Lipid Peroxidation Product 4-Hydroxy-trans-2-nonenal Causes Endothelial Activation by Inducing Endoplasmic Reticulum Stress*

Elena Vladykovskaya,1,5 Sinivas D. Sithu,1,5 Petra Haberzettel,1,5 Nalinie S. Wickramasinghe,1,5 Michael L. Merchant,6 Bradford G. Hill1,5 James McCracken,1,5 Abhinav Agarwal,1,5 Susan Dougherty,1,5 Sharon A. Gordon,6 Dale A. Schuschte,1,5 Oleg A. Barski1,5, Timothy O’Toole1,5, Stanley E. D’Souza1,5, Aruni Bhatnagar1,5, and Sanjay Srivastava1,5,2

From the 1Diabetes and Obesity Center and the Departments of 2Medicine, 3Physiology and Biophysics, and 4Biochemistry and Molecular Biology, University of Louisville, Louisville, Kentucky 40202

Background: Oxidized lipids cause endothelial activation.
Results: Endothelial activation by the lipid peroxidation product, 4-hydroxy-trans-2-nonenal, was associated with ER stress and was prevented by chaperones of protein folding.
Conclusion: ER stress regulates endothelial activation by oxidized lipids.
Significance: Vascular inflammation caused by oxidized lipids could be attenuated by decreasing ER stress.

Lipid peroxidation products, such as 4-hydroxy-trans-2-nonenal (HNE), cause endothelial activation, and they increase the adhesion of the endothelium to circulating leukocytes. Nevertheless, the mechanisms underlying these effects remain unclear. We observed that in HNE-treated human umbilical vein endothelial cells, some of the protein-HNE adducts colocalize with the endoplasmic reticulum (ER) and that HNE forms covalent adducts with several ER chaperones that assist in protein folding. We also found that at concentrations that did not induce apoptosis or necrosis, HNE activated the unfolded protein response, leading to an increase in XBP-1 splicing, phosphorylation of protein kinase-like ER kinase and eukaryotic translation initiation factor 2α, and the induction of ATF3 and ATF4. This increase in eukaryotic translation initiation factor 2α phosphorylation was prevented by transfection with protein kinase-like ER kinase siRNA. Treatment with HNE increased the expression of the ER chaperones, GRP78 and HERP. Exposure to HNE led to a depletion of reduced glutathione and an increase in the production of reactive oxygen species (ROS); however, glutathione depletion and ROS production by tert-butyl-hydroperoxide did not trigger the unfolded protein response. Pretreatment with a chemical chaperone, phenylbutyric acid, or adenoviral transfection with ATF6 attenuated HNE-induced monocyte adhesion and IL-8 induction. Moreover, phenylbutyric acid and taurine-conjugated ursooxycholic acid attenuated HNE-induced leukocyte rolling and their firm adhesion to the endothelium in rat cremaster muscle. These data suggest that endothelial activation by HNE is mediated in part by ER stress, induced by mechanisms independent of ROS production or glutathione depletion. The induction of ER stress may be a significant cause of vascular inflammation induced by products of oxidized lipids.

Extensive experimental and clinical evidence suggests that atherosclerosis begins with the subendothelial accumulation of lipoproteins in lesion-prone sites (1). Within the acellular space of the subintima, which is devoid of antioxidants, unsaturated lipoproteins in lesion-prone sites (1). Within the acellular space of the subintima, which is devoid of antioxidants, unsaturated lipoproteins undergo extensive oxidation and aggregation. It is currently believed that the reactive products generated within oxidized lipoproteins cause endothelial activation, leading to an increase in endothelial adhesion and cytokine production. Activation of the endothelium is one of the earliest events in atherogenesis that results in the recruitment of monocytes to sites of lesion initiation associated with the accumulation of oxidized lipoproteins. However, the mechanisms by which lipoprotein oxidation products cause endothelial activation remain unclear.

Oxidation of lipids by reactive oxygen species (ROS)3 generates several reactive molecules of which aldehydes are major end products (2). These aldehydes are more stable than ROS, and therefore they can diffuse from their site of formation to propagate and amplify oxidative injury. Among the products generated in oxidized lipids, the C9 unsaturated aldehyde 4-hydroxy-trans-2-nonenal (HNE) is the most abundant unsaturated aldehyde. It is generated by the oxidation of α-6 polyunsaturated (arachidonate, γ-linolenate, linoleate) fatty acids and under some conditions constitutes a majority of the unsaturated aldehydes present in oxidized lipids (3).

The abbreviations used are: ROS, reactive oxygen species; HNE, 4-hydroxy-trans-2-nonenal; HUVEC, human umbilical vein endothelial cells; ER, endoplasmic reticulum; HERP, homocysteine inducible endoplasmic reticulum protein; UPR, unfolded protein response; PERK, protein kinase-like ER kinase; eIF2α, eukaryotic translation initiation factor 2α; PBA, phenylbutyric acid; TUDCA, taurine-conjugated ursooxycholic acid; HBSS, Hanks’ balanced salt solution; SEC, size exclusion chromatography; MCB, monochlorobimane; t-BHB, tert-butyl-hydroperoxide; ICAM-1, intercellular adhesion molecule 1.

1 The abbreviations used are: ROS, reactive oxygen species; HNE, 4-hydroxy-trans-2-nonenal; HUVEC, human umbilical vein endothelial cells; ER, endoplasmic reticulum; HERP, homocysteine inducible endoplasmic reticulum protein; UPR, unfolded protein response; PERK, protein kinase-like ER kinase; eIF2α, eukaryotic translation initiation factor 2α; PBA, phenylbutyric acid; TUDCA, taurine-conjugated ursooxycholic acid; HBSS, Hanks’ balanced salt solution; SEC, size exclusion chromatography; MCB, monochlorobimane; t-BHB, tert-butyl-hydroperoxide; ICAM-1, intercellular adhesion molecule 1.

* This work was supported, in whole or in part, by National Institutes of Health Grants ES17260, HL95593, HL55477, HL59378, and RR 24489.

1 To whom correspondence should be addressed: Diabetes and Obesity Center, 580 S. Preston St., Delia Baxter Bldg., Rm. 204F, Louisville, KY 40202. Fax: 502-852-3663; E-mail: s0sriv01@exchange.louisville.edu.
HNE is a highly reactive aldehyde that avidly attacks nucleophilic centers in proteins, phospholipids, and nucleotides (2). Several studies have shown that HNE-modified proteins and DNA accumulate in diseased and injured tissues (4). Significantly, high levels of protein–HNE adducts are also generated in oxidized LDL (5), and HNE-modified proteins have been detected in atherosclerotic lesions (6) and inflammation (7). Exposure to HNE has been shown to increase monocyte adhesion (8) and to stimulate cytokine production (9) in endothelial cells. Nonetheless, the mechanisms by which HNE causes endothelial activation remain unclear. Therefore, the current study was designed to identify the molecular and cellular mechanisms that contribute to HNE-induced endothelial activation. Our results show that exposure to HNE leads to the modification of ER-resident proteins and that HNE-induced endothelial activation is mediated in part by the unfolded protein response (UPR) to ER stress.

