4-Hydroxynonenal Induces G₂/M Phase Cell Cycle Arrest by Activation of the Ataxia Telangiectasia Mutated and Rad3-related Protein (ATR)/Checkpoint Kinase 1 (Chk1) Signaling Pathway*

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Background: HNE is an important signaling molecule.

Results: HNE induces G₂/M cell cycle arrest and phosphorylation of H2A.X. ATR/Chk1-mediated regulation of Cdc25C and activation of p21 is the predominant mechanism of HNE-induced cell cycle arrest. GSTA4-4 overexpression inhibits HNE-induced cell arrest.

Conclusion: HNE causes DNA damage and G₂/M arrest.

Significance: HNE and GSTA4-4 play a role in the maintenance of genomic integrity.

4-Hydroxynonenal (HNE) has been widely implicated in the mechanisms of oxidant-induced toxicity, but the detrimental effects of HNE associated with DNA damage or cell cycle arrest have not been thoroughly studied. Here we demonstrate for the first time that HNE caused G₂/M cell cycle arrest of hepatocellular carcinoma HepG2 (p53 wild type) and Hep3B (p53 null) cells that was accompanied with decreased expression of CDK1 and cyclin B1 and activation of p21 in a p53-independent manner. HNE treatment suppressed the Cdc25C level, which led to inactivation of CDK1. HNE-induced phosphorylation of Cdc25C at Ser-216 resulted in its translocation from nucleus to cytoplasm, thereby facilitating its degradation via the ubiquitin-mediated proteasomal pathway. This phosphorylation of Cdc25C was regulated by activation of the ataxia telangiectasia and Rad3-related protein (ATR)/checkpoint kinase 1 (Chk1) pathway. The role of HNE in the DNA double strand break was strongly suggested by a remarkable increase in comet tail formation and H2A.X phosphorylation in HNE-treated cells in vitro. This was supported by increased in vivo phosphorylation of H2A.X in mGsta4 null mice that have impaired HNE metabolism and increased HNE levels in tissues. HNE-mediated ATR/Chk1 signaling was inhibited by ATR kinase inhibitor (caffeine). Additionally, most of the signaling effects of HNE on cell cycle arrest were attenuated in hGSTA4 transfected cells, thereby indicating the involvement of HNE in these events. A novel role of GSTA4-4 in the maintenance of genomic integrity is also suggested.

Polysaturated fatty acids in the membrane lipid bilayer are one of the early targets of the reactive oxygen species generated during metabolic processes or due to the exposure to radiation, heat shock, and xenobiotics. Reactive oxygen species initiate an autocatalytic chain of lipid peroxidation of polysaturated fatty acids, resulting in the formation of large amounts of toxic electrophilic species and free radicals that may play important roles in various human diseases, including carcinogenesis (1–5). Our previous studies have shown that even a minimal transient exposure of cells to stress agents, such as UV, H₂O₂ or oxidant chemicals, causes substantial lipid peroxidation, leading to a significant rise in the level of lipid peroxidation end product, 4-hydroxynonenal (HNE), which is considered to be one of the most abundant cytotoxic aldehydes (6, 7). HNE reacts not only with DNA but also with proteins and other molecules containing thiol and other nucleophilic groups (1, 4, 5, 8, 9). HNE forms a bulky exocyclic DNA adduct, 6-(1-hydroxyhexanyl)-8-hydroxy 1, 5, 8, 9. HNE forms a bulky exocyclic DNA adduct, 6-(1-hydroxyhexanyl)-8-hydroxy 1, 5, 8, 9. HNE-dG adduct is a strong mutagen and is found in various normal tissues of humans and rats (8, 10–14). HNE-dG adduct is a strong mutagen and induces mainly G:C to T:A mutations in human cells. HNE-dG adduct preferentially forms at the third base of codon 249 (-AGG-) of the p53 gene, a mutational hotspot in human hepatocellular carcinoma and cigarette smoke-related lung cancer (3, 11, 15–18), suggesting that HNE could be involved in the etiology of smoking-related carcinogenesis.

Under the normal physiological conditions, the cellular concentration of HNE ranges from 0.1 to 3 μM (1, 2, 4, 5). Thus, the

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1 The abbreviations used are: HNE, 4-hydroxy-2-nonenal; HNE-dG, 6-(1-hydroxyhexanyl)-8-hydroxy 1, N²-propano-2⁻-deoxyguanosine (HNE-dG); HNE causes DNA damage and G₂/M arrest.

