JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Influence of Delphinidin-3-glucoside on Oxidized Low-Density Lipoprotein-Induced Oxidative Stress and Apoptosis in Cultured Endothelial Cells

Xueping Xie, Ruozhi Zhao, and Garry X. Shen*

Departments of Internal Medicine and Physiology, University of Manitoba, Winnipeg, Manitoba, Canada

ABSTRACT: Increased oxidative stress and apoptosis were detected in atherosclerotic lesions. Oxidized low-density lipoprotein (oLDL) may induce oxidative stress and apoptosis via multiple pathways in vascular endothelial cells (EC). Delphinidin-3-glucoside (D3G), an anthocyanidin glycan enriched in dark-skin berries, may neutralize those effects of oLDL in EC. The present study demonstrated that oLDL increased the generation of intracellular NADPH-dependent superoxide and impaired redox status in cultured porcine aortic EC (PAEC). The activities of mitochondrial respiratory chain complex I–IV and the contents of NADH dehydrogenase (ND)1, ND6 (complex I enzyme subunits), or cytochrome *b* (complex III enzyme subunit) were significantly reduced in PAEC treated with oLDL compared to controls. Treatment with oLDL significantly increased the abundances of NADPH oxidase (NOX)2, NOX4, and p22phox in PAEC. oLDL reduced cell viability and the protein content of B-cell lymphoma (Bcl)-2, but increased the content of caspase 3 in PAEC. Co-treatment with D3G prevented oLDL-induced increases in intracellular superoxide or in the protein content of NOX2, NOX4, p22phox, or caspase 3, inhibited the impairment of redox statues or cell viability, and prevented the attenuation of mitochondrial enzyme activities and the reductions of Bcl-2, ND1, or cytochrome *b* contents in PAEC. The findings suggest that oLDL induced oxidative stress and apoptosis in EC, which was associated with the activation of NOX, the impairment of mitochondrial respiration chain enzymes, and the disorder of key regulators for apoptosis. D3G neutralized the harmful effects of oLDL on oxidative stress, mitochondrial dysfunction, and apoptosis in cultured vascular EC.

KEYWORDS: *delphinidin-3-glucoside, oxidized LDL, mitochondrial respiratory chain enzymes, NADPH oxidase, apoptosis, vascular endothelial cells*

INTRODUCTION

Oxidized low-density lipoprotein (oLDL) is a biomarker of oxidative stress in the body.[1] Elevated levels of the markers for oLDL were detected in the blood circulation of patients with coronary artery disease (CAD).[2] Increased oxidative stress and apoptosis were observed in the aortic wall of atherosclerotic animals.[3] Antigens of oLDL were presented in atherosclerotic lesions in CAD patients or in experimental animal models.[4]

Endothelium is a monolayer of cells located between blood components and other vascular tissues. Interactions between blood components and endothelial cells (EC) may play critical roles in the pathophysiology of atherosclerosis or thrombosis, the two major pathological findings in CAD. Accumulated lines of evidence suggest that reactive oxygen species (ROS) mediate multiple important cellular processes related to the etiology of atherosclerosis.

Reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) and mitochondria are two major sources of ROS in vascular EC. NOX complexes locate in the membrane of intracellular organelles and generate superoxide via the catabolism of NADPH.[5] Mitochondria generate ROS during intracellular respiration. Previous studies in our laboratory demonstrated that oLDL increased the release of superoxide and hydrogen peroxide (H_2O_2) from EC, which was associated with transient increases in the activities of multiple redox enzymes.[6] Treatment with oLDL increased the contents of components of NOX complexes in EC.[7] Our recent studies demonstrated that oLDL reduced enzymatic activities of mitochondrial respiratory chain complex I–IV, which were associated with elevated levels of mitochondria-associated ROS in cultured EC.[8] Sustained mitochondrial dysfunction may lead to apoptosis. Previous studies demonstrated that oLDL increased caspase-mediated apoptosis in EC, which was associated with the decreased expression of B-cell lymphoma-2 protein (Bcl-2), a negative regulator of apoptosis.[9]

Previous studies indicated that a variety of dark-skin berries yielded antioxidant effects in multiple experimental models.[10,11] Anthocyanidins are a class of color pigments in plants. Delphinidin is one of the most abundant types of anthocyanidins in dark-skin berries.[12,13] Delphinidin inhibited apoptosis induced by 7β-hydrocholesterol, a component of oLDL, in EC[10,11] and attenuated doxorubicin-induced cardiotoxicity in cardiomyocytes.[14] Underlying mechanisms for the cardioprotective, antioxidant, and antiapoptotic effects of delphinidin remain unclear. Delphinidin-3-glucoside (D3G) is an abundant glycan of delphinidin in dark-skin plant products. D3G extracted from eggplants had potent radical scavenger capacity for superoxide.[15]

The present study examined the effects of D3G on ROS production, the activities and contents of mitochondrial respiratory chain complex enzymes, NOX complex compo-

Received: November 2, 2011
Revised: January 23, 2012
Accepted: January 25, 2012
Published: January 25, 2012

dx.doi.org/10.1021/jf204461z | J. Agric. Food Chem. 2012, 60, 1850–1856

nents, cell viability, and apoptosis regulators induced by oLDL in porcine aortic EC (PAEC).

MATERIALS AND METHODS

Isolation and Modification of Lipoproteins. LDL (density = 1.019−1.063) was isolated from the 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.[16] The oxidation of LDL was verified using a thiobarbituric acid reactive substance assay and nondenatured gel electrophoresis. Lipoproteins were stored in sealed tubes under a layer of nitrogen at 4 °C in the dark to prevent autoxidation.

Cell Culture. PAEC were obtained from Dr. P. E. DiCorleto of the Cleveland Clinic Research Institute. Cells were grown in Dulbecco's modified Eagle medium (Invitrogen, ON, Canada) supplemented with 10% fetal bovine serum and 1% penicillin−streptomycin (Invitrogen) in an incubator at 37 °C and maintained in a humidified atmosphere containing 5% CO_2.[17]

Experimental Incubation. D3G (Polyphenols Laboratories, Sandnes, Norway) was dissolved in 0.01% HCl as instructed by the manufacturer. D3G and its solution were handled without direct exposure to light. PAEC were pretreated with indicated concentrations of D3G or vehicle for 30 min at 37 °C under 5% CO_2 before the addition of lipoproteins.