**EXPERIMENTAL PROCEDURES**

**Chemical Reagents and Antibodies**—[3H]HNE and HNE were synthesized as described (10). The polyclonal antibody against keyhole limpet hemocyanin-protein–HNE was raised as described before (15). Phycoerythrin-conjugated rat anti-mouse CD31 was obtained from BD Pharmingen (San Diego, CA) and Serotec (Raleigh, NC), respectively. Monoclonal anti-KDEL and ATF6 (activation transcription factor 6) antibodies were obtained from Stressgen (Ann Arbor, MI) and IMGENEX (San Diego, CA), respectively. Polyclonal anti-actin antibody was purchased from Sigma-Aldrich. Polyclonal antibodies against ATF3 and phospho-PERK (Thr981) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz). Anti-eIF2α and phospho-eIF2α (Ser51), GRP94 and protein-disulfide isomerase (PDI) antibodies, and HRP-linked secondary goat anti-rabbit or goat anti-mouse antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-ICAM-1 monoclonal antibody was purchased from BD Biosciences. Alexa Fluor 488-labeled anti-rabbit IgG and Texas Red-labeled anti-mouse IgG antibodies and the Slow Fade Gold antifade reagent. Images were acquired on a fluorescent microscope.

**Liquid Chromatography-Mass Spectrometric Analysis of HNE-modified Proteins**—HUVEC cultured in T75 flasks to ~80% confluence were incubated without or with HNE for 30 min in HBSS at 37 °C and frozen at −80 °C until used for proteomic analyses. Cells were thawed on ice, lysed in a hypotonic buffer (10 mm Hepes, pH 7.4, 1.5 mm MgCl2, 0.1% Nonidet P-40, 1× Roche Phosphostop phosphatase inhibitor mixture, and 1× Roche Complete mini protease inhibitor mixture), transferred to a 1.5-ml microcentrifuge tube, and gently triturated using a pipette tip several times. The lysates were stored on ice for 10 min and then spun at 600 × g for 3 min to pellet nuclei. The cytosolic fraction was transferred to a fresh tube and stored on ice prior to protein assay.

The cytoplasmic cell fraction (200 μl) was loaded onto a size exclusion chromatography (SEC) column (BioSep-SEC-S4000, Phenomenex (Torrance, CA)) and eluted isocratically in 0.1 M phosphate buffer at a flow rate of 1 ml/min using an Ultimate™ 3000 (Dionex, Sunnyvale, CA) HPLC. The elution was monitored with an Ultimate 3000 dual wavelength UV-visible detector operating at 280 nm. Fractions (1 ml) were collected and digested overnight at 37 °C with shaking, using mass spectrometry grade trypsin (without reduction or alkylation) following buffer adjustment to pH 8.0. The digested protein sample was trap-cleaned (MicroTrap, Michrom Bioresources, Auburn, CA), concentrated, and, following lyophilization, redissolved in 2% acetonitrile, 0.05% formic acid.

The digested samples (1–5 μg) of five SEC fractions were analyzed using one-dimensional reversed phase HPLC-MS/MS as described (13). The sample fractions containing stress response proteins, including HSP90, HSP70, and protein-disulfide isomerasers were further characterized using two-dimensional liquid chromatography (LC) of strong cation exchange (SCX) followed with reverse phase prior to MS/MS analysis using a Thermo Scientific LTQ ion trap mass spectrometer interfaced to the Ultimate™ 3000 (Dionex) using a nanospray source operated in a data-dependent manner. Tandem mass spectra.
were extracted without charge state deconvolution or deisotoping. All MS/MS samples were analyzed using Sequest (Thermo Fisher Scientific (San Jose, CA), version 27, rev. 11). Sequest was set up to search the human_refseq_20110926 database (unknown version, 32,972 entries) assuming the digestion enzyme trypsin (maximum missed cleavages of 2). Sequest was searched with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 1.2 Da. Modification of cysteine, histidine, and lysine by 4-hydroxynonenal (+156, +78, +52 for +1, +2, or +3 charge states) or dehydrated HNE (+138, +69, +46 for +1, +2, or +3 charge states) on cysteine, histidine, and lysine were specified in Sequest as variable modifications. Scaffold (version Scaffold_3.1.4.1, Proteome Software Inc. (Portland, OR)) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at >95.0% probability as specified by the Peptide Prophet algorithm. Protein identification was accepted if it could be established at >95.0% probability and contained one or more identified peptides below the 1% false discovery rate. Protein probabilities were assigned by the Protein Prophet algorithm. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis were grouped to satisfy the principles of parsimony.

**Apoptosis**—Apoptosis in HNE-treated cells was examined by fluorescence-assisted cell sorting (FACS) using the Annexin V apoptosis detection kit FITC from eBioscience (San Diego, CA) as per the manufacturer’s instructions, on an LSRII flow cytometer. Data were analyzed using the FACSDiva software (BD Biosciences).

**Western Blot Analysis**—Western blotting was performed in total cell lysates as described (14). To detect protein–HNE adducts, DTT was omitted from the lysis and sample buffers.

**Genetic Ablation Using Small Interfering RNA (siRNA)**—IRE-1 (inositol-requiring ER-to-nucleus protein-1), eIF2α, and PERK mRNA in HUVEC cells were knocked down using an RNAi method following an siRNA transfection protocol provided by Invitrogen. The siRNAs were purchased from Qiagen (Valencia, CA), each as a pool of four target-specific 20–25-nucleotide siRNAs. Scrambled siRNA, purchased from Qiagen, contained non-targeting 20–25-nucleotide RNA and was applied to control cells as a negative control. Briefly, after culturing the cells in antibiotic-free growth medium at 37 °C in a humidified atmosphere of 5% CO₂ for 24 h, siRNA diluted in Opti-MEM (Invitrogen) and preincubated with the transfection agent oligofectamine (Invitrogen) was added. After transfection with scrambled or target-specific siRNA for 24 h for (RNA) or 48 h for (protein), the medium was replaced with HBSS and treated with HNE as indicated. RNA was isolated using the RNeasy minikit (Qiagen), and quantitative RT-PCR was performed to measure specific mRNA expression.

**RNA Isolation and PCR Analysis**—XBP-1 (X-box protein-1) splicing was analyzed by regular PCR as described (14). Quantitative real-time PCR was performed as described (14), using the following primer sets: TNF-α, 5’-TGA TCC CTG ACA TCT GGA ATCTG-3’ (forward primer) and 5’-GCT GGG CTC CGT GTC TCA-3’ (reverse primer); IL-8, 5’-CCA CAC TGC GCC AAC ACA A-3’ (forward primer) and 5’-TCA CTG ATT CTT GGAT ACC ACA GAG A-3’ (reverse primer); GRP78, 5’-CGG GCA AAG ATG TCA GGA AAG-3’ (forward primer) and 5’-TTC TGG ACG GGC TTC ATA GTA GAC-3’ (reverse primer); and GAPDH, 5’-CGC TCT CGT CTC CTC CGT TT-3’ (forward primer) and 5’-CCA TGG TGT CTG AGC GAT GT-3’ (reverse primer). Primer pairs for the amplification of GRP94, PDI, Erp72, calnexin, HERP, and IL-6 were purchased from SAbiosciences (Frederick, MD).

**Reduced Glutathione Measurement**—Reduced glutathione (GSH) concentration in HUVEC was determined by FACS as described (15). Briefly, HUVEC (3–4×10⁵ cells) were incubated with monochlorobimane (MCB; 10 μM) for 15 min at 37 °C. The cells were then incubated with HNE (25 μM), nonanal (25 μM), or tert-butyl-hydroperoxide (t-BHP) (100 μM) in 0.5 ml of HBSS for 15, 30, and 60 min at 37 °C. Samples were analyzed on an LSRII flow cytometer.

**Surface Expression of ICAM-1**—HUVEC were incubated without or with HNE in HBSS for 2 h at 37 °C followed by incubation in endothelial cell culture medium for 4 h at 37 °C. Cells were harvested in 5 mM EDTA and incubated with anti-ICAM-1 antibody for 1 h on ice. After incubation, the cells were pelleted, resuspended in PBS containing an Alexa488 conjugated anti-mouse IgG, and incubated for an additional 30 min. After washing, the fluorescence properties of these cells were analyzed on an LSR II flow cytometer (BD Biosciences). Cells stained with an isotype control antibody were used to detect background staining, and the mean fluorescence intensity of these samples was subtracted from the mean fluorescence intensity of anti-ICAM-1-stained cells.

