

Liraglutide Treatment Reduces Endothelial Endoplasmic Reticulum Stress and Insulin Resistance in Patients With Diabetes Mellitus

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Background—Prior studies have shown that nutrient excess induces endoplasmic reticulum (ER) stress in nonvascular tissues from patients with diabetes mellitus (DM). ER stress and the subsequent unfolded protein response may be protective, but sustained activation may drive vascular injury. Whether ER stress contributes to endothelial dysfunction in patients with DM remains unknown.

Methods and Results—To characterize vascular ER stress, we isolated endothelial cells from 42 patients with DM and 37 subjects without DM. Endothelial cells from patients with DM displayed higher levels of ER stress markers compared with controls without DM. Both the early adaptive response, evidenced by higher phosphorylated protein kinase-like ER eukaryotic initiation factor-2 α kinase and inositol-requiring ER-to-nucleus signaling protein 1 ($P=0.02$, $P=0.007$, respectively), and the chronic ER stress response evidenced by higher C/EBP α -homologous protein ($P=0.02$), were activated in patients with DM. Higher inositol-requiring ER-to-nucleus signaling protein 1 activation was associated with lower flow-mediated dilation, consistent with endothelial dysfunction ($r=-0.53$, $P=0.02$). Acute treatment with liraglutide, a glucagon-like peptide 1 receptor agonist, reduced p-inositol-requiring ER-to-nucleus signaling protein 1 ($P=0.01$), and the activation of its downstream target c-jun N-terminal kinase ($P=0.025$) in endothelial cells from patients with DM. Furthermore, liraglutide restored insulin-stimulated endothelial nitric oxide synthase activation in patients with DM ($P=0.019$).

Conclusions—In summary, our data suggest that ER stress contributes to vascular insulin resistance and endothelial dysfunction in patients with DM. Further, we have demonstrated that liraglutide ameliorates ER stress, decreases c-jun N-terminal kinase activation and restores insulin-mediated endothelial nitric oxide synthase activation in endothelial cells from patients with DM. (*J Am Heart Assoc.* 2018;7:e009379. DOI: 10.1161/JAHA.118.009379.)

Key Words: endothelium • nitric oxide • vascular function

Diabetes mellitus (DM) type 2 is a key public health problem worldwide, predicted to affect more than 500 million people by 2035.¹ DM is a major risk for cardiovascular diseases (CVDs) including coronary heart disease, stroke, peripheral artery disease, heart failure, and atrial fibrillation; and DM was listed as the primary or secondary cause of >200 000 deaths in the United States in 2014.² The

dramatic increase in the incidence of DM has resulted in a critical need for novel therapies to protect the vasculature and prevent the prevalence of cardiovascular disease in DM.

Endothelial dysfunction has been described as a hallmark and a predictor of CVD.^{3–5} However, our understanding the molecular mechanisms underlying insulin resistance and endothelial dysfunction in the vasculature of patients with DM is still uncertain. A greater understanding of the mechanisms that lead to an impairment in endothelial function and the subsequent development of CVDs is necessary to identify new targets for the treatment of CVD in DM.

Over the past decade, the endoplasmic reticulum (ER) has emerged as an important regulator of metabolic processes and recently has been implicated in the pathogenesis of endothelial dysfunction.^{6–8} The ER is a large, membrane-enclosed cellular compartment, critical to the physiologic regulation of many cellular processes, including protein folding, lipid biosynthesis and redox homeostasis.^{9–11} Under physiologic conditions, the ER's protein load and folding

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Clinical Perspective

What Is New?

- We have demonstrated that liraglutide ameliorates endoplasmic reticulum stress and restores insulin-mediated eNOS activation in endothelial cells from patients with diabetes mellitus.

What Are the Clinical Implications?

- This study provides evidence for an acute beneficial effect of liraglutide on endoplasmic reticulum stress and endothelial phenotype in patients with diabetes mellitus.
- Our findings support the possibility that endoplasmic reticulum stress may be a mechanism for the cardiovascular effects of liraglutide in the vasculature, and treatments with endoplasmic reticulum stress inhibitors may be a novel strategy to restore endothelial function in human diabetes mellitus.

capacity are in balance; however, ER overload produces an accumulation of misfolded proteins in the ER, leading to the activation of the unfolded protein response (UPR), a process that is known as ER stress.¹² UPR is initiated by the activation of 3 transmembrane proteins: (1) RNA-dependent protein kinase-like ER eukaryotic initiation factor-2a kinase (PERK); (2) inositol-requiring ER-to-nucleus signaling protein 1 (IRE1 α); and (3) activating transcription factor 6 (ATF6).^{13,14} Acute ER stress activation has protective properties; however, chronic activation of the UPR leads to adverse cellular consequences. Specifically, chronic activation of the UPR promotes increased oxidative stress and inflammation and often results in cell death in endothelial cells.^{15,16} Emerging preclinical and clinical evidence support the notion that pharmacologic modulators of ER stress have therapeutic potential as novel treatments for metabolic diseases.¹⁰ Experimental studies have demonstrated that the glucagon-like peptide 1 (GLP-1) receptor agonist, liraglutide, downregulates high glucose-induced UPR activation and ER stress in cultured endothelial cells.¹⁷ Further, liraglutide improves cardiovascular function in animal models.¹⁸ However, limited translational research has addressed the involvement of ER stress activation in the vasculature, and the contribution of ER stress to endothelial dysfunction in human DM is not known.

In the present study, we explore the activation of ER stress in the vasculature of DM patients, and investigate whether chronic ER stress activation is involved in vascular endothelial dysfunction and insulin resistance in human DM. We hypothesize that ER stress activation could function as a central regulatory node mediating abnormal endothelial phenotype in human DM that might serve as a novel treatment target for vascular protection.

Methods

Beyond the descriptions provided here, data, analytical methods, and study materials will not be available.

Reagents

Insulin (#I9278), Glucose (#G8769), Exendin fragment 9–39 (E7269), Tunicamycin (#11089-65-9) were from Sigma, and liraglutide from American Peptide Company (#46-1-48).

