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# Palmitate-Associated ET-1 and PAI-1 Transcriptional Responses Under High-Glucose Conditions in HUVECs: An Exploratory Glucolipotoxic Stress Model

**Cem Abidoğlu**
Hatay Mustafa Kemal University
**Ebru Derici Eker**
Mersin University
**Meral Urhan Küçük**
mukucuk@mku.edu.tr

Hatay Mustafa Kemal University

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## Abstract

# Background

Hyperglycemia and elevated saturated free fatty acids are key metabolic stressors implicated in vascular injury. However, the early transcriptional responses of endothelial cells to combined glucose and lipid stress remain incompletely defined. This exploratory in vitro study investigated whether high-glucose conditioning modifies palmitate-associated oxidative and endothelial stress-related gene expression responses in human umbilical vein endothelial cells (HUVECs).

# Methods

HUVECs were cultured under low-glucose (LG; 1 g/L) or high-glucose (HG; 4.5 g/L) conditions and exposed to palmitic acid (Pal; 0.25, 0.5, or 1 mM) for 24 hours. Lipid peroxidation was assessed by measuring malondialdehyde (MDA) levels. The mRNA expression levels of endothelial stress-related and inflammatory markers, including EDN1/ET-1, NOS3/eNOS, VCAM1, SERPINE1/PAI-1, TNF, and IL6, were evaluated by RT-qPCR.

# Results

Under LG conditions, Pal exposure increased MDA levels, particularly at 0.25 and 0.5 mM, whereas the HG condition showed a more complex non-linear oxidative stress response. In the HG environment, Pal exposure significantly increased ET-1 expression across all tested concentrations and significantly upregulated PAI-1 expression, while eNOS and VCAM-1 mRNA levels did not show significant changes. TNF-α and IL-6 displayed concentration-dependent and biphasic responses, particularly at higher Pal concentrations.

# Conclusion

These findings suggest that high-glucose conditioning selectively modifies palmitate-associated endothelial stress-related transcriptional responses in HUVECs, particularly through ET-1 and PAI-1 upregulation. However, because NO bioavailability, protein-level validation, cell viability, osmotic control, and functional endothelial assays were not included, the

## INTRODUCTION

The endothelium is a specialized tissue of mesodermal origin composed of a single layer of squamous endothelial cells lining the lumen of blood vessels [1–2]. Once regarded as a passive structural barrier, the vascular endothelium is now recognized as a highly active and dynamic interface that plays a crucial role in the regulation of numerous physiological processes. These include the control of vascular tone,

maintenance of hemostatic balance, modulation of immune and inflammatory responses, regulation of coagulation, and coordination of angiogenesis [3–4].

Endothelial tissue regulates vascular homeostasis through the coordinated synthesis and release of multiple bioactive mediators, including nitric oxide (NO), endothelins (ETs), prostaglandins (PGs), and components of the angiotensin system, all of which are produced by endothelial cells. Under physiological conditions, the endothelial nitric oxide synthase (eNOS) gene is the primary source of vascular NO, a key mediator with potent vasodilatory properties that is essential for the maintenance of normal vascular tone [3–6].

Impaired nitric oxide (NO) synthesis, reduced NO bioavailability, or accelerated NO degradation is frequently associated with a decline in endothelium-dependent vasodilatory function. As the capacity for vasodilation diminishes, circulating NO levels decrease, while the expression and release of endothelin-1 (ET-1), a potent endothelium-derived vasoconstrictor, are enhanced [3–5]. This imbalance, characterized by a predominance of vasoconstrictive over vasodilatory mediators, disrupts vascular homeostasis, resulting in impaired vascular relaxation and the development of endothelial dysfunction [3, 7].

Endothelial dysfunction is widely recognized as a hallmark of numerous cardiovascular-related diseases, including dyslipidemia, diabetes mellitus, obesity, arterial hypertension, and atherosclerosis, as well as physiological conditions such as aging [4–6,]. One of the most common and central mechanisms underlying this complex process, in which normal endothelial function is disrupted, is oxidative stress, which plays a pivotal role in the initiation and progression of endothelial impairment [4].

Oxidative stress is defined as a disruption of the balance between the production of reactive oxygen species (ROS) and the capacity of the antioxidant defense system to neutralize them, resulting in excessive accumulation of ROS. Under physiological conditions in aerobic organisms, ROS are continuously generated as byproducts of cellular oxygen metabolism; however, when antioxidant defenses are overwhelmed or impaired, ROS levels rise and exert deleterious effects on cellular function [3–8].

Elevated ROS levels reduce the bioavailability of nitric oxide (NO) and promote the activation of inflammatory gene expression, processes that are central to the initiation and progression of atherosclerosis. In addition, ROS influence key cellular mechanisms, including cell proliferation and apoptosis, cell migration, and extracellular matrix remodeling, all of which contribute to vascular pathology [6]. Experimental and clinical studies have associated increased ROS production or inadequate ROS clearance with the development of endothelial dysfunction [9].

