Effects of extensively oxidized low-density lipoprotein on mitochondrial function and reactive oxygen species in porcine aortic endothelial cells

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Atherosclerotic cardiovascular disease is the leading cause of morbidity and mortality in most industrialized countries. An elevated level of LDL-cholesterol is a classic risk factor for coronary artery disease (CAD) (39). Oxidation of LDL contributes to atherosclerosis (22). Increased levels of biomarkers for oxidized LDL and reactive oxygen species (ROS) were detected in the circulation of patients with CAD (10, 42). Several groups, including ours, demonstrated that oxidized LDL increases the generation of superoxide or H2O2 from vascular endothelial cells (EC) (9, 51). It is widely accepted that oxidative stress and endothelial dysfunction play important roles in the pathogenesis of atherosclerosis (40). The regulatory mechanism for oxidized LDL-induced ROS generation from EC remains unclear.

Mitochondria, a potential intracellular source of ROS (25), convert up to 4% of intracellular molecular oxygen to ROS as by-products of oxidative phosphorylation in the mitochondrial respiratory chain (20). Increased production of ROS in mitochondria is associated with cardiovascular disease (4). A recent study suggests that mitochondrial function may be impaired in metabolic syndrome and, thus, linked to diabetes and hyperlipidemia (30). The initial products of ROS are superoxide anions, which can be converted to H2O2 or other species of ROS in mitochondria (14). Dysfunction of electron transport in mitochondria promotes the generation of excessive amounts of ROS (18). Notably, oxidative modifications of mitochondrial lipids, proteins, and DNA may impair mitochondrial function (25). At moderate concentrations, ROS play an important role as a regulatory mediator in signaling processes (11). Increased levels of cholesterol or oxidatively modified lipoproteins in blood circulation are associated with mitochondrial dysfunction (33). LDL or its degradation products can be oxidized by mitochondria-derived ROS in vivo (24). Oxidized LDL and glycated HDL impair various aspects of endothelial function, including decreased mitochondrial respiratory chain activities and production of ROS (13, 28, 52). Oxidized lipids have been shown to induce EC apoptosis and reduce mitochondrial function (37). Caesar et al. (7) demonstrated that nonapoptotic oxidized LDL increases complex I activity in human umbilical vein EC (HUV EC). Recent studies demonstrated that oxidized LDL increases cytochrome c release, reduces mitochondrial membrane potential, or induces mitochondrial dysfunction in EC or macrophages (1, 6, 26, 44). The effect of oxidized LDL on mitochondrial activity may depend on the intensity of oxidation. The impact of extensively oxidized LDL (eoLDL) on oxygen consumption and mitochondrial respiratory chain enzyme activity in EC has not been documented.

In the present study, we used multiple approaches, including analysis of oxygen consumption by highly sensitive oxygraphy, assays for the activities of enzymes in mitochondrial respiratory chain complexes by spectrophotometry, and assays for mitochondria-associated ROS in EC by fluorescent and confocal microscopy, to examine the effects of eoLDL on mitochondrial function in cultured porcine aortic EC (PAEC).

MATERIALS AND METHODS

Isolation and modification of lipoproteins. LDL (density 1.019–1.063) was isolated from freshly separated plasma of healthy donors by sequential floating ultracentrifugation (36). EoLDL was prepared by incubation of LDL with 5 μM CuSO4 at 22°C for 24 h. In a parallel...
Cells were cultured at 37°C in a humidified atmosphere with 5% CO₂. 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen). Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with DiCorleto (Cleveland Clinic Foundation). PAEC were maintained in Research Ethics Board of the University of Manitoba. by all donors. The protocol and consent form were approved by the Research Ethics Board of the University of Manitoba. Informed consents were signed from different healthy donors. This study was conducted in accordance with the Helsinki Declaration. Informed consents were signed by all donors. The protocol and consent form were approved by the Research Ethics Board of the University of Manitoba.

