

Original Research Paper

Progressive LDL Reduction to Very Low Levels Improves Dimeric Nitric Oxide Synthase, Nitric Oxide Bioavailability and Reduces Peroxynitrite in Endothelial Cells during Hyperglycemia

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Abstract: Circulating levels of glucose and LDL influence Endothelial Cell (EC) function in a highly interactive manner as evidenced by nitric oxide production and coupling of endothelial Nitric Oxide Synthase (eNOS) dimer. Here, we report on the status of eNOS function in Human Umbilical Vein Endothelial Cells (HUVECs) cultured under normo- and hyperglycemic (250 mg/dL) condition followed by exposure to increasing LDL concentrations (10-150 mg/dL). Production of bioavailable, cytoprotective NO and cytotoxic peroxynitrite (ONOO⁻) were measured in endothelium with nanosensors. Coupling efficiency of dimeric eNOS was measured using immunochemistry. The ratio of cytoprotective NO and cytotoxic ONOO⁻ concentrations ([NO]/[ONOO⁻]) was used as a marker for eNOS and endothelial function. The normal ratio for [NO]/[ONOO⁻] is 2.5-5.0. A ratio of 1.0 or below is an indicator of modest to severe endothelial dysfunction. Under normoglycemic conditions (up to 100 mg/dL glucose) [NO]/[ONOO⁻] can reach the 0.50 value with the LDL concentration of about 110 mg/dL, while in hyperglycemia (250 mg/dL glucose), the 0.50 value was reached at about 50 mg/dL. Reduction of LDL down to 50 mg/dL, in hyperglycemia, helps prevent severe dysfunction in endothelium by enhancing eNOS dimerization, increasing NO production and decreasing the concentration of cytotoxic ONOO⁻, which may significantly reduce CV risk factors.

Keywords: Endothelium, LDL, Nitric Oxide Synthase, Hyperglycemia

Introduction

As a Cardiovascular (CV) risk factor, Diabetes Mellitus (DM) confers more than a two-fold increase in absolute adjusted risk of CV death. There is also a log linear relationship between cholesterol levels and CV death for subjects with DM that is independent of baseline LDL levels (Stamler *et al.*, 1993). The Collaborative Atorvastatin Diabetes Study (CARDS) demonstrated that patients with type 2 diabetes and other risk factors for CVD benefited from statin treatment regardless of their baseline LDL level (Colhoun *et al.*, 2004). The Cholesterol Treatment Trialists (CTT) Collaborators showed a significant reduction in CV events with statin therapy in individuals with DM even without other risk

factors (Cholesterol Treatment Trialists *et al.*, 2008). Beyond LDL reductions, certain benefits of statins may be attributed to pleiotropic mechanisms (Bonetti *et al.*, 2003; Consentino *et al.*, 2002; Mason *et al.*, 2005a). Of particular interest, statins increase the levels of eNOS and NO production, a regulator of thrombus development and endothelial function (Laufs *et al.*, 1998; Mason *et al.*, 2008; Treasure *et al.*, 1995). As a result of the clinical evidence, ADA guidelines indicate that individuals with DM should be treated with a statin for primary and secondary prevention of cardiovascular events.

While statins have had a major impact on reducing the burden of atherosclerotic disease, there is still substantial CVD risk, especially in higher risk subjects, such as those with DM (Fruchart *et al.*, 2014). For this

reason, there is now emerging clinical evidence that further reductions in LDL, below optimal levels (considered to be 70 mg/dL), may provide additional reductions in CVD risk. With the recent availability of PCSK-9 inhibitors like evolocumab for intensive LDL reduction beyond statins, this hypothesis was recently addressed in the FOURIER trial (Sabatine *et al.*, 2017). With over 27,000 subjects, it found that reducing LDL levels to an average of 30 mg/dL using evolocumab was associated with an overall 15-20% CVD risk reduction in an incremental manner.