Furthermore, heightened oxidative stress is closely associated with several major cardiovascular risk factors implicated in the pathophysiology of atherosclerosis, including diabetes mellitus, hypercholesterolemia, chronic kidney disease, aging, hypertension, and smoking [3, 10]. Collectively, these findings underscore oxidative stress as a central mechanism linking metabolic and environmental risk factors to endothelial injury and vascular disease progression.

Hyperglycemia induces excessive production of reactive oxygen species (ROS), reduces the activity of enzymes involved in molecular oxygen binding, and promotes the generation of superoxide anions ($O_2^-$). This cascade of events leads to reduced nitric oxide (NO) bioavailability and ultimately results in endothelial dysfunction. In parallel with hyperglycemia, type 2 diabetes mellitus is well recognized as a major contributor to cardiovascular disease development [11].

Similarly, in hyperlipidemia, endothelial cells experience increased oxidative stress in response to elevated circulating levels of free fatty acids (FFAs). This lipotoxic environment has been shown to upregulate endothelin-1 (ET-1) expression while simultaneously downregulating NO production and impairing insulin signaling pathways. Such alterations are closely associated with the cardiovascular complications of type 2 diabetes, including atherosclerosis, which frequently coexist with hyperlipidemia. Moreover, hyperlipidemia is considered a key factor contributing to the development and progression of type 2 diabetes [12].

Endothelial dysfunction arises at an early stage of the atherogenic process and plays a critical role in the initiation, progression, and complication of atherosclerotic plaque formation. Notably, endothelial dysfunction has been demonstrated in individuals who present with cardiovascular risk factors but do not yet exhibit clinical manifestations of atherosclerosis, highlighting its role as an early and sensitive marker of vascular disease [13–14].

Glucose concentration and lipid exposure are important metabolic stressors that can influence endothelial cell responses in HUVECs under in vitro conditions, even in the absence of additional cellular stimuli. To characterize these responses, the present study investigated the expression levels of selected endothelial stress-related genes, including endothelial nitric oxide synthase (eNOS), endothelin-1 (ET-1), vascular cell adhesion molecule-1 (VCAM-1), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and plasminogen activator inhibitor-1 (PAI-1), a marker associated with thrombosis and atherosclerosis.

This exploratory study aimed to evaluate whether high-glucose conditioning modifies palmitate-associated oxidative stress and endothelial stress-related transcriptional responses in HUVECs. By characterizing these early transcriptional patterns, the study may provide a preliminary biological context for future mechanistic investigations into glucolipotoxic endothelial stress and its potential relevance to metabolic vascular complications.

## MATERIALS AND METHODS

# Cell Culture and Experimental Design

Human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection (ATCC). Cells were cultured in DMEM/F12 medium (Gibco, Grand Island, NY, USA) supplemented with 1% penicillin/streptomycin, 20% fetal bovine serum (FBS), 0.3 ng/mL epidermal growth factor (EGF), and 10 mg/mL heparin. Cells were maintained in a humidified incubator at 37°C

with 5% $CO_2$. The culture medium was changed every two days, and cells were subcultured when they reached 70–80% confluence.

## Preparation of the Palmitate-BSA Complex

Palmitic acid (Pal) was conjugated with bovine serum albumin (BSA; Cat. No. A3059; Sigma-Aldrich) according to a previously described protocol with minor modifications. Briefly, BSA was dissolved in 150 mM NaCl and incubated at 37°C with gentle agitation for 1 hour. In parallel, Pal was dissolved in 150 mM NaCl by heating at 70°C until a clear solution was obtained. The Pal solution was then cooled to 37°C and gradually added to the BSA solution, followed by gentle shaking at 37°C for an additional 1 hour to allow conjugation. The pH of the resulting Pal-BSA complex was adjusted to 7.4, and the solution was sterilized through a 0.22 μm membrane filter. The final Pal concentrations used for cell treatments were 0.25, 0.5, and 1 mM. Corresponding control groups received the same final concentration of BSA used in the Pal-BSA-treated groups.

## Establishment of the Glucolipotoxic Stress Model

HUVECs were seeded into 75 cm² culture flasks at a density of 25,000 cells/mL. After initial attachment and expansion, cells were maintained under two glucose conditions: low glucose (LG; 1 g/L glucose) or high glucose (HG; 4.5 g/L glucose) using DMEM/F12 medium. To maintain stable glucose exposure, the culture medium was refreshed every 48 hours for approximately 5–8 days. When cells reached approximately 70–80% confluence, Pal treatments were initiated. HUVECs in both LG and HG conditions were incubated in reduced-serum medium containing 1% FBS and treated with 0, 0.25, 0.5, or 1 mM Pal for 24 hours. The 0 mM Pal groups served as glucose-condition-specific controls. Lipid peroxidation was assessed by measuring malondialdehyde (MDA) levels, and endothelial stress-related transcriptional responses were evaluated by RT-qPCR.