**Adhesion Assay**—HUVEC (5×10⁴ cells) cultured for 24 h in 96-well plates were incubated with HNE in HBSS for 2 h at 37 °C. Cells were then incubated in endothelial cell culture medium for 18 h. THP-1 cells were labeled with calcein acetoxymethyl ester (Invitrogen) (10 μM; 30 min at 37 °C) and allowed to adhere to the endothelial monolayer (50,000 THP-1 cells/well; 1 h at 37 °C in HBSS with 0.1% glucose). The unadhered THP-1 cells were washed four times with PBS, and the adhesion was measured using a florescent plate reader (16).

**Transmigration Assay**—The HUVEC (4×10⁴ cells) were incubated with HNE (25 μM) in HBSS for 2 h at 37 °C in transwell chambers. THP-1 cells (2×10⁵) were added to the transwells and transmigration was measured as described before (17).

**Adenoviral Transfection**—The adenovirus expressing ATF6 was kindly provided by Dr. Ron Prywes (Columbia University). HUVEC were transfected with the indicated amount of adenovirus in 0.5 ml of serum-free cell culture medium for 30 min in 12-well plates (18). Subsequently, culture medium was aspirated, and fresh cell culture medium with serum was added to the cells. Cells were incubated for 24 h. Transfected cells were then incubated without or with HNE for the indicated time in HBSS, and mRNA expression and protein abundance were measured by quantitative real-time PCR and Western blotting, respectively.

**Intravital Microscopy**—Sprague-Dawley rats (200–250 g) were administered PBS (vehicle; 250 μl intravenously in tail vein) or HNE (1 mg/kg in 250 μl of PBS, intravenously in tail vein). After 30 min, the concentration of HNE in the plasma was measured by gas chromatography-mass spectrometry as
described (6). To examine the effect of HNE on endothelial function, the rats were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally) 18 h after treatment with HNE. The trachea and the carotid artery were cannulated to maintain airway patency and to directly monitor blood pressure, respectively. The cremaster muscle was prepared for *in vivo* microcirculatory observation as described previously (19) and positioned over an optic port in a specially designed plexiglass bath. The bath was filled with 60 ml of modified Kreb’s solution that was maintained at a temperature of 35 ± 0.5 °C and a pH of 7.4 ± 0.5. The rat or the tissue bath was placed on the modified stage of a Nikon MM-11 microscope so that the microcirculation could be observed by transillumination of the cremaster muscle. Closed circuit television microscopy was used to observe and quantify the diameters of single, unbranched third order venules that had basal diameters of 25–35 μm. The video system was calibrated with a stage micrometer, and the vessel diameters were measured with a video caliper. The adhesion interactions between leukocytes and the vascular endothelium were measured by quantifying the number of transiently (rolling) and firmly (sticking) adherent leukocytes. Leukocyte rolling was determined by counting the number of leukocytes passing an arbitrary reference point, and leukocyte adhesion was quantified by counting, for each vessel, the number of adherent leukocytes in a 100-μm length. To study the effect of chemical chaperones on leukocyte rolling and adhesion to cremaster endothelium *in vivo*, rats were administered PBA (0.25 g/kg in PBS, by gavage) or TUDCA (0.25 g/kg in PBS intraperitoneally) three times at 12-h intervals prior to HNE treatment. PBS, by gavage) or TUDCA (0.25 g/kg in PBS intraperitoneally) three times at 12-h intervals prior to HNE treatment.

**RESULTS**

**Protein-HNE Adducts Colocalize with ER**—To study the effects of HNE on endothelial activation, we first determined the concentrations at which HNE does not induce cell death. HNE is highly toxic and has been shown to trigger apoptosis and necrosis in a variety of cell types (20, 21). Hence, to identify the concentrations at which HNE does not induce cell death. HNE in normal plasma and tissue is between 0.1 and 6 μM (2, 22). However, between 2 and 40 μM HNE has been detected in diseased tissue under oxidative stress (2). Hence, HNE concentrations of <25 μM are within the range expected under oxidative stress *in vivo*, and the effects observed at these concentrations are unlikely to be overwhelmed by the activation of cell death pathways.

Because HNE and related aldehydes form covalent adducts with proteins, and several of the effects of HNE could be related to protein modification (2), we studied the modification of endothelial proteins by HNE. Incubation of HUVEC with [3H]HNE (5 nmol in 1.0 ml of HBSS; 30 min at 37 °C) resulted in the binding of 43 ± 2 pmol of HNE with cell proteins (Fig. 1A, i). Incubation with a higher concentration of HNE (10–25 μM) increased protein-HNE adduct formation in a concentration-dependent manner (Fig. 1A, ii). Western blot analysis showed that incubation with HNE (25 μM; 30 min at 37 °C in HBSS) resulted in the appearance of several proteins that displayed positive reactivity with anti-protein HNE antibodies (Fig. 1A, i), indicating that HNE causes extensive modification of endothelial proteins.

To examine the cellular localization of HNE-modified proteins, the cells were incubated with HNE, and protein-HNE adducts were visualized by immunocytochemical analysis and confocal microscopy. Only sparse staining was observed in untreated cells. Treatment with HNE resulted in a large increase in antibody staining of the cell. To examine whether treatment with HNE also modifies ER proteins, the cells were stained with the anti-KDEL antibody, which binds to ER-resident proteins. The difference in staining from one cell to the next reflected the extent of ER in different cells. After comparing several such cells, we did not see a significant difference in the ER staining between untreated and HNE-treated cells. Confocal overlay images showed significant co-localization of the protein-HNE adducts with the ER (Fig. 1B). These data suggest that HNE forms covalent adducts with several endothelial proteins and that some of these adducts co-localize with the ER.

To identify which proteins were modified by HNE, we analyzed proteins extracted from HNE-treated HUVEC by one-dimensional LC-MS/MS. Analysis of SEC-HPLC fractions collected from the separation of HNE-treated cell cytoplasm using one-dimensional LC-MS/MS proteomic methods identified 101 proteins by one peptide or more at the 0.3% protein and peptide false discovery rate. Molecular chaperones, including HSP90, HSP70, and PDI along with structural proteins (actin and vimentin) comprised the five most abundant proteins (by a label-free spectral counting approach). These chaperones were localized to two SEC-HPLC fractions (retention times of 4 and 5 min). Further analysis of these two fractions using a two-dimensional LC-MS/MS approach, used to achieve a greater sensitivity to low abundance post-translational modifications, identified a total of 243 proteins by one peptide or more at the 0.2% peptide and 0.3% protein false discovery rate. HNE and dehydrated HNE modifications of lysine, histidine, and cysteine side chains were detected on 58 peptides derived from 27 proteins, including six ER-associated proteins and 21 non-ER-associated proteins (Table 1). As shown in Fig. 1C, HNE formed a Michael adduct with the lysine residue of the peptide NQLTSNPENTVFADK* (+156) of ER chaperone GRP78. Similar Michael adducts were also formed with ER-associ-
HNE Causes Endothelial Activation

HNE Triggers Unfolded Protein Response—Given the observations that protein-HNE adducts co-localize with ER proteins and that several ER proteins were modified by HNE, we examined the possibility that HNE induces ER stress. Indeed, microarray analysis of RKO cells treated with HNE was shown to increase in ER stress genes, such as HERP, ATF3, and GADD34 (23), and treatment with HNE has been reported to increase the phosphorylation of IRE1 and eIF2α in human endothelial cells (24). Nevertheless, the impact of HNE on the UPR in its entirety has not been assessed, and the role of ER stress in mediating the biological response to HNE has not been studied. Hence, we examined the effect of HNE on each of the three canonical arms of the UPR: XBP-1 splicing, the activation of the PERK-eIF2α and ATF6, and the transcription factors ATF3 and ATF4, which are induced by ER stress (25).