Measurement of mitochondrial oxygen consumption by oxygraphy. Oxygen consumption was determined at 37°C using highly sensitive Oxygraphy-2K (Oroboros, Innsbruck, Austria) (8). PAEC were trypsinized and counted using a hemocytometer. Freshly harvested cells were resuspended in KC1 medium (80 mM KCl, 10 mM Tris-HCl, 3 mM MgCl₂, 1 mM EDTA, and 5 mM potassium phosphate, pH 7.4) at 1.5 × 10⁶ cells/ml. EC were permeabilized using digitonin (25 μg/10⁶ cells); the plasma membrane of the cells was permeabilized while the mitochondrial membrane remained intact, as previously described (8, 12). Various substrates and inhibitors for mitochondrial electron transport chain (ETC) complexes were used as indicated in Figs. 1 and 4. Glutamate (10 mM) + malate (5 mM), succinate (10 mM), and ascorbate (5 mM) + N,N,N’,N’-tetramethyl-p-phenylenediamine dihydrochloride (TMPD, 0.5 mM) were used as substrates for complex I, II, III, and IV, respectively. Rotenone (1 μM), antimycin A (1 μg/ml), and potassium cyanide (KCн, 0.25 mM) were used as inhibitors for complex I, III, and IV, respectively. Oligomycin (1 μM) was used as an ATP synthase inhibitor, and carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP, 1 μM) was used as a mitochondrial uncoupler. Unless otherwise stated, all chemicals were obtained from Sigma (St. Louis, MO). Oroboros DatLab software was used for calculation and graphic presentation of oxygen consumption. Oxygen consumption was normalized by cell numbers and is presented as picomoles per second per 10⁶ cells. Respiratory control index (RCI) was assessed from oxygen consumption in mitochondria of digitonin-permeabilized EC induced by addition of glutamate and malate in the presence or absence of ADP.

NADH-ubiquinone dehydrogenase activity. NADH-ubiquinone dehydrogenase (ND) activity (complex I) was measured as described previously (5). Mitochondrial extracts (50 μg) were added to a buffer containing 25 mM potassium phosphate (pH 7.2), 5 mM MgCl₂, 2 mM KCн, 2.5 mg/ml bovine serum albumin (fraction V), 2 μg/ml antimycin A, 0.1 mM NADH, and 50 μg decylubiquinone. The cells were subjected to three 20-s cycles of sonication ice, which ruptures the mitochondrial membrane and allows the access of substrates to the intermembrane space. Measurement of ND activity was started at 3 min before addition of rotenone (2 μg/ml) and continued for an additional 3 min at 340 nm with an Ultrospec 2000 UV-visible spectrophotometer equipped with Biochrom Swift II software (Biopharmacia Biotech, Uppsala, Sweden) (8).

Succinate cytochrome c reductase activity. For evaluation of the enzymatic activity of succinate cytochrome c reductase (SCCR, complex II/III), the rate of reduced cytochrome c formation was monitored using succinate as a substrate. The reaction mixture contained 10 mM potassium phosphate (pH 7.4), 2 mM EDTA, 0.01% bovine serum

Fig. 1. O₂ consumption in mitochondria of porcine aortic endothelial cells (PAEC) treated for 12 h with 100 μg/ml of extensively oxidized LDL (eoLDL) or LDL. O₂ consumption was assessed in PAEC treated with vehicle (A), LDL (B), or eoLDL (C) by oxygraphy. O₂ consumption in response to complex-specific substrates (i.e., O₂ slope) was verified by addition of complex-specific inhibitors: rotenone (Rot, 1 μM) for complex I, antimycin A (AA, 1 μg/ml) for complex III, or potassium cyanide (KCн, 0.25 mM) for complex IV. Dig, digitonin (25 μg/10⁶ cells); G, glutamate (10 mM); M, malate (5 mM); ADP, 2 mM ADP, Succ, succinate (10 mM); Asc, ascorbate (5 mM); TMPD, N,N,N’,N’-tetramethyl-p-phenylenediamine dihydrochloride (0.5 mM). Y1, O₂ slope in the chamber, in black curves; Y2, O₂ slope or consumption, in gray curves.

