

Intravenous Lipid Infusion Induces Endoplasmic Reticulum Stress in Endothelial Cells and Blood Mononuclear Cells of Healthy Adults

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Background—Endoplasmic reticulum (ER) stress and the subsequent unfolded protein response may initially be protective, but when prolonged, have been implicated in atherogenesis in diabetic conditions. Triglycerides and free fatty acids (FFAs) are elevated in patients with diabetes and may contribute to ER stress. We sought to evaluate the effect of acute FFA elevation on ER stress in endothelial and circulating white cells.

Methods and Results—Twenty-one healthy subjects were treated with intralipid (20%; 45 mL/h) plus heparin (12 U/kg/h) infusion for 5 hours. Along with increased triglyceride and FFA levels, intralipid/heparin infusion reduced the calf reactive hyperemic response without a change in conduit artery flow-mediated dilation consistent with microvascular dysfunction. To investigate the short-term effects of elevated triglycerides and FFA, we measured markers of ER stress in peripheral blood mononuclear cells (PBMCs) and vascular endothelial cells (VECs). In VECs, activating transcription factor 6 (ATF6) and phosphoinositol requiring kinase 1 (pIRE1) proteins were elevated after infusion (both $P < 0.05$). In PBMCs, ATF6 and spliced X-box-binding protein 1 (XBP-1) gene expression increased by 2.0- and 2.5-fold, respectively (both $P < 0.05$), whereas CHOP and GADD34 decreased by $\approx 67\%$ and 74% , respectively (both $P < 0.01$). ATF6 and pIRE1 protein levels also increased (both $P < 0.05$), and confocal microscopy revealed the nuclear localization of ATF6 after infusion, suggesting activation.

Conclusions—Along with microvascular dysfunction, intralipid infusion induced an early protective ER stress response evidenced by activation of ATF6 and IRE1 in both leukocytes and endothelial cells. Our results suggest a potential link between metabolic disturbances and ER stress that may be relevant to vascular disease. (*J Am Heart Assoc.* 2016;5:e002574 doi: 10.1161/JAHA.115.002574)

Key Words: endoplasmic reticulum stress • endothelium • free fatty acids • leukocyte

Free fatty acids (FFAs) are elevated in obesity and type 2 diabetes mellitus and are associated with hypertension, insulin resistance, and atherosclerosis.¹ More specifically, increased plasma levels of FFA either postprandial or after lipid infusion result in endothelial dysfunction in healthy subjects.^{2–4} However, the exact mechanisms linking FFA with

vascular disease remain incompletely understood. Previous studies have shown that FFA induce production of reactive oxygen species (ROS) and proinflammatory cytokines and impair nitric oxide bioactivity in the vascular wall.^{1,2,5} In macrophages and endothelial cells, saturated FFAs activate nuclear factor kappa B (NF- κ B) through Toll-like receptor 4 (TLR4) and promote inflammation and endothelial dysfunction.⁶ Recent studies point to endoplasmic reticulum (ER) stress as an important mechanism for mononuclear cell activation by FFA.^{7–11}

The ER is responsible for the synthesis and assembly of proteins and contributes to calcium homeostasis and lipid biosynthesis.¹² Cellular stress disrupts ER function and leads to the accumulation of misfolded or unfolded proteins and activation of 3 molecular sensors collectively known as the unfolded protein response.¹³ These include protein kinase-like ER kinase (PERK), inositol requiring kinase 1 (IRE1), and activating transcription factor 6 (ATF6). PERK phosphorylates eukaryotic initiation factor alpha (eIF2 α), which suppresses protein translation, and induces activating transcription factor

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4 (ATF4).¹³ IRE1 functions as an endoribonuclease leading to splicing and production of the transcription factor X-box-binding protein 1 (XBP-1).¹⁴ ATF6 translocates to the Golgi, where it is cleaved to an active transcription factor (ATF6 α). All 3 pathways, particularly ATF6 and IRE1, are involved in an early adaptive response that tends to alleviate ER stress. Prolonged or more-pronounced ER stress induces apoptosis through ATF4 and CCAAT/enhancer-binding protein (CHOP) and through activation of apoptosis signal-regulating kinase 1 (ASK1) and c-Jun N-terminal kinase (JNK) by IRE1 (Figure 1).^{8,13,15}

Previous experimental and human studies suggest that metabolic disturbances trigger ER stress. For example, ER stress is induced in cultured endothelial and β -pancreatic cells exposed to fatty acids, oxysterols, and oxidized low-density lipoprotein (LDL) and in macrophages in lipid-rich atherosclerotic plaques obtained at autopsy.^{8,9,16–18} Expression of ER stress markers is increased in adipose tissue and endothelial cells from obese humans^{19–21} and in leukocytes from patients with metabolic syndrome and type 2 diabetes mellitus.^{22,23} Previous human studies of ER stress, however, were mainly cross-sectional in design and examined patients with chronic conditions. The present intervention study investigated early activation of ER stress in humans. We recruited 21 healthy volunteers and used a well-established intralipid/heparin infusion protocol to study the same individuals before and after induction of ER stress, reducing the effects of confounding risk factors.

Materials and Methods

Study Subjects

We recruited 21 healthy volunteers by advertisement. Eligible subjects were taking no medications, had blood pressure <140/90 mm Hg, fasting LDL cholesterol <160 mg/dL, and fasting glucose <100 mg/dL, and had never smoked or had stopped smoking for >1 year before enrollment. The Boston Medical Center Institutional Review Board approved the study, and all participants provided written informed consent.