To examine whether HNE increases XBP-1 splicing, the cells were incubated with 5–25 μM HNE for 2 h, and changes in the splicing of XBP-1 mRNA were measured. As shown in Fig. 2A, treatment with HNE led to a marked increase in the intensity of the spliced form. In untreated cells, there was no increase in the spliced form of XBP-1, which was robustly increased upon treatment with the known UPR inducer thapsigargin. These data suggest that HNE stimulates the IRE-1 pathway of the UPR.

To examine how HNE affects the PERK-eIF2α pathway, the cells were incubated with HNE for 30 min and stained with anti-phospho-PERK antibody. Treatment with HNE led to a 2-fold increase in PERK phosphorylation (Fig. 2B, i). HNE also led to a concentration-dependent increase in eIF2α phosphorylation (Fig. 2B, ii). These results show that HNE-induced ER stress leads to the activation of the PERK-eIF2α pathway of the UPR. However, under conditions of nutrient deprivation and viral infection (26), PERK could also be activated by a PERK-independent mechanism. Therefore, to determine the relationship between PERK and eIF2α phosphorylation, we examined the effect of PERK gene silencing on HNE-induced phosphorylation of eIF2α. As shown in Fig. 2B, iii, transfection of HUVEC with PERK siRNA decreased the abundance of PERK protein by ~70% and inhibited HNE-induced eIF2α phosphorylation by

![Image](https://example.com/image.png)

**FIGURE 1.** Protein-HNE adducts accumulate in the endoplasmic reticulum. A, modification of endothelial proteins by HNE. i, protein-bound radioactivity recovered from HUVEC incubated with [3H]HNE. Values are mean ± S.E., n = 6. ii, Western blots of lysates prepared from cells incubated without or with HNE (25 μM) in HBSS for 30 min developed using the anti-keyhole limpet hemocyanin-HNE antibodies. B, localization of protein-HNE adducts. Confocal images of HUVEC left untreated or treated with 25 μM HNE. The cells were stained with anti-keyhole limpet hemocyanin-HNE primary antibody and Alexa 488 (green)-conjugated secondary antibody (left panels). For staining the ER-resident chaperones, anti-KDEL primary antibody and Texas Red-conjugated secondary antibody (middle panels) were used. Merged images demonstrate the localization (yellow; right panel) of protein-HNE adducts and ER-resident proteins in the perinuclear area. C, mass spectrometric identification of HNE-modified proteins. Nanospray MS/MS spectrum of the [M + 2H]2+ ion at m/z 1835.19 was obtained in a data-dependent fashion from two-dimensional LC-MS/MS analysis of the cytoplasmic fraction of HUVEC treated with HNE (25 μM) for 30 min. The MS2 spectrum collected included extensive peptide fragmentation data allowing for >93% sequence coverage, including the +2 b15-NH3-NH3 ion and sequence identification after database searching corresponding to the ER-associated protein GRP78. Similar data are provided for assignment of other HNE-modified ER proteins listed in Table 1.

- HNE Causes Endothelial Activation
- HNE Triggers Unfolded Protein Response
- PERK-eIF2α Pathway
- ATF6 and ATF3 Pathway
- XBP-1 Splicing
- PERK Gene Silencing
- HNE-Induced ER Stress
- Nutrient Deprivation and Viral Infection
- PERK-independent Mechanism
- PERK-eIF2α Pathway
- HNE-induced eIF2α Phosphorylation
HNE Causes Endothelial Activation

Table 1: Modification of proteins by HNE in HUVEC

Site-specific modification of cysteine (C), histidine (H), or lysine (K) amino acid side chains by HNE forming a stable Michael adduct has been noted with lower-case letters (c, h, or k). Site-specific modification of C, H, or K amino acid side chains by HNE with subsequent loss of water to form the Schiff-base (HNE-ΔH2O) has been noted with lower-case and bolded letters (c, h, or k). Amino acids that appear prior to and following the peptide are denoted using parentheses.

| Sequence number | Proteins | Accession number | Peptide sequence | Modification |
|-----------------|----------|------------------|------------------|--------------|
| 1               | GRP78    | 16507237         | (K)QNLTSNPENTVFDAAEk(R) | HNE          |
| 2               | GP96     | 4507677          | (K)FTFIENPRHPLIR(D) | HNE-ΔH2O     |
| 3               | Pro-collagen-lysine, 2-oxoglutarate 5-deoxyglucose 2 (isoform 1/2/3/4) | 33636742 | (K)TVFADADGWPDk(R) | HNE          |
| 4               | Protein glutamine γ-glutamyltransferase 2 (isoform a) | 39777997 | (R)EAFTRANIAKk(N) | HNE-ΔH2O     |
| 5               | Ribosome-binding protein-1 | 110611218 | (K)BBPAPAEPSDSLAKk(R) | HNE          |
| 6               | Stress-induced phosphoryl protein-1 | 5803181 | (K)KRETKEPEMEDLPk(N) | HNE-ΔH2O     |

Table 2: ER-associated proteins

| Sequence number | Proteins | Accession number | Peptide sequence | Modification |
|-----------------|----------|------------------|------------------|--------------|
| 7               | Actin cytoplasmic 1 | 4501885 | (K)DVDIRDLILYANTVGLGTTMYPIGADMR(K) | HNE-ΔH2O     |
| 8               | α-Tocopherol transfer protein-like | 85861243 | (i)KASHTFPIFk(A) | HNE          |
| 9               | Annexin A6 (isoform 1) | 71773329 | (K)EDYHSLDAISSLTSGGHFRk(P) | HNE-ΔH2O     |
| 10              | Caspase-8 (isoform C) | 12205478 | (i)KSPKRGCyLINk(N) | HNE and HNE-ΔH2O |
| 11              | Coiled-coil domain-containing 168 | 226246554 | (K)KTYLSLEAKk(P) | HNE and HNE-ΔH2O |
| 12              | DBF4-type zinc finger-containing protein 2 | 151301098 | (i)KTVTVEHoKk(V) | HNE and HNE-ΔH2O |
| 13              | Desmoscin-3 (isoform Dsc3a) | 148539846 | (K)ATANHyrk(M) | HNE and HNE-ΔH2O |
| 14              | DNA endonuclease RBP8 (isoform a) | 42718015 | (i)KPSLQk(ENV)k(F) | HNE and HNE-ΔH2O |
| 15              | DNA repair protein complementing XP-C cells (isoform 1) | 224809295 | (i)KJKMSkDGKEAKk(R) | HNE          |
| 16              | E3 Sumo protein ligase PIA53 | 115298686 | (i)KGTk(G)kTGk(PPS) | HNE          |
| 17              | E3 ubiquitin ligase HECTD1 | 118498337 | (K)MMk(He)SeAllk(E)k(R) | HNE          |
| 18              | Filamin-B | 105900514 | (R)LIALLkELk(Q)k(R) | HNE          |
| 19              | HSC71 (isoform 1) | 5729877 | (K)RiGk(D)kAAQk(N)k(AMP)k(T) | HNE          |
| 20              | HSP75 mitochondrial precursor | 155723983 | (K)Rk(D)kAEk(SP)k(IAKk(D)) | HNE and HNE-ΔH2O |
| 21              | NCK-associated protein 5 (isoform 1) | 126362967 | (K)TPLDKk(A)k(R)k(SS) | HNE and HNE-ΔH2O |
| 22              | Neuroblast differentiation-associated protein AHNk (isoform 1) | 61743954 | (K)GGVGYk(T)kPVYk(V)k(G)k(V) | HNE(CHK) |
| 23              | Protein Bassoon | 91208420 | (i)Rk(A)kEQk(Q)kAASk(AA)k(A)k(KPk(T)) | HNE and HNE-ΔH2O |
| 24              | Protein MICAL3 (isoform 3) | 170172518 | (i)Kk(Fe)kGAlk(A)kDk(H)k(S)k(R)k(Q)k(Q) | HNE and HNE-ΔH2O |
| 25              | Transferrin receptor protein | 118958887 | (K)k(R)kRk(IGk(V)k(Vk(G)k(Q))k(R)k(PR) | HNE and HNE-ΔH2O |
| 26              | Xyloolitrarase 1 | 28269693 | (K)k(V)kDkWkGkCsSnk(Dk(F)k(P)k | HNE and HNE-ΔH2O |
| 27              | Zinc finger protein 615 (isoform 1) | 313569854 | (i)Kk(S)kDkEaKFTk(K)k(S) | HNE and HNE-ΔH2O |

