4-Hydroxynonenal Self-Limits Fas-Mediated DISC-Independent Apoptosis by Promoting Export of Daxx from the Nucleus to the Cytosol and Its Binding to Fas†

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ABSTRACT: Previously, we have shown that 4-hydroxynonenal (4-HNE) induces Fas-mediated apoptosis in HLE B-3 cells through a pathway which is independent of FasL, FADD, procaspase 8, and DISC (Li, J., et al. (2006) *Biochemistry 45*, 12253−12264). The involvement of Daxx has also been suggested in this pathway, but its role is not clear. Here, we report that Daxx plays an important regulatory role during 4-HNE-induced, Fas-mediated apoptosis in Jurkat cells. 4-HNE induces Fas-dependent apoptosis in procaspase 8-deficient Jurkat cells via the activation of ASK1, JNK, and caspase 3, and the apoptosis can be inhibited by masking Fas with the antagonistic anti-Fas antibodies. We demonstrate that 4-HNE exposure to Jurkat cells leads to the induction of both Fas and Daxx. 4-HNE binds to both Fas and Daxx and promotes the export of Daxx from the nucleus to the cytosol, where it binds to Fas and inhibits apoptosis. Depletion of Daxx results in an increase in the activation of ASK1, JNK, and caspase 3 along with exacerbation of 4-HNE-induced apoptosis, suggesting that Daxx inhibits apoptosis by binding to Fas. 4-HNE-induced translocation of Daxx is also accompanied by the activation of the transcription factor HSF1. The results of these studies are consistent with a model in which, by interacting with Fas, 4-HNE promotes proapoptotic signaling via ASK1, JNK, and caspase 3. In parallel, 4-HNE induces Daxx and promotes its export from the nucleus to the cytosol, where it interacts with Fas to self-limit the extent of apoptosis by inhibiting the downstream proapoptotic signaling. Cytoplasmic translocation of Daxx also results in up-regulation of HSF1-associated stress-responsive genes.

4-Hydroxynonenal (4-HNE),[1] one of the major products of lipid peroxidation generated during oxidative stress, is invariably present in aerobic organisms (*1*). At higher concentrations, 4-HNE has been shown to be an inducer of apoptosis in various cell types (*2*−*7*). Because apoptotic stressors such as UV radiation, heat shock, and many xenobiotics markedly elevate the intracellular concentration of 4-HNE above its basic constitutive level in cells, it has been suggested that 4-HNE plays a crucial role in the mechanisms of programmed cell death (*3*−*5*). A number of studies have revealed that depending on the intracellular concentrations, 4-HNE is also involved in a wide variety of signaling processes including the activation of tyrosine kinase receptors on the cell membrane (*8*, *9*). The role of 4-HNE in signal transduction is complex; at "subphysiologic" concentrations it appears to promote cell survival signals, while at high concentrations it induces cell death (*10*).

Cellular apoptotic signals have been suggested to be of two types, extrinsic and intrinsic. Extrinsic apoptotic signaling is mediated by the activation of cell surface death receptors that transmit the signals after ligation with specific ligands. Fas (Apo1/CD95) is one of the death receptors belonging to the tumor necrosis factor receptor (TNFR) gene superfamily (*11*, *12*). Fas and other members of the TNFR family consist of cysteine-rich extracellular subdomains which recognize their ligands with specificity, resulting in the trimerization and activation of the respective death receptor. Subsequent signaling is mediated by the cytoplasmic part of the death receptor, which contains a conserved sequence termed as the death domain (DD). Adapter molecules such as FADD are recruited to the DD of the activated death receptor, thereby forming death-inducing signaling complex (DISC). In addition to DD, the adaptor FADD also contains a death effector domain (DED) which sequesters procaspase 8 in the DISC (*12*). Recruitment of procaspase 8 to the DISC leads to its autocatalytic activation and release of active caspase 8, which then processes downstream effector caspases, leading to cell death.

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[1] Abbreviations: 4-HNE, 4-hydroxynonenal; DISC, death-inducing signaling complex; ASK1, apoptosis signal-regulating kinase; JNK, c-jun N-terminal kinase; DOX, doxorubicin; TNFR, tumor necrosis factor receptor; RTK, receptor tyrosine kinase; Daxx, death domain-associated protein; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; HSF, heat shock factor 1; PKC, protein kinase C.

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Recent studies in our laboratory have demonstrated that 4-HNE affects a number of signaling pathways (*3*−*5*, *13*) including signaling for apoptosis mediated by Fas. Our studies revealed that the regulation of Fas-mediated apoptotic signaling by 4-HNE in human lens epithelial (HLE B-3) cells was independent of the classical extrinsic apoptotic pathway (*13*) summarized above and suggested an involvement of another death-associated protein, Daxx (*14*). Daxx is primarily a nuclear protein which translocates to the cytoplasm during stress and acts as a death receptor adaptor at the cell surface (*15*−*18*). Nuclear Daxx can associate directly with different DNA-binding transcription factors and has been shown to be a transcriptional repressor (*15*−*18*). Previous studies by other investigators have suggested that Daxx binds to Fas and activates Fas-mediated apoptosis via the apoptosis signal-regulating kinase 1 (ASK1), which in turn activates the c-jun N-terminal kinase (JNK) pathway, and that this pathway is independent of the Fas−FADD−caspase 8 pathway operative in various cell types (*14*).

The critical role of caspase 8 in Fas-mediated apoptosis has been demonstrated in a number of studies involving caspase 8 mutant cells (*19*, *20*). In these cells, procaspase 8 is inactivated by mutations at the active site that render it incapable of interacting with FADD. One such mutant cell line, CRL2571 (clone I 9.2), has been developed from the wild-type Jurkat cell line A3 (*19*). CRL2571 cells have been shown to be resistant to apoptosis caused by toxic Fas antibodies and are also partially resistant to apoptosis caused by UV radiations and doxorubicin (DOX) (*19*). Because 4-HNE induces apoptosis in HLE B-3 cells through a caspase 8/DISC-independent pathway, we used CRL2571 cells to examine the role of the Fas−Daxx−ASK1−JNK pathway in 4-HNE-triggered apoptotic signaling. The results of these studies indicate that exposure of CRL2571 cells to 4-HNE causes a dose- and time-dependent apoptosis in these cells through activation of Fas via a direct interaction of 4-HNE with the Fas receptor. This is accompanied by activation of ASK1 and JNK and results in the cleavage of the effector procaspase 3 and PARP. 4-HNE also induces an activation and translocation of Daxx from the nucleus to the cytoplasm. Surprisingly, silencing of Daxx rendered the cells more susceptible to 4-HNE-triggered apoptosis, suggesting that Daxx plays a regulatory role during 4-HNE-induced signaling for apoptosis.

MATERIALS AND METHODS

Materials. 4-Hydroxynonenal was purchased from Cayman Chemical (Ann Arbor, MI). Bradford reagent, bisacrylamide, and SDS for SDS−PAGE were obtained from BioRad (Hercules, CA). The apoptosis detection system (CaspACE FITC-VAD-FMK in situ marker) was purchased from Promega Inc. (Madison, WI). The cytoplasmic and nuclear protein extraction kit was acquired from Imgenex Co. (San Diego, CA), protein A/G−agarose from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), JNK inhibitor SP6000125 from A-G Scientific (San Deigo, CA), and Western blot stripping buffer from Pierce Co. (Rockford, IL). All other reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO). The cell culture medium and fetal bovine serum were from GIBCO (Invitrogen, Carlsbad, CA).

Cells. CRL2571(ATCC), a caspase 8 mutant cell line developed from Jurkat cells was purchased from ATCC (Manassas, VA) and maintained in RPMI medium supplemented with 10% FBS, 300 mg/L L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, and 1% penicillin and streptomycin.

Jurkat cells were purchased from the Tissue Culture Core Facility of the University of Texas Medical Branch, Galveston, and maintained in RPMI medium supplemented with 300 mg/L L-glutamine, 10% FBS, and 1% penicillin and streptomycin. Both cell lines were maintained at 37 °C in a 5% CO_2 atmosphere.

Antibodies. Antibodies against Fas receptor (IgM, CH11) were purchased from MBL International Corp. (Woburn, MA), whereas Fas mouse monoclonal (B-10) and polyclonal antibodies against PARP, caspase 3, Daxx, p53, HSF1, and Bax were purchased from Santa Cruz Biotechnology. c-jun fusion protein bound to agarose beads and phospho-c-jun antibodies were procured from Cell Signaling Technologies (Boston, MA). Polyclonal antibodies against 4-HNE−protein adducts (4-HNE 11-S) used in this study were from Alpha Diagnostics (San Antonio, TX). Daxx siRNA (h), a pool of 3 target-specific 20−25-nucleotide siRNAs designed to knock down Daxx gene expression, was purchased from Santa Cruz Biotechnology, and siRNA-A (sc-37007), a nontargeting 20−25-nucleotide siRNA, was used as a negative control.

Preparation of Cell Extracts. Cells were pelleted at 357*g*, washed twice with cold PBS, and resuspended in radioimmunoprecipitation assay (RIPA) buffer containing 1× phenylmethanesulfonyl fluoride (PMSF) and 2 μg/mL pepstatin. To prepare cytoplasmic protein extracts, the cells were washed with ice-cold PBS and resuspended in hypotonic lysis buffer (Imgenex) for 15 min, mixed with 30 μL of 10% NP-40, and vortexed for 10 s. The cell lysate was centrifuged for 30 s at 10000*g*, and the supernatant was collected. The pellet was extracted in 100 μL of nuclear extraction buffer, vortexed, and incubated at 4 °C for 30 min on a shaker. The suspension was once again vortexed for 30 s and centrifuged at 10000*g* for 10 min, and the supernatant containing nuclear extract was collected. Cytoplasmic and nuclear extracts were then used for further analysis.

