SUPPORTING INFORMATION

Mechanisms of 4-Hydroxy-2-nonenal Induced Pro- and Anti-Apoptotic Signaling

Pankaj Chaudhary, Rajendra Sharma*, Abha Sharma, Rit Vatsyayan, Sushma Yadav, Sharad S. Singhal, Navin Rauniyar, Laszlo Prokai, Sanjay Awasthi, and Yogesh C. Awasthi*

Department of Molecular Biology and Immunology, University of North Texas Health Science Center, Fort Worth, Texas 76107

---

*To either of whom the correspondence and reprint requests should be addressed:

Department of Molecular Biology and Immunology,

University of North Texas Health Science Center,

Fort Worth, Texas 76107.

Tel: 817-735-2366; Fax: 817-735-2118;

Email: Yogesh.Awasthi@unthsc.edu, or Rajendra.Sharma@unthsc.edu

Materials and Methods:

Antibodies- Antibodies against Fas receptor (CH11) were obtained from MBL International Corp. (Woburn, MA), whereas Fas (B-10), Daxx, p-Daxx (Ser671), p53, p21, HSF1, Hsp70, FADD, FasL, JNK, ASK1, Bcl-xL and Bax were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies against 4-HNE−protein adducts (4-HNE 11-S) used in this study were from Alpha Diagnostics (San Antonio, TX). Antibodies to phosphorylated SEK1 (Thr261), JNK (Thr183/Tyr185), p53 (Ser15) and ASK1 (Thr845) were from Cell Signaling Technology (Danvers, MA) and antibodies to phospho Daxx (Ser668) were from Abcam Inc. (Cambridge, MA).

Cytotoxicity assay- The sensitivity of the HepG2 cells to 4-HNE was measured by the MTT assay (*1*). 2×10^4 cells in an aliquot of 190 μL of full serum medium were seeded in 96-well flat bottomed microtiter plates for 24 h to allow attachment to the culture plates. After incubation, 10 μL of PBS containing various amounts of 4-HNE (0-100 μM) were added. Four replicate wells were used for each concentration of 4-HNE in these studies. After 2 h incubation of the plates at 37°C, 10 μL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium Bromide (MTT) solution (5 mg/mL in PBS) was added to each well, and the plate was incubated for an additional 4 h at 37°C. The plate was then centrifuged at 2,000 *g* for 10 min and the medium was aspirated from each well. Dimethylsulfoxide (100 μL) was added to each well and the formazan dye crystals formed in cells were dissolved by shaking the plates at room temperature for 2 h. The absorbance of formazan at 562 nm was measured using a microplate reader (ELx 808 BioTek Instruments, Inc). A dose-response curve was plotted and the concentration of 4-

HNE resulting in a 50% decrease in formazan formation was calculated as the IC_{50} value of 4-HNE.

Apoptosis detection by flow cytometry- For apoptosis detection, both non-adherent and adherent HepG2 cells were collected after 2 h of treatment with 0 to 100 μM 4-HNE. Using an Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, Palo Alto, CA), cells were stained with Annexin V-FITC and propidium iodide (PI) according to the manufacturer's instructions. After incubation at room temperature for 20 min, cells were analyzed by flow cytometry using the FITC signal detector (FL1) and the PI detector (FL3) in Beckman Coulter FC500 flow cytometer. Cells negative for both Annexin V and PI staining are live cells; Annexin V-positive- and PI-negative-stained cells are early apoptotic cells; Annexin V- and PI-positive stained cells are necrotic and/or late apoptotic cells; and PI-positive and Annexin V-negative stained cells are necrotic cells. For each measurement, at least 50,000 cells were counted.

Co-Immunoprecipitation studies- Cells were washed with cold PBS and pellets were resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.0 mM Na_2EDTA, 1.0 mM EGTA, 1% Triton, 2.5 mM $Na_4P_2O_7$, 1.0 mM β-glycerophosphate, 1.0 mM Na_3VO_4, 1.0 μg/ml leupeptin, 1.0 mM PMSF). Following sonication on ice, the homogenates were centrifuged and aliquots of the supernatant containing 500μg protein were incubated with anti-Fas or anti-Daxx antibodies (1:100) at 4°C overnight. Protein A/G-PLUS agarose beads (50 μL) were then added to the reaction mixture and incubated for 4 h at 4°C. The agarose beads were collected, washed three times with lysis buffer, resuspended in 60 μL of 2× sample buffer, and boiled for 5 min to dissociate the

immunocomplexes from the beads. The supernatant collected after centrifugation was subjected to Western blot analysis.

