin increases the protein expression of phosphor$^{(tyr)}$-IR and phosphor$^{(tyr)}$-IRS-1, production of NO, protein expression of phosphor (^{ser1177})-eNOS and activity of eNOS, and downregulates the mRNA level of ET-1 in a dose-dependent manner. The HUVECs were pretreated with 30 mM glucose and 10^{-5} M insulin, and then with atorvastatin (0, 10^{-6}, 10^{-5} and 10^{-4} M). (A) Cell proliferation rates were analyzed using an MTS assay. Western blot analysis of (B) phosphor $^{(tyr)}$IR and (C) phosphor $^{(tyr)}$IRS-1. (D) Nitrate reduction assay of NO concentrations and reverse transcription-quantitative polymerase chain reaction analysis of ET-1 mRNA. The values for ET-1 mRNA in the IR vec cells without atorvastatin are defined as 1. $^{\#\#}$P<0.01 vs. 1. (E) Western blot analysis of phosphor $^{(ser1177)}$ eNOS and (F) assessment of eNOS activity. (A-F) **P<0.01 and ***P<0.001 vs. untreated IR vec. The data are presented as the mean ± standard deviation of three independent experiments. Nor vec, cells cultured in normal media; IR vec, cells cultured in media containing 30 mM glucose and 10^{-5} M insulin; AT, atorvastatin; HUVECs, human umbilical vein endothelial cells; phosphor/P, phosphorylated; t, total; IR, insulin receptor; IRS-1, insulin receptor substrate-1; NO, nitric oxide; eNOS, endothelial NO synthase.

glucose, either alone or in combination with 10^{-5} M insulin, for 24 h. No differences in cell proliferation or cell morphology were observed between the cells cultured in different concentrations of glucose with or without 10^{-5} M insulin (data not shown). The cells were then treated with insulin at an effective stimulus concentration of 10^{-5} M for 6 h. As shown in Fig. 1A and B, 30 mM glucose significantly decreased the protein levels of phosphor$^{(tyr)}$-IR and phosphor$^{(tyr)}$-IRS-1 by 60 and 40%, respectively, compared with the level in the cells cultured with 5 mM glucose. Of note, when the cells were treated with the high dose (30 mM) of glucose and 10^{-5} M insulin, there were further decreases in phosphor$^{(tyr)}$-IR and phosphor$^{(tyr)}$-IRS-1 (P<0.05), compared with the cells treated with 30 mM glucose alone. Specifically, the expression of phosphor$^{(tyr)}$-IR was reduced by 60% and the expression of phosphor$^{(tyr)}$-IRS-1 was reduced by 40%, compared with the level in the cells cultured with 5 mM glucose. The addition of 10^{-5} M insulin alone did not alter the level of phosphor$^{(tyr)}$-IR, but decreased the level of phosphor$^{(tyr)}$-IRS-1 in the cells cultured with 5 mM glucose.

To confirm that the endothelial dysfunction was induced by high levels of glucose and insulin, the total NO concentration and mRNA level of ET-1 were measured. The protein expression of eNOS was also evaluated. NO production was reduced in the 30 mM glucose group (53.69±3.45 μM) compared with the 5 mM glucose group (116.17±4.93 μM), and 30 mM glucose combined with 10^{-5} M insulin caused a further reduction in NO production (36.26±5.24 μM; Fig. 1C). By contrast, the combination treatment of 30 mM glucose and 10^{-5} M insulin induced an increase of ~13 fold in the mRNA level of ET-1, compared with the cells treated with 5 mM glucose.

The protein expression of eNOS was further assessed in the present study. As shown in Fig. 1D, glucose and insulin treatment did not alter the expression level of total eNOS, however, higher doses of glucose decreased the protein expression of phosphor$^{(ser1177)}$-eNOS, which was further augmented by the presence of insulin. The phosphorylation level of the cell cultured in 30 mM glucose and 10^{-5} M insulin decreased to 22% of that of the cells cultured in 5 mM glucose. It was also found that the activity of eNOS altered in parallel with the protein expression of phosphor$^{(ser1177)}$-eNOS. The combination of 30 mM glucose and 10^{-5} M insulin caused a significant reduction, to 25% of tn the cells cultured in 5 mM glucose (Fig. 1E).