Study Protocol

Study subjects underwent initial screening and baseline collection of endothelial cells, peripheral blood mononuclear cells (PBMCs), and serum samples after an overnight fast. Briefly, subjects fasted overnight before all study visits. Baseline collection of laboratory values, endothelial cells and leukocytes, and vascular testing was performed in the morning. Fat emulsion was infused in a forearm vein at 45 mL/h for 5 hours, and heparin 12 U/kg/h was coinfused to activate lipoprotein lipase and increase circulating FFA. Fat emulsion contained linoleic acid 44% to 62%, oleic acid 20% to 30%, palmitic acid 7% to 15%, linolenic acid 4% to 10%, stearic acid 1% to 5.5%, and egg yolk phospholipids 1% (20% Intralipid; Baxter Healthcare Corp, Deerfield, IL). Endothelial cell, PBMC, and serum collection and vascular function testing were repeated immediately after infusion. Serum samples were processed for

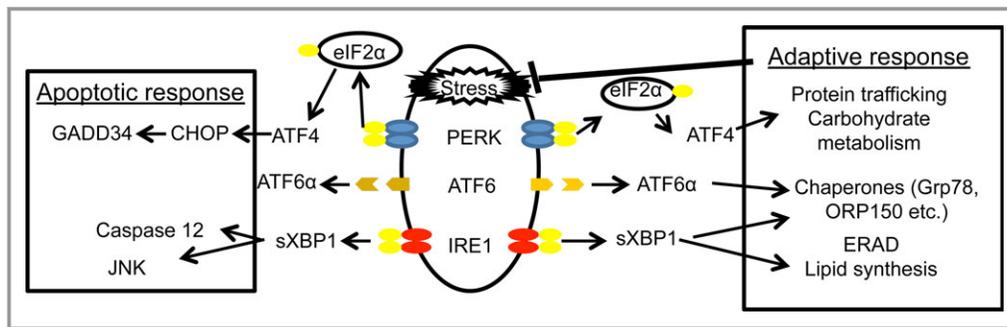


Figure 1. Adaptive and proapoptotic ER stress pathways. The early adaptive response involves activation of ATF6 and IRE1. ATF6 is transferred to the Golgi apparatus, where it is cleaved to form the transcription factor, ATF6 α . IRE1 is dimerized and phosphorylated and then functions as an endoribonuclease leading to the splicing and production of the transcription factor, sXBP-1. These pathways increase the production of chaperones improving the ER's protein folding capacity and activate ERAD, which limits ER stress. More-prolonged ER stress activates PERK, which phosphorylates eIF2 α and downregulates overall protein synthesis and also induces the transcription factor, ATF4. During prolonged or severe ER stress, the PERK/eIF2 α /ATF4 branch becomes more important and leads to an apoptotic response, mainly through activation of CHOP/GADD34. Additionally, sXBP1 can also induce apoptosis under the same conditions through activation of JNK and caspase 12. ATF6 indicates activating transcription factor 6; eIF2 α , eukaryotic initiation factor alpha; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated protein degradation; IRE1, inositol requiring kinase 1; JNK, c-Jun N-terminal kinase; PERK, protein kinase-like ER kinase; sXBP1, spliced X-box-binding protein 1.

glucose, insulin, FFA, and lipid profile in the Clinical Chemistry Laboratory of Boston Medical Center. The assay used for the measurement of triglycerides did not measure glycerol. Plasma FFA concentrations were measured using a colorimetric method (kit from Wako Diagnostics, Richmond, VA).

Vascular Function Testing

We measured conduit artery vascular function using high-resolution ultrasound to assess brachial artery flow-mediated dilation (FMD) induced by a 5-minute cuff occlusion on the upper arm as described previously.²⁴ All images were analyzed using customized software (Medical Imaging Applications LLC, Coralville, IA) by individuals blinded to clinical and laboratory status of subjects. To assess microvascular responses that may be particularly sensitive to early metabolic changes, we measured reactive hyperemia in the leg. Because the later stages of reactive hyperemia reflect nitric oxide production, we assessed the entire hyperemic response using venous occlusion plethysmography to measure leg blood flow using a mercury-in-silastic strain gauge placed on the calf, upper thigh, and ankle cuffs and a computerized plethysmograph (Hokanson, Inc., Bellevue, WA).²⁴ To exclude flow to the foot, an ankle cuff was inflated to suprasystolic pressure before flow measurements. Baseline flow was measured 5 times with thigh cuff inflation to 40 mm Hg. After 5 minutes of thigh cuff inflation to suprasystolic pressures, blood flow recordings were made every 15 seconds for a total of 1.5 minutes.

