Heat Shock Factor 1 Attenuates 4-Hydroxynonenal-mediated Apoptosis

CRITICAL ROLE FOR HEAT SHOCK PROTEIN 70 INDUCTION AND STABILIZATION OF Bcl-X$_L$

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Lipid peroxidation is a consequence of both normal physiology and oxidative stress that generates various reactive metabolites, a principal end product being 4-hydroxynonenal (HNE). As a diffusible electrophile, HNE reacts extensively with cellular nucleophiles. Consequently, HNE alters cellular signaling and activates the intrinsic apoptotic cascade. We have previously demonstrated that in addition to promoting apoptosis, HNE activates stress response pathways, including the antioxidant, endoplasmic reticulum stress, DNA damage, and heat shock responses. Here we demonstrate that activation of the heat shock response by HNE is dependent on the expression and nuclear translocation of heat shock factor 1 (HSF1), which promotes the expression of heat shock protein 40 (Hsp40) and Hsp70-1. Ectopic expression and immunoprecipitation of c-Myc-tagged Hsp70-1 indicates that HNE disrupts the inhibitory interaction between Hsp70-1 and HSF1, leading to the activation heat shock gene expression. Using siRNA to silence HSF1 expression, we observe that HSF1 is necessary for the induction of Hsp40 and Hsp70-1 by HNE, and the lack of Hsp expression is correlated with an increase in apoptosis. Nrf2, the transcription factor that mediates the antioxidant response, was also silenced using siRNA. Silencing Nrf2 also enhanced the cytotoxicity of HNE, but not as effectively as HSF1. Silencing HSF1 expression facilitates the activation of JNK pro-apoptotic signaling and selectively decreases expression of the anti-apoptotic Bcl-2 family member Bcl-X$_L$. Overexpression of Bcl-X$_L$ attenuates HNE-mediated apoptosis in HSF1-silenced cells. Overall, activation of HSF1 and stabilization of Bcl-X$_L$ mediate a protective response that may contribute significantly to the cellular biology of lipid peroxidation.

The oxidation of membrane phospholipids is an inescapable consequence of normal cellular respiration. A major route for lipid oxidation involves the abstraction of bis-allenic hydrogen atoms from polyunsaturated fatty acids by reactive oxygen species. The resulting lipid radicals react readily with dioxygen to form lipid hydroperoxides, which are mainly reduced to their corresponding hydroxyl species. A number of xenobiotic metabolites, anti-neoplastic drugs, transition metals, and disease states promote increased levels of both reactive oxygen species and oxidized lipids. The decomposition of oxidized lipids yields a variety of diffusible electrophiles, including the $\alpha$, $\beta$-unsaturated aldehyde, 4-hydroxynonenal (HNE). Diffusible lipid electrophiles are capable of modifying nucleophilic sites on DNA and proteins throughout the cell. Modification of cellular targets by HNE is found in multiple disease states, including atherosclerosis, ischemia-reperfusion injury, Parkinson disease, and Alzheimer disease (1-5). Plasma conjugates of HNE have been observed at low micromolar concentrations in healthy adults, implying the detectable generation of HNE under normal physiological conditions, although absolute steady-state concentrations are less clear (6, 7). At the cellular level, elevated levels of HNE promote cell cycle arrest and programmed cell death. Studies in a variety of cultured cell systems, including: neuronal (PC12); endothelial human umbilical vein endothelial cells; myeloid (K562, HL-60); lymphoid (MOLT-4, Reh); and colorectal cell lines (RKO) have verified the induction of apoptosis by HNE (8-13). Reports have indicated that apoptosis is mediated in part by the activation of c-Jun N-terminal kinase (JNK) and that HNE toxicity is reduced by the JNK-specific inhibitor SP600125 (9, 14-16).