Atorvastatin increases the protein expression of phosphor$^{(tyr)}$-IR and phosphor$^{(tyr)}$-IRS-1, production of NO, protein expression of phosphor $^{(ser1177)}$-eNOS and activity of eNOS, and downregulates the mRNA level of ET-1 in a dose-dependent manner. Prior to examining the effect of atorvastatin on the production of NO, the present study recorded the effect of atorvastatin

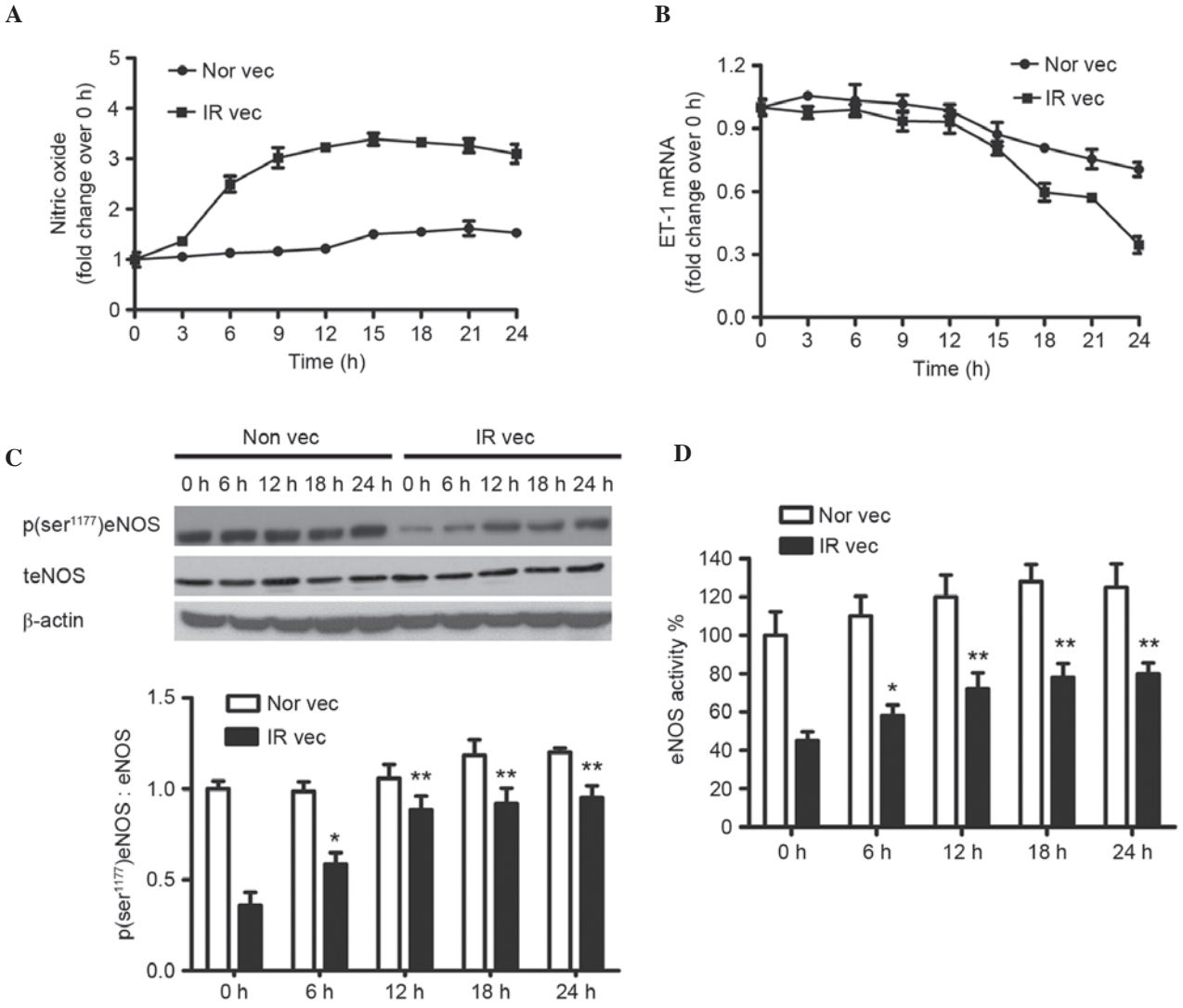

Figure 3. Atorvastatin increases the production of NO, the protein expression of phosphor $^{(ser1177)}$-eNOS and the activity of eNOS, and downregulates the mRNA expression of ET-1 in a time-dependent manner. HUVECs were cultured in normal media (Nor vec) and pretreated with 30 mM glucose and 10^{-5} M insulin for 24 h (IR vec) with or without 10^{-4} M atorvastatin (A) Nitrate reduction assay of NO concentrations and (B) reverse transcription-quantitative polymerase chain reaction analysis of ET-1 mRNA were performed every 3 h. (C) Western blot analysis of protein levels of phosphor $^{(ser1177)}$-eNOS and total eNOS; (D) eNOS activity was measured at the indicated time points. The data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01 vs. IR vec at 0 h. AT, atorvastatin; HUVECs, human umbilical vein endothelial cells; phosphor/P, phosphorylated; t, total; IR, insulin receptor; IRS-1, insulin receptor substrate-1; NO, nitric oxide; eNOS, endothelial NO synthase.

on cell proliferation to assess the toxicity of the atorvastatin. As shown in Fig. 2A, no differences in cell proliferation were observed between the Nor vec, IR vec and IR vec cells incubated with increasing doses of atorvastatin for 48 h (P>0.05).