Western Blot Analysis. Cell extracts containing 50−60 μg of protein were separated on SDS−polyacrylamide gels (4−20%) and transferred onto nitrocellulose (BioRad) or PVDF (Millipore) membranes. The membranes were blocked with 1% fat-free milk and 1% BSA at room temperature for 30 min and incubated overnight at 4 °C with the appropriate primary antibody in 1% milk and 1% BSA in Tris-buffered saline (TBS) containing 50 mM NaF and 0.05% Tween 20 (T−TBS). After being washed with T−TBS, the membrane was incubated with the appropriate secondary antibodies at room temperature for 1 h. After being washed again with T−TBS, the membrane was treated with SuperSignal "West Pico" chemiluminescent reagent (Pierce) as per the manufacturer's instructions and exposed to Hyperfilm ECL film (Amersham) at room temperature.

Detection of PARP. For the detection of PARP, 1×10^7 cells were suspended in 100 μL of denaturing lysis buffer containing 62.5 mM Tris−HCl, pH 6.8, 6.0 M urea, 2% SDS, 10% glycerol, 1.4 mM β-mercaptoethanol, 0.00125% bromphenol blue, 0.5% Triton X-100, and 1 mM PMSF. The cells were sonicated (3 × 5 s) on ice to disrupt protein−DNA binding and incubated at 65 °C for 15 min. Samples

containing 30 μg of protein were applied to 10% SDS−PAGE gels, and Western blot analysis was performed using PARP antibodies.

In Situ Caspase 3 Assay for Apoptosis. A total of 1×10^5 CRL2571 cells were treated with 0−20 μM 4-HNE or with Fas-agonistic CH11 antibodies (250 ng/mL) for 2 h at 37 °C. Apoptotic cells were detected by staining with 5 or 10 μM CaspACE FITC-VAD-FMK (Promega) in situ marker for 30 min in the dark. The cells were cytospun on polylysine-coated glass slides at 500 rpm (5 min). The slides were fixed with 4% paraformaldehyde for 1 h, rinsed with PBS twice, mounted in a medium containing DAPI (1.5 μg/mL), and observed under a fluorescence microscope (Nikon, Japan).

Immunoprecipitation Studies. The cells were washed twice with cold PBS, and the pellets were resuspended in RIPA buffer containing 1× PBS, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, and 2 μg/mL pepstatin. Following sonication on ice, the homogenates were centrifuged at 10000*g* for 15 min at 4 °C, and aliquots of the supernatant containing 500 μg of protein were incubated with anti-Fas or anti-Daxx antibodies (1:100) at 4 °C overnight. A 50 μL portion of protein A/G−agarose beads was then added to the reaction mixture and the resulting mixture incubated again overnight at 4 °C. The agarose beads were collected by pulse centrifugation (5 s at 14000 rpm in a microfuge), washed three times with ice-cold RIPA 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 (10000*g*) was subjected to Western blot analysis using specific antibodies.

Cytotoxicity Assay. The sensitivity of the cells to 4-HNE was measured by the MTT assay as described by Mosmann (*21*) with slight modifications. Briefly, 2×10^4 cells in 190 μL of medium were plated to each well in 96-well flat-bottomed microtiter plates. A 10 μL portion of PBS containing various amounts of 4-HNE was added. Eight replicate wells were used for each concentration of 4-HNE used in these experiments. Following incubation of the plates at 37 °C for 2 h, 10 μL of MTT solution (5 mg/mL in PBS) was added to each well, and the plates were incubated for an additional 4 h at 37 °C. The plates were centrifuged at 2000*g* for 10 min. The medium within the wells was aspirated, and 100 μL of dimethyl sulfoxide (DMSO) was added to each well. The intracellular formazan dye crystals were dissolved by shaking the plates at room temperature for 2 h. The absorbance of formazan at 562 nm was measured using a microtiter plate reader (Elx808 BioTek Instruments, Inc.). The concentration of 4-HNE resulting in a 50% decrease in formazan formation (IC_{50}) was obtained by plotting a dose−response curve.

Kinase Assays. Solid-phase kinase assays were performed by the method of Uchida et al. (*22*). Briefly, the cells were treated with 20 μM 4-HNE for different time periods. Whole-cell lysates were prepared in lysis buffer containing 20 mM Tris−HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM PMSF, and 1 μg/mL leupeptin. JNK activity was precipitated from cell lysates by incubating overnight at 4 °C with 2 μg of GST c-jun (1−89) fusion protein linked to GSH−agarose beads. The beads were then washed twice with lysis buffer and then twice with kinase buffer (25 mM Tris−HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium vanadate, 10 mM $MgCl_2$) and resuspended in 50 μL of kinase buffer containing 100 μM ATP for 30 min at 37 °C. The solid-phase kinase reaction was terminated by the addition of Laemmli sample buffer, and phophorylation of GST c-jun on Ser-63 was examined after SDS−PAGE and immunoblotting with anti-phospho (Ser-63) c-jun antibodies.

Transfection of Daxx siRNA in CRL2571 Cells. The Daxx siRNA transfection was essentially carried out according to the manufacturer's instructions. Briefly, CRL2571 cells (2×10^5 cells per well) were plated in a six-well tissue culture plate in 2 mL of normal growth medium supplemented with FBS. The cells were cultured at 37 °C until 60−80% confluency. For each transfection, 50 pmol of siRNA was diluted with 100 μL of siRNA transfection medium (solution A), and 6 μL of siRNA transfection reagent was diluted with 100 μL of siRNA transfection medium (solution B). Solution A was directly added to solution B, they were mixed gently, and the mixture was incubated for 30 min at room temperature. The cells were washed with 2 mL of siRNA transfection medium. A 0.8 mL portion of siRNA transfection medium was added to the mixture of solutions A and B and the resulting combination gently mixed and overlaid onto the washed cells. The cells and overlaid mixture were incubated for 24 h at 37 °C. After 24 h, 2 mL of fresh normal medium was added to each well, and the cells were further incubated for 48 h. Control cells were treated in a similar manner with a mixture of scrambled siRNA. The cells were harvested at appropriate time points, and the silencing of Daxx was examined.

Protein Estimation. Bradford (*23*) assay was used to determine the protein concentration in the cell lysates.

Statistical Analysis. The data are expressed as the mean ± SD for each group. The statistical significance was determined by Student's *t* test and was set at 0.05.

RESULTS

4-HNE Causes Apoptosis in Wild-Type as Well as Caspase 8 Mutant Cells. CRL2571 cells have been used to establish the requirement of procaspase 8 in Fas-mediated apoptosis through the extrinsic pathway involving DISC formation (*19*). In these cells, the active wild-type procaspase 8 of Jurkat cells has been replaced by an inactive procaspase 8 mutant. These cells are resistant to apoptosis induced by toxic Fas antibodies (CH11) which cause apoptosis in wild-type Jurkat cells. In the course of the present studies, the results of experiments comparing the effect of CH11 antibodies confirmed that these antibodies were more toxic to wild-type Jurkat cells than to CRL2571 cells (Figure 1A). CH11 antibodies induced apoptosis only in the wild-type cells as indicated by characteristic morphological changes such as cell shrinkage, nuclear condensation (data not presented), and the activation of caspase 3. In the caspase 8 mutant CRL2571 cells these antibodies did not cause apoptosis as confirmed by the lack of caspase 3 activation (Figure 2). These results confirmed the previous findings that the CH11 antibodies do not cause apoptosis in CRL2571 cells and established the validity of this cell model for use in the present studies. In contrast to the differential cytotoxicity of CH11 antibodies to the wild-type and caspase 8 mutant (CRL2571) Jurkat

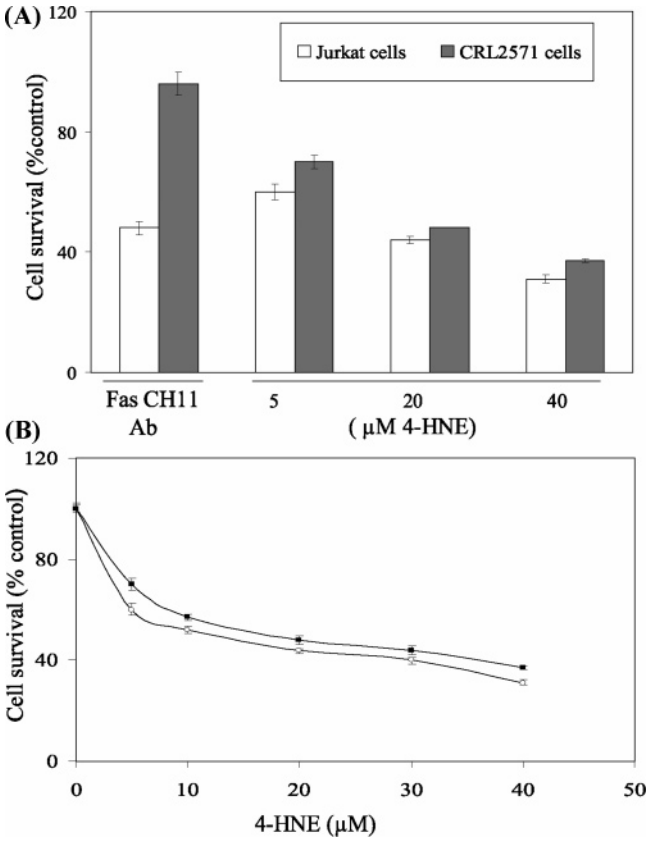

FIGURE 1: Cytotoxicity of 4-HNE to wild-type (WT) Jurkat and CRL2571 cells. The cells (2×10^4) were plated in 96-well plates in complete growth medium and treated with an increasing concentration of 4-HNE (0−40 μM) for 2 h at 37 °C. The same number of cells in duplicate columns of 96-well plates were separately treated for 2 h with Fas-agonistic CH11 antibodies (50 ng/well), and an MTT cytotoxicity assay was performed as described in the Materials and Methods. (A) Bar graph showing the percentage of cell survival after exposure of Jurkat and CRL2571 cells to anti-Fas CH11 antibodies or different concentrations of 4-HNE. (B) Cell survival plots for 4-HNE-treated WT Jurkat and CRL2571 cells ($n = 4$).

cells, 4-HNE had similar cytotoxic effects on both cells as indicated by comparable IC_{50} values of 4-HNE (14.3 ± 1.2 μM vs 16.8 ± 2.3 μM) for these cell lines (Figure 1B). 4-HNE also induced apoptosis in both wild-type and mutant (CRL2571) cells. However, these results strongly indicated that the wild-type Jurkat cells were noticeably more sensitive to 4-HNE-induced apoptosis as compared to the CRL2571 cells. In situ immunohistochemical analysis to assess the activation of caspase 3 as the end point of apoptosis showed that when exposed to 10 μM 4-HNE for 2 h, the wild-type cells had a noticeably higher number of apoptotic cells as compared to CRL2571 cells (Figure 2A). This inference was further supported by the results of Western blot analyses which showed only a minimal activation of caspase 3 in 10 μM 4-HNE-treated CRL2571 cells and a robust activation of caspase 3 in the wild-type cells under identical conditions. Furthermore, exposure to 5 μM 4-HNE caused a clearly noticeable activation of caspase 3 only in the wild-type cells and not in CRL2571 cells (Figure 2B). The differential effect of 4-HNE showing that CRL2571 cells are relatively more resistant to 4-HNE-induced apoptosis as compared to the wild-type cells parallels the reported relatively higher resistance of the CRL2571 cells to UV and DOX-induced apoptosis as compared to that of the wild-type cells (*19*). This is consistent with the earlier studies (*3*−*9*, *24*) suggesting that 4-HNE is involved in the mechanisms of stress signaling.

