Covalent Modification of Epithelial Fatty Acid-binding Protein by 4-Hydroxyynonenal in Vitro and in Vivo

EVIDENCE FOR A ROLE IN ANTIOXIDANT BIOLOGY

Assumpta Bennaars-Eiden, LeeAnn Higgins, Ann V. Hertzell, Rebecca J. Kapphahn, Deborah A. Ferrington, and David A. Bernlohr

From the Departments of Biochemistry, Molecular Biology and Biophysics, and Ophthalmology, University of Minnesota, Minneapolis, Minnesota 55455

4-Hydroxynonenal (4-HNE) is a cytotoxic \( \alpha,\beta \)-unsaturated acyl aldehyde that is naturally produced from lipid peroxidation and cleavage in response to oxidative stress and aging. Such reactive lipids covalently modify cellular target proteins, thereby affecting biological structure and function. Herein we report the identification of the epithelial fatty acid binding protein (E-FABP) as a molecular target for 4-HNE modification both in vitro and in vivo. 4-HNE covalently modified \( \tau_{1/2} < 60 \) s E-FABP in vitro, as revealed by a combination of matrix-assisted laser desorption ionization-time of flight mass spectrometry and immunological reactivity using antibodies directed to 4-HNE-protein conjugates. Identification of Cys-120 as the major site of modification was determined through tandem mass spectral sequencing of tryptic peptides, as well as analysis of proteins by two-dimensional gel electrophoresis using anti-4-HNE antibodies reacting with a 15-kDa protein that was identified by electrospray ionization and matrix-assisted laser desorption ionization-time of flight mass spectrometry as E-FABP. Evaluation of retinal pigment epithelial cell extracts derived from E-FABP null mice by two-dimensional gel electrophoresis using anti-4-HNE antibodies revealed increased modification in the null cells relative to those from wild type cells. These results indicate that E-FABP is a molecular target for 4-HNE modification and the hypothesis that E-FABP functions as an antioxidant protein by scavenging reactive lipids through covalent modification of Cys-120.

The mammalian intracellular lipid-binding proteins (LBP) are expressed from a large multigene family and encode \( \sim 15 \) kDa proteins found dispersed within the cytoplasm and nucleus (1). Because of their high affinity for hydrophobic molecules, they play a variety of roles in intracellular fatty acid, retinoid, bile acid, and sterol trafficking (2, 3). Each protein encoded by an LBP gene folds into a conserved structure consisting of 10 anti-parallel \( \beta \) strands that form a large interior water-filled cavity that serves as the hydrophobic ligand-binding site. Despite a wide variance in amino acid identity between family members (20–70%), x-ray crystallographic and high field NMR data demonstrate that all LBP forms exhibit the same \( \beta \)-barrel fold and that their \( \alpha \)-carbon backbones are virtually superimposable (4, 5). The epithelial fatty acid-binding protein (E-FABP) is unique among the LBPs because of the presence of six cysteine residues, two of which, Cys-120 and Cys-127, are modeled to form a disulfide bond within the ligand binding cavity (6, 7). Importantly, the side chains of Cys-120 and Cys-127 lie within 4.5 \( \AA \) of the C2–C4 methylene region of the \( \alpha,\beta \)-unsaturated acyl aldehydes, the most reactive oxidants produced by membrane phospholipids during oxidative stress and the generation of reactive oxygen species. These include chemical, molecular (9), or viral transformation; disease states associated with inflammatory diseases (psoriasis) (10, 11); and physical or chemical damage (nerve crush, kainic acid release) (12–14). Reactive oxygen species can be produced exogenously or from a variety of intracellular processes collectively linked to the generation of superoxide anions, hydroxyl radicals, and hydrogen peroxide (15). Such reactive oxidants chemically modify a variety of biological molecules, including polyunsaturated acyl chains of membrane phospholipids generating a family of lipid hydroperoxides. In addition, enzymatic oxidation of membrane phospholipids through the lipoxygenase enzyme systems also generates bioactive lipid hydroperoxides. The combination of chemical and enzymatic lipid hydroperoxide-generating systems provide substrates for the Hock cleavage, thereby generating a variety of hydroperoxides. This paper is available on line at http://www.jbc.org

The abbreviations used are: LBP, lipid-binding protein; FABP, fatty acid-binding protein; E-FABP, epithelial fatty acid-binding protein; 4-HNE, 4-hydroxynonenal; MALDI, matrix-assisted laser desorption ionization; TOF, time of flight; MS, mass spectrometry; ESI, electrospray ionization; MS/MS, tandem mass spectrometry; A-FABP, adipocyte fatty acid-binding protein; FA, fatty acid; CMF-PBS, Ca\(^{2+}\)/Mg\(^{2+}\)-free phosphate-buffered saline; DTT, dithiothreitol; 4-HNE, 4-hydroxynonenal; RPE, retinal pigment epithelial.

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† To whom correspondence should be addressed: Dept. of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, 321 Church St. S.E., Minneapolis, MN 55455. Tel.: 612-624-2712; E-mail: bernlo101@umn.edu.

‡ The abbreviations used are: LBP, lipid-binding protein; FABP, fatty acid-binding protein; E-FABP, epithelial fatty acid-binding protein; 4-HNE, 4-hydroxynonenal; MALDI, matrix-assisted laser desorption ionization; TOF, time of flight; MS, mass spectrometry; ESI, electrospray ionization; MS/MS, tandem mass spectrometry; A-FABP, adipocyte fatty acid-binding protein; FA, fatty acid; CMF-PBS, Ca\(^{2+}\)/Mg\(^{2+}\)-free phosphate-buffered saline; DTT, dithiothreitol; 4-HNE, 4-hydroxynonenal; RPE, retinal pigment epithelial.
ally considered to be cytotoxic because of its ability to co-
valently modify a variety of biomolecules via Michael addition
reactions across the double bond and Schiff base formation at
the carbonyl (18, 19). 4-HNE-modified DNA is associated with
defects in replication (20, 21), interference with cell cycle reg-
ulation (22), and the induction of apoptosis (23, 24). On pro-
teins, 4-HNE reacts with a variety of amino acid side chains
through amine and/or sulfhydryl modification, often affecting
the biochemical structure and activity of target proteins (17, 18).
Because reactive oxygen species are linked to oxidative stress
and aging (25–27), the identification of cellular targets for
4-HNE modification has been a subject of intense investigation.
Moreover, analysis of cellular mechanisms linked to the anti-
oxidant response may provide mechanistic clues as to the reg-
ulation of macromolecule turnover.