Individuals with DM are at higher risk for atherosclerosis, a disease process characterized by inflammation, oxidative stress and EC dysfunction (Cavender *et al.*, 2015; Harrison *et al.*, 1987; Liao, 1998; Oemar *et al.*, 1998; Williams *et al.*, 1996). EC dysfunction is observed in patients and animal models of diabetes and indicated by a loss of NO release (Kurioka *et al.*, 2000; Mason *et al.*, 2009; Node *et al.*, 1997). The net concentration of NO in the circulation is dependent on the balance between the enzymatic production of NO through the activity of eNOS and the production of superoxide (O_2^-) (Harrison, 1997; Dawoud and Malinski, 2018). Attenuated NO release contributes to platelet aggregation, leukocyte adhesion and loss of normal vasodilation (Paniagua *et al.*, 2001; Panza *et al.*, 1990). When the levels of co-factors are insufficient, the dimeric form of eNOS is uncoupled and there is a reduction of molecular oxygen, followed by the oxidation of L-arginine. This results in the concomitant generation of O_2^- and NO, which can react rapidly to produce cytotoxic ONOO⁻ (Harrison, 1997; Mason *et al.*, 2009; Node *et al.*, 1997). In this study, we tested the hypothesis that LDL reductions below 70 mg/dL enhance endothelial NO bioavailability in a progressive manner due to improved coupling efficiency of dimeric eNOS, while simultaneously decreasing the production of cytotoxic ONOO⁻ – a main component of oxidative stress. We tested this hypothesis with the simultaneous *in situ* measuring of NO and ONOO⁻ release in human ECs exposed to high and normal glucose concentrations, as well as monitoring eNOS dimerization via immunocytochemistry under both LDL and glycemic conditions. The results provide new insights into the relationship between cellular NO release, eNOS coupling and LDL levels under normal and hyperglycemic conditions.

Materials and Methods

Materials

LDL was isolated from healthy volunteers.

Cell Culture

Primary human umbilical vein endothelial cells (HUVECs; Lonza, Walkersville, MD) were cultured in complete EC growth medium and incubated at 37°C in

95% air/ 5% CO₂ according to the distributor's recommendations. Medium was replaced every other day and cells were propagated using a tryptic procedure. Before amperometric measurement of NO/ONOO⁻, cells were exposed to LDL (150 to <10 mg/dL) at either normal glucose or elevated glucose levels for 1 hour.

NO and ONOO⁻ Measurement

NO and ONOO⁻ release were measured from the surface of a monolayer of ECs using amperometric nanosensors as previously described (Malinski and Taha, 1992; Mason *et al.*, 2005b). Carbon fibers were fed through pulled capillaries and coupled to copper wire using conductive silver epoxy (EpoTek). The exposed tip of the fiber was insulated with nonconductive wax epoxy and flame sharpened to produce an electroactive sensing tip (4-5 μm length, 200-300 nm diameter). The tip was then chemically modified by depositing conductive polymeric porphyrinic films via cyclic voltammetry on a Gamry 600 potentiostat (Gamry Instruments, Warminster, PA). For NO nanosensors, the sensing material deposited was nickel (II) tetrakis (3-methyl-4-hydroxyphenyl) porphyrin, while ONOO⁻ nanosensors were modified with manganese (III) – [2,2] paracyclophenyl porphyrin. Finally, ion exchangers were dip coated on the sensors surface to reduce interference of measuring the analyte; Nafion (Sigma) was used for the NO nanosensor and polyvinyl pyridine for ONOO⁻ nanosensor. Electrodes were calibrated by linear regression with standard solutions of both NO and ONOO⁻ before and after cellular measurements and the detection limit of each electrode was on the order of 10⁻⁹ M.

Finished nanosensor tandems were lowered 5±2 μm from the surface of the ECs in HBSS using a micromanipulator (Sensapex, Finland) and NO/ONOO⁻ release was stimulated with calcium ionophore A21387 (CaI, 1 μmol/L). Amperometric responses were obtained using a Gamry Reference 600 dual potentiostat (Gamry Instruments, Warminster, PA).