Purification of bacterially expressed Daxx- *Escherichia coli* strain BL21(DE3) was transformed with a pET-30a expression vector (Novagen, USA) containing the full length *Daxx* cDNA ligated in frame to the plasmid encoding a 6x histidine tag at the C-terminus of expressed proteins. Briefly, the bacteria were grown at 37^0C until A_{600} reached to 0.6, and then induced with 1.0 mM IPTG at 37^0C for another 3 h. The induced culture was harvested and resuspended in 10 mL lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 8.0, 1 mM PMSF, and 10 mg lysozyme) and incubated on ice for 30 min. The cell suspension was lysed by sonication and supernatant was collected by centrifugation. The supernatant was passed through TALON® His-Tag purification resins (Clontech). After washing the column with washing buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, pH 8.0), the bound protein was eluted with elution buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 250 mM imidazole) and stored at -20^0C. The eluted protein was concentrated using Amicon Centriprep concentrators followed by sequential dialysis against buffer containing 0.1 M phosphate buffer, pH 7.4, authenticated for its purity by SDS-PAGE and Western blot analyses, before being used for further studies.

References:

(1) Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods 65*, 55-63.

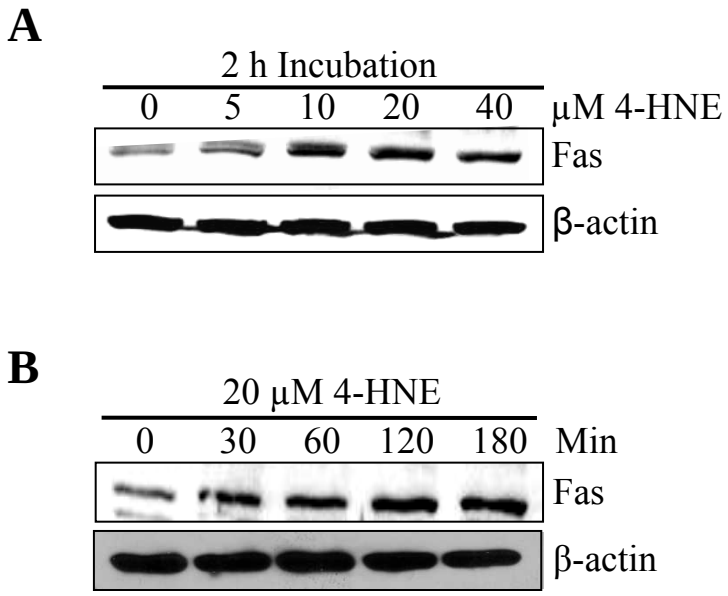


Figure S1. Effect of 4-HNE on Fas mediated apoptosis in HepG2 cells. (A) Cells were treated with 0, 5, 10, 20 and 40 μM 4-HNE for 2 h at 37^0C. Total protein lysates (30 μg) were analyzed by western blotting for Fas. β-actin was used as a loading control. (B) The cells were treated with 20 μM 4-HNE for 0, 30, 60, 120, 180 min at 37^0C. Total protein lysates were subjected to western blot analysis for Fas expression. β-actin was used as a loading control.