There was an apparent decrease in the insulin-stimulated expression of phosphor$^{(tyr)}$-IR in the IR vec cells, which increased following treatment with atorvastatin. There were significant differences in the expression of phosphor$^{(tyr)}$-IR between the Nor vec (1.00±0.07), untreated IR vec (0.38±0.04) and IR vec cells treated with increased doses of atorvastatin (10^{-6} M, 0.68±0.01; 10^{-5} M, 0.62±0.03; 10^{-4} M, 0.63±0.04). The expression trend of phosphor$^{(tyr)}$-IRS-1 was similar to phosphor$^{(tyr)}$-IR. There was a significant increase when the IR vec cells were treated with increased doses of atorvastatin (10^{-6} M, 0.57±0.05; 10^{-5} M, 0.55±0.05; 10^{-4} M, 0.60±0.03), compared with the untreated IR vec cells (0.36±0.04). Increasing doses of atorvastatin did not alter the protein expression levels of either phosphor $^{(tyr)}$-IR or phosphor $^{(tyr)}$-IRS-1 (P>0.05).

The effect of atorvastatin on NO production was also evaluated. As shown in Fig. 2D, atorvastatin significantly increased NO production, compared with the untreated IR vec group, in a dose dependent manner (untreated IR vec, 49.10±4.23 μM; 10^{-6} M, 63.69±5.19 μM; 10^{-5} M, 77.43±5.56 μM; 10^{-4} M, 90.37±7.92 μM). By contrast, atorvastatin reduced the mRNA expression of ET-1, compared with the untreated IR vec group. Among the four treatment concentrations, the maximal inhibition rate occurred at the dose of 10^{-4} M (76%), however, inhibition was readily detectable at 10^{-6} M (24%). Atorvastatin significantly increased the protein expression of phosphor $^{(ser1177)}$-eNOS in a dose-dependent manner, compared with the untreated IR vec group: Untreated IR vec, 0.24±0.02; 10^{-6} M, 0.52±0.04; 10^{-5} M, 0.62±0.03; 10^{-4} M, 0.72±0.06). Atorvastatin significantly increased the activity of eNOS,

