Endonuclease G mediates endothelial cell death induced by carbamylated LDL

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End-stage kidney disease (ESKD) is a terminal stage of chronic kidney disease (CKD), which is associated with high incidence of cardiovascular disease (CVD). The CVD frequently results from endothelial injury caused by carbamylated LDL (cLDL), the product of LDL modification by urea-derived cyanate. Our previous data suggested that cLDL induces mitogen-activated protein kinase (MAPK)-dependent mitotic DNA fragmentation and cell death. However, the mechanism of this pathway is unknown. The current study demonstrated that cLDL-induced endothelial mitotic cell death is independent of caspase-3. Expression of endonuclease G (EndoG), the nuclease implicated in caspase-independent DNA fragmentation, was significantly increased in response to cLDL exposure to the cells. Inhibition of EndoG by RNAi protected cLDL-induced DNA fragmentation, while overexpression of EndoG induced more DNA fragmentation in endothelial cells. Ex vivo experiments with primary endothelial cells isolated from wild-type (WT) and EndoG knockout (KO) mice demonstrated that EndoG KO cells are partially protected against cLDL toxicity compared to WT cells. To determine cLDL toxicity in vivo, we administered cLDL or native LDL (nLDL) intravenously to the WT and EndoG KO mice and then measured floating endothelial cells in blood using flow cytometry. The results showed an increased number of floating endothelial cells after cLDL vs nLDL injection in WT mice but not in EndoG KO mice. Finally, inhibitors of MEK-ERK1/2 and JNK-c-jun pathways decreased cLDL-induced EndoG overexpression and DNA fragmentation. In summary, our data suggest that cLDL-induced endothelial toxicity is caspase-independent and results from EndoG-dependent DNA fragmentation.
INTRODUCTION

Cardiovascular disease (CVD) is the major complication in patients with end-stage kidney disease (ESKD) (15, 40). The risk of CVD among patients with ESKD is about 10-30 times of that among the general population, and CVD remains the major cause of mortality among ESKD patients (1, 18, 38). Endothelial injury plays a crucial role in the development of CVD (22, 37). Recent studies identified carbamylated low-density lipoprotein (cLDL) as an important factor that is detected in healthy individuals and patients with chronic kidney disease (CKD), causes endothelial injury in vitro, and leads to atherosclerosis in vivo (3, 5, 10, 19, 23, 36). cLDL is produced by chemical modification of low-density lipoprotein (LDL) by cyanate derived from urea or thiocyanate (11, 28). Because of chronic elevation of urea, CKD patients have more protein carbamylation (28) and increased plasma cLDL (5). cLDL uses several scavenger receptors on endothelial cells and causes induction of adhesion molecules and monocyte adhesion, suggesting its importance for atherosclerosis (4, 6, 8, 36). cLDL was also shown to induce endothelial cell proliferation followed by cell death that occurred predominantly in mitotic cells (2). Prevention of endothelial proliferation by DNA polymerase or mitogen-activated protein kinases (MAPK) inhibitors protected cells from cLDL-induced cell death. The cLDL-induced cell death was associated with DNA fragmentation measured by using the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL). The nuclease that produced cLDL-induced DNA fragmentation and death of endothelial cells is unknown; however, one potential candidate is endonuclease G (EndoG). It was previously described as a nuclear DNA-coded mitochondrial manganese-dependent endonuclease that has a unique site-selectivity, initially attacking poly(dG).poly(dC) sequences in double-stranded DNA (39). EndoG is known of being released from mitochondria during apoptosis and cleaving DNA without sequence specificity at higher degrees of DNA degradation (30). It can be localized in nuclei during apoptotic DNA fragmentation confirmed by the TUNEL assay (29). EndoG is an important participant of the caspase-independent mitotic cell death (27, 34), which was detected in several models of endothelial injury (32, 42).

Therefore, in this study, we hypothesized that EndoG is the mediator of cLDL-induced mitotic death of endothelial cells.

