Redox Regulation of 4-Hydroxy-2-nonenal-mediated Endothelial Barrier Dysfunction by Focal Adhesion, Adherens, and Tight Junction Proteins*

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4-Hydroxy-2-nonenal (4-HNE), one of the major biologically active aldehydes formed during inflammation and oxidative stress, has been implicated in a number of cardiovascular and pulmonary disorders. 4-HNE has been shown to increase vascular endothelial permeability; however, the underlying mechanisms are unclear. Hence, in the current study, we tested our hypothesis that 4-HNE-induced changes in cellular thiol redox status may contribute to modulation of cell signaling pathways that lead to endothelial barrier dysfunction. Exposure of bovine lung microvascular endothelial cells (BLMVECs) to 4-HNE induced reactive oxygen species generation, depleted intracellular glutathione, and altered cell-cell adhesion as measured by transendothelial electrical resistance. Pretreatment of BLMVECs with thiol protectants, N-acetylcysteine and mercaptopropionyl glycine, attenuated 4-HNE-induced decrease in transendothelial electrical resistance, reactive oxygen species generation, Michael protein adduct formation, protein tyrosine phosphorylation, activation of ERK, JNK, and p38 MAPK, and actin cytoskeletal rearrangement. Treatment of BLMVECs with 4-HNE resulted in the redistribution of FAK, paxillin, VE-cadherin, β-catenin, and ZO-1, and intercellular gap formation. Western blot analyses confirmed the formation of 4-HNE-derived Michael adducts with the focal adhesion and adherens junction proteins. Also, 4-HNE decreased tyrosine phosphorylation of FAK without affecting total cellular FAK contents, suggesting the modification of integrins, which are natural FAK receptors. 4-HNE caused a decrease in the surface integrin in a time-dependent manner without altering total α5 and β3 integrins. These results, for the first time, revealed that 4-HNE in redox-dependent fashion affected endothelial cell permeability by modulating cell-cell adhesion through focal adhesion, adherens, and tight junction proteins as well as integrin signal transduction that may lead dramatic alteration in endothelial cell barrier dysfunction during heart infarction, brain stroke, and lung diseases.

The endothelium functions as a semipermeable barrier between plasma and interstitium to macromolecules and circulating blood components. Maintenance of barrier integrity is a crucial physiological process for vessel wall homeostasis and lung function under normal physiological and pathophysiologival states. Among various circulating edemic agents, ROS2 generated at sites of inflammation and injury by activated leukocytes or endothelial cells (ECs) play an important role in the disruption of barrier function. 4-HNE is one of the biologically active major aldehydes of membrane lipid peroxidation, formed during inflammation and oxidative stress, and can accumulate in certain tissues up to concentrations of 10 μM to 5 mM (1–4). Recent studies have demonstrated a key role of 4-HNE in cardiovascular and lung disorders (5). Experimental ischemia or ischemia/reperfusion has been shown to induce early generation of 4-HNE and protein modification in lung (6) or isolated rat heart (7). Significant changes induced by 4-HNE in cells and tissues have been described under several conditions, such as ozone exposure (8) and chronic obstructive pulmonary disease (9). Infusion of 4-HNE (50 μM) into rat lungs has been shown to cause perivascular edema with vascular compression and early EC disruption (10). Treatment of human umbilical vein ECs with 4-HNE has been reported to increase albumin transport through cell monolayers (10) and to alter capillary permeability leading to disturbances in blood-brain barrier function (11). However, the role of 4-HNE in EC barrier dysfunction and underlying cell signaling pathway(s) leading to permeability changes have not been well characterized. We have recently demonstrated that exposure of lung microvascular endothelial cells to 4-HNE (25–100 μM) mediated the activation of mitogen activated protein kinases (MAPks), including p38 MAPK, extracellular signal-regulated kinases 1 and 2 (ERK1/2) and c-Jun NH2-terminal kinase (JNK), cytoskeletal remodeling, and alterations in EC barrier function (12). Moreover, activation of focal adhesion kinase (FAK) by oxidative stress is an important event in ROS-mediated vascular EC bar-