4-HNE Causes Up-Regulation of Fas and Induces Fas-Mediated Apoptosis. To examine the possible role of Fas in 4-HNE-induced apoptosis in Jurkat cells, two series of experiments were carried out. First, we determined the effect of 4-HNE on the expression of Fas. As shown in Figure 3A,B, 4-HNE treatment caused a time- and concentration-dependent increase of Fas. These results are consistent with the reported induction of Fas by 4-HNE in HLE B-3 cells (*13*) and suggest that induction of Fas by 4-HNE is not limited to a particular cell type. These results, however, did not provide information about the involvement of Fas in 4-HNE-induced apoptosis. To address this question, a second set of experiments were conducted in which the cells were coated with the antagonistic monoclonal antibodies (B-10) to mask Fas. Unlike CH11, B-10 antibodies do not induce apoptosis even though they bind to Fas (*25*). The results of these experiments showed that cells preincubated with B-10 antibodies acquired resistance to apoptosis induced by 4-HNE (Figure 3E). The combined results of the above experiments indicated that Fas was induced by 4-HNE and that it was required for 4-HNE-induced apoptosis in Jurkat cells through a pathway which was independent of procaspase 8.

4-HNE Activates ASK1 and JNK. Previous studies have shown that binding of Daxx to Fas is observed during stress-induced, Fas-mediated apoptosis in several cell types (*14*−*18*, *26*) and is accompanied by activation of ASK1 and JNK, the kinases involved in the downstream signaling for apoptosis. We have therefore examined the effect of 4-HNE on these parameters in CRL2571 cells and observed a dose-dependent activation of ASK1 and JNK (Figure 4). The activation of both ASK1 and JNK by 4-HNE was rapid and sustained as indicated by our results showing that, for ASK1, activation was maximal at 30 min and was sustained for both kinases for at least 120 min. These results are consistent with earlier studies (*2*−*5*) suggesting that a sustained activation of JNK is required for 4-HNE-induced apoptosis. This contention was further supported by the results of experiments showing that pretreatment of CRL2571 cells with the JNK inhibitor SP600125 made these cells resistant to apoptosis by 4-HNE (Figure 4C).

Effect of 4-HNE on Daxx. Daxx has been shown to bind to the cytosolic domain of Fas during FADD−DISC-independent apoptosis caused by oxidative stress in Jurkat, HeLa, and HEK-293 cells (*14*−*18*). Therefore, we studied the effect of 4-HNE on Daxx and its interaction with Fas. The results of these experiments showed that 4-HNE treatment caused a rapid and sustained up-regulation of Daxx expression in CRL2571 cells (Figure 5). The induction of Daxx by 4-HNE was rapid and reached a maximum within 30−60 min of exposure to 4-HNE (Figure 5B). Daxx is known to be essentially localized in the nucleus associated with PML and nuclear 10 domains (*14*−*18*). When analysis of Daxx was performed in the nuclear and cytoplasmic compartments of 4-HNE-treated cells, an increase of Daxx in the cytoplasmic compartment was observed (Figure 5C), indicating that 4-HNE treatment facilitated the export of Daxx from the nucleus to the cytoplasm. Since 4-HNE induces Fas-mediated apoptosis and causes a concomitant induction and translocation of Daxx to the cytoplasm, we

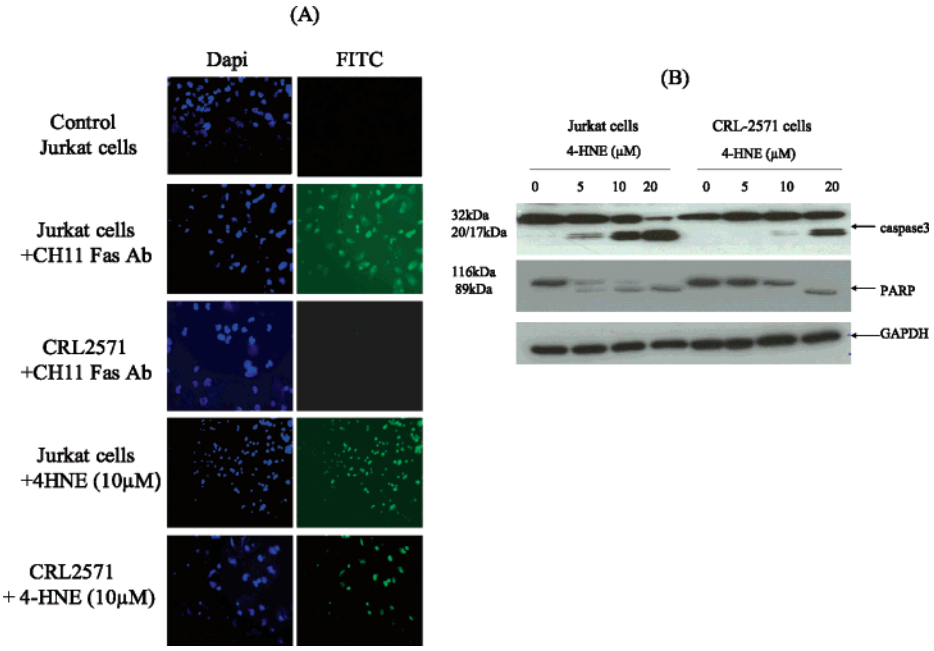

FIGURE 2: 4-HNE- and anti-Fas CH11 antibody-induced apoptosis in WT and CRL2571 Jurkat cells. (A) In situ activation of caspase 3. The cells (2×10^5) were treated either with anti-Fas CH11 antibodies (250ng/mL) or with 10 μM 4-HNE for 2 h at 37 °C in 8 mL of complete growth medium. The activation of caspase was examined by staining with 10 μM CaspACE FITC-VAD-FMK in situ marker as per the manufacturer's instructions. The slides were mounted with Vectashield DAPI mounting medium and observed under a fluorescence microscope (Nikon). The photographs were taken with a 20× objective lens. DAPI- and FITC-stained cells are appropriately marked in the panels. (B) Western blot analysis showing the activation of caspase 3 and PARP cleavage. WT and CRL2571 Jurkat cells (2×10^5) were separately treated with different concentrations of 4-HNE (0−20 μM) for 2 h at 37 °C in complete growth medium. The cells were pelleted and washed 2× with PBS, and the cell lysates were prepared as described in the Materials and Methods. The cell extracts (50 μg of protein) were then subjected to Western blot analysis using anti-caspase 3 or anti-PARP antibodies. Anti-GAPDH antibodies were used as the loading control.

examined the interaction of 4-HNE with Fas and Daxx and the relevance, if any, of these interactions to the mechanisms of 4-HNE-induced apoptosis in Jurkat cells. Whole-cell lysates of 4-HNE-treated cells were immunoprecipitated with Fas antibodies, and these immunoprecipitates were probed with anti-4-HNE 11-S antibodies (Figure 6). The appearance of bands corresponding to the molecular weights of Fas and Daxx in the Western blots indicated the presence of the adducts of 4-HNE with Fas and Daxx in the immunoprecipitates. The immunoprecipitates obtained with Fas antibodies were also probed with anti-Daxx antibodies. The results of these experiments showed that Daxx was immunoprecipitated with Fas (Figure 6), confirming the previously reported binding of Daxx to Fas (*14*−*18*). Increased binding of Daxx to Fas was observed with the time of exposure of the cells to 4-HNE (not shown). FADD was not detected in the Fas immunoprecipitates, indicating no interaction of Fas with FADD during 4-HNE-induced apoptosis (Figure 6). Collectively, the results of these experiments indicate that 4-HNE-induced, Fas-mediated apoptosis of Jurkat cells is independent of FADD and procaspase 8 and that it is accompanied by (i) binding of 4-HNE to Fas and Daxx, (ii) induction of both Fas and Daxx, (iii) translocation of Daxx to the cytoplasm, (iv) increased binding of Daxx to Fas, and (v) a rapid and sustained activation of ASK1 and JNK. It should be, however, noted that these results demonstrate a correlation but not necessarily a cause−effect relationship between binding of Daxx to Fas and the activation of downstream signaling kinases ASK1 and JNK.