Endothelial and Leukocyte Isolation From Study Subjects

Peripheral venous endothelial cell biopsy was performed as previously described.^{25,26} Briefly, a 20-gauge intravenous catheter was inserted into a superficial forearm vein under aseptic technique. A 0.018-in J-wire (Arrow International, Reading PA) was introduced through the catheter, and endothelial cells were collected by gentle abrasion of the vessel wall. Endothelial cells along with blood leukocytes for fluorescent imaging were recovered from the wire tip by centrifugation in a dissociation buffer and plated on poly-L-lysine-coated microscope slides (Sigma-Aldrich, St. Louis, MO). Once plated, cells were fixed immediately in 4% paraformaldehyde and stored at -80°C before staining. PBMCs were isolated by density gradient centrifugation in Vacutainer cell preparation tubes (CPT Tubes with sodium citrate; Becton Dickinson Co., Franklin Lakes, NJ), treated with an RNA stabilizer (RNA protect cell reagent; Qiagen, Inc., Valencia, CA), and stored at -80°C before RNA isolation. Fluorescence-activated cell sorting analysis was performed in the Boston Medical Center Hematology Laboratory and showed a cell population of $82\pm 4\%$ lymphocytes (CD3-positive cells)

and $14\pm 2\%$ monocytes (CD14-positive cells), with the remaining 4% comprised of other mononuclear cells ($n=5$).

Materials for Assessment of Gene and Protein Expression

The following primary antibodies were used: anti-ATF6 (Abcam, Cambridge, MA); anti-AFT4 (Abcam); anti-Grp78 (Abcam); anti-IRE1 alpha (p Ser724; Novus Biological, Littleton, CO); anti-GADD34 (S-20; Santa Cruz Biotechnology, Santa Cruz, CA); and anti-von Willebrand factor (vWF; Dako, Carpinteria, CA). Alexa Fluor-488- and Alexa Fluor-594-conjugated secondary antibodies were obtained from Invitrogen (Carlsbad, CA), and Vectashield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) was obtained from Vector Laboratories, Inc. (Burlingame, CA). TaqMan gene expression assays were all obtained from Applied Biosystems (Carlsbad, CA).

RNA Isolation and Quantitative Gene Expression

Total RNA was isolated from PBMCs with the miRNeasy Mini kit (Qiagen) according to the manufacturer's instructions. An RNase inhibitor (Applied Biosystems) was used and RNA was reverse transcribed to cDNA with TaqMan Reverse Transcription Reagents followed by cDNA preamplification with TaqMan PreAmp Master Mix (Applied Biosystems), according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction was performed with TaqMan Gene Expression Assays ATF6 (Hs00232586 m1), ATF4 (Hs00909569 g1), spliced-XBP1 (Hs03929085 g1), Grp78 (Hs99999174_m1), CHOP (Hs01090850_m1), GADD34 (Hs00169585_m1), ORP150 (Hs00197328 m1), aP2 (Hs01086177 m1), and GAPDH (Hs 99999905 m1). GAPDH was used as a housekeeping gene, and results were interpreted by the relative quantity method ($\Delta\Delta\text{Ct}$).

Immunofluorescence Staining and Microscopy

Fixed samples were rehydrated with PBS containing glycine 50 mmol/L and permeabilized with 0.1% Triton X-100. After nonspecific binding sites were blocked with 0.5% BSA, slides with PMBCs were incubated with one of the following antibodies: anti-ATF6 (1:200); anti-ATF4 (1:100); anti-Grp78 (1:200); anti-pIRE1 (1:100); or anti-GADD34 (1:100). Staining of endothelial cells was performed with anti-vWF (1:300) with either anti-ATF6 or pIRE1. Slides were then incubated with corresponding Alexa Fluor-488 and Alexa Fluor-594 secondary antibodies and mounted with Vectashield containing the nuclear stain, DAPI. All slides were examined for quantitative immunofluorescence with a fluorescence microscope (Nikon Eclipse TE2000-E; Nikon Instruments Inc., Melville, NY) at $\times 20$ magnification, and cellular images were digitally captured by a Photometric CoolSnap HQ2 camera

(Photometrics, Tucson, AZ). Thirty mononuclear cells and 20 endothelial cells in each patient sample were analyzed based on previous reproducibility evaluation and the intensity was measured with NIS Elements AR Software (Nikon Instruments) and averaged. Intensity quantification was performed blinded to subject identity. Staining of PBMCs was performed in 1 batch to avoid any day-to-day variability in staining sessions, and all images were captured at the same exposure time and presented values are corrected for local background fluorescence. In order to minimize batch-to-batch variability in staining intensity, fluorescence quantification for each patient sample was normalized to the intensity of human aortic endothelial cells (HAECs) staining performed simultaneously as described previously^{25,26} For endothelial cells, intensity is expressed in arbitrary units (au) calculated by dividing the average fluorescence intensity from the patient sample by the average fluorescence intensity of the HAEC sample and multiplying by 100. Confocal microscopy was performed using a Zeiss LSM 710 Live Duo scan microscope (Carl Zeiss, Oberkochen, Germany) with a $\times 60$ objective.

Statistical Analyses

Statistical analyses were completed with SPSS software (version 20.0; SPSS, Inc., Chicago, IL). Data are reported as mean \pm SD unless otherwise noted. Variables with normal distribution (assessed by the Shapiro–Wilk test) were compared using paired *t* tests. Variables that were not normally distributed were compared with the Wilcoxon signed-rank test for paired samples. Calf hyperemic response was compared using 2-way repeated-measures ANOVA including study condition (before or after intralipid), time interval (before and 15-second intervals after cuff release), and study condition by time interval interaction. In exploratory analyses limited by the study sample size, potential for effect modification by sex was evaluated by testing for sex by study condition (before or after intralipid) interaction in a repeated-measures ANOVA. Two-sided *P* values of <0.05 were considered statistically significant.