Study Participants

Adult patients with type 2 DM were enrolled from the clinical practices at Boston Medical Center. Controls were enrolled through advertisement or through participation in prior research studies. DM was defined as fasting glucose ≥ 126 mg/dL and glycated hemoglobin A_{1c} $\geq 6.5\%$, or ongoing pharmacologic treatment for type 2 DM. Patients with DM treated with GLP-1 receptor agonists or dipeptidyl peptidase-4 (DPP4) inhibitors were excluded from the study. Control individuals without DM were defined as fasting glucose < 100 mg/dL. Fasting glucose and lipids were measured in the Boston Medical Center Clinical Laboratory.

Vascular Function Testing

We measured brachial artery flow-mediated dilatation in each patient as previously described.^{19,20} Briefly, high-resolution ultrasound was used to measure brachial artery diameter before and 1 minute after induction of hyperemic response by 5-minute cuff occlusion of the upper arm. Doppler flow signals were recorded from the brachial artery before and after cuff occlusion to measure reactive hyperemia.²¹ Afterwards, we measured nitroglycerin-mediated dilatation, which reflects non-endothelium-dependent dilatation.

Fresh Isolation of Human Endothelial Cells

Peripheral venous endothelial cell biopsies were performed as previously described.^{22,23} Briefly, a 20-gauge intravenous catheter was placed in a superficial forearm vein using aseptic technique. Endothelial cells were collected by gentle abrasion of the vessel wall with a 0.018-inch J-wire introduced through the catheter. Endothelial cells were recovered from the wire by centrifugation in a dissociation buffer and plated on poly-L-lysine-coated microscope slides. Once plated, cells were either directly fixed with 4% paraformaldehyde immediately, or fixed after insulin, liraglutide, or exendin stimulation. Slides were then washed with phosphate-buffered saline, fixed, and stored at -80°C until further processing. Acute treatments were up to 1.5 hours to preserve the in vivo phenotype.

Endothelial Cell Culture and Treatment

Human aortic endothelial cells (HAECs) were purchased from Lonza and maintained with endothelial growth medium-2 containing 5 mmol/L glucose in a standard incubator (37°C, 5% CO₂). Cells from passages 4 to 7 and 90% confluence were used for the experiments after being starved for 3 hours in serum-free medium. For drug treatment, cultured endothelial cells were incubated with 100 nmol/L liraglutide for 15 minutes, which is within the therapeutic range achieved in humans with 1.8 µg/day treatment with liraglutide, and a standard dose used in different types of endothelial cells including human umbilical vein endothelial cells,¹⁷ HAECs,²⁴ and cardiac microvascular endothelial cells.²⁵ To investigate the role of GLP-R1, the GLP-1R inhibitor Exendin fragment (9–39) (10 nmol/L) was incubated for 30 minutes before liraglutide treatment, as previously described.²⁵

Western Blot Analysis

After drug treatment, endothelial cells were washed in phosphate-buffered saline, trypsinized, and the pellet was lysed in ice-cold lysis buffer (20 mmol/L Tris-HCl, pH 7.4, 150 Na₂EDTA, 1% Triton) and protease/phosphatase inhibitor cocktail (following the instructions provided by the supplier). Protein content in the samples was quantified by bicinchoninic acid protein assay (Pierce), and 20 to 35 µg protein was subjected to electrophoresis in 4% to 15% sodium dodecyl sulfate–polyacrylamide gels, under reducing conditions, and then transferred to a polyvinylidene difluoride membrane using the Bio-Rad Transblot Turbo Transfer System. Membranes were blocked with 3% bovine serum albumin for 1 hour at room temperature, and incubated overnight with the respective primary antibody at 4°C (1:1000–1:5000). Blots were probed with anti-eNOS (BD Transduction, #610296); antiphospho-eNOS (Ser1177, #9571), CCAAT/enhancer binding protein homologous protein (#28945) and IRE1α from Cell Signaling (#3294) and p-IRE1α (ab124945) from Abcam. Immunoblots were normalized to monoclonal β-actin antibody (Sigma-Aldrich, #A1978). Horseradish peroxidase–conjugated secondary antibodies were incubated for 1 hour at room temperature (antirabbit #HAF008 or antimouse #HAF007, R&D Systems). Immune complexes were visualized with Amersham ECL Western Blotting Detection Reagents. Membranes were stripped with Restore Western Blot Stripping Buffer (ThermoFisher) for 10 minutes at 37°C, and reprobed with either total protein or housekeeping proteins to verify equal loading. Resulting bands were quantified by densitometry.

Quantitative Immunofluorescence Staining

Fixed samples were thawed and rehydrated with 50 mmol/L glycine in phosphate-buffered saline. Cells were permeabilized

with 0.1% Triton X-100, and nonspecific binding sites were blocked in 0.5% bovine serum albumin. Slides were incubated overnight at 4°C with primary antibodies (1:100) against the following targets: phospho-eNOS (Ser1177, Abcam #ab184154), phospho-c-jun N-terminal kinase (JNK) (Thr183/Tyr185, Cell Signaling #9255), CHOP (Cell Signaling #2895); pIRE1 (Abcam #ab48187); IRE1 (Novus Biologicals #NB100-2324); p-PERK (SantCruz #sc-32577); PERK (ThermoFisher #PA5-38811); ATF4 (Abcam #ab50546); ATF6 (Abcam #122897); GRP78 (Abcam #108615). All cells were also stained with anti-von Willebrand factor antibody (1:200) for endothelial cell identification. Slides were incubated with corresponding Alexa Fluor-488 and Alexa-Fluor-594 antibodies (1:200) for 45 minutes at 37°C. Coverslips were mounted with Vectashield containing 4',6-diaminido-2-phenylindole for nuclear identification. Slides were imaged with a confocal microscope (Leica SP5) at 63× magnification. All images were captured at the same exposure time and corrected for background fluorescence. Fluorescence intensity was quantified with NIS Element AR Software. The fluorescence intensity of at least 20 cells from each patient and protein of interest was averaged and normalized to the intensity of HAEC, simultaneously stained to adjust for any variation in antibody and staining conditions. We stain the same batch and passage of cultured aortic endothelial cells to normalize for each protein of interest. Intensity quantification was performed blinded to participant identity and DM status.