Western Blot Analysis

Mouse anti-eNOS antibody and HRP goat anti-mouse Ig were purchased from BD Biosciences, San Jose, CA). Anti-B-actin was purchased from Sigma (St. Louis, MO). RIPA lysis buffer was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Dimeric eNOS was detected with Western blot using SDS-PAGE at low temperature (LT-SD-PAGE) as previously described (Benson *et al.*, 2013). Briefly, after treatments cells were lysed with RIPA buffer supplemented with protease inhibitor at 4°C. Protein lysate were mixed 1:1 with Laemmli buffer and loaded onto a 6% polyacrylamide gel and subjected to SDS-PAGE with at low temperature. After electrophoresis, proteins were transferred onto Polyvinyl Difluoride (PVDF) membrane

at 4°C overnight. Following the transfer, membranes were incubated with 5% non-fat milk /TBSP blocking buffer for 2 h at room temperature and washed three times in TBST buffer. Blots were incubated with anti-eNOS antibody at 4°C overnight and washed with TBST three times before conjugation with the secondary HRP-IgG antibody for 1.5 hours at room temperature. Blots were washed and visualized using enhanced chemiluminescence (ChemiDoc MP System; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and analyzed and quantified using Image Lab Version 5.2.1; Bio-Rad Laboratories, Inc.).

Calculations and Statistical Analysis

All data are presented as mean \pm Standard Deviation (SD) of the mean of $n > 3$. Statistical analysis of the mean difference between multiple groups was performed using one-way Analysis of Variance (ANOVA) with Student-Newman-Keuls multiple comparisons post hoc analysis; and between two groups, using Student's *t*-test. The alpha level for all the tests was 0.05. A P value < 0.05 was considered to be statistically significant. All statistical analyses were performed using Origin (v 6.1 for Windows; OriginLab, Northampton, MA) and

GraphPad Prism (v. 5.00 for Windows; GraphPad Software, San Diego, CA).

Results

Nitric Oxide and Peroxynitrite Release from Endothelium

Figure 1 shows a typical amperogram (concentration proportional to current vs. time) recorded by NO and ONOO⁻ nanosensors, positioned about $5 \pm 2 \mu\text{m}$ from the surface of the HUVECs. After stimulation by calcium ionophore, a rapid release of both [NO] and [ONOO⁻] was observed (Fig. 1). After about 2-5 seconds, maximum concentration was reached, followed by a gradual decrease in both [NO] and [ONOO⁻]. In the presence of LDL, maximum [NO] decreased vs. control by about 30%. A further decrease in maximal [NO] was observed for HUVECs treated with both LDL and elevated glucose (Fig. 1a). The change in maximal [ONOO⁻] were opposite of that observed for [NO]. In the presence of LDL, maximal [ONOO⁻] increased by about 25-35%. A further significant increase in [ONOO⁻] was also observed in the presence of elevated glucose (250 mg/dL) and LDL (30 mg/dL) (Fig. 1b).

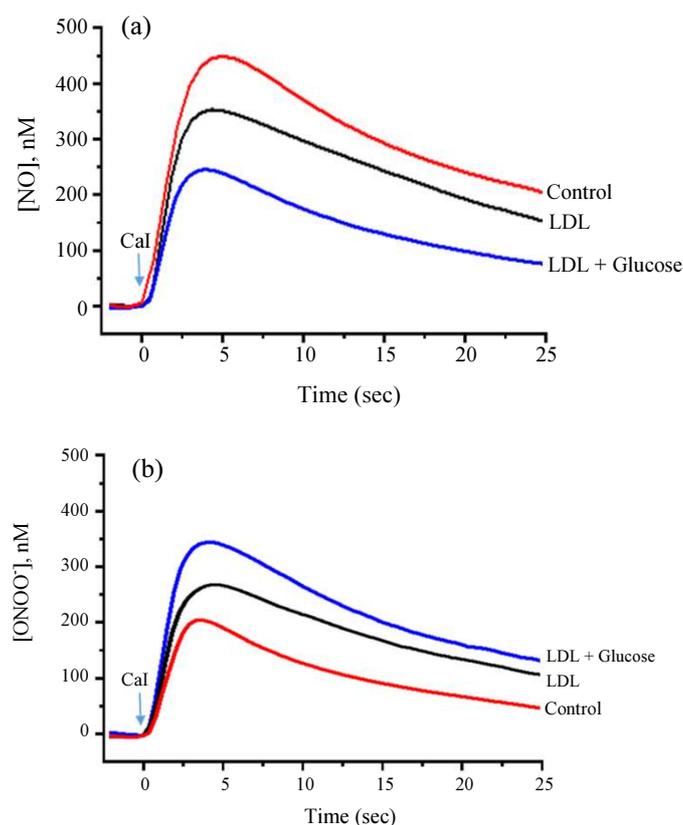


Fig. 1: Typical amperogram showing changes of nitric oxide concentrations [NO] (a) and peroxynitrite concentrations [ONOO⁻] (b), with time. NO and ONOO⁻ production was measured simultaneously with nanosensors after stimulation with calcium ionophore (CaI, 1.0 μM). HUVECs were incubated with LDL (30 mg/dL) or with LDL (30 mg/dL) and elevated glucose (250 mg/dL) for 1 h

