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Impact of diabetes-associated lipoproteins on oxygen consumption and mitochondrial enzymes in porcine aortic endothelial cells*

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Impairments in mitochondrial function have been proposed to play an important role in the pathogenesis of diabetes. Atherosclerotic coronary artery disease (CAD) is the leading cause of mortality in diabetic patients. Mitochondrial dysfunction and increased production of reactive oxygen species (ROS) are associated with diabetes and CAD. Elevated levels of glycated low density lipoproteins (glyLDL) and oxidized LDL (oxLDL) were detected in patients with diabetes. Our previous studies demonstrated that oxLDL and glyLDL increased the generation of ROS and altered the activities of antioxidant enzymes in vascular endothelial cells (EC). The present study examined the effects of glyLDL and oxLDL on mitochondrial respiration, membrane potential and the activities and proteins of key enzymes in mitochondrial electron transport chain (mETC) in cultured porcine aortic EC (PAEC). The results demonstrated that glyLDL or oxLDL significantly reduced oxygen consumption in Complex I, II/III and IV of mETC in PAEC compared to LDL or vehicle control using oxygraphy. Incubation with glyLDL or oxLDL significantly reduced mitochondrial membrane potential, the activities of mitochondrial ETC enzymes — NADH dehydrogenase (Complex I), succinate cytochrome *c* reductase (Complex II + III), ubiquinol cytochrome *c* reductase (Complex III), and cytochrome *c* oxidase (Complex IV) in PAEC compared to LDL or control. Treatment with oxLDL or glyLDL reduced the abundance of subunits of Complex I, ND1 and ND6 in PAEC. However, the effects of oxLDL on mitochondrial activity and proteins were not significantly different from glyLDL. The findings suggest that the glyLDL or oxLDL impairs mitochondrial respiration, as a result from the reduction of the abundance of several key enzymes in mitochondria of vascular EC, which potentially may lead to oxidative stress in vascular EC, and the development of diabetic vascular complications.

Key words: low density lipoprotein, respiration chain, mitochondrial oxygen, consumption, mitochondrial membrane potential, vascular endothelial cells

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INTRODUCTION

Hyperglycemia and dyslipidemia are two major biochemical markers of diabetes. Cardiovascular diseases are the predominant cause of death in diabetic patients. The most common cardiovascular complication in diabetic patients is atherosclerotic coronary artery disease (Bartnik *et al.,* 2007). Elevated low density lipoprotein (LDL) is a classical risk factor for atherosclerotic cardiovascular disease. Increased levels of oxidized LDL (oxLDL) and glycated LDL (glyLDL) have been detected in diabetic patients (Lyons, 1993; Tsimikas *et al.,* 2003). Our previous studies demonstrated that oxLDL and glyLDL increased the generation of reactive oxygen species (ROS) in vascular endothelial cells (EC) (Zhao & Shen, 2005). ROS is implicated in endothelial dysfunction and diabetic vascular complications. Mitochondria are an important source of ROS in cells. We hypothesize that oxLDL or glyLDL may impair mitochondrial respiratory function in vasculature. Endothelium is a single layer cellular barrier between blood components and vascular wall. Interactions between modified LDL and vascular EC play important roles in the pathophysiology of atherosclerosis and thrombosis. Our group recently demonstrated that oxLDL or glyLDL impaired mitochondrial electron transport chain (mETC) complexes activity in EC (Roy Chowdhury *et al.*, 2010; Sangle *et al.*, 2010). The effects of oxLDL and glyLDL on mETC activity in EC have not been compared in parallel. The impact of glyLDL or oxLDL on the proteins of the key enzymes in mETC in EC has not been documented. The present study compared the effects of oxLDL and glyLDL on mitochondrial activities and examined the impact of diabetes-associated LDLs on the abundance of key enzymes in mitochondrial ETC in cultured porcine aortic EC (PAEC).