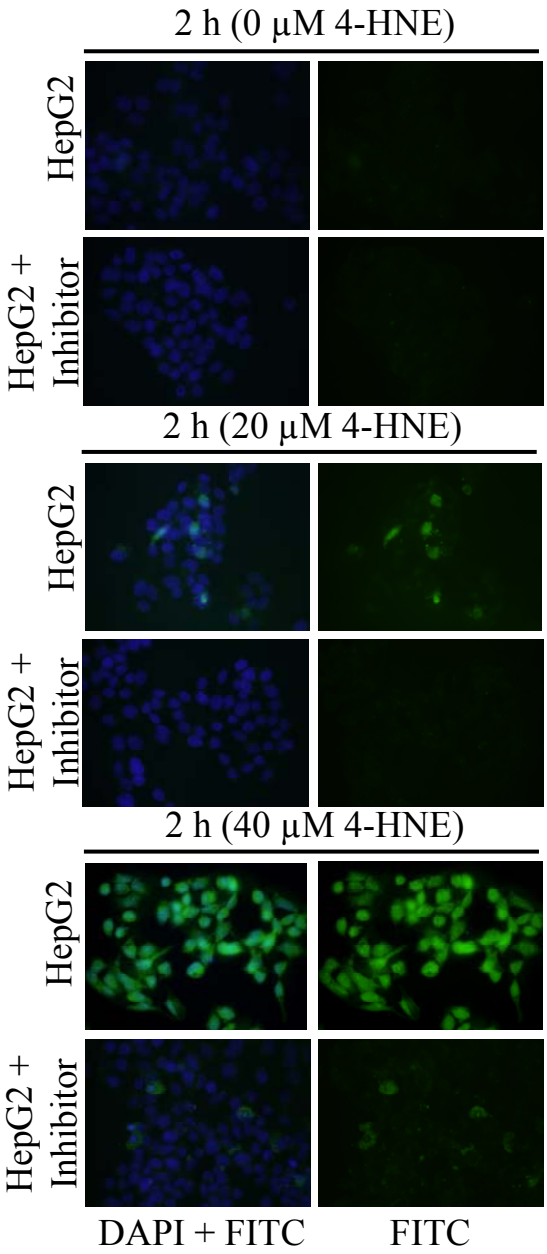


Figure S2. Inhibition of 4-HNE induced apoptosis by pretreatment of HepG2 cells with JNK inhibitor SP600125. Approximately 2 x 10^5 cells were grown on glass cover slides and after pretreatment with JNK inhibitor SP600125 (40 nM), the cells were treated with 0, 20 and 40 μM 4-HNE for 2 h. The activation of activated caspase-3 was examined by staining with 10 μM CaspACETM FITC-VAD-FMK *in situ* marker in HepG2 cells. The slides were mounted with Vectashield DAPI mounting medium and observed under a fluorescence microscope (Olympus). The photographs were taken at 400x magnification.