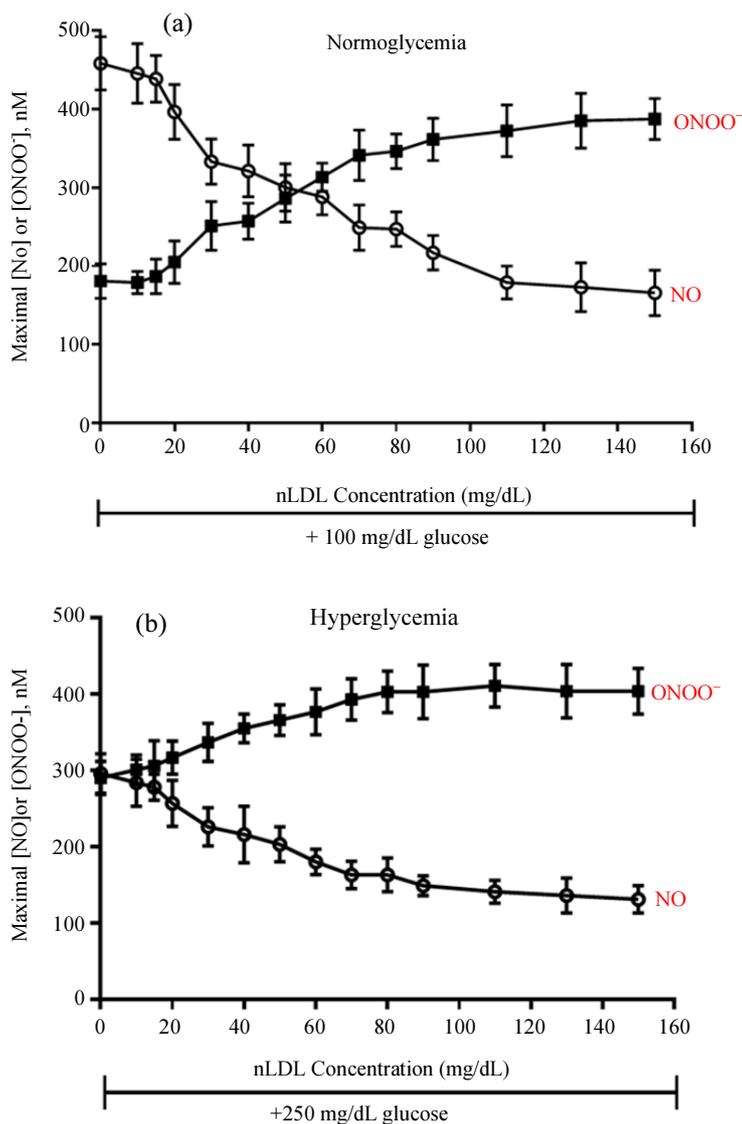


Fig. 2: Maximal nitric oxide (open circles) and peroxynitrite (solid square) concentrations at different LDL levels. HUVECs were incubated with normal glucose levels (100 mg/dL) (a) and elevated glucose (250 mg/dL) (b) in the presence of LDL, ranging from 10 to 150 mg/dL

Effects of Progressive LDL Reductions on NO and ONOO⁻ Release Under Normo- and Hyperglycemic Conditions

A significant effect of LDL changes on the release of NO and ONOO⁻ was observed under normo- and hyperglycemic conditions (Fig. 2). With increasing LDL levels, NO decreased exponentially, while ONOO⁻ increased in a similar manner, under conditions of both normo- and hyperglycemia. However, the decrease of NO and the increase in ONOO⁻ originated from significantly different levels. With normal glucose, maximal NO is 458±34 while in hyperglycemia it is reduced to 296±26. Also, in hyperglycemia, the level of

ONOO⁻ is about 60% higher than with normal glucose. Therefore, the contribution of elevated LDL on the balance of [NO]/[ONOO⁻], is much more apparent under conditions of hyperglycemia.