MATERIALS AND METHODS

Isolation and modification of lipoproteins. LDL (density 1.019–1.063) was isolated from plasma of healthy donors using sequential density floatation ultracentrifugation. LDL was oxidized through dialysis against 5 μM $CuSO_4$ for 24 h at 22 °C (Ren *et al.*, 1997). The oxidation of LDL was verified using thiobarbituric acid reactive substance assay and non-denatured gel electrophoresis. GlyLDL was prepared by incubation of LDL with 50 mM glucose and 50 mM sodium cyanoborohy-

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Abbreviations: CS, citrate synthase; EC, endothelial cells; glyLDL, glycated LDL; LDL, low density lipoprotein; mETC, mitochondrial electron transport chain; ND, NADH dehydrogenase; oxLDL, oxidized LDL; PAEC, porcine aortic endothelial cells; ROS, reactive oxygen species; SCCR, succinate cytochrome *c* reductase; TMRM, tetramethylrhodamine metyl ester

dride in the presence of 0.01% EDTA for 2 weeks at 37°C as previously described (Zhang *et al.*, 1998). Free glucose or chemicals in glyLDL preparation was removed *via* dialysis. Approximately 60% of lysine residues were glycated in the preparations of glyLDL used in following experiments assessed using trinitrobenzenesulfonic acid assay. The level of endotoxin in lipoproteins was monitored using E-Toxate kit with a threshold of 0.05 ng/ml (Sigma, St. Louis, MO, USA). Lipoproteins were stored in sealed tubes under a layer of nitrogen at 4°C in dark to prevent auto-oxidation.

Cell culture and experimental incubation. PAEC were obtained from Dr. P. E. DiCorleto at the Cleveland Clinic Foundation (Shen *et al.*, 1989). PAEC were grown in Dulbecco's modified Eagle medium (Invitrogen, ON, Canada) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen). Cells were cultured in an incubator at 37°C and maintained in a humidified atmosphere containing 5% CO_2. PAEC were treated with vehicle, lipoproteins at indicated concentration and time at 37°C under 5% CO_2.

Measurement of mitochondrial oxygen consumption using oxygraphy. Oxygen consumption was determined at 37°C using OROBOROS Oxygraphy-2K (Oroboros, Innsbruck, Austria) (Chowdhury *et al.,* 2000). PAEC were trypsinized and counted using hemocytometer. Cells were resuspended in the KCl medium (80 mM KCl, 10 mM Tris/HCl, 3 mM $MgCl_2$, 1 mM EDTA, 5 mM potassium phosphate, pH 7.4) to 1.5×10^6 cells/ml. To permeabilize plasma membrane for the access of substrates to mitochondria, EC were treated with digitonin (25 μg/2 ml for 10^6 cells). At this concentration of digitonin, intact mitochondria in EC were maintained. Various substrates and inhibitors for mitochondrial respiratory chain complexes were added as described in Fig. 1. Unless mentioned otherwise, all the chemicals were obtained from Sigma (Sigma, MO, USA). Oroboros DatLab software was used for the calculation and graphic presentation of oxygen consumption. Oxygen consumption was normalized by cell numbers and presented as pmol O_2/s per 10^6 cells.

Assessment of mitochondrial membrane potential. PAEC were treated with 100 μg/ml of oxLDL or glyLDL for 12 h. After treatment, cells were harvested by trypsinisation, washed three times in cold phosphate-buffered saline, counted and resuspended in the KCl at a protein concentration of 1 mg/ml. Digitonin was added at standardized concentration (0.02 mg digitonin/mg protein), cells were incubated for 5 min on ice and spin for 5 min at $600 \times g$. The supernatant was removed after centrifugation and cells were resuspended in 0.5 ml KCl medium. The permeabilised cells were incubated with 20 nM tetramethylrhodamine methyl ester (TMRM), a mitochondrial membrane potential probe (Molecular Probes, Eugene, OR, USA), for 10 min at room temperature. Mitochondrial membrane potential ($\Delta\Psi_m$) measurements were performed in the presence of 10 mM succinate and 1 μM rotenone using MoFloXDP Coulter flow cytometer (Beckman) equipped with an argon laser 488 nm (Floryk & Houstěk, 1999). TMRM signal was analyzed in the FL2 channel, equipped with band pass filter 575 ± 25 nm. Approximately 23000 cells were used for each measurement. Data were acquired and analyzed in log scale using Summit 5.2 software (Beckman). Arithmetic mean values of TMRM fluorescence signal in arbitrary units were calculated for each condition for graphic representation.

NADH dehydrogenase (ND, Complex I) activity. ND activity was measured as described previously (Birch-Machin *et al.,* 1994). Mitochondrial fraction of PAEC (50 μg) was added to a buffer containing 25 mM potassium phosphate (pH 7.2), 5 mM $MgCl_2$, 2 mM KCN, 2.5 mg/ml bovine serum albumin (fraction V), 2 μg/ml antimycin A, 0.1 mM NADH, and 50 μM decylubiquinone. The measurement of Complex I activity was started at 3 min before the addition of rotenone (2 μg/ml) and continued for another 3 min at 340 nm using an Ultrospec 2000 UV-visible spectrophotometer equipped with Biochrom Swift II software (Biopharmacia Biotech, Uppsala, Sweden).