In addition to initiating apoptosis, HNE activates various protective, stress response pathways. We previously used microarray analysis to explore transcriptional pathways activated by HNE in RKO cells and found that HNE induces the DNA damage, endoplasmic reticulum stress, antioxidant, and heat shock responses (17). Among the pathways activated by HNE, the heat shock response is particularly robust. The heat shock response mediates the induction of a highly conserved set of heat shock proteins (Hsps) (18). The inducible expression of Hsps is mediated by heat shock transcription factor 1 (HSF1), which translocates to the nucleus upon activation and enhances the expression of genes from promoters containing heat shock elements (HSE) (19, 20). A principal function of Hsps is to chaperone other proteins, binding to nascent polypeptide chains as...
well as to unfolded and damaged proteins. Their function as protein chaperones aids in the recovery of cells from thermal and chemical-induced damage (21, 22). Moreover, Hsps regulate a diverse set of signaling pathways via their interactions with “client” proteins (23). In addition, although Hsp70 does not interact directly with Bax, it does inhibit Bax translocation to the mitochondrion and attenuates stress-induced apoptosis (24).

Here we investigate activation of the heat shock response by HNE in detail. Treatment of a colorectal cancer cell line with HNE elicits a concentration-dependent activation of HSF1, associated with a robust increase in Hsp40 and Hsp70-1 (inducible Hsp70, Hsp72) expression. Utilizing an siRNA-based method to silence HSFI1, we attenuate the heat shock response and examine the effect on cell survival. Our data indicate that heat shock response protecst against HNE-induced apoptosis and that HSF1 activation is critical in mediating this effect. The degree of cytoprotection afforded by the heat shock response exceeds the anti-apoptotic effects of Nrf2 activation. We find that treatment with HNE causes both activation of JNK signaling and a specific reduction in Bcl-XL expression, both of which are exacerbated by silencing HSF1. Ectopic expression of Bcl-XL confers resistance to HNE-mediated apoptosis and partial reverses the sensitization to HNE caused by HSF1 siRNA. These results indicate a critical role for HSF1 activation in resistance to stress-mediated apoptosis and suggest a novel role for Hsp70-1 as a regulator of mitochondria-mediated cell death.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatment**—RKO cells, a poorly differentiated, p53(−) colon carcinoma cell line widely used for studies on DNA damage and cellular signaling of electrophiles, was obtained from American Type Culture Collection and maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Atlas), 1% antibiotic/antimycotic (Invitrogen), and 25 mM HEPES buffer. HNE (Axxora) was quantified by treating cell pellets with an argon stream and reconstituted in Me2SO. The cells were treated with HNE or Me2SO. After 48 h the cells were split into DMEM containing 200 μg/ml hygromycin, and stably expressing clones were selected. For transient transfection of Bcl-XL, RKO cells at 70% confluence in 10-cm dishes were transfected using either the Bcl-XL construct or empty pcDNA3.1 Hygro(+) and Lipofectamine 2000. After 24 h the cells were treated with HNE for 48 h then resuspended in 10-cm dishes were transfected using either the Bcl-XL construct or empty pcDNA3.1 Hygro(+) and Lipofectamine 2000, and the experiments were performed 24 h after transfection.

**Viability Assays**—RKO cells were seeded in 96-well plates at a density of 7.5 × 10^3 cells/well. After adhering overnight, the cells were treated with HNE or Me2SO. After 48 h of incubation, the medium was aspirated, washed once in Dulbecco’s phosphate-buffered saline (D-PBS) and replaced with a solution of 2 μg/ml calcine-AM (Molecular Probes) in D-PBS. After 30 min, fluorescence was read using a SpectraMax multwell plate reader (Molecular Devices) with an excitation λ of 494 nm and an emission λ of 517 nm.

**siRNA Transfections**—For siRNA transfections, RKO cells were seeded at 25% confluence in 10-cm dishes. After adhering overnight, the cells were washed once with D-PBS, and 4 ml of Opti-MEM (Invitrogen) was added per dish. The transfections were performed with 0.2 nmol of Stealth siRNA (HSF1 sense strand sequence, 5’-CGGAUUCAGGAAGCAGCUG-GUGCA-3’; Nrf2 sense strand sequence, 5’-CCAGUUGACAGUGAACUCUUAAAU-3’) and Lipofectamine 2000 (Invitrogen) per dish according to the manufacturer’s instructions. After 24 h the cells were split at a ratio of 1:4 and transfected a second time the following day. The cells were split, allowed 24 h to adhere, and then treated with HNE or Me2SO.