Effect of 4-HNE on Daxx-Deficient CRL2571 Cells. Since 4-HNE-induced binding of Daxx to Fas was accompanied by signaling for apoptosis, we anticipated that deletion of Daxx will prevent 4-HNE-triggered apoptosis. Furthermore, if binding of Daxx to Fas contributed to the activation of ASK1 and JNK, then these kinases should not be activated upon 4-HNE treatment in Daxx-depleted cells. To test these predictions, we silenced the expression of Daxx by siRNA. As shown in Figure 7A, in CRL2571 cells transfected with Daxx siRNA, the level of Daxx was only about 5% of the cells transfected with scrambled siRNA. Contrary to our expectations, treatment of Daxx-depleted cells with 4-HNE resulted in a remarkable potentiation of cytotoxic (Figure 7B,C) and apoptotic effects of 4-HNE as compared to control cells (Figure 8). Also, 4-HNE caused a more pronounced activation of ASK1 and JNK in Daxx-depleted than in control cells (Figure 9). Moreover, CRL2571 cells that were resistant to apoptosis triggered by CH11 Fas antibodies became sensitized to these antibodies by Daxx depletion, as indicated by the onset of apoptosis in Daxx-deficient cells, which was absent in the control cells with normal Daxx expression (Figure 8A). Together, these results suggest that the binding of Daxx to Fas is not necessary for either activation of ASK1, JNK, and caspase 3 or the onset of 4-HNE-induced apoptosis. Increased sensitivity of Daxx-deficient cells to 4-HNE-induced apoptosis along with comparatively more facile activation of ASK1, JNK, and caspase 3 suggests that, at least in Jurkat cells, the binding of Daxx to Fas plays an inhibitory role rather than that of the propagator of proapoptotic signals by 4-HNE. This inhibitory role of Daxx in the mechanisms of signaling for apoptosis is further indicated by abrogation of the resistance of Daxx-depleted CRL2571 cells to apoptosis by CH11 Fas antibodies. It is to be noted

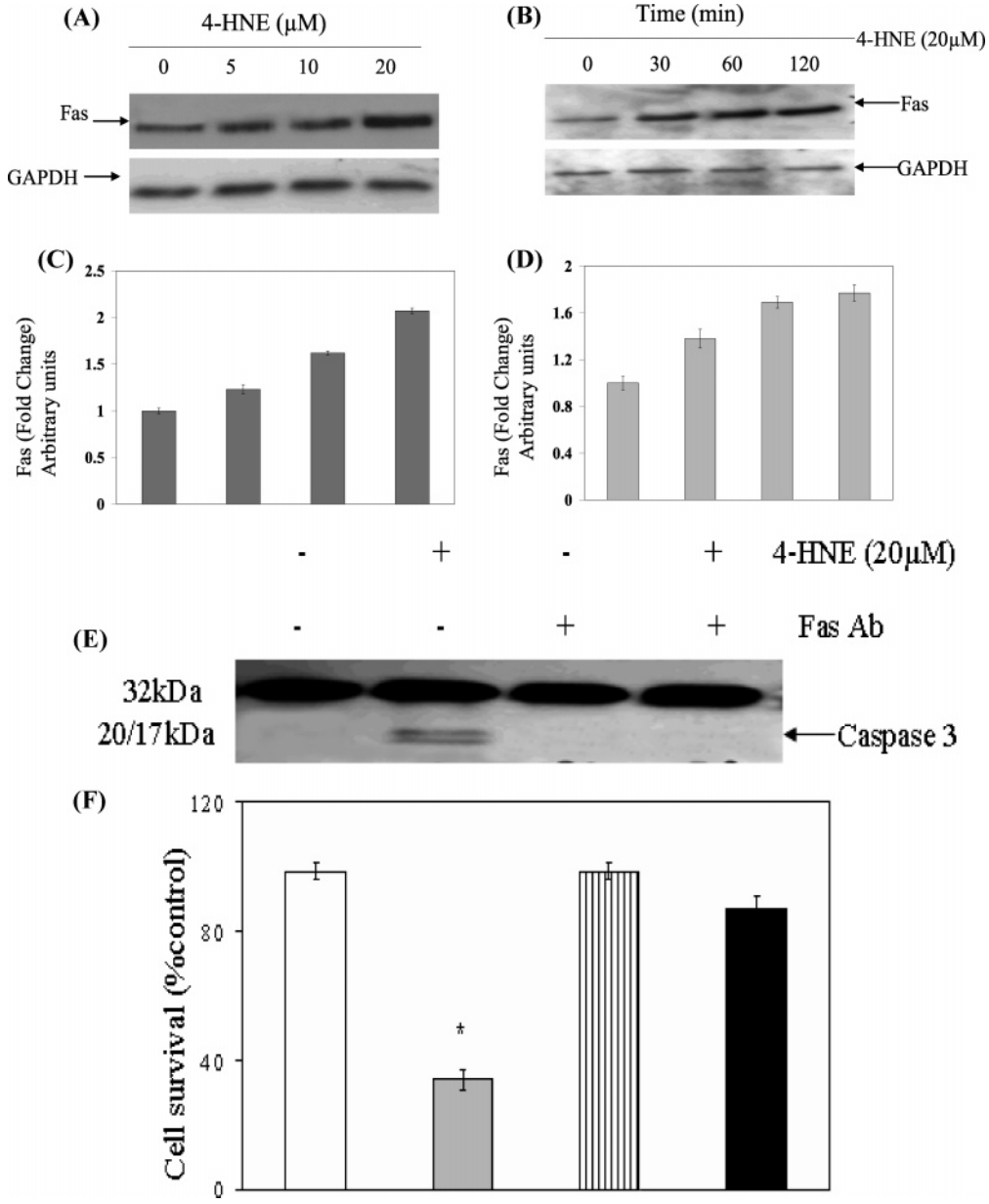

FIGURE 3: Effect of 4-HNE on the expression of Fas in CRL2571 cells. To examine the dose-dependent (A) or time-dependent (B) effect of 4-HNE, the cells (2×10^5) were treated with 4-HNE (0−20 μM) for 2 h or with 20 μM 4-HNE at different time points (0−120 min) in complete growth medium at 37 °C. After incubation the cells were pelleted, washed 2× with PBS, homogenized in radioimmunoprecipitation assay (RIPA) buffer, and centrifuged at 10000*g*. Supernatants containing 50 μg of protein were subjected to Western blot analysis using anti-Fas monoclonal antibodies (B-10) as described in the Materials and Methods. The blots were developed by West Pico chemiluminescence reagent (Pierce). (C, D) Bar graphs showing the fold change (mean ± SD, $n = 3$) in densitometric analysis of the Fas bands relative to GAPDH bands using Kodak 1D 3.6 image analysis software. (E) Effect of antagonistic anti-Fas antibodies on 4-HNE- induced caspase 3 activation. The cells (2×10^5) were plated in Petri dishes and pretreated with anti-Fas (B-10) monoclonal antibodies (2 μg of IgG protein) for 2 h followed by 20 μM 4-HNE treatment for another 2 h at 37 °C. The cells were washed with PBS (2×) and counted in a hemocytometer using the trypan blue dye exclusion method. The rest of the cell pellets were extracted in RIPA buffer as described above, and the extracts were analyzed for the activation of caspase 3 on immunoblots using anti-caspase 3 antibodies. (F) Attenuation of 4-HNE toxicity in anti-Fas antibody (B-10) coated cells: bar chart showing the percentage of cell survival in the control and Fas antibody-treated cells after treatment with 4-HNE. Data presented are the mean ± SD of two separate experiments done in triplicate (the asterisk indicates results significantly different from those of the control cells, $p < 0.01$).

that activation of caspase 3 and hence apoptosis is not completely inhibited in Daxx-deficient 4-HNE-treated cells (Figure 7). This may be expected because, in parallel to the extrinsic pathway, 4-HNE also causes activation of the p53-mediated intrinsic apoptotic pathway as described later in this section.

4-HNE Causes Translocation of HSF1 to the Nucleus and Up-Regulation of Expression of Hsp70. Previous studies (*27*) have suggested that stress-induced export of Daxx from the nucleus to the cytoplasm leads to the activation of HSF1. Therefore, we examined whether 4-HNE-induced transport of Daxx to the cytoplasm had any effect on HSF1. The results presented in Figure 10 show that, concomitant with the cytoplasmic export of Daxx in 4-HNE-treated CRL2571 cells, enhanced accumulation of HSF1 in the nucleus was observed (Figure 10A−C) in a time-dependent manner, suggesting an increase in the transcription of stress-responsive heat shock proteins. This was confirmed by the