## Determination of Lipid Peroxidation (MDA Levels)

Lipid peroxidation was assessed by measuring malondialdehyde (MDA) levels as thiobarbituric acid reactive substances (TBARS). Following 24 hours of Pal exposure, cells were washed with Hank's Balanced Salt Solution (HBSS), lysed with RIPA buffer, and stored at − 80°C until biochemical analysis. Total protein concentrations were determined using the BCA protein assay, and MDA levels were normalized to total protein content and expressed as nmol/mg protein.

The TBARS assay was performed according to the method described by Ohkawa et al. (1979), with minor modifications for 96-well plate adaptation [15]. Briefly, samples or standards were mixed with sodium dodecyl sulfate, acetic acid, thiobarbituric acid, and distilled water, followed by incubation at 95°C for 30 minutes. After cooling, the MDA-TBA adduct was extracted using n-butanol/pyridine, centrifuged, and the absorbance of the organic phase was measured at 532 nm using a microplate

reader. A standard curve was generated using 1,1,3,3-tetraethoxypropane, and final MDA concentrations were calculated accordingly.

## RNA Extraction and Quantitative Real-Time PCR (RT-qPCR)

For each experimental group, approximately 1 × $10^6$ cells were harvested for RNA isolation. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Catalog No. 74104) according to the manufacturer's instructions. RNA concentration and purity were assessed spectrophotometrically, and samples with an A260/A280 ratio between 1.8 and 2.0 were used for downstream analysis. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Cat. No. 4368814). Quantitative real-time PCR was performed using the Rotor-Gene Q system (Qiagen) with Power SYBR Green PCR Master Mix. The reaction mixture contained SYBR Green master mix, 200 nM forward and reverse primers, and 2 µL of cDNA template. The primer sequences for EDN1/ET-1, NOS3/eNOS, TNF, IL6, SERPINE1/PAI-1, and VCAM1 are provided in Table 1. GAPDH was used as the internal reference gene. The qPCR cycling protocol consisted of an initial denaturation at 95°C for 15 minutes, followed by 40 cycles of denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. Melting curve analysis was performed to verify amplification specificity. All experiments were conducted in three independent biological replicates, and each sample was analyzed in technical duplicate. Relative expression levels were calculated using the $2^{-}\Delta\Delta Ct$ method and expressed as fold-change relative to the corresponding control group.

Table 1
Primer sequences used for RT-qPCR analysis

| Gene Symbol | Full Gene Name | Accession Number | Primer Sequence (5' → 3') |
|---|---|---|---|
| ET-1 | Endothelin-1 | NM_001955.5 | F: GACATC ATTTGGGTCAACACTC<br>R: GGCATCTATTTTCACGGTCTGT |
| eNOS | Endothelial nitric oxide synthase | NM_000603.5 | F: GTTTGTCTGCGGCGATGT<br>R: GTGCGTATGCGGCTTGTC |
| TNF-α | Tumor necrosis factor alpha | NM_000594.4 | F: CTCTTCTGCCTGCTGCACTTTG<br>R: ATGGGCTACAGGCTTGTCACTC |
| IL-6 | Interleukin-6 | NM_000600.5 | F: AGACAGCCACTCACCTCTTCAG<br>R: TTCTGCCAGTGCCTCTTTGCTG |
| PAI-1 | Plasminogen activator inhibitor-1 | NM_000602.5 | F: CCTCAGGAAGCCCCTAGA<br>R: TGGAGAGGCTCTTGGTCT |
| VCAM-1 | Vascular cell adhesion molecule 1 | NM_001078.4 | F: TCTGGAAATGCAACTCTCACC<br>R: CAAAACTCACAGGGCTCAGG |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase | NM_002046.7 | Commercially validated (Qiagen) |

### 2.6. Statistical Analysis

**Statistical analyses were performed using GraphPad Prism version 5.01. Data are** presented as mean ± standard deviation (SD) or median where appropriate. All experiments were conducted in at least three independent biological replicates, with technical duplicate measurements for RT-qPCR analyses. For MDA measurements, data distribution was assessed for normality. Because MDA data did not meet parametric assumptions, pairwise comparisons were performed using the Mann–Whitney U test. For RT-qPCR data, relative gene expression levels were calculated using the $2^{-}\Delta\Delta Ct$ method, and treatment groups were compared with their corresponding glucose-condition-specific controls using one-way ANOVA followed by Tukey's post hoc test. A fold-change threshold of > 1.5 or < 0.67 together with $p < 0.05$ was considered biologically and statistically relevant. A p-value < 0.05 was considered statistically significant.