Based on previous data from our laboratory, the study was designed to provide 93% power ($\alpha=0.05$) to detect a 2 percentage point change in the vascular endpoint of brachial flow-mediated dilation (eg, from 11% to 9%) with a sample size of 21 subjects.

Results

Study Subjects and Metabolic and Vascular Response to Intralipid Infusion

We enrolled 21 healthy adults (age 31 ± 11 years; 42% female; body mass index, 23.3 ± 3.0 kg/m²). As shown in Table, baseline lipid profile and insulin and glucose levels were not

Table. Effect of Intralipid Infusion on Metabolic and Vascular Measures

	Before Lipid Infusion	After Lipid Infusion	<i>P</i> Value
Metabolic parameters			
Triglycerides, mg/dL	90 \pm 41	230 \pm 199	0.003
Free fatty acids, mmol/L	0.43 \pm 0.12	2.09 \pm 0.62	<0.001
Total cholesterol, mg/dL	182 \pm 34	176 \pm 33	0.04
HDL, mg/dL	54 \pm 10	48 \pm 14	0.02
Insulin, mU/L	3.0 (2.0–5.75)	2.0 (2.0–2.75)	0.06
Glucose, mg/dL	63 (57–81)	75 (71–81)	0.06
Vascular measures			
Systolic blood pressure, mm Hg	117 \pm 10	118 \pm 13	0.67
Diastolic blood pressure, mm Hg	69 \pm 8	69 \pm 8	0.97
Baseline brachial diameter, mm	3.53 \pm 0.76	3.56 \pm 0.74	0.43
Flow-mediated dilation, %	11.1 \pm 4.6	11.8 \pm 4.2	0.32
Baseline flow, mL/min	65 (51–161)	95 (55–170)	0.51
Hyperemic flow, mL/min	1065 \pm 574	1095 \pm 596	0.73
Nitroglycerin-mediated dilation, %	18.8 \pm 6.2	16.4 \pm 4.3	0.16

Mean \pm SD or median (interquartile range). HDL indicates high-density lipoprotein.

elevated. We treated subjects with intralipid for 5 hours because earlier studies have shown that infusion of a similar emulsion for more than 4 hours in healthy volunteers can induce changes in vascular parameters.⁴ As shown, the 5-hour intralipid infusion induced a 2.5-fold increase in serum triglycerides and a 4.2-fold increase in FFA similar to previous studies.²⁷ There were decreases in total serum cholesterol and high-density lipoprotein (HDL) levels during infusion, as expected, given that elevated triglyceride levels are associated with increased HDL metabolism.²⁸ Control experiments using heparin plus normal saline infusion were not performed; however, heparin infusion alone has not been shown to have any significant effect on the FFAs and triglyceride levels in humans.²⁹ Acute FFA elevation after intralipid infusion was associated with a modest reduction of the hyperemic response in the leg (Figure 2), suggestive of microvascular dysfunction without a change in FMD of the brachial artery (Table). In exploratory analyses, there were no significant sex by intralipid interactions (all $P>0.1$) for any of the metabolic parameters and vascular measures; however, the sample size

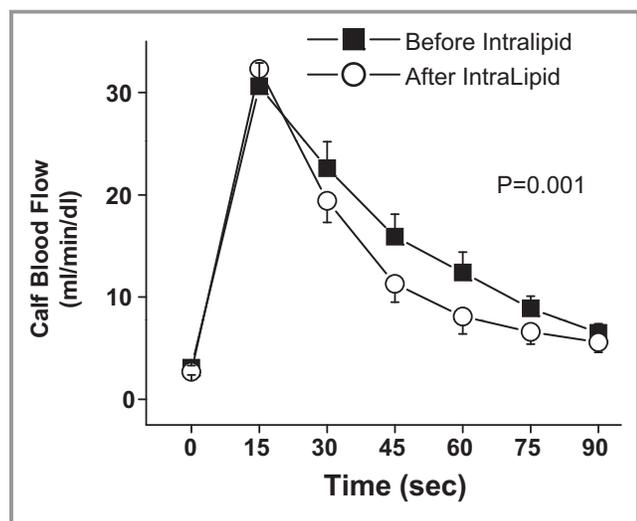


Figure 2. Effects of intralipid on microvascular function. Reactive hyperemia was assessed in the lower extremity using venous occlusion plethysmography at baseline and after intralipid infusion. Intralipid infusion impaired the hyperemic response ($P=0.001$ by repeated-measures ANOVA; $N=12$).

precludes definitive findings regarding potential sex-based differences in the response to intralipid.

Intralipid Infusion Increases the Expression of ER Stress-Related Genes in PBMCs

The effect of the 5-hour intralipid/heparin infusion on the expression of ER stress genes is shown in Figure 3. mRNA levels of ATF6 and spliced-XBP-1, which participate in the early adaptive response to ER stress, increased 2- and 2.5-fold, respectively (Figure 3A). Downstream components of the adaptive response (Grp78 and ORP150) did not change significantly (Figure 3A). There was no change in ATF4 expression. There were significant decreases in the expression of CHOP and GADD34 (Figure 3B), suggesting that exposure to intralipid downregulated the apoptotic ER stress response potentially as a protective mechanism.