Statistical Analysis

Statistical analyses were performed using SPSS version 20.00 (IBM Corporation, Armonk, NY). Data are expressed as mean±standard deviation, unless otherwise noted. Variables were evaluated for normality by the Shapiro-Wilk test. Participants with DM and controls were compared using the unpaired Student *t* test for normally distributed continuous variables or Mann-Whitney *U* test for those not normally distributed; chi-squared tests were used for discrete variables. Paired (pre/post) samples were compared using the paired Student *t* test for normally distributed continuous variables or the Wilcoxon signed rank test for variables not normally distributed.

Spearman correlation coefficients were used to correlate measures of vascular function and expression of proteins of interest. A 2-tailed *P*<0.05 was considered to be statistically significant. To understand the magnitude of the differences found, the estimated effect size (Cohen's *d*) was calculated using an online tool that takes into account unpaired and paired data (https://memory.psych.mun.ca/models/stats/efect_size.shtm). Cohen's *d* effect size >0.5 was considered a moderate difference, and >0.8 was considered a large difference.²⁶

Table. Clinical and Vascular Characteristics

	Nondiabetic (n=37)	Diabetic (n=42)	P Value
Clinical characteristics			
Age, y	49±8	51±9	0.34
Female sex, %	32	55*	0.048
Black race, %	73	76	0.795
Body mass index, kg/m ²	30.9±7.9	37.3±8.9*	0.001 [†]
Total cholesterol, mg/dL	189±44	190±51	0.942
LDL cholesterol, mg/dL	125±31	119±41	0.471
HDL cholesterol, mg/dL	52±15	44±13*	0.06 [†]
Triglycerides, mg/dL	93.9±43	133±86*	0.07 [†]
Fasting glucose, mg/dL	86.7±11	190±99*	<0.001 [†]
Hemoglobin A _{1c} , %	5.4±0.3	8.5±2.5*	<0.001 [†]
Systolic blood pressure, mm Hg	133±17	133±17	0.777 [†]
Diastolic blood pressure, mm Hg	83±13	81±13	0.383 [†]
Hypertension, %	13	67*	0.000 [†]
Smoking, %	38	38	0.981
Antiplatelet therapy, %	3	17*	0.038
Lipid-lowering therapy, %	8	45*	<0.001
ACE inhibitor or ARB therapy, %	5	50*	<0.001
Metformin, %	0	69*	<0.001
Sulfonylureas, %	0	24*	0.002
Insulin therapy, %	0	26*	0.001
Vascular function			
Baseline diameter, mm	4.20±1.1	4.32±0.8	0.93 [†]
Baseline flow, mL/min	150±149	172±104	0.079 [†]
Hyperemic flow, mL/min	1206±797	1055±594	0.461 [†]
Flow-mediated dilation, %	9.8±5.7	6.1±3.3*	0.03 [†]
Flow-mediated dilation, mm	0.41±0.05	0.25±0.03	0.004 [†]
Nitroglycerin-mediated dilation	11.7±7.6	11.7±5.9	0.975
Baseline flow velocity	15.2±10.6	22.3±34.5	0.185 [†]
Hyperemic flow velocity	110.8±52.7	91.8±70.9	0.118 [†]

Data are expressed as mean±SD. ACE indicates angiotensin-converting enzyme; ARB, angiotensin receptor blocker; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

*A two-tailed $P<0.05$ was considered to be statistically significant.

[†]Mann-Whitney test to compare patients with diabetes mellitus and controls; others by chi-square test or t test as appropriate.

Study Approval

The study protocol was approved by the Boston Medical Center Institutional Review Board (H-26605), and all participants provided written informed consent before inclusion in the study.

Results

Study Participants and Vascular Function

We enrolled 42 subjects with DM and 37 subjects without DM of similar age and sex. The clinical characteristics and measures of vascular function are shown in the Table. As expected, patients with DM had clinical parameters consistent with metabolic abnormalities including higher fasting glucose, higher body mass index, and higher hemoglobin A_{1c}. Likewise, patients with DM had lower low-density lipoprotein levels, likely reflecting the use of cholesterol-lowering medications. Endothelial-dependent flow-mediated dilatation of the brachial artery was significantly lower in the patients with DM, consistent with the presence of endothelial dysfunction. There were no differences in nitroglycerin-mediated dilatation among groups, suggesting the absence of smooth muscle dysfunction.

DM Is Associated With Elevated ER Stress in the Vascular Endothelium

To characterize vascular ER stress, we studied the acute and chronic ER stress response in freshly isolated venous endothelial cells from patients with DM and patients without DM. We first evaluated total protein expression of the 3 ER membrane-localized proteins considered to be “acute sensors” of ER stress: IRE1 α , PERK, and ATF6. As shown in Figure S1, expression of these proteins was similar between the patients without DM and participants with DM.