MATERIALS AND METHODS
**LDLs**

Human native LDL (nLDL) was purchased from Intracel (Frederick, MD) and all other chemicals were purchased from Sigma (St. Louis, MO), unless stated otherwise. Carbamylated LDL was prepared by chemical modification, as previously described (2). Carbamylation of LDL was verified by the colorimetric method using diacetyl monoxime (41). Electrophoretic mobility of nLDL and cLDL was determined in 0.5% agarose gel and 0.2% bovine serum albumin (w/v), as described by Noble (35). All LDLs were diluted with phosphate-buffered saline (PBS) containing 200 µM EDTA, kept at 4°C away from light and used within 3 weeks after preparation.

**Cell cultures**

Human coronary artery endothelial cells (HCAECs) were purchased from Lonza (Walkersville, MD) and propagated in EGM-2 medium (Lonza) for up to three passages. Unless stated otherwise, HCAECs were treated with 200 µg/ml cLDL for 24 hours, and control HCAECs were treated with either equal amount/volume of nLDL or vehicle (PBS, 200 µM EDTA), as previously described (36). The vehicle did not show any cellular toxicity and did not induce any DNA fragmentation. For all experiments, HCAECs were plated 4-6×10³ cells/cm².

Cell death was tested by LDH release assay, for which cells were plated in 96-well plates and treated with LDLs for 24 hours. The experiments were performed using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI), as described previously (2).

For experiments with MAPK inhibitors, two hours prior to the LDL exposure, HCAECs were pretreated with U0126 (MEK inhibitor) or SP600125 (JNK inhibitor) at final concentrations of 10 µM and 1 µM, respectively, as elaborated before (2).

Primary mouse aortic endothelial cells were isolated, as previously reported (31). Briefly, mice were exsanguinated and perfused through the heart with ice-cold Hank’s buffered saline solution (HBSS). The thoracic aorta was isolated from the body, separated from heart, and incubated with 0.1% collagenase IV in MCDB-131 complete medium from Vec Technologies (Rensselaer, NY) for 3 min. Then, the aorta was segmented and immobilized in 34-mm Petri dishes coated with 0.2% gelatin. Endothelial cells were allowed to proliferate for 72 to 96 hours in complete MCDB-131, after which aortic segments were removed and endothelial cells were isolated by fluorescein-
activated cell sorting after staining with Dil-Ac-LDL (Biomedical Technologies, Stoughton, MA) (31).

**Animal experiments**

All experiments with animals were approved by the Animal Care and Use Committee of the Central Arkansas Veterans Healthcare System. EndoG knockout (KO) mice (C57BL6/J background) were obtained from Lieber and collaborators (24). Wild-type (WT) and EndoG KO mice were subjected to a single intravenous administration of cLDL or nLDL (40 mg/kg). Control mice were injected with the equal volume of vehicle (200 μM EDTA in saline). Twenty-four hours later, mice were euthanized and exsanguinated. From each animal, 1 ml of whole blood was collected in 1-ml LH Lithium heparin mini collect vials from Greiner Bio-One (Monroe, NC) and used for flow cytometry.

**Cytochemistry and image analyses**

TUNEL assay, coupled with immunocytochemical double staining of LDL-treated cells with anti-BrdU (Oncogene, Cambridge, MA) and anti-EndoG antibody (Millipore, Billerica, MA), was performed, as previously described (7). HCAECs were plated in 8-well slide chambers and treated with 200 μg/ml LDL, as described above. BrdU label (Oncogene) was added to the medium 4 hours after the start of cLDL treatment. In 24 hours, the cells were washed with ice-cold PBS, fixed with 4% formaldehyde (pH 7.0), and probed overnight with the mouse anti-BrdU and rabbit anti-EndoG at 1:100 and 1:300 dilution, respectively. The primary antibodies were detected with 1:400 diluted anti-mouse IgG-AlexaFluor 594 and anti-rabbit IgG-AlexaFluor 647 conjugates (Invitrogen, Carlsbad, CA), respectively. Subsequently, DNA fragmentation was measured with TUNEL assay using an *in situ* cell death detection kit (Roche). Cells were then washed, counterstained with 4’,6-diamidino-2-phenylindol (DAPI), mounted under cover slips with Prolong® Antifade kit (Invitrogen), and acquired using the Olympus IX-81 inverted microscope (Olympus America, Center Valley, PA) equipped with Hamamatsu ORCA-ER monochrome camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan). Image analysis was performed using the SlideBook 4.2 software (SciTech Pty Ltd., Australia). Quantification was done as described before (44).
siRNA and EndoG-CFP overexpression