∼70%, whereas cells transfected with the oligofectamine (vehicle) or non-targeting siRNA (NT-siRNA) were not affected. These data indicate that HNE stimulates eIF2α phosphorylation via PERK.

Incubation of the cells with HNE led to a 4-fold increase in ATF4 protein (data not shown). Moreover, as shown before (23), treatment with HNE led to a 4-fold increase in ATF3 protein (data not shown). Transfection of HUVEC with PERK or eIF2α siRNA attenuated HNE-induced ATF3 up-regulation (data not shown), suggesting that HNE-induced increase in ATF3 is via the PERK/eIF2α pathway. Treatment with HNE also led to a significant increase in CHOP (data not shown). Collectively, these observations suggest that HNE activates the PERK/eIF2α pathway, leading to an increase in transcription factors that are activated upon ER stress.

To examine the effect of HNE on the adaptive phase of the UPR, we examined changes in ATF6. Whereas control cells had minimal ATF6 in the nuclei (Fig. 2C, i), incubation of the cells with HNE resulted in a 6-fold increase in the nuclear translocation of ATF6 as compared with untreated cells, suggesting that HNE activates ATF6. To examine whether activation of ATF6 leads to the induction of ER-resident chaperones, which ameliorate ER stress and attenuate the UPR, cells were incubated without or with HNE, and expression of mRNA of ER chaperones was examined by quantitative RT-PCR. As shown in Fig. 2C, ii, treatment with HNE led to a significant increase in the expression of GRP78 and HERP mRNA. HNE-induced increase in GRP78 mRNA was accompanied by a >2-fold increase in GRP78 protein in cells treated with HNE (data not shown). These data suggest that in addition to triggering the alarm phase of the UPR, HNE also activates adaptive UPR responses. Taken together, these results indicate that HNE activates all three of the canonical pathways of the UPR.

Depletion of Reduced Glutathione Is Not Sufficient to Cause ER Stress—Having found that HNE induces ER stress, we next examined the effect of HNE on ROS formation, activation of ROS-dependent phase II detoxification enzyme, heme oxygenase-1, [Ca2+]i, and glutathione levels in endothelial cells. Treatment of HUVEC with HNE significantly increased the formation of ROS and heme oxygenase-1, but it did not affect...
[Ca\(^{2+}\)]_i (data not shown). Because increased ROS formation lowers GSH levels, we asked whether HNE triggers the UPR by depleting GSH. Due to its strong electrophilic nature, HNE readily reacts with GSH, and this reaction is further accelerated by glutathione S-transferases (27). Therefore, some of the cellular effects of HNE could be secondary to GSH depletion. To examine this possibility, HUVEC were incubated with HNE, and GSH content was measured by FACS using MCB as a fluorescent probe for detecting reduced thiols. Incubation with HNE led to a steady decline in MCB fluorescence such that after 60 min, >30% of MCB fluorescence was lost (Fig. 3A). Similar to HNE, t-BHP (25 μM), which oxidizes GSH to GSSG (28), also caused a large decrease in MCB fluorescence. These observations suggest that both HNE and t-BHP lead to a decrease in intracellular thiols, mainly GSH. To examine whether thiol depletion is sufficient to cause ER stress, we measured the splicing of XBP-1 and phosphorylation of eIF2α. However, although t-BHP caused GSH depletion (Fig. 3A), it did not increase XBP-1 splicing (Fig. 3B) or eIF2α phosphorylation (Fig. 3C). Treatment of cells with the...
HNE Causes Endothelial Activation

Effects cannot be entirely excluded. Therefore, for evaluating 

vation is mediated in part by ER stress.

duced increase in the adhesion of THP-1 cells to HUVEC 

Importantly, preincubation with PBA prevented HNE-in-

crease in ATF3 (Fig. 5

with HNE led to a concentration-dependent increase in the 

surface expression of ICAM-1 and the adhesion of THP-1 

aldehyde nonanal did not cause GSH depletion (Fig. 3A), XBP-1 splicing (Fig. 3B), or eIF2α phosphorylation (Fig. 3C). Together, these observations suggest that although the electrophilic nature of HNE is required for inducing ER stress, depletion of GSH or an increase in ROS formation is not sufficient to trigger the UPR by HNE.

HNE Causes Endothelial Activation—To elucidate the role of ER stress in HNE-induced endothelial activation, we tested whether inhibition of ER stress by chemical chaperones would prevent endothelial activation by HNE. Treatment with HNE led to a concentration-dependent increase in the surface expression of ICAM-1 and the adhesion of THP-1 cells to HUVEC (Fig. 4A, i and ii). HNE also increased the transmigration of THP-1 cells (Fig. 4A, iii) and the production of proinflammatory cytokines (Fig. 4B). Preincubation with the chemical chaperone PBA prevented HNE-induced increase in ATF3 (Fig. 5A), ATF4 and heme oxygenase-1 protein (data not shown), suggesting that an increase in protein folding capacity diminishes HNE-induced ER stress. Importantly, preincubation with PBA prevented HNE-induced increase in the adhesion of THP-1 cells to HUVEC (Fig. 5B). It also prevented the increase in the production of IL-8 in HNE-treated cells (Fig. 5C). These observations are consistent with the view that HNE-induced endothelial activation is mediated in part by ER stress.

Although PBA is an effective chaperone, its nonspecific effects cannot be entirely excluded. Therefore, for evaluating

saturated aldehyde nonanal did not cause GSH depletion (Fig. 3A), XBP-1 splicing (Fig. 3B), or eIF2α phosphorylation (Fig. 3C). Together, these observations suggest that although the electrophilic nature of HNE is required for inducing ER stress, depletion of GSH or an increase in ROS formation is not sufficient to trigger the UPR by HNE.

HNE Causes Endothelial Activation—To elucidate the role of ER stress in HNE-induced endothelial activation, we tested whether inhibition of ER stress by chemical chaperones would prevent endothelial activation by HNE. Treatment with HNE led to a concentration-dependent increase in the surface expression of ICAM-1 and the adhesion of THP-1 cells to HUVEC (Fig. 4A, i and ii). HNE also increased the transmigration of THP-1 cells (Fig. 4A, iii) and the production of proinflammatory cytokines (Fig. 4B). Preincubation with the chemical chaperone PBA prevented HNE-induced increase in ATF3 (Fig. 5A), ATF4 and heme oxygenase-1 protein (data not shown), suggesting that an increase in protein folding capacity diminishes HNE-induced ER stress. Importantly, preincubation with PBA prevented HNE-induced increase in the adhesion of THP-1 cells to HUVEC (Fig. 5B). It also prevented the increase in the production of IL-8 in HNE-treated cells (Fig. 5C). These observations are consistent with the view that HNE-induced endothelial activation is mediated in part by ER stress.