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albumin (fatty acid-free), 0.2 mM ATP, 1 mM KCN, 5 μM rotenone, and 10 mM succinate. Sonicated cell lysates (0.2 mg protein) were incubated in the reaction mixture for 3 min, and 40 μM oxidized cytochrome c was subsequently added as previously described (8). Changes in absorbance were monitored at 30°C using a spectrophotometer for 5 min at 550 nm (35).

**Ubiquinone cytochrome c reductase activity.** The enzymatic activity of ubiquinone cytochrome c reductase (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 MgCl₂, 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 (5).

**Cytochrome c oxidase activity.** For measurement of cytochrome c oxidase (complex IV) activity at 30°C, the rate of oxidation of reduced cytochrome c at 550 nm was monitored. The assay was performed in the presence of 40 μM reduced cytochrome c, 20 mM phosphate buffer, 0.1 mg of protein from freshly cultured cells, and 16 mg of lauryl maltoside per milligram of protein (0.16%) (45).

**Citrate synthase activity.** Citrate synthase (CS) activity was evaluated in a medium containing 150 mM Tris·HCl (pH 8.2), 0.16% lauryl maltoside, 0.1 mM dithionitrobenzoic acid, and 0.1 mg of cell lysates. The reaction was initiated with the addition of 300 μM acetyl-CoA, and changes in absorbance were measured at 412 nm for 1 min. This rate was subtracted from that obtained with addition of 0.5 mM oxalacetic acid. CS activity was used to estimate the mitochondrial content in cells (38).

**NAD⁺/NADH assay.** The levels of NAD⁺ and its reduced form NADH in lysates of EC were measured using EnzyChrom NAD⁺/NADH assay kits (BioAssay System, Haywood, CA) and detected at 565-nm optical density.

**Detection and quantification of mitochondria-derived ROS.** Lipoprotein-treated cells were loaded with a fluorogenic probe for ROS, MitoSOX Red (2.5 μM; Invitrogen), for 20 min (34). For confirmation of localization of ROS to mitochondria, cells were also labeled with MitoTracker Green (20 nM; Invitrogen) for 20 min. Cells were washed with Hank's balanced salt solution and fixed with 3% paraformaldehyde for 15 min. Fluorescent images were captured using an Olympus IX70 inverted microscope coupled to an Olympus Fluoview confocal imaging system (23). Quantification of MitoSOX Red fluorescence after treatment of live cells with the dye and subsequent fixation with 3% paraformaldehyde was performed using an iCys laser scanning cytometer (CompuCyte) and associated system hardware. Cell nuclei were labeled using H-33342 (1 μg/ml) for 30 s. For these studies, images were captured using a ×20 objective with a 5-μm scan step. For all analyses, red fluorescence (MitoSOX), blue fluorescence (nuclei), and forward light scatter were captured to generate shaded relief contrast images. For determination of mean integrated fluorescence intensity of MitoSOX Red in each scan area (5 mm²), the fluorescence signal from a nonoverlapping matrix of ~780 circular “phantom contour” events (each 50 μm diameter) was captured. Data were captured from duplicate experiments using three different cell cultures. For comparison between treatments and experiments, MitoSOX Red fluorescence was normalized on the basis of the number of cells per field, as determined by primary event counts, with contouring on DNA fluorescence.

**Measurement of ROS released from EC.** H₂O₂ levels in postculture media of PAEC were measured using reagents of the PeroxiDetect kit (Sigma). The measurement was based on the fact that peroxides convert Fe²⁺ to Fe³⁺ at acidic pH. Fe³⁺ forms a color adduct with xylenol orange, which was detected at 560 nm (27, 51).