Succinate cytochrome *c* reductase (SCCR, Complex II/III) activity. SCCR activity was measured by monitoring the rate of reduced cytochrome *c* formation using succinate as substrate. The reaction mixture contained 10 mM potassium phosphate (pH 7.4), 2 mM EDTA, 0.01% bovine serum albumin (fatty acid free), 0.2 mM ATP, 1 mM KCN, 5 μM rotenone, and 10 mM succinate (Chowdhury *et al.*, 2005). Sonicated total cellular proteins (0.2 mg protein) were incubated with the reaction mixture for 3 min and the reaction was started by the addition of 40 μM oxidized cytochrome *c*. Changes in absorbance were monitored at 30°C using a spectrophotometer for 5 min at 550 nm.

Ubiquinol cytochrome *c* reductase (UCCR, Complex III) activity. UCCR activity was evaluated using 100 μg of cell lysates with a reaction mixture containing 25 mM potassium phosphate (pH 7.4), 5 mM $MgCl_2$, 2 mM KCN, 2 μg/ml rotenone, 2.5 mg/ml bovine serum albumin, and 50 μM cytochrome *c* in a final volume of 1 ml. After a 2-min equilibration period, the reaction was started by the addition of 50 μM ubiquinol-2 and the increase in absorbance at 550 nm was monitored using a spectrophotometer (Vondra *et al.,* 1977).

Cytochrome *c* oxidase (COX, Complex IV) activity. COX activity was examined at 30°C by following the rate of oxidation of reduced cytochrome *c* at 550 nm. The assay in cultured cells was performed in the presence of 40 μM reduced cytochrome *c,* 20 mM phosphate buffer, 0.1 mg of protein from cultured cells, and 16 mg of lauryl maltoside/mg protein (0.16%) (Chowdhury *et al.* 2007).

Citrate synthase (CS) activity. CS activity was determined at 30°C in a medium containing 150 mM Tris/HCl (pH 8.2), 0.16% of lauryl maltoside, 0.1 mM dithionitrobenzoic acid, and 0.1 mg protein from PAEC. The reaction was started by the addition of 300 μM acetyl-CoA, and changes in absorbance at 412 nm were measured for 1 min. This rate was subtracted from that with the addition of 0.5 mM oxalacetic acid. CS activity was used to determine the amount of functional mitochondria in cells (Srere, 1969).

Western blotting. Western blotting analysis was performed as previously described (Sangle *et al.,* 2010). Equal amounts of total cellular proteins were run on 12% SDS/PAGE and electrotransferred to nitrocellulose membrane using antibodies against subunits of NADH dehydrogenase (ND1, ND6) (Santa Cruz, CA, USA) or porin (Abcam, Cambridge, MA, USA). Enhanced chemiluminescence reagents (Amersham, Piscataway, NJ, USA) were used for detecting targeted antigens on membrane. The densities of antigens were analyzed using Chemi-Doc system with Quantity-One software (Bio-Rad, Hercules, CA, USA). The abundance of targeted proteins on membrane was normalized with the level of porin, a control protein in mitochondria.

Statistical analysis. The data were presented as means of three replicates ± standard deviation (S.D.). All

results were processed by the one-way variance analysis (ANOVA). Differences at $P<0.05$ were considered as significant.

RESULTS

Effects of oxLDL and glyLDL on mitochondrial oxygen consumption

The oxygen consumption of mitochondrial complexes in digitonin-permeabilized PAEC was examined using oxygraphy with complex-specific substrates and inhibitors. The oxygen consumption of Complex I was determined as rotenone-sensitive respiration in the presence of NADH-dependent substrates. The consumption of Complexes II and III were evaluated as succinate-antimycin A-sensitive respiration in the presence of flavoprotein-dependent substrates. The respiratory capacity of Complex IV was evaluated in the presence of ascorbate and *N,N,N′,N′*-tetramethyl-*p*-phenylendiamine dihydrochloride (TMPD). Our previous studies demonstrated that 100 μg/ml of oxLDL or glyLDL for < 24 h induced increase of ROS production without detectable cellular injury of PAEC (Zhao & Shen, 2005). PAEC were treated with 100 μg/ml of LDL, oxLDL of glyLDL for 2–24 h. OxLDL or glyLDL significantly reduced oxygen consumption in Complex I, II+III or IV in EC after 12–24 h of incubation compared to control ($P<0.05$, Fig. 1A–C). LDL treatment moderately, but not significantly, reduced oxygen consumption in Complex I, II+III or IV. Treatment with oxLDL or glyLDL for 24 h induced significant decreases in oxygen consumption in Complex I, II+III or IV compared to LDL ($P<0.05$, Fig. 1A-C).

