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Ibrolipim attenuates high glucose-induced endothelial dysfunction in cultured human umbilical vein endothelial cells via PI3K/Akt pathway

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Objective: Endothelial dysfunction is a key event in the onset and progression of atherosclerosis associated with diabetes. Increasing cell apoptosis may lead to endothelial dysfunction and contribute to vascular complications. Therefore, we aimed to elucidate the possible role and mechanism of ibrolipim in preventing endothelial dysfunction induced by high glucose. *Methods:* Human umbilical vein endothelial cells (HUVECs) were cultured respectively under normal glucose level (5.5 mM), high glucose level (33 mM), and high glucose level with ibrolipim treatment. Endothelial dysfunction was identified by the expression of ET-1 and vWF through reverse transcription PCR (RT-PCR). HUVECs apoptosis was assessed by fluorescent staining with Hoechst 33258. Akt activity was analyzed by western blot. *Results:* High glucose condition significantly increased the rate of apoptotic cells, weakened cell viability, and decreased the expression of ET-1 and vWF. Ibrolipim treatment significantly attenuated these alterations of endothelial dysfunction. The lower concentrations (2, 4, 8 μM) of ibrolipim inhibited apoptosis of cultured HUVECs, improved cell viability, down-regulated the mRNA levels of ET-1, vWF, and attenuated the cytotoxicity; however, higher concentration (16, 32 μM) of ibrolipim aggravated the damage of HUVECs cultured under high glucose level. Meanwhile, high glucose induced a decrease of Akt activity which led to apoptosis, and ibrolipim prevented the decrease and attenuated apoptotic effect induced by high glucose. Furthermore, the PI3K inhibitor LY294002 significantly abolished the anti-apoptotic effect of ibrolipim, and decreased Akt phosphorylation. Although, the expression of Akt mRNA and total protein were not altered in cultured HUVECs. *Conclusion:* Ibrolipim at lower concentrations can inhibit high glucose-induced apoptosis in cultured HUVECs, which might be related to the alternation of Akt activity. Ibrolipim has the potential to attenuate endothelial dysfunction and lower the risk of diabetes-associated vascular diseases. And it might be a therapeutic agent for diabetic vascular complications.

1. Introduction

Diabetes mellitus can cause a wide variety of vascular complications and cardiovascular dysfunction. Dyslipidemia, hyperglycemia and increased oxidative stress have been proposed to explain the acceleration of atherosclerosis in diabetes mellitus(Renard and Van Obberghen 2006; Sudano et al. 2006). The importance of hyperglycemia is becoming increasingly evident in this process. Population studies show that higher levels of blood glucose are associated with an incremental risk of cardiovascular disease and hyperglycemia is thought to be a key factor in the development of endothelial dysfunction (1998). Endothelial dysfunction might be the beginning of atherosclerosis in diabetes mellitus (Kosuge et al. 2005). The mechanisms of hyperglycemia-related endothelial dysfunction and clinical complications still remain unclear. Some studies show that cell apoptosis is an important factor contributing to the increased vascular risk associated with Diabetes mellitus, and high glucose can accelerate cell apoptosis, which can aggravate this process (Ho et al. 2006; Sheu et al. 2005). Therefore, prevention of high glucose-induced endothelial-cell dysfunction may be a new to treat diabetes-associated atherosclerosis.

Increased cell apoptosis (Bannerman et al. 1998), and upregulation of endothelial specific markers such as von Willebrand factor (vWF) and endothelin-1 (ET-1) are considered characteristics of endothelial dysfunction (Bousette and Giaid 2003; Chang et al. 2002; Chen et al. 2003; Lam 2001; Schneider et al. 2002). Vascular endothelial dysfunction of Diabetes mellitus type 2 is concerned with protein kinase B activity inhibition (Kobayashi et al. 2004). Protein kinase PKB/Akt is an important anti-apoptotic protein, and mediates the pathological process of tumors and diabetes (Amaravadi and Thompson 2005; Fulton et al. 1999). Evidence indicates that PI3K/Akt plays an important role in preventing high glucose-induced endothelial cell injury.

It has been reported that ibrolipim ([4-(4-bromo-2-cyanophenylcarbamoyl) benzyl]-phosphoric acid diethyl ester, NO-1886) (Fig. 1) can decrease triglycerides, glucose and FFAs level in plasma, with an increase of lipoprotein lipase (LPL) activity.

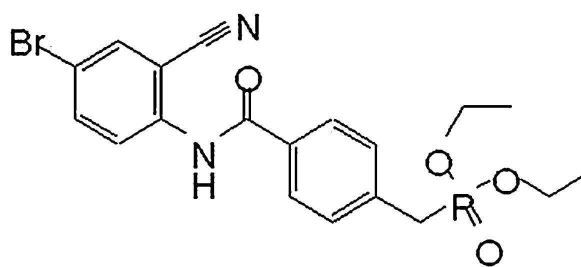
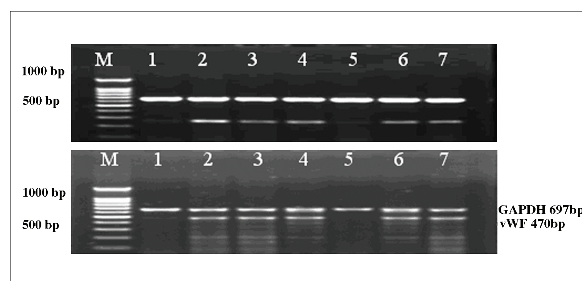


Fig. 1: Ibrolipim (NO-1886), [4-(4-bromo-2-cyano-phenylcarbamoyl)-benzyl]-phosphonic acid diethyl ester

Meanwhile ibrolipim up-regulates high density lipoprotein cholesterol levels in rats, rabbits and minpigs. Furthermore, ibrolipim can prevent fat accumulation in high fat-fed rats, reduce insulin resistance (Kusunoki et al. 2000), and inhibit atherosclerosis lesions in diabetic experimental animals (Yin et al. 2002).