Given the lipid binding properties of E-FABP, its up-regula-
tion in response to conditions linked to oxidative stress, and
structural positioning of redox-sensitive thiols within the bind-
ing cavity near the bound lipid, we have pursued the hypoth-
esis that the protein is an endogenous target for 4-HNE mod-
ification. Herein we report that cysteine 120 is covalently
modified by 4-HNE in vitro as well as the properties of such
modification. Moreover, the protein is modified with 4-HNE in
vitro and retinal epithelial cell lines derived from E-FABP null
mice exhibit an up-regulation of 4-HNE-modified proteins.
These results indicate that E-FABP is a molecular target for
4-HNE modification and the hypothesis that the protein serves
as an antioxidant by scavenging reactive lipids from the cellu-
lar environment.

EXPERIMENTAL PROCEDURES

Materials—4-HNE was purchased from Cayman Chemical Co. (Ann Arbor, MI). Polyclonal antibody against 4-HNE-protein adducts was obtained from Alpha Diagnostics (San Antonio, TX). 1-Anilinonaphtalene-8-sulfonate was purchased from Biomol, Inc. Modified sequence grade trypsin was obtained from Promega. α-Cyano-4-hydroxycinnamic acid and dihydroxybenzoic acid in methanol were purchased from Agi-
ilent Technologies (Palo Alto, CA). Bio-Spin columns loaded with Bio-Gel P-6 were obtained from Bio-Rad. Enhanced chemiluminescence re-
agents were purchased from Amersham Biosciences.

Preparation of E-FABP derived from Escherichia coli—Bacterially expressed E-FABP was purified to homogeneity as previously described (28) through a combination of acid fractionation and gel filtration chromatography. Homogeneous protein eluting from the Sephadex G-75 column was concentrated and dialyzed extensively against the standard buffer. Purified E-FABP null mice exhibit an up-regulation of 4-HNE-modified proteins. These results indicate that E-FABP is a molecular target for 4-HNE modification and the hypothesis that the protein serves as an antioxidant by scavenging reactive lipids from the cellu-
lar environment.

Construction of E-FABP Cysteine Mutants—To prepare mutants of E-FABP with various cysteine to alanine substitutions, the murine E-FABP cDNA was PCR-amplified as a BamHI/EcoRI fragment and cloned into the MS2 plasmid resulting in the expression vector pHVSET-His6-E-FABP. After induction of expression in E. coli, a recombinant protein with an amino terminus containing a six-histidine tract, a flexible linker region, and a protease cleavage site amino to the E-FABP coding region is produced. The resulting protein has an amino termi-
nus of MGSHHHHHHHGMAPGQGDNMRGLYDDDKRWSGM, where the His6 is added to generate a soluble protein extract. The soluble protein extract containing various E-FABP forms was used for 4-HNE modifi-
cation reactions. Because the cysteine to alanine mutations have no effect on fatty acid binding activity, wild type and E-FABP mutants associate with a variety of endogenous E. coli fatty acids, the most prevalent being palmitic and oleic acid. On average, recombinant proteins were expressed between 0.4 and 0.6 of the total cell protein. As such, protein used in crude extracts or purified without acidification is considered largely holoprotein.

In Vitro Modification of E-FABP by 4-HNE—Purified E-FABP (10 µm) in 10 mM potassium phosphate, 150 mM NaCl (pH 7.4) (standard buffer) was incubated with 4-HNE at 22 °C for various times. Aliquots were removed and immediately applied to a P-6 Bio-Spin column and centrifuged for 4 min to separate modified protein eluting in the void volume from any free unreacted 4-HNE remaining on the column. Protein eluting from the Bio-Spin column was analyzed by a combina-
tion of MALDI-TOF MS and electrospray MS including tandem mass spectrometry, or by immunochemical methods. To evaluate the influ-
ence of noncovalently bound fatty acids on 4-HNE-modified E-
FABP (10 µm) was incubated with oleic acid (10- and 25-fold molar ratio to E-FABP) and equilibrated for 2 min to saturate the protein (30) prior to addition of 4-HNE, and then treated identically.

For immunochemical detection of 4-HNE bound to protein, the sam-
ple were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membrane was blocked in phosphate-buffered saline
containing 0.05% Tween 20 and 5% nonfat dry milk and incubated with rabbit polyclonal antibody directed toward 4-HNE protein adducts (1:1000 dilution) at 4 °C for 16 h. The membranes were then washed in the same buffer and incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:25,000 dilution) for 1 h at room temperature. The immunoreactivity was detected with enhanced chemiluminescence according to the instructions from the manufac-
turer (31). For some experiments, goat anti-rabbit alkaline phospha-
tase-conjugated secondary antibody (1:3000 dilution) was used in con-
junction with the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium to visualize the immunoreaction.

Guaniidine Hydrochloride-induced Protein Denaturation—Guaniidine hydrochloride-induced protein unfolding was performed as previously described (28). Briefly, E-FABP (0.5 µm) in a potassium phosphate buffer (pH 7.4) was mixed with increasing concentrations of guani-
didine hydrochloride from 0 to 3 M and the intrinsic tryptophan fluorescence measured using a Spex Fluoromax II fluorometer. Tryptophan fluores-
cence intensity was measured at 295 nm, and denaturation was de-
ermined by the red-shifting of the fluorescence emission maximum from 336 to 356 nm.