Assessment of Redox Status. Intracellular redox status was assessed using 2,7-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Molecular Probes, Eugene, OR) assay to avoid colorimetric interference of anthocyanin. PAEC were seeded into 96-well plates (2×10^5 cells/well) and allowed to grow for 24 h. After treatment with D3G and/or lipoproteins, cells were incubated with 20 μM H_2DCF-DA in HEPES-buffered salt solution (HBSS, pH 7.4) for 30 min. At the end of incubation, medium was removed and cells were washed with HBSS three times. Intensities of fluorescence in cells were measured at 485/530 nm (excitation/emission) using a fluorescence microplate reader (FLUOStar OPTIMA, BMG Lab Technologies, GmbH, Ortenberg, Germany). Relative redox state in EC was expressed as percentage of control.[18]

Intracellular Superoxide Determination. The levels of intracellular superoxide were measured using enhanced lucigenin assay as previously described.[19] After experimental incubation, cells were homogenized in lysis buffer (50 mM KH_2PO_4, pH 7.0, 1 mM EGTA, 10 μg/mL of aprotinin, 0.5 μg/mL of leupeptin, 1 μg/mL of pepstatin, and 0.5 mM phenylmethanesulfonyl fluoride). Aliquots of 50 μg of cellular proteins were added in an assay buffer (50 mM KH_2PO_4, pH 7.0, 1 mM EGTA, 150 mM sucrose, and 100 μM NADPH). The reaction was started by the addition of 25 μM lucigenin. The level of NADPH-dependent superoxide was assessed according to chemoluminescence in tested samples detected using a photon counter (Lumat LB 9507) for 30 min.[20]

Western Blotting. Western blotting analysis was performed as previously described.[16] Equal amounts of cellular lysate were run on 12% SDS-PAGE and electrotransferred to nitrocellulose membrane. Proteins were identified using antibodies against reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase (ND)1, ND6, cytochrome *b*, p22phox (Santa Cruz, CA), β-actin (Sigma, St. Louis, MO), porin (Abcam, Cambridge, MA), Bcl-2, or caspase 3 (Cell Signaling, Pickering, ON, Canada). Enhanced chemiluminescence reagents (Amersham, Piscataway, NJ) were used for detecting targeted antigens on membrane. Densities of antigens were analyzed using Chemi-Doc system with Quantity-One software (Bio-Rad, Hercules, CA). Abundances of targeted proteins were normalized with the levels of control protein (porin for mitochondrial proteins and β-actin for nonmitochondrial cellular proteins).

ND Activity. ND (complex I enzyme) activity was measured as described previously.[21] Mitochondrial fractions of PAEC (50 μg) were added to 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).[22]

Succinate Cytochrome *c* Reductase (SCCR) Activity. SCCR (complex II/III enzymes) activity was measured by monitoring the rate of reduced cytochrome *c* formation using succinate as substrate. Sonicated cell lysates (0.2 mg of protein) were preincubated with a reaction mixture (10 mM potassium phosphate, pH 7.4, 2 mM EDTA, 0.01% bovine serum albumin, 0.2 mM ATP, 1 mM KCN, 5 μM rotenone, and 10 mM succinate) for 3 min, and reaction was started by the addition of 40 μM oxidized cytochrome *c*.[23] Changes in absorbance were monitored at 30 °C using a spectrophotometer for 5 min at 550 nm.[22]

Ubiquinol Cytochrome *c* Reductase (UCCR) Activity. The activity of UCCR (complex III enzyme) 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, reaction was started by the addition of 50 μM ubiquinol-2. Changes in absorbance at 550 nm were monitored.[24]

Cytochrome *c* Oxidase (COX) Activity. COX (complex IV enzyme) activity was examined at 30 °C by measuring the rate of oxidation of reduced cytochrome *c* at 550 nm. Assay was performed in the presence of 40 μM reduced cytochrome *c*, 20 mM phosphate buffer, 0.1 mg of protein from PAEC, and 16 mg of lauryl maltoside/mg protein (0.16%).[25]

Citrate Synthase (CS) Activity. CS activity was determined at 30 °C 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 cellular protein from EC. The reaction was started by adding 300 μM acetyl-CoA. Changes in absorbance were measured at 412 nm for 1 min. The rate of absorbance change was subtracted from that with the addition of 0.5 mM oxalacetic acid. CS activity was used as a control of mitochondrial enzymes in cells.[26]

Cell Viability Assay. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. PAEC were seeded in 96-well plates (1×10^4/well) cultured to achieve 70−80% confluence. Cells were incubated with indicated agents for 12−60 h. Media were replaced by fresh medium containing 0.5 mg/mL MTT, and the incubation was continued for 2 h. At the end of incubation, medium containing MTT was removed, and insoluble formazan crystals formed in cells were dissolved in 150 μL of dimethyl sulfoxide (Sigma). The absorbance was measured at 570 nm using a 96-well plate FLUOstar Optima.[11]

Statistical Analysis. Data are presented as the mean of values from three independent experiments ± standard deviation (SD). Data from multiple groups were analyzed using the one-way variance assay followed with posthoc tests. Differences at $p < 0.05$ were considered to be significant.

RESULTS

Effect of D3G on Redox Status in EC. The effect of D3G (25−150 μM for 30 min) on intracellular redox status was characterized in PAEC. D3G at the levels of 75−125 μM significantly reduced H_2DCF-DA fluorescence intensity in PAEC in a concentration-dependent manner ($p < 0.05$ or 0.01). The maximal inhibition on redox states was detected in cultures treated with 100 μM D3G for 30 min (Figure 1A). In a separate set of experiments, oLDL at 100 μg/mL for 1−24 h significantly increased the fluorescence intensity of H_2DCF-DA in PAEC ($p < 0.05$ or 0.01). The incubation of 100 μM D3G alone for 0.5−24 h significantly reduced H_2DCF-DA intensity in PAEC ($p < 0.05$ or 0.01). Co-treatment of D3G with oLDL for 2−24 h significantly reduced oLDL-induced increases of H_2DCF-DA intensity in PAEC ($p < 0.05$ or 0.01, Figure 1B).