**Statistical analysis.** Values are means ± SE. Statistical significance was determined using one-way ANOVA followed by Tukey’s post hoc test for comparisons among multiple groups. The level of significance was defined as P < 0.05.

**RESULTS**

Complex-specific oxygen consumption in mitochondria of aortic EC. Oxygen consumption of mitochondrial complexes in PAEC was assayed using oxygraphy with complex-specific substrates and inhibitors. Polarographic detection of oxygen consumption in digitonin-permeabilized EC enables the evaluation of the function of respiratory complexes in intact mitochondria in living cells. The function of complex I was determined as rotenone-sensitive respiration in the presence of NADH-dependent substrates. Complex II and III activities were evaluated as succinate-antimycin A-sensitive respiration in the presence of FADH-dependent substrates. Complex IV activity was evaluated in the presence of ascorbate and TMPD and verified by the addition of KCN (a complex IV inhibitor).

![Fig. 2. Effect of eoLDL on O₂ consumption in complex I, II + III, and IV in mitochondria of PAEC. PAEC were treated with 100 μg/ml of LDL or eoLDL for 2–24 h, and O₂ consumption in PAEC was measured using oxygraphy as in Fig. 1. A: O₂ consumption in complex I was assessed in the presence of glutamate + malate + ADP followed by addition of rotenone. B: complex II + III respiration was assessed by antimycin A-sensitive O₂ consumption induced by succinate. C: complex IV respiration measured by KCN-sensitive O₂ consumption induced by ascorbate + TMPD. Values (means ± SE of 3 experiments using different batches of LDL) were normalized to number of cells. CTL, control. *P < 0.05; **P < 0.01 vs. control. +P < 0.05 vs. LDL.
Effects of eoLDL on oxygen consumption in mitochondrial complexes. PAEC were treated with 100 μg/ml of LDL or eoLDL for 2–24 h, and respiratory control index was measured from O₂ consumption in PAEC by oxygraphy. Values (means ± SE of 3 experiments using different batches of LDL) are expressed as ratios. *P < 0.05; **P < 0.01 vs. control. +P < 0.05 vs. LDL.

Effects of eoLDL on oxygen consumption in mitochondrial complexes. PAEC were treated with 100 μg/ml of LDL or eoLDL for 2–24 h. Treatment with eoLDL reduced oxygen consumption in complex I, II + III, and IV of EC was significantly reduced by eoLDL after 12–24 h of incubation compared with control (P < 0.05; Fig. 2). LDL treatment moderately, but not significantly, reduced oxygen consumption in complex I, II + III, and IV (Fig. 2). Treatment with eoLDL for 24 h induced significant decreases in oxygen consumption in complex I, II + III, and IV compared with LDL (P < 0.05; Fig. 2, A and C).

Effect of eoLDL on RCI. RCI reflects the tightness of coupling between respiration and oxidative phosphorylation in mitochondria. Treatment with LDL or eoLDL for 12 or 24 h significantly reduced RCI in PAEC compared with control (P < 0.05). A significantly greater impairment of RCI in EC was induced by 24 h of treatment with eoLDL than LDL (P < 0.05; Fig. 3).

Effect of eoLDL on mitochondrial oxygen consumption in the presence of uncoupler. PAEC were treated with 100 μg/ml of eoLDL or vehicle (control) for 12 h. The cells were trypsinized, resuspended in KCl medium, and used for measurement of oxygen consumption by oxygraphy as described in MATERIALS AND METHODS. Treatment with eoLDL significantly reduced ADP-induced oxygen consumption in EC compared with control (50.8 ± 1.2 vs. 87.7 ± 5 pmol O₂·s⁻¹·10⁶ cells⁻¹, n = 3). Oxygen consumption induced by ADP was decreased by oligomycin in control and eoLDL-treated cells. The addition of FCCP, a mitochondrial uncoupler, stimulated oxygen consumption to a greater extent in control than in eoLDL-treated cells (210.9 ± 27.3 vs. 102.6 ± 3.1 pmol O₂·s⁻¹·10⁶ cells⁻¹, n = 3; Fig. 4).