Fatty acid-binding protein aP2 has specifically been implicated in fatty acid-induced ER stress in murine models.^{7,8} We were unable to detect an increase in aP2 levels in human PBMCs after intralipid/heparin infusion. Collectively, these changes in gene expression suggest early induction of the adaptive, but not the apoptotic, ER stress response in PBMCs isolated from healthy subjects exposed to increased FFA for 5 hours.

Intralipid Infusion Altered the Protein Levels of Early-Activated Branches of ER Stress

Although the duration of the intralipid/heparin infusion was relatively short, we sought to determine whether ER stress was manifested at the protein level. Consistent with the

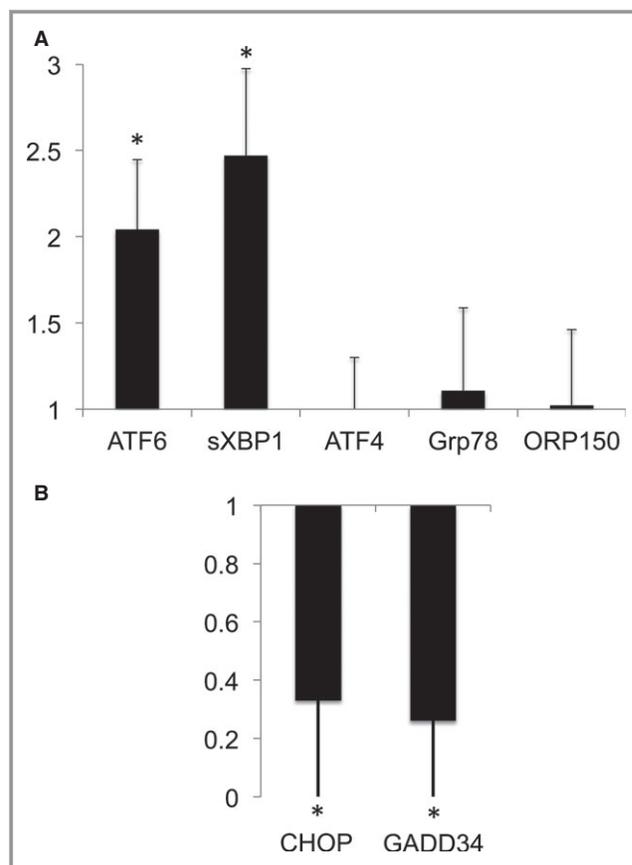


Figure 3. Changes in ER stress-related genes after 5 hours of intralipid/heparin infusion in healthy subjects. A, Gene expression was assessed by real-time quantitative PCR in peripheral blood mononuclear cells, as described in Methods. ATF6 ($P=0.027$) and spliced XBP1 ($P=0.039$) increased after intralipid/heparin infusion. B, CHOP ($P=0.005$) and GADD34 ($P=0.005$) decreased after infusion. Data are mean±SEM ($N=12$ for each gene; * $P<0.05$). ATF6 indicates activating transcription factor 6; ER, endoplasmic reticulum; PCR, polymerase chain reaction; XBP1, X-box-binding protein 1.

observed increase in ATF6 mRNA expression, we observed a 40% increase in ATF6 protein in PBMCs. Interestingly, there also was increased nuclear localization of ATF6, consistent with activation (Figure 4). There was no change in expression of Grp78, a key effector of the adaptive ER stress response that is downstream of ATF6 (Figure 4).

The early phase of the ER stress response is associated with phosphorylation of IRE1, which is upstream of sXBP1 (Figure 1).³⁰ Consistent with activation of this pathway, we observed a 50% increase in leukocyte phospho-IRE1 levels after intralipid/heparin infusion (Figure 4C through 4E). Finally, we observed no significant changes in PBMC protein expression of ATF4, Grp78, or GADD34 (Figure 4F). Similar to the changes in gene expression, the observed changes in protein expression in human PBMCs also suggested an early adaptive ER stress response and lack of activation of the apoptotic ER stress response after intralipid/heparin infusion.