**Plasmid Constructs and Transfections**—Clones for Bcl-Xl (NCBI accession BC019307) and Hsp70-1 (NCBI accession BC009322) were obtained from the Vanderbilt Microarray Shared Resource clone collection. Bcl-Xl was excised from pCMV-SPORT6 using the restriction endonuclease BglII and ligated into the BamHI site of pcDNA3.1 Hygro(+). Hsp70-1 was excised from pOTB7 using MscI and XhoI. The excised fragment and a double-stranded oligonucleotide encoding the c-Myc tag (5’-P-GCATGGAGCAAAAGCTCATTTCTGAA-3’) were ligated into the EcoRV and XhoI sites of pcDNA3.1-Hygro(+) and Lipofectamine 2000, and the experiments were performed 24 h after transfection.

**Protein Extraction and Western Blotting**—For total protein extraction the cells were scraped in cold D-PBS and collected by centrifugation. Cold mammalian protein extraction reagent (M-Per) lysis buffer (Pierce) containing protease and phosphatase inhibitors (Sigma) was added to cell pellets. Protein lysates were cleared by centrifugation and stored at −80 °C. For nuclear proteins, the cells were collected by centrifugation and then resuspended in 800 μl of ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride), and 50 μl of Igepal CA-630 (Nonidet P-40) was added. The nuclei were pelleted by centrifugation at 1000 × g for 5 min at 4 °C and washed twice with buffer A. Nuclear proteins were extracted by the addition of buffer B (10 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM MgCl2, 0.1 mM EDTA, 0.1 mM EGTA, 25% glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and centrifuged at 14,000 × g for 10 min at 4 °C to remove debris. Protein concentrations were determined using Bradford protein assay (Bio-Rad). For Western blot, equal amounts of protein were resolved by SDS-PAGE, transferred onto a 0.2-μm nitrocellulose membrane, and incubated in blocking buffer (20 mM Tris, pH 7.6, 140 mM NaCl, 0.05% Tween 20, 5% nonfat dry milk) prior to the addition of primary antibody. Following incubation with primary and secondary antibodies, Luminol-based detection was performed. Primary antibodies were obtained from the following sources: HSF1, c-Myc tag, PARP, caspase 3, caspase 3 cleavage products, JNK, phospho-JNK, Bad, Bax, Bcl-2, and Bcl-Xl from Cell Signaling; Hsp27, Hsp40, Hsp70, Hsp90, and Hsp105 from BD Biosciences; α-tubulin from Sigma-Aldrich; and Nrf2, actin, and all secondary antibodies from Santa Cruz Biotechnology.
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**Immunoprecipitation**—RKO cells were transfected with empty pcDNA3.1-Hygro(+) vector, or with the c-Myc-Hsp70-1 expression construct. After 24 h total proteins were collected in mammalian protein extraction reagent (M-PER) lysis buffer containing protease inhibitors. The lysates were cleared by centrifugation, and protein concentrations were determined by Bradford protein assay. Protein samples were treated with either 0.1% Me2SO (vehicle) or 15 μM or 45 μM HNE for 1 h, and then 400 μg of total protein was immunoprecipitated using the ProFound c-Myc tag immunoprecipitation kit (Pierce). Following elution in 25 μl of SDS loading buffer, the samples were resolved by SDS-PAGE and analyzed by Western blot.

**Luciferase Assay**—An HSE-bearing luciferase construct was generated by insertion of a double-stranded oligonucleotide (5’T-GCTAGCCTTCTGGAAACCTTCTCTCTG-GAACTTCTGGAAACCTTCTCTCTCTGGAAACCTTCTCTCTCTGGAAACCTTCTGAG-OH3’) into the Smal site of pGL3-Promoter (Promega). The cells were seeded at a density of 25% confluence in 12-well plates and co-transfected with 200 ng of pcDNA3.1-Hygro(+) and 5 ng of Renilla luciferase (pRL-SV40)(Promega) using Lipofectamine 2000 and grown for an additional 24 h. The cells were treated with 8 h with Me2SO or HNE at indicated concentrations and then collected in passive lysis buffer. Analysis of luciferase activity was measured using a dual luciferase assay system (Promega).