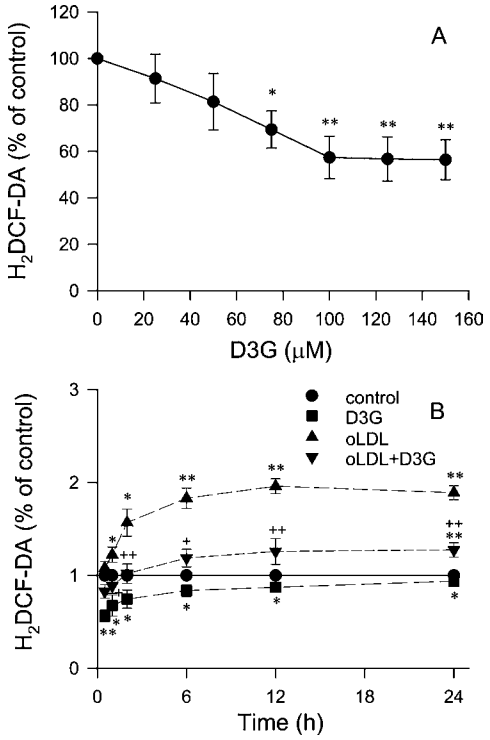

Figure 1. Effect of delphinidin-3-glucoside (D3G) on oxidized LDL (oLDL)-induced impairment of redox status in porcine aortic endothelial cells (PAEC): (A) PAEC were treated with 0−150 μM D3G for 30 min; (B) PAEC were treated with vehicle (control), 100 μM D3G, or 100 μg/mL oLDL or D3G + oLDL for 0.5−24 h. At the end of treatment, cells were incubated with 20 μM H_2DCF-DA for 30 min. Redox states were measured using H_2DCF-DA-derived fluorescence in cells at 485/530 nm (excitation/emission). Values are expressed as the mean ± SD in percentage of control (n = 3 independent experiments). (*, **) $p < 0.05$ or 0.01 versus control; (+, ++) $p < 0.05$ or 0.01 versus oLDL.

Effects of D3G on the Activities of Mitochondrial Respiratory Chain Complex Enzymes in oLDL-Treated EC. Our previous studies demonstrated that oLDL (100 μg/mL) decreased the activities of ND and SCCR in PAEC after ≥12 h of incubation and that of UCCR or COX after ≥6 h of incubation.[8] The present study examined the effect of D3G on the activities of the mitochondrial enzymes in EC exposed to 100 μg/mL oLDL for 12 h. Treatment with oLDL significantly reduced the activities of ND, SCCR, UCCR, and COX, but did not affect the activity of CS (a control mitochondrial enzyme) in PAEC compared to vehicle control ($p < 0.05$ or 0.01). Co-treatment with 100 μM D3G inhibited oLDL-induced reduction in ND and UCCR activities compared to oLDL-treated EC ($p < 0.05$), but did not significantly alter the levels of SCCR and COX in PAEC (Figure.2).

Effects of D3G on Protein Contents of Subunits in Mitochondrial Respiratory Chain Enzymes. To determine whether oLDL affects the contents of mitochondrial respiratory chain enzyme complex subunits in EC, the levels of ND1 and ND6 in complex I were analyzed using Western blotting. Treatment with oLDL at 100 μg/mL for 12 h significantly reduced levels of ND1 and ND6 in PAEC after normalization with the levels of porin in corresponding samples ($p < 0.05$). D3G (100 μM) alone did not significantly alter the levels of ND1 or ND6 in EC. Co-treatment with D3G prevented oLDL-induced decrease in the content of ND1, but not ND6, in PAEC ($p < 0.05$). oLDL inhibited the content of cytochrome b in complex III in PAEC ($p < 0.05$). D3G inhibited oLDL-induced decrease in the content of cytochrome b in EC ($p < 0.01$, Figure 3).

Effect of D3G on oLDL-Induced Intracellular Superoxide. The condition for oLDL-induced ROS production in EC was optimized in previous studies.[5] Treatment with 100 μg/mL oLDL for 2 h increased intracellular superoxide in PAEC by more than 2-fold ($p < 0.01$). D3G alone at 100 μM reduced intracellular superoxide by >50% compared to control ($p < 0.01$). Co-treatment of D3G and oLDL prevented oLDL-induced increase of superoxide in EC compared to oLDL alone ($p < 0.05$, Figure 4).

Effects of D3G on oLDL-Induced Increases in NOX Complex Components. Our previous studies demonstrated that oLDL (100 μg/mL for 6 h) increased the protein contents of NOX2 and p22phox, an essential component of NOX complex, in human EC.[6] The present study demonstrated that oLDL (100 μg/mL for 6 h) increased the content of NOX2, NOX4, and p22phox in PAEC ($p < 0.05$ or 0.01). D3G alone (100 μM) did not significantly alter the level of NOX2, NOX4, and p22phox. Co-treatment with D3G inhibited the increases of the contents of NOX2, NOX4, and p22phox induced by oLDL in PAEC ($p < 0.05$ or 0.01, Figure 5).

Effect of oLDL on Cell Viability. The effect of oLDL on cell viability was examined in PAEC treated with 100 μg/mL oLDL for 12−60 h. The cell viability of PAEC was significantly decreased by treatment with 100 μg/mL oLDL for ≥48 h ($p < 0.01$), but not in cells treated with oLDL for ≤24 h. Treatment with 100 μM D3G prevented oLDL-induced impairment of cell viability at 48 and 60 h compared to that exposed to oLDL alone ($p < 0.05$, Figure 6).

Effects of oLDL on Apoptosis Regulators. oLDL treatment (100 μg/mL) for 24 h significantly increased the expression of caspase 3, a mitochondria-dependent apoptosis agonist, and decreased the content of Bcl-2, a negative regulator for apoptosis, in PAEC compared to controls ($p < 0.01$). Co-treatment with 100 μM D3G inhibited oLDL-induced changes in caspase 3 and Bcl-2 in PAEC compared to that of oLDL alone ($p < 0.05$). D3G alone did not significantly alter the content of caspase 3 or Bcl-2 in PAEC (Figure.7).