Despite recent advances in therapy, prognosis for diabetes-associated vascular diseases is poor. So there remains a need for more effective and less harmful treatments. Therefore, investigating the molecular mechanism of diabetes-associated vascular diseases is important.

In this study, we aim to confirm whether ibrolipim attenuates high glucose-induced endothelial dysfunction in human umbilical vein endothelial cells (HUVECs).



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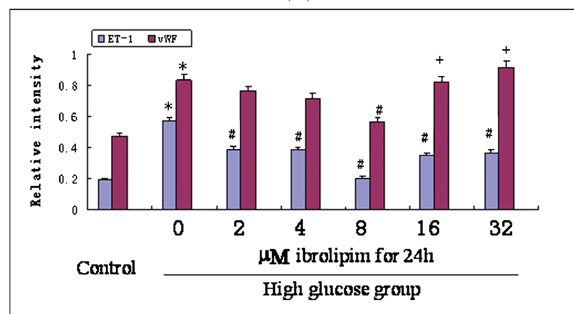


(B)

Fig. 2: High glucose increased the mRNA levels of ET-1 and vWF in HUVECs. Ibrolipim (2, 4, 8 μM) gradually decreased the mRNA levels of ET-1 and vWF, but the mRNA levels of ET-1, vWF were significantly increased at 16, 32 μM ibrolipim. Values are mean ± S.D. from 3 independent experiments. *$p < 0.05$ compared with control group. # < 0.05 compared with high glucose group. + $p < 0.05$ compared with 8 μM ibrolipim plus high glucose group

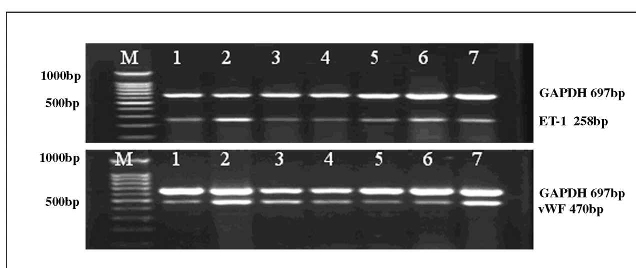
2. Investigations and results

2.1. *Effects of ibrolipim on the gene expression of ET-1 and vWF*

In this study, we used RT-PCR to examine the concentration dependence effects of ibrolipim on ET-1 and vWF mRNA levels in HUVECs treated with high glucose concentration. The results showed that after pretreatment with high glucose for 72 h, the mRNA levels of ET-1 and vWF mRNA were significantly increased, by 297.1% and 177.8% compared with the control group respectively, and ibrolipim (2, 4, 8 μM) attenuated the mRNA expression levels induced by high glucose (Fig. 2). The 8 μM ibrolipim treated group showed more significant effects than the 0 μM treated ibrolipim group. The mRNA levels of ET-1 and vWF showed no significant difference between the control group and the 8 μM ibrolipim group. However, compared with the 8 μM ibrolipim treated group, the mRNA levels of ET-1, vWF were significantly increased in both the 16 and 32 μM ibrolipim treated groups, and there was no significant difference between the high glucose group and the 32 μM ibrolipim treated group. The results indicated that the effects of ibrolipim on the mRNA levels of ET-1 and vWF in HUVECs treated by high glucose were related to ibrolipim concentration, and the 8 μM ibrolipim treated group led to the most significant decrease.

2.2. *Time dependent effect of 8 μM ibrolipim on the gene expression of ET-1 and vWF*

The time course effects of ET-1 and vWF mRNA levels in HUVECs treated by high glucose and 8 μM ibrolipim were analyzed by the RT-PCR method. As shown in Fig. 3, compared with the high glucose model group, mRNA levels of ET-1 and vWF showed a significant decrease, but there was the biggest decline in the 24-h exposure group. There were no significant differences between the control group and the 24-h exposure group. ET-1 and vWF mRNA levels in the 24-h exposure group decrease to 30.0% and 42.9% of the control group, respectively. However, compared with the 24-h group, the mRNA levels increased significantly in the 48-h and 72-h exposure groups. Experimental results indicated that the protective effect of 8 μM ibrolipim on endothelial dysfunction induced by high glucose was concerned with intervention time, 24-h while the exposure group showed the best protective effects.