Effect of eoLDL on ND activity and NAD⁺-to-NADH ratio. Treatment with 100 μg/ml of eoLDL or LDL for 12 or 24 h significantly decreased ND activity in PAEC compared with control (P < 0.05 or 0.01). A greater inhibition of ND activity was induced by eoLDL than LDL (P < 0.05; Fig. 5A). To verify the effect of eoLDL on ND activity, we analyzed the effect of eoLDL (100 μg/ml) on the abundance of NAD⁺ and NADH and the NAD⁺-to-NADH ratio in total cell lysates in PAEC, since complex I is an intracellular source of NAD⁺ converted from NADH (15). Treatment with eoLDL for 6, 12, or 24 h significantly reduced the NAD⁺-to-NADH ratio compared with control (P < 0.05). LDL at the same concentration reduced the NAD⁺-to-NADH ratio after 12 or 24 h of incubation compared with control (P < 0.05). A significantly greater reduction in the NAD⁺-to-NADH ratio was induced by eoLDL than LDL after 12 or 24 h of incubation (P < 0.05; Fig. 5B).
Effect of eoLDL on SCCR activity. SCCR activity was significantly reduced in PAEC treated with eoLDL for 12 or 24 h compared with control or LDL (P < 0.05 or 0.01; Fig. 5C).

Effect of eoLDL on UCCR activity. UCCR activity was significantly reduced in PAEC after treatment with LDL or eoLDL for 6, 12, or 24 h (P < 0.05 or 0.01). Treatment with eoLDL significantly reduced UCCR activity in EC after 6, 12, or 24 h of incubation compared with LDL (P < 0.05; Fig. 5D).

Effect of eoLDL on cytochrome c oxidase activity. Cytochrome c oxidase activity was significantly reduced in PAEC after treatment with LDL or eoLDL for 6, 12, or 24 h (P < 0.05 or 0.01). A greater decrease in cytochrome c oxidase activity in EC was induced after 6–24 h of incubation with eoLDL than LDL (P < 0.05; Fig. 5E).

Effect of eoLDL on CS activity. CS, a mitochondrial matrix-soluble enzyme, is relatively insensitive to the effects of oxidants (27). CS activity reflects mitochondrial mass in cells (45). The effects of LDL or eoLDL (100 μg/ml, 2–24 h) on CS activity were determined in PAEC as an internal control for the activity of other mitochondrial enzymes. CS activity was not significantly altered in EC after exposure to LDL or eoLDL at conditions tested in the present study (Fig. 5F).
Effect of moLDL on ND, SCCR, UCCR, and cytochrome c oxidase activity. ND, UCCR, and cytochrome c oxidase, but not SCCR, activity in PAEC was moderately decreased by 12 h of treatment with 100 μg/ml of moLDL compared with control (Fig. 6; \( P < 0.05 \) or \( P < 0.01 \)); this effect was similar to that of unmodified LDL. Significantly greater inhibition of ND, SCCR, UCCR, and cytochrome c oxidase activity was induced by 12 h of incubation with 100 μg/ml of eoLDL than moLDL or LDL (Fig. 6; \( P < 0.05 \) or \( P < 0.01 \)).

Effect of antioxidant on eoLDL-induced impairment of ND and UCCR activity. Our previous studies demonstrated that 80 μM butylated hydroxytoluene (BHT), a potent antioxidant, prevented oxidized LDL-induced \( \text{H}_2\text{O}_2 \) production in EC (50). To determine whether antioxidant prevents eoLDL-induced mitochondrial activity, we examined the effect of BHT on eoLDL-induced impairment of ND and UCCR activity in PAEC. BHT normalized the eoLDL-induced reduction of ND and UCCR activity in EC (Fig. 7; \( P < 0.05 \) or \( P < 0.01 \)).