■ DISCUSSION

Major novel findings of the present study include the following: (a) D3G prevented oLDL-induced impairment in redox status and increase in NADPH-dependent superoxide in PAEC. (b) D3G neutralized oLDL-induced inhibition on activities of mitochondrial respiratory chain complex I or III enzymes and the attenuation of the contents of ND1 and cytochrome b in PAEC. (c) D3G inhibited oLDL-induced up-regulation of NOX4, NOX2, and p22phox in PAEC. (d) D3G prevented oLDL-induced impairment of cell viability and imbalance between caspase 3 and Bcl-2 in PAEC.

Previous studies demonstrated that anthocyanidin-rich berries reduced the susceptibility of EC to ROS or tumor necrosis factor-α.[27] *Artisotelia chileansis* berry juice decreased intracellular oxidative stress induced by H_2O_2 in cultured EC.[28] Mulberry extracts inhibited LDL oxidation in vitro.[29] Grape seed supplementation protected cardiac function by reducing lipid peroxidation and blocking apoptotic signaling.[30] The results of the present study demonstrated that D3G alone reduced intracellular superoxide in EC at basal condition, which supported findings from previous studies.

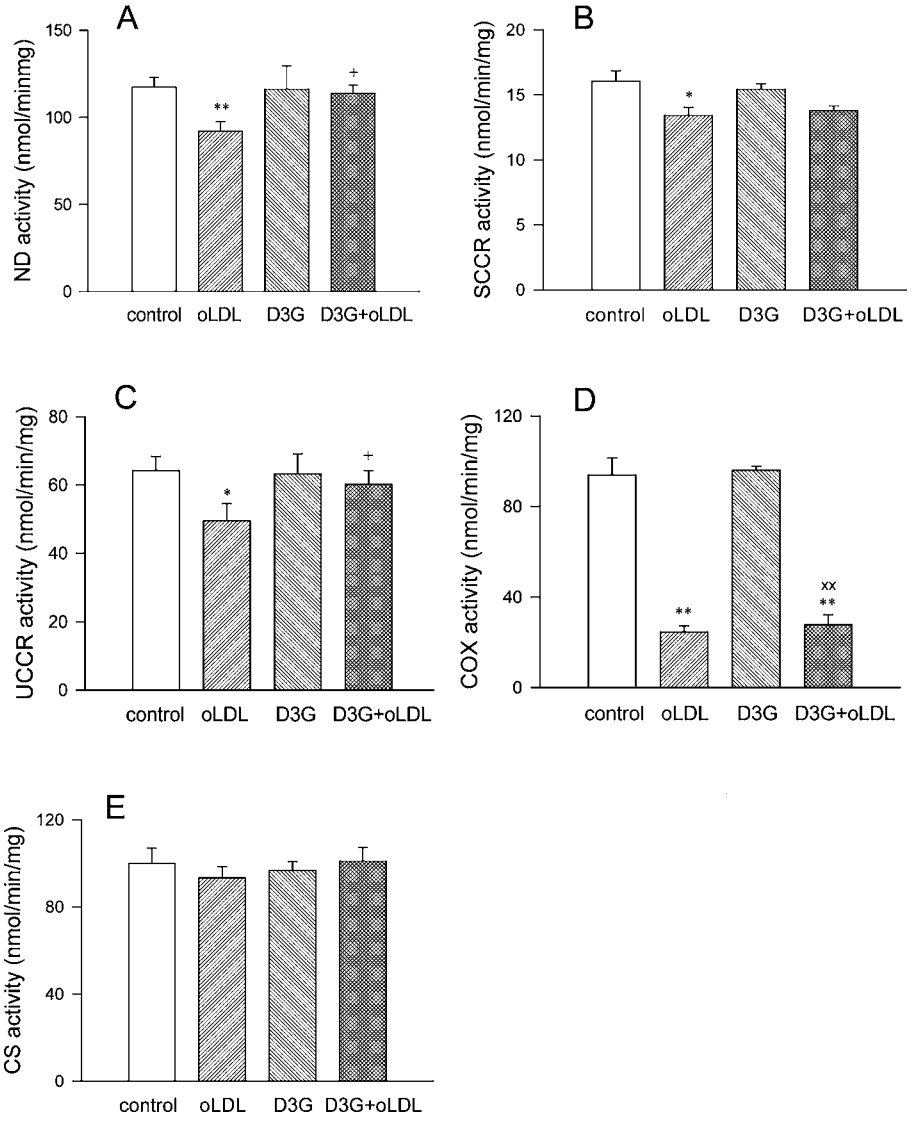

Figure 2. Effects of D3G on the activities of enzymes in mitochondrial respiratory chain complexes in PAEC. PAEC were treated with vehicle (control), 100 μM D3G, or 100 μg/mL oLDL or D3G + oLDL for 12 h. Activities of NADH dehydrogenase (ND; A), succinate cytochrome *c* redutase (SCCR; B), ubiquinol cytochrome *c* reductase (UCCR; C), cytochrome *c* oxidase (COX; D), and citrate synthase (CS; E) in EC were analyzed as described under Materials and Methods. Values are expressed as the mean ± SD in nmol/min/mg protein (n = 3 independent experiments). (*, **) $p < 0.05$ or 0.01 versus control; (+) $p < 0.05$ versus oLDL; (××) $p < 0.01$ versus D3G.

Mitochondrion is an important source of ROS in cells. A small portion of electron may leak during mitochondrial respiration and form ROS in mitochondrial matrix at physiological condition. The production of ROS in mitochondria may be enhanced when respiratory chain activity is impaired. Our recent studies demonstrated that oLDL increased mitochondria-associated ROS in EC, which may result from the impairment of the activities of mitochondrial respiratory chain enzymes.[8] The particles of oLDL contain multiple forms of active lipid peroxidation products, including conjugated dienes and peroxy radicals,[31] which may directly increase intracellular oxidative stress and subsequently impair mitochondrial respiratory chain activity. The effect of delphinidin glycan on mitochondrial activity has not been documented. The results of the present study originally demonstrated that D3G inhibited oLDL-induced impairment of redox status and prevented the deterioration of multiple subunits of key mitochondrial respiratory chain enzymes for ROS generation in EC. The beneficial effect of D3G may help to prevent vascular endothelial injury induced by oLDL.