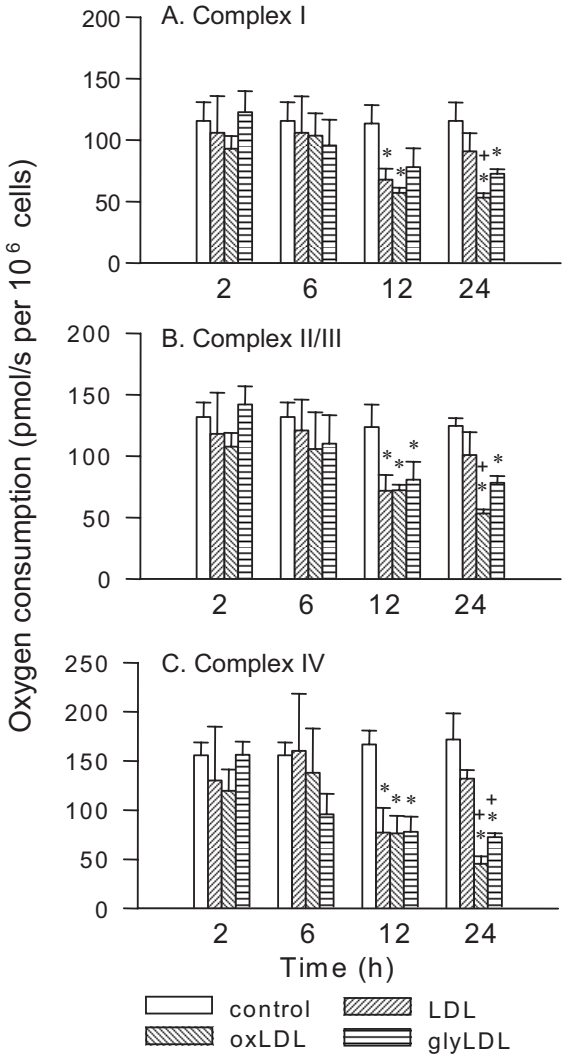

Figure 1. Effect of oxLDL and glyLDL on oxygen consumption in porcine aortic endothelial cells (PAEC)
Confluent PAEC were treated with 100 μg/ml of LDL, oxLDL or glyLDL for 24 h. Cells were trypsinized and counted. Cell suspension (3×10^6 cells) in the KCl buffer was added in a chamber of oxygraphy. Oxygen concentration (left axis) and oxygen consumption rate or slope (right axis) were instantly analyzed. **(A)** Complex I oxygen consumption was assessed from rotenone-sensitive oxygen consumption induced by glutamate + malate. **(B)** Complex II/III activity was assessed from succinate-induced oxygen consumption. **(C)** TMPD-induced oxygen consumption. Values were presented in mean ± S.D. (n = 3) after justification with cell numbers. *$P<0.05$ *versus* control; +$P<0.05$ *versus* LDL.

Effects of oxLDL and glyLDL on mitochondrial membrane potential

Our earlier study demonstrated that glyLDL reduced mitochondrial membrane potential in EC (Sangle *et al.,* 2010). The present study compared the effects of oxLDL and glyLDL on membrane potential in PAEC. Fig. 2 demonstrated that the effect of oxLDL on mitochondrial membrane potential was comparable to that of glyLDL. Both types of diabetes-associated lipoproteins significantly reduced mitochondrial membrane potential in PAEC ($P<0.01$, Fig. 2).