Although PBA is an effective chaperone, its nonspecific effects cannot be entirely excluded. Therefore, for evaluating

saturated aldehyde nonanal did not cause GSH depletion (Fig. 3A), XBP-1 splicing (Fig. 3B), or eIF2α phosphorylation (Fig. 3C). Together, these observations suggest that although the electrophilic nature of HNE is required for inducing ER stress, depletion of GSH or an increase in ROS formation is not sufficient to trigger the UPR by HNE.

HNE Causes Endothelial Activation—To elucidate the role of ER stress in HNE-induced endothelial activation, we tested whether inhibition of ER stress by chemical chaperones would prevent endothelial activation by HNE. Treatment with HNE led to a concentration-dependent increase in the surface expression of ICAM-1 and the adhesion of THP-1 cells to HUVEC (Fig. 4A, i and ii). HNE also increased the transmigration of THP-1 cells (Fig. 4A, iii) and the production of proinflammatory cytokines (Fig. 4B). Preincubation with the chemical chaperone PBA prevented HNE-induced increase in ATF3 (Fig. 5A), ATF4 and heme oxygenase-1 protein (data not shown), suggesting that an increase in protein folding capacity diminishes HNE-induced ER stress. Importantly, preincubation with PBA prevented HNE-induced increase in the adhesion of THP-1 cells to HUVEC (Fig. 5B). It also prevented the increase in the production of IL-8 in HNE-treated cells (Fig. 5C). These observations are consistent with the view that HNE-induced endothelial activation is mediated in part by ER stress.

Although PBA is an effective chaperone, its nonspecific effects cannot be entirely excluded. Therefore, for evaluating

saturated aldehyde nonanal did not cause GSH depletion (Fig. 3A), XBP-1 splicing (Fig. 3B), or eIF2α phosphorylation (Fig. 3C). Together, these observations suggest that although the electrophilic nature of HNE is required for inducing ER stress, depletion of GSH or an increase in ROS formation is not sufficient to trigger the UPR by HNE.

HNE Causes Endothelial Activation—To elucidate the role of ER stress in HNE-induced endothelial activation, we tested whether inhibition of ER stress by chemical chaperones would prevent endothelial activation by HNE. Treatment with HNE led to a concentration-dependent increase in the surface expression of ICAM-1 and the adhesion of THP-1 cells to HUVEC (Fig. 4A, i and ii). HNE also increased the transmigration of THP-1 cells (Fig. 4A, iii) and the production of proinflammatory cytokines (Fig. 4B). Preincubation with the chemical chaperone PBA prevented HNE-induced increase in ATF3 (Fig. 5A), ATF4 and heme oxygenase-1 protein (data not shown), suggesting that an increase in protein folding capacity diminishes HNE-induced ER stress. Importantly, preincubation with PBA prevented HNE-induced increase in the adhesion of THP-1 cells to HUVEC (Fig. 5B). It also prevented the increase in the production of IL-8 in HNE-treated cells (Fig. 5C). These observations are consistent with the view that HNE-induced endothelial activation is mediated in part by ER stress.

Although PBA is an effective chaperone, its nonspecific effects cannot be entirely excluded. Therefore, for evaluating

saturated aldehyde nonanal did not cause GSH depletion (Fig. 3A), XBP-1 splicing (Fig. 3B), or eIF2α phosphorylation (Fig. 3C). Together, these observations suggest that although the electrophilic nature of HNE is required for inducing ER stress, depletion of GSH or an increase in ROS formation is not sufficient to trigger the UPR by HNE.
in an increase in adhesion of THP-1 cells; however, this was significantly less in cells infected with the ATF6 virus (Fig. 6C).

Similarly, HNE increased the transcription of IL-8 gene by 20-fold in cells infected with empty vector, whereas infection with the ATF6 virus significantly decreased the induction of IL-8 by HNE (Fig. 6D).

To determine whether HNE activates the endothelium in vivo, 1 mg/kg HNE was injected in the tail vein of adult male Sprague-Dawley rats. Thirty minutes after injection, the plasma HNE concentration was ~0.1 μM. In vehicle-treated rats, the plasma concentration of HNE was 0.015 μM, indicating that the injection led to a marked increase in plasma HNE concentration.

Eighteen hours after treatment, ER stress was appreciably increased in the cremasta of HNE-treated rats versus rats that were treated with the vehicle alone as evident by the increased staining for KDEL (Fig. 7A). Moreover, both the rolling of leukocytes (Fig. 7B) and their firm adhesion to the endothelium (Fig. 7C) was increased by HNE. Pretreatment with PBA (0.25 g/kg) or TUDCA (0.25 g/kg) attenuated both ER stress and leukocyte rolling (Fig. 7B) as well as leukocyte adhesion (Fig. 7D).
HNE Causes Endothelial Activation

DISCUSSION

The findings of this study suggest that the lipid peroxidation product HNE induces ER stress, which results in the activation of both the alarm and the adaptive phases of the UPR. We found that treatment of endothelial cells with HNE stimulates all three of the canonical pathways of the UPR, leading to the activation of several stress-sensitive transcription factors, such as ATF3, ATF4, and CHOP. Although treatment with HNE led to the modification of several proteins, changes in the ER appear to be particularly significant because inhibition of ER stress or an increase in the protein folding capacity of the cell prevented HNE-induced increases in endothelial adhesion and cytokine production. Collectively, these observations reveal a new mechanism of action of lipid peroxidation products, and they indicate a novel link between ER stress and endothelial activation caused by lipid peroxidation products, such as HNE.

ER stress and the UPR are increasingly recognized as important regulators of cell function (26). Conditions under which the influx of nascent, unfolded proteins exceeds the folding capacity of the ER trigger the UPR to restore homeostasis. To match demand to capacity, protein translation and transcription of secreted proteins are transiently inhibited, whereas the degradation of unfolded proteins is increased. Changes in the protein folding status are transmitted to the nucleus by the UPR, which results in ER expansion and an increase in the protein folding capacity of the cell. Conditions under which homeostasis cannot be restored lead to apoptosis. Multiple conditions can trigger the UPR, including viral infections, glucose deprivation, and changes in ATP or calcium levels. Our work adds to the growing list of these conditions, by demonstrating that electrophilic injury by lipid peroxidation products like HNE is a potent, robust, and complete initiator of the UPR.

Although redox status has been considered to be an important regulator of the UPR, our results show that treatment with t-BHP did not trigger the UPR, although it depleted GSH and increased ROS production. Therefore, GSH depletion and ROS production in HNE-treated cells are unlikely to be important mediators of the UPR. Instead, it seems that the ability of HNE to cause ER stress may be related to the modification of multiple proteins that could overwhelm the total proteolytic capacity of the cell and thereby trigger the UPR. Previous studies have shown that inhibition of the proteosome can be propagated to the ER to cause ER stress and activate the UPR (26). However, the observation that HNE stimulates the UPR suggests that ER itself may be a direct target of electrophilic injury. The cytosol, the mitochondria, and the nuclei maintain a high GSH/GSSG ratio (100:1), which prevents electrophilic protein modification and oxidative protein damage. In contrast, the ER maintains a highly oxidized state (GSH/GSSG ratio ~100:1), which prevents electrophilic protein modification and oxidative protein damage. In contrast, the ER maintains a highly oxidized state (GSH/GSSG ratio 1:1–3:1) (29), and therefore proteins in the ER may be particularly vulnerable to oxidative damage or modification by electrophiles, such as HNE. Moreover, the cytosol contains several aldehyde-metabolizing enzymes (aldehyde dehydrogenases and reductases and GSTs) and cofactors (NAD, NADPH, and GSH) required to metabolize and detoxify aldehydes. These defenses are not present in the ER; therefore, even low levels of lipid peroxidation products could readily diffuse into the ER and modify proteins without encountering significant defense.