To evaluate IRE1 α and PERK activation, we performed quantitative immunofluorescence to measure the phosphorylation at the activation sites (Serine (Ser) 724 and Threonine (Thr) 981, respectively). Endothelial cells from patients with DM had elevated levels of activated IRE1 α (** $P=0.007$) and PERK ($*P=0.02$) compared with controls without DM, suggesting an elevated ER stress response in patients with DM (Figure 1). Notably, we found a positive correlation between IRE1 α and PERK activation in the endothelium ($r=0.54$, $*P=0.03$). Interestingly, higher levels of activated IRE1 α were associated with lower flow-mediated dilatation, consistent with a link between ER stress activation and impaired endothelial vasodilator function ($r=0.53$, $*P=0.02$). (Figure 1C). Although acute ER stress activation could be beneficial for the cell, chronic ER stress results in an

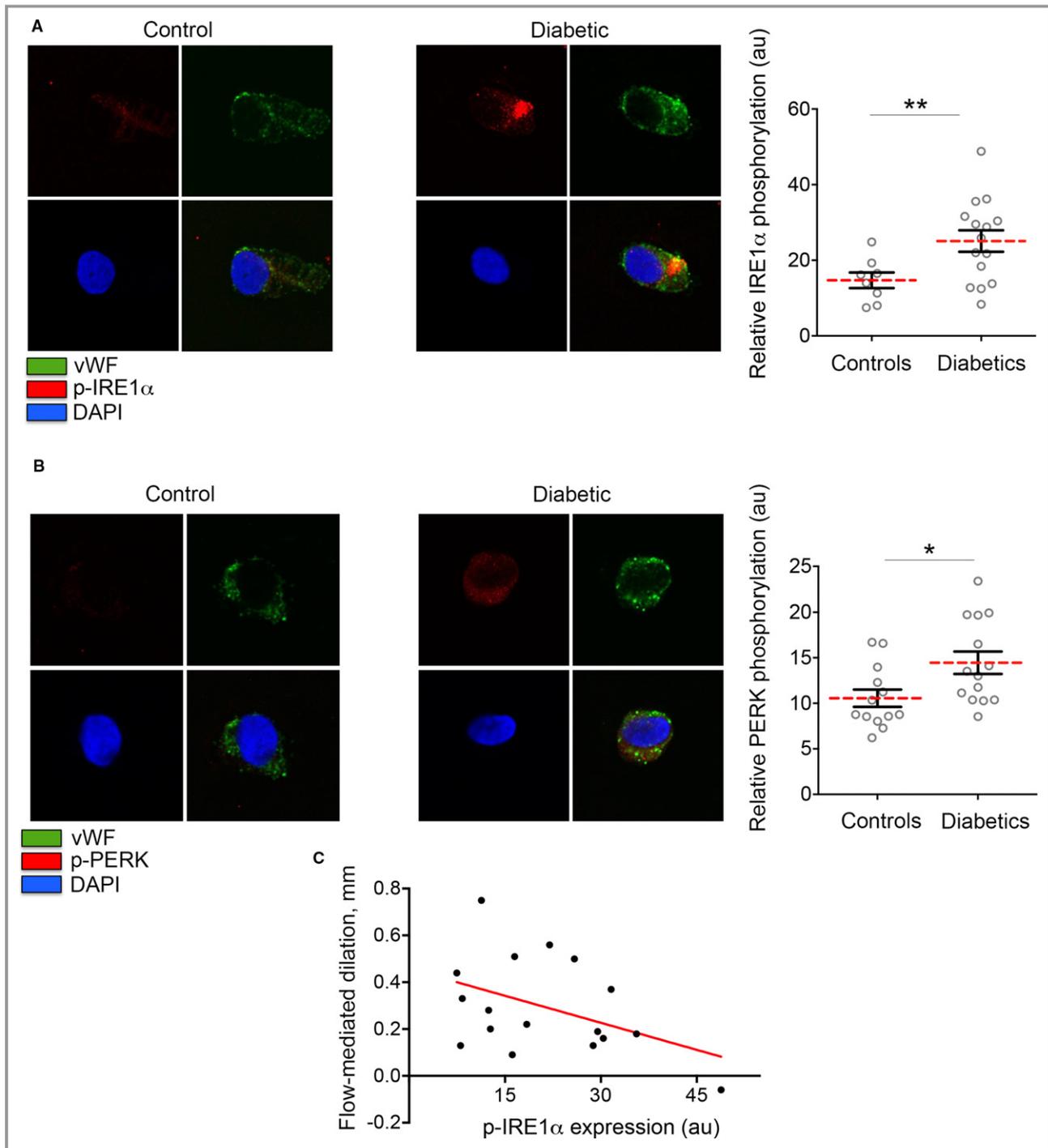


Figure 1. Activation of the unfolded protein response in vascular endothelial cells of patients with diabetes mellitus (DM). Venous endothelial cells patients with and without DM were freshly isolated as described in the Materials and Methods section of this article. Endothelial cells were identified by von Willebrand factor staining and nuclear morphology. A, Inositol-requiring ER-to-nucleus signaling protein 1 (IRE1 α) activation was evaluated as IRE1 α phosphorylation at Ser 724. Representative cell from a patient with DM (right) shows higher activation of IRE1 α when compared with a representative cell from a controls without DM (left). Pooled data showed that the IRE1 α phosphorylation at its activatory site is enhanced in patients with DM (n=15) compared with the controls without DM (n=8) (** $P=0.007$, Cohen's $d=1.08$). B, RNA-dependent protein kinase-like ER eukaryotic initiation factor-2a kinase (PERK) activation was evaluated as PERK phosphorylation at Thr 981. Representative cells from patients without DM (left) and patients with DM (right) are shown. Pooled data showed that PERK phosphorylation is higher in patients with diabetes mellitus compared with controls without diabetes mellitus (n=14/13, * $P=0.020$, Cohen's $d=0.954$). Variables were compared using the t test. C, Higher IRE1a activation in freshly isolated venous endothelial cells from patients were associated with lower mediated dilation ($r=0.53$, * $P=0.02$). DAPI indicates 4',6-diamidino-2-phenylindole.

increased translation of ATF4, which promotes the upregulation of genes involved in oxidative stress and apoptosis. Chronic ER stress results in an elevated expression of the proapoptotic transcription factor CHOP.²⁷ Endothelial cells from patients with DM showed higher CHOP expression compared with controls without DM ($*P=0.02$) (Figure 2), suggesting that DM is associated with a chronic ER stress activation and the activation of apoptotic signaling pathways in the vascular endothelium. Moreover, higher CHOP expression was associated with higher activation of IRE1 α ($r=0.82$, $*P=0.04$) and PERK ($r=0.59$, $*P=0.03$).

Collectively, our findings suggest that DM is associated with an aberrant activation of ER stress in the vascular endothelium.