Effect of antioxidant on eoLDL-induced impairment of complex I and III activity in PAEC. PAEC were treated with 100 μg/ml of moLDL, eoLDL, or LDL for 12 h, and cell lysates were assayed spectrophotometrically for ND (A), SCCR (B), UCCR (C), and cytochrome c oxidase (D) activity. Values (means ± SE of 3 experiments using different batches of LDL) were normalized to cellular proteins. *\( P < 0.05 \); **\( P < 0.01 \) vs. control. +\( P < 0.05 \); +\( P < 0.01 \) vs. LDL. #\( P < 0.05 \); ##\( P < 0.01 \) vs. moLDL.

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Fig. 8. Effects of eoLDL on mitochondria-derived reactive oxygen species (ROS) generation from PAEC. A: confocal images showing PAEC stained with MitoTracker Green and MitoSOX Red. Merged image shows significant overlapping staining and confirms specificity of MitoSOX staining for mitochondria-derived ROS. B: PAEC were treated with vehicle (control) or 100 μg/ml of LDL or eoLDL for 2 h and then incubated with MitoSOX Red (red staining) before fixation and staining of DNA with H-33342 dye (blue staining). Shaded relief contrast images are typical of individual scan fields captured using an iCys laser scanning cytometer for cultures treated with vehicle (control), LDL, and eoLDL. Background autofluorescence of cells before MitoSox Red staining was also captured (not shown). C: mean integrated fluorescence (MIFL) for MitoSOX Red for each condition in B. Autofluorescence (black line) and control MIFL (red line) are shown at left, middle, and right. Blue lines show signals measured after LDL (middle) and eoLDL (right) treatment. D: MitoSOX Red MIFL in control and LDL-treated PAEC was obtained by laser scanning cytometry. Values are means ± SE (n = 3 experiments using different batches of LDL). *P < 0.05 vs. control. +P < 0.05 vs. LDL.
Effect of eoLDL on mitochondria-associated ROS. To determine the involvement of mitochondria in eoLDL-induced ROS generation, PAEC were treated with 100 μg/ml of LDL or eoLDL for 2 h and then labeled with MitoSOX Red. High-resolution confocal microscopy was used to confirm that labeling with MitoSOX Red was specific for mitochondria-derived ROS (Fig. 8A). Indeed, the ROS signal was entirely overlapped by staining with MitoTracker Green, a mitochondria-specific dye. Confirming the validity of MitoSOX Red for measurement of mitochondria-specific ROS generation, we used quantitative imaging by laser scanning cytometry to measure the increase in mitochondria-derived ROS after eoLDL treatment. With eoLDL exposure, the number of cells that generate mitochondrial ROS was nearly doubled compared with control or LDL-treated PAEC (*P < 0.05; Figs. 8B and 5D).

Effect of eoLDL on ROS release. Previous studies by our group demonstrated that treatment with eoLDL increases the release of H₂O₂ from HUVEC (51). The effect of eoLDL on the release of ROS from PAEC has not been documented. PAEC were treated with 100 μg/ml of LDL or eoLDL for 2 h. The present study demonstrated that eoLDL significantly increased the release of H₂O₂ from PAEC compared with LDL or control (Fig. 9; *P < 0.05) in addition to the increase of intracellular ROS associated with mitochondria.

DISCUSSION

The present study, for the first time, demonstrated that eoLDL significantly reduces mitochondrial complex-specific oxygen consumption in PAEC. The results are consistent with the impairment of the activity of key enzymes in complexes I–IV of mitochondria in PAEC induced by eoLDL.