That the ER is a direct target of HNE is consistent with our immunohistochemical data indicating a significant overlap between protein-HNE and ER staining and with our mass spectrometric data showing HNE adduction of several ER proteins. The vulnerability of the ER to HNE is further supported by the robust activation of all three of the pathways of the UPR (IRE-1,
PERK, and ATF6) in HNE-treated cells. These signaling events elicit the adaptive, alarm, and apoptotic responses in an attempt to restore ER function. Because XBP-1 splicing occurs exclusively by IRE-1 activation, the formation of the spliced form of XBP-1 is considered an essential mediator of ER stress (26). Our data showing that HNE causes the cleavage of the XBP-1 mRNA suggest that HNE-induced ER stress activates the IRE-1 pathway of the UPR. Knockdown of IRE-1 completely prevented the induction of IL-8 in HNE-treated cells, indicating that the activation of IRE-1 upon ER stress is an obligatory requirement for IL-8 production in HNE-treated cells.

In addition to IRE-1, the PERK/eIF2α pathway of the UPR was also activated by HNE. Under most conditions, PERK is activated exclusively by ER stress; however, its downstream effector kinase eIF2α could also be phosphorylated by other kinases, including PKR, GCN2, and HRI (26). Our data show that transfection with PERK siRNA attenuated the HNE-induced activation of eIF2α, suggesting that HNE causes the phosphorylation of eIF2α via PERK. Whereas eIF2α phosphorylation suppresses global protein synthesis to decrease ER load, it selectively up-regulates ATF4. This in turn can activate the transcription factor ATF3, which is a negative regulator of LPS-induced cytokine production (30). We observed that HNE up-regulates both ATF4 and ATF3. These observations are in agreement with an earlier report that HNE induces ATF3 in RKO human colorectal carcinoma cells, albeit at a much higher concentration (23). A comparison of these data suggests that endothelial cells might be more sensitive to HNE-induced ATF3 induction than cancer cells. Moreover, because HNE-induced up-regulation of ATF3 was attenuated by PBA and knockdown of PERK and eIF2α, it appears that HNE induces ATF3 by activating the PERK/eIF2α pathway of the UPR.

ATF6 is the transducer of the protective pathway of the UPR. It increases the transcription of genes encoding molecular chaperones and those involved in protein folding. ATF6 also induces the transcription of XBP-1 (31). Our data show that HNE activates ATF6 as evinced by the nuclear translocation of ATF6 and the increase in the transcription of HERP and GRP78 genes in HNE-treated endothelial cells. Significantly, we observed that adenoviral infection of endothelial cells with ATF6 the up-regulated molecular chaperones GRP78, GRP94, and PDI and diminished HNE-induced IL-8 expression, suggesting that activation of this protective pathway of the UPR plays a critical role in preventing aldehyde-induced endothelial activation.

Our observation that HNE causes endothelial activation by inducing ER stress suggests that conditions that are associated with an increase in lipid peroxidation could lead to vascular inflammation via ER stress. Notably, the accumulation of proteins modified by lipid peroxidation products in atherosclerotic lesions is associated with an increase in the expression of ATF3 and ATF4 specifically in areas that colocalize with aldehyde-modified proteins (32). Although a causative role of ER stress in atherosclerotic lesion formation remains to be established, the findings of this study support the possibility that induction of the UPR in the endothelium by products of lipid peroxidation may be an early initiating event in atherogenesis.

In addition to atherogenesis, several other disease conditions, such as heart failure, Parkinson and Alzheimer diseases (33), obesity, and insulin resistance (34) are associated with tissue accumulation of HNE-modified proteins, indicating ongoing oxidative damage. Significantly, these pathological states are also associated with ER stress (35). That ER stress contributes to the development of these conditions is suggested by the observation that treatment with chemical chaperones inhibits atherosclerosis (36) and prevents insulin resistance and restores glucose levels in ob/ob mice (12). Similarly, ER stress has also been implicated in the development of neurodegenerative disorders, such as Alzheimer and Parkinson diseases (37). Because these disease states induce oxidative injury, it is likely that aldehydes generated by oxidized lipid may be important triggers of ER stress during the manifestation/progression of these diseases.

REFERENCES

1. Skålen, K., Gustafsson, M., Rydberg, E. K., Hultén, L. M., Wiklund, O., Innerarity, T. L., and Borén, J. (2002) Subendothelial retention of atherogenic lipoproteins in early atherosclerosis. Nature 417, 750–754
2. Esterbauer, H., Schaur, R. J., and Zollner, H. (1991) Chemistry and biochemistry of 4-hydroxyynonalenal, malonaldehyde, and related aldehydes. Free Radic. Biol. Med. 11, 81–128
3. Benedetti, A., Comporti, M., and Esterbauer, H. (1982) Identification of a cytotoxic product originating from the peroxidation of liver microsomal lipids. Biochim. Biophys. Acta 620, 281–296
4. LoPachin, R. M., Gavin, T., Petersen, D. R., and Barber, D. S. (2009) Molecular mechanisms of 4-hydroxy-2-nonenal and acrolein toxicity. Nucleophilic targets and adduct formation. Chem. Res. Toxicol. 22, 1499–1508
5. Annangudi, S. P., Deng, Y., Gu, X., Zhang, W., Crabb, J. W., and Salomon, R. G. (2008) Low-density lipoprotein has an enormous capacity to bind (E)-4-hydroxyynonenal (HNE). Detection and characterization of lysyl and histidyl adducts containing multiple molecules of HNE. Chem. Res. Toxicol. 21, 1384–1395
6. Srivastava, S., Vladykovskaya, E., Barski, O. A., Spite, M., Kaiserova, K., Petrasr, J. M., Chung, S. S., Hunt, G., Dawn, B., and Bhatnagar, A. (2009) Aldose reductase protects against early atherosclerotic lesion formation in apolipoprotein E-null mice. Circ. Res. 105, 793–802
7. Rittner, H. L., Hafner, V., Klimiuk, P. A., Szewda, L. I., Goronzy, J. J., and Weyand, C. M. (1999) Aldose reductase functions as a detoxification system for lipid peroxidation products in vasculitis. J. Clin. Invest. 103, 1007–1013
8. Go, Y. M., Halvey, P. J., Hansen, J. M., Reed, M., Pohl, J., and Jones, D. P. (2007) Reactive aldehyde modification of thioredoxin-1 activates early steps of inflammation and cell adhesion. Am. J. Pathol. 171, 1670–1681
9. Henriksen, P. A., Hitt, M., Xing, Z., Wang, J., Haslett, C., Riemersma, R. A., Webb, D. J., Kotelvetsev, Y. V., and Sallenave, J. M. (2004) Adenoviral gene delivery of eflam and secretory leucocyte protease inhibitor attenuates NF-kB-dependent inflammatory responses of human endothelial cells and macrophages to atherogenic stimuli. J. Immunol. 172, 4535–4544
10. Srivastava, S., Chandra, A., Wang, L. F., Seifert, W. E., Jr., DaGia, B. B., Ansari, N. H., Srivastava, S. K., and Bhatnagar, A. (1998) Metabolism of the lipid peroxidation product, 4-hydroxy-trans-2-nonenal in isolated perfused rat heart. J. Biol. Chem. 273, 10893–10900
11. Baba, S. P., Barski, O. A., Ahmed, Y., O’Toole, T. E., Conklin, D. J., Bhatnagar, A., and Srivastava, S. (2009) Reductive metabolism of AGE precursors. A metabolic route for preventing AGE accumulation in cardiovascular tissue. Diabetes 58, 2486–2497
12. Ozcan, U., Yilmaz, E., Ozcan, L., Furuhashi, M., Vaillancourt, E., Smith, R. O., Görgün, C. Z., and Hotamisligil, G. S. (2006) Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. Science 313, 1137–1140
13. Merchant, M. L., Powell, D. W., Wilkey, D. W., Cummins, T. D., Deegens,
HNE Causes Endothelial Activation