## RESULTS

# Determination of Oxidative Stress Levels via MDA Analysis

To evaluate lipid peroxidation under glucolipotoxic stress conditions, HUVECs were cultured in two glucose environments: low glucose (LG; 1 g/L) and high glucose (HG; 4.5 g/L). Following 24 hours of exposure to palmitic acid (Pal; 0.25, 0.5, and 1 mM) in the presence of BSA, malondialdehyde (MDA) levels were quantified as a lipid peroxidation marker. In the LG group, Pal treatment significantly increased MDA levels compared with the LG-BSA control group. Specifically, exposure to 0.25 mM Pal ($p < 0.01$) and 0.5 mM Pal ($p < 0.001$) resulted in significant elevations in MDA levels, whereas no significant change was observed in the 1 mM Pal subgroup (Fig. 1a). In the HG group, Pal exposure showed a non-linear MDA response. While 0.25 mM Pal significantly increased MDA levels relative to the HG-BSA control ($p < 0.001$), MDA levels were significantly reduced in the 0.5 mM Pal subgroup ($p < 0.001$). Exposure to 1 mM Pal did not result in a statistically significant difference compared with the HG-BSA control (Fig. 1b). Comparison of the two glucose conditions showed that MDA levels were significantly higher in the LG groups than in the corresponding HG groups across Pal exposure concentrations (0.25, 0.5, and 1 mM; $p < 0.0001$) (Fig. 2). This difference was also observed between the baseline BSA control groups. Overall, these findings suggest that Pal exposure was associated with a more pronounced lipid peroxidation response under LG conditions than under HG conditions in this experimental setting. Because cell viability or cytotoxicity assays were not performed, the responses observed at 1 mM Pal should be interpreted cautiously, as this concentration may represent a high lipotoxic stress range.

## Gene Expression Levels of Endothelial Stress-Related and Inflammatory Markers

The effects of palmitic acid (Pal) on the mRNA expression levels of selected endothelial stress-related markers (ET-1, eNOS, VCAM-1) and inflammatory/prothrombotic markers (IL-6, TNF-alpha, PAI-1) were evaluated in HUVECs cultured under LG and HG conditions.

# Endothelial Stress-Related Markers

Under LG conditions, Pal exposure (0.25, 0.5, and 1 mM) did not induce statistically significant changes in the mRNA expression levels of eNOS, ET-1, or VCAM-1 compared with the LG control group (Fig. 3a; $p > 0.05$). In contrast, HUVECs maintained under HG conditions showed a more pronounced ET-1 transcriptional response to Pal exposure. ET-1 mRNA levels were significantly increased by 2.10-fold ($p = 0.0048$), 2.19-fold ($p = 0.0031$), and 1.97-fold ($p = 0.0387$) in the 0.25, 0.5, and 1 mM Pal groups, respectively (Fig. 3b). No significant alterations were observed in eNOS or VCAM-1 mRNA expression under HG conditions.

# Inflammatory and Prothrombotic Markers

Under LG conditions, Pal exposure did not result in significant changes in the mRNA expression levels of IL-6, PAI-1, or TNF-α compared with the LG control group (Fig. 4a; $p > 0.05$). In the HG group, however, Pal exposure was associated with concentration-dependent and non-linear transcriptional responses (Fig. 4b). IL-6 expression was significantly reduced at higher Pal concentrations, with a 0.18-fold change at 0.5 mM and a 0.21-fold change at 1 mM Pal ($p < 0.001$). TNF-α showed a biphasic response; expression was significantly increased at 0.5 mM Pal ($p < 0.001$), whereas a significant decrease was observed at 1 mM Pal ($p < 0.01$). PAI-1 mRNA levels were significantly increased in all Pal-treated subgroups under HG conditions, with an average increase of approximately 1.62-fold ($p < 0.001$), particularly in the 1 mM Pal group.

# DISCUSSION

In this exploratory *in vitro* study, we evaluated whether high-glucose conditioning modifies palmitate-associated oxidative stress and endothelial stress-related transcriptional responses in HUVECs. The experimental model involved exposing HUVECs cultured under either high-glucose (HG) or low-glucose (LG) conditions to palmitic acid (Pal), a representative saturated free fatty acid. The main finding was that Pal exposure under HG conditions selectively increased ET-1 and PAI-1 mRNA expression, whereas eNOS and VCAM-1 mRNA levels did not show significant changes within the 24-hour exposure period. In contrast, MDA-based lipid peroxidation responses were more pronounced under LG conditions than under HG conditions. These findings suggest that glucose availability may differentially influence lipid peroxidation and endothelial stress-related transcriptional remodeling.

The vascular endothelium plays a pivotal role in maintaining vascular homeostasis by regulating vascular tone, platelet activation, leukocyte adhesion, inflammatory signaling, and fibrinolytic balance. Endothelial dysfunction is generally characterized by impaired endothelium-dependent vasodilation, reduced nitric oxide (NO) bioavailability, oxidative stress, and inflammatory activation [16–18]. Oxidative stress is considered one of the central contributors to endothelial impairment because excessive reactive oxygen species (ROS) production can reduce NO bioavailability, promote inflammatory gene expression, and contribute to vascular remodeling and atherogenic processes [3–6]. In the present study, MDA analysis revealed a non-linear lipid peroxidation response to Pal exposure. Under LG conditions, 0.25 and 0.5 mM Pal significantly increased MDA levels, whereas 1 mM Pal did not produce a significant increase. Under HG conditions, 0.25 mM Pal increased MDA levels, while 0.5 mM Pal was associated with reduced MDA levels and 1 mM Pal showed no significant difference compared with the HG-BSA control. Moreover, direct comparison between the glucose conditions showed that MDA levels were higher under LG than HG conditions across the Pal exposure subgroups. This finding indicates that Pal-associated lipid peroxidation and endothelial stress-related transcriptional responses may not proceed in a simple parallel manner.