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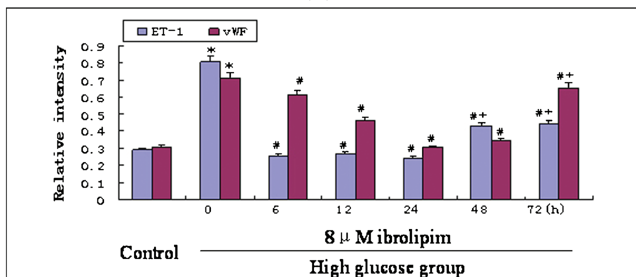

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Fig. 3: High glucose increased the mRNA levels of ET-1 and vWF in HUVECs. 8 μM ibrolipim exposure could significantly decrease the mRNA levels of ET-1 and vWF induced by high glucose treatment. And there was the biggest decline in the 24 h group. However, the mRNA levels of ET-1 and vWF were significantly increaced in 48 h and 72 h group compared with 24 h group. Values are mean ± S.D. from 3 independent experiments. *$p < 0.05$ compared with control group. #$p < 0.05$ compared with high glucose group. + $p < 0.05$ compared with 8 μM ibrolipim for 24 h plus high glucose group

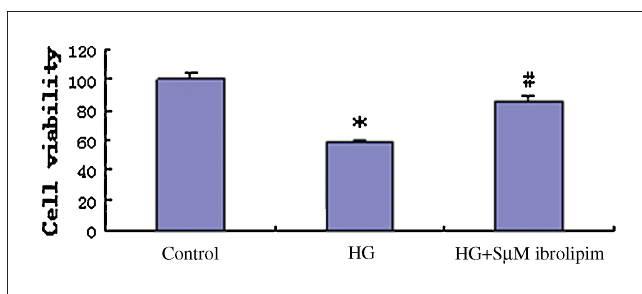


Fig. 4: Effect of 8 μM ibrolipim on high glucose-induced loss of cell viability. HUVECs pretreated with 8 μM ibrolipim were exposed to high glucose for 72 h. Cell viability was determined by MTT assay. Results are expressed as percentages of control. Values are mean ± S.D. from 3 independent experiments. *$p < 0.05$ compared with control group. #$p < 0.05$ compared with high glucose group.

2.3. *Effect of ibrolipim on high glucose-induced loss of HUVECs viability*

To determine the effects of glucose and 8 μM ibrolipim on HUVECs, we first performed a MTT assay to measure cell viability. As illustrated in Fig. 4, compared with normal glucose, exposure of HUVECs to high glucose concentration for 72 h resulted in a significant decrease in cell viability to approximately 57.67%, which was consistent with previous reports (Ho et al. 2006; Ido et al. 2002). Pretreatment with 8 μM ibrolipim improved cell viability, which increased to 86.06% of the control group.

2.4. *Iibrolipim inhibited cells apoptosis under high glucose condition*

HUVECs apoptosis were confirmed by Hoechst 33258 staining. As shown in Fig. 5A, C, HUVECs nuclei were round and stained with Hoechst 33258. Fig. 5B, D showed manifest fragment of DNA in nuclei. High glucose induced significant apoptosis (Fig. 5B), whereas ibrolipim significantly attenuated high glucose -induced apoptosis (Fig. 5C), and LY294002 abolished the anti-apoptosis effect of ibrolipim (Fig. 5D). Marked apoptotic morphologic alterations including membrane and nuclear condensation were also observed by fluorescence microscopy.

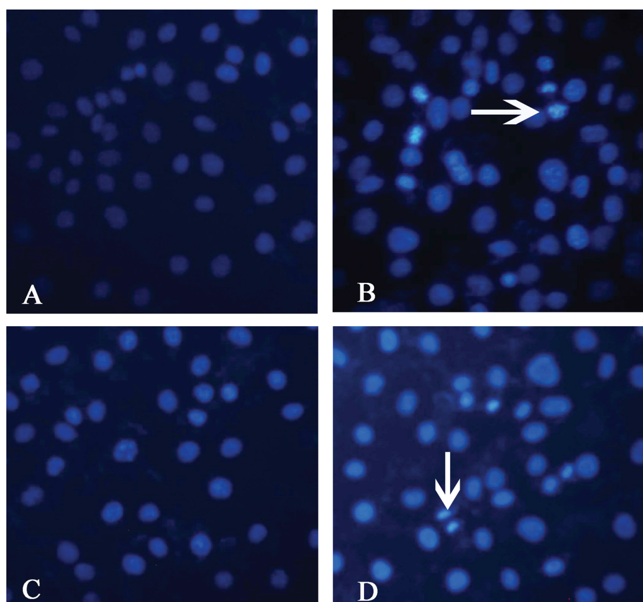
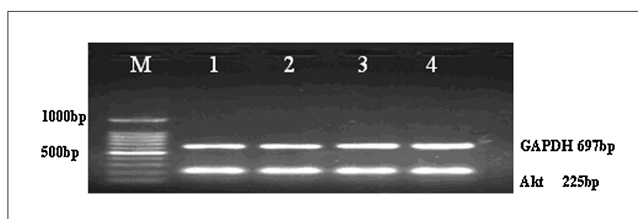


Fig. 5: Induction of nuclear fragmentation in HUVECs (2×10^5 cells/ml) were treated with high glucose for 72 h, 8 μM ibrolipim for 24 h and LY294002 preconditioning for 30 min, then stained with Hoechst 33258. (A) Control group; (B) High glucose group; (C) 8 μM ibrolipim plus high glucose group; (D) 10 μM LY294002 plus 8 μM ibrolipim plus high glucose group. Cell morphology was observed under a fluorescence microscope (× 200). Normal cells: structure integrated, normal size, and uniformly blue (A, C). Apoptotic cells: containing bright blue patches in the nuclei (B, D).