The Effect of Progressive Elevation of LDL on [NO]/[ONOO⁻] Ratio Under Normal and Hyperglycemic Conditions

The [NO]/[ONOO⁻] ratio is an indicator of eNOS efficiency and endothelial function. At higher [NO]/[ONOO⁻] ratios, the bioavailability of cytoprotective NO is higher relative to cytotoxic ONOO⁻, the main component of nitroxidative stress.

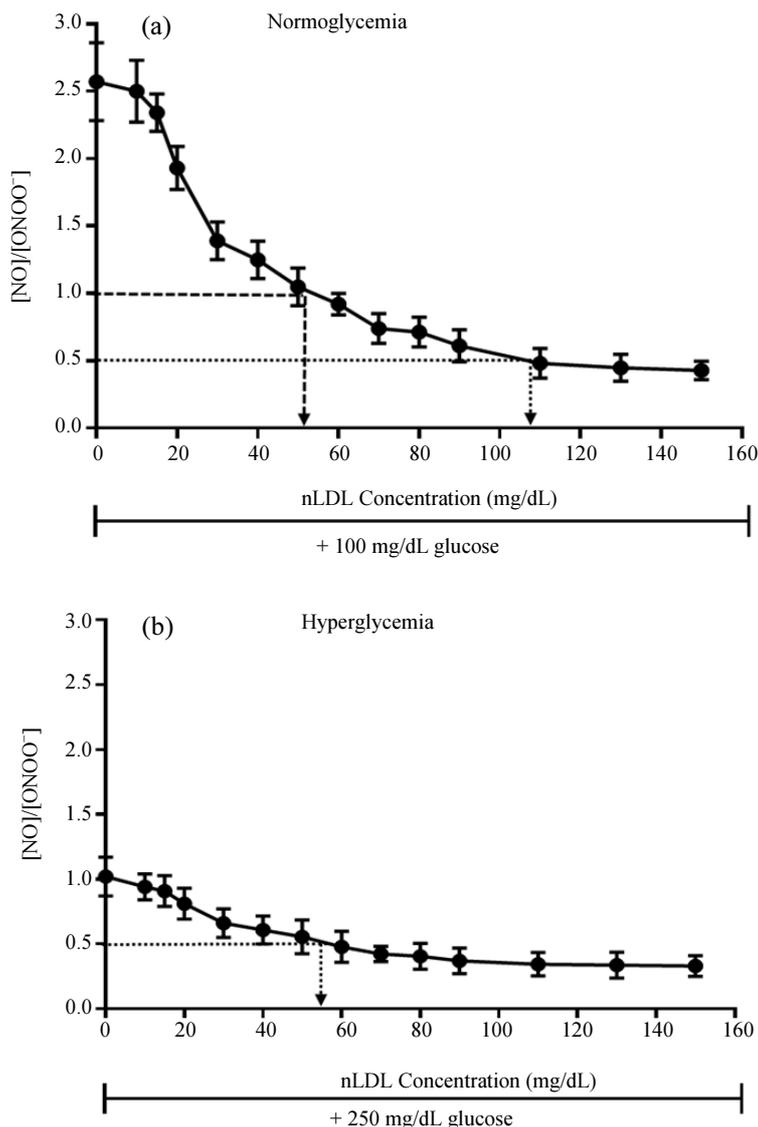


Fig. 3: The ratio of maximal [NO]/[ONOO⁻] produced by HUVECs under normal glucose levels (100 mg/dL) (a) and elevated glucose (250 mg/dL) (b) in the presence of LDL, ranging from 10 to 150 mg/dL. Arrows indicate extrapolated concentration of LDL at [NO]/[ONOO⁻] = 1.0 and [NO]/[ONOO⁻] = 0.50