Effects of oxLDL and glyLDL on enzymatic activity of mETC

Treatment with 100 μg/ml of oxLDL, glyLDL or LDL for 12 h significantly decreased the enzymatic activity of Complex I in PAEC compared to control (Fig. 3A). OxLDL or glyLDL induced significantly greater decrease in Complex I activity compared to LDL ($P<0.05$, Fig. 3A). OxLDL, glyLDL or LDL significantly reduced SCCR activity compared to control ($P<0.05$). OxLDL, but not glyLDL, induced significantly greater inhibition on SCCR activity compared to LDL ($P<0.05$, Fig. 3B). OxLDL,

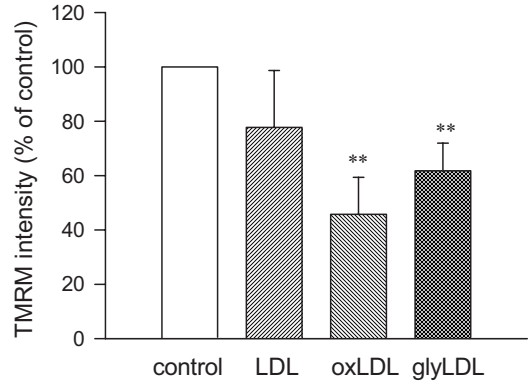

Figure 2. Effects of oxLDL and glyLDL on mitochondrial membrane potential in PAEC
PAEC cultures were treated with vehicle (control), 100 μg/ml of oxLDL or glyLDL for 12 h. Cells were harvested and treated with 0.02 mg/mg protein of digitonin, then incubated with TMRM. Cytofluorimetric analysis was undertaken using flow cytometer. Changes in TMRM intensity were expressed in mean ± S.E. in percent of control (n = 3 experiments). **$P<0.01$ *versus* control.