**RNA Extraction and Real Time PCR**—Total RNA was collected using RNeasy RNA collection kit (Qiagen). Digestion of trace DNA was performed by incubation with DNase using DNA-free reagent (Ambion). 1 μg of total RNA was used in each reverse transcription reaction with iScript reagent, (Bio-Rad). One-tenth of each reaction volume (2 μl) was used per well in subsequent real time PCR analysis, using iQ SYBR Green Supermix (Bio-Rad). Real time reactions were performed using a Bio-Rad iCycler real time PCR machine. Standard curves were generated by the amplification of target sequences that were previously cloned into pGEM-T (Promega), in dilution series from 10⁻¹ to 10⁻⁶ fmol/well.

**RESULTS**

**HNE Induces Nuclear HSF1 Expression and Promotes Heat Shock Protein Expression**—Our previous observation that HNE activates the heat shock response led us to examine the underlying mechanism in detail. RKO cells were treated with either Me2SO (vehicle) or HNE for various times. Nuclear proteins were collected and analyzed for HSF1 expression by Western blot. Me2SO-treated cells showed little nuclear HSF1 expression. In contrast, the addition of 5, 15, or 45 μM HNE induced a robust accumulation of HSF1 within the nucleus (Fig. 1). Expression of HSF1 in nuclear extracts was noticeable within 15 min following HNE addition. To examine the magnitude of heat shock protein induction, RKO cells were treated with 30–60 μM HNE and total protein extracts collected at 8 h. The lysates were analyzed by Western blot for the expression of various heat shock proteins. HNE induced a robust, concentration-dependent increase in the expression of both Hsp40 and Hsp70-1 (Fig. 2). Only modest expression of Hsp40 and Hsp70-1 was observed in Me2SO-treated cells. Also, HNE did not significantly influence the expression of other heat shock proteins, including Hsp27, Hsc70 (Hsp73), Hsp90, and Hsp105.

**Interaction of Hsp70-1 with HSF1**—Previous work has established that Hsp70 is a negative regulator of HSF1 transcriptional activity (25, 26). Furthermore, others have shown that HNE modifies Hsp70-1 in vitro, hindering its function as a protein chaperone (27). Therefore, we hypothesized that HNE disrupts the inhibitory interaction between Hsp70-1 and HSF1. RKO cells were transiently transfected with either empty pcDNA3.1 Hygro(+) or an N-terminally c-Myc-tagged Hsp70-1 expression construct. Western blotting revealed that
immunoprecipitation of c-Myc-Hsp70-1 resulted in co-immunoprecipitation of HSF1. Preincubation of protein lysates with either an empty pcDNA3.1-Hygro (−) or an N-terminally c-Myc-tagged Hsp70-1 expression construct (+) after 24 h total proteins were collected. The protein lysates were treated with either 0.1% Me₂SO (0), 15 μM, or 45 μM HNE for 1 h. The lysates (400 μg) were immunoprecipitated using anti-c-Myc conjugated beads. The samples were resolved by SDS-PAGE and analyzed by Western blot for expression of the c-Myc tag (c-Myc-Hsp70-1) and HSF1.

FIGURE 3. HNE treatment inhibits the interaction of Hsp70-1 with HSF1. The cells were transiently transfected with either empty pcDNA3.1-Hygro (−) or an N-terminally c-Myc-tagged Hsp70-1 expression construct (+). After 24 h total proteins were collected. The protein lysates were treated with either 0.1% Me₂SO (0), 15 μM, or 45 μM HNE for 1 h. The lysates (400 μg) were immunoprecipitated using anti-c-Myc conjugated beads. The samples were resolved by SDS-PAGE and analyzed by Western blot for expression of the c-Myc tag (c-Myc-Hsp70-1) and HSF1.

FIGURE 4. Silencing HSF1 expression in RKO cells by siRNA. A. The cells were transfected with either a negative control siRNA or an HSF1-specific siRNA. Total RNA was collected and analyzed by quantitative real time reverse transcription-PCR for expression of HSF1 as described under “Experimental Procedures.” The data are represented in fmol of HSF1 cDNA/μg of total RNA. The error bars represent the mean values ± S.D. of four samples for each condition. B, cells were transfected with either negative control (−) siRNA or HSF1 siRNA (+). Total protein lysates were collected and analyzed by Western blot for HSF1 expression. α-Tubulin was included as a loading control.