The changes in the ratio of cytoprotective [NO] to cytotoxic [ONOO⁻] are depicted in Fig. 3. With normal glucose, [NO]/[ONOO⁻] was 2.57 ± 0.29 and by adding 10 mg/dL LDL decreased this ratio slightly (by about 3%). As the LDL lever further increased, [NO]/[ONOO⁻] reached a value 1.0 at about 50 mg/dL. A [NO]/[ONOO⁻] ratio of 1.0 reflects on the equimolar concentrations of NO and ONOO⁻, which is the prime indicator of endothelial dysfunction and eNOS uncoupling. When [NO]/[ONOO⁻] is below 1, the cellular environment is controlled by oxidative stress, imposed by ONOO⁻. When [NO]/[ONOO⁻] = 0.50 and the concentration of ONOO⁻ is nearly double to NO, this is a sign of a further significant increase of oxidative stress. In

normoglycemic conditions, this level of [NO]/[ONOO⁻] was reached at about 110 mg/dL of LDL (Fig. 3a).

Under hyperglycemic conditions, the ratio of [NO]/[ONOO⁻] is already about 1.0, which is about 160% lower than in normoglycemia. Therefore, in the presence of LDL, a further rapid decrease in [NO]/[ONOO⁻] was observed. However, in contrast to normoglycemia, the effect of LDL is now imposed on a significantly dysfunctional endothelium. Therefore, the ratio of [NO]/[ONOO⁻] = 0.5 is reached at an LDL concentrations of about 50 mg/dL (Fig. 3b). A further increase in LDL, up to 150 mg/dL, decreased [NO]/[ONOO⁻] to about 0.40, further increasing oxidative stress. Therefore, in hyperglycemia, the

moderate oxidative stress in endothelium can be maintained only at LDL levels lower than 50 mg/dL.

Changes in eNOS Dimer Formation with Different Levels of LDL with Normal and Elevated Glucose

In addition to measuring NO and ONOO⁻ production with varying LDL levels, we also examined eNOS dimer formation with and without high glucose. Under normoglycemic conditions, uncoupling of eNOS and the concentration of dimer decreased gradually with an increase in LDL concentration (Fig. 4a). At about 110-120 mg/dL of LDL, the concentration of eNOS dimer was about 50% of that observed in the absence of LDL.

Under hyperglycemic conditions, eNOS dimer concentrations were already decreased by about 50%. The addition of the effect of LDL on the dimeric eNOS markedly decreased its concentration. At a concentration of LDL around 30-50 mg/dL, the dimer concentration dropped to about 15% compared to levels observed under normoglycemic conditions (Fig. 4b). There is a significant correlation between the presence of functional dimeric eNOS and the levels of [NO] and [ONOO⁻]. It appears that an LDL level of 50 mg/dL has to be maintained in order to prevent a further decay of dimeric eNOS and endothelial function in hyperglycemia.