J. K., Rood, I. M., McAfee, K. J., Fleischcr, C., Klein, E., and Klein, J. B. (2010) Microfiltration isolation of human urinary exosomes for characterization by MS. Proteomics Clin. Appl. 4, 84–96

14. Haberzettt, P., Vladykowska, E., Srivastava, S., and Bhatnagar, A. (2009) Role of endoplasmic reticulum stress in acrolein-induced endothelial activation. Toxicol. Appl. Pharmacol. 234, 14–24

15. Sebastià, J., Cristòfol, R., Martín, M., Rodríguez-Farré, E., and Sanfeliu, C. (2003) Evaluation of fluorescent dyes for measuring intracellular glutathione content in primary cultures of human neurons and neuroblastoma SH-SYSY. Cytometry A 51, 16–25

16. Tsakadze, N. L., Sithu, S. D., Sen, U., English, W. R., Murphy, G., and D’Souza, S. E. (2006) Tumor necrosis factor-α-converting enzyme (TACE/ADAM-17) mediates the ectodomain cleavage of intercellular adhesion molecule-1 (ICAM-1). J. Biol. Chem. 281, 3157–3164

17. Sithu, S. D., English, W. R., Olson, P., Krubasik, D., Baker, A. H., Murphy, G., and D’Souza, S. E. (2007) Membrane-type 1-matrix metalloproteinase regulates intracellular adhesion molecule-1 (ICAM-1)-mediated monocyte transmigration. J. Biol. Chem. 282, 25010–25019

18. Watson, L. M., Chan, A. K., Berry, L. R., Li, J., Sood, S. K., Dickhout, J. G., Xu, L., Werstuck, G. H., Baijar, L., Klamut, H. J., and Austin, R. C. (2003) Overexpression of the 78-kDa glucose-regulated protein/immunoglobulin-binding protein (GRP78/BiP) inhibits tissue factor procoagulant activity. J. Biol. Chem. 278, 17438–17447

19. Schuschke, D. A., Saari, J. T., and Miller, F. N. (1995) A role for dietary copper in nitric oxide-mediated vasodilation. Microcirculation 2, 371–376

20. Herbst, U., Toborek, M., Kaiser, S., Mattson, M. P., and Hennig, B. (1999) 4-Hydroxynonenal induces dysfunction and apoptosis of cultured endothelial cells. J. Cell Physiol. 181, 295–303

21. West, J. D., Ji, C., Duncan, S. T., Amarnath, V., Schneider, C., Rizzo, C. J., Brash, A. R., and Marnett, L. J. (2004) Induction of apoptosis in colorectal carcinoma cells treated with 4-hydroxy-2-nonenal and structurally related aldehydic products of lipid peroxidation. Chem. Res. Toxicol. 17, 453–462

22. Gruene, T., Michel, P., Sitte, N., Eggert, W., Albrecht-Nebe, H., Esterbauer, H., and Siems, W. G. (1997) Increased levels of 4-hydroxy-2-nonenal modified proteins in plasma of children with autoimmune diseases. Free Radic. Biol. Med. 23, 357–360

23. West, J. D., and Marnett, L. J. (2005) Alterations in gene expression induced by the lipid peroxidation product, 4-hydroxy-2-nonenal. Chem. Res. Toxicol. 18, 1642–1653

24. Sanson, M., Augé, N., Vindis, C., Muller, C., Bando, Y., Thiers, J. C., Marchet, M. A., Zarkovic, K., Sawa, Y., Salvayre, R., and Nègre-Salvayre, A. (2009) Oxidized low-density lipoproteins trigger endoplasmic reticulum stress in vascular cells. Prevention by oxygen-regulated protein 150 expression. Circ. Res. 104, 328–336

25. Zhang, K., and Kaufman, R. J. (2008) From endoplasmic-reticulum stress to the inflammatory response. Nature 454, 455–462

26. Marciniak, S. J., and Ron, D. (2006) Endoplasmic reticulum stress signaling in disease. Physiol. Rev. 86, 1133–1149

27. Awashti, Y. C., Yang, Y., Tiwari, N. K., Patrick, B., Sharma, A., Li, J., and Awashti, S. (2004) Regulation of 4-hydroxynonenal-mediated signaling by glutathione S-transferases. Free Radiol. Biol. Med. 37, 607–619

28. Bhatnagar, A., Srivastava, S. K., and Szabo, G. (1990) Oxidative stress alters specific membrane currents in isolated cardiac myocytes. Circ. Res. 67, 535–549

29. Fewell, S. W., Travers, K. J., Weissman, J. S., and Brodsky, J. L. (2001) The action of molecular chaperones in the early secretory pathway. Annu. Rev. Genet. 35, 149–191

30. Whitmore, M. M., Iparraguirre, A., Kubelka, L., Weninger, W., Hai, T., and Williams, B. R. (2007) Negative regulation of TLR-signaling pathways by activating transcription factor-3. J. Immunol. 179, 3622–3630

31. Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., and Mori, K. (2001) XBPI mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 107, 881–891

32. Gargalovic, P. S., Gharavi, N. M., Clark, M. J., Pagnon, J., Yang, W. P., He, A., Truong, A., Baruch-Oren, T., Berliner, J. A., Kirchgessner, T. G., and Lusis, A. J. (2006) The unfolded protein response is an important regulator of inflammatory genes in endothelial cells. Arterioscler. Thromb. Vasc. Biol. 26, 2490–2496

33. Srivastava, S., Ramana, K. V., Bhatnagar, A., and Srivastava, S. K. (2010) Synthesis, quantification, characterization, and signaling properties of glutathionyl conjugates of enals. Methods Enzymol. 474, 297–313

34. Singh, S. P., Niemczyk, M., Saini, D., Awashti, Y. C., Zammit, L., and Zammit, P. (2008) Role of the electrophilic lipid peroxidation product 4-hydroxynonenal in the development and maintenance of obesity in mice. Biochemistry 47, 3900–3911

35. Ozcan, U., Cao, Q., Yilmaz, E., Lee, A. H., Ikawashi, N. N., Ozdelen, E., Tuncman, G., Görgün, C., Glimcher, L. H., and Hotamisligil, G. S. (2004) Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science 306, 457–461

36. Erbay, E., Babaev, V. R., Mayer, J. R., Makowski, L., Charles, K. N., Snitow, M. E., Fazio, S., Wiest, M. M., Watkins, S. M., Linton, M. F., and Hotamisligil, G. S. (2009) Reducing endoplasmic reticulum stress through a macrophage lipid chaperone alleviates atherosclerosis. Nat. Med. 15, 1383–1391

37. Lindholm, D., Wootz, H., and Korhonen, L. (2006) ER stress and neurodegenerative diseases. Cell Death Differ. 13, 385–392