One possible explanation for the non-linear MDA response at higher Pal concentrations is that high-dose Pal exposure may shift cells from adaptive stress signaling toward severe lipotoxic or cytotoxic stress. High concentrations of palmitic acid have been reported to reduce cell survival and increase membrane

damage indicators in cellular models [19]. In addition, saturated fatty acids can promote mitochondrial dysfunction, mitochondrial fission, and loss of membrane integrity, which may alter measurable oxidative stress markers [20]. Therefore, the reduced or plateaued MDA levels observed at higher Pal concentrations may reflect altered cellular viability, reduced metabolic activity, or impaired retention/generation of intracellular lipid peroxidation products rather than restoration of oxidative balance. However, because cell viability, LDH release, or apoptosis assays were not performed, this interpretation remains speculative and should be considered a limitation of the present study.

Saturated free fatty acids, particularly palmitic acid, have been associated with endothelial inflammation, oxidative stress, insulin signaling impairment, and vascular metabolic stress. Previous studies have shown that palmitic acid can induce ROS production, activate inflammatory pathways, impair insulin signaling, and contribute to endothelial stress through pathways involving NF-κB, IRS-1, PI3K/Akt/eNOS, and related mediators [21–23]. Batumalaie et al. (2016) reported that palmitic acid-induced endothelial insulin resistance is associated with oxidative stress and inflammatory activation [24]. Similarly, Zhang et al. (2018) demonstrated that palmitic acid can increase ET-1 expression in vascular endothelial cells through endoplasmic reticulum stress and protein kinase C-related signaling [25]. Liu et al. (2012) further showed that palmitate-associated endothelial stress and insulin resistance may involve inflammatory pathway activation and disruption of PI3K/Akt/eNOS signaling [26]. These studies provide a biological context for interpreting the Pal-associated transcriptional changes observed in the present study.

In our study, the transcriptional response of HUVECs to Pal exposure was appeared to be influencedby the glucose environment. Under LG conditions, Pal did not significantly alter the mRNA expression levels of ET-1, eNOS, VCAM-1, IL-6, TNF-α, or PAI-1. In contrast, under HG conditions, Pal exposure significantly increased ET-1 expression across all tested concentrations and increased PAI-1 expression. Hyperglycemia has been linked to activation of protein kinase C, increased hexosamine pathway flux, oxidative stress, and endothelial transcriptional changes, including pathways that may influence ET-1 regulation [27–30]. Therefore, the present findings suggest that high-glucose conditioning may sensitize HUVECs to Pal-associated vasoconstrictive and prothrombotic transcriptional responses.

ET-1 is a potent endothelial-derived vasoconstrictive mediator and is closely related to vascular tone regulation and metabolic vascular stress. The observed ET-1 upregulation under HG + Pal conditions may indicate an early vasoconstrictive transcriptional shift in HUVECs. However, because ET-1 protein levels or secretion were not measured, this finding should be interpreted at the mRNA level only. Similarly, PAI-1 is associated with impaired fibrinolytic balance, thrombosis risk, and atherosclerosis-related vascular complications. The increase in PAI-1 mRNA expression under HG conditions suggests that Pal exposure in a high-glucose environment may promote a prothrombotic transcriptional profile. Taken together, the ET-1 and PAI-1 findings support the presence of endothelial stress-associated transcriptional remodeling rather than definitive functional endothelial dysfunction.

The inflammatory markers showed a concentration-dependent and non-linear response. TNF-α increased at 0.5 mM Pal under HG conditions but decreased at 1 mM Pal, whereas IL-6 expression was reduced at

higher Pal concentrations. This pattern may reflect a biphasic response to Pal exposure, in which moderate lipotoxic stress activates inflammatory transcriptional signaling, while higher Pal concentrations may suppress transcriptional activity as a consequence of severe cellular stress or reduced viable cell number. While saturated fatty acids are known to activate inflammatory signaling through pathways such as TLR4/NF-κB, the present data do not allow us to distinguish between active inflammatory downregulation and cytotoxicity-related transcriptional suppression. Therefore, the high-dose 1 mM Pal results should be interpreted cautiously.

No significant changes were observed in eNOS or VCAM-1 mRNA expression under the experimental conditions used in this study. This is an important finding because endothelial dysfunction is commonly associated with reduced NO bioavailability and altered eNOS activity. However, eNOS function is regulated not only at the transcriptional level but also through post-translational mechanisms, particularly phosphorylation at Ser1177 and Thr495. Therefore, the absence of significant eNOS mRNA changes does not exclude possible changes in eNOS activity or NO production. Nevertheless, because NO bioavailability, total eNOS protein, phosphorylated eNOS, and functional endothelial assays were not assessed, the present study cannot confirm functional impairment of the NO pathway.