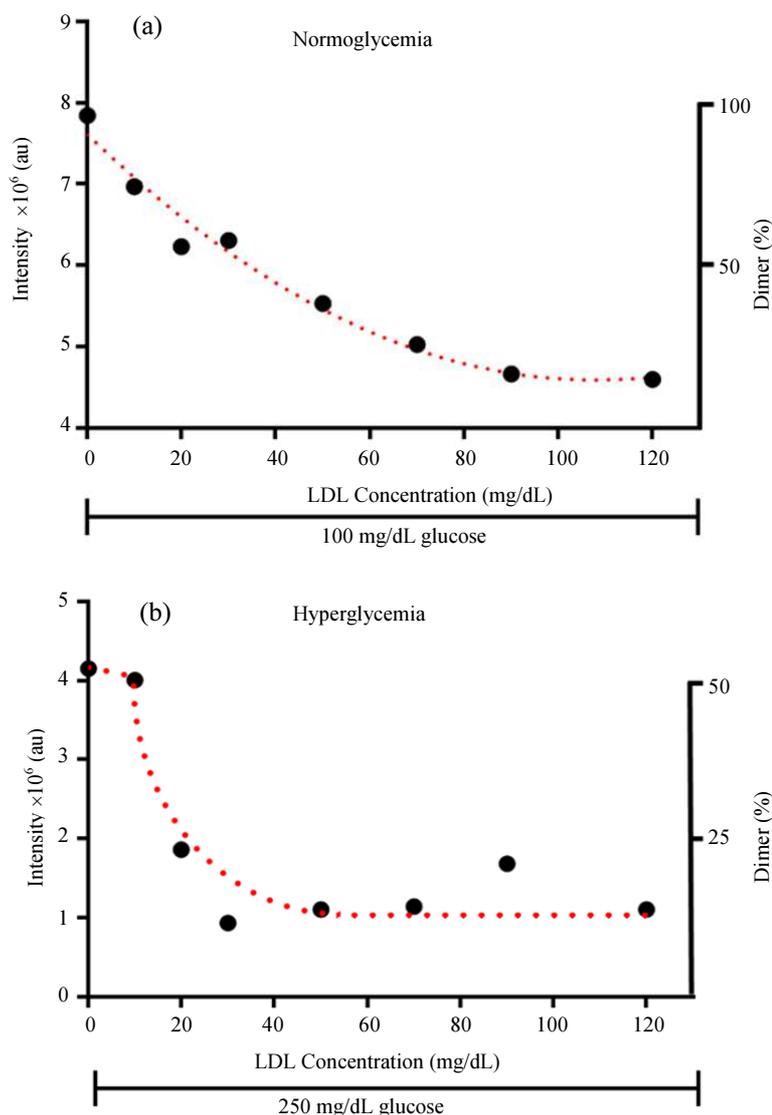


Fig. 4: Chemiluminescence intensity (arbitrary units) proportional to dimer concentration. As the function of LDL concentration. HUVECs were exposed to normal (100mg/dL) (a) and elevated glucose levels (250 mg/dL) (b) for 1hr, in the presence of different LDL concentrations. The dimer concentration measured under normoglycemic condition in the absence of LDL was assumed as 100%

Discussion

The key finding from this study is that a reduction in LDL levels to very low levels is a necessity to limit endothelial dysfunction, oxidative stress and vascular damage under conditions of hyperglycemia. Even at LDL levels of 50 mg/dL, which are well under the widely accepted target of 70 mg/dL, we observed uncoupled eNOS as an efficient generator of ONOO⁻, especially under conditions of high glucose. These findings show that uncoupled, monomeric, eNOS can produce both NO and O₂⁻ in close proximity which can react rapidly in a diffusion-controlled manner to produce ONOO⁻. ONOO⁻ is a powerful biological oxidant that at high concentrations (~100 nM) can induce endothelial dysfunction (Chen *et al.*, 2014; Stuehr *et al.*, 2004; Vasquez-Vivar *et al.*, 2003). NO is derived from the oxidation of L-arginine to L-citrulline by the enzymatic activity of eNOS. The activity of this electron transport enzyme requires calcium/calmodulin, flavin adenine dinucleotide, flavin mononucleotide and tetrahydrobiopterin (BH₄) as cofactors. Efficient eNOS activity (i.e., optimal generation of NO) is dependent upon the dimerization of the enzyme, along with enzymatic substrates and adequate co-factors, as these play key roles in stabilizing the functional dimer. When levels of substrates (L-arginine and O₂) or co-factors, like BH₄, are insufficient, uncoupled eNOS reduces molecular oxygen to superoxide (Harrison, 1997; Mason *et al.*, 2009; Node *et al.*, 1997). As a result, NO is scavenged by O₂⁻ and ONOO⁻ is generated while eNOS loses its cytoprotective actions within the cell that leads to endothelial dysfunction.

Using nanosensors, we measured the concentrations of NO and ONOO⁻ from ECs following maximal stimulation with calcium ionophore. Based on these measurements, we were able to calculate the ratio of [NO]/[ONOO⁻], which reflects the balance between the concentrations of cytoprotective NO and cytotoxic ONOO⁻, the main source of nitroxidative stress. The balance of [NO]/[ONOO⁻] is directly related to the relative dimeric and monomeric forms of eNOS. Under normal conditions, the [NO]/[ONOO⁻] ratio in functional ECs varies from 2.5-5 and is indicative of efficient eNOS coupling. However, if the [NO]/[ONOO⁻] ratio falls below this value, it is evidence of progressive eNOS uncoupling, resulting in low bioavailability of NO and an increase in oxidative stress, which leads to endothelial dysfunction. Peroxynitrite becomes the dominating factor when [NO]/[ONOO⁻] falls below 1.0, resulting in an unfavorable redox environment. If [NO]/[ONOO⁻] falls below 0.5, it produces a severe oxidative stress that contributes to mechanisms of disease.²⁴ We found that it is not the absolute level of NO or ONOO⁻, but the ratio between them that is the determinant of the cellular redox environment (Burewicz *et al.*, 2013; Mason *et al.*, 2015; Khan *et al.*, 2018; Sambe *et al.*, 2018).