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2 The abbreviations used are: ROS, reactive oxygen species; BLMVEC, bovine lung microvascular endothelial cell; EC, endothelial cell; ERK, extracellular signal-regulated kinase; GSH, glutathione; 4-HNE, 4-hydroxy-2-nonenal; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; MEM, minimum essential media; MPG, mercaptopropionyl glycine; NAC, N-acetylcysteine; TER, transendothelial electrical resistance; DCFDA, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid.
rier function that is regulated by cell-cell and cell-matrix contacts (13). Because 4-HNE not only forms Schiff base adducts with -NH₂ residues of proteins (14) but also alters cellular redox status due to loss of cellular sulfhydryl compounds (2), in our present study, we investigated the mechanisms of redox regulation of 4-HNE-mediated EC barrier dysfunction. The present study shows that: (i) 4-HNE, in a dose-dependent fashion, modulates cell-cell adhesion contacts as measured by transendothelial electrical resistance (TER); (ii) thiol protectants, such as N-acetylcysteine (NAC) and mercaptopropionyl glycine (MPG), attenuate 4-HNE-mediated ROS formation, activation of ERK, JNK, and p38 MAPKs signaling pathways, Michael protein adduct formation, and cytoskeleton rearrangement; and (iii) 4-HNE affects focal adhesion, adherent and tight junction proteins in lung microvascular ECs involving integrin signal transduction.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine lung microvascular ECs (BLMVECs, passage number 7) were purchased from Cell Systems. Cell lysis buffer was obtained from Cell Signaling Technology (Beverly, MA). 4-HNE was obtained from Biomol Research Laboratory (Plymouth Meeting, PA). NAC and MPG, trans-2-nonenal, nonylaldehyde, allopurinol, rotenone, apocynin, triethanolamine, 2-vinylpyridine, sodium borohydride, butylated hydroxytoluene, and metaphosphoric acid were from Sigma. 6-Carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCFDA) was obtained from Molecular Probes. A glutathione assay kit was purchased from Cayman Chemical. Accutase was obtained from Innovative Cell Technologies, Inc. (San Diego, CA). Lab-Tek II 8-well glass slide chambers (cat. no. 154534) were obtained from Nalge Nunc Int. Precast gels for SDS-PAGE was from Invitrogen. Transfer membrane, Immobilon-P polyvinylidene difluoride (0.45 mm) was obtained from Millipore, UK. An enhanced chemiluminescence kit (ECL) was from Amersham Biosciences.

**Cell Culture**—BLMVECs were cultured in MEM supplemented with 10% fetal bovine serum, antibiotics, and growth factors as described previously (12). All experiments were carried out between five and nine passages. Cells from each flask were detached with 0.05% trypsin, resuspended in fresh medium, and cultured on gold electrodes for TER measurements, on 100-mm dishes for Western blotting, or on glass coverslips for immunocytochemistry studies.

**Antibodies**—Anti-HNE Michael adducts antibody (cat. nos. 393205 or 393207) was obtained from Calbiochem. Antibodies for FAK (cat. no. 610087), paxillin (cat. no. 610619), and β-catenin (cat. no. 610153) were from BD Biosciences. VE-cadherin antibody (cat. no. 160840) was from Cayman Chemical. Anti-ERK1 (cat. no. sc-93), anti-ERK2 (cat. no. sc-154) and anti-phosphospecific-ERK (cat. no. sc-7383), anti-JNK (cat. no. sc-571) and anti-phosphospecific-JNK (cat. no. sc-6254), anti-p38 (cat. no. sc-535) and anti-phosphospecific-p38 (cat. no. sc-7973), and anti-FAK (cat. no. sc-558), integrin α5 (cat. no. sc-10729) antibodies, Protein A/G plus-agarose (cat. no. sc-2003), and immunoprecipitation reagents and bovine serum albumin (cat. no. sc-2323), were purchased from Santa Cruz Biotechnology. Integrin β3 antibody (cat. no. 4702) was from Cell Signaling. Anti-phosphotyrosine (monoclonal, cat. no. 05–321) and anti-phospho-FAK (Tyr-397, polyclonal, cat. no. 07–012) antibodies were obtained from Upstate Biotechnology or from Chemicon International (monoclonal, cat. no. MAB1144). Anti-ZO-1 (cat. no. 61–7300) was procured from Zymed Laboratories Inc.. Alexa Fluor 488 (mouse, cat. no. A11029 and rabbit, cat. no. A11034), Alexa Fluor Phalloidin 568 (cat. no. A12380), Mitotracker (cat. no. M-7512) were obtained from Molecular Probes. Secondary anti-rabbit- (cat. no. 170–6515), or anti-mouse-IgG (cat. no. 170–6516), (H+L) horseradish peroxidase conjugates were obtained from Bio-Rad. FITC-conjugated integrin αβ3 complex (monoclonal, cat. no. 555505) and CD51/CD61 integrin αβ3 complex (monoclonal, cat. no. 555504) antibodies were kindly provided by BD Biosciences.

**Determination of EC GSH**—GSH content in lysates of BLMVECs was determined by using a glutathione assay kit according to the manufacturer’s protocol. Briefly, after 4-HNE treatment, cells were washed twice with PBS, collected with a rubber policeman into 0.3 ml of 50 mM MES buffer (pH 7.4) containing 1 mM EDTA, sonicated 2 × 10 s, and centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant was used immediately for GSH determination or stored at −20 °C. After protein estimation equal amounts of protein from each sample were treated for 5 min at room temperature with freshly prepared 10% metaphosphoric acid for deproteinization and centrifuged for 5 min at 2,000 × g, and supernatants were used for further analyses or stored at −20 °C. To determine GSH, the pH was adjusted with triethanolamine according to manufacturer protocol, and 40 mM 2-vinylpyridine was added to inhibit color development in the assay mixture. Glutathione level was estimated by using the assay kit mixture that included 5,5′-dithiobis-2-nitrobenzoic acid and glutathione reductase. After incubation for 25 min at room temperature, the absorbance was monitored at 405 nm in a plate reader. The GSH concentration was calculated from a glutathione standard curve and expressed as micromolar per mg of protein.