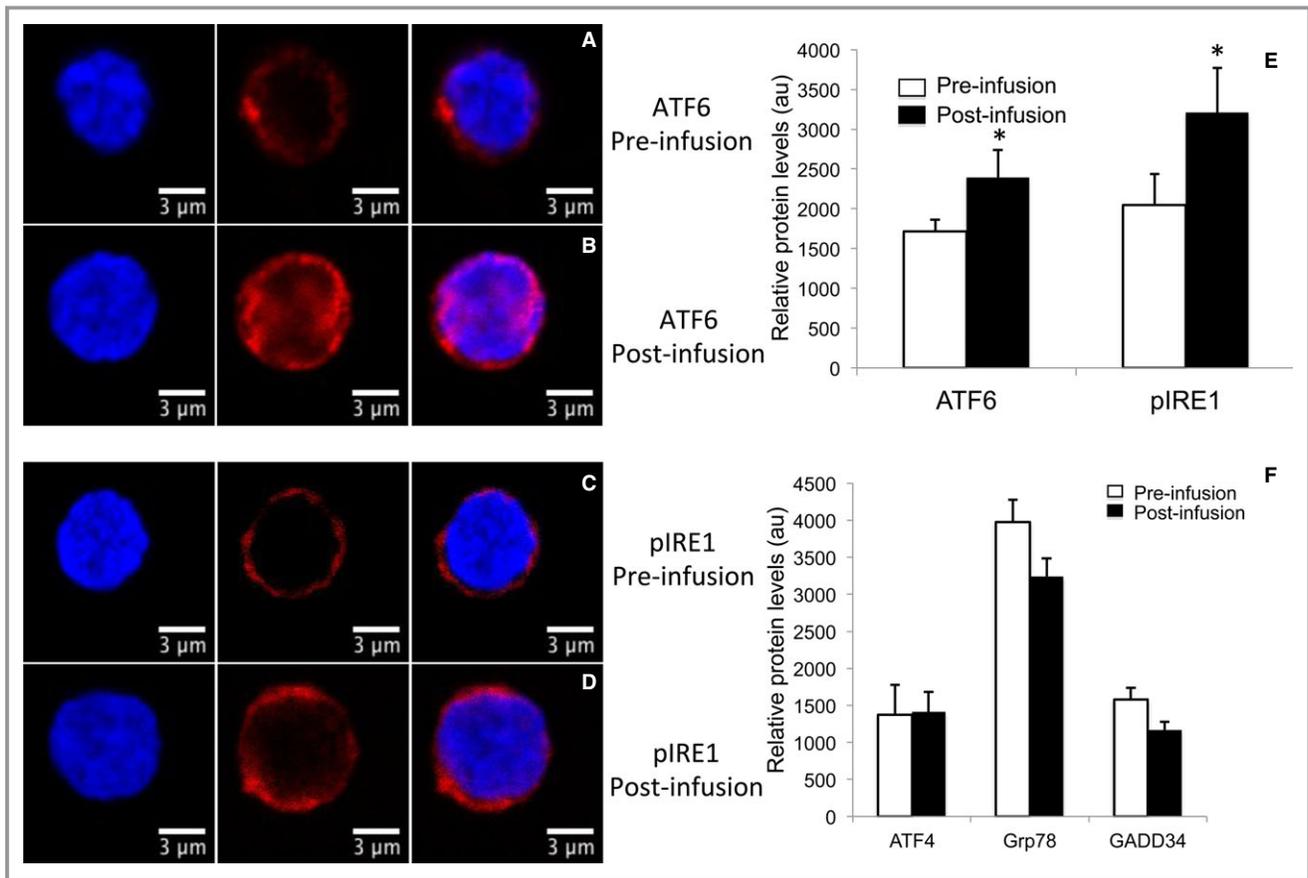


Figure 4. Effects of intralipid/heparin infusion on ER stress-related proteins ATF6 and phospho-IRE1 in PBMCs after 5 hours of intralipid/heparin infusion in healthy subjects. PBMCs were isolated and immunofluorescence microscopy was performed as described in Methods. Nuclei were labeled with DAPI (blue) and ATF6 (A and B) or phospho-IRE1 (C and D) were labeled with red. As shown, intralipid/heparin infusion was associated with an increase in ATF6 expression and nuclear localization and an increase in phospho-IRE1 expression (E) without changes in ATF4, Grp78, or GADD34 (F). Data are mean±SEM (n=10 for each protein; *P<0.05). ATF6 indicates activating transcription factor 6; DAPI, 4',6-diamidino-2-phenylindole; ER, endoplasmic reticulum; IRE1, inositol requiring kinase 1; PBMCs, peripheral blood mononuclear cells.

Because ER stress has been implicated in endothelial cell dysfunction and subsequently in development of atherosclerosis,^{31,32} we investigated the effect of intralipid on the early protein sensors of ER stress. In freshly isolated endothelial cells, we observed an increase of the early activated ER stress factor, ATF6, by 45% and of phosphorylated IRE1 by 23% after intralipid infusion (Figure 5).

Discussion

In the present study, we used a well-established intralipid/heparin infusion protocol to increase FFA levels in healthy adults. As has been previously reported, intralipid/heparin infusion induced abnormalities in vascular function. Furthermore, FFA elevation was associated with activation of early, and probably adaptive, ER stress-related pathways in PBMCs and venous endothelial cells. In particular, we observed increased protein and gene expression of ATF6, phosphorylation of IRE1, and increased expression of sXBP1 in PBMCs and upregulation of ATF6 and pIRE1 proteins in endothelial cells.

Additional evidence of ATF6 activation was provided by the observation that the intralipid infusion increased nuclear localization in PBMCs. Proapoptotic ER stress pathways were not altered, including ATF4, and interestingly, we observed significant decreases in downstream components, including CHOP and GADD34. These findings add to our understanding of the consequences of elevated FFAs and postprandial dyslipidemia in inflammatory and endothelial cells.

Intralipid infusion has previously been shown to induce endothelial dysfunction in patients with obesity, diabetes,³³ and in healthy adults.^{2,4} Our overall findings are similar with previous reports though demonstrating abnormal microvascular responses after intralipid/heparin infusion.^{34,35} Previous studies indicate associations of cardiovascular risk factors with reduced hyperemic response and other interventions, including physical inactivity impairing microvascular