ND, the complex I enzyme, mediates the transfer of electron from NADH to coenzyme Q. It is one of the major sites of the generation of superoxide in mitochondria. ND complex is composed of 43 subunits. ND1−6 proteins are encoded by mitochondrial DNA. Complex III enzyme, UCCR, is another important source of ROS in mitochondria. UCCR is composed of 11 subunits, but only cytochrome *b* is encoded by mitochondrial DNA. Mitochondrial DNA attaches to the matrix side of the inner membrane of mitochondria, where it may directly contact with ROS in matrix released from mitochondrial respiratory chain. Mitochondrial DNA is susceptible to attack by ROS in mitochondrial matrix partially due to the lack of protection of histone as nuclear DNA.[32] Although oLDL reduced activities of enzymes in all four mitochondrial respiratory chain complexes, D3G treatment inhibited oLDL-induced decreases of activities only in complex I and III, but not in complex II or IV, in PAEC. In addition,

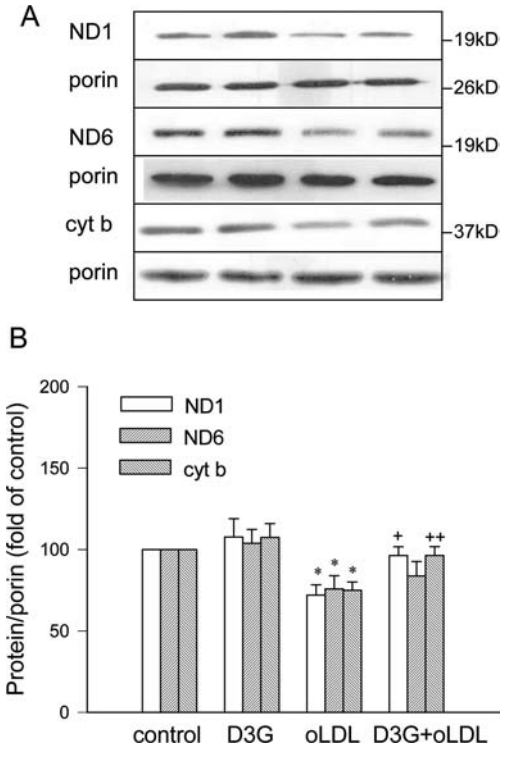

Figure 3. Effects of D3G on oLDL-induced attenuation in contents of mitochondrial respiratory chain complex I and III subunits in PAEC. Cells were treated with vehicle (control), 100 μM D3G, or 100 μg/mL oLDL or D3G + oLDL for 12 h. Contents of NADH dehydrogenase 1 (ND1), ND6, cytochrome *b* (cyt b), and porin (control for mitochondrial proteins) were assessed using Western blotting. Sizes of molecular weight standards are indicated beside blots when a protein first appeared in this or following figures. Values are expressed as the mean ± SD in percentage of control after normalization with porin (n = 3 independent experiments). (*) p < 0.05 versus control; (+, ++) p < 0.05 or 0.01 versus oLDL.

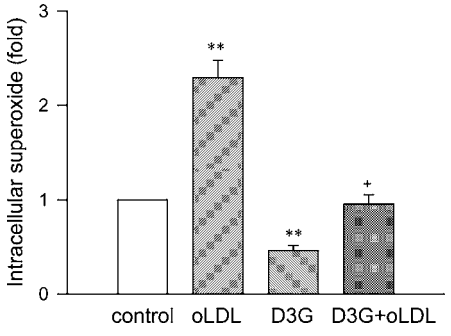

Figure 4. Effect of D3G on intracellular superoxide in EC. PAEC were treated with vehicle (control), 100 μM D3G, or 100 μg/mL oLDL or D3G + oLDL for 6 h. Intracellular superoxide was measured using lucigenin assay as described under Materials and Methods. Values are expressed as the mean ± SD in folds of control (n = 3 independent experiments). (**) p < 0.01 versus control; (+) p < 0.05 versus oLDL.

D3G prevented the reduction of the abundances of ND1 and cytochrome *b*, but not that of ND6, induced by oLDL in PAEC. The findings suggest that the protective effects of D3G on oLDL-induced mitochondrial dysfunction are related to multiple mitochondrial respiratory chain enzymes and several subunits encoded by mitochondrial DNA. The involvement of mitochondrial proteins encoded by nuclear DNA in oLDL or D3G-induced changes in mitochondrial enzyme activity in EC has not been excluded. The underlying mechanism for the beneficial effects of D3G on mitochondrial transcriptional machinery remains to be investigated in subsequent studies.

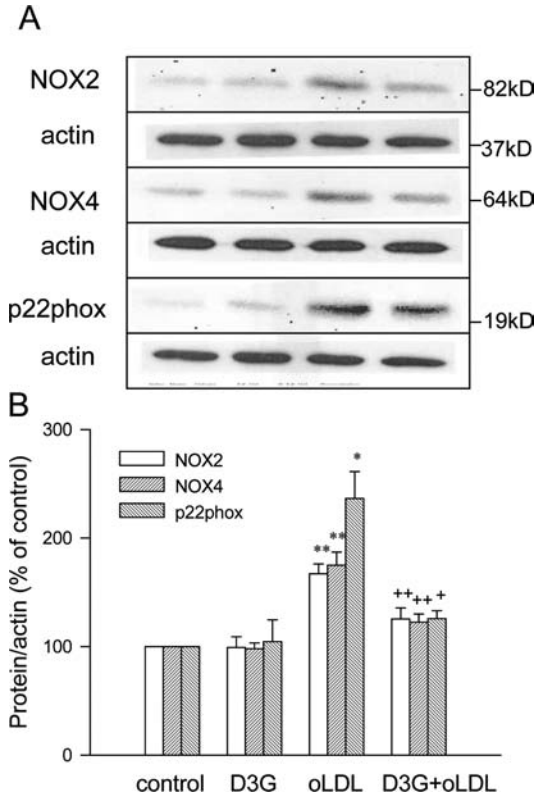

Figure 5. Effects of D3G on the contents of NOX2, NOX4, and p22phox in EC. PAEC were treated with vehicle (control), 100 μM D3G, or 100 μg/mL oLDL or D3G + oLDL for 6 h. Contents of NOX2, NOX4, p22phox, and β-actin (actin) in cellular proteins were assessed using Western blotting. Values are expressed as the mean ± SD in folds of control after normalization with actin (n = 3 independent experiments). (*, **) p < 0.05 or 0.01 versus control; (+, ++) p < 0.05 or 0.01 versus oLDL.