**Measurement of ROS in Endothelial Cells**—4-HNE-induced ROS formation in cells was detected and quantified by fluorescence microscopy as described previously (12). Briefly, BLMVECs (~90% confluence) grown in 35-mm dishes were pretreated with 1 mM NAC or MPG, or allopurinol (100 μM), rotenone (2 μM), apocynin (30 μM), or MitoTracker (50 nM, 15 min) in basal MEM and loaded with DCFDA (10 μM) for 30 min at 37 °C in a 95% air-5% CO₂ environment. Following removal of medium containing DCFDA by careful aspiration, ECs were washed once with warm MEM and treated with 4-HNE as indicated. At the end of incubation, cells were washed twice with warm PBS and were examined under Nikon Eclipse TE 2000-S fluorescence microscope and pictures were captured on a Hamamatsu digital charge-coupled device camera (Japan) using a 20× objective lens. The fields were randomly chosen, and statistical analyses were performed by MetaVue software (Universal Imaging Corp.).

**Measurement of TER as an Index of Barrier Function**—BLMVECs were seeded on gold electrodes (eight wells, ten electrodes per well) to ~95% confluence as described earlier (12). TER was measured on a electrical cell-substrate impedance...
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sensing system (ECIS, Applied Biophysics Inc.) as described previously (12). The total endothelial electrical resistance, as measured across the EC monolayers, was determined by the combined resistance between the basal and/or cell matrix adhesion (15, 16). To estimate differences between cell-cell and cell-matrix components, total TER was resolved into values reflecting resistance to current flow beneath the cell layer (α) and resistance to current flow between adjacent cells (Rb), utilizing the method of Giaever and Keese (16), which models the endothelial monolayer mathematically.

Preparation of Cell Lysates and Western Blotting—BLMVECs grown on 100-mm dishes to ~95% confluence were pretreated with NAC or MPG in MEM for 1 h before stimulation with 25 μM 4-HNE as indicated. The cells were washed once in ice-cold PBS containing 1 mM orthovanadate, scraped into 0.5 ml of lysis buffer (20 mM Tris-HCl buffer, pH 7.4, containing 0.5% deoxycholate, 0.5% SDS, 1% Triton X-100, 1% Nonidet P-40, 1 mM sodium orthovanadate, and protease inhibitor mixture). Cell lysates were prepared by sonicating the cells (3×10 s) with a probe sonicator and subsequent centrifugation at 5000 × g for 5 min at 4 °C. Protein content in cell lysates was determined by protein assay (Pierce Chemicals). For preparation of immunoprecipitates, cell lysates (0.5–1 mg of protein/ml) were mixed, with appropriate antibodies at 4 °C for 18 h. Protein A/G (20 μl) was then added, and the mixture was incubated for an additional 2 h at 4 °C. The antibody complex was obtained as a pellet by centrifugation at 5000 × g for 10 min, and the pellet was washed thrice in ice-cold PBS for further SDS-PAGE and Western blotting analysis. Cell lysates containing equal amounts of protein (1 mg/ml) or immunoprecipitates, following dissociation in Laemmli buffer by boiling for 5 min, were loaded onto 10% gels and subjected to SDS-PAGE. After SDS-PAGE, the proteins were transferred to Immobilon-P membranes by electro-blotting, blocked for 1 h with TBST (Tris-buffered saline with 0.1% Tween 20) containing 1% bovine serum albumin, and probed with primary and secondary antibodies according to the manufacturer’s protocol and immunodetected by enhanced chemiluminescence’s kit (Amersham Biosciences). The blots were scanned (UMAX Power Look II) and quantified by automated digitizing system UN-SCAN-IT GEL (Silk Scientific Corp.).

Immunofluorescence and Confocal Microscopy—BLMVECs grown on coverslips or 8-well glass slide chambers to ~95% confluence were treated as indicated in the figures, rinsed twice with PBS, and fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. Then cells were rinsed three times with PBS and permeabilized for 5 min with 0.25% Triton X-100 in Tris-buffered saline containing 0.01% Tween 20 (TBST). After washing, cells were incubated for 30 min at room temperature in TBST blocking buffer containing 1% bovine serum albumin and then incubated with primary antibodies in blocking buffer for 1 h. Cells were thoroughly rinsed with TBST (3×5 min) followed by staining with Alexa Fluor 488 or 568 (1:200 dilution in blocking buffer, 1 h) as secondary antibodies. Actin stress fibers were determined by staining the cells on coverslips with Alexa Fluor Phallolidin 568. Slides were prepared with mounting medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories, cat. no. H-1200) to stain nucleus. Cells were examined by Nikon Eclipse TE 2000-S fluorescence microscope with a Hamamatsu digital camera (Japan) using a 60× oil immersion objective and MetaVue software (Universal Imaging Corp.). Confocal images were captured using a 63×, numerical aperture 1.3 glycerol objective on a Leica SP2 AOPS system with 405 and 488 nm excitation lines and narrow bandwidths for 4′,6-diamidino-2-phenylindole and Alexa Fluor 488 with identical gain settings between samples using Leica software (Exton, PA). Color overlays and three-dimensional reconstructions were created using ImageJ (Wayne Rasband).