Mapping the Site of Covalent Modification of E-FABP by 4-HNE—4-
HNE-modified protein prepared as described from the P-6 column was dia-
mixed to 100 mM ammonium bicarbonate buffer (pH 8) containing 6 M guanidine hydrochloride, 10 mM EDTA at 4 °C to denature the protein. The protein was incubated with 10 mM dithiothreitol (DTT) for 1 h at 56 °C and subsequently cooled to room temperature. To modify the reduced thiols groups, iodoc acidic acid was added to a final concen-
tration of 20 mM and incubated for 30 min at 25 °C in the dark. For rat retinal proteins, iodoc acidamide was used for cysteine alkylations. The protein was then dialyzed exhaustively against 100 mM ammonium bicarbonate buffer (pH 8.0) and treated with 0.016 nmol of trypsin in 50 mM ammonium bicarbonate buffer (pH 8.0) containing 1 mM CaCl2 at 37 °C for 16 h. The reaction was stopped by the addition of glacial acetic acid and samples stored at –80 °C prior to mass spectral analysis. For some analyses, 4-HNE-modified E-FABP was subjected to SDS-polyacrylamide gel electrophoresis and silver-
staining for interest of mass. The expressed and purified proteins bands were excised and processed for trypsin in-gel digestion as described (32). Prior to protease digestion, the cysteine residues were reduced and alkylated using iodoc acidic acid. Peptides were extracted by sequential extraction with 50% ammonium bicarbonate buffer and 45% acetonitrile, 5% formic acid. The peptides were then evaporated to near dry-
mixing and stored at –80 °C as lyophilized residue.

Matrix-assisted Laser Desorption Ionization–Time of Flight Mass Spec-
trometry (MALDI-TOF MS)—Prior to MALDI-TOF MS analysis, a portion of the peptide mixture was desalted using Millipore C18 ZipTips using the protocol of the manufacturer. Full scans from 500 to 3500 m/z

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of the tryptic peptide mixtures and intact protein data from 4000 to 20,000 m/z were collected on a Bruker Biflex III MALDI-TOF mass spectrometer equipped with a N2 laser (337 nm, 3-ns pulse length) and a microchannel plate detector. The peptide data were collected in the reflectron mode, positive polarity, with an accelerating potential of 19 kV using a -cyano-4-hydroxycinnamic acid diluted 1:1 (v/v) with acetonitrile:nanopure water (50:50), 0.1% trifluoroacetic acid. Whole protein masses were collected in linear mode using sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) as the matrix with an accelerating potential of 19 kV. Each spectrum was the accumulation of ~200 laser shots. External calibration for peptide analysis was performed using human angiotensin II (monoisotopic [MH+] 1046.5 m/z; Sigma) and adrenocorticotropic hormone fragment 18-39 (monoisotopic [MH+] 2492.2 m/z; Sigma); external calibration for intact protein analysis was performed with myoglobin (average [MH+] 16,952 m/z) and cyctochrome c (average [MH+] 12,361 m/z).

Full scan and tandem mass spectral data of select ions of the peptide mixture were collected on a QSTAR Pulsar quadrupole time-of-flight mass spectrometer (Applied Biosystems Inc., Foster City, CA) with a MALDI source using dihydroxybenzoic acid as the matrix. The TOF region acceleration voltage was 4 kV, and the injection pulse repetition rate was 6.0 kHz. Laser pulses were generated with a nitrogen laser at 337 nm, 33 microjoules of laser energy using a laser repetition rate of 20 Hz. Mass spectra were the average of ~50 laser shots collected in positive mode from 500 to 3500 m/z. External calibration was performed with the same standards used on the Biflex, described above.

Electrospray Mass Spectral Analysis: Ion Trap—The tryptic peptides in a portion of the sample were separated on a 75-μm internal diameter C18 capillary column with a ThermoFinnigan (San Jose, CA) LCQ Classic ion trap mass spectrometer equipped with a nanoelectrospray source from New Objective (34). Tandem MS data were searched against a subset protein data base containing Mus mus mus entries using Sequest™, the ThermoFinnigan peptide MS/MS data interpretation software (34). The variable modification HNE (δ mass 156 if a Michael addition occurs on a cysteine, histidine or lysine residue and 138 if a Schiff base reaction occurs on lysine residue) (19) was entered into the appropriate Sequest parameter field.

Electrospray Mass Spectral Analysis: Quadrupole TOF—An aliquot of the tryptic peptides was desalted using Poros R2 (ABI, Foster City, CA) in a glass purification capillary (Protana, Odense, Denmark). Briefly, the peptide mixture was loaded onto the R2, washed three times with 7 μl of H2O, acetonitrile (95:5) and 5% formic acid, and eluted with 1.5 μl of H2O/acetone/acid (30:70) and 0.5% formic acid into a coated nanoelectrospray capillary (Protana). Tandem mass spectra were collected in an information-dependent acquisition scan mode during nanospray infusion of peptides at ~5 nl/min flow rate with a spray voltage of 1000 V and TOF parameters as described above. Tandem mass spectra were searched using BioAnalyst (ABI) and Mascot (www.matrixscience.com).

Isolation of Rat Retinal Extracts and Marine Retinal Pigment Epithelial Cell Lines—Experiments were performed on retinas obtained from 10-month-old Fischer 344 Brown Norway F1 hybrid male rats. Retinal isolates were prepared by homogenizing the retinas from two rats in 1 ml of homogenization buffer (20% sucrose, 2 mM MgCl2, 10 mM glucose, 20 mM Tris-acetate (pH 7.2), and 0.05% Nonidet F-40) and centrifuged for 15 min at 100 × g. The supernatant was collected and the pellet re-homogenized in an additional 1 ml of homogenization buffer. The supernatants were combined and centrifuged at 600 × g for 15 min. The supernatant containing soluble retina proteins was retained, and aliquots were stored at ~80 °C.

To prepare retinal pigment epithelial (RPE) cell lines, wild type C57Bl/6J animals harboring a targeted disruption of the Fabp5 gene encoding E-FABP were anesthetized with 5 mg of ketamine and 1 mg of xylazine per mouse and sacrificed with a lethal injection of Euthasol (200 μl). Epithelial cells from the retina were harvested and cultured as described (35) with minor modifications. Briefly, the eyes were enucleated and washed twice with Ca2+/Mg2+-free phosphate buffer (CMF-PBS) and then digested in a digest solution containing 105 units/ml collagenase and 50 units/ml hyaluronidase in CMF-PBS for 30 min at 37 °C. The tissues were washed twice with CMF-PBS and digested a second time with 0.05% (w/v) trypsin containing 0.5 mM EDTA for 15 min at 37 °C. Trypsin was inactivated by washing the tissues with Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum, 2 mM t-glutamine, 100 μM/ml penicillin, 100 μg/ml streptomycin. Using a dissecting microscope, the cornea and lens were aseptically excised from the eyes and the retina with the layer of RPE cells still attached was gently lifted away from the choroid.