ER Stress Activation Induces Endothelial Dysfunction

A number of experimental studies in cultured endothelial cells and in animal models suggest a role for ER stress in endothelial dysfunction.⁷ We were interested in whether acute stimulation of ER stress would produce similar effects in a healthy endothelium as the phenotype observed in the endothelium of patients with DM. To study the relationship between ER stress and endothelial dysfunction in our model, we conducted in vitro studies with commercially available HAECs in the presence and absence of an ER stress activator, tunicamycin. Tunicamycin treatment-induced ER stress as demonstrated by enhanced activation of IRE-1 α and increased expression of CHOP (Figure S2A). To investigate the importance of ER stress activation in endothelial dysfunction and insulin resistance, we treated HAECs with insulin in the presence or absence of tunicamycin and quantified eNOS phosphorylation at its activation site, Ser 1177. As shown in Figure S2B, ER stress activation by tunicamycin treatment impairs insulin-induced eNOS phosphorylation at its activation site in endothelial cells in culture.

GLP-1 Analogue, Liraglutide, Reduces Acute ER Stress in EC from Patients With DM

To determine whether ER stress contributes to endothelial dysfunction in the setting of DM, we utilized liraglutide, a GLP-1 analogue, which has been demonstrated to reduce ER stress in cultured endothelial cells exposed to high glucose¹⁷ and to exert favorable effects on cardiovascular function in both preclinical and clinical studies.^{18,28,29}

To gain insight into the potential role of liraglutide in the vasculature of patients with DM, we evaluated the acute effect of liraglutide on ER stress activation. We treated freshly isolated endothelial cells from patients with DM, treated with liraglutide for 45 minutes, and evaluated acute ER stress

activation by PERK and IRE1 α activation. As shown in Figure S3, acute treatment with liraglutide did not induce a change in PERK activation; however, we observed a decrease not only in IRE1 α activation but also in the activation of its downstream target, JNK (Figure 3). Interestingly, we have previously demonstrated the important role of JNK in endothelial dysfunction and insulin resistance in human DM.³⁰

Collectively, the findings in freshly isolated endothelial cells from patients with DM suggest that liraglutide reduces acute ER stress activation in the endothelium of diabetic patients with DM.

Liraglutide Restores Insulin Response and Endothelial Function in Patients With DM

To determine whether liraglutide improves endothelial function in DM, we first studied the effect of liraglutide on eNOS activation. GLP-1 analogues have been described previously to induce a transient phosphorylation of eNOS at Ser1177.³¹ As shown in Figure S4, and concordant with our previous reports, patients with DM had higher basal levels of activated eNOS at Ser1177.³² We found that liraglutide treatment induced an increase in eNOS phosphorylation at its activation residue (Ser1177) in endothelial cells isolated from patients without DM. However, liraglutide did not promote eNOS activation in endothelial cells freshly isolated from patients with DM.

Next, we evaluated whether liraglutide and ER stress inhibition ameliorate endothelial dysfunction by studying insulin-mediated eNOS activation in endothelial cells from patients with DM in the presence and absence of the ER stress inhibitor. As shown in Figure 4 and consistent with our previous reports, insulin stimulation did not induce eNOS phosphorylation in patients with DM.³² Pretreatment with liraglutide for 15 minutes before insulin stimulation (10 nmol/L, 30 minutes) restored eNOS activation and endothelial function in endothelial cells isolated from patients with DM ($n=10$; $P=0.019$).

Collectively, our results suggest that liraglutide treatment restores vascular insulin sensitivity and this effect was independent of a direct activation of eNOS by the GLP-1 analogue.

Liraglutide-Induced Amelioration of Endothelial Dysfunction in DM is Dependent on GLP-1 Receptor

Liraglutide is a GLP-1 receptor agonist described to activate different signaling pathways via GLP-1R-dependent or independent signals.¹⁸ To determine if the effects of liraglutide on the vascular endothelium are mediated through GLP-1R, freshly isolated endothelial cells from patients with DM were