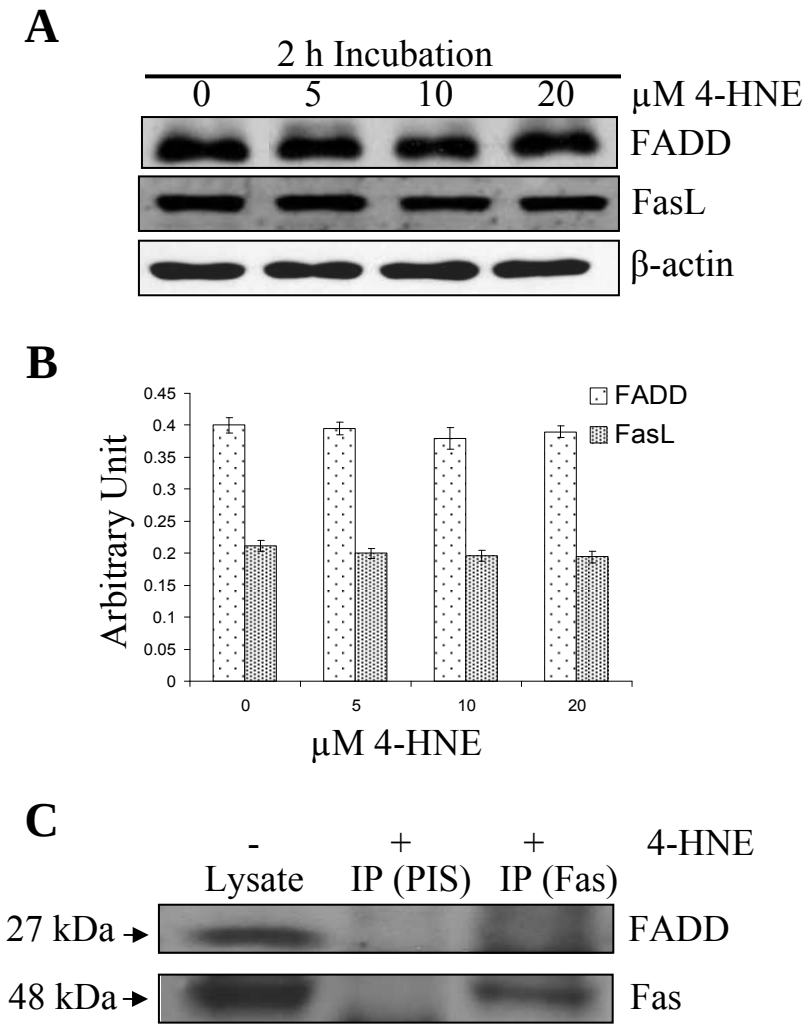
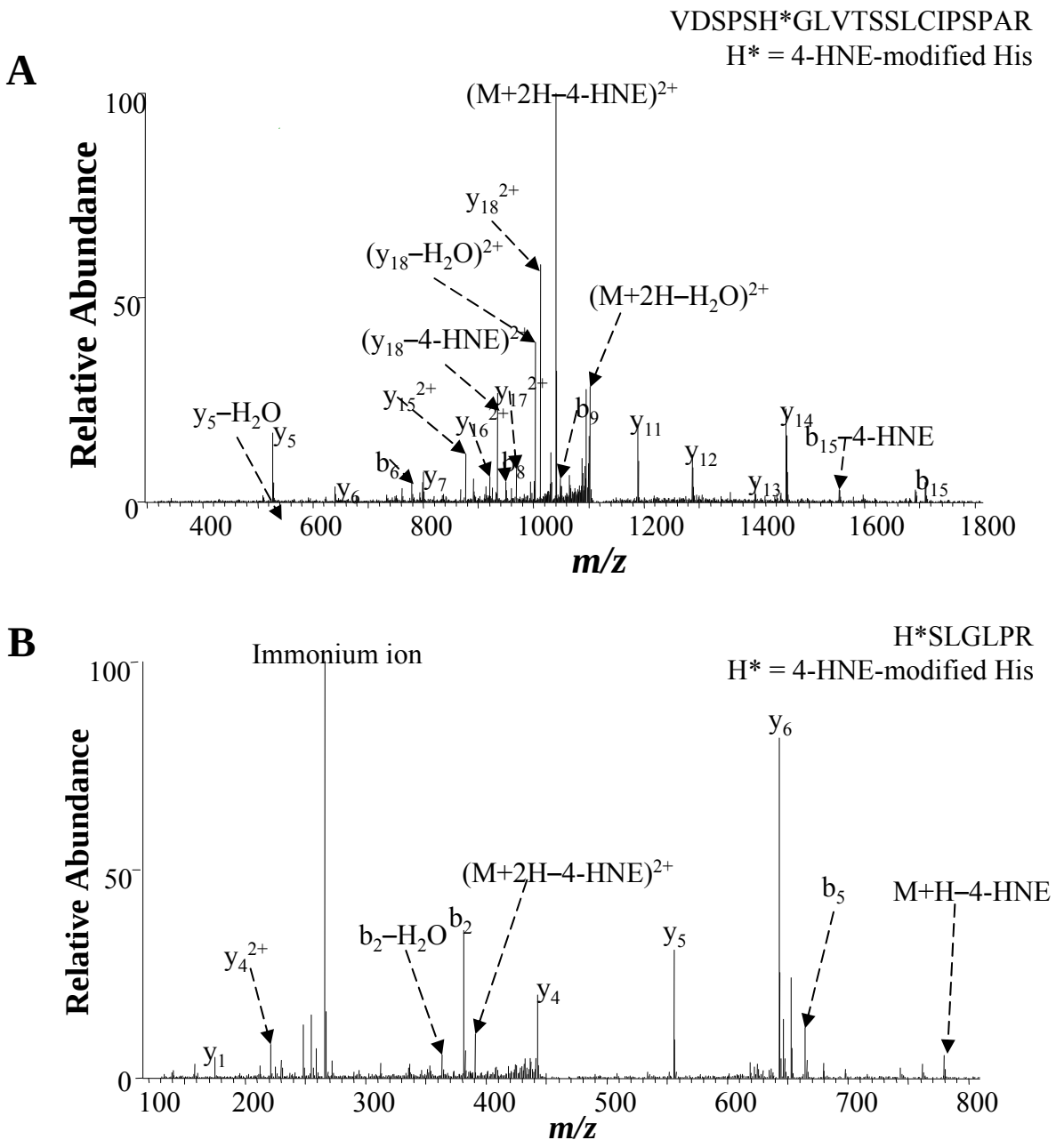