HSF1 Silencing Attenuates HSE-driven Gene Expression and Heat Shock Protein Induction—To investigate the biological consequences of HSF1 activation and the resulting induction of the heat shock response, an siRNA-based approach was used to selectively inhibit HSF1 expression. RKO cells were transfected with either an HSF1-specific siRNA or a negative control siRNA (scrambled sequence). Real time PCR was performed to determine the extent of HSF1 mRNA reduction. The level of HSF1 mRNA in cells transfected with HSF1 siRNA was reduced to ~20% of the level in control cells (Fig. 4A). Western blotting indicates complete silencing of HSF1 protein expression (Fig. 4B). A luciferase-based reporter construct also was employed to verify the inhibition of heat shock-mediated gene expression. Cells treated with siRNA (HSF1 siRNA) were subsequently transfected with the HSE-bearing firefly luciferase construct (pGL3-HSE). As a control, cells were co-transfected with a constitutive Renilla luciferase expression vector (pSV40-RL). Following treatment with 30–60 μM HNE, the samples were collected in passive lysis buffer. Analysis of normalized luciferase activity (firefly/Renilla activity) showed that HNE elicited a concentration-dependent increase in expression from the pGL3-HSE construct (Fig. 5A), and a maximal 21-fold increase in normalized luciferase was observed with 60 μM HNE. In contrast, silencing HSF1 nearly abolished HNE-induced luciferase expression from pGL3-HSE. Inhibition of the heat shock response was also evaluated by Western blot. Induction of both Hsp40 and Hsp70-1 by HNE was robust in control siRNA-transfected cells (Fig. 5B); however, their expression was completely attenuated in cells transfected with HSF1 siRNA, further verifying successful knockdown of the heat shock response.

Nrf2 Silencing Suppresses Activation of the Antioxidant Response—The induction of heat shock proteins is a cytoprotective mechanism that enhances cell survival in the wake of
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Silencing Nrf2 expression in RKO cells by siRNA. The cells were transfected twice with either a negative control (−) or Nrf2-specific siRNA (+). A, cells were treated with 45 μM HNE for indicated times, and nuclear protein lysates were collected as described under “Experimental Procedures.” The lysates were analyzed by Western blot for Nrf2 expression. B, cells were treated with either MeSO (lane 0) or 30 or 45 μM HNE for 24 h, and total protein lysates were collected. The lysates were analyzed by Western blot for HO-1 expression. Actin was included as a loading control.

FIGURE 6. Silencing Nrf2 expression in RKO cells by siRNA. The cells were transfected twice with either a negative control (−) or Nrf2-specific siRNA (+). A, cells were treated with 45 μM HNE for indicated times, and nuclear protein lysates were collected as described under “Experimental Procedures.” The lysates were analyzed by Western blot for Nrf2 expression. B, cells were treated with either MeSO (lane 0) or 30 or 45 μM HNE for 24 h, and total protein lysates were collected. The lysates were analyzed by Western blot for HO-1 expression. Actin was included as a loading control.

Silencing of HSF1 or Nrf2 expressions in cells treated with HNE. The cells were transfected with either negative control, HSF1-specific, or Nrf2-specific siRNA. Subsequently, the cells were seeded in 96-well plates at a density of 7.5 × 103 cells/well. After adhering overnight, the cells were treated with various concentrations of HNE (5–100 μM) in DMEM containing 10% fetal bovine serum. After 48 h cell viability was determined with calcein-AM, and fluorescence was measured using a multiwell plate reader. The data are represented as a percentage of control fluorescence (ratio of arbitrary fluorescence units of HNE-treated sample to MeSO-treated sample × 100). The error bars represent standard deviations (n = 5).