The connection between the retina and RPE was disrupted by incubating the tissue at 37 °C in trypsin/EDTA until the RPE cells dissociated from the retina. After removing the retina, the remaining supernatant containing RPE cells was washed two times with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and incubated as described above with the additions of 0.05% dimethyl sulfoxide (Me2SO) and 50 ng/ml mouse epidermal growth factor at 37 °C. The RPE cells were immortalized ~5 days after harvest with the papilloma virus E6a and E7 genes via a retrovector product vector in PA317 packaging cells.

Two-dimensional Gel Electrophoresis—To separate rat retinal or marine RPE proteins, two-dimensional gel electrophoresis consisting of first dimension isoelectric focusing and second dimension SDS-PAGE was utilized. 100 μg of soluble protein was organically precipitated according to the method of Wessel and Flugge (36), dried under nitrogen, and rehydrated into an immobilized pH gradient strip (pH 3–11) using 250 μl of 10 mM Tris (pH 7.5), 2% Triton X-100, 8 mM urea, and 10 mM DTT for 14 h. Following hydration, samples were focused at 250 V for 15 min, at which time the voltage was linearly increased to 8000 V over 2.5 h and held to reach a total of 35,000 Vh. For the second dimension, the focused strips were equilibrated for 10 min in 6 μl urea, 2% SDS, 375 mM Tris-HCl (pH 8.8), 20% glycerol, 100 mM DTT; rinsed; and incubated for 10 min in 6 μl urea, 2% SDS, 375 mM Tris-HCl (pH 8.8), 20% glycerol, 135 mM iodoacetamide. The equilibrated strips were embedded in 0.5% (w/v) agarose on the top of 10% acrylamide gel, and second dimension SDS-PAGE was performed (37). Proteins in gels were either stained (32) or transferred to polyvinylidene difluoride membranes for immunochemical analysis.

RESULTS

High resolution NMR structures of E-FABP with a bound fatty acid have revealed that the C2–C4 methylene region of the acyl chain is adjacent to the Cys-120-Cys-127 disulfide bond (8). In addition, E-FABP is up-regulated in response to a variety of stimuli correlated with oxidative damage. Because oxidative damage is often mechanistically linked to the generation of reactive oxygen species and lipid peroxidation with subsequent production of reactive α,β-unsaturated medium chain acyl aldehydes, we hypothesized that E-FABP may bind such lipids, even though their chain length is generally considered too short to be bound by FABPs. Moreover, given the proximity of the Cys-120–Cys-127 disulfide to the putative position of the bound unsaturated aldehyde, we conjectured that, if such binding were to occur, a Michael addition reaction may proceed producing a covalent protein-lipid conjugate. To that end, we carried out preliminary experiments by incubating 4-HNE, as a model α,β-unsaturated aldehyde, and homogeneous E-FABP and analyzed the product following separation using P-6 Bio-Spin column chromatography by MALDI-TOF MS. The calculated molecular mass of murine E-FABP

FIG. 1. MALDI-TOF MS linear mass spectrum of a mixture of native and 4-HNE-modified E-FABP. E-FABP was modified with 4-HNE, separated from any unreactive lipid by spin column chromatography, desalted, mixed with sinapinic acid, and subjected to MALDI-TOF MS analysis. The raw data of mass intensity versus m/z were smoothed using the Gelay-Savitzky formula with 15 smoothing points using the Xmass software package (Bruker). The peak at m/z 15,001 corresponds to unmodified E-FABP, at 15,155 to singly 4-HNE-modified E-FABP, and at 15,206 to the sinapinic acid adduct of FABP.
lacking the initiating methionine is 15,006 Da. Fig. 1 shows that three major mass forms of E-FABP were detected. An ion at \( m/z \) of 15,001 \pm 5 corresponds to the native protein, and an ion observed at \( m/z \) of 15,155 \pm 5 is consistent with the calculated mass for E-FABP plus one 4-HNE molecule covalently bound. The third ion with a \( m/z \) of 15,206 \pm 5 represents a sinapinic acid adduct of E-FABP normally seen with high resolution mass spectrometry. The indicated errors in \( m/z \) value reflect the expected mass accuracy of the Biflex MALDI-TOF MS. Control experiments carried out by incubating E-FABP with long chain fatty acids (oleic acid) did not generate higher molecular mass forms, suggesting that the \( m/z \) 15,155 product represented a covalent lipid-protein conjugate that survives mass spectral analysis as opposed to noncovalently bound lipid.

As an alternate method to assess lipid-protein conjugate formation independently from mass spectrometry, we employed immunoochemical identification using antibodies directed to 4-HNE protein conjugates (31). As shown in Fig. 2, incubation of E-FABP with either 4-HNE or with oleic acid followed by SDS-PAGE and immunoblotting shows that the anti-HNE antibody is reactive only with the samples containing 4-HNE and not with fatty acids, consistent with a covalent lipid-protein conjugate being formed.

To determine the time course of 4-HNE modification of E-FABP, the protein was incubated with 10-fold molar excess of the lipid at 22 °C and at various times aliquots removed, the reaction stopped, and protein analyzed by MALDI-TOF MS to detect the presence of 4-HNE covalently bound to E-FABP. Within the first 1 min, \(-40\%\) of the protein was modified with a single HNE molecule (Fig. 3). This suggests that the initial modification of E-FABP by 4-HNE is an extremely rapid process (\( t_{1/2} \) of \( \leq 60 \) s). The rapid modification was followed by the much slower addition of a second and third molecule of 4-HNE on E-FABP over 20–120 min. Prolonged incubation (16 h) resulted in the addition of up to 6 molecules of 4-HNE on E-FABP (data not shown). These results suggest that the covalent modification of E-FABP by 4-HNE is kinetically biphasic with the rapid incorporation of a single HNE molecule on a seconds time scale followed by the slow addition of several other 4-HNEs over the course of several hours.

To assess the concentration dependence of the 4-HNE modification, E-FABP was incubated for 10 min at 22 °C with increasing molar equivalents of 4-HNE and the progress of the modification assessed immunoochemically. As shown in Fig. 4, the modification of E-FABP with 4-HNE and the subsequent formation of lipid-protein conjugates was concentration-dependent. Combining the results of the time course and the concentration dependence, standard conditions were adopted in which a 10-fold molar excess of 4-HNE was incubated with E-FABP for 10 min at 22 °C. Typically, reactions were stopped by P-6 Bio-Spin chromatography or, in some cases, by simple addition of SDS-PAGE sample buffer.