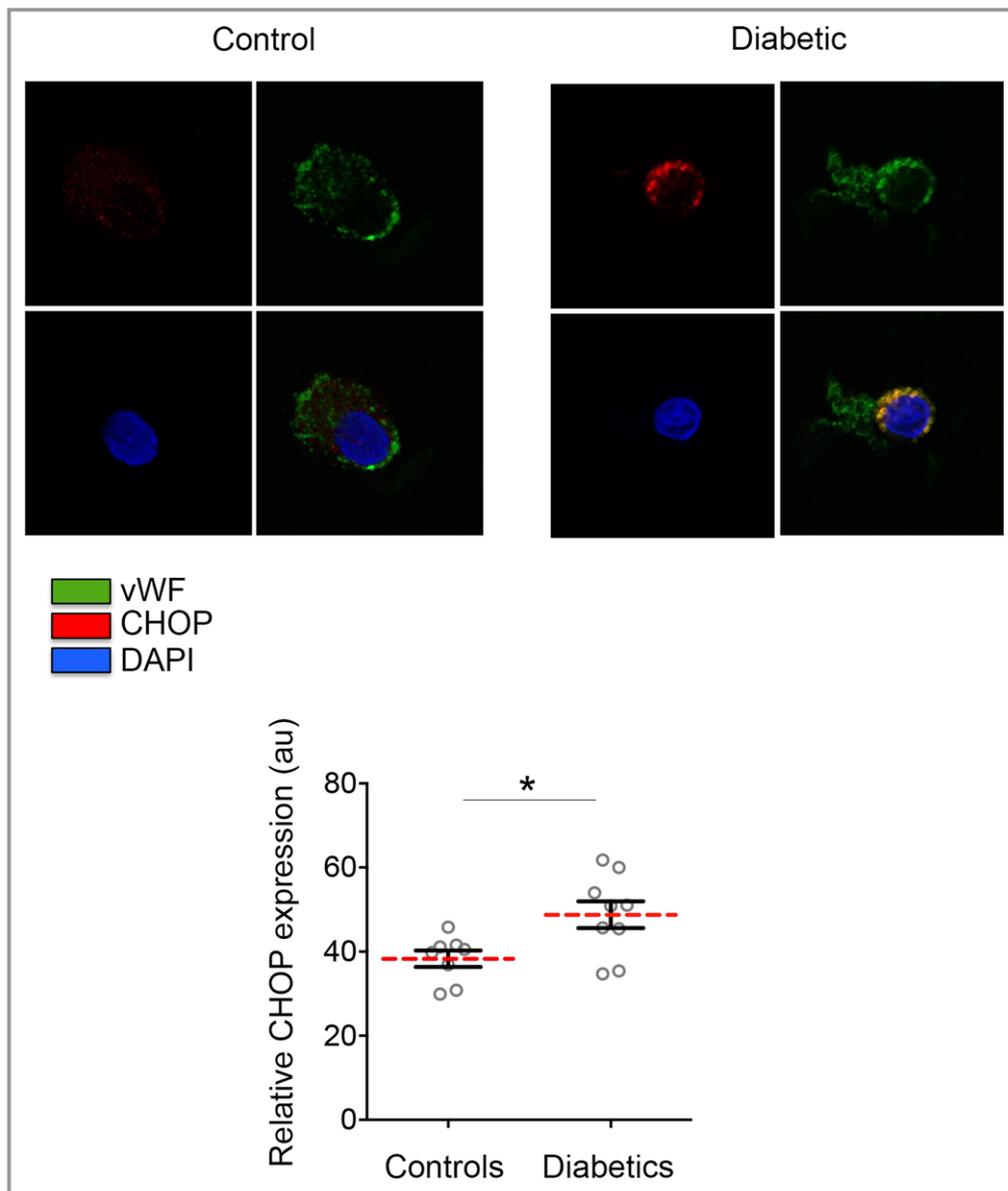


Figure 2. Diabetes mellitus (DM) is associated with a chronic activation of endoplasmic reticulum (ER) stress in the vasculature. Venous endothelial cells from patients with and without DM were freshly isolated and identified by von Willebrand factor staining and nuclear morphology. Representative cell from a diabetic patient (right) shows higher expression of the chronic ER stress marker CCAAT/enhancer binding protein homologous protein (CHOP) compared with a representative cell from a control without DM (left). Pooled data showed that CHOP is higher expressed in endothelial cells from patients with DM ($n=9$) when compared with patients without DM ($n=8$) ($*P=0.015$; Cohen's $d=1.322$). Variables were compared using the t test. DAPI indicates 4',6-diamidino-2-phenylindole.

pretreated for 30 minutes with the GLP-1R antagonist exendin fragment (9–39) before liraglutide and insulin sequential stimulation. Exendin fragment (9–39) abolished liraglutide-driven restoration of insulin sensitivity in endothelial cells from patients with DM ($n=5$; $P=0.02$) (Figure 5).

Our findings indicate that the beneficial effect of liraglutide on DM induced ER stress in endothelial cells is dependent on GLP-1R.

Discussion

In the present study, we demonstrated the involvement of ER stress in endothelial dysfunction and insulin resistance in patients with DM. Using the venous endothelial biopsy procedure, we first demonstrated an enhanced and aberrant ER stress activation in the vasculature of diabetic patients when compared with controls without DM. Further, we found that