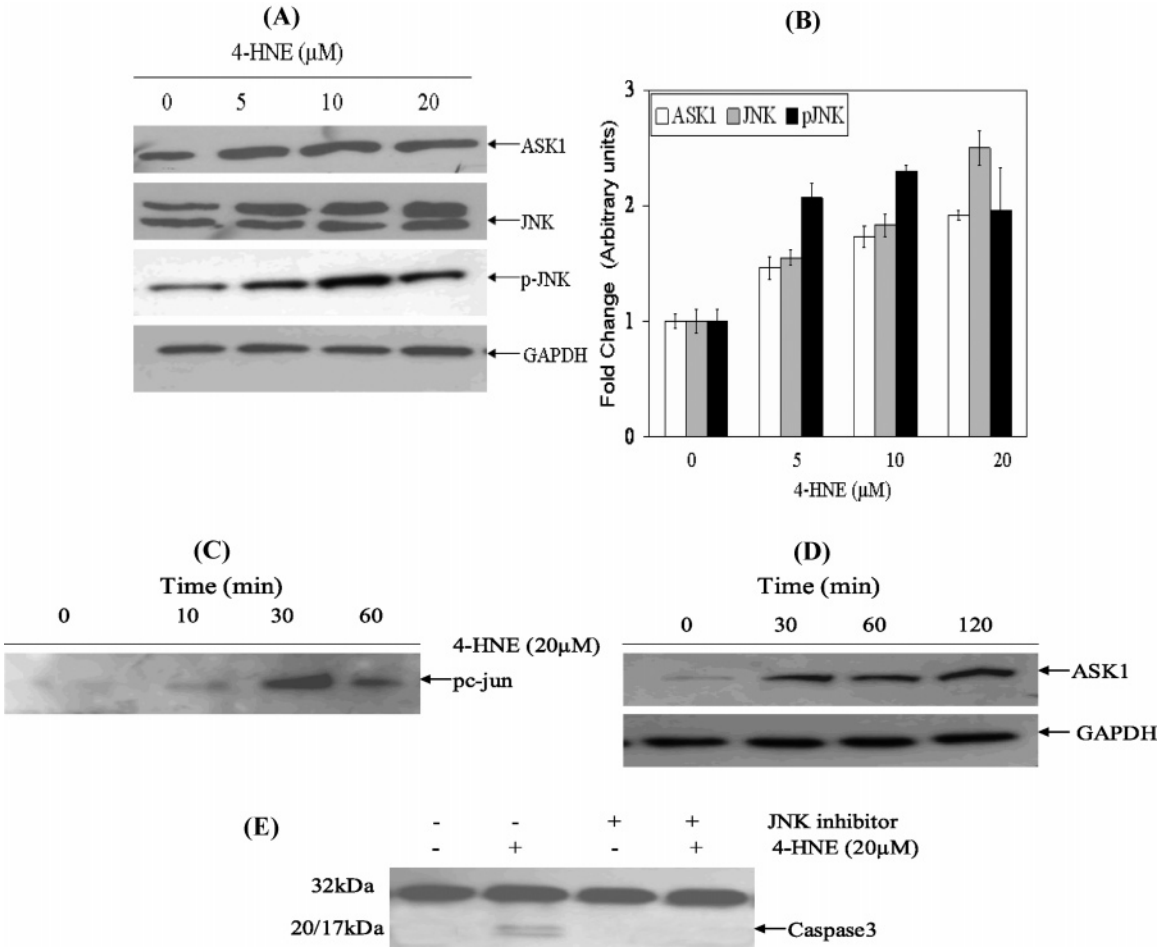

FIGURE 4: Activation of ASK1 and JNK by 4-HNE and inhibition of 4-HNE-induced apoptosis by JNK inhibitor in CRL2571 cells. For concentration dependence, the cells (2×10^5) were treated with 0−20 μM 4-HNE for 2 h. For time course studies, the cells were incubated with 20 μM 4-HNE for the indicated time points at 37 °C and processed as described in the Materials and Methods. (A) Dose-dependent activation of ASK1, JNK, and pJNK by 4-HNE. (B) Histogram showing the fold change in the densitometric analysis of the bands of the Western blots obtained using Kodak ID 3.6 image analysis software. The values (mean ± SD, $n = 3$) of different bands are normalized with those obtained from GAPDH bands used as the loading control. (C, D) Time course of phospho-c-jun induction and ASK1 activation upon treatment with 20 μM 4-HNE. (E) Inhibition of 4-HNE-induced caspase 3 activation by pretreatment of cells with JNK inhibitor SP600125 (40 nM).

results showing that the treatment of cells with 4-HNE caused a significant increase in the expression of Hsp70 in the cells (Figure 10D).

Effect of 4-HNE on p53. The contribution of 4-HNE to apoptosis through the Fas-dependent extrinsic pathway and its ability to interact with the nuclear protein Daxx in a parallel manner prompted us to investigate any possible effect of 4-HNE on the p53-mediated intrinsic pathway for apoptosis (*28*, *29*). We therefore measured p53 in the cytoplasmic and nuclear compartments of CRL2571 cells treated with different concentrations of 4-HNE for 2 h. The results of these experiments, presented in Figure 11A, indicated a dose-dependent increase in p53 expression in both cytosolic and nuclear fractions. Induction of Bax has been reported during the p53-mediated intrinsic apoptotic pathway. We therefore compared the expression of Bax in control and 4-HNE-treated CRL2571 cells. Similar to p53, a consistent increase in the expression of Bax was observed in 4-HNE-treated cells (Figure 11B). These results suggest that, besides activating the Fas-linked pathway, 4-HNE may also contribute to apoptosis via the p53-dependent intrinsic pathway, thus supporting the existence of multiple pathways for 4-HNE-induced apoptosis, similar to mechanisms reported for UV-induced apoptosis in HeLa cells (*30*).

DISCUSSION

The results of our present work are consistent with previous studies suggesting that 4-HNE acts as an important signaling molecule and that it can induce apoptosis in a wide variety of cells through the activation of JNK and caspase 3 (*2*−*5*, *30*−*34*). We have now demonstrated that, in Jurkat cells, 4-HNE induces apoptosis through a pathway which is independent of Fas ligand and of DISC formation. CRL2571 cells express an inactive mutant of procaspase 8 (*19*) and are resistant to apoptosis caused by FasL and by toxic Fas antibodies CH11 (*19*) because of an inability to form DISC. Since 4-HNE causes apoptosis in both wild-type and procaspase 8 mutant CRL2571 Jurkat cells, 4-HNE-induced apoptosis in these cells is independent of the canonical extrinsic pathway initiated by the interaction of Fas with FasL. Our present studies suggest that 4-HNE-induced Fas-mediated apoptosis is not limited to a particular cell type because we have earlier shown that 4-HNE causes Fas-dependent apoptosis in HLE B-3 cells (*13*).

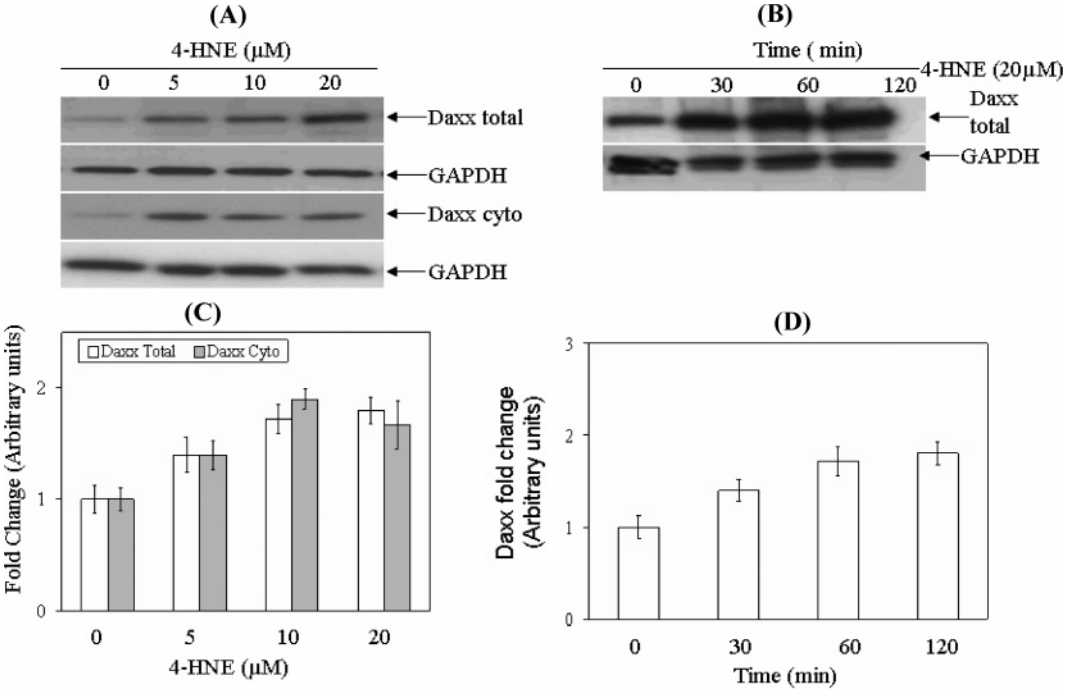

FIGURE 5: 4-HNE-mediated induction of Daxx and its cytoplasmic export and 4-HNE-mediated export of Daxx from the nucleus to the cytoplasm. (A) CRL2571 (5×10^5) cells were treated with 4-HNE (0−20 μM) at 37 °C for 2 h. (B) The cells were treated with 4-HNE (20 μM) for the indicated time periods. The cells were washed, pelleted, and extracted in RIPA buffer. The cell extracts (50 μg of protein) were resolved on 4−20% gel and immunoblotted on PVDF membranes, and the immunoblots were probed with anti-Daxx polyclonal antibodies. To assess the 4-HNE-mediated cytoplasmic export of Daxx, cytoplasmic and nuclear extracts of the cells were prepared by using the Imgenex cell processing kit and subjected to Western blot analysis, and the immunoblots were probed with anti-Daxx polyclonal antibodies. The blots were developed by West Pico chemiluminescence reagent (Pierce). The blots were probed with GAPDH and used as loading controls. **(**C, D**)** Bar charts showing the results of the densitometric analysis (Kodak 1D 3.6 software) of the bands in panels A and B.

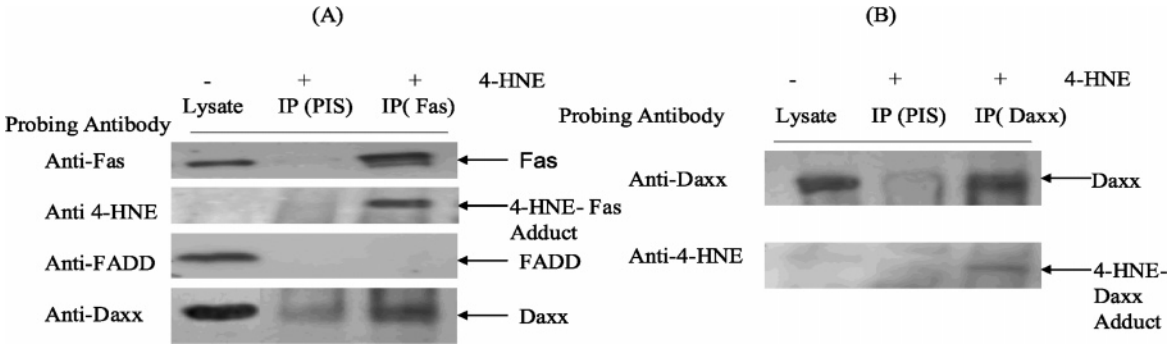

FIGURE 6: Analysis of immunoprecipitates obtained with Fas and Daxx antibodies. The whole-cell lysates of control and 4-HNE-treated (20 μM, 2 h) cells were immunoprecipitated with anti-Fas and anti-Daxx antibodies as described in the Materials and Methods. Immunoprecipitates with preimmune serum (PIS) were included as controls. (A) The immunoprecipitate of Fas (IP Fas) probed with antibodies indicated in the panel shows the presence of Fas, 4-HNE−Fas adduct, and Daxx and the absence of FADD. (B) The immunoprecipitate of Daxx (IP Daxx) probed with antibodies indicated in the panel shows the presence of Daxx and 4-HNE−Daxx adduct.