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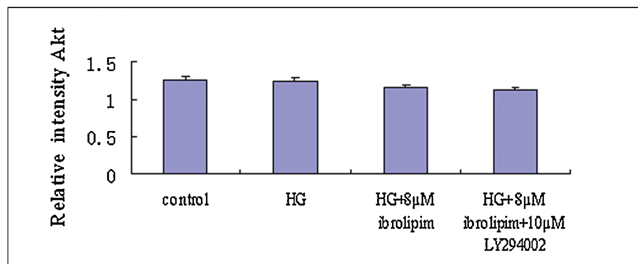

(B)

Fig. 6: HUVECs pretreated with or without ibrolipim were exposed to high glucose with or without inhibitors for 72 h. High glucose, 8 μM ibrolipim and 10 μM LY294002 did not alter the expression of Akt mRNA in cultured HUVECs. Values are mean ± S.D. from 3 independent experiments.

2.5. *Effect of ibrolipim on high glucose-induced Akt mRNA expression*

In the study, we used RT-PCR to examine the effect of ibrolipim on Akt mRNA levels in HUVECs treated by high glucose. As shown in Fig. 6, the mRNA levels of Akt showed no significant difference between control group and high glucose group. However, pretreatment with 8 μM ibrolipim for 24 h showed no significant effect of Akt mRNA levels. Meanwhile, there was no alteration of Akt mRNA levels in cultured HUVECs pretreating with 10 μM LY294002 for 30 minutes.

2.6. *Ibrolipim promoted Akt activity under high glucose condition*

To explore the possible mechanism of the anti-apoptotic effect of ibrolipim on high glucose-induced HUVECs, we investigated the effect of ibrolipim on Akt activity. High glucose decreased the ratio of p-Akt (Ser-473) and p-Akt (Thr-308) to Akt by 74.4% and 80.6%, compared with the control group, respectively. Ibrolipim increased the ratio of p-Akt (Ser-473) and p-Akt(Thr-308) to Akt by 119.7% and 114.7%, compared with the HG group, respectively (Fig. 7). The protective effect of ibrolipim was inhibited by LY204002, by 86.7% and 83.3%, respectively. High glucose and ibrolipim did not alter the expression of total Akt.

3. Discussion

Diabetes mellitus is a major risk factor for the development of vascular complications leading to a threefold increase in death relative to nondiabetic patients. Complications are the major contributors to morbidity and mortality in diabetes (Ido et al. 2002). Hyperglycemia can accelerate endothelial dysfunction (Datta et al. 1999). And endothelial dysfunction is a key event in the onset and progression of atherosclerosis associated with diabetes (Sheu et al. 2005). Furthermore, increasing cell apo-

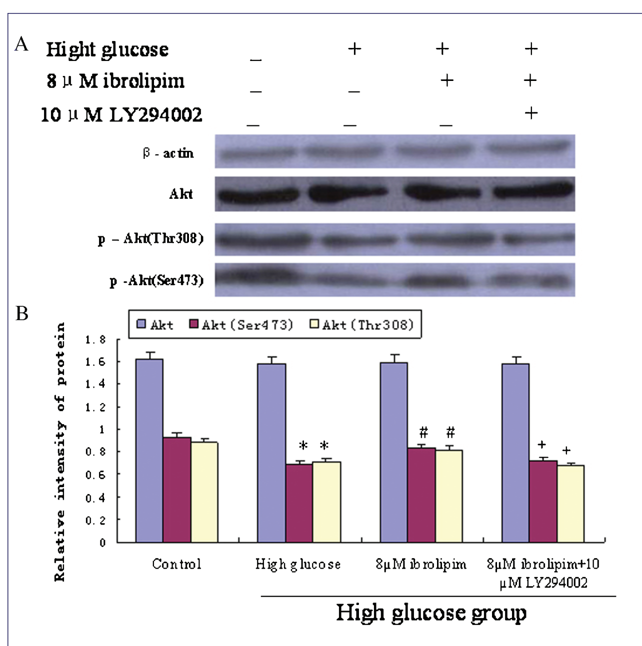

Fig. 7: Effect of ibrolipim on PI3k/Akt pathway under high-glucose condition in HUVECs. We used the ratio of phosphorylation to total protein expression to evaluate Akt activity. HUVECs were incubated with or without ibrolipim (8 μM), high glucose (33 mM), inhibitors for 72 h. Values are mean ± S.D. from 3 independent experiments. *$p < 0.05$ compared with control group. #$p < 0.05$ compared with high glucose group. +$p < 0.05$ compared with 8 μM ibrolipim plus high glucose group.

ptosis may contribute to the loss of endothelial integrity and lead to vascular complications (Sheu et al. 2005). High glucose accelerates cell apoptosis (Burgering and Coffer 1995; He et al. 2006). We aimed to elucidate the possible role and mechanism of ibrolipim in preventing endothelial dysfunction induced by hyperglycemia. In this study, high glucose enhanced the expression of vWF and ET-1, promoted cell apoptosis and inhibited Akt activity in cultured HUVECs, thus resulting in endothelial dysfunction. However, ibrolipim, a lipoprotein lipase activator, could prevent the negative effects induced by high glucose, thereby inhibiting the accelerated endothelial dysfunction process induced by high glucose. This effect was associated with an upregulated PI3K/Akt pathway.