Flow Cytometry—BLMVECs grown on 35-mm dishes to ~95% confluence were treated with 25 μM 4-HNE for different time intervals in serum-free media, washed twice with 2 ml of PBS, and then cells were detached with 0.3 ml of Accutase. Integrin staining with FITC-conjugated integrin αβ3 complex was performed according to manufacturer’s protocol. Briefly, cell suspension was centrifuged (5 min, 500 × g), pellets were resolved in 0.2 ml of PBS, and the mixture was incubated in the dark for 20 min at room temperature in the presence of 10 μl of FITC-conjugated anti-integrin antibody. Then cells were centrifuged and washed twice with PBS. Finally, pellets were resuspended in 0.3 ml of PBS and analyzed on Cyan ADP flow cytometer (DakoCytomation, Ft. Collins, CO) equipped with a 488 nm laser and 530/40 band pass filter for FITC emission. 10,000 live cells were collected and analyzed for FITC fluorescence.

Post-acquisition analysis was done with Flowjo analysis software (Treestar, Ashland, OR). A region was set around live cells, FITC fluorescence of the live cells was plotted on a histogram, and the median fluorescence intensity was recorded for each data file.

Statistics—Analysis of variance with Student-Newman-Keel’s test was used to compare two or more different treatment groups. The level of significance was taken to be p < 0.05 unless otherwise stated. Data are expressed as means ± S.E.

RESULTS

4-HNE Modulates Cell-Cell Adhesion Contacts—4-HNE is one of the major biologically active aldehydes generated from peroxidation of membrane lipids (1). We and others have shown that 4-HNE causes actin cytoskeleton remodeling, MAPK activation, protein tyrosine phosphorylation, and EC barrier disruption (12, 17–21). We recently demonstrated that exposure of ECs to oxidant stress modulates barrier function through the changes in cell-cell adhesion interactions (13). To further elucidate the mechanism(s) of 4-HNE-mediated EC barrier dysfunction, BLMVECs were challenged with varying concentrations of 4-HNE (10, 25, and 50 μM), and relative contributions of intercellular junctions and focal adhesions were resolved to Rb (cell-cell) and α (cell-matrix). As shown in Fig. 1A, decrease in TER was due to changes in Rb with minimal contributions due to α.

Specificity of 4-HNE on TER Changes and ROS Production in Endothelial Cells—In addition to 4-HNE, lipid peroxidation generates other aldehydes of varying chain lengths. Therefore, we examined the specificity of 4-HNE effects on TER and ROS generation. BLMVECs were challenged with 4-HNE or trans-2-nonenal or nonenal, and as shown in Fig. 1B, only 4-HNE, but
not trans-2-nonenal or nonanal, caused EC barrier dysfunction. Furthermore, 4-HNE enhanced ROS production as measured by DCFDA oxidation (Fig. 2), which is consistent with the previously reported study on 4-HNE-mediated ROS production in ECs (48, 52). These results suggest that the presence of a hydroxyl group at carbon 4, in addition to a trans double bond at carbon 2, is essential for maximal changes in TER and ROS production in ECs.

Mitochondrial Electron Transport as the Source of 4-HNE-induced ROS in ECs—To address the possible sources of ROS generation we used specific inhibitors for xanthine oxidase (allopurinol), mitochondrial electron transport chain (rotenone), and NADPH-oxidase (apocynin). As shown in Fig. 3A, only rotenone significantly attenuated 4-HNE-mediated ROS production. Analysis of living cells by immunofluorescence microscopy revealed that 4-HNE enhanced the level of DCFDA oxidation, which colocalized with MitoTracker, a marker for mitochondria (Fig. 3B). Furthermore, pretreatment with rotenone attenuated the colocalization of green fluorescence of oxidized DCFDA with red fluorescence of MitoTracker (data not shown). These data are consistent with a recent study showing 4-HNE modulated an electrophile-dependent ROS production in mitochondria (52).

4-HNE Mediates Thiol Modification—As a highly reactive electrophile, 4-HNE may affect EC functions by depletion of...
cellular thiols, such as GSH (2, 22). Treatment of BLMVECs with 4-HNE, in dose- and time-dependent fashion, decreased intracellular GSH (Fig. 4). These results further demonstrate that 4-HNE caused depletion of cellular GSH and causes cellular thiol redox status in BLMVECs.

Thiol-protective Agents Attenuate 4-HNE-induced ROS Production and EC Barrier Dysfunction—In our recent studies we have shown that 4-HNE (12) or diamide (23), an agent that oxidizes GSH to oxidized glutathione and protein thiols, in a dose- and time-dependent fashion, caused barrier dysfunction in lung microvascular EC monolayers. Therefore, we hypothesized that 4-HNE-induced ROS formation and changes in cellular thiols may contribute to the alteration of EC barrier function. To test this hypothesis, BLMVECs were pretreated with 1 mM NAC or MPG for 1 h prior to challenge with 4-HNE (25 μM). As shown in Fig. 5, NAC and MPG attenuated the 4-HNE-induced changes in ROS production (Fig. 5A) and cell-cell adhesion contacts (Fig. 5B). These data suggest the involvement of cellular thiol redox status in 4-HNE-mediated barrier dysfunction in microvascular ECs.