Figure S3. Effect of 4-HNE on the expression of FADD and FasL in HepG2 cells. (A) The cells were grown and treated with different concentrations of 4-HNE (0-40 μM) for 2h at 37^0C at the end of which they were collected and washed with ice cold PBS. Cell lysates were prepared as described in materials and methods. The cells extracts (50 μg of protein) were then analyzed by western blot analysis using anti-FADD and anti-FasL antibodies. Anti-β-actin antibody was used as the loading control. (B) Bar graph showing the fold change in the densitometric analysis of the bands of the Western blots obtained in panel A (C) Co-immunoprecipitation of Fas. Total protein lysates were collected from 4-HNE-treated (20 μM 4-HNE for 2h) HepG2 cells. The cell lysates were immunoprecipitated with anti-Fas antibodies as described in the materials and methods followed by Western blotting to check the expression of FADD and Fas. Immunoprecipitation with pre-immune serum (PIS) was used as control.


A
VDSPSH*GLVTSSLCIPSPAR
H* = 4-HNE-modified His
Relative Abundance
m/z
(M+2H–4-HNE)2+
y18 2+
(y18–H2O)2+
(M+2H–H2O)2+
(y18–4-HNE)2+
y5–H2O
y5
y6
b6
y7
b9
y11
y12
y13
y14
b15–4-HNE
b15
B
H*SLGLPR
H* = 4-HNE-modified His
Immonium ion
Relative Abundance
m/z
y6
(M+2H–4-HNE)2+
b5
M+H–4-HNE
b2–H2O
b2
y5
y4 2+
y4
y1