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concentration of this endogenously generated DNA-damaging agent in cells is relatively high as compared with the concentrations of the exogenous DNA-damaging agents that cells may normally encounter in the environment. Moreover, under oxidative stress conditions, HNE can accumulate in membranes at even higher concentrations that may range from 10 μM to 5 mM (2, 4, 5). In Fisher rats exposed to CCl₄, a significant amount of HNE-dG adduct (≥100 nmol/mol, 37-fold increase) is formed in the liver, accompanied by a remarkable increase in the levels of HNE-protein adducts, and these rats have a high incidence of liver cancer (10, 14, 19). Besides DNA, HNE can also react with the sulfhydryl group of cysteine, the amino group of lysine, and the imidazole group of histidine in proteins by Michael addition (2, 9). Thus, it is likely that proteins involved in DNA repair may be adducted by HNE, resulting in the impairment of DNA repair mechanisms that may contribute to cytotoxicity and carcinogenicity.

Recent studies have established that, besides exerting toxicity, HNE plays a key role in stress-induced signaling for the regulation of gene expression, for induction of cell cycle arrest and apoptosis, and also for the activation of defense mechanisms against oxidative stress (20–25). Although HNE is known to cause DNA base modifications and strand breaks (8, 11, 13), the mechanism of HNE-induced DNA damage and its effects on cell cycle signaling are poorly understood. The cellular response to DNA damage is complex and involves the functions of gene products that recognize DNA damage and signal for the inhibition of proliferation (26), for stimulation of repair mechanisms (27), or ultimately for the induction of apoptosis (28). In general, the cellular response to DNA damage and the resulting interference in replication involve the activation of signal transduction pathways known as checkpoints that inhibit cell cycle progression and induce the expression of genes that facilitate DNA repair (26, 27) to ensure high fidelity during DNA replication and chromosome segregation. Defects in these checkpoint responses can result in genomic instability, cell death, and predisposition to cancer (28–30). The present studies were designed to elucidate the mechanisms involved in HNE-induced cell cycle arrest. The results of these studies show that HNE causes G₂/M phase cell cycle arrest in liver-derived hepatocellular carcinoma cell lines, and this is associated with a marked decrease in the expression of key G₂/M transition regulatory proteins, including CDK1 and cyclin B1. These studies, for the first time, report a link between HNE-induced G₂/M cell cycle arrest and the ATR/Chk1 signaling pathway in hepatocellular carcinoma cells. Furthermore, we demonstrate that Chk1-mediated phosphorylation of Cdc25C and activation of p21 are important events associated with this phenomenon.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture Conditions**—The HepG2 and Hep3B cells purchased from the American Type Culture Collection were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1% of a stock solution containing 10,000 IU/ml penicillin, and 10 mg/ml streptomycin in an incubator at 37 °C under a humidified atmosphere containing 5% CO₂.

**Materials**—4-Hydroxynonenal was purchased from Cayman Chemical (Ann Arbor, MI). The cell culture medium RPMI 1640, Geneticin (G418), Lipofectamine 2000 transfection reagent, and fetal bovine serum were from Invitrogen. Antibodies against p53, p21, cyclin B1, CDK1, and β-actin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), whereas p-ATR (Ser-428), p-Chk1 (Ser-296), Cdc25C, Cdc25C (Ser-216), p-CDK1 (Tyr-15), p-CDK1 (Thr-161) and p-H2A.X (Ser-139) were from Cell Signaling Technology (Danvers, MA). All other reagents and chemicals were purchased from Sigma-Aldrich.

**Preparation of Cell Extracts and Western Blot Analysis**—Cells were lysed in 200 μl of radioimmune precipitation lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 1 mg ml⁻¹ aprotinin, 1 mg ml⁻¹ leupeptin, 1 mM Na₃VO₄, 1 mM NaF). Protein concentrations were determined by the Bradford assay (31) as described in the standard protocol. Western blot analysis was performed as described previously (32). Isolation of nuclear and cytoplasmic enriched fractions was by the Imgenex nuclear extraction kit as per the manufacturer’s instructions (Imgenex, San Diego, CA). After treatment, normalized amounts of cell lysate were processed for the immunoprecipitation study with appropriate antibody, as described previously (32), and analyzed by Western blotting.

**Cytotoxicity Assay**—The sensitivity of the HepG2 or Hep3B cells to HNE was measured by the MTT assay as described previously (32). A dose-response curve was plotted, and the concentration of HNE resulting in a 50% decrease in formazan formation was calculated as the IC₅₀ value of HNE.