These studies indicate that large reductions in LDL are necessary to decrease eNOS uncoupling, thereby preventing the generation of ONOO⁻ and preserving bioavailable NO. The redox balance between NO and ONOO⁻ can be changed with decreases in LDL that lead to improved vasorelaxation of the cardiovascular system, while concomitantly reducing vasoconstriction induced by ONOO⁻ and preventing nitroxidative damage to the endothelium. Previously, we also found improved eNOS function in obese Zucker rats by treating ECs with superoxide dismutase or an inhibitor of NADPH oxidase (Mason *et al.*, 2011).

The most important finding of these studies is that hyperglycemia contributes strongly to endothelial dysfunction at LDL concentrations higher than 50mg/dL. Therefore, a significant reduction of LDL, even from the current target of 70 mg/dL, is associated with reversal of endothelial damage under conditions of hyperglycemia.

Type 2 DM in eNOS knockout mice results in vascular abnormalities typically associated with insulin resistance, including hyperinsulinemia (Duplain *et al.*, 2001). Also, an endogenous eNOS inhibitor, Asymmetrical Dimethylarginine (ADMA), has been shown to reduce insulin sensitivity in a transgenic animal model (Shankar *et al.*, 2000). This is consistent with the observation that patients with type 2 DM have elevated plasma ADMA levels linked to reduced vascular compliance and glucose uptake (Stuhlinger *et al.*, 2002). Impaired endothelial function has been shown to predict atherosclerotic disease progression and its clinical manifestations (Schächinger *et al.*, 2000).

The clinical implications of this study suggest that current LDL target levels may not be aggressive enough to fully restore vascular function and reverse atherosclerosis. Despite reducing the progression of coronary atherosclerosis and associated clinical events, statins do not fully address residual CV risk even in patients who achieve optimal LDL levels (Fruchart, 2014). Consistent with the observations in this study, there is now evidence that lipid therapy beyond statins can help further reduce CV risk by lowering LDL levels well below target levels (Bosi, 2009; Kinaan *et al.*, 2015).

Limitations of this analysis include uncertainty about whether these results are directly applicable to humans with type 2 DM. This was a cell culture study and therefore the results need to be confirmed in human subjects.

Conclusion

This study showed a strong link between LDL reduction and improved eNOS function in hyperglycemia. Lowering LDL levels below 50 mg/dL significantly improved eNOS coupling, resulting in increased NO production and mitigated production of cytotoxic ONOO⁻ in hyperglycemic cells, while the same benefits, albeit in a more moderate manner, were observed under normoglycemic conditions at LDL levels below 110 mg/dL. Taken together, these results illustrate

the importance of LDL lowering in patients with comorbid CV risk factors like DM.

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Authors Contributions

R. Preston Mason: Was responsible for project design and writing.

Hazem Dawoud: Was responsible for data collection and analysis.

Samuel C.R. Sherratt: Was responsible for data collection and analysis

Michael R. Wagner: Was responsible for data collection and analysis.

Tadeusz Malinski: Was responsible for project design, writing and managing the experiments.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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Abbreviations and Units

AMDA	Asymmetric dimethylarginine
BH4	Tetrahydrobiopterin
CaI	Calcium ionophore
CARDS	Collaborative atorvastatin diabetes study
CTT	Cholesterol treatment trialists
CVD	Cardiovascular disease
DM	Diabetes mellitus
EC	Endothelial cell
eNOS	Endothelial nitric oxide synthase
HBSS	Hank's balanced salt solution
HUVECs	Human umbilical vein endothelial cells
LDL	Low-density lipoprotein
LT-SD-PAGE	Low temperature sodium dodecyl sulfate polyacrylamide gel electrophoresis
NO	Nitric oxide
O2-	Superoxide
ONOO-	Peroxonitrite
RIPA	Radioimmunoprecipitation assay buffer
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis