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Original Contribution

Impairment of mitochondrial respiratory chain activity in aortic endothelial cells induced by glycated low-density lipoprotein

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ABSTRACT

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 LDL (glyLDL) were detected in patients with diabetes. Our previous studies demonstrated that glyLDL increased the generation of ROS and altered the activities of antioxidant enzymes in vascular endothelial cells (EC). This study examined the effects of glyLDL on oxygen consumption in mitochondria and the activities of key enzymes in the mitochondrial electron transport chain (ETC) in cultured porcine aortic EC. The results demonstrated that glyLDL treatment significantly impaired oxygen consumption in Complexes I, II/III, and IV of the mitochondrial ETC in EC compared to LDL or vehicle control detected using oxygraphy. Incubation with glyLDL significantly reduced the mitochondrial membrane potential, the NAD^+/NADH ratio, and the activities of mitochondrial ETC enzymes (NADH-ubiquinone dehydrogenase, succinate cytochrome *c* reductase, ubiquinone cytochrome *c* reductase, and cytochrome *c* oxidase) in EC compared to LDL or control. The abundance of mitochondria-associated ROS and the release of ROS from EC were significantly increased after glyLDL treatment. The findings suggest that glyLDL attenuates the activities of key enzymes in the mitochondrial ETC, decreases mitochondrial oxygen consumption, reduces mitochondrial membrane potential, and increases ROS generation in EC, which potentially contribute to mitochondrial dysfunction in diabetic patients.

Diabetes mellitus (DM) is one of most rapidly growing metabolic disorders in the world and has been projected to afflict more than 300 million individuals worldwide by the year 2025 [1]. Of even greater concern, diabetes is an important risk factor for coronary artery disease (CAD) [2]. One of the several hypotheses on the linkage between hyperglycemia and accelerated atherogenesis is the increased glycation of proteins and lipids in diabetic patients [3]. Glycation is the nonenzymatic glycosylation of protein or lipid molecules by sugars. Advanced glycation end products (AGEs) are the results of a chain of chemical reactions after initial glycation [4]. Glycation promotes the oxidation of proteins and lipids. Increased levels of glycated low-density lipoprotein (glyLDL), one type of AGE, have been detected in diabetic patients [3]. It is now widely accepted that oxidative stress is a key player in the development of diabetic cardiovascular complications, including CAD [5]. Elevated levels of glyLDL and reactive oxygen species (ROS) were detected in the circulation of patients with diabetes [6,7].

Mitochondria are one of the major intracellular sources of ROS in cells and regulate the generation of superoxide, a by-product generated from the electron transport chain (ETC) [8]. DM is associated with mitochondrial dysfunction that may result in increased ROS generation and impaired bioenergetics [9]. Chronic hyperglycemia is often associated with increased glycation of mitochondrial proteins [10]. Hyperglycemia-induced generation of superoxide within mitochondria plays a major role in the development of diabetic vascular complications [11]. Several groups including ours demonstrated that glyLDL or AGEs increased the generation of superoxide and/or hydrogen peroxide (H_2O_2) in vascular endothelial cells (EC) [12–14].

Increased production of ROS and impaired mitochondrial function are associated with diabetes and CAD [15–17]. Hyperglycemia promotes the glycation of LDL. Glucose modification accelerates the

Abbreviations: DM, diabetes mellitus; CAD, coronary artery disease; AGE, advanced glycation end product; ETC, electron transport chain; EC, endothelial cells; PAEC, porcine aortic endothelial cell; glyLDL, glycated low-density lipoprotein; NAD^+, nicotinamide adenine dinucleotide; ND, NADH-ubiquinone dehydrogenase; SCCR, succinate cytochrome *c* reductase; UCCR, ubiquinone cytochrome *c* reductase; CS, citrate synthase; RCI, respiratory control index; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reactive substance; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride; TMRM, tetramethylrhodamine methyl ester.