EndoG silencing by siRNA was performed, as described earlier (9). HCAECs were grown in 96-well plates to 70-80% confluence and transfected with designed siRNA duplexes (sense 5’-AUGCCUGGAACACCUGGAdTdT-3’ and antisense 3’-UCCAGGUUGUCCAGGCAUdTdT-5’) or Control Non-Targeting siRNA#1 from Dharmaco (Lafayette, CO) in mixtures containing 50 nM siRNA and TransIT-TKO transfection reagent (Mirus, Houston, TX) in serum-free medium for 48 hours. Cells were then washed and exposed with 200 µg/ml cLDL for an additional 24 hours. Control cells were treated with nLDL or vehicle (PBS, 200 µM EDTA). After the treatment, cell death was assessed using the LDH release assay.

For EndoG expression experiments, an EndoG-CFP vector was prepared. Human mature EndoG (amino acids 45-294) coding cDNA was cloned in mammalian expression vector pECFP.N1 (BD Biosciences Clontech, Franklin Lakes, NJ) with restriction sites Bgl II and Age I. The primers used for the construction were 5’-TCAGATCTCGAGATGGCCGATCTTCCCGC and 5’-GGTGCGGCCGTCGTTGCTGCCAGCAGTG. Seventy percent confluent HCAECs in 8-well chamber slides were transfected with the EndoG-CFP using Lipofectamin LTX Plus (Invitrogen) for 16 hours. Control cells were treated with the vector that expresses CFP only. Next, cells were exposed with either cLDL or nLDL (200 µg/ml) for 24 hours, then fixed in 4% formaldehyde and used for TUNEL staining, cytochemistry, and microscopy.

Real-time reverse transcriptase PCR

Total RNA was isolated using the RNeasy Mini kit from Qiagen (Valencia, CA). The reverse transcription reaction was performed using 0.5 µg total RNA, Oligo d(T)16 and the GeneAmp Gold RNA PCR core kit (Applied Biosystems, Carlsbad, CA). The real time PCR was performed as elaborated before (43). Briefly, cDNA samples were diluted for real-time 1:5 and 1:200 for EndoG and 18s, respectively. The reaction mix was prepared using Platinum SYBR Green qPCR Supermix-UDG (Invitrogen) according to the manufacturer recommendations. Primers were: 5’-CTACCTGAGCAACGTCG-3’ and 5’-TCCAGGTTGTTCCAGGCA-3’. 18s ribosomal subunit RNA was amplified in
parallel reaction using primers 5'-TTGAAACGTCTGCCTATCAA-3' and 5'-
ATGGTAGGCACGGCGACTA-3'. Two-temperature cycles with annealing/extension
temperature at 62°C for EndoG and 64°C for 18s were applied to samples using
SmartCycler (Cepheid, Sunnyvale, CA). The melting curve analysis was performed
between 60°C and 95°C to assess the quality of final PCR products. Calculation of the
relative RNA concentration was performed using Cepheid SmartCycle software (Version
2.0d). Data are presented as a ratio of EndoG/18s mRNA.