Silencing HSF1 Enhances HNE-induced Apoptosis—Exposure of RKO cells to elevated concentrations of HNE induces apoptotic cell death, as evidenced by cytochrome c release, internucleosomal chromatin cleavage (DNA laddering), and cleavage of PARP and caspase 3 (13). Suppression of HSF1 levels by siRNA significantly increases apoptotic cell death. Following treatment with 45 μM HNE, expression of the 85-kDa PARP fragment was elevated in HSF1 siRNA-treated cells compared with control (Fig. 8A). Levels of the catalytically active 17- and 12-kDa fragments of cleaved caspase 3 were also elevated. In addition, HNE-induced expression of the pro-apoptotic proteins CHOP (GADD153) and ATF3, which are associated with the activation of endoplasmic reticulum stress-mediated apoptosis, were enhanced in HSF1-deficient cells (Fig. 8B). These data establish the crucial role of HSF1 in mediating multiple aspects of the anti-apoptotic response in cells exposed to HNE.

Silencing HSF1 Enhances JNK Phosphorylation and Decreases Bcl-XL Expression—To explore the mechanisms underlying HNE-induced cell death, we have evaluated the signaling processes controlling the initiation of apoptosis. Previous reports have correlated HNE-induced apoptosis with activation of the pro-apoptotic, stress-activated mitogen-activated protein kinase, JNK. In support of earlier reports, our data confirm that HNE (45 μM) promotes sustained JNK phosphorylation on residues Thr-183/Tyr-185, corresponding to the catalytically active form of the protein. Moreover, this process appears to be inhibited by the heat shock response, because silencing of HSF1 drastically enhanced the level of phospho-JNK in HNE-treated cells and lowered the threshold for JNK phosphorylation from 45 to 15 μM (Fig. 9).

The pro-apoptotic consequences of JNK activation are likely mediated through a variety of downstream pathways, one being the modulation of proteins that regulate the mitochondrial
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To test the hypothesis that decreased Bcl-X \(_{L} \) levels facilitate HNE-mediated apoptosis, Bcl-X \(_{L} \) was stably overexpressed in RKO cells utilizing a hygromycin-selectable expression construct, and the cells were subsequently treated with 15–45 \( \mu \)M HNE. Western blots show that over-expression of Bcl-X \(_{L} \) attenuated apoptosis in HSF1-deficient cells, as evidenced by a reduction in PARP and caspase 3 cleavage products (Fig. 11), supporting a role for Bcl-X \(_{L} \) down-regulation in HNE-mediated cell death. These data also suggest a role for Hsp70-1 and Hsp40 as negative regulators of the intrinsic apoptotic pathway via stabilization of Bcl-X \(_{L} \) levels.

DISCUSSION

HNE is an electrophilic lipid oxidation product and is one of the major constituents responsible for the toxic effects of lipid peroxidation (35). In addition to being cytotoxic, HNE promotes the activation of various signaling networks related to cell stress, including the DNA damage, endoplasmic reticulum stress, antioxidant, and heat shock response pathways (17). Previously, HNE has been shown to promote heat shock gene expression in the human hepatoma cell line, HepG2 (36). HNE has likewise been shown to induce the nuclear expression of an HSE-binding protein in HeLa cells (presumably, HSF1) (37). Here, we addressed the role of HSF1 in the cellular response to HNE and examined the mechanistic relationship between induction of the heat shock response and HNE-mediated apoptosis.

Our data reveal a role for the heat shock response in counteracting the pro-apoptotic effects to HNE (Fig. 12). Using siRNA against HSF1 significantly enhanced the induction of apoptosis. Hence, activation of the heat shock response significantly attenuated the degree of cell death at certain levels of HNE exposure. Based on our data, HSF1-deficient cells at 50 \( \mu \)M HNE were <5% viable, compared with control cells, which were >50% viable. By using siRNA against Nrf2, we also silenced the antioxidant response, which likewise sensitized cells to HNE. Our data, however, indicate that the degree of cytoprotection afforded by the heat shock response exceeded the effect of the antioxidant response.

A principal step in the activation of HSF1 is translocation to the nucleus following heat shock or chemical stress (20, 38). In unstressed cells, HSF1 is inhibited through its associations with Hsp90, Hsp70, and additional co-chaperones (25, 26, 39–42). Mapping experiments have revealed that the interaction of HSF1 with Hsp70 is mediated by a 15-amino acid sequence (43). A mechanism for the HNE-mediated activation of HSF1 has not been established. One possibility involves direct modification of Hsp90 or Hsp70 residues by HNE, thereby abolishing their inhibition of HSF1. In support of this hypothesis, HNE has been shown to inhibit the chaperone activities of both Hsp90 and Hsp70 in vitro. Moreover, inhibition of Hsp90 and Hsp70 was correlated with the identification of specific HNE amino acid adducts by tandem mass spectrometry analysis (27, 44). Our
data using co-immunoprecipitation of c-Myc-tagged Hsp70 suggest that HNE treatment inhibits the interaction of Hsp70 with HSF1, which we suspect liberates HSF1 and facilitates its nuclear accumulation.