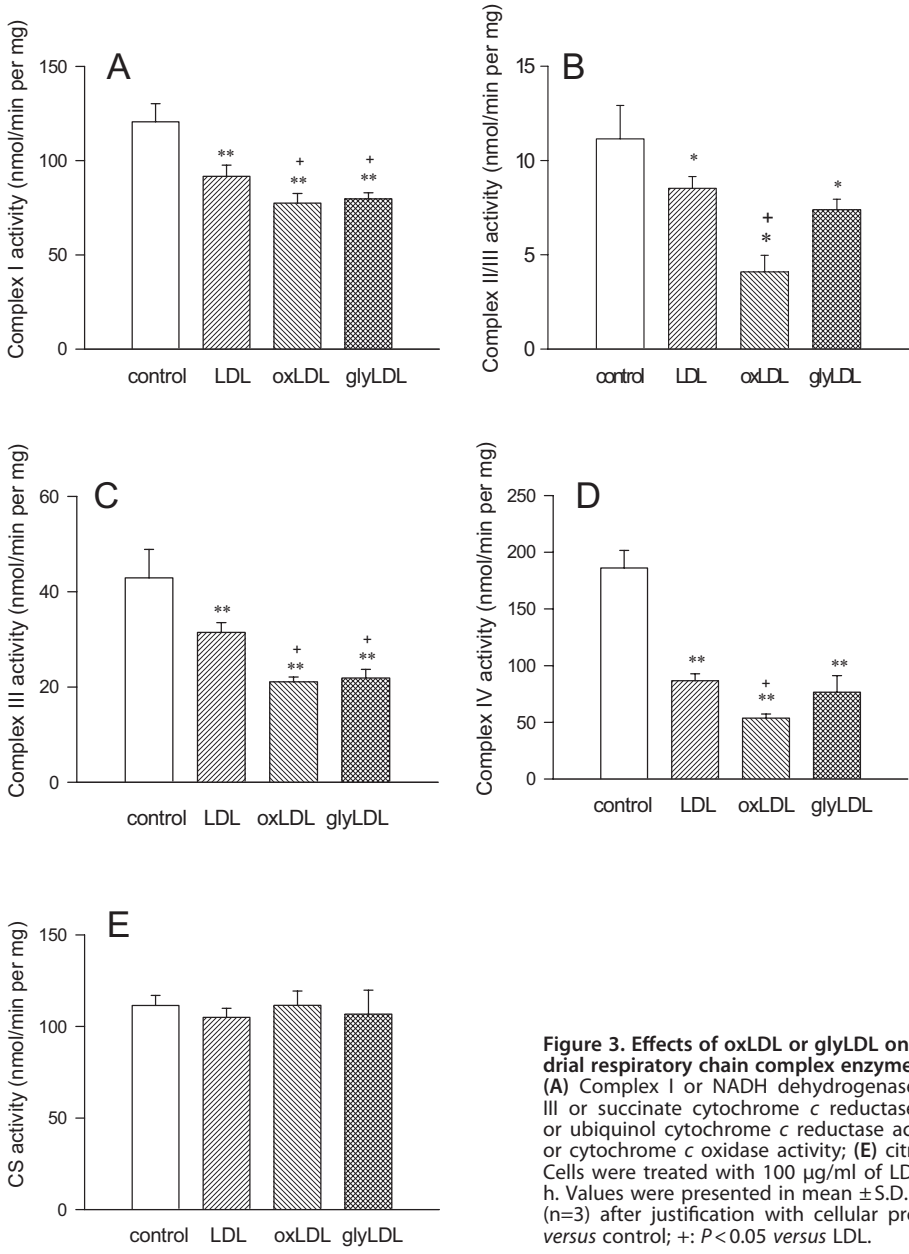

Figure 3. Effects of oxLDL or glyLDL on the activity of mitochondrial respiratory chain complex enzyme activities in PAEC

(A) Complex I or NADH dehydrogenase activity; **(B)** Complex II/III or succinate cytochrome *c* reductase activity; **(C)** Complex III or ubiquinol cytochrome *c* reductase activity and **(D)** Complex IV or cytochrome *c* oxidase activity; **(E)** citrate synthase (CS) activity. Cells were treated with 100 μg/ml of LDL, oxLDL or glyLDL for 12 h. Values were presented in mean ± S.D. nmol/min per mg protein (n=3) after justification with cellular proteins. *,**$P<0.05$ or 0.01 *versus* control; +: $P<0.05$ *versus* LDL.

glyLDL or LDL significantly reduced Complex III activity compared to control ($P<0.01$). The reductions in Complex III activity in PAEC exposed to oxLDL or glyLDL were significantly greater compared to LDL ($P<0.05$, Fig. 3C). OxLDL, glyLDL or LDL treatment significantly deceased the activity of Complex IV by >50% ($P<0.01$). OxLDL, but not glyLDL, induced greater decrease in the activity of cytochrome *c* oxidase compared to LDL ($P<0.05$, Fig. 3D). In comparison, LDL or its modified forms did not significantly alter the activity of CS (Fig. 3E), which is a mitochondrial matrix-soluble enzyme and relatively insensitive to the effects of oxidants (Masaki *et al.,* 1995).

Effects of oxLDL and glyLDL on abundance of subunits of NADH-dehydrogenase, ND1 and ND6

To address the question whether oxLDL or glyLDL reduces the levels of key mETC complex enzymes, the abundances of components of Complex I, ND1 and ND6 were examined. The results indicated that oxLDL and glyLDL significantly decreased the abundances of ND1, and ND6 compared to vehicle control, but did not evidently alter the level of porine in PAEC (Fig. 4).

DISCUSSION

The present study demonstrated that oxLDL and glyLDL significantly inhibited oxygen consumption, membrane potential, mETC enzymatic activity and the abundances of ND1 and ND6 compared to control in cultured PAEC.

Elevated levels of oxLDL and glyLDL are detected in diabetic patients (Lyons, 1993; Tsimikas *et al.,* 2003). Previous studies demonstrated that hyperglycemia increased the oxidation of LDL (Lyons, 1993). Our group previously reported that increased lipid peroxides were detected in glyLDL. Prolonged incubation with EC increased

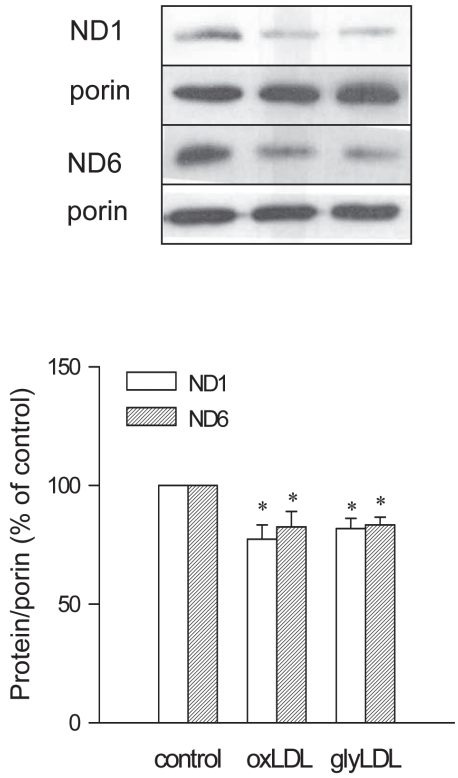

Figure 4. Effects of oxLDL and glyLDL on the abundance of mitochondrial proteins

PAEC were incubated with 100 μg/ml of oxLDL, glyLDL or vehicle (control) for 12 h. Cellular proteins were analyzed using Western blotting with antibody for NADH dehydrogenase 1 and 6 (ND1, ND6) or porin. The levels of ND1 or ND6 were normalized with porin in corresponding lanes. Values were expressed in mean ± S.D. % of control (n = 3 experiments). *$P < 0.05$ *versus* control.