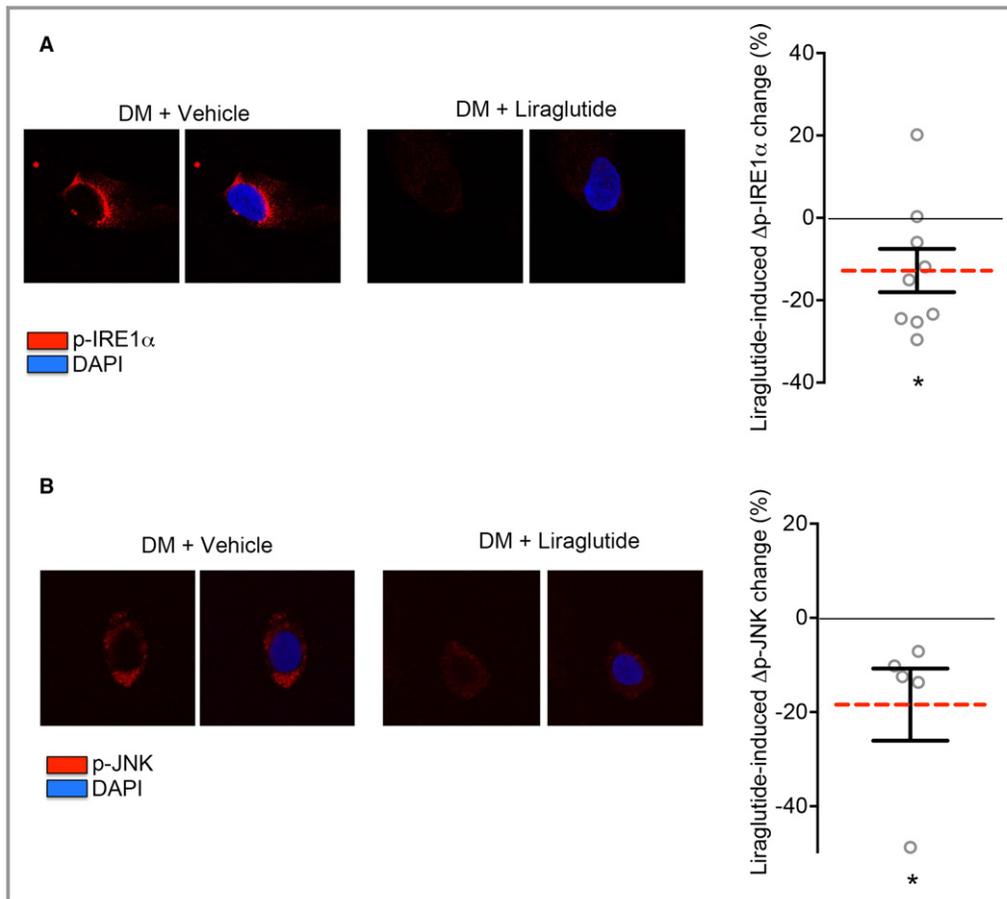


Figure 3. Liraglutide reduces endoplasmic reticulum (ER) stress acute activation and the subsequent inflammatory response in endothelial cells from patients with diabetes mellitus (DM). Venous endothelial cells were isolated from patients with DM and acute ER stress activation was studied in the presence and absence of liraglutide. A, Inositol-requiring ER-to-nucleus signaling protein 1 (IRE1 α) phosphorylation was studied in endothelial cells from patients with DM in the presence of liraglutide (0–100 nmol/L). Representative cells from the same patient are shown. Pooled data showed that the IRE1 α activation decrease after liraglutide treatment (n=9; * P =0.01; Cohen's d =1.125). B, phospho-c-jun N-terminal kinase (JNK) activation was evaluated as JNK phosphorylation at Thr183/Tyr185. Representative cells from a patient with DM treated with liraglutide (0–100 nmol/L) are shown. Pooled data demonstrated a reduction in JNK activation in endothelial cells from patients with DM in the presence of liraglutide (n=5; * P =0.025; Cohen's d =1.563). Variables were compared using the paired t test. DAPI indicates 4',6-diamidino-2-phenylindole.

IRE1 α activation was associated with endothelial dysfunction in patients with DM. We demonstrated that ER stress activation induced insulin resistance in commercially available endothelial cells, recapitulating the phenotype we observed in the endothelial cells from patients with DM. Furthermore, the GLP-1 analogue liraglutide ameliorated ER stress and JNK activation in endothelial cells from patients with DM. Liraglutide treatment also restored insulin-mediated eNOS activation in endothelial cells freshly isolated from patients with DM in a GLPR-1 dependent manner. Taken together, our findings suggest that aberrant ER stress activation contributes to insulin resistance and endothelial dysfunction in the vasculature of patients with DM and that ER stress inhibition has potential as a strategy to improve vascular health in patients with DM.

Prior studies have demonstrated that ER overload produces an accumulation of misfolded proteins, UPR activation, and ER stress, which contributes to the development and progression of chronic disorders including neurodegeneration, atherosclerosis, liver disease, and cancer, among others.^{33,34} In the past decade, the ER has been identified as an important regulator of metabolic processes,³⁵ and Gargalovic et al were among the first to directly link ER stress to endothelial disturbances.³⁶ Experimental studies in cultured endothelial cells have demonstrated that chemically induced ER stress causes endothelial dysfunction and insulin resistance.^{37,38} Further, a number of studies in animal models have provided additional insights into the molecular mechanisms linking ER stress induction and endothelial dysfunction.^{39,40} Nevertheless,

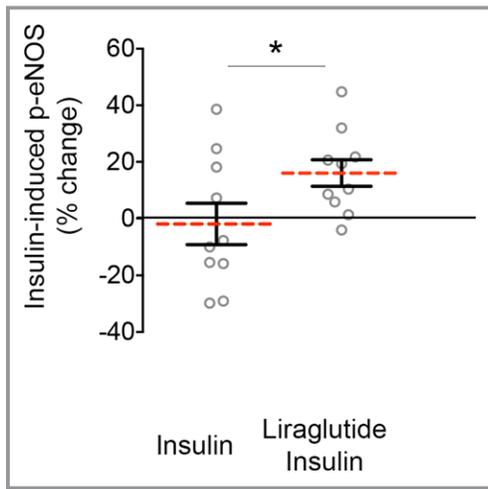


Figure 4. Liraglutide treatment restores endothelial function and insulin response in endothelial cells from patients with diabetes mellitus (DM). Freshly endothelial cells isolated from patients with DM were treated with 0 or 10 nmol/L insulin for 30 minutes in the absence or in the presence of liraglutide and then fixed. Endothelial nitric oxide (eNOS) activation was evaluated as eNOS phosphorylation at Ser1177. Pooled data demonstrated that liraglutide restores insulin-induced eNOS phosphorylation in endothelial cells isolated from patients with DM ($n=10$, $*P=0.041$; Cohen's $d=0.752$). Variables were compared using the paired t test.

limited translational research has addressed the impact of ER stress in the vasculature,^{41,42} and the contribution of ER stress to endothelial dysfunction in human DM is not known. Our finding that ER stress is aberrantly activated in endothelial cells from patients with DM lends additional support for the concept that ER stress plays a role in vascular endothelial dysfunction in patients with DM.

Currently, much of the data on the regulation of ER stress in human metabolic diseases and endothelial function have been focused on the involvement of the IRE1 α and PERK branches of the UPR.³⁵ In this study, we have demonstrated an enhanced activation of acute ER stress markers IRE1 α and PERK, and a lack of differential expression in ATF6 in patients with DM when compared with controls without DM. However, further studies focused in the activation or subcellular location of ATF6 would be necessary to discard the activation of ATF6 branch in the vasculature of patients with DM. During ER stress, IRE1 α and PERK-ATF4 trigger the activation of JNK, a key inflammatory signaling pathway.^{43–45} Interestingly, we have previously reported an enhanced activation of JNK in patients with DM, that contributes to reduced nitric oxide production and lower flow-mediated dilatation.³⁰ In the present study, we develop evidence that ER stress pathways may be an important regulator of JNK activation in the vasculature in patients with DM by demonstrating increased activation of both the IRE1 α and