Using the standard reaction protocol, the pH and temperature dependence of the reaction was analyzed immunoochemically using antibody directed to 4-HNE-protein adducts. The modification of 4-HNE with E-FABP was unaffected by the pH of the buffer over the pH range of 6.4 to 8.4; similarly, the formation of 4-HNE protein adducts was not affected by the temperature of the reaction from 4 to 37 °C (results not shown).

Previous studies have shown that 4-HNE can covalently modify proteins at histidine, lysine, and cysteine residues (19). E-FABP contains six cysteine residues, two of which (Cys-120 and Cys-127) are located within the ligand-binding cavity and form a disulfide bond. In an effort to determine the site of 4-HNE modification of E-FABP, the lipid-protein conjugate prepared using the standard protocol was digested with trypsin and analyzed by MALDI-TOF MS. A theoretical digest of the protein with trypsin and no missed cleavage sites yields 12 different peptide fragments with molecular masses ranging from 537 to 2390 Da. Peptide mass fingerprinting of the 4-HNE E-FABP conjugate by MALDI-TOF MS identified peptides rep-
Covalent Modification of E-FABP by 4-HNE

4-HNE-modified E-FABP was digested with trypsin and the peptide fragments analyzed by MALDI-TOF (Biflex III and QSTAR Pulsar quadrupole; mass accuracies of <150 and 40 ppm, respectively) and ESI (unit mass accuracy) mass spectrometry.

| No. | MC | Amino acid position | Theoretical [MH]+ | Sequence | Identification |
|-----|----|---------------------|-------------------|----------|---------------|
|     |    | From | To | m/z |                     | Peptide m/z | MS/MS spectrum |
| 1   | 0  | 13   | 24 | 1500.6503 | LMESHGFEEYMK | X |
| 2   | 0  | 13   | 24 | 1656.7653 | LMESHGFEEYMK(hne) | X |
| 3   | 0  | 25   | 33 | 927.5827  | ELGVGLAR | X |
| 4   | 1  | 25   | 33 | 1055.6577 | ELGVGLAR | X |
| 5   | 0  | 35   | 55 | 2029.0499 | MAAMAKPDCHTCGDNGNITV | X |
| 6   | 0  | 35   | 55 | 2235.0593 | MAAMAKPD(cm)IIITC(cm)GDNGNITV | X |
| 7   | 0  | 62   | 72 | 1198.5778 | TTFSNCNLGEK | X |
| 8   | 0  | 62   | 72 | 1296.5833 | TTFS(cm)NLGEK | X |
| 9   | 1  | 62   | 81 | 2190.9977 | TTFSNLGEKFDTEDTA | X |
| 10  | 1  | 62   | 81 | 2249.0032 | TTFS(cm)NLGEKFDTEDTA | X |
| 11  | 2  | 62   | 82 | 2319.0921 | TTFSNLGEKFDTEDTA | X |
| 12  | 2  | 62   | 82 | 2377.9776 | TTFS(cm)NLGEKFDTEDTA | X |
| 13  | 0  | 73   | 81 | 1011.4983 | FDTEDTA | X |
| 14  | 1  | 82   | 103 | 2519.1899 | KTETVCTFQDGALVHQQW | X |
| 15  | 1  | 82   | 103 | 2577.2044 | KTETVCM(cm)TFQDGALVHQQW | X |
| 16  | 0  | 83   | 103 | 2391.1039 | TETVCTFQDGALVHQQW | X |
| 17  | 0  | 83   | 103 | 2433.1094 | TETVCM(cm)TFQDGALVHQQW | X |
| 18  | 1  | 83   | 103 | 3136.4640 | TETVCM(cm)TFQDGALVHQQW | X |
| 19  | 2  | 83   | 110 | 3284.5589 | TETVCM(cm)TFQDGALVHQQW | X |
| 20a | 1  | 113  | 129 | 2156.9800 | DGK(hne)MIVEC(cm)VMNNATC(cm)TR | X |
| 21  | 0  | 116  | 129 | 1584.7007 | MIVECMNNATC | X |
| 22  | 0  | 116  | 129 | 1642.7962 | MIVECMNNATC(cm)TR | X |
| 23  | 0  | 116  | 129 | 1700.7117 | MIVECM(cm)VMNNATC(cm)TR | X |
| 24d | 0  | 116  | 129 | 1798.8212 | MIVECM(hne)VMNNATC(cm)TR | X |

a MC, number of trypsin missed cleavage sites in peptide.

b Modifications indicated in parentheses, positioned COOH-terminal to the relevant amino acid; hne = 4-hydroxyenonal; cm = carboxymethyl.

c Proposed HNE modification based on peptide m/z value. Signal/noise (S/N) for the monoisotopic peaks of peptide numbers 2 and 20 were 10 and 18, respectively.

d Confirmed HNE modification based on MS/MS sequence information. Some peptides listed in the table were detected with oxidized methionines in addition to the forms detected above, but the m/z values are not shown.

The tandem mass spectral analysis revealed similar coverage (76%) of the primary sequence of E-FABP. Incorporation of a single 4-HNE onto proteins yields a mass addition of 156 and is consistent with a Michael addition reaction. Manual interpretation of the MALDI-TOF MS data showed that two ions correspond to singly charged m/z values of 4-HNE-modified peptides. An abundant ion (85% relative abundance by MALDI-TOF MS) with a m/z of 1798.8 corresponds to a single carboxymethylated cysteine (587 Da) and a single 4HNE (156 Da) adduct on the FABP peptide from amino acids 116--129 (peptide 24; Table I) with a mass accuracy of 5 ppm.