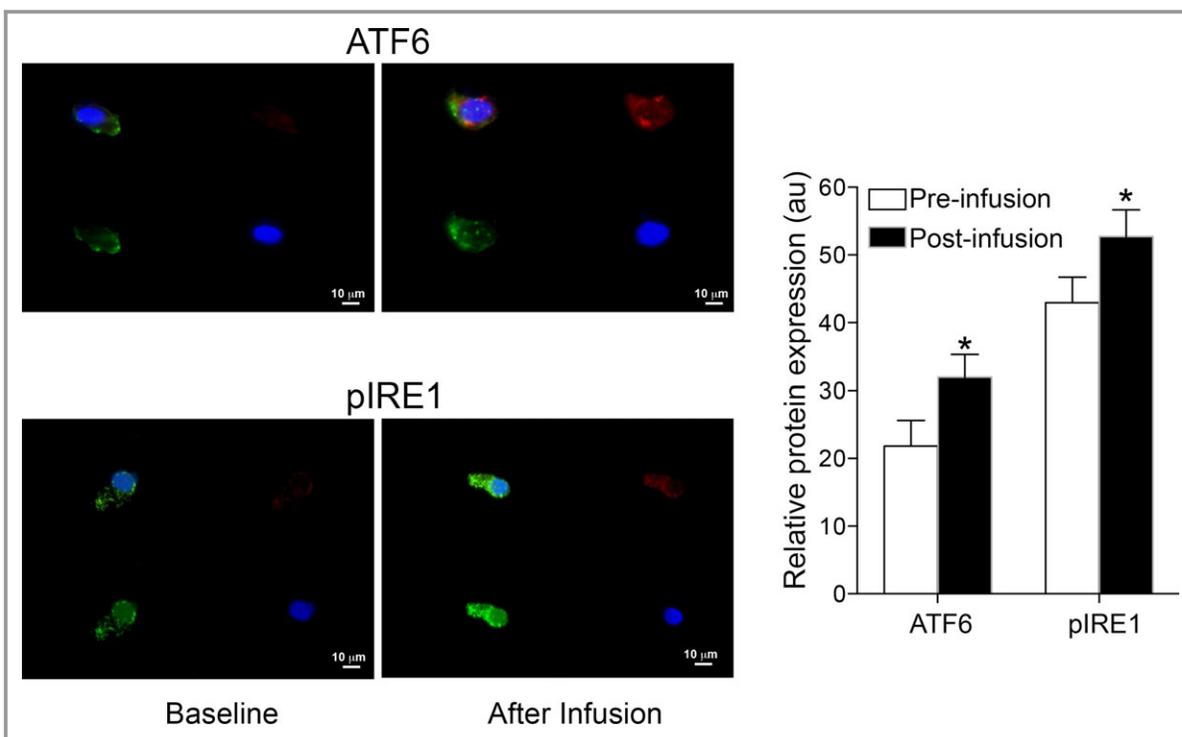


Figure 5. Changes in ER stress-related proteins in endothelial cells after 5 hours of intralipid/heparin infusion in healthy subjects. Endothelial cells were fixed on microscope slides and protein expression measured by quantitative immunofluorescence as described in Methods. Representative images show higher ATF6 and pIRE1 after intralipid/heparin infusion (green= von Willebrand’s factor; blue=DAPI; red=ATF 6 or pIRE1). Pooled data show higher ATF6 ($P=0.048$) and pIRE1 ($P<0.005$) protein expression after infusion. Data are mean±SEM ($n=9$ for each protein; $*P<0.05$). ATF6 indicates activating transcription factor 6; DAPI, 4’,6-diamidino-2-phenylindole; ER, endoplasmic reticulum; pIRE1, phospho-inositol requiring kinase 1.

responses in healthy individuals.^{24,36} Overall, the hyperemic response involves multiple ischemia-mediated vasodilator substances, including nitric oxide.³⁷ In longitudinal studies, lower hyperemic response is associated with vascular risk.³⁸ The lack of change in FMD in the present study may reflect differences in the duration and intensity of intralipid infusion or the relatively well-preserved baseline vasodilator responses in the present sample. The distinct effects on conduit, compared to microvascular, responses are similar to a previous study of short-term physical inactivity and may reflect differential impact of metabolic factors on particular arterial responses.^{24,39}

Several previous studies have suggested increased ER stress in the setting of dyslipidemia in humans. Sage et al. reported markers of ER stress in mononuclear cells isolated from patients with metabolic syndrome and elevated FFA.²² In addition, oral glucose challenge activated the unfolded protein response and increased expression of inflammatory cytokines in leukocytes of healthy adults.²² PBMCs isolated from patients with diabetes mellitus also had elevated ER stress proteins, altered ER morphology, and impaired phagocytotic activity.²³ Furthermore, coronary artery atherosclerotic specimens obtained at autopsy in humans showed activation of ER

stress and apoptosis in macrophages from lipid-rich ruptured plaques and not in the fibrous caps of thick-cap atheromas.^{18,40} Similarly, in human atherosclerotic plaques, increased endothelial ER stress proteins were colocalized with oxidized phospholipids.¹⁷ Moreover, increased expression of ER stress chaperones was identified in the adipose tissue and endothelial cells of obese humans.²⁰ Finally, ER stress-related proteins were decreased in adipose and liver tissue of obese humans subjects 1 year after gastric bypass surgery.⁴¹ One study demonstrated higher ER stress in endothelial cells in obese adults.²¹ The present study adds to our understanding of ER stress in humans because it provides a direct assessment of the association of elevated FFAs commonly observed in insulin-resistant states and the ER stress-related pathways in PBMCs and endothelial cells of healthy subjects.