**Western blotting**

Western blotting was performed as described before (44). Briefly, proteins were
separated in 12% gel, transferred onto the nitrocellulose membrane in Novex
transferring buffer (Invitrogen) at 40V for 3 hours. Blocked membrane was incubated
with polyclonal anti-EndoG (Millipore) diluted 1:800 and washed in Tris-buffered saline
(TBS), and primary antibodies were detected with anti-rabbit IgG-horseradish peroxidase
(HRP) using a SuperSignal chemiluminescent kit (Thermo Fisher Scientific, Rockford,
IL). The load control was performed by stripping the membranes using Restore Plus
Western Blot Stripping Buffer (Thermo Fisher Scientific, Rockford, IL) and probing with
anti-β-actin (1:1000) antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

**Cell ELISA**

Cell ELISA was performed as described before (6). Briefly, HCAECs were
seeded in a 96-well plate, treated with MAPK inhibitors and cLDL/nLDL, washed, and
fixed (4% w/v formaldehyde, 0.012% saponin, PBS) for 10 minutes at room temperature.
After blocking, cells were probed with anti-EndoG antibody (1:800) (Millipore). The
primary antibodies were detected with anti-rabbit antibody (1:1000) conjugated with
horseradish peroxidase (HRP) (Invitrogen). The HRP activity was measured using
3,3',5,5'-tetramethylbenzidine substrate solution (Sigma) at delta 450-540 nm. Total
protein was normalized by β-actin expression measured with the rabbit polyclonal anti-β-
actin antibody conjugated with FITC (Santa Cruz Biotechnology). All measurements
were done in quadruplicates per one marker per one time/concentration point and were
repeated at least three times in different plates. For negative control, the primary
antibody was substituted with blocking buffer.
Whole blood fluorescein-activated cell sorting (FACS) analysis

Freshly collected blood was treated with red blood cell lysis buffer (0.83% NH₄Cl, 0.1% KHCO₃, 0.01% EDTA) for 15 min at room temperature, and cells were precipitated at 220×g for 5 min. After two subsequent washings with PBS, blood cells were resuspended in an original volume of 2% BSA in PBS containing 1:500-diluted anti-CD31-FITC (Millipore) for 1 hour. To control cell labeling, a separate batch of blood cells from WT mice was treated with the same solution without antibody. After incubation, cells were precipitated at 220×g for 5 min, and the hybridization solution was removed. After two washings with PBS, cells were fixed with 4% formaldehyde and analyzed using the Becton Dickinson BD FACS Calibur flow cytometer (San Jose, CA). The cutoff limit for non-nuclear cells and non-specific autofluorescence (based on the negative control) was applied to cell sorting setting, which resulted to exclusion of more than 99.9% of cells. The percentage of CD-31-positive cells was calculated using the Becton Dickinson CELLQUEST software package.

Statistics

Statistical analysis was performed using ANOVA and Student's *t*-test. Results were expressed as mean ± SEM. P<0.05 was considered significant.

RESULTS

cLDL induces EndoG overexpression and mitotic death in endothelial cells.

Our previous reports suggested that cLDL induces endothelial injury that preferentially occurs in the proliferating cells (2, 43). In our first experimental setting, we tested whether the cLDL-induced DNA fragmentation (detectable by TUNEL) occurred in proliferating cells (detectable by BrdU incorporation). Our secondary goal was to determine if the cLDL-induced DNA fragmentation was dependent on EndoG or caspase-3. Our results showed that vast majority of the TUNEL-positive cells had BrdU label, suggesting that mitotic cell death occurred in proliferating cells in response to cLDL treatment (Figs. 1A and 1B). In the most of the cases, the TUNEL-positive/BrdU-positive cells had a strong association with EndoG overexpression rather than with cleaved caspase-3. Overall, EndoG expression was significantly higher in all cLDL-treated endothelial cells compared to nLDL- or vehicle-treated cells. To rule out the
possible problem with cleaved caspase-3 immunostaining, positive control (HCAECs treated with 200 µM H$_2$O$_2$) was successfully used during the immunocytochemical staining (Fig. 1C). cLDL-induced elevation of EndoG mRNA and protein expression were confirmed by real-time RT-PCR and Western blotting, respectively (Figs. 1D and 1E). Therefore, our data suggest that EndoG and caspase-independent pathway of cell death are likely the mechanisms of cLDL-induced endothelial injury.