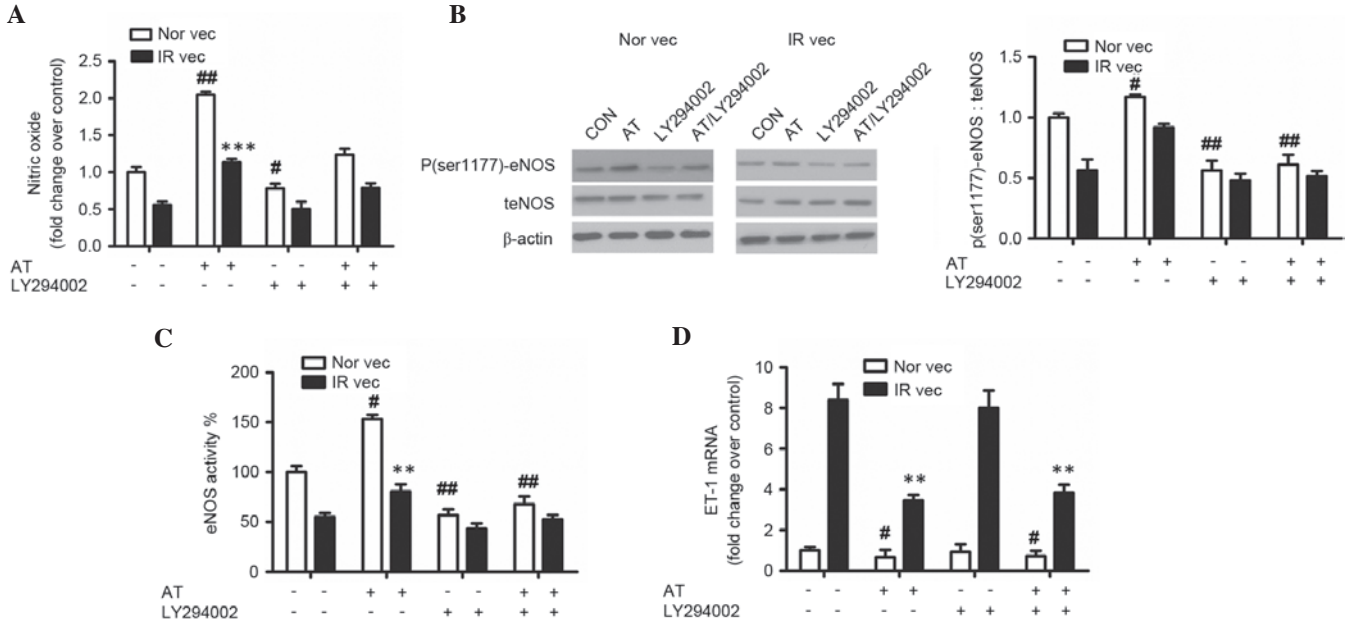

Figure 4. PI3K inhibitor,LY29004, decreases the effect of atorvastatin on NO production, the protein expression of phosphor $^{(ser1177)}$-eNOS and activity of eNOS, but does not affect the mRNA level of ET-1. HUVECs cultured in normal media (Nor vec) and pretreated with 30 mM glucose and 10^{-5} M insulin media (IR vec) were treated with PBS (control), 10^{-4} M atorvastatin, 25 μM LY29004, or a combination of 10^{-4} M atorvastatin and 25 μM LY29004, respectively. (A) Nitrate reduction assay of NO concentrations; (B) western blot analysis of the protein expression levels of phosphor $^{(ser1177)}$ eNOS and total eNOS; (C) assessment of eNOS activity, (D) reverse transcription-quantitative polymerase chain reaction analysis of ET-1 mRNA. The data are presented as the mean ± standard deviations, *P<0.05, **P<0.01 and ***P<0.001 vs. IR vec treated with PBS; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. Nor vec treated with PBS. PI3K, phosphatidylinositol 3-kinase; AT, atorvastatin HUVECs, human umbilical vein endothelial cells; phosphor/P, phosphorylated; t, total; IR, insulin receptor; IRS-1, insulin receptor substrate-1; NO, nitric oxide; eNOS, endothelial NO synthase; CON, control.

compared with the untreated IR vec group: Untreated IR vec, 42.33±4.49%; 10^{-6} M, 61.67±5.43%; 10^{-5} M, 66.14±4.92%; 10^{-4} M, 74.67±3.68% (Fig. 2D and E).

Atorvastatin increases the production of NO, protein expression of phosphor $^{(ser1177)}$-eNOS and activity of eNOS, and downregulates the mRNA expression of ET-1 in a time-dependent manner. To further examine the effect of atorvastatin, the levels of NO produced in the Nor vec and IR vec cells were measured every 3 h. As shown in Fig. 3A, in the IR vec cells, atorvastatin induced the production of NO, the level of which was highest at 9 h and was maintained during the duration of the asssessment. By contrast no significant alterations were observed in the Nor vec cells. Atorvastatin caused a reduction in the mRNA levels of ET-1 in the Nor vec and IR vec cells. The effect of atorvastatin on the activity of eNOS was also measured. As shown in Fig. 3C, atorvastatin did not alter the expression level of total eNOS in either the Nor vec and IR vec cells during the assessment, however, atorvastatin increased the protein expression of phosphor $^{(ser1177)}$-eNOS in a time dependent manner. In addition, eNOS activity in the IR vec cells increased following atorvastatin treatment, but remained at the same level in the Nor vec cells in 24 h.