Effect of NAC and MPG on 4-HNE-mediated Michael Adduct Formation in ECs—One of the initial reactions of 4-HNE in cells is the protein modification by the formation of Michael adducts (7, 8, 12, 20). Exposure of BLMVECs to 4-HNE caused a 2-fold increase in the Michael adducts level as compared with the untreated control cells. When BLMVECs were pretreated with 1 mM NAC or MPG prior to treatment with 4-HNE, Michael adduct formation, as analyzed by Western blotting and immunofluorescence microscopy, was completely abolished (Fig. 6, A and B). These results demonstrate that 4-HNE induces Michael adduct formation in EC proteins, which is completely blocked by pretreatment of cells with thiol protectants, further suggesting that intracellular thiols are targets for 4-HNE leading to the formation of Michael adducts.

Effect of NAC and MPG on 4-HNE-mediated Protein Tyrosine Phosphorylation and MAPK Activation—To elucidate the putative signal transduction mechanisms that promote 4-HNE-induced EC barrier dysfunction, we examined the ability of 4-HNE to induce protein tyrosine phosphorylation. As shown in Fig. 7A, 4-HNE stimulated tyrosine phosphorylation of EC proteins in the molecular mass range of 20–203 kDa, with intense phosphorylation around 35–40, 60–80, and 120–203 kDa. Both the thiol protectants, NAC and MPG, almost completely and significantly attenuated 4-HNE-mediated protein tyrosine phosphorylation in BLM-
VECs, further suggesting the involvement of intracellular thiol redox status in 4-HNE-induced protein tyrosine phosphorylation. Analyses by immunofluorescence microscopy showed that phosphotyrosine was mostly associated with focal adhesions in control cells (Fig. 7B), whereas 4-HNE caused redistribution of tyrosine phosphorylated proteins from cell periphery. Furthermore, pretreatment with NAC or MPG attenuated 4-HNE-induced phosphotyrosine redistribution to focal adhesions.

Recently, it has been observed that 4-HNE modulates MAPKs cell signaling in mammalian cells (4, 12, 20), which play an important regulatory role in the physiological functions of the endothelium (24, 25). To determine if activation of MAPKs by 4-HNE is under redox regulation in lung microvascular ECs, we studied the effect of NAC and MPG on 4-HNE-induced MAPK phosphorylation. As shown in Fig. 7 (C–E) pretreatment of BLMVEC, with 1 mM NAC or MPG for 1 h, completely and significantly attenuated the 4-HNE-induced phosphorylation of ERK, JNK, and p38 MAPKs. These results indicate that MAPK activation by 4-HNE is redox-dependent.
4-HNE Suppresses Tyrosine Phosphorylation of Focal Adhesion Kinase—Among the protein complexes associated with focal adhesions, focal adhesion kinase (FAK) plays an important role in the transmission of integrin-induced cytoplasmic signals and in the reorganization of actin cytoskeleton (33, 34). Elevation of FAK is associated with enhanced focal adhesion and increased phosphorylation of adherens and tight junction proteins, leading to EC monolayer tightening (29). As shown in Fig. 8, treatment of ECs with 4-HNE (25 μM) decreased FAK tyrosine phosphorylation in a time-dependent fashion without altering the levels of non-phosphorylated FAK as compared with the control. NAC and MPG attenuated reduction in FAK phosphorylation by 4-HNE (Fig. 8B). These results suggest that 4-HNE suppresses FAK phosphorylation, which in turn may affect focal adhesion, adherens, and tight junction proteins, cell-cell contacts and gap formation and ultimately EC barrier function.

4-HNE Modulates Focal Adhesion, Adherent and Tight Junction Proteins, and Cytoskeleton—Because it was evidenced from the earlier experiments of this study that 4-HNE modulated cell-cell adhesion contact and FAK phosphorylation, we investigated the localization of focal adhesion, adherens, and tight junction proteins that play a pivotal role in EC barrier function (26, 27, 29, 32). Cells were treated with 4-HNE (25 μM) for 30 min and then were immunostained to visualize FAK, paxillin, VE-cadherin, β-catenin, and ZO-1. As shown in Figs. 9–11, 4-HNE modulated redistribution of these proteins and caused intercellular gap formation. NAC and MPG attenuated 4-HNE-induced redistribution of focal adhesion, adherens, and tight

FIGURE 7. Effect of NAC and MPG on 4-HNE-mediated protein tyrosine phosphorylation and MAPK activation in ECs. In A, BLMECs grown to ~95% confluence in 100-mm dishes were challenged with 4-HNE (25 μM) for 30 min. Cell lysates (20 μg of protein) were subjected to 10% SDS-PAGE and probed with anti-phospho-tyrosine (A and B), anti-phospho-ERK and pan ERK antibodies (C), anti-phospho-JNK or pan JNK antibodies (D), or anti-phospho-p38 MAPK and anti-p38 MAPK antibodies (E) as described under “Experimental Procedures.” Shown are representative blots from three different experiments. Values are means ± S.E. Changes compare with control calculated from the left panel. *, significantly different from non-treated cells (p < 0.05); **, significantly different from 4-HNE treatment (p < 0.05). Cells on the glass slide chambers were fixed, permeabilized as described under “Experimental Procedures,” probed with phospho-tyrosine primary antibody and Alexa Fluor 488 secondary antibody, and nuclei were stained with 4’,6-diamidino-2-phenylindole. Slides were examined by immunofluorescence microscopy using a 60× oil objective. Shown is a representative image from three independent experiments.