To verify the location of the 4-HNE modification on the peptide 116--129 peptide, the ion with a m/z of 1798.8 was selected for tandem spectral analysis by MALDI. The sequence of the peptide (116--129) was confirmed as MIVECVMNNATC, which includes the identification of a carboxymethylated cysteine at amino acid 127 and 4-HNE-modified cysteine at amino acid 120 (Fig. 5). In the MS/MS spectrum, the observed monoisotopic product ions y3 (m/z 437.23), y4 (m/z 538.22), y5 (m/z 609.25), y6 (m/z 723.31), y7 (m/z 837.32), and y9 (m/z 1067.47) correspond to carboxymethyl cysteine-containing fragments generated from the precursor 1798.8 (see Ref. 38 for fragment ion nomenclature). The precursor ion m/z 1798.8, as well as the monoisotopic m/z value of 1642.74, which pertains to the carboxymethylated peptide 116--129 with the loss of 4-HNE (9 mass 156), each provide evidence for covalent modification of cysteine by 4-HNE in a Michael addition reaction. The cysteine residue that is modified is clearly indicated by the y10 (m/z 1326.60) and y10-H2O (m/z 1308.57) fragment ions and the internal fragment C5hneVMN-CO (m/z 576.25), which pertains to peptide 120--124 with the loss of 28 Da (CO = carbon monoxide). The mass difference between the y9 and y10 ions, 259 Da, corresponds to the mass of a cysteine residue (103 Da) + the delta mass value for 4-HNE (156). The average mass accuracy for all fragment ion m/z values (all reported as monoisotopic values) is 16 ppm.

A second less abundant ion (15% relative abundance by MALDI-MS) at 2156.97 m/z corresponds to peptide from amino acids 113--129 with two carboxymethyl cysteines and one
C120A, C127A, and C120A/C127A with bound fatty acids were used in the crude bacterial extracts. Covalent Modification of E-FABP by 4-HNE

4-HNE (δ 156) (peptide 20; Table I). This ion was not observed using either ESI or MALDI sources on the QSTAR mass spectrophotometer; therefore, no tandem mass spectral data could be acquired for sequence confirmation. Overall, tandem mass spectra data (40 ppm mass accuracy) provided evidence for the confirmation of 13 peptide sequences. Consistent with only 40% of the protein modified with 4-HNE, MS/MS data revealed a mixture of peptides with both fully and partially carboxymethylated cysteines (Table I).

As an independent method to identify which cysteine was modified, and to determine whether both cysteine residues (Cys-120 and Cys-127) within the ligand binding cavity of E-FABP were required for 4-HNE modification, a series of cysteine to alanine E-FABP mutants were constructed. Soluble proteins from E. coli extracts expressing wild type E-FABP, C120A, C127A, and C120A/C127A with bound fatty acids were incubated with a 10-fold molar equivalent of 4-HNE for 10 min at 22 °C. Immunoblot analysis using the antibody against 4-HNE protein adducts (normalized to total E-FABP expression) revealed that, in the C120A mutant, no modification was detected (Fig. 6), whereas modification was detected in the C127A mutant. Therefore, the presence of a nearby second thiol is not necessary for 4-HNE covalent modification of E-FABP. Interestingly, modification was also detected at very low levels (less than 13% that of C127A mutant) in the double mutant C120A/C127A. Although not characterized, this trace modification may correspond to the Cys-127 and/or Lys-115 modifications.

To that end, E-FABP was preincubated with either a 10- or 25-fold molar excess of oleic acid (Kd = 248 nM yielding an occupancy of 99.7 and 99.9%, respectively) and then 4-HNE was added to the same level as fatty acid. As shown in Fig. 7, the modification was markedly enhanced, relative to the apo-protein, by the presence of fatty acids within the lipid-binding cavity. This suggests that, in crude extracts, the specific modification of E-FABP cysteine mutants extracts (Fig. 6) may be in part caused by the presence of bound endogenous fatty acids. Currently, we are not able to separate 4-HNE-bound E-FABP from unmodified E-FABP. As such, we cannot determine the fatty acid binding properties of 4-HNE-modified E-FABP.

To confirm that, in the presence of bound FA, the site of 4-HNE modification was Cys-120, peptide mass fingerprinting of the 4-HNE E-FABP conjugate by MALDI-TOF MS as well as peptide sequencing by MS/MS was carried out. 82% coverage of E-FABP primary sequence was identified as well as the site of modification in the presence of fatty acids. Consistent with the apoprotein modification, Cys-120 was the site of modification when short term incubations with 4-HNE were carried out (Table II). At longer time points (60 min), minor secondary modifications (<5%), likely to be at Cys-127 and/or Lys-115, were indicated. As such, both in the absence and presence of fatty acids, Cys-120 is the site of 4-HNE modification.

Work with fatty acids has suggested that the presence of a covalently bound ligand stabilizes the protein structure to chemical denaturation. However, this point cannot be adequately addressed for the fatty acid rapidly exchanges from the protein during analysis. To address this point with covalently bound 4-HNE, the protein prepared using the standard protocol was subjected to increasing concentrations of guanidine hydrochloride and the red-shifting of the emission maximum was used as an indicator of protein unfolding (28). As shown in Fig. 8, there is a shift in the progress curve to increasing concentrations of guanidine hydrochloride when using the lipid-protein conjugate relative to native protein. Because the sample contains a mixture of unmodified and modified protein, we cannot determine a true ∆G° unfolding for the reaction. However, it is clear that the presence of covalently bound 4-HNE stabilizes the protein structure to chemical denaturation.

To determine whether a lipid-protein conjugate could be detected in vivo, two-dimensional polyacrylamide gel electrophoresis was used to separate proteins from crude rat retinal tissue extracts where E-FABP is expressed to high levels. Despite the numerous proteins present in the sample, only 1% of these spots were immunoreactive using an antibody directed toward 4-HNE-protein adducts (Fig. 9). Using a combination of MALDI-TOF MS and electrospray ionization MS/MS analysis of tryptic digests of the resolved proteins, several spots were identified. The protein spot with a molecular mass of 15 kDa
was identified as E-FABP. By combining the results obtained using MALDI-TOF MS and ESI mass spectrometry, 70.4% of the primary sequence of E-FABP was covered by a peptide map (Table III) and four peptides were sequenced by MS/MS. No peptide fragment corresponding to 4-HNE covalently bound to Cys-120 was identified, although peptide 116–129 (without HNE) was sequenced by MS/MS. The data imply either that the abundance of the modification was too low to detect the lipid-protein conjugate or that the modification in vivo occurs on a residue(s) distinct from Cys-120.