**Flow Cytometric Analysis**—The effect of HNE on cell cycle arrest was determined by flow cytometric analysis. The cells were treated with 40 μM HNE for 24 h at 37 °C. Appropriate controls were also set up. After treatment, floating and adherent cells were collected, washed with PBS, and fixed with 70% ethanol. The cell suspensions were counted, and ~60,000 cells were resuspended in 500 μl of PBS in flow cytometry tubes. Cells were treated with 2.5 μl of RNase (20 mg/ml) and incubated at 37 °C for 30 min, after which they were treated with 5 μl of a propidium iodide (1 mg/ml) solution and incubated at room temperature for 30 min in the dark. The stained cells were analyzed using the Beckman Coulter Cytomics FC500 flow cytometry analyzer. CXP2.2 analysis software from Beckman Coulter was used to deconvolute the cellular DNA content histograms to quantitate the percentage of cells in the respective phases (G₁, S, and G₂/M) of the cell cycle. The appearance of the sub-G₁/G₀ peak indicates cells undergoing apoptosis.

**Immunofluorescence Studies**—The immunofluorescence studies were performed as described previously (32). HepG2 cells were exposed to 20 and 40 μM HNE. Treated and untreated cells were fixed with 4% paraformaldehyde and then permeabilized. After blocking, slides were incubated with anti-phospho-H2A.X (Ser-139) antibody diluted 1:100 in PBS for overnight at 4 °C. After washing with PBS, the coverslips were incubated with FITC-labeled goat anti-rabbit immunoglobulin G (Southern Biotech) diluted 1:400 in PBS for 2 h at room temperature in the dark. The coverslips were then washed with PBS and mounted on glass slides with VectaShield medium containing DAPI (1.5 μg/ml) (Vector Laboratories, Inc.). The slides were bated at 37 °C for 30 min, after which they were treated with 5 μl of a propidium iodide (1 mg/ml) solution and incubated at room temperature for 30 min in the dark. The stained cells were analyzed using the Beckman Coulter Cytomics FC500 flow cytometry analyzer. CXP2.2 analysis software from Beckman Coulter was used to deconvolute the cellular DNA content histograms to quantitate the percentage of cells in the respective phases (G₁, S, and G₂/M) of the cell cycle. The appearance of the sub-G₁/G₀ peak indicates cells undergoing apoptosis.
were examined using LSM 510 Meta confocal system (Carl Zeiss) equipped with an inverted microscope (Axio Observer Z1, Carl Zeiss).

**Immunohistochemical Localization of p-H2A.X in Liver Tissue**—Formalin-fixed liver tissues from mGsta4 (∼−/−) and wild-type (+/+ ) mice were sectioned and processed using standard histologic techniques. The slides were incubated with anti-phospho-H2A.X (Ser-139) antibody (1:100 dilution) overnight at 4 °C in a humidifier chamber. The primary antibody was washed off with PBS. Goat anti-rabbit FITC-conjugated antibody was added. After 2 h at room temperature, unbound secondary antibody was removed by washing with PBS, and slides were mounted with coverslips with Vectashield DAPI mounting medium and analyzed under an LSM 510 Meta confocal system (Carl Zeiss) and photographed. Photographs were taken at ×200 magnification.

**p53 Small Interfering RNA (siRNA) Transfection in HepG2 Cells**—siRNA transfection experiments against p53 were performed using double-stranded RNA synthesized by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Briefly, HepG2 cells were cultured in a 6-well plate at 37 °C until 60–80% confluence. For each transfection, 100 nm double-stranded non-targeting control siRNA or p53-specific siRNA was transfected into HepG2 cells using siRNA transfection reagent according to the manufacturer’s protocol. Cells were harvested at appropriate time points, and the silencing of p53 was examined by Western blotting.

**Neutral Comet Assay**—HepG2 cells were treated with 0–40 μM HNE for 8 h. The presence of DNA damage was assessed by single cell gel electrophoresis and performed using the Trevigen comet assay kit (Trevigen Inc., Gaithersburg, MD) according to the manufacturer’s instructions. Comet tails were stained with SYBR Green and analyzed by a fluorescent microscope.

**Transient Transfection with pTarget and hGSTA4**—HepG2 and Hep3B cells were plated in a 100-mm Petri dish. Petri dishes having >60% confluent cells were used for the transfection. The cells were transiently transfected with 24 μg of either empty pTarget-T vector (VT) or the pTarget vector with the open reading frame (ORF) of the hGSTA4 sequence (hGSTA4-Tr), using Lipofectamine 2000 reagent (Invitrogen) as per the manufacturer’s instructions.