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oxidation of LDL [18]. Mitochondrial dysfunction has been detected in EC under hyperglycemic conditions, in which the generation of ROS from mitochondria plays a crucial role [19]. Reduced activities of mitochondrial enzymes were detected in patients with type 2 DM [20]. Mitochondrial oxidative activity and adenosine triphosphate (ATP) synthesis were decreased in insulin-resistant subjects [21]. Recent studies suggest that diabetes is associated with alterations in the activities of mitochondrial respiratory chain enzymes in kidneys [9]. Glycated high-density lipoprotein induces apoptosis of EC as a result of deterioration of mitochondrial function [22]. Hyperglycemia increases oxidative stress and EC apoptosis through ROS overproduction in the mitochondrial ETC [23]. GlyLDL increased ROS generation in vascular cells, including EC [14,24]. A previous study demonstrated that nonapoptotic oxidized LDL increased Complex I activity in human umbilical vein EC (HUVEC) [25], but the effects of glyLDL on mitochondrial function or respiratory chain enzyme activity in EC have not been documented.

This study examined the effects of glyLDL on mitochondrial oxygen consumption, mitochondrial membrane potential, the activities of key enzymes in the mitochondrial respiratory chain, and ROS production in cultured porcine aortic EC (PAEC).

Materials and methods

Isolation and modification of LDL

LDL (density 1.019–1.063) was isolated from freshly separated plasma of healthy donors by sequential floating ultracentrifugation [26]. LDL was glycated by incubation with 50 mM glucose and 50 mM sodium cyanoborohydride, or modified with 50 mM sodium cyanoborohydride alone (as control), for 2 weeks at 37°C [27]. The extent of glycation was estimated using trinitrobenzenesulfonic acid assay [28]. The oxidation status of glyLDL (lipid peroxidation) was estimated using the thiobarbituric acid-reactive substance (TBARS) assay [29]. The levels of TBARS in glyLDL used in this study were 11.4 ± 1.4 nmol/mg protein compared to 5.1 ± 0.3 nmol/mg protein ($n = 3$) in native LDL. GlyLDL and LDL preparations were stored at 4°C in the dark in sealed tubes overlaid with nitrogen to prevent autoxidation [27].

Cell culture

PAEC were obtained from Dr. P.E. DiCorleto at the Cleveland Clinic Foundation. 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.

Measurement of oxygen consumption using oxygraphy

Highly sensitive Oroboros Oxygraphy-2K (Oroboros, Innsbruck, Austria) was used to determine oxygen consumption at 37°C in EC [30]. For each measurement, PAEC were trypsinized and counted using a hemocytometer. Freshly harvested cells were resuspended in KCl medium (80 mM KCl, 10 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM EDTA, 5 mM potassium phosphate, pH 7.4) at a concentration of 1.5×10^6 cells/ml. EC were permeabilized using digitonin (25 μg/10^6 cells) to permeabilize the plasma membrane of the cells, maintaining intact mitochondrial membrane as previously described [30]. Various substrates and inhibitors for the mitochondrial ETC complexes were used as indicated in Fig. 1. Glutamate (10 mM) + malate (5 mM), succinate (10 mM), or ascorbate (5 mM) + TMPD (0.5 mM) were used as substrates for Complex I, II/III, or IV, respectively. Rotenone (1 μM), antimycin A (1 μg/ml), or potassium cyanide (0.25 mM) was used as inhibitor for Complex I, III, or IV, respectively. Oroboros DatLab software was used for analysis and graphic presentation of experimental data. Oxygen consumption was normalized to cell number and expressed as pmol/s/10^6 cells. The respiratory control index (RCI) was determined from oxygen respiration in the mitochondria of digitonin-permeabilized EC induced by adenosine diphosphate (ADP; 2 mM) [30].

NADH-ubiquinone dehydrogenase (ND) activity

ND activity (Complex I) was measured as described previously [31]. Mitochondrial extracts (50 μg) were 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 ND 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) [30].

NAD^+/NADH assay

The levels of nicotinamide adenine dinucleotide (NAD^+) and its reduced form, NADH, in cell lysates were analyzed using EnzyChrom NAD^+/NADH assay kits from BioAssay System (Haywood, CA, USA). Changes in absorbance were measured under 565 nm using spectrophotometer.

Succinate cytochrome c reductase (SCCR) activity

SCCR activity (Complex II/III) 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 [32]. Sonicated cell lysates (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 [30].

Ubiquinone cytochrome c reductase (UCCR) activity

The enzymatic activity of UCCR (Complex III) 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 [20].

Cytochrome c oxidase activity

Cytochrome *c* oxidase (Complex IV) 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%) [30,33].

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 EC. 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 [34].