The plasma levels of vWF are elevated in response to endothelial damage, hypoxia, inflammatory cytokines, thrombin, and adrenalin. And levels of vWF are a biomarkers highly associated with endothelial dysfunction (Hogikyan et al. 1998). Increasing vWF in cardiovascular disease patients forecast the major clinical events such as the occurrence of death and myocardial infarction (Lunetta et al. 1998). ET-1, a 21-amino acid peptide produced primarily in endothelial cells, is the most powerful endogenous vasoconstrictive agent and has been identified as a key player of endothelial dysfunction resulting from endothelial cells activation even injury (Thompson et al. 1995). ET-1 participates in the entire atherosclerosis process (Ihling et al. 2004) as well as being an important index of endothelial dysfunction. It also plays an important role in atherosclerosis, especially diabetes-associated vascular diseases and indicates endothelial function in vasoconstriction and vasodilation and the injury of HUVECs exposed to high glucose.

High glucose results in endothelial cells injury even death, which leads to the secretion of ET-1 and vWF by Ecs (Varga-Szabo et al. 2008). To investigate the release of ET-1 and vWF by HUVECs subjected to high glucose, the expression of ET-1 and vWF were measured. Results showed that hyperglycemia induced the expression of ET-1 and vWF in HUVECs compared with control groups. After pretreatment with ibrolipim for 24 h, the expression of ET-1 and vWF significantly decreased compared with high glucose group. The lower concentrations (2, 4, 8 μM) of ibrolipim decreased the expression of ET-1 and vWF induced by high glucose, and attenuated the cytotoxicity; in contrast, higher concentrations (16, 32 μM) of ibrolipim aggravated the damage of HUVECs induced by high glucose. Whether there is an interaction with high glucose, such as increasing oxidative stress reaction etc, which has not been seen. And the specific mechanism needs further clarification. Our results show that ibrolipim inhibited the expression of ET-1 and vWF and subsequently more effectively reversed the changes of ET-1 and vWF, and ameliorated endothelial dysfunction that occurred during hyperglycemia.

Akt is a downstream effector of PI3K and a multifunctional regulator of priming apoptosis, cell survival, cell cycle, anti-oxidation, angiogenesis and protein synthesis, for example. Meanwhile Akt mediates tumor and diabetes pathological processes in carcinogenesis and diabetes (Abraham and Dashwood 2008). Akt regulates cell apoptosis and metabolism by Akt-Thr308 and Akt-Ser473 sites phosphorylation (Berk et al. 2001; Coutinho et al. 1999). Phosphorylation of Ser473 is very important to maximize Akt activity (Franke et al. 1997). Recent studies show that the PI3k/Akt pathway plays an important role in preventing high glucose-induced cell injury.

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Akt activity reduced by high glucose results in the inhibition of GLUT4-mediated glucose transportation (Hernandez et al. 2001; Tremblay et al. 2001). It is plausible that hyperglycemia-induced uncoupling of post-receptor insulin signaling at PI3k-Akt in ECs may be responsible for the development of vascular complications in diabetes. High glucose concentration has been reported to increase ECs apoptosis (Liu et al. 2004). The need for research into the role of Akt signaling in the pathophysiology of complications of diabetes mellitus has been emphasized (Zdychova and Komers 2005).

Apoptosis is particularly prominent in models of hyperglycemic injury, affecting a significant proportion of vascular endothelium in the tissue damage (Ceriello et al. 2002; Nakagami et al. 2001; Zou et al. 2002). Apoptosis is implicated in hyperglycemia-induced endothelial dysfunction (Ayalasomayajula and Kompella 2003; Gryglewski et al. 2001; Meeking et al. 2000). It is likely that hyperglycemia inhibits the PI3k/Akt pathway in ECs, thus resulting in reduced ECs viability and proliferation, with increased ECs apoptosis. ECs apoptosis contributes to the pathogenesis of atherosclerosis and thromboembolization.

Recent studies have shown that certain factors, by up-regulating PI3K/Akt pathway, promoted Akt phosphorylation, decreased cells apoptosis, attenuated endothelial dysfunction. And application of PI3K inhibitors inhibited Akt activity and enhanced cells apoptosis. In our study, we gained some similar results. Our results showed unequivocally the role of Akt in high glucose induced effects in HUVECs. Expectedly, we observed that the treatment of HG in HUVECs induced apoptosis, decreased cell viability, and demonstrated that high glucose could promote cell apoptosis. However, whether ibrolipim attenuates cell apoptosis induced by high glucose is unclear. In our study, we chose an appropriate concentration of ibrolipim (8 μM) to carry on a follow-up study through in a dose- and time-dependent manner experiment. At first, we investigated the effect of five different concentrations (2 μM, 4 μM, 8 μM, 16 μM, 32 μM) of ibrolipim and five different time points (6, 12, 24, 48, 72 h) of 8 μM ibrolipim on high glucose accelerated endothelial cell dysfunction. We also found anti-apoptotic effect of 8 μM ibrolipim for 24 h was best. We found that ibrolipim could inhibit cell apoptosis induced by high glucose in HUVECs (Fig. 5C), improved cell viability (Fig. 4), and alleviated the direct toxicity of high

Table: Primer sequence for RT-PCR

Gene Name	Forward primer	Reverse primer	Annealing (°C)	Product (bp)	Accession no.
ET1	TGACCCACAACCGAGCACA	TCCCCAGATGAAAGAAGAGACC	57	258	NM001955
vWF	AAGAAGAGGAAGGGCGAGTAGG	CGAGGTGAGCATTGCGTGAC	58	470	NM199121
Akt	CTGTGGCACTCCAGAATA	CAAGAGCCCTGAAAGCAA	56	225	NM005465
GAPDH	TCACCATCTTCCAGGAGCGAG	TGTCGCTGTTGAAGTCAGAG	60	697	NM002046

glucose and its metabolites on endothelial cells. Moreover, pretreatment with the PI3K/Akt inhibitor LY294002 significantly increased the rate of cell apoptosis (Fig. 5D).