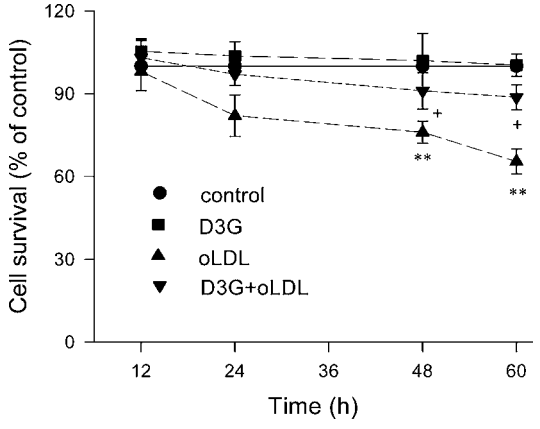

Figure 6. Effect of D3G on oLDL-induced changes in cell viability in EC. PAEC were treated with vehicle (control), 100 μM D3G, or 100 μg/mL oLDL or D3G + oLDL for 12−60 h. Cell viability was measured using MTT assay as described under Materials and Methods. Values are expressed as the mean ± SD in percentage of control (n = 3 independent experiments). (**) p < 0.01 versus control with corresponding incubation time; (+) p < 0.05 versus oLDL.

dx.doi.org/10.1021/jf204461z | J. Agric. Food Chem. 2012, 60, 1850−1856

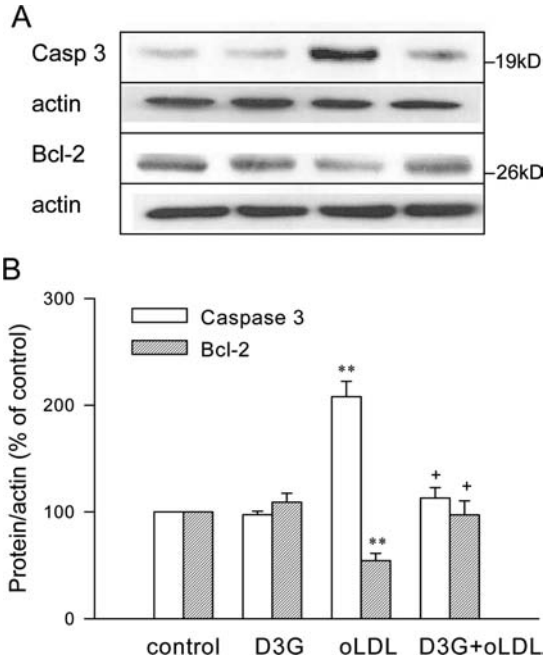

Figure 7. Effects of D3G on oLDL-induced changes in the contents of caspase 3 (Casp 3) and B cell lymphoma-2 (Bcl-2) protein in EC. PAEC were treated with vehicle (control), 100 μM D3G, or 100 μg/mL oLDL or D3G + oLDL for 24 h. The contents of Casp 3, Bcl-2, and β-actin were assessed using Western blotting. Values are expressed as the mean ± SD in percentage of control (n = 3 independent experiments). (**) $p < 0.01$ versus control; (+) $p < 0.05$ versus oLDL.

NOX complexes are another important source of ROS in EC. Our recent studies demonstrated that oLDL increased levels of ROS and the expression of NOX2 and p22phox in human EC[6] and the content of NOX4 in mouse embryo fibroblasts.[20] The results of the present study demonstrated that cotreatment of D3G prevented oLDL-induced increases in intracellular superoxide and the components of NOX complexes, including NOX2, NOX4, and p22phox, in EC. NOX4 is the only NOX complex detected in mitochondria.[33] Our findings indicate that the increases of the contents of NOX components were prior to changes in the activities or proteins of mitochondrial respiratory chain enzymes in oLDL-treated EC. NOX4-derived ROS in mitochondria induced by oLDL may result in the impairment of mitochondrial respiratory chain enzymes activity and further increase of ROS generation in EC. The findings suggest that D3G may down-regulate oxidative stress in vascular EC through multiple pathways.

Increased ROS in mitochondria may cause caspase-dependent apoptosis of EC. Results of previous studies suggested that the effects of delphinidin glycans on apoptosis were cell-type specific. Delphinidin-sambubioside induced apoptosis in HL-60 leukemia cells through a ROS-mediated pathway.[18] Delphinidin inhibited cell viability and increased the activation of caspase 3 in HCT-16 colon cancer cells.[34] Delphinidin increased DNA degradation in lymphocytes via a ROS-dependent mechanism.[35] In HT29 colon cancer cells, delphinidin prevented oxidative DNA damage through a catalase-dependent pathway.[36] Delphinidin inhibited apoptosis in EC induced by 7β-hydrocholesterol via a nitric oxide-dependent pathway.[10] A recent study demonstrated that delphinidin restored oLDL-induced decrease of cell viability in human EC, which was associated with the up-regulataion of Bcl proteins.[11] The results of the present study suggested that D3G may protect EC from oLDL-induced apoptosis. We further demonstrated that D3G inhibited oLDL-induced increase in caspase 3 protein in addition to the neutralization of oLDL-induced reduction of Bcl-2 levels in EC. The effect of D3G on oLDL-induced apoptosis is possibly related to its effect on NOX activation and mitochondrial dysfunction. oLDL also triggers endoplasmic reticulum (ER) stress in EC.[37] ER stress may play an interactive role in mitochondrial dysfunction-induced apoptosis and cell injury.[38] The results of the present study have not excluded possible involvement of ER stress regulators in the protective effects of D3G on oLDL-induced mitochondrial dysfunction or apoptosis in EC.