**EndoG mediates cLDL-induced endothelial cell death in vitro.** To study whether observed EndoG induction plays a causative role in cLDL-induced DNA fragmentation and endothelial toxicity, we inhibited EndoG expression using specific siRNA. Efficiency of anti-EndoG siRNA was initially tested in HCAECs, and the results suggested that EndoG is silenced by ~50% (Fig 2A). In subsequent experiments, EndoG silencing was shown to be significantly protective against cLDL-induced cell death (Fig 2B). We then tested whether EndoG overexpression would make HCAECs more susceptible to cLDL toxicity. HCAECs were transfected with pECFP-N1 that expresses EndoG-CFP fused protein. As determined by TUNEL assay, endothelial cells that overexpressed EndoG were more sensitive to cLDL (Fig. 3A). Quantification of the TUNEL data suggested that EndoG overexpression significantly exacerbated the cLDL-induced DNA fragmentation and cell death (Fig. 3B). Hence, both in vitro models suggested a strong positive link between EndoG expression and cLDL toxicity toward the endothelial cells.

**EndoG knockout protects endothelial cells from cLDL-induced cell death ex vivo and in vivo.** We next examined whether or not primary EndoG KO endothelial cells are protected against cLDL toxicity. For this, primary mouse aortic endothelial cells were prepared from WT and EndoG KO mice, and their injury after cLDL treatment ex vivo was assessed by LDH release. Our data suggested that there was significant induction of endothelial cell death after cLDL exposure compared to nLDL in both WT and EndoG KO cells (Fig. 4). However, compared to WT cells, EndoG KO cells were partially protected from the injury induced by cLDL. Afterward, our experiments were extended to live animals. For this, WT and EndoG KO mice were subjected to single tail vein administration of cLDL or nLDL. Twenty-four hours later, floating CD31+ (endothelial) nuclear cells were assessed by FACS analysis of the whole blood. Our data suggested a significant increase of floating endothelial cells in circulation of WT mice, while EndoG KO mice were insensitive to cLDL (Fig. 5). Thus, our in vivo results
confirmed in vitro and ex vivo data, suggesting that EndoG plays a critical role in mediating the cLDL-induced endothelial cell death.

Inhibition of ERK1/2 or JNK protects endothelial cells from EndoG overexpression and death. Our previous report showed that MEK–ERK1/2 and JNK–c-jun pathways are involved in the cLDL-induced death of proliferating endothelial cells (2). Because both MAPK and EndoG seem to be responsible for cLDL cytotoxicity, in the current study, their relation was assessed in cLDL-treated HCAECs. Our data showed that cLDL-induced EndoG overexpression was either partially or completely prevented by U0126 and SP600125, the inhibitors of MEK and JNK, respectively (Fig. 6A). However, cLDL-induced DNA fragmentation in endothelial cells was more efficiently decreased by MEK inhibitor compared to JNK inhibitor (Fig. 6B). Taken together with our previous publication, these results suggest that in response to cLDL impact to endothelial cells, EndoG overexpression is primarily dependent on JNK–c-jun mechanism, however DNA fragmentation and endothelial cell death relies on both MEK–ERK1/2 and JNK–c-jun pathways, which may be tentatively explained by other mechanisms that regulate for example nuclear translocation of EndoG or directly cause DNA damage.

DISCUSSION

Endothelial injury plays a critical role in the disturbance of vascular homeostasis and significantly contributes to the development of CVD (22, 37). ESKD patients are known to be prone for the endothelial dysfunction and injury, which may be a key process in their greater predisposition to cardiovascular complications in comparison to the general population (1, 18, 38). Despite the fact that cLDL is elevated in the plasma of CKD patients and experimental uremia-induced atherosclerosis is associated with cLDL, the mechanisms of cLDL-induced endothelial injury and atherosclerosis are not well understood. In the physiological concentrations, which were registered in ESKD patients (5), cLDL induces both proliferation and injury of endothelial cells (2, 21). The coincidence of the two seemingly controversial processes suggests that there may be a link between them. Indeed, our first study in this direction suggested this (2), and the current study confirmed that cLDL-induced proliferation and cell death are related. Prevention of the cell cycle drift to S-G2 or mitosis significantly reduced the cell death caused by cLDL. Similar events were also observed in oxLDL-treated endothelial cells
implicating that this is a potentially universal phenomenon in the pathogenesis of endothelial injury. Cell death associated with proliferation is called “mitotic cell death” (AKA mitotic catastrophe or reproductive or proliferating cell death). The term describes cell death that occurs in the cell cycle stages that follow the one during which the cell is impacted with an injuring agent (16). Mitotic cell death usually has the morphological features of apoptosis and necrosis and results from DNA damage in vital genes or shows contradictory biological stimuli and inability of the cell to pass a certain checkpoint (16). Mitotic cell death is a common cellular phenomenon that occurs in endothelial cells in response to the number of impacts or treatments (20, 25, 26).