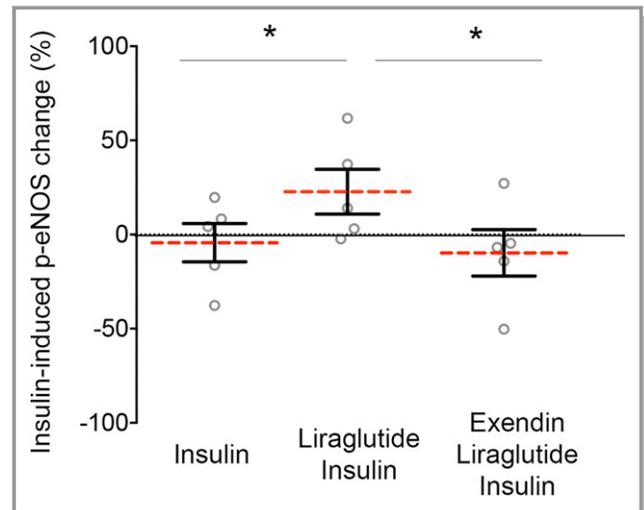


Figure 5. Liraglutide effect on endothelial function in diabetes mellitus (DM) is dependent on the glucagon-like peptide 1 receptor (GLP-1R). Venous endothelial cells from patients with DM were freshly isolated as described in the Materials and Methods section of this article and were treated with GLP-1R antagonist, exendin fragment (9–39) (10 nmol/L, 30 minute), before the addition of liraglutide (100 nmol/L, 15 minutes) and insulin (10 nmol/L 30 minutes) sequential treatment. Pooled data demonstrated that exendin (9–39) impaired liraglutide-driven restoration of insulin sensitivity in diabetic patients ($n=5$; $*P=0.020$; Cohen's $d=1.676$). Variables were compared using the paired t test.

PERK pathways. Prolonged ER stress results in the induction of the pro-apoptotic transcription factor CHOP, regarded as a key mediator of cell death, endothelial impairment, and disease in response to ER overload.^{27,46–48} In the present study, we showed an enhanced expression of CHOP in the vascular endothelium of patients with DM when compared with controls without DM. Furthermore, we found that CHOP abundance was associated with UPR activation in the vasculature.

GLP-1 is a gut hormone secreted in a nutrient-dependent manner that stimulates insulin secretion and inhibits glucagon secretion, thereby reducing postprandial glycemia. Native GLP-1 has limited therapeutic potential because it is rapidly degraded by DPP4, and truncated GLP-1 is unable to interact with its receptor and exert its function. Two strategies have been developed to sustain GLP-1-mediated effects over a longer period: inhibition of DPP4 and the development of GLP-1 receptor agonists.⁴⁹ Clinical trials have investigated the efficacy of agents altering GLP-1 signaling in DM to reduce cardiovascular complications.^{50,51} DPP4 inhibitors were predicted to have beneficial effects on the cardiovascular system; however, clinical trials with several DPP4 inhibitors have not shown reduction of cardiovascular events.^{52–55} However, there is evidence that GLP-1 receptor agonists have cardiovascular protective effects.^{50,56,57} In the recent LEADER (Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results) trial, liraglutide significantly

reduced the risk of the 3 major adverse cardiovascular events (death from cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke) in patients with DM who were at high risk for cardiovascular events.⁵⁸ Furthermore, the addition of liraglutide to antidiabetic therapies improved several cardiovascular risk markers in clinical studies.^{59–65} However, the mechanism by which liraglutide confers cardiovascular benefits has not been fully elucidated. Experimental studies have demonstrated that GLP-1 agonists have anti-inflammatory effects on endothelial cells. Treatment with the GLP-1 receptor agonist liraglutide inhibited tumor necrosis factor- α , intracellular adhesion molecule-1 and vascular cell adhesion molecule-1 in human endothelial cells.^{66,67} In animal models, liraglutide has been shown to reduce serum levels of several inflammatory markers such as C-reactive protein, interleukin-6, tumor necrosis factor- α and the plasmin plasminogen activator inhibitor-1.⁶⁸

Interestingly, liraglutide was recently shown to downregulate ER stress in cultured endothelial cells and in animal diabetic models.^{17,69,70} In the present study, we provide evidence that liraglutide reduced acute ER stress activation (IRE1 α) and JNK phosphorylation in endothelial cells freshly isolated from patients with DM. A previous study in patients with DM showed a trend in liraglutide-mediated endothelium-dependent vasodilation.⁷¹ Here, we demonstrate that liraglutide has the potential to restore insulin action in the endothelium of patients with DM, probably due to the lower variability in the response in our ex vivo study. Moreover, in the present study we demonstrate that the beneficial effect on the vasculature is dependent on the GLP-1 receptor.

Some limitations of our study should be addressed. This study was performed in venous endothelial cells; arterial endothelial cells may be more directly relevant to the development of cardiovascular disease. However, both venous and arterial endothelial cells are exposed to similarly abnormal metabolic abnormalities in DM. Patients with DM have multiple systemic risk factors that likely contribute together to the endothelial phenotype observed, including differences in lipid profile. Prior studies have shown that administration of GLP-1 agonists has an impact on lipid homeostasis.^{72–75} Liraglutide administration appears to have beneficial effects on plasma lipids and lipoproteins, including a reduction in total cholesterol, triglycerides, and low-density lipoprotein cholesterol, and an increase in high-density lipoprotein cholesterol.^{76–78} Moreover, we have demonstrated that intravenous lipid infusion induces ER stress in endothelial cells.⁴¹ Additional studies will be required to determine the complete set of regulators of ER stress involved in its abnormal activation that lead to endothelial dysfunction and cardiovascular events in patients with DM.

Although recent trials have described the beneficial role of novel drugs in the treatment of DM in reducing the risk of

cardiovascular complications in patients with DM, the mechanism remains incompletely elucidated. Our findings provide evidence for an acute beneficial effect of liraglutide on endothelial phenotype including ER stress activation in cells isolated from patients with DM. Our work supports further studies to determine whether vascular ER stress activation may be a mechanism for the cardiovascular benefit of liraglutide and a therapeutic target in DM.

Author Contributions

RBR performed the experiments, analyzed the data and wrote the manuscript. RMW, BF and DK collected human samples. MH recruited participants. MMS performed vascular function analysis in patients. JLF and JZ contributed to review the manuscript. NMH directed the study and reviewed the manuscript.

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Disclosures

None.

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