The presence of 4-HNE−Fas adducts in the immunoprecipitates of the lysates from 4-HNE-treated cells obtained with Fas antibodies indicates that 4-HNE does indeed modify Fas. The formation of 4-HNE adducts on Fas is required to transduce signaling for apoptosis, because when Fas is masked by coating with nonapoptotic, antagonistic anti-Fas antibodies, the cells acquire resistance to 4-HNE-induced apoptosis. Though not investigated in detail, the present studies suggest that 4-HNE also induces p53-mediated apoptosis in Jurkat cells. Further studies are under way to explore this possibility. Our results showing modification of Fas by 4-HNE and subsequent activation of the Fas-L-independent pathway for apoptosis lend support to the idea that 4-HNE can affect cellular signaling by mimicking the effects of receptor ligands (*13*, *35*). It has been demonstrated that 4- HNE can activate membrane tyrosine kinase receptors (RTK) and mimic the effect of their ligands on the downstream signaling events (*8*, *35*−*37*). Classically, the epidermal growth factor receptor (EGFR) is activated by binding its ligand EGF. This interaction leads to dimerization of the receptor and activation of its intrinsic cytosolic tyrosine kinase, resulting in the transautophosphorylation of tyrosine residues and activation of downstream signaling events (*35*−*37*). It has been shown that reaction of 4-HNE with EGFR leads to its aggregation (*38*), which can affect the EGFR-mediated signaling events somewhat similarly to binding of EGF (*35*−*37*). The induction of Fas-mediated signaling for apoptosis by 4-HNE reported here indicates that membrane

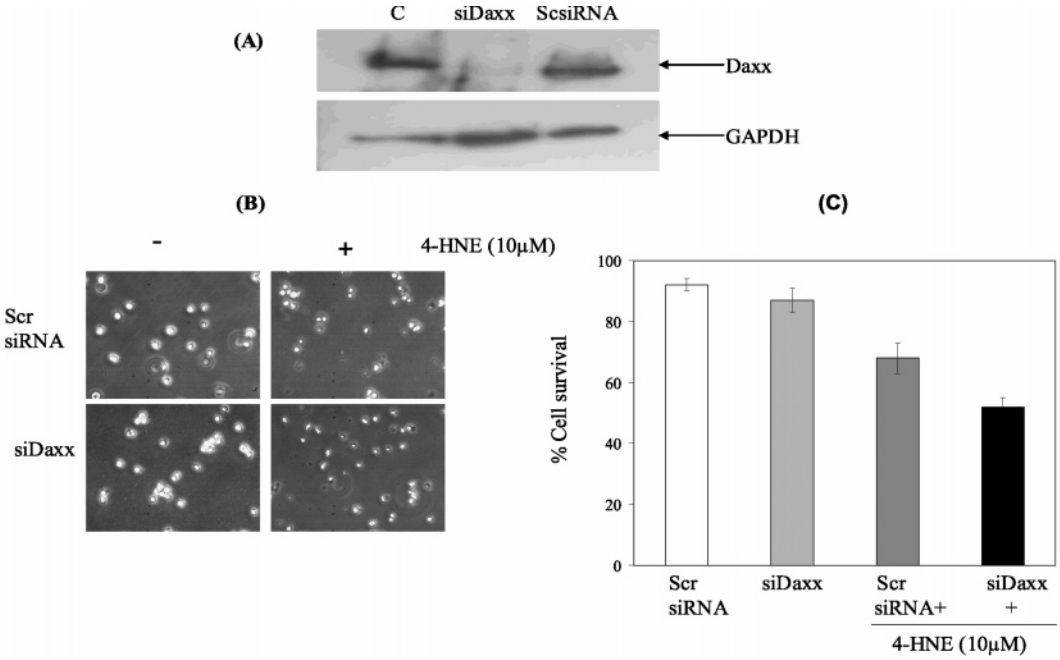

FIGURE 7: Potentiation of the 4-HNE cytotoxicity to CRL2571 cells upon silencing of Daxx with siRNA. (A) Silencing of Daxx. The cells were transfected with Daxx siRNA duplex (50 pmol) as per the manufacturer's instructions (Santa Cruz Biotechnology), while the controls were treated with scrambled siRNA duplex in a similar manner. The treated cells were harvested 48 h post-transfection, and silencing of Daxx expression was examined by Western blot analysis of the cell extracts using anti-Daxx antibodies. Daxx siRNA (middle lane) caused almost complete silencing of Daxx expression. After silencing of Daxx expression was ascertained, Daxx-depleted cells (2×10^5) were then grown in six-well plates and treated with 10 μM 4-HNE for 2 h. (B) Phase contrast micrographs showing the effect of 4-HNE (100×) and (C) bar graph showing the percent cell survival determined by Trypan blue dye exclusion of Daxx-depleted and scrambled siRNA-transfected cells in control and 4-HNE-treated cells. Values are the mean ± SD of two separate experiments done in triplicate (the asterisk indicates there are significant differences observed in the control and treated cells, $p < 0.01$).

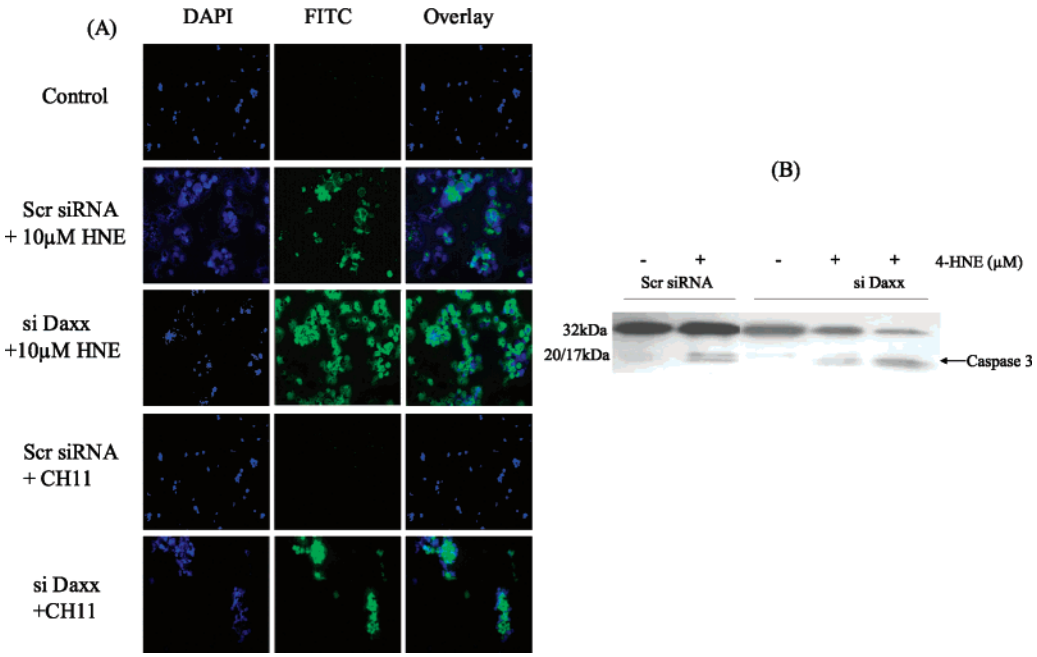

FIGURE 8: Potentiation of apoptosis by 4-HNE and CH11 through caspase 3 activation in Daxx-depleted CRL2571 cells. (A) In situ immunofluorescence analysis. The cells (2×10^5) were grown in six-well plates and treated with either 50 ng/well Fas-agonistic antibodies or 10 μM 4-HNE for 2 h at 37 °C in complete growth medium. The activation of caspase 3 in these cells was examined by staining with 10 μM CaspACE FITC-VAD-FMK in situ marker as per the manufacturer's instructions. After treatment with the in situ marker, the cells were washed twice with PBS and cytospun on polylysine-coated slides at 500 rpm for 5 min. The cells were fixed in 4% paraformaldehyde, washed 3× with PBS, mounted with Vectashield DAPI mounting medium, and observed under a fluorescence microscope (Nikon). The photographs were taken with a 40× objective lens. Respective labels of DAPI- and FITC-stained cells in control and 4-HNE treated cells or Fas CH11 antibodies are shown in different panels. (B) Western blots showing the caspase 3 activation in control and Daxx-depleted cells exposed to 10 and 20 μM 4-HNE.

receptors of different gene families (e.g., death receptor, RTK) can be modulated by 4-HNE. Therefore, the possibility of 4-HNE interacting with other membrane receptors should be vigorously explored. In addition to its interactions with