We explored the possible mechanism of the anti-apoptotic effect of ibrolipim on high glucose-induced HUVECs. In this study, we found that lower concentration ibrolipim attenuated high glucose-induced apoptosis in cultured HUVECs by upregulation Akt phosphorylation of threonine 308 and serine 473, increased Akt activity, and inhibited apoptosis. The study indicated that Akt (Thr308) and Akt (Ser473) might be signal cascade sensitive components in ibrolipim influenced apoptosis of endothelial cells. Pretreatment with inhibitors LY294002 obviously blocked Akt phosphorylation to levels similar to those under high glucose alone, inhibiting ibrolipim antiapoptotic function, and leading to cell apoptosis. It has been shown that Akt activation has an antiapoptotic effect in endothelial cells, ibrolipim might exert antiapoptotic effects under hyperglycemic conditions at least in part through PI3-K/Akt pathways. Ibrolipim might directly enhance Akt activity to inhibit apoptosis and might markedly decrease the expression of endothelial markers such as vWF and ET-1, so endothelial function was improved. Therefore, ibrolipim may exert a beneficial effect in preventing diabetes-associated cardiovascular complications by increasing Akt activity and antiapoptotic effect. Therefore, further studies will be needed to clarify the mechanism of ibrolipim effects on endothelial dysfunction induced by high glucose.

In summary, endothelial dysfunction induced by high glucose might be an important mechanism in the pathological changes of cardiovascular complications in diabetes. We found that ibrolipim, a lipoprotein lipase activator, decreased the expression of ET-1 and vWF, inhibited high glucose-induced apoptosis of cultured HUVECs through upregulating Akt activity. And we demonstrated that ibrolipim had the potential to attenuate endothelial dysfunction and lower the risk of diabetes-associated vascular diseases. Further studies are required to determine the effect of ibrolipim on the development of endothelial dysfunction.

4. Experimental

4.1. Reagents

Agent ibrolipim (NO-1886), [4-(4-bromo-2-cyano-phenylcarbamoyl)-benzyl]- phosphonic acid diethyl ester, CAS 133208–93–2, Lot.No. C00C99H74SM, was synthesized in the new drug research laboratory of Otsuka pharmaceutical factory Inc., (Tokushima, Japan). DMEM medium and fetal bovine serum were purchased from GIBCO/BRL. D-glucose was obtained from Sigma Chemicals (St. Louis, MO, USA). RevertAidTM First Strand cDNA Synthesis Kit was purchased from Fementas Inc., (MD, USA). Antibodies of Akt, P-Akt (Ser473) and P-Akt (Thr308) were purchased from Cell Signaling Technology (Danvers, MA, USA). Enhanced chemiluminescence (ECL) detection kit was purchased from Santa Cruz Biotechnology, Inc. (CA, USA). LY294002, antibodies ET-1, vWF and anti-β-actin, Hoechst33258 fluorescein stain apoptosis Kit were obtained from Beyotime Institute Biotechnology. All other reagents used were high grade commercially available products.

4.2. Cell culture

HUVECs-12 were obtained from Central South University (CSU) (Changsha, China) and were cultured in normal glucose (5.5 mM) Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin and maintained at 37 °C in a humidified 5% CO_2 in air incubator. Cells at passages 2–5 were used in this study. After serum-starvation for 12 h, HUVECs were incubated in normal glucose (control group, 5.5 mM) for 72 h, high glucose (HG group, 33 mM) for 72 h, ibrolipim (2, 4, 8, 16, 32 μM) for 24 h plus high glucose, 8 μM ibrolipim (6, 12, 24, 48, 72 h) plus high glucose, pretreated with LY294002 (10 μM) for 30 min before ibrolipim (8 μM) plus high glucose.

4.3. Reverse transcription-PCR

Total RNA was isolated from the cells using Trizol reagent according to the manufacturer's instructions. Two μg of total RNA was used for reverse transcription in a total volume of 20 μl with the RevertAid^{TM} First Strand cDNA synthesis Kit. Aliquots of 2 μl cDNA were subsequently amplified in a total volume of 20 μl using the MasterMix PCR kit following conditions recommended by the manufacturer. The sense and antisense primer sequences and PCR conditions are listed in the Table. PCR products were analyzed by electrophoresis on a 1.5% agarose gel viewed by ethidium bromide (EB) staining. The data were acquired with Alpha Imager 2200 software.

4.4. MTT assay

Cell viability was assessed by the 3-(4,5-dimethylthiazol-z-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. Briefly, cells seeded on 96-well microplates at 2000 cells/well were incubated with the test compounds for indicated period. After exposure, 20 μL of the MTT solution (5 mg/mL) was added into each well, and the plates were incubated for an additional 4 h. After removing the medium, DMSO (150 μL) was added into each well. The plates were read on a enzyme immunoassay analyzer (Bio-Tek Inc., ELx-800, USA) at 490 nm.

4.5. Cell apoptosis

HUVECs were collected, cells were washed with cold PBS twice and then fixed with 4% formaldehyde for 10 min at 4 °C. After fixation, the cells were washed with PBS twice and stained with Hoechst 33258 in the dark to counterstain nuclei. Cells were observed and photographed under a Nikon fluorescence microscope.