D3G may be obtained through a variety of food sources, such as wild blueberries, Saskatoon berries, raspberries,[12] dark-skin grapes, and red wine,[13] for the purpose of prevention of mitochondrial dysfunction or oxidative stress in individuals with risk of CAD, especially hypercholesterolemia. However, the dosage of D3G administration for mitochondrial protection or antioxidative stress in vivo needs to be verified and optimized in proper animal models and human studies.

The present study demonstrated that D3G neutralized the effects of oLDL on NOX activation, mitochondrial dysfunction, and caspase/Bcl-2 related apoptosis in cultured vascular EC. The findings may increase understanding of the cardiovascular protective effect of anthocyanidins and the development of potential natural cardioprotective antioxidants.

AUTHOR INFORMATION

Corresponding Author

*Postal address: Diabetes Research Group, University of Manitoba, 835-715 McDermot Avenue, Winnipeg, MB R3E 3P4, Canada. Phone: (204) 789-3816. Fax: (204) 789-3987. E-mail: gshen@ms.umanitoba.ca.

Funding

We are grateful for the operating grants from the Canadian Diabetes Association, the Heart and Stroke Foundation of Manitoba, and the Canadian Institutes of Health Research for G.X.S.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We appreciate the research space provided by the Health Sciences Centre Foundation.

REFERENCES

(1) Diaz, M. N.; Frei, B.; Vita, J. A.; Keaney, J. F. Jr. Antioxidants and atherosclerotic heart disease. *N. Engl. J. Med.* **1997**, *3376*, 408−416.

(2) Tsimikas, S.; Bergmark, C.; Beyer, R. W.; Patel, R.; Pattison, J.; Miller, E.; Juliano, J.; Witztum, J. L. Temporal increases in plasma markers of oxidized low-density lipoprotein strongly reflect the presence of acute coronary syndromes. *J. Am. Coll. Cardiol.* **2003**, *41*, 360−370.

(3) Yu, Y. M.; Chang, W. C.; Wu, C. H.; Chiang, S. Y. Reduction of oxidative stress and apoptosis in hyperlipidemic rabbits by ellagic acid. *J. Nutr. Biochem.* **2005**, *16*, 675−681.

(4) Ylä-Herttuala, S.; Palinski, W.; Rosenfeld, M. E.; Parthasarathy, S.; Carew, T. E.; Butler, S.; Witztum, J. L.; Steinberg, D. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J. Clin. Invest.* **1989**, *84*, 1086−1095.