Similarly, the lack of significant VCAM-1 mRNA alteration suggests that the 24-hour HG + Pal exposure may not be sufficient to induce a detectable adhesion-related transcriptional response under the present experimental conditions. VCAM-1 induction may require longer exposure durations, additional inflammatory stimuli, or protein-level assessment to detect biologically relevant changes. This may explain why ET-1 and PAI-1 responded more clearly to HG + Pal exposure, whereas VCAM-1 did not show significant transcriptional alteration during the same exposure window.

Taken together, the present findings suggest that high-glucose conditioning does not uniformly amplify all Pal-induced stress responses in HUVECs. Instead, HG appears to selectively modify endothelial stress-related transcriptional outputs, particularly ET-1 and PAI-1 expression. The discordance between MDA responses and gene expression patterns indicates that lipid peroxidation and endothelial transcriptional remodeling may represent partially distinct layers of glucolipotoxic stress. Therefore, the current model is best interpreted as an exploratory transcriptional model of glucolipotoxic endothelial stress rather than as definitive evidence of endothelial dysfunction.

# LIMITATIONS AND FUTURE PERSPECTIVES

This study has several limitations. First, endothelial dysfunction-related changes were evaluated using selected endothelial stress-related transcriptional markers rather than being directly confirmed by functional assays. NO bioavailability, monocyte-endothelial adhesion, endothelial permeability, tube formation, and endothelium-dependent functional responses were not assessed. Therefore, the present findings should be interpreted as exploratory evidence of endothelial stress-associated transcriptional remodeling rather than definitive proof of endothelial dysfunction. Second, the study relied primarily on RT-qPCR-based mRNA analysis. Protein-level validation of ET-1, PAI-1, VCAM-1, total eNOS, and

phosphorylated eNOS was not performed. This is particularly important for eNOS, which is strongly regulated through post-translational modifications, including phosphorylation at Ser1177 and Thr495. Accordingly, the lack of significant eNOS mRNA alteration cannot exclude changes in eNOS activity or NO bioavailability. Third, cell viability, LDH release, and apoptosis assays were not included. This limitation is especially relevant for the 1 mM Pal condition, where non-linear or suppressed transcriptional responses may reflect high-dose lipotoxic or cytotoxic stress rather than specific regulatory downregulation. Future studies should include cytotoxicity assays to distinguish adaptive endothelial stress responses from loss of viable and transcriptionally active cells. Fourth, an osmotic control using mannitol was not included; therefore, glucose-specific responses cannot be fully distinguished from potential osmolarity-related effects. In addition, although BSA was used as the Pal carrier, vehicle control conditions should be carefully standardized in future experimental designs to fully control for the carrier effect of the Pal-BSA complex. Finally, the HUVEC model provides a controlled in vitro system for investigating endothelial stress responses but does not replicate the full complexity of vascular physiology, including shear stress, circulating immune cells, metabolic hormones, and tissue-specific endothelial heterogeneity. Future studies incorporating NO measurement, protein-level validation, cytotoxicity assessment, osmotic and vehicle controls, and functional endothelial assays are required to confirm the mechanistic significance of the present transcriptional findings.

# CONCLUSION

In conclusion, this exploratory in vitro study shows that high-glucose conditioning modifies palmitate-associated transcriptional responses in HUVECs, particularly by increasing ET-1 and PAI-1 expression under combined glucolipotoxic stress. Although Pal-induced lipid peroxidation was more pronounced under LG conditions, the HG environment appeared to be associated with a more pronounced vasoconstrictive and prothrombotic transcriptional profile. These findings suggest that glucose availability may differentially influence oxidative stress and endothelial stress-related transcriptional remodeling. However, because NO bioavailability, protein-level validation, cell viability, osmotic control, and functional endothelial assays were not included, the present findings should be interpreted as exploratory transcriptional evidence rather than definitive proof of endothelial dysfunction. Future studies incorporating these additional mechanistic and functional assessments are required to validate the biological significance of this model.

# Declarations


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# Author Contribution

C.A. performed the experiments. E.D.E. and M.U.K. conceived and designed the study, analyzed the data, prepared the figures, and wrote the main manuscript text. All authors reviewed the manuscript.