lipid peroxidation in glyLDL. Antioxidants reduced the formation of lipid peroxides in LDL or glyLDL (Ren *et al.*, 2000). The present study compared the effects of oxLDL and glyLDL on mitochondrial oxygen consumption and enzymatic activities of mETC Complex I–IV in PAEC in parallel. The results indicated that both oxLDL and glyLDL decreased mitochondrial oxygen consumption and mETC enzymes activities. The findings suggest that both oxLDL and glyLDL impair mitochondrial respiration. Persistent hyperglycemia and hypercholesterolemia may contribute to the deterioration of mitochondrial activity in diabetic patients.

Complex I and III have been considered as the major sources of ROS in mETC. Previous studies demonstrated that hyperglycemia and diabetic conditions may reduce Complex III activity (Kowluru *et al.,* 2006; Munusamy *et al.,* 2009). Ceaser *et al.* (2003) found that non-apoptic oxLDL increased Complex I enzyme activity in EC. Earlier study in our laboratory demonstrated that extensively oxLDL, in an extent of oxidation similar as that used in the present study, reduced Complex I activity compared to control of LDL, which suggest the inhibition of oxLDL on Complex I activity may depend on the extensity of oxidation in LDL. In the presence of uncoupler, ADP-induced oxygen consumption increased by 50%, which suggests a large portion of oxLDL-induced decrease in mitochondrial respiration is not due to the reduction of ATP synthesis in PAEC (Roy Chowdhury *et al.,* 2010). The present study demonstrated that oxLDL reduced the activities of Complex I, II/III, III and IV compared to vehicle or LDL. GlyLDL treatment induced significant decreases in activities of enzymes in Complex I–IV compared to control, but only had significant reduction in Complex I and III activities compared to LDL in PAEC. The results of the present study are consistent to our earlier findings that both oxLDL and glyLDL increased the generation of ROS from EC (Zhao & Shen, 2005) and also supports previous findings of the reduction of Complex III activity in diabetes (Kowluru *et al.,* 2006; Munusamy *et al.,* 2009).

ND complex contains more than 40 subunits. Seven of Complex I subunits, including ND1 and ND6, are encoded by mitochondrial DNA (mtDNA) (DiMauro & Andreu, 2000). Unlike nuclear DNA, mtDNA lacks of the protection of histone that might contribute to enhanced susceptibility of mtDNA to oxidative damage. Proximity of mtDNA to ROS production site in mitochondria is a possible risk for high rate of mtDNA mutation. In addition, mtDNA attaches to inner membrane and close to ROS in matrix generated from mitochondrial respiration. Mitochondrial enzyme subunits encoded by mtDNA (including ND1–6) are expected to be more susceptible to oxidative stress than nuclear DNA encoded mETC subunits. The present study demonstrated that abundances of ND1, and ND6 proteins were significantly reduced by oxLDL or glyLDL. The results suggest that the reduction of Complex I activities induced by oxLDL or glyLDL may result from the decreases in the levels of mtDNA encoded Complex I enzyme subunits, including, but not limited to, ND1 and ND6, in PAEC. Relationship between the diabetes-associated LDLs and subunits in other mtDNA encoded mETC enzymes remains to be investigated in subsequent studies. OxLDL or glyLDL contains lipid peroxidative products, which may impair mitochondrial respiration. Increased ROS production *via* mitochondrial respiration may damage mtDNA and reduce the content of mtDNA encoded mETC enzymes in PAEC exposed to oxLDL or glyLDL.

In conclusion, the present study for the first time directly compared the effects of oxLDL and glyLDL on mitochondrial oxygen consumption, membrane potential and enzymatic activities in EC, and originally demonstrated that the diabetes-associated lipoproteins reduced the abundances of two subunits of mETC Complex I enzyme, ND1 and ND6, in PAEC. These changes in mitochondrial activities may contribute to the development of cardiovascular complications in diabetic patients.

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