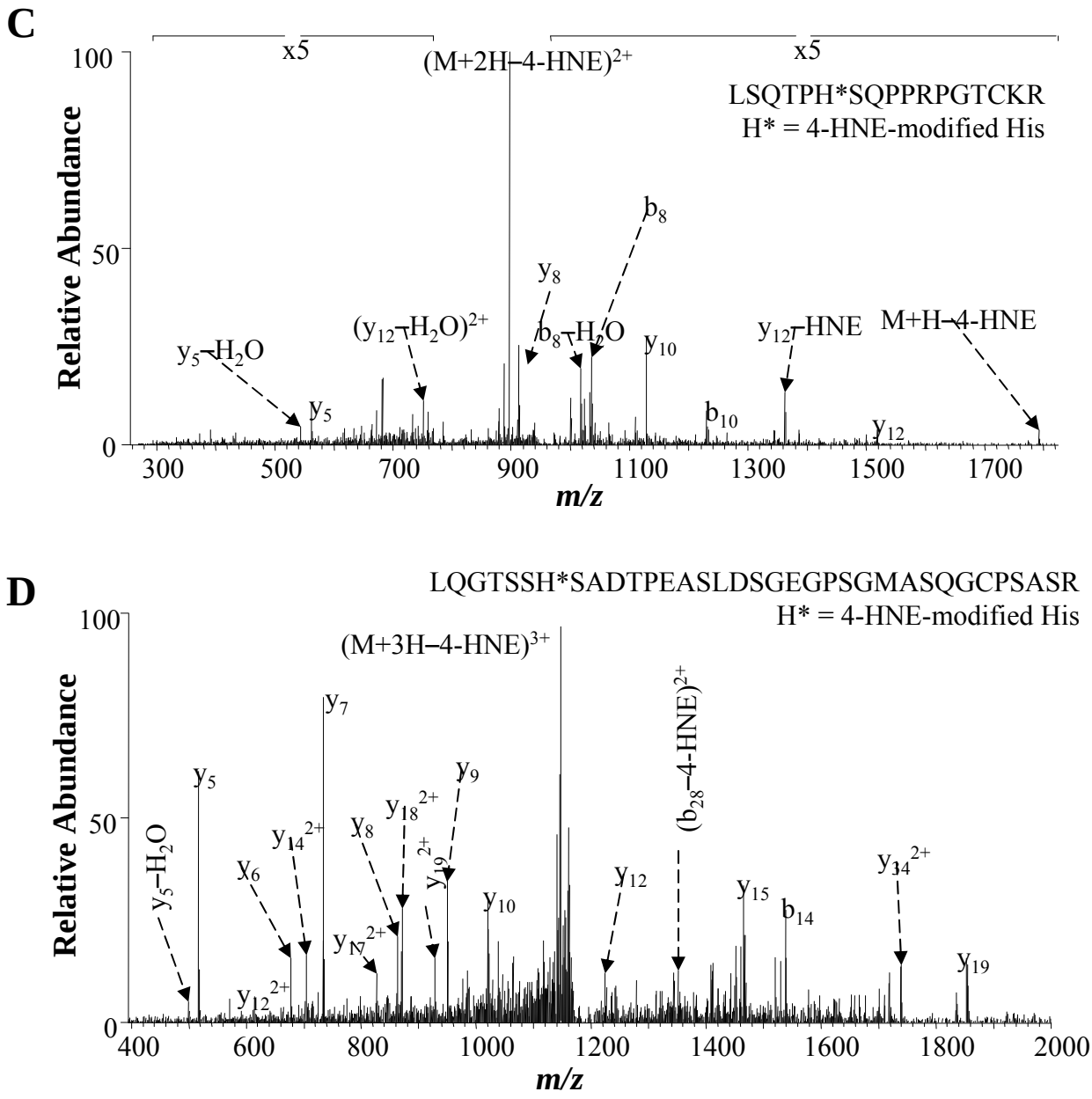


Figure S4. Tandem mass spectra of the tryptic peptides showing 4-HNE-modifications of Daxx obtained by data-dependent acquisition during online liquid chromatography. (A) CID-MS/MS spectrum of the doubly-charged 4-HNE-modified Daxx tryptic peptide VDSPSH*GLVTSSLCIPSPAR (*m/z* 1118.58); **(B)** CID-MS/MS spectrum of the doubly-charged 4-HNE-modified Daxx tryptic peptide, H*SLGLPR (at *m/z* 468.29); **(C)** CID-MS/MS spectrum of the 4-HNE-modified Daxx tryptic peptide LSQTPH*SQPPRPGTCK (*m/z* 974.01); **(D)** CID-MS/MS spectrum of the triply-charged 4-HNE-modified Daxx tryptic peptide-LQGTSSH*SADTPEASLDSGEGPSGMASQGCPSASR (*m/z* 1197.87). Histidine (H) marked with the asterisk sign (*) denote side-chain 4-HNE modification (Michael adduct). Methionine (M) oxidation and cysteine (C) carbamidomethylation introduced during sample preparation, when these residues were present; therefore, they were considered irrelevant from the study's point of view and not indicated.

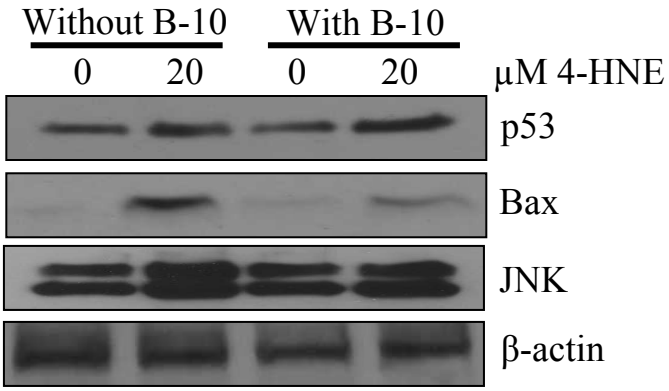


Figure S5. Effect of 4-HNE on p53 mediated pathway in antagonistic anti-Fas (B-10) antibody coated HepG2 cells. HepG2 cells were grown on 6 well plate and after pretreatment with or without antagonistic anti-Fas (B-10) antibody for 2 h (250 ng/mL), cells were treated with 0 and 20 μM 4-HNE for 2 h at 37^0C. Total protein lysates (30 μg) were analyzed by western blotting for p53, Bax and JNK. β-actin was used as a loading control.