Hotamisligil and colleagues, as well as Owada et al. (39), have independently reported the development of E-FABP null mice. The phenotype of the null mice is subtle; however, Owada et al. have reported that in E-FABP null mice the skin epithelial cell membranes are metabolically compromised such that there is a marked decrease in transepidermal water loss. Using such E-FABP null mice and their wild type littermates, we have developed immortalized lines of retinal pigment epithelial cells and analyzed soluble cell extracts for 4-HNE-protein conjugates using two-dimensional gel electrophoresis followed by immunological analysis. Ferrington and colleagues (40) have shown in retinal extracts that several proteins are 4-HNE-modified and that their abundance increases with increasing age. As shown in Fig. 10, there are a number of proteins endogenously modified by 4-HNE. Moreover, there is an up-regulation of both the number and intensity of the spots corresponding to 4-HNE-modified proteins in the E-FABP null cells (see white arrows). Overall, there was more than a doubling of the number of 4-HNE-modified proteins in the E-FABP null cells compared with wild type epithelial cells. In such immortalized cells, the abundance of E-FABP is markedly down-regulated such that, although E-FABP is a rather major protein in retinal extracts (Fig. 9A), E-FABP in the immortalized epithelial cells is a quite minor protein (determined from Western blotting; results not shown). This may explain why we do not see a 15-kDa protein modified by 4-HNE in the immortalized cells as compared with the rat retinal extracts. However, it is clear that the loss of E-FABP in the null cells results in an increased level of 4-HNE-modified cellular proteins.

**DISCUSSION**

The up-regulation of E-FABP in response to conditions linked to oxidative stress and the presence of redox-sensitive sulfhydryl residues within the lipid-binding cavity led to the hypothesis that E-FABP maybe an endogenous target for

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**Table II**

| No. | Peptide amino acids | Observed [M+H]⁺ | Sequence | Abundance* |
|-----|--------------------|----------------|----------|------------|
| 1   | 116–129            | 1798.82        | MIVEC(hne)VMNNATC(cm)/TR | 0.3380    |
| 2   | 116–129            | 1896.91        | MIVEC(hne)VMNNATC/hne/ | 0.0318    |
| 3   | 113–129            | 2157.01        | DGK(hne)MIVEC(cm)/VMNNATC(cm)/TR | 0.0190    |

*Peptide abundance relative to peak intensities for complete field.

bPeptide observed in all conditions: 10-min and 60-min time point with both 10 and 25 nmol of oleic acid and 4-HNE.

cPeptides observed only at 60-min time point with 25 nmol of oleic acid and HNE.
<p>Covalent Modification of E-FABP by 4-HNE</p>

**TABLE III**

| Amino acids | Sequence | Observed mass<sup>a</sup> | Theoretical mass |
|-------------|----------|--------------------------|------------------|
| 13–24       | LVESHGFEDYM<sup>a</sup> | 1469.64                  | 1469.64          |
| 25–33       | ELGVGLALR<sup>a</sup>  | 926.43                   | 926.55           |
| 35–55       | MGAMAKPDEITLDGNLTVK    | 2280.68                  | 2281.12          |
| 62–72       | TTVFSTDLEGK<sup>a</sup>| 1241.59                  | 1241.59          |
| 73–81       | FDFTTAADR             | 1010.25                  | 1010.43          |
| 73–82       | FDFTTAADR             | 1138.27                  | 1138.53          |
| 82–99       | KTETVCTFDGalVQHQR     | 2061.6                   | 2062.02          |
| 83–99       | TETVCTFDGalVQHQR      | 1953.53                  | 1953.92          |
| 116–129     | MVVCVMMNACTR<sup>a</sup> | 1695.43                  | 1695.76          |

<sup>a</sup> Peptides sequenced by tandem mass spectrometry.

<sup>b</sup> Observed mass of peptide identified by MALDI-TOF MS.

4-HNE modification and that the protein functions to remove chemically reactive lipids from the cellular environment. Three lines of evidence support the conclusion that E-FABP is a target for covalent modification, both in vitro and in vivo, and that the protein may play a role in antioxidant biology. First, the properties of the in vitro reaction of 4-HNE with E-FABP have been characterized using a combination of mass spectrometry and immunochemical analysis. Second, two-dimensional electrophoresis of crude retinal lysates identified E-FABP as a target for covalent modification, both in the presence and absence of fatty acids. This result was corroborated with evidence obtained from the E-FABP cysteine mutants, where no modification with 4-HNE was detected with C120A; only trace modification was revealed with C120A/C127A relative to C127A modification. In vitro, a second minor ion (2156.97 m/z) was detected whose mass was consistent with amino acids 113–129 plus two carbonylations and one HNE-modified lysine residue (possibly Lys-115). It is possible that the 4-HNE modification observed in the C120A/C127A mutant is on Lys-115. This point has yet to be established but is consistent with the binding data as well as NMR structure analysis of E-FABP with bound palmitic acid.

**FIG. 10.** Western blot analysis of proteins derived from epithelial cells of wild type and E-FABP null mice. Soluble proteins (100 μg) from epithelial cells derived from wild type and E-FABP null mice were separated by two-dimensional isoelectric focusing/SDS-PAGE, transferred to polyvinylidene difluoride membrane, and analyzed immunologically with antibody against 4-HNE-modified proteins. White arrows indicate 4-HNE-modified proteins for which abundance is increased in E-FABP null cells, whereas a black arrow indicates a modified protein that decreases in abundance. Numbers on the left hand border represent the molecular mass of proteins in kDa.

The presence of six cysteine residues in E-FABP distinguishes it from the other lipid-binding protein family members (6, 8). High resolution x-ray and NMR structures reveal that the six cysteines are found as three clustered pairs: Cys-43 and Cys-47 found on the surface of the protein, Cys-67 and Cys-87 buried in the hydrophobic core, and Cys-120 and Cys-127 present in the ligand binding cavity as a disulfide bond (8). Analysis of fatty acid binding (palmitic) within the cavity of E-FABP indicates that the lipid adopts a U-shaped conformation with the carboxyl head group coordinated between two arginine residues (109 and 129) and Tyr-131 in a hydrogen-bonding network (7). The disulfide bond between Cys-120 and Cys-127 of E-FABP is in close proximity to the C2-C4 region of the bound fatty acid but is not directly involved in fatty acid binding. Cys-120 of E-FABP is homologous to Cys-117 of A-FABP, a residue for which the side chain also lies within close proximity to the bound fatty acid. Covalent modification of Cys-117 of A-FABP with small adducts (methylmethane thiolsulfonate) has no effect on fatty acid binding; however, large adducts (N-ethylmaleimide) inhibit association (44). As such, steric factors around Cys-120 play a role in access to the H-bonding network involving the fatty acid carboxylate and Arg-109, Arg-129, and Tyr-131. In the absence of fatty acids, the carbonyl group of 4-HNE could H-bond with Tyr-131, providing some stabilization energy. Interestingly, the observation that, in vitro, 4-HNE modification of E-FABP was stimulated by oleic acid implies that both a fatty acid and 4-HNE are bound simultaneously. The presence of a fatty acid adjacent to Cys-120 could dynamically alter the electrostatic micro-environment within the cavity, thereby affecting the pK<sub>a</sub> of Cys-120, thus making it more reactive toward 4-HNE modification. Alternatively, fatty acid bound may increase the generalized hydrophobicity of the cavity by displacing water thereby increasing the binding of the hydrophobic medium chain aldehyde, a reaction not normally carried out by FABPs. The combination of Cys-120 modification, potential for hydrogen bonding to Tyr-131, and stimulation by fatty acids suggests that the aldehyde is coordinated in a similar, but not identical, manner to fatty acids. Utilization of C120A mutant of E-FABP in future experiments will facilitate dissection of the binding reaction in the
absence of catalysis and the structural organization of the binding cavity with two ligands present.