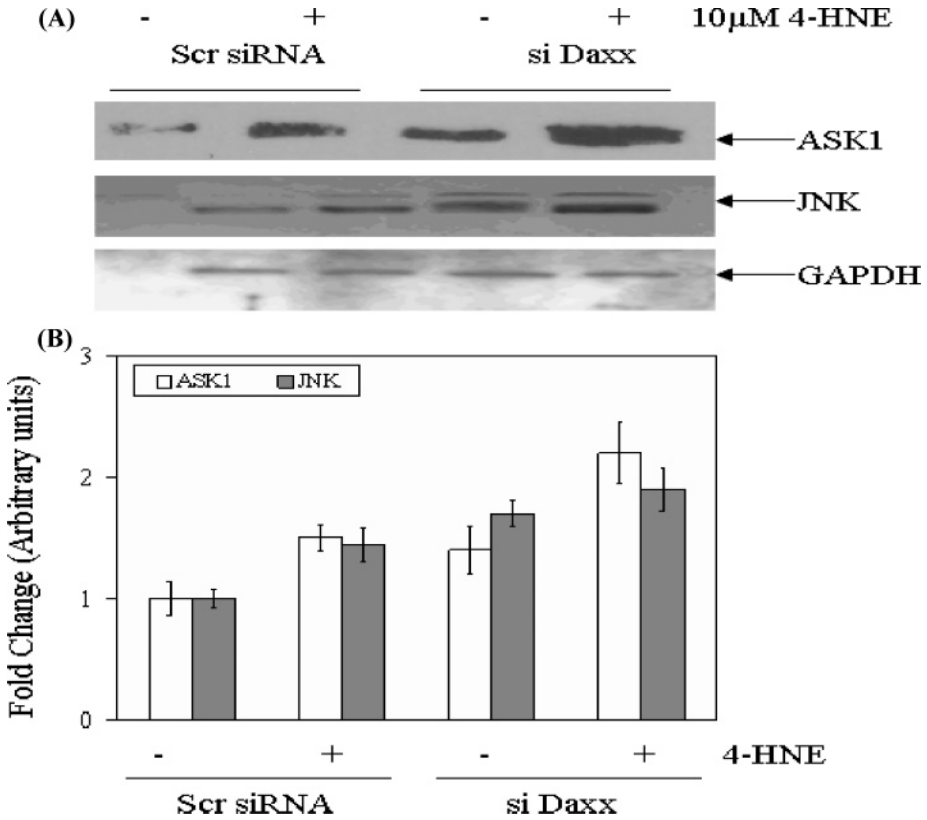

FIGURE 9: Potentiation of 4-HNE-induced activation of ASK1, JNK, and caspase 3 in Daxx-depleted CRL2571 cells. **(A)** Scrambled siRNA- and Daxx-si RNA-transfected cells (2×10^5) were separately incubated with or without 10 μM 4-HNE for 2 h at 37 °C. The extracts of the cells (60 μg of protein) were subjected to Western blot analysis. The immunoblots were probed with anti-ASK1 and anti-JNK antibodies and developed by West Pico chemiluminescence's reagent (Pierce). **(B)** Bar charts showing the results of the densitometric analysis (Kodak 1D 3.6 software) of bands of immunoblots obtained from three different experiments (mean ± SD, $n = 3$).

membrane receptors, 4-HNE can also exert a regulatory role on cellular signaling through its interactions with nuclear proteins as indicated by its antiapoptotic effect on the transcription repressor Daxx and a simultaneous proapoptotic effect on p53. Such interactions of 4-HNE may explain a wide spectrum of effects of 4-HNE on cellular events reported over the years (*1*, *24*, *30*−*32*).

Earlier studies, including our own (*13*−*18*), have suggested the involvement of Daxx in a DISC-independent pathway for apoptosis such as that seen in the present studies. Binding of Daxx to Fas is accompanied by the activation of ASK1 and subsequent downstream signals for the execution of apoptosis (*14*). Our present studies reaffirm the binding of Daxx to Fas and demonstrate further that 4-HNE enhances this binding by facilitating the export of Daxx from the nucleus to the cytoplasm. However, while the binding of Daxx to Fas has been conclusively demonstrated (*14*−*18*), there is no strong evidence that this interaction is required for downstream signals for apoptosis. In Fas-mediated apoptosis caused by UV, the suggested role of Daxx is controversial. While some studies indicate that the binding of Daxx to Fas is required for the activation of ASK1 and JNK and subsequent apoptosis (*14*−*18*, *30*), in other studies an inhibitory role of Daxx has been suggested (*39*). Our studies of procaspase 8-deficient Jurkat cells clearly demonstrate that the silencing of Daxx leads to a potentiation of 4-HNE-induced apoptosis in these cells, as well as to the activation of ASK1, JNK, and caspase 3. These results indicate that binding of Daxx to Fas inhibits 4-HNE-triggered signaling from Fas to ASK1 and JNK that is required for apoptosis in these cells. CRL2571 cells are resistant to apoptosis caused by the "toxic" Fas antibodies (CH11) which can induce apoptosis in the wild-type cells. Upon silencing of Daxx, CRL2571 cells become sensitive to apoptosis by toxic Fas antibodies. This is consistent with the interpretation that binding of Daxx to Fas is not only unnecessary for Fas-mediated signaling for apoptosis, but also actually inhibitory to Fas-mediated apoptosis. This inhibitory role of Daxx in Fas-mediated apoptosis upon exposure to 4-HNE agrees with some of the previous studies (*30*, *39*) showing that Daxx silencing promotes Fas-mediated apoptosis caused by UV or oxidative stress-causing agents (e.g., doxorubicin) in HeLa cells (*30*, *39*).

Our results showing ASK1, JNK, and caspase 3 activation in CRL2571 cells by 4-HNE are also consistent with numerous other studies on various cell types in which a sustained activation of JNK has been demonstrated during 4-HNE- or stress-induced (UV, H_2O_2, superoxide anion, heat, DOX) apoptosis (*2*, *3*, *24*). It has been suggested that activation of ASK1 is dependent on the cellular redox status regulated by the thioredoxin and thioredoxin reductase system (*40*). The reduced form of thioredoxin binds to ASK1, leading to inhibition of its kinase activity. However, during oxidative stress dissociation of thioredoxin from ASK1 causes the activation of ASK1 by oligomerization, which leads to the activation of JNK and apoptosis (*41*). The inhibition of thioredoxin reductase through direct interaction with 4-HNE has also been shown both in vitro and in vivo (*42*). ASK1-independent activation of JNK during oxidative stress has also been shown to be regulated by GST-π (*43*).

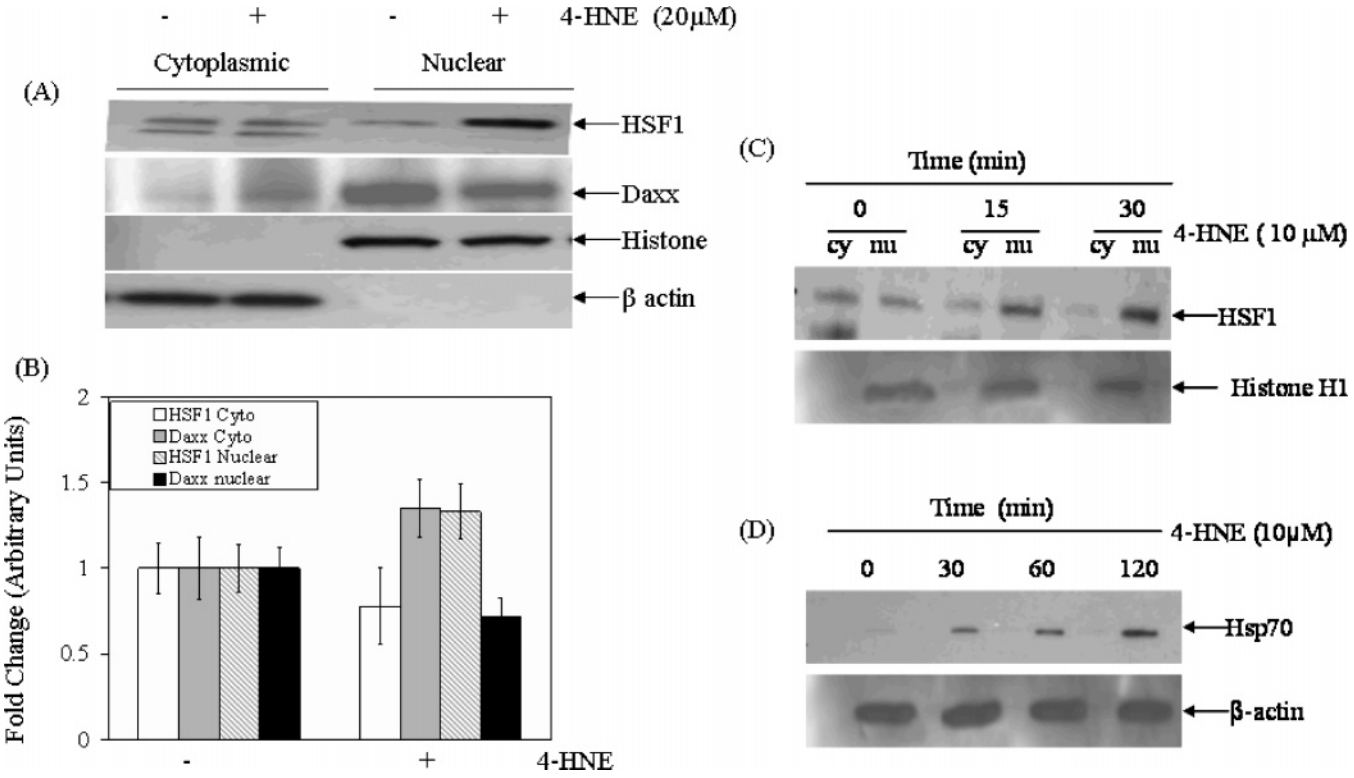

FIGURE 10: 4-HNE-induced cytoplasmic export of nuclear Daxx in CRL2571 cells is accompanied by the activation of HSF1 and up-regulation of Hsp70. (A) The cells (2×10^5) were treated with 20 μM 4-HNE for 2 h at 37 °C. After completion of incubation, the cells were washed 2× in PBS and pelleted by centrifugation. Nuclear and cytoplasmic extracts of the cell pellets were prepared by using the Imgenex cell processing kit. The extracts (50 μg of protein) were subjected to Western blot analysis by using anti-HSF1 and anti-Daxx antibodies as described in the Materials and Methods. The immunoblots were also probed with β-actin and histone H1 antibodies as loading controls and to ascertain the purity of the cytoplasmic and nuclear fractions. (B) Bar chart showing the densitometric analysis of HSF1 and Daxx bands of immunoblots obtained from three different experiments expressed as the fold change (mean ± SD, $n = 3$). (C) Time course of 4-HNE-induced nuclear accumulation and activation of HSF1. (D) Time course of 4-HNE-induced activation of Hsp70 in CRL2571 cells.