In addition to acting as protein chaperones, Hsps inhibit cell death by directly inhibiting a variety of pro-apoptotic mediators. For example, Hsp70 associates with and inhibits the activation of JNK1 (45). By inhibiting JNK1, Hsp70 prevents Bid cleavage, thereby decreasing expression of the pro-apoptotic tBid fragment and consequent cytochrome c release (46). Our data showing enhanced JNK phosphorylation in HSF1-deficient cells support these observations. Apoptosis is also inhibited downstream of cytochrome c release by the direct interactions of Hsp70 and Hsp90 with apoptotic peptidase activating factor 1, thereby hindering formation of the apoptosome (47, 48). Furthermore, Hsp70 inhibits apoptosis-inducing factor, a mitochondrial flavoprotein whose nuclear expression in apoptotic cells promotes PARP cleavage and chromatin condensation (49). Recently, Hsp70 was shown to inhibit the cleavage of the transcription factor GATA-1 by activated caspase 3, suggesting that the protection of client proteins from caspase-mediated proteolysis may be an additional means of inhibiting apoptosis (50).

Evidence suggests that Bcl-XL is critical in mediating the resistance of eukaryotic cells to chemical and redox stress (51, 52). Previously, induction of the heat shock response was shown to enhance Bcl-XL expression, and conversely, silencing Hsp70 with antisense RNA lead to diminished levels of Bcl-XL (53, 54). We found that pro-apoptotic concentrations of HNE caused a dramatic decrease in Bcl-XL expression, whereas Bcl-2, Bad, and Bax were unaffected. Moreover, silencing HSF1 exacerbated the decrease in Bcl-XL levels by HNE. The specific down-regulation of Bcl-XL is recently emerging as a consequence of exposure of cells to chemotherapeutics, including cisplatin, bischloronitrosourea (carmustine), retinoic acid as well as to UV light (55–58). Our finding that HSF1-mediated gene expression stabilizes Bcl-XL levels implies that activation of the heat shock response may partly mediate chemotherapeutic resistance.

The consequences of heat shock response activation and Hsp induction likely extend beyond cytoprotection from electrophilic lipids. For example, Hsp70 has been shown to directly inhibit a variety of signaling pathways, including the lipopolysaccharide-induced innate immune response. As a conse-
quence, Hsp70 attenuates the induction of pro-inflammatory genes, including cyclooxygenase-2, inducible nitric oxide synthase, tumor necrosis factor α, interleukin-1, and interleukin-6 (59–62). In a study conducted using Hsp70-1 knock-out mice (Hsp70.1/−/−), an increase in mortality caused by septic shock was observed over their wild type counterparts, and the absence of Hsp70 was correlated with increases in NF-κB activation, inflammatory cytokine expression, profound lung injury, and enhanced mortality (63).

Our findings indicate that a fundamental consequence of promoting HSF1-mediated gene expression is enhanced cell survival under otherwise lethal concentrations of HNE. In our experiments, the magnitude of cytoprotection exceeded the contribution of NrF2 and induction of the antioxidant response. In view of the complex biology of HNE and similar endogenous metabolites, the role of HSF1 in a physiological context is undoubtedly significant. For example, the modification of DNA by HNE yields potentially mutagenic lesions such as N2-etheno-dG and cyclic N2-propano-dG adducts, which have been found at significant levels in disease states including chronic pancreatitis, ulcerative colitis, and Crohn disease (64–68). In such a context, the combined effects of DNA damage and cytoprotection imply a heightened potential for the accumulation of mutations. Overall, through its ability to enhance cell survival by inhibiting pro-apoptotic pathways, we speculate that the heat shock response contributes significantly in the cellular biology and pathophysiology of HNE and similar oxidized lipid electrophiles.

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