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# Figures

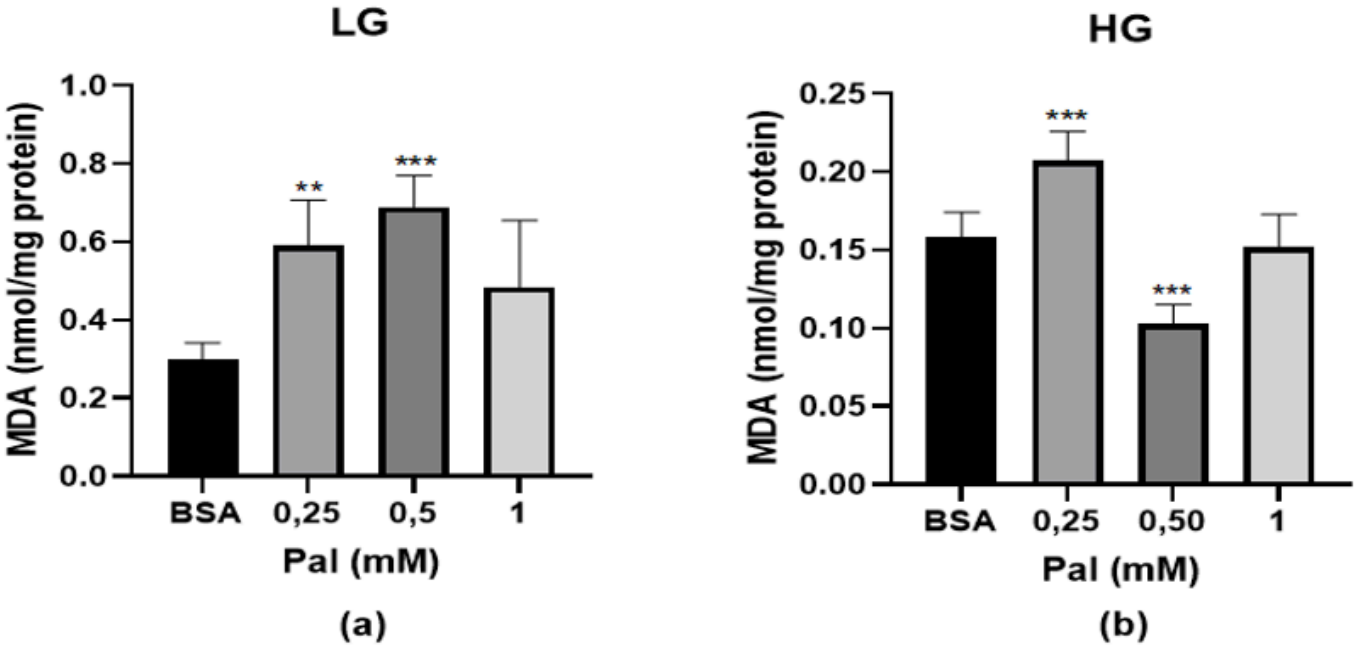



**Figure 1**

Effect of palmitic acid on MDA levels in HUVECs after 24 h treatment under (a) LG and (b) HG conditions. Pal: palmitic acid; BSA: bovine serum albumin ([2.5% or 5%; use the actual value]); LG: low glucose (1 g/L); HG: high glucose (4.5 g/L). **p < 0.001, ***p < 0.0001.

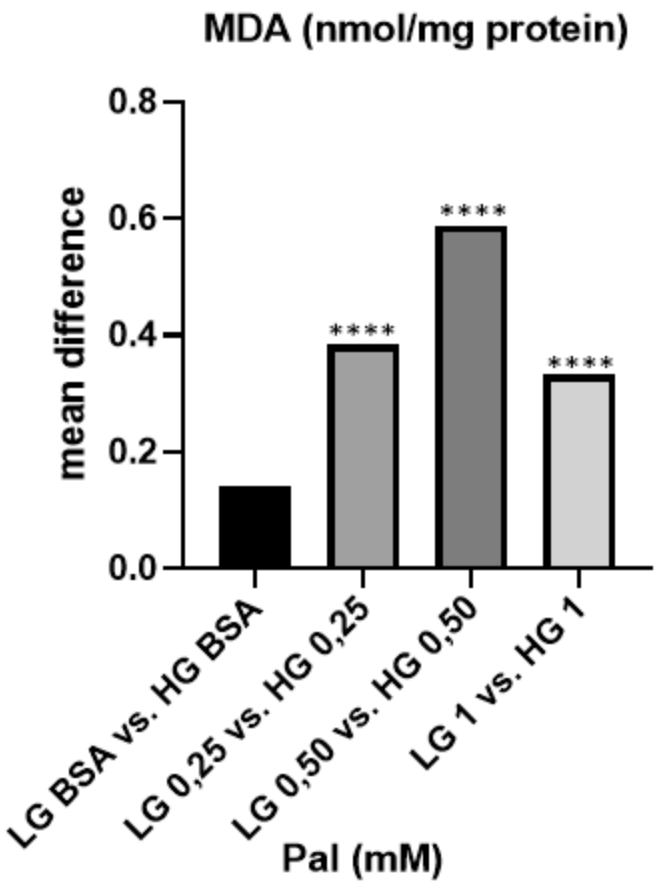



**Figure 2**

Comparison of MDA levels between LG and HG conditions across corresponding Pal exposure subgroups in HUVECs. Pal: palmitic acid; BSA: bovine serum albumin ([2.5% or 5%; use the actual value]); LG: low glucose (1 g/L); HG: high glucose (4.5 g/L). ***p < 0.0001.