FIGURE 8. Effect of 4-HNE on FAK phosphorylation. In A, BLMECs grown to ~95% confluence in 100-mm dishes were challenged with 4-HNE (25 μM) for different time periods, and cell lysates were subjected to 10% SDS-PAGE and Western blotted with anti-phospho-FAK or anti-FAK antibodies as described under “Experimental Procedures.” Shown are representative blots from three different experiments. Values are means ± S.E.*, significantly different from control (p < 0.05). In B, BLMECs grown to ~95% confluence in 100-mm dishes were pretreated with 1 mM NAC or MPG for 1 h prior to challenge with 4-HNE (25 μM) for 30 min. Cell lysates (20 μg of protein) were subjected to SDS-PAGE and probed with anti-phospho-FAK or anti-FAK antibodies as described above. Shown are representative blots from three different experiments. Values are means ± S.E., and changes as compared with control were calculated after densitometry. *, significantly different from control (p < 0.05).
junction proteins. Actin microfilaments play a critical role in EC barrier regulation (26–29). It has been shown that actin is the primary target for 4-HNE (30, 31), and 4-HNE modulates actin cytoskeleton (12, 17, 18, 30, 31). Because intracellular thiols are involved in 4-HNE-induced activation of MAPKs, here we examined the role of thiol protectants, NAC and MPG, on 4-HNE-mediated actin stress fiber formation in ECs. BLMVECs were pretreated with 1 mM NAC or MPG prior to 4-HNE challenge, and cells were examined for filamentous F-actin by immunofluorescence microscopy. As shown in Fig. 11B, pretreatment of BLMVECs with NAC or MPG attenuated 4-HNE-mediated dissolution of the dense peripheral actin bands, assembly of actin stress fibers, and formation of actin aggregates in the cytosol. Furthermore, Western blot analyses revealed that 4-HNE formed Michael adducts with FAK, β-catenin, paxillin, VE-cadherin, ZO-1, and actin cytoskeleton (Fig. 12), which was blocked by NAC or MPG (data not shown). These results suggest that 4-HNE modulated focal adhesion, adherens, and tight junction proteins and cytoskeleton suggesting their involvement in 4-HNE-induced alterations of cell-cell adhesion in ECs.

4-HNE Modulates Integrins in ECs—Because integrin plays a crucial role in the regulation of FAK (33–38) and endothelial function (29, 39–41), we hypothesized that 4-HNE-mediated suppression/inhibition of FAK phosphorylation could very well be the outcome of integrin modification. To test this possibility, BLMVECs were treated with 25 μM 4-HNE for different time intervals and then analyzed for αvβ3 integrin complex by immunofluorescence microscopy and flow cytometry. As shown in Fig. 13A, treatment of ECs with 4-HNE resulted in a rapid and time-dependent decrease in integrin levels on the cell surface. The decrease in integrin level was statistically significant as early as 15 min of 4-HNE treatment as analyzed by flow cytometry (Fig. 13B). Confocal microscopic analysis and three-dimensional reconstruction demonstrated the integrin localization and clustering near the periphery of ECs (Fig. 13C). Exposure ECs to 4-HNE resulted in a time-dependent decrease and redistribution of integrin as early as 15 min of treatment (shown is 15 min of the treatment). These results are consistent with our observations that 4-HNE caused a reduction in FAK tyrosine phosphorylation after 15-min treatment. Next we tested if 4-HNE induced integrins degradation in ECs. Analysis of total cell lysates by Western blotting with anti-integrin α5 or integrin β3 antibody showed that treatment of BLMVECs with 4-HNE did not alter total integrin levels (Fig. 13D); however, formation of 4-HNE-dependent Michael adducts with integrin α5 and integrin β3 was observed (Fig. 13D). Pretreatment of cells with NAC and MPG had no effect on integrin α5 and integrin β3 levels but blocked the Michael adduct formation (data not shown). These results suggest that 4-HNE altered EC permeability by modulating cell-cell adhesion involving focal adhesion, adherens, and tight junction proteins as well as integrin signal transduction.
DISCUSSION

The purpose of this study was to elucidate the mechanism(s) underlying the 4-HNE-mediated barrier dysfunction in ECs. The present study shows that in BLMVECs (i) 4-HNE-induced changes in cell-cell adhesion contacts are dependent on ROS formation and depletion of intracellular GSH level; (ii) thiol protectants attenuate 4-HNE-induced formation of Michael adduct proteins; (iii) 4-HNE-induced protein tyrosine phosphorylation and MAPKs activation is redox-sensitive; (iv) 4-HNE-mediated actin cytoskeletal remodeling in ECs is redox-dependent; and (v) 4-HNE affects EC permeability by modulating cell-cell adhesion involving focal adhesion, adherens, and tight junction proteins as well as integrin signal transduction (Fig. 14).