Several mechanisms might account for induction of ER stress in PBMCs and endothelial cells in our study. Oxidative stress is a known inducer of ER stress,⁴² and Tripathy et al. observed NF-κB activation and increased production of reactive oxygen species in mononuclear cells in healthy subjects exposed to a similar intralipid infusion protocol.⁴ In endothelial cells palmitate activated NFκB through the

production of ROS and through the TLR4-signaling pathway.¹⁰ Another proposed mechanism for FFA-mediated ER stress is the incorporation of palmitate and other lipids into the ER membrane, which can change membrane morphology and fluidity, alter protein folding, and trigger ER stress.^{9,43} Experimental studies have shown that palmitate induces ER stress in macrophages through fatty acid-binding protein 4 (aP2), a lipid chaperone known to regulate lipid metabolism,⁷ although we were unable to detect a significant change in aP2 in our study.

As noted above, the observed changes in ER stress genes in the present study are consistent with activation of an early, presumably adaptive response. Increased activation of ATF6 and IRE1 and the suppression of CHOP and GADD34 are expected to alleviate ER stress and prevent apoptosis in PBMCs and endothelial cells (Figure 1).⁴⁴ Our results are consistent with the findings of Cnop et al., who showed that FFAs induce apoptosis through CHOP in cultured β -pancreatic cells and that IRE1 and ATF6 have an early protective role.¹⁶ Moreover, several previous studies have demonstrated that induction of apoptosis through CHOP depends on the intensity of the stressor and requires prolonged ER stress.^{30,44} Depending on the stressor, components of the unfolded protein response have different activation and deactivation timing and IRE1 and ATF6 are usually affected earliest.⁴⁵⁻⁴⁷ Additionally, GADD34 has been shown to activate protein phosphatase 1, which dephosphorylates p-eIF2 α and blocks an early adaptive response inducing apoptosis.³⁰ Therefore, it seems possible that more-prolonged intralipid infusion would induce the PERK/ATF4/CHOP/GADD34 pathway, leading to impairment of the function of PBMCs and endothelial cells and apoptosis. This contention is supported by the finding that CHOP and Grp78 are elevated in monocytes from type 2 diabetic patients, who have chronically elevated triglycerides and FFA.²³ These mechanisms may be relevant to the pathogenesis of cardiovascular disease given that experimental evidence shows increased CHOP expression and ER stress-mediated activation in macrophages isolated from vulnerable atherosclerotic plaques and in the endothelium of atherosusceptible regions.^{8,18,48}

In addition to the short infusion duration, another possible explanation for selective activation of an adaptive response is the fatty acid composition of the lipid emulsion used in our study. Previous *in vitro* studies have evaluated the relative effects of saturated (palmitic acid) and unsaturated (oleic and linoleic acid) fatty acids on isolated cells.^{49,50} In those analyses, palmitic acid was shown to induce ER stress and CHOP-mediated apoptosis. Interestingly, this response was prevented by co- or pretreatment with an unsaturated fatty acid. Therefore, the relatively large amount of unsaturated fatty acid of our intralipid emulsion (60% to 90%) might explain the lack of PERK/ATF4/CHOP activation in PBMCs.

We acknowledge that our study has a number of limitations. First, intralipid apart from triglycerides contains small amounts of phospholipids (1.2%) and glycerol (2.25%) and we were unable to isolate the effect of triglycerides in the present study.²⁷ Additional studies would be required to isolate the effects of specific components of intralipid on ER stress. We studied the short-term responses to FFA, which limits our ability to detect changes in protein expression. Furthermore, our short-term study may have less relevance to the chronic effects of metabolic diseases. Our study may also be limited by the use of PBMCs, which represent a mixture of lymphocytes and monocytes, and it is possible the findings might differ in a pure monocyte preparation. We assessed venous, and not arterial, endothelial cells that are primarily involved in atherosclerosis. However, venous endothelial cells are similarly exposed to systemic metabolic conditions and previously published studies suggest correlation in venous and arterial protein expression.⁵¹ The study design evaluated subjects before and after intralipid infusion without comparison to a control infusion; thus, it is not possible to adjust for changes that may be observed with time. However, previous studies with control infusions have observed vascular effects of intralipid infusion.³⁴ The sample size precludes definitive evaluation of the potential sex-based differences in the response to intralipid infusion. These limitations are balanced by the study design, which allowed us to evaluate the same individuals before and after induction of alterations in FFA levels. In addition, this design allows us to differentiate the effects of FFA from the confounding effects of concomitant risk factors and medications that were present in cross-sectional studies that compared diabetics or obese patients to healthy controls. Because the intralipid infusion likely has multiple potential and observed effects, our study design precludes the establishment of a definitive causal relationship between ER stress and vascular dysfunction.

In conclusion, our study shows that acute FFA elevation by 5-hour intralipid/heparin infusion in healthy volunteers that is associated with vascular dysfunction induces parallel upregulation of 2 ER stress sensors associated with an early, presumably adaptive response in PBMCs and endothelial cells and suppresses expression of 2 ER stress mediators of apoptosis in PBMCs. Prolonged or repeated ER stress induction may overwhelm the adaptive response, leading to mononuclear and endothelial cell dysfunction and apoptosis. Our results suggest a direct role for metabolic dysfunction as an inducer of ER stress in mononuclear and endothelial cells in humans and a potential contributor to vascular diseases.

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Disclosures

None.

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