Given the homologous cysteine of A-FABP (Cys-117) and its similar fatty acid binding character, we have preliminarily evaluated whether 4-HNE could covalently modify this protein. Interestingly, attempts to covalently modify A-FABP in vitro with 4-HNE were unsuccessful (results not shown) under a limited set of experimental conditions. This may indicate that the covalent modification of E-FABP by 4-HNE is more complex than simply thiol availability. The modification of E-FABP C127A by 4-HNE implies that redox chemistry involving a structurally vicinal thiol is not likely to be involved in the modification; however, this point has not been analyzed in detail. As such, the molecular determinants that define binding versus catalysis are obscure. It will be interesting to analyze the potential covalent modification of other FABPs that have a cysteine at a homologous position (myelin P2, testis lipid-binding protein) to determine whether they too are reactive.

The role of E-FABP as an antioxidant protein is consistent with the cell and/or tissue types in which it is expressed. E-FABP is found in cells exposed to high oxidative stress, including the retina, lens, lung, and tongue (45). In these cells, E-FABP may play a role in limiting the amount of oxidative damage to proteins by scavenging 4-HNE-like molecules. Herein we report using crude rat retinal lysates the in vitro identification of E-FABP as a molecular target for HNE modification. The outer rod segment membranes of the retina in particular are highly enriched with long chain polyunsaturated fatty acids including arachidonic acid (20:4) and docosahexaenoic acid (22:6), thus making them highly susceptible to lipid peroxidation (46). A recent study has demonstrated that in vitro induced lipid peroxidation of rod outer segment membranes was reduced by the addition of increasing concentrations of retinal extracts containing a low molecular mass FABP (46). The mechanism of this protection is currently not clear, but it is possible that the FABP acts by binding the free fatty acids preventing their oxidation. Alternatively, as demonstrated herein, protection of rod outer segments may be through the covalent modification (scavenging) of E-FABP by 4-HNE. This point is currently under investigation.

Using retinal pigment epithelial cell lines derived from wild type and E-FABP null mice, the abundance of soluble 4-HNE-modified proteins was evaluated immunchemically. Typically, FABPs are expressed to very high levels in cells in vivo (2) and generate a cytoplasmic pool of fatty acid for metabolic utilization. However, in cultured epithelial cells, E-FABP expression is markedly down-regulated, and we did not observe any 4-HNE-modified E-FABP in the culture system, whereas we did identify the protein-lipid conjugate in retinal extracts. The inability to detect 4-HNE-modified E-FABP in cultured cells may be caused by any of several reasons. For example, E-FABP with covalently bound 4-HNE could be a substrate for the proteasome (40) such that the modified protein is cleared quickly from cells. An alternate possibility is that the covalent modification of E-FABP by 4-HNE could be reversed within the cellular context by protein glutathionylation. Indeed, Fratelli et al. (47) have reported that in oxidatively stressed T lymphocytes E-FABP is glutathionylated, although the site of modification was not reported. Current experiments are focused on evaluating these possibilities.

Even with a low level of E-FABP expression in the wild type cells, there was a marked up-regulation in the modification of soluble proteins in cell lines derived from E-FABP null mice. There are several potential mechanisms to explain the up-regulation of 4-HNE-modified proteins in the E-FABP null cells. The most direct explanation is that the increase in 4-HNE modification of epithelial cell proteins may be a result of the lack of the proposed protective effects of E-FABP via covalent modification by 4-HNE. The absence of E-FABP may result in 4-HNE modification of alternate targets such as those seen in Fig. 10. Alternatively, increased modification by 4-HNE could be caused by increased lipid oxidation as a result of redistribution of endogenous polyenoic fatty acids from the E-FABP solubile pool to the membrane, where they could be more accessible to peroxidation.

In sum, we have shown that HNE can rapidly modify Cys-120 of E-FABP in vitro as shown by a combination of mass spectral analysis and immunochemically with an antibody directed toward 4-HNE protein adducts. E-FABP was also identified as a cellular target for HNE modification in vivo as demonstrated by two-dimensional analysis of rat retinal lysates. In vitro, the presence of fatty acids within the lipid-binding cavity greatly enhanced the covalent modification of E-FABP by 4-HNE, suggesting that the holoprotein is the target for 4-HNE modification in vivo. As such, the protein does not undergo suicide inactivation because the noncovalent fatty acid binding function is not affected. The absence of E-FABP in retinal pigment epithelial cell lines resulted in an increase in the number and abundance of proteins modified by 4-HNE. Thus, the role of E-FABP can be expanded beyond simple fatty acid trafficking to a novel protective/antioxidant function. This marks the first report, to our knowledge, of a role for any FABP in the covalent binding of lipids and suggests the hypothesis that E-FABP functions as an antioxidant protein, protecting the integrity of the cellular environment through inactivation of reactive lipids.

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Covalent Modification of Epithelial Fatty Acid-binding Protein by 4-Hydroxynonenal in Vitro and in Vivo: EVIDENCE FOR A ROLE IN ANTIOXIDANT BIOLOGY
Assumpta Bennaars-Eiden, LeeAnn Higgins, Ann V. Hertzel, Rebecca J. Kapphahn, Deborah A. Ferrington and David A. Bernlohr

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