4.6. Western blot analysis

The cells were rapidly washed with ice cold PBS and lysed in sample buffer. Lysates were boiled for 5 min, and equal amounts of total cell protein were separated with 10% SDS-PAGE under denaturing conditions. After the proteins were electrotransferred to nitrocellulose, the membranes were soaked in 5% nonfat dry milk for 4 h and incubated overnight at 4 °C with the anti-p-Akt (Ser-473) or anti-p-Akt (Thr-308) polyclonal antibody (diluted 1:500) or anti- Akt or anti-β-actin. After incubation with horseradish peroxidase-conjugated secondary antibody (diluted 1:5000) for 2 h at room temperature, the immune complexes were visualized by enhanced chemiluminescence methods; the band intensity was measured and quantitated. The p-Akt or Akt band intensity was relative to the β-actin band intensity. The resulting digital images were quantified using Alpha Imager 2200 software.

4.7. Statistical analysis

All data were presented as mean ± S.D., and statistical significance was analyzed with one-way ANOVA followed by LSD and Dunnett's T3 test using SPSS 12.0. *P value* < 0.05 was deemed statistically significant in all experiments.

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References

Abraham D, Dashwood M (2008) Endothelin - role in vascular disease. Rheumatology 47 Suppl 5: v23–24.

Amaravadi R, Thompson CB (2005) The survival kinases Akt and Pim as potential pharmacological targets. J Clin Invest 115: 2618–2624.

Ayalasomayajula SP, Kompella UB (2003) Celecoxib, a selective cyclooxygenase-2 inhibitor, inhibits retinal vascular endothelial growth factor expression and vascular leakage in a streptozotocin-induced diabetic rat model. Eur J Pharmacol 458: 283–289.

Bannerman DD, Sathyamoorthy M, Goldblum SE (1998) Bacterial lipopolysaccharide disrupts endothelial monolayer integrity and survival signaling events through caspase cleavage of adherens junction proteins. J Biol Chem 273: 35371–35380.

Berk BC, Abe JI, Min W, Surapisitchat J, Yan C (2001) Endothelial atheroprotective and anti-inflammatory mechanisms. Annal NY Acad Sci 947: 93–109; discussion 109–111.

Bousette N, Giaid A.(2003) Endothelin-1 in atherosclerosis and other vasculopathies. Canad J Physiol Pharmacol 81: 578–587.

Burgering BM, Coffer PJ (1995) Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature 376: 599–602.

Ceriello A, Quagliaro L, D'Amico M, Di Filippo C, Marfella R, Nappo F, Berrino L, Rossi F, Giugliano D (2002) Acute hyperglycemia induces nitrotyrosine formation and apoptosis in perfused heart from rat. Diabetes 51: 1076–1082.

Chang CZ, Winardi D, Lin CL, Kwan AL, Jeng AY, Kassell NF, Howng SL, Lee KS (2002) Attenuation of hemolysate-induced cerebrovascular endothelial cell injury and of production of endothelin-1 and big endothelin-1 by an endothelin-converting enzyme inhibitor. Surgical Neurol 58: 181–187; discussion 187–188.

Chen Y, McCarron RM, Golech S, Bembry J, Ford B, Lenz FA, Azzam N, Spatz M (2003) ET-1- and NO-mediated signal transduction pathway in human brain capillary endothelial cells. Amer J Physiol 284: C243–249.

Coutinho M, Gerstein HC, Wang Y, Yusuf S (1999) The relationship between glucose and incident cardiovascular events. A metaregression analysis of published data from 20 studies of 95,783 individuals followed for 12.4 years. Diabetes Care 22: 233–240.

Datta SR, Brunet A, Greenberg ME (1999) Cellular survival: a play in three Akts. Genes Devel 13: 2905–2927.

Franke TF, Kaplan DR, Cantley LC (1997) PI3K: downstream AKTion blocks apoptosis. Cell 88: 435–437.

Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC (1999) Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature 399, 597–601.

Gryglewski RJ, Chlopicki S, Uracz W, Marcinkiewicz E (2001). Significance of endothelial prostacyclin and nitric oxide in peripheral and pulmonary circulation. Med Sci Monit 7: 1–16.

He L, Simmen FA, Mehendale HM, Ronis MJ, Badger TM (2006) Chronic ethanol intake impairs insulin signaling in rats by disrupting Akt association with the cell membrane. Role of TRB3 in inhibition of Akt/protein kinase B activation. J Biol Chem 281: 11126–11134.

Hernandez R, Teruel T, Lorenzo M (2001) Akt mediates insulin induction of glucose uptake and up-regulation of GLUT4 gene expression in brown adipocytes. FEBS Lett 494: 225–231.

Ho FM, Lin WW, Chen BC, Chao CM, Yang CR, Lin LY, Lai CC, Liu SH, Liau CS (2006) High glucose-induced apoptosis in human vascular endothelial cells is mediated through NF-kappaB and c-Jun NH2-terminal kinase pathway and prevented by PI3K/Akt/eNOS pathway. Cellular Signal 18: 391–399.

Hogikyan RV, Galecki AT, Pitt B, Halter JB, Greene DA, Supiano MA (1998) Specific impairment of endothelium-dependent vasodilation in subjects with type 2 diabetes independent of obesity. J Clin Endocrinol Metabol 83: 1946–1952.