Several previous reports suggested that mitotic cell death may employ caspase-independent mechanisms (27, 33, 34). The current study revealed that cLDL-induced endothelial mitotic cell death is rather caspase-independent and associated with induction of EndoG, one of the major effectors of caspase-independent cell death (33, 45). Both in vitro and ex vivo experiments with EndoG modulation proved that EndoG is causatively involved in the cLDL-induced endothelial cell injury. Furthermore, our in vivo experiments performed in transgenic mice strongly supported the hypothesis that EndoG mediates the cLDL-induced endothelial cell injury. To the best of our knowledge, this is the first report that suggests the causative role of EndoG in mitotic endothelial cell death induced by cLDL or any other modified LDL. Interestingly, Diener and coauthors (17) recently suggested that under normal physiological conditions, EndoG protects endothelial cells from apoptosis and, at the same time, potentiates necrosis. This contradiction with our results perhaps can be explained by the difference in cell death mechanisms between normal conditions and conditions resulting from cLDL toxicity. Similar contradictory results on the effect of EndoG withdrawal were previously shown in other models (7, 24, 46).

MAPKs seems to be largely responsible for a great number of endothelial cell functions, including cell proliferation, growth arrest, differentiation, and cell death (42). Our previous report suggested that both MEK-ERK1/2 and JNK-c-jun pathways, but not the MAPK p38 pathway, are responsible for mediating mitotic cell death induced by cLDL (2). In the current study, MEK and JNK inhibitors significantly reduced EndoG expression and DNA fragmentation in endothelial cells during cLDL-induced injury. Interestingly, while inhibition of JNK caused stronger downregulation of EndoG than inhibition of MEK, the MEK inhibitor was more efficient in protection against cLDL-
induced DNA fragmentation as compared to JNK inhibitor. Detected link between JNK and EndoG is in agreement with studies, which showed similar link between EndoG and JNK, but not with ERK1/2 activation in apoptosis (12-14). Higher efficiency of MEK inhibitor in prevention of cLDL-induced DNA fragmentation compared to JNK inhibitor is also in agreement with our previous report that demonstrated higher efficiency of MEK inhibition above JNK inhibitor in endothelial cells’ rescue from cLDL toxicity (2). The observed cell protection by MEK-ERK1/2 pathway inhibition is likely resulted from other than EndoG mechanism(s). It is possible that cLDL-induced ERK1/2-mediated TUNEL and cytotoxicity use some known or unknown endonuclease that causes DNA fragmentation. Because ERK1/2 (unlike JNK) participates in scheduled DNA synthesis and cell proliferation (2), it makes DNA even more susceptible for degradation and cells more prone to cell death. Therefore, we may speculate that cLDL-induced DNA fragmentation and endothelial cell toxicity use at least two potential pathways: 1) JNK-c-jun-dependent pathway with EndoG as a major executive endonuclease, and 2) MEK-ERK1/2-dependent pathway with one or more apoptotic endonucleases that cause DNA degradation without great involvement of EndoG (Figure 7).