Several in vivo and in vitro studies have demonstrated that 4-HNE is a key player in cardiovascular and lung disorders. The formation and accumulation of 4-HNE in tissues have been shown under different pathophysiological conditions. Formation of 4-HNE and enhanced levels of 4-HNE-modified proteins have been described in ischemic rat hearts (7) or airway and alveolar epithelial cells and in ECs of subjects with chronic obstructive pulmonary disease (9). In human subjects, ozone exposure causes formation of protein adducts, induction of stress proteins, and apoptosis in airway and lung cells (8). It has been proposed that 4-HNE formed during shock, sepsis, and...
FIGURE 13. 4-HNE mediates redistribution of integrins and formation of Michael adducts. BLMVECs grown on glass coverslips or in 35-mm dishes to 95% confluence were treated with 4-HNE (25 μM) for different time intervals as indicated. In A, cells were analyzed by immunofluorescence microscopy using CD51/CD61 integrin αvβ3 complex as a primary antibody and Alexa Fluor 488 as a secondary antibody. In B, cells were analyzed by flow cytometry using FITC-conjugated integrin αvβ3 complex as described under “Experimental Procedures.” Shown are representative tracings from three different experiments. a, unstained cells (vehicle); b, stained cells (vehicle); c, stained cells (4-HNE treatment, 15 min). Time course of 4-HNE-induced alterations in total integrin was calculated from flow cytometry measurements. *, significantly different from control (p < 0.05). In C, BLMVECs were treated with 4-HNE (25 μM) for 15 min, and confocal images were collected using a 63×, numerical aperture 1.3 glycerol objective on a Leica SP2 AOB system. Shown is a representative image from three independent experiments (bars, 10 μm). In D, BLMVECs grown to 95% confluence in 60-mm dishes were challenged with 4-HNE (25 μM) for 30 min, and cell lysates were Western blotted with anti-integrin α5 (6% SDS-PAGE) or anti-integrin β3 (10% SDS-PAGE) antibodies, or immunoprecipitated (0.5 mg protein) with anti-integrin α5 or anti-integrin β3 antibodies and probed with 4-HNE Michael adducts antibody as described under “Experimental Procedures.” Shown are representative blots from three different experiments.
ischemia/reperfusion may be causally related to lung injury (6). Interestingly, 4-HNE applied exogenously elicits similar effect to the 4-HNE formed endogenously. Infusion of low doses of 4-HNE into isolated rat lungs results in perivascular edema with vascular compression and early EC disruption (6). 4-HNE increases paracellular transport of albumin across the human umbilical EC monolayer (10) or increases blood-brain barrier permeability (11). However, the molecular mechanisms of 4-HNE-induced EC barrier dysfunction have not been thoroughly defined.

Accumulating evidence indicates that 4-HNE is not only the cytotoxic end product of lipid peroxidation but is capable of invoking a wide range of biological activities by modulation of different cell signaling pathways (1–4, 42, 43). Others and we have shown the ability of 4-HNE to modulate MAPKs, including p38 MAPK, ERK, and JNK (4, 12, 20). Moreover, we have reported that the inhibition of ERK, JNK, and p38 MAPK attenuates 4-HNE-induced MAPKs phosphorylation, actin cytoskeletal rearrangement, and barrier function in lung microvascular ECs (12). In similar studies, we have also shown that oxidants (H$_2$O$_2$ and diamide) deplete intracellular GSH, modulate MAPK activation, and alter EC barrier function (51). One possible mechanism may involve 4-HNE-dependent ROS formation and MAPK activation (12, 20, 44). The present study demonstrates the ability of 4-HNE to induce ROS formation in lung ECs confirming the earlier reports of 4-HNE-mediated formation of ROS (20, 52). Furthermore, in bovine aortic ECs, 4-HNE-induced generation of ROS was mainly localized in mitochondria due to direct interaction of electrophilic lipid oxidation products with the mitochondria (52). As shown here, 4-HNE-mediated increase in DCFDA fluorescence was almost completely abolished by rotenone (Fig. 3A) suggesting involvement of mitochondrial complex I in ROS generation, as suggested for bovine aortic ECs (52). Colocalization DCFDA fluorescence with specific mitochondrial marker MitoTracker (yellow color, Fig. 3B) further confirmed the role of mitochondria in ROS generation.