Multiple lines of evidence suggest that oxidative stress, characterized by an elevated generation of ROS, is involved in the pathogenesis of CAD (4, 16), which implies that oxidized LDL may promote the development of cardiovascular complications through oxidative stress (46). Earlier studies demonstrated that oxidized LDL increases ROS generation from EC (51). Mitochondria are an important source of ROS and contribute to oxidative stress in cells under pathological conditions (43). The present study reveals that eoLDL increases the intracellular abundance of ROS and the release of ROS from aortic EC. ROS in PAEC treated with eoLDL were closely colocalized with mitochondrial marker. This suggests that mitochondria may also be an important source of ROS in EC exposed to eoLDL.

NAD⁺ and its reduced form NADH are mediators of various biological processes, including energy metabolism, mitochondrial electron transport, oxidative stress, aging, and cell death (47, 48). The NAD⁺-to-NADH ratio is an index of cellular reducing potential and may be altered under various pathological conditions. Complex I is one of the main consumers of NAD⁺ in cells (48). Previous studies demonstrated that diabetes is associated with decreased NAD⁺-to-NADH ratio in retinas of mice (31). Dysfunction of complex I may affect NADH oxidation, reverse electron transfer, and ROS generation (15). A recent study demonstrated that the inhibition of complex I reduces NAD⁺ levels, which is associated with enhanced superoxide production (19). The results of the present study, for the first time, demonstrate that eoLDL significantly reduces the NAD⁺-to-NADH ratio in EC compared with LDL or control. NAD⁺ and NADH may also be generated from other intracellular sources in addition to mitochondria. We hypothesize that eoLDL-induced reduction of the NAD⁺-to-NADH ratio may be partially due to the dysfunction of mitochondrial complex I in EC. This hypothesis is supported by our findings that eoLDL reduces ND activity (Fig. 5A) and rotenone-inhibited mitochondrial oxygen consumption induced by malate + glutamate in EC (Fig. 1).

Ceaser et al. (7) demonstrated that treatment with noncytotoxic oxidized LDL (≤150 μg/ml) increases complex I activity in HUVEC. A large volume of evidence suggests that eoLDL is proapoptotic (6, 26) and causes mitochondrial dysfunction (1, 44). Previous studies demonstrated that minimally oxidized LDL (TBARS ~8 nmol/mg protein) is antiapoptotic for EC (29), whereas eoLDL (TBARS >30 nmol/mg protein) has proapoptotic effects in EC (6). Although Ceaser et al. did not report the extent of oxidation of the nonapoptotic oxidized LDL, we assume that their oxidized LDL preparations were not extensively oxidized, since an antiapoptotic effect of the oxidized LDL was detected in EC. Preparations of eoLDL used in the present study were extensively oxidized (TBARS >30 nmol/mg protein) and may be assumed to be apoptotic on the basis of the results of previous studies (6). Preliminary studies in our laboratory indicate that eoLDL is proapoptotic in a time-dependent manner. Treatment with eoLDL for ≥48 h reduced cell viability in HUVEC. No significant change in cell viability was detected in EC treated with eoLDL for ≤24 h (unpublished observation). The results of the present study demonstrate that treatment with eoLDL for 12 or 24 h causes mitochondrial dysfunction, including the impairment of complex I activity. In addition, treatment with moLDL, which is less oxidized than eoLDL, induces weaker inhibition on mitochondrial enzyme activity in PAEC. Antioxidant (BHT) treatment normalized eoLDL-induced inhibition of mitochondrial enzyme activities in EC. However, the results do not contradict the stimulating effect of nonapoptotic oxidized LDL on complex I activity described by Ceaser et al. and suggest that oxidized LDL may have dual effects on mitochondrial respiratory chain activity in EC, depending on the extent of oxidation.