(5) Jiang, F.; Zhang, Y.; Dusting, G. J. NADPH oxidase-mediated redox signaling: roles in cellular stress response, stress tolerance, and tissue repair. *Pharmacol. Rev.* **2011**, *63*, 218−242.
(6) Zhao, R.; Shen, G. X. Functional modulation of antioxidant enzymes in vascular endothelial cells by glycated LDL. *Atherosclerosis* **2005**, *179*, 277−284.
(7) Zhao, R.; Ma, X.; Xie, X.; Shen, G. X. Involvement of NADPH oxidase in oxidized LDL-induced upregulation of heat shock factor-1 and plasminogen activator inhibitor-1 in vascular endothelial cells. *Am. J. Physiol.: Endocrinol. Metab.* **2009**, *297*, E104−111.
(8) Roy Chowdhury, S. K.; Sangle, G. V.; Xie, X.; Stelmack, G. L.; Halayko, A. J.; Shen, G. X. Effects of extensively oxidized low-density lipoprotein on mitochondrial function and reactive oxygen species in porcine aortic endothelial cells. *Am. J. Physiol: Endocrinol. Metab.* **2010**, *298*, E89−E98.
(9) Chen, J.; Mehta, J. L.; Haider, N.; Zhang, X.; Narula, J.; Li, D. Role of caspases in Ox-LDL-induced apoptotic cascade in human coronary artery endothelial cells. *Circ. Res.* **2004**, *94*, 370−376.
(10) Martin, S.; Giannone, G.; Andriantsitohaina, R.; Martinez, M. C. Delphinidin, an active compound of red wine, inhibits endothelial cell apoptosis via nitric oxide pathway and regulation of calcium homeostasis. *Br. J. Pharmacol.* **2003**, *139*, 1095−1102.
(11) Chen, C. Y.; Yi, L.; Jin, X.; Mi, M. T.; Zhang, T.; Ling, W. H.; Yu, B. Delphinidin attenuates stress injury induced by oxidized low-density lipoprotein in human umbilical vein endothelial cells. *Chem.−Biol. Interact.* **2010**, *183*, 105−112.
(12) Hossenian, F. S.; Beta, T. Saskatoon and wild blueberries have higher anthocyanin contents than other Manitoba berries. *J. Agric. Food Chem.* **2007**, *55*, 10832−10838.
(13) Garcia-Alonso, M.; Minihane, A. M.; Rimbach, G.; Rivas-Gonzalo, J. C.; de Pascual-Teresa, S. Red wine anthocyanins are rapidly absorbed in humans and affect monocyte chemoattractant protein 1 levels and antioxidant capacity of plasma. *J. Nutr. Biochem.* **2009**, *20*, 521−529.
(14) Choi, E. H.; Chang, H. J.; Cho, J. Y.; Chun, H. S. Cytoprotective effect of anthocyanins against doxorubicin-induced toxicity in H9c2 cardiomyocytes in relation to their antioxidant activities. *Food Chem. Toxicol.* **2007**, *45*, 1873−1881.
(15) Noda, Y.; Kneyuki, T.; Igarashi, K.; Mori, A.; Packer, L. Antioxidant activity of nasunin, an anthocyanin in eggplant peels. *Toxicology* **2000**, *148*, 119−123.
(16) Ren, S.; Shatadal, S.; Shen, G. X. Protein kinase C-β mediates lipoprotein-induced generation of PAI-1 from vascular endothelial cells. *Am. J. Physiol.: Endocrinol. Metab.* **2000**, *278*, E656−E662.
(17) Sangle, G. V.; Chowdhury, S. K.; Xie, X.; Stelmack, G. L.; Halayko, A. J.; Shen, G. X. Impairment of mitochondrial respiratory chain activity in aortic endothelial cells induced by glycated low-density lipoprotein. *Free Radical Biol. Med.* **2010**, *48*, 781−790.
(18) Hou, D.; Tong, X. H.; Terahara, N.; Luo, D.; Fujiim, M. Delphinidin 3-sambubioside, a hibiscus anthocyanin, induces apoptosis in human leukemia cells through reactive oxygen species-mediated mitochondrial pathway. *Arch. Biochem. Biophys.* **2005**, *440*, 101−109.
(19) Gilleron, M.; Marechal, X.; Montaigne, D.; Franczak, J.; Neviere, R.; Lancel, S. NADPH oxidase participates to doxorubicin-induced cardiac myocyte apoptosis. *Biochem. Biophys. Res. Commun.* **2009**, *388*, 727−731.
(20) Zhao, R.; Moghadasian, M. H.; Shen, G. X. Involvement of NADPH oxidase in up-regulation of plasminogen activator inhibitor-1 and heat shock factor-1 in mouse embryo fibroblasts induced by oxidized LDL and in apolipoprotein E-deficient mice. *Free Radical Res.* **2011**, *45*, 1013−1023.
(21) Birch-Machin, M. A.; Briggs, H. L.; Saborido, A. A.; Bindoff, L. A.; Turnbull, D. M. An evaluation of the measurement of the activities of complexes I−IV in the respiratory chain of human skeletal muscle mitochondria. *Biochem. Med. Metab. Biol.* **1994**, *51*, 35−42.
(22) Chowdhury, S. K.; Drahota, Z.; Floryk, D.; Calda, P.; Houstek, J. Activities of mitochondrial oxidative phosphorylation enzymes in cultured amniocytes. *Clin. Chim. Acta* **2000**, *298*, 157−173.
(23) Rustin, P.; Chretien, D.; Bourgeron, T.; Gérard, B.; Rötig, A.; Saudubray, J. M.; Munnich, A. Biochemical and molecular investigations in respiratory chain deficiencies. *Clin. Chim. Acta* **1994**, *228*, 35−51.
(24) Vondra, K.; Rath, R.; Bass, A.; Slabochová, Z.; Teisinger, J.; Vitek, V. Enzyme activities in quadriceps femoris muscle of obese diabetic male patients. *Diabetologia* **1977**, *13*, 527−529.
(25) Kirkland, R. A.; Franklin, J. L. Evidence for redox regulation of cytochrome *c* release during programmed neuronal death: antioxidant effects of protein synthesis and casepase inhibition. *J. Neurosci.* **2001**, *21*, 1949−1963.
(26) Srere, P. A. Citrate synthase. *Methods Enzymol.* **1969**, *13*, 3−26.
(27) Youdim, K. A.; McDonald, J.; Kalt, W.; Joseph, J. A. Potential role of dietary flavonoids in reducing microvascular endothelium vulnerability to oxidative and inflammatory insults. *J. Nutr. Biochem.* **2002**, *13*, 282−288.
(28) Miranda-Rottmann, S.; Aspillaga, A. A.; Pérez, D. D.; Vasquez, L.; Martinez, A. L.; Leighton, F. Juice and phenolic fractions of the berry *Aristotelia chilensis* inhibit LDL oxidation in vitro and protect human endothelial cells against oxidative stress. *J. Agric. Food Chem.* **2002**, *50*, 7542−7547.
(29) Liu, L. K.; Lee, H. J.; Shih, Y. W.; Chyau, C. C.; Wang, C. J. Mulberry anthocyanin extracts inhibit LDL oxidation and macrophage-derived foam cell formation induced by oxidative LDL. *J. Food Sci.* **2008**, *73*, H113−121.
(30) Bagchi, D.; Sen, C. K.; Ray, S. D.; Das, D. K.; Bagchi, M.; Preuss, H. G.; Vinson, J. A. Molecular mechanisms of cardioprotection by a novel grape seed proanthocyanidin extract. *Mutat. Res.* **2003**, *523−524*, 87−97.
(31) Ren, S.; Shen, G. X. Impact of antioxidants and HDL on glycated LDL-induced generation of fibrinolytic regulators from vascular endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **2000**, *20*, 1688−1693.
(32) Bonawitz, N. D.; Clayton, D. A.; Shadel, G. S. Initiation and beyond: multiple functions of the human mitochondrial transcription machinery. *Mol. Cell* **2006**, *24*, 813−825.
(33) Graham, K. A.; Kulawiec, M.; Owens, K. M.; Li., X.; Desouki, M. M.; Chandra, D.; Singh, K. K. NADPH oxidase 4 is an oncoprotein localized to mitochondria. *Cancer Biol. Ther.* **2010**, *10*, 223−231.
(34) Yun, J. M.; Afaq, F.; Khan, N.; Mukhtar, H. Delphinidin, an anthocyanidin in pigmented fruits and vegetables, induces apoptosis and cell cycle arrest in human colon cancer HCT116 cells. *Mol. Carcinog.* **2009**, *48*, 260−270.
(35) Hanif, S.; Shamim, U.; Ullah, M. F.; Azmi, A. S.; Bhat, S. H.; Hadi, S. M. The anthocyanidin delphinidin mobilizes endogenous copper ions from human lymphocytes leading to oxidative degradation of cellular DNA. *Toxicology* **2008**, *249*, 19−25.
(36) Fritz, J.; Roth, M.; Holbach, P.; Esselen, M.; Marko, D. Impact of delphinidin on the maintenance of DNA integrity in human colon carcinoma cells. *J. Agric. Food Chem.* **2008**, *56*, 8891−8896.
(37) Sanson, M.; Augé, N.; Vindis, C.; Muller, C.; Bando, Y.; Thiers, J. C.; Marachet, M. A.; Zarkovic, K.; Sawa, Y.; Salvayre, R.; Nègre-Salvayre, A. Oxidized low-density lipoproteins trigger endoplasmic reticulum stress in vascular cells: prevention by oxygen-regulated protein 150 expression. *Circ. Res.* **2009**, *104*, 328−336.
(38) Timmins, J. M.; Ozcan, L.; Seimon, T. A.; Li, G.; Malagelada, C.; Backs, J.; Backs, T.; Bassel-Duby, R.; Olson, E. N.; Anderson, M. E.; Tabas, I. Calcium/calmodulin-dependent protein kinase II links ER stress with Fas and mitochondrial apoptosis pathways. *J. Clin. Invest.* **2009**, *119*, 2925−2941.