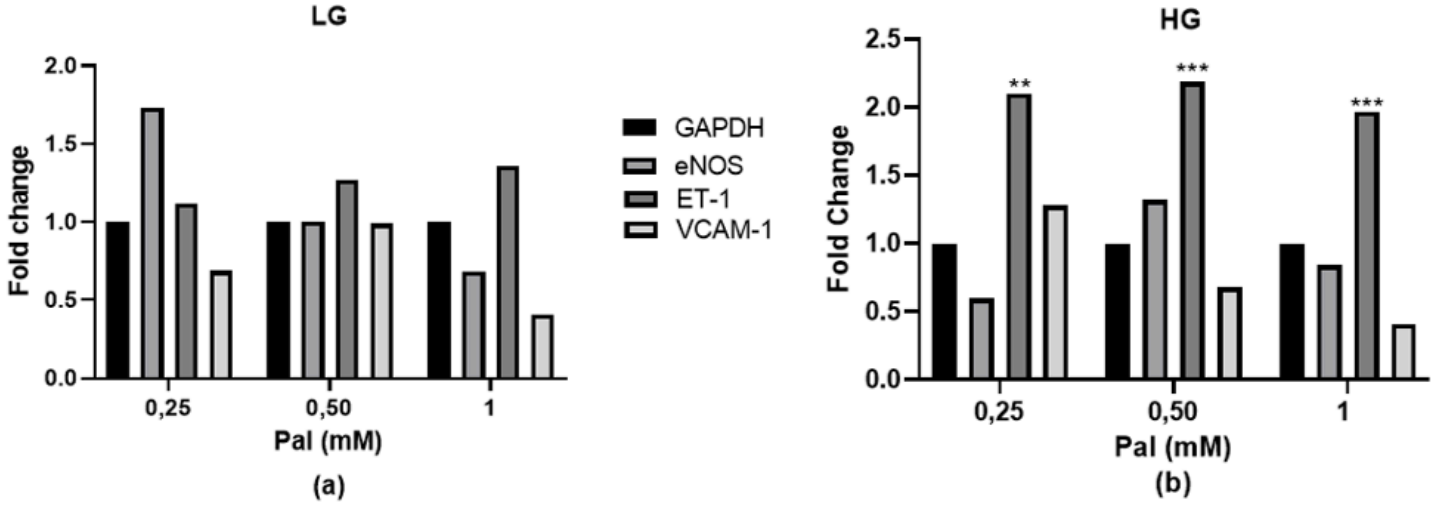


**Figure 3**

Gene expression levels of selected endothelial stress-related markers, including eNOS, ET-1, and VCAM-1, after Pal exposure under (a) LG and (b) HG conditions. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test (n = 3). Pal: palmitic acid; BSA: bovine serum albumin ([2.5% or 5%; use the actual value]); LG: low glucose (1 g/L); HG: high glucose (4.5 g/L). **p < 0.01, ***p < 0.001.

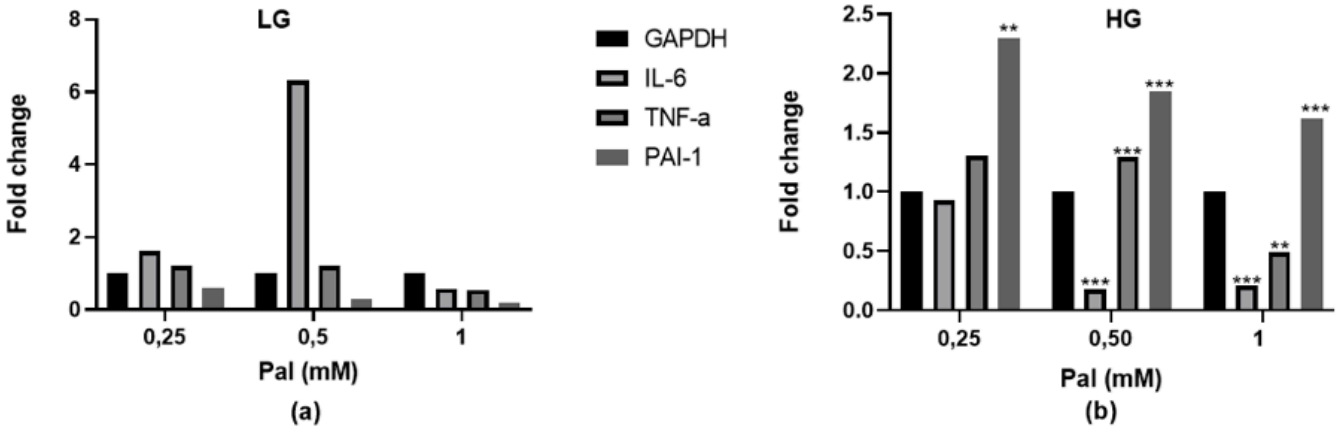



**Figure 4**

Gene expression levels of inflammatory and prothrombotic markers, including IL-6, TNF-α, and PAI-1, after Pal exposure under (a) LG and (b) HG conditions. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test (n = 3). Pal: palmitic acid; BSA: bovine serum albumin ([2.5% or 5%; use the actual value]); LG: low glucose (1 g/L); HG: high glucose (4.5 g/L). **p < 0.01, ***p < 0.001.