It is important to acknowledge that all described in vitro and in vivo phenomena were observed with the chemically modified cLDL at concentration of 200 µg/ml used in several studies (2, 4, 6). Although in vivo produced cLDL may differ from artificially modified one in degree of carbamylation, our previous report suggested that uremic patients have serum cLDL that is equal to the average concentration of 281.5±46.9 µg/ml of chemically modified cLDL (5). Therefore the used concentration of cLDL is close to the physiologically relevant level observed in chronic uremia. It may be speculated that with different degree of carbamylation and longer duration of treatment, cLDL may have alternative pathways of vascular injury.

In conclusion, this study revealed the mechanistic link between MAPK and EndoG in caspase-independent mitotic endothelial cell death induced by cLDL. In the future, this mechanism may be used as a new therapeutic target for the prevention and treatment of CVD associated with chronic uremia.

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Caspase-independent death of Leber's hereditary optic neuropathy cybrids is driven by

FIGURE LEGENDS

**Figure 1.** EndoG is upregulated in the cLDL-induced mitotic death of endothelial cells. Co-localization of BrdU (red color), TUNEL (green color), and EndoG (**A**) or cleaved caspase-3 (**B**) (purple color) in HCAECs treated for 24-hours with vehicle, nLDL, or cLDL (200 µg/ml each) in serum-free medium. Counterstaining with DAPI (blue color). Scales – 20 µm. Immunocytochemical reaction with cleaved caspase-3 was positively controlled using the same endothelial cells treated with 200 µM H₂O₂ (**C**). EndoG mRNA expression (**D**) and protein expression (**E**) in HCAECs treated with nLDL or cLDL (200 µg/ml each) by real-time RT-PCR and Western blotting, respectively. n = 4 per point. *P<0.05 compared with nLDL.

**Figure 2.** EndoG inactivation protects endothelial cells from the cell death induced by cLDL. Effect of EndoG silencing by anti-EndoG siRNA (**A**) and endothelial protection from cLDL toxicity as measured by LDH release assay (**B**). n = 3 per point. *P<0.05 compared with nLDL; #P<0.05 compared with control siRNA- and vehicle-treated cells.

**Figure 3.** EndoG overexpression (cyan color) induces DNA fragmentation assessed by TUNEL (red color) in the cLDL- or nLDL-treated (200 µg/ml, 24 hours) HCAECs. Representative images (**A**) and quantification (**B**). Scales – 20 µm. n = 4 per point. *P<0.05 compared with nLDL.

**Figure 4.** Endothelial cells with genetic lack of EndoG are less sensitive to cLDL toxicity. Primary mouse aortic endothelial cells isolated from WT and EndoG KO mice were treated with cLDL (200 µg/ml, 24 hours) and cell death was measured by LDH release assay. n = 6 per point. *P<0.05 compared with nLDL; #P<0.05 compared with cLDL-treated WT cells.

**Figure 5.** Detection of floating CD31-positive endothelial cells by flow cytometry in whole blood from WT and EndoG KO mice after a single intravenous injection of cLDL. Representative images (**A**) and quantification (**B**). n = 8 per point. *P<0.05 compared with nLDL; #P<0.05 compared with cLDL-treated WT animals.

**Figure 6.** Inhibition of MAPK pathway prevents EndoG upregulation and cLDL-induced DNA fragmentation. EndoG protein expression measured by direct cell ELISA (**A**) and DNA fragmentation measured by quantitative TUNEL assay (**B**) in HCAECs treated with cLDL after inhibition of MEK and JNK kinases. n = 3-4 per point. *P<0.05 compared with nLDL; #P<0.05 compared with DMSO and cLDL-treated cells.
Figure 7. Schematic of the proposed pathway of cLDL-induced endothelial cell death.
Figure 1. Apostolov et al. “Endonuclease G mediates endothelial cell death induced by carbamylated LDL”
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Figure 6. Apostolov et al. “Endonuclease G mediates endothelial cell death induced by carbamylated LDL”
Figure 7. Apostolov et al. “Endonuclease G mediates endothelial cell death induced by carbamylated LDL”

Carbamylated LDL

MEK → pERK1/2

JNK → p-c-Jun

DNA synthesis

another DNA damage mechanism

EndoG

DNA fragmentation

Cell death