Mitochondria are considered an important target for ROS, including mitochondrial lipids, enzymes, and DNA. Oxidized components of eoLDL are possible candidates for inducing mitochondrial dysfunction and apoptosis in EC (4). Previous
studies by our group and others demonstrated that eoLDL increases ROS generation in EC (51, 52). A recent study found that oxidized LDL-induced ROS generation from mitochondria is associated with complex II (24). The results of the present study demonstrate that eoLDL significantly reduces the enzymatic activity of SCCRs, a key enzyme for complex II in EC. Oxidative stress mediates a cascade of events in EC, including mitochondrial damage (3). Previous studies suggest that electrons derived from FADH₂ via complex II can undergo “reverse electron transport” into complex I (21). Findings from the present study suggest that complex II may also be an important target of eoLDL in mitochondria. Previous studies demonstrated that mitochondrial complex III is a major site for ROS production in mitochondria (25). Findings of the present study demonstrate that eoLDL significantly impairs enzymatic activity of UCCRs in EC compared with LDL or control. Moreover, results from cytometry-based assay demonstrate that mitochondria are an important source of ROS in EC exposed to eoLDL. The eoLDL-induced impairment of complex II and III may enhance mitochondrial dysfunction in EC.

Cytochrome c oxidase is coded by nuclear and mitochondrial DNA (7, 49). Deficiency or reduced activity of any component of the mitochondrial respiratory chain can affect ATP production; however, the dysfunction of complex IV interrupts the final step of the mitochondrial respiration chain and promotes ROS production (2). Impaired activity of complex IV is associated with aging and is believed to play a critical role in oxidative injury of senescent EC (49). Ceaser et al. (7) found that nonapoptotic oxidized LDL does not significantly alter complex IV activity in HUVEC. The present study is the first to show that eoLDL inhibits complex IV activity in EC as determined using two independent approaches: 1) ascorbate/TMPD-induced, KCN-sensitive oxygen consumption and 2) cytochrome c oxidase activity. The effect of oxidized LDL on the expression of key enzymes of mitochondrial respiration chain enzymes has not been documented; however, this possibility cannot be excluded and may be investigated in subsequent study.

In intact mitochondria of living cells, the addition of ADP causes a sudden burst of oxygen uptake, which is likely due to increased oxidative phosphorylation and formation of ATP. The present study demonstrates that treatment with eoLDL for ≥12 h significantly reduces ADP-induced oxygen uptake and RCI in EC. RCI reflects the relationship between ATP synthesis and oxygen consumption in mitochondria. ATP levels were evidently decreased in atherosclerotic lesions that were associated with hypercholesterolemia (17). Previous studies demonstrated that oxidized LDL treatment significantly reduces ATP production in vascular smooth muscle cells (41). Results of the present study demonstrate that FCCP, a mitochondrial uncoupler, records <50% of the eoLDL-induced decrease in oxygen consumption, which suggests that a large portion of the eoLDL-induced decrease in mitochondrial respiration is not due to the reduction of ATP synthase (Fig. 4). Mitochondrial dysfunction is involved in reduced energy output and increased mitochondrial oxidative stress (4). The results of the present study suggest that eoLDL may simultaneously impair energy generation and oxygen consumption in EC. RCI may be considered a marker for mitochondrial dysfunction in EC induced by increased levels of eoLDL.

Our previous study demonstrated that BHT, a potent antioxidant, inhibits oxidized LDL-induced H₂O₂ release in vascular EC (50). The results of the present study demonstrate that BHT normalizes eoLDL-induced changes in complex I and III activity. Generally, complex I and III are considered major sites for ROS production (25). The results provide additional evidence that the extent of oxidation is important in LDL-induced deterioration of mitochondrial activity in EC.

In summary, the results of the present study indicate that eoLDL reduces the activities of multiple enzymes in mitochondrial respiratory chain complexes and corresponding complexes specific oxygen consumption in EC, which may contribute to the increased ROS generation from EC. Mitochondrial dysfunction and ROS production induced by eoLDL may promote endothelial dysfunction in the vasculature of patients with atherosclerotic vascular disease.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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Disclosures

No conflicts of interest are declared by the author(s).

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