Ido Y, Carling D, Ruderman N (2002) Hyperglycemia-induced apoptosis in human umbilical vein endothelial cells: inhibition by the AMP-activated protein kinase activation. Diabetes 51: 159–167.

Ihling C, Bohrmann B, Schaefer HE, Technau-Ihling K, Loeffler BM (2004) Endothelin-1 and endothelin converting enzyme-1 in human atherosclerosis – novel targets for pharmacotherapy in atherosclerosis. Curr Vasc Pharmacol 2: 249–258.

Kobayashi T, Taguchi K, Yasuhiro T, Matsumoto T, Kamata K (2004) Impairment of PI3-K/Akt pathway underlies attenuated endothelial function in aorta of type 2 diabetic mouse model. Hypertension 44: 956–962.

Kosuge M, Kimura K, Kojima S, Sakamoto T, Matsui K, Ishihara M, Asada Y, Tei C, Miyazaki S, Sonoda M, Tsuchihashi K, Yamagishi M, Ikeda Y, Shirai M, Hiraoka H, Inoue T, Saito F, Ogawa H (2005). Effects of glucose abnormalities on in-hospital outcome after coronary intervention for acute myocardial infarction. Circ J 69, 375–379.

Kusunoki M, Hara T, Tsutsumi K, Nakamura T, Miyata T, Sakakibara F, Sakamoto S, Ogawa H, Nakaya Y, Storlien LH (2000) The lipoprotein lipase activator, NO-1886, suppresses fat accumulation and insulin resistance in rats fed a high-fat diet. Diabetologia 43: 875–880.

Lam HC (2001) Role of endothelin in diabetic vascular complications. Endocrine 14: 277–284.

Liu B, Bhat M, Nagaraj RH (2004) AlphaB-crystallin inhibits glucose-induced apoptosis in vascular endothelial cells. Biochem Biophys Res Comm 321: 254–258.

Lunetta M, Infantone L, Calogero AE, Infantone E (1998) Increased urinary albumin excretion is a marker of risk for retinopathy and coronary heart disease in patients with type 2 diabetes mellitus. Diab Res Clin Pract 40: 45–51.

Meeking DR, Browne DL, Allard S, Munday J, Chowienczyck PJ, Shaw KM, Cummings MH (2000) Effects of cyclo-oxygenase inhibition on vasodilatory response to acetylcholine in patients with type 1 diabetes and nondiabetic subjects. Diabetes Care 23: 1840–1843.

Nakagami H, Morishita R, Yamamoto K, Yoshimura SI, Taniyama Y, Aoki M, Matsubara H, Kim S, Kaneda Y, Ogihara T (2001) Phosphorylation of p38 mitogen-activated protein kinase downstream of bax-caspase-3 pathway leads to cell death induced by high D-glucose in human endothelial cells. Diabetes 50: 1472–1481.

Renard C, Van Obberghen E (2006) Role of diabetes in atherosclerotic pathogenesis. What have we learned from animal models? Diabetes Metabol 32: 15–29.

Schneider JG, Tilly N, Hierl T, Sommer U, Hamann A, Dugi K, Leidig-Bruckner G., Kasperk C (2002). Elevated plasma endothelin-1 levels in diabetes mellitus. Amer J Hypertens 15: 967–972.

Sheu ML, Ho FM, Yang RS, Chao KF, Lin WW, Lin-Shiau SY, Liu SH (2005) High glucose induces human endothelial cell apoptosis through a phosphoinositide 3-kinase-regulated cyclooxygenase-2 pathway. Arterioscler, Thrombos Vasc Biol 25: 539–545.

Sudano I, Spieker LE, Hermann F, Flammer A, Corti R, Noll G, Luscher TF (2006) Protection of endothelial function: targets for nutritional and pharmacological interventions. J Cardiovasc Pharmacol 47 Suppl 2: S136–150; discussion S172–136.

Thompson SG, Kienast J, Pyke SD, Haverkate F, van de Loo JC (1995) Hemostatic factors and the risk of myocardial infarction or sudden death in patients with angina pectoris. European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group. New Engl J Med 332: 635–641.

Tremblay F, Lavigne C, Jacques H, Marette A (2001). Defective insulin-induced GLUT4 translocation in skeletal muscle of high fat-fed rats is associated with alterations in both Akt/protein kinase B and atypical protein kinase C (zeta/lambda) activities. Diabetes 50: 1901–1910.

Varga-Szabo D, Pleines I, Nieswandt B (2008) Cell adhesion mechanisms in platelets. Arterioscler, Thromb Vasc Biol 28: 403–412.

Yin W, Tsutsumi K, Yuan Z, Yang B (2002) Effects of the lipoprotein lipase activator NO-1886 as a suppressor agent of atherosclerosis in aorta of mild diabetic rabbits. Arzneim.- Forsch 52: 610–614.

Zdychova J, Komers R (2005) Emerging role of Akt kinase/protein kinase B signaling in pathophysiology of diabetes and its complications. Physiol Res/Academia Scientiarum Bohemoslovaca 54: 1–16.

Zou MH, Shi C, Cohen RA (2002) High glucose via peroxynitrite causes tyrosine nitration and inactivation of prostacyclin synthase that is associated with thromboxane/prostaglandin H (2) receptor-mediated apoptosis and adhesion molecule expression in cultured human aortic endothelial cells. Diabetes 51: 198–203.