LY29004, a PI3K inhibitor, decreases the effect of atorvastatin on NO production, protein expression of phosphor $^{(ser1177)}$-eNOS and activity of eNOS, but not the mRNA level of ET-1. To determine whether atorvastatin stimulates eNOS activation in a PI3K/Akt/eNOS-dependent manner, the cells were treated with the PI3K inhibitor, LY294002. As shown in Fig. 4A, atorvastatin induced an increase in the production of NO in the Nor vec and IR vec cells. This effect was reduced by LY294002 (P<0.05). Similarly, the effects of atorvastatin on the protein expression of phosphor (ser1177)-eNOS and activity of eNOS were reduced by LY294002 in the Nor vec and IR vec cells (P<0.05; Fig. 4B and C). However, the redutcion observed in the mRNA expression of ET-1 was not affected by LY294002 (P>0.05).

Discussion

Impairment of vascular endothelial cell structure and function is a common pathological basis of cardiovascular disease. The predominant factors, which lead to vascular endothelial dysfunction include dyslipidemia, impairment of endothelium-dependent vasodilation and inflammation (1). Insulin resistance, the early stage of type 2 diabetes, is frequently associated with endothelial dysfunction as an early predictor of atherosclerosis and risk of cardiovascular disease (13,26). However, the mechanism of interaction between insulin resistance and endothelial dysfunction remains to be elucidated. Atorvastatin is a vessel protective drug, which has been confirmed to protect endothelial function by increasing NO production, and reducing circulating levels of interleukin (IL)-6 and tumor necrosis-factor (TNF)-α independent of changes in plasma cholesterol (27,28). In the present study, an insulin resistant-endothelial dysfunction model was established by treating the HUVECs with high concentrations of glucose and insulin for 24 h. Compared with normal culture media, high concentrations of glucose and insulin interfered with insulin signaling by decreasing insulin-stimulated phosphor $^{(tyr)}$-IR and downstream IRS-1. This result is consistent with previous reports that hyperglycemia can induce insulin resis-

tance by inhibiting phosphor $^{(tyr)}$-IRS-1, which in turn negatively regulates its function (29,30).

In individuals with insulin-resistance, the impairment of insulin signal transduction via the PI3K pathway can down-regulate the level of phosphor $^{(ser1177)}$-eNOS and decrease NO production (31). In the present study, impairment of endothelial insulin signaling was accompanied by a reduction in tyrosine phosphorylation of the insulin receptor substrate. Previous reports have indicated that the expression of TNF-α is significantly increased in insulin resistance by decreasing the tyrosine kinase activity of the insulin receptor (32,33). In addition, TNF-α induces the activation of NAD(P)H oxidase, leading to endothelial dysfunction with increased ET-1 availability (34,35). Thus, the present study hypothesized that superphysiological concentrations of insulin and glucose increase the expression of ET-1 through the downstream effects of the upregulation of TNF-α on NAD(P)H oxidase and superoxide anion production. The effects of atorvastatin on insulin sensitivity have been reported in previous years. Wong *et al* reported that atorvastatin induced insulin sensitization in Zucker lean and fatty rats (36). Atorvastatin also reverses the reduction of phosphor $^{(tyr)}$-IR and phosphor $^{(tyr)}$-IRS-1 in animal models of insulin resistance or impairment of insulin signaling (37,38). However, the function of atorvastatin on insulin sensitivity in insulin resistant-endothelial dysfunction cell models has not been reported. The data obtained in the present study are the first, to the best of our knowledge, to show that atorvastatin reversed the inhibition of phosphor $^{(tyr)}$-IR and phosphor $^{(tyr)}$-IRS-1 induced by high glucose in combination with high insulin. In addition, atorvastatin increased the production of NO and dow-regulated the expression of ET-1 in a dose and time dependent manner.

In the present study, the PI3K inhibitor, Ly294002, was used. The production of NO, protein expression of phosphor $^{(ser1177)}$-eNOS and activity of eNOS were significantly decreased when the cells were treated with LY294002, which indicated that PI3K was a specific upstream effector to phosphorylate the eNOS on ser1177. In conclusion, the present study provided the first evidence, to the best of our knowledge, that a high concentration of glucose in combination with a high concentration of insulin stimulated endothelial insulin resistance in vascular endothelial cells. In addition, atorvastatin ameliorated these effects, primarily via the PI3K/Akt/eNOS pathway. These findings provide further evidence that atorvastatin is useful for patients with insulin resistance.

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