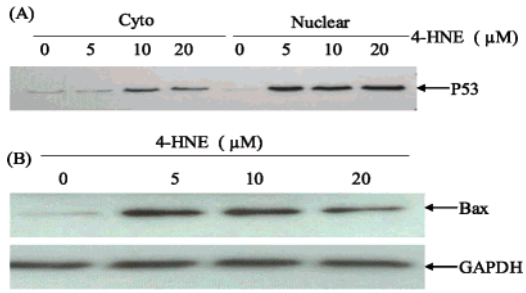

FIGURE 11: Effect of 4-HNE on the expression of p53 and Bax in CRL2571 cells. The cells (2×10^5) were treated with 4-HNE (0−20 μM) for 2 h at 37 °C in complete growth medium. (A) Nuclear and cytoplasmic extracts of the cells were prepared by the Imgenex cell processing kit, and 50 μg of protein from each extract was subjected to the Western blot analysis by using anti-p53 antibodies. (B) Immunoblots of the whole-cell extract (50 μg of protein) prepared in RIPA lysis buffer probed with anti-Bax antibodies. Blots probed with anti-GAPDH antibodies are shown as the loading control.

It is possible that the 4-HNE-induced activation of ASK1 and JNK observed during the present studies in CRL2571 may be due to the modulation of the thioredoxin−thioredoxin reductase system by 4-HNE. Further studies are required to explore this possibility. 4-HNE-induced apoptosis in CRL-2571 cells can be blocked by inhibiting JNK, which indicates that the activation of JNK is an obligatory step in the process. Since 4-HNE is almost always formed in cells subjected to stress through UV, chemicals, heat, starvation, etc., 4-HNE is likely to be a major player in signaling initiated by these agents, in agreement with several earlier studies (*2*−*5*) where 4-HNE has been shown to be a common denominator in signaling for apoptosis by oxidants and UV radiation in various cell types.

An interesting parallel can be drawn between 4-HNE signaling and signaling mediated by hydrogen peroxide (H_2O_2) (*44*, *45*). Both H_2O_2 and 4-HNE cause proliferation at lower concentrations and apoptosis at higher concentrations. Both these messengers are reactive small molecules with a biological half-life comparable to the time scale of many biological processes. This allows for diffusibility over distances needed to transmit a signal, but also assures a physiologically appropriate rate of signal termination. The moderate reactivity of both H_2O_2 and 4-HNE provides a balance between signaling efficiency and selectivity. However, these similarities of H_2O_2 and 4-HNE signaling do not necessarily mean that the mechanisms are redundant. Sulfhydryl groups are the main targets of H_2O_2-mediated oxidation, whereas modification by 4-HNE is characterized by a broader set of targets, including amino groups in addition to sulfhydryls, and a wider range of reaction types, including Michael addition and Schiff base formation. Therefore, during signaling H_2O_2 and 4-HNE are likely to target different proteins, and the resulting modifications are chemically and functionally distinct from each other.

Our present studies show that 4-HNE causes a dose- and time-dependent up-regulation of Fas and Daxx. Moreover,

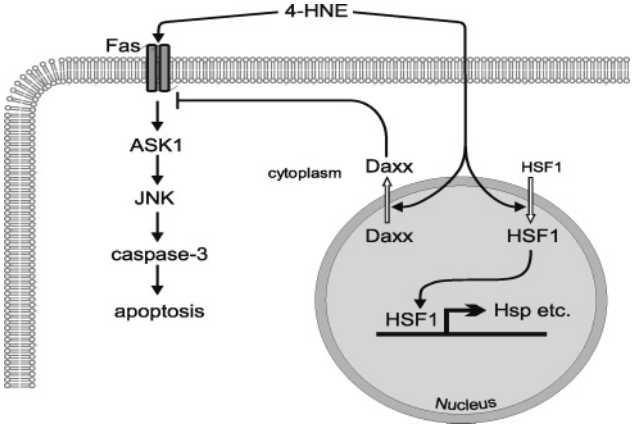

FIGURE 12: Schematic representation of a model showing the role of 4-HNE in Fas-mediated signaling. 4-HNE promotes apoptosis by directly interacting with Fas, resulting in the activation of downstream kinases ASK1 and JNK, leading to the proteolytic cleavage of caspase 3, culminating in apoptosis. On the other hand, a direct interaction of 4-HNE with Daxx facilitates its export from the nuclear compartment to the cytosol, where it binds to Fas and inhibits Fas-mediated apoptosis. Translocation of Daxx also leads to the activation of transcription factor HSF1 to transcribe stress-responsive heat shock proteins.

we demonstrate for the first time that 4-HNE also promotes the translocation of Daxx to the cytosol, where it binds to Fas. Such translocation has been previously documented (*14*−*18*) for cells stressed with UV or DOX exposure, i.e., under conditions that trigger 4-HNE generation (*24*). These findings are consistent with a hypothetical model in which 4-HNE would act as one of the chemical proapoptotic signals elicited by a variety of different stressors.

The effects of 4-HNE on the expression of Fas and on the expression and compartmentalization of Daxx suggest the presence of a self-limiting regulatory circuit. The physiologic significance and the mechanistic details of the inhibitory Daxx−Fas interaction still need to be clarified, particularly in terms of the concentration dependence and time course relative to that of the activation of Fas by 4-HNE. However, according to our proposed model (Figure 12), moderate levels of 4-HNE could trigger the translocation of Daxx to the cytoplasm, where Daxx would inhibit Fas-mediated apoptosis. During severe stress, elevated 4-HNE could cause both an overexpression of Fas and its modification by 4-HNE, leading to the onset of Fas-mediated signaling. This signaling could reach the threshold necessary to overcome the Daxx inhibition and lead to massive apoptosis.

Because of the diffusibility of 4-HNE, a proapoptotic signal could spread beyond the initial site of the insult to adjacent cells or tissues. Therefore, a 4-HNE-triggered export of Daxx to the cytoplasm that serves to control and limit a runaway apoptosis process would be of special importance to adjacent "bystander" cells. Such a protective role of 4-HNE may be augmented by 4-HNE-linked translocation of HSF1 into the nucleus as observed during the present studies. In a complex with remaining nuclear Daxx (*27*), trimeric HSF1 would activate the transcription of several heat shock proteins and thus contribute to protection of the cells. Such seemingly opposite effects of 4-HNE on cellular signaling have been reported previously in studies showing that 4-HNE can either activate or inhibit PKC in a concentration-dependent manner (*46*, *47*).

It is noteworthy that the already mentioned similarities in H_2O_2 and 4-HNE signaling extend to the logic of their respective regulatory circuits (albeit not to the underlying biochemical reactions). Specifically, we postulate that the inhibitory action of Daxx dampens the apoptotic response to 4-HNE at low (subthreshold) but not at high (above-threshold) 4-HNE concentrations and that the system becomes self-limiting, at least spatially, because diffusion of 4-HNE into adjacent cells elicits Daxx-mediated inhibition of apoptosis. This nonlinear response of the 4-HNE circuit parallels that of H_2O_2 (*48*): below a certain threshold, peroxiredoxin efficiently removes any H_2O_2 that is generated and inhibits signaling; when H_2O_2 levels rise above that threshold, peroxiredoxin is oxidatively inactivated, thus opening the "floodgates" and allowing H_2O_2 signaling to proceed (*48*). Similarly, it is possible that the inhibition of the thioredoxin−thioredoxin reductase system by 4-HNE above a certain threshold concentration may profoundly affect the apoptotic signal transduction through the ASK1−JNK−caspase 3 pathway. The striking functional similarity of mechanisms that utilize different signaling molecules, different chemistries, and different target proteins suggests that the resulting nonlinear regulatory circuits are robust and biologically useful.

Stress-induced activation and nuclear translocation of heat shock transcription factor 1 (HSF1), resulting in enhanced expression of heat shock proteins, is one of the major mechanisms for cells to recover from oxidative damage. It has been suggested that in unstressed cells the activation of HSF1 is inhibited through its association with heat shock proteins such as Hsp70, Hsp90, and other cochaperones (*49*, *50*). Under the conditions of oxidative stress this inhibition of HSF1 has been suggested to be abrogated/attenuated by the direct interaction of 4-HNE with Hsp70 and/or Hsp90 (*51*, *52*). Recently, through immunoprecipitation and c-myc-tagged Hsp70 data, it has been shown that 4-HNE treatment inhibits the interaction of Hsp70 and HSF1, leading to enhanced accumulation of HSF1 and increased transcription of heat shock proteins (*53*). Our results showing 4-HNE-induced translocation of Daxx from the nucleus and HSF1 into the nucleus may have major implications for the mechanisms of stress-mediated signaling, particularly by stressors such as exposure to UV radiation, heat shock, or oxidants, which invariably increase the intracellular concentration of 4-HNE in cells. Daxx is a transcription repressor which can regulate the expression of stress-responsive genes. It has also been shown that release of Daxx from the nuclear compartment to the cytoplasm leads to the activation of transcription factor HSF1 that up-regulates the expression of HSF1-associated genes (*27*). Likewise, it is known that interaction of Daxx with other transcription factors such as ETS1, Pax 3, Pax5, smad4, and p53 also affects the expression of associated genes (*46*, *54*−*57*). The involvement of 4-HNE in promoting the cytoplasmic export of Daxx with concomitant activation and accumulation of HSF1 and the activation of Hsp70 in CRL2571 demonstrated during the present studies suggests a novel mechanism in stress-mediated signaling through which 4-HNE can influence a large set of fundamental cellular processes including proliferation, cell cycle events, and apoptosis, in part by profoundly affecting the expression of numerous genes (*10*, *34*). For example, 4-HNE can modulate the expression of genes

including p53, c-myc, AKT, PKC, TGFβ, ASK1, JNK, Nrf2, integrins, connexins, ERK pathway genes, aldose reductase, glycogen synthase kinase, γ-GGT, adenylate cyclase, RalBP-1, and heat shock proteins among others and affect cellular processes associated with these genes (*1*, *33*, *34*, *58*). The present studies, demonstrating the effects of 4-HNE on the transcription repressor Daxx, may provide a clue to the mechanism(s) through which 4-HNE could affect such a multitude of cellular events.

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