It has been shown that activation of FAK by ROS is an important event in oxidant-mediated vascular EC barrier dysfunction regulated by cell-cell and cell-matrix contacts (13). Here we hypothesized that 4-HNE-induced changes in cellular thiol redox status may contribute to modulation of cell signaling pathways and endothelial barrier dysfunction involving focal adhesion, adherens, and tight junction proteins as well as integrin signal transduction. There are probably multiple mechanisms by which 4-HNE can alter EC permeability, which include: (i) modulation of proteins/enzymes activity by Michael adducts formation (7–9, 12, 20); (ii) enhancing the level of protein tyrosine phosphorylation of the target proteins (4, 12, 20, 45) by the inhibition of phosphatases (45–48); (iii) redox-dependent mechanisms due to the depletion of cellular thiols (6, 22, 31, 44, 49, 50); and (iv) integrin-mediated regulation of cell-cell adhesion (29, 33, 34). Here, we showed that well established thiol protectants, such as NAC, MPG (51, 53), attenuated 4-HNE-induced Michael adduct proteins formation, protein tyrosine phosphorylation, MAPKs activation, and actin remodeling in BLMVECs. Moreover, pretreatment of ECs with NAC and MPG attenuated 4-HNE-induced changes in TER.

Our in vitro cell culture experiments presented here are consistent with the in vivo or animal and organ model studies on the pathophysiological effects of 4-HNE. 4-HNE formed under oxidative stress, inflammation, or under pathophysiological conditions may very well modify -SH groups, particularly by depletion of intracellular GSH level (22, 49). GSH is a vital protective intracellular antioxidant, and changes in cellular GSH or GSH/oxidized glutathione ratio may be a key factor in many cardiovascular diseases (22). Glutathione S-transferase, a major enzyme that detoxifies cellular 4-HNE by conjugation with GSH reduces accumulation of lipid hydroperoxides, including reactive aldehydes (1, 32, 54–56). In addition to acting via the constitutively expressed glutathione S-transferase, oxidants such as H$_2$O$_2$, 4-HNE, lipid hydroperoxides or UV-light treatment can induce glutathione S-transferase to detoxify the excess oxidants generated in mammalian cells (54). Substances containing -SH groups such as aminoethanethiol, dimethylthiourea, NAC, and MPG may confer protective antioxidants effects (51). NAC blocks 4-HNE-induced depletion in cellular glutathione (2, 19, 20), apoptosis (53), suppresses activation of MAPKs (20), and phospholipase D activation (14, 57). MPG administration to the heart, immediately before ischemia, reduces 4-HNE protein adducts by 75% (7). Finally, there is evidence for beneficial effects of thiol protectants in clinical pharmacology of cardiovascular diseases (51). Additionally, cytoskeletal protein modification by 4-HNE, including actin (12, 17, 30, 31, 36) and microtubules (17, 58–59), may play an important role in the regulation of cell-cell contacts and EC barrier function (13, 60). Cross-talk between FAK, integrin, and cytoskeleton has been proposed to be responsible for FAK activation and autophosphorylation by integrin in cell adhesion (33). Integrins are a family of transmembrane $\alpha$ and $\beta$ heterodimers consisting of matrix binding sites for collagen, lamin-
nin, vitronectin, and fibronectin (33, 35, 37). The cytoplasmic domain of integrin interacts with a number of signaling and adaptor proteins that link to focal adhesion and cytoskeleton (29, 33, 34, 38). As cell-matrix adhesion and cell shape are mediated by integrins, loss of this binding could result in intercellular gaps and disruption of EC barrier function. In the current study we have demonstrated for the first time that 4-HNE caused a rapid (15 min) decline in surface integrins and tyrosine phosphorylation of FAK, which may explain a rapid and dramatic alteration of EC barrier dysfunction leading to tissue damage and edema during heart infarction, brain stroke, and lung diseases. A recent study (61) has demonstrated that integrins are critical mediators of lung vascular permeability and acute lung injury. The one or more mechanisms by which 4-HNE affects integrins signaling are not completely understood. One of the possible pathways may involve 4-HNE-induced activation of Ephrin kinases, which in turn can inhibit integrin-mediated cell adhesion (62). Integrin-mediated ROS production requires inhibition of a low molecular weight phosphotyrosine phosphatase, which prevents dephosphorylation and activation of FAK (63). However, it is unclear if such a mechanism is involved in attenuation of tyrosine phosphorylation of FAK by 4-HNE in lung ECs. Because 4-HNE formed Michael adducts with focal adhesion and adherens junction proteins as well as with α5 and β3 integrins, further studies are necessary to establish mechanisms of 4-HNE-induced integrin signaling in EC barrier dysfunction.

In conclusion, we have shown that 4-HNE induced GSH depletion, formation of Michael adducts proteins, protein tyrosine phosphorylation, MAPK activation, actin remodeling, and cell monolayer permeability changes in BLMVEC, which were all attenuated by pretreatment with thiol protectants, suggesting an important role of thiols redox in regulation of EC barrier function under 4-HNE challenge. Furthermore, 4-HNE caused the redistribution of FAK, β-catenin, paxillin, VE-cadherin, and ZO-1, a decrease in surface integrins levels and induction of tyrosine phosphorylation of FAK. We, for the first time, also report here an important role of integrins in 4-HNE-mediated EC permeability by modulating cell-cell adhesion involving focal adhesion, adherens, and tight junction proteins.

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REFERENCES


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