**Statistical Analysis**—The data are expressed as the mean ± S.E. for each group. The statistical significance was determined by Student’s t test and was set at p < 0.05.

**RESULTS**

**HNE Induces Cell Cycle Arrest in G₂/M Phase in HepG2 and Hep3B Cells**—In our previous studies, we have shown that in addition to Fas-mediated apoptosis, HNE also activates the p53-mediated intrinsic apoptotic pathway (32). These studies show that HNE also induces the expression of p21, which is needed for the induction of cell cycle arrest in either G₀/G₁ or G₂/M phase. During the present studies, we have examined the effects of HNE on cell cycle in human hepatocellular carcinoma HepG2 cells. Additionally, the p53 null Hep3B cell line was used to determine the possible role of p53 in HNE-induced cell cycle arrest. The cytotoxicity of HNE to HepG2 and Hep3B cells was evaluated by an MTT assay. In the MTT assay (Fig. 1A), HNE concentrations ranging from 0 to 100 μM gradually decreased HepG2 and Hep3B cell viability, corresponding to IC₅₀ values of 49.7 ± 3.43 and 42.6 ± 2.39 μM (n = 8), respectively, after 24 h of treatment. Based on these results, HNE concentrations of 5–40 μM were used in the present study to examine its effect on cell cycle signaling in both cell types. Cell cycle distribution patterns were compared in control and HNE-treated (40 μM, 24 h) cells by measuring the DNA content by flow cytometric analysis of propidium iodide-stained cells. Results of these experiments presented in Fig. 1 showed a statistically significant increase in G₂/M phase cells in both HepG2 (Fig. 1, B and D) and Hep3B (Fig. 1, C and E) cells after exposure to HNE. An average of cell cycle distribution determined in three independent experiments showed that although the majority of HepG2 or Hep3B cells were in G₀/G₁ phase in the absence of HNE, ~35–45% of the cells were arrested in G₂/M phase in both cell lines after 24 h of sustained exposure to HNE. This arrest in G₂/M phase was accompanied with a concomitant decrease in G₀/G₁ phase cells (Fig. 1, D and E). HNE-induced G₂/M phase arrest and corresponding decrease in G₀/G₁ phase cells in both cell lines was time- and concentration-dependent (data not presented). In addition, a small (statistically insignificant) increase in S phase cells was also observed in both cell lines upon HNE treatment, suggesting that the S phase entry of the cells was not significantly affected in response to HNE, but the exit from S phase might be partially impaired, causing a slight increase in S phase cells.

**HNE Affects Expression of Cyclin B1 and CDK1**—Cell cycle progression is regulated by a sequential activation of cyclin-dependent kinases (CDKs). The activity of CDKs is dependent upon their association with regulatory cyclins (29, 33, 34), and each of the CDKs can associate with different cyclins, which determines which of the proteins are to be phosphorylated by a particular CDK-cyclin complex. The CDK1-cyclin B1 complex has been shown to be important for entry into mitosis (29, 33). Therefore, to elucidate the mechanism of HNE-induced G₂/M phase arrest, we compared the levels of cyclin B1 and CDK1 proteins in whole lysates prepared from the controls and HNE-treated cells by Western blot analyses. The blots presented in Fig. 1F showed that in HepG2 as well as in Hep3B cell lines, the levels of cyclin B1 and CDK1 were consistently down-regulated by HNE in a time-dependent manner. In HepG2 cells (Fig. 1G), 49 and 51% reduction was observed in cyclin B1 and CDK1 expression, respectively, after 24 h of 40 μM HNE treatment. Likewise, in Hep3B cells (Fig. 1G), 49 and 65% reduction was observed in the expression of cyclin B1 and CDK1 (40 μM, 24 h), respectively. Together, these results suggested that HNE did indeed down-regulate cyclin B1 and CDK1 expression and caused the G₂/M phase cell cycle arrest in both HepG2 and Hep3B cells.

**HNE Affects Phosphorylation Status of CDK1 for the Down-regulation of Its Activity**—The activity of CDK1-cyclin B1 complex is dependent on the phosphorylation/dephosphorylation status of CDK1 (26, 29, 33). The entry of eukaryotic cells into mitosis is regulated by CDK1 activation, which is controlled at several steps, including the binding of CDK1 to cyclin B1, its phosphorylation at Thr-161, and dephosphorylation at Thr-14/Tyr-15 residues (29, 33). The phosphorylation of CDK1 at Thr-161 is required for the activation of CDK1-cyclin B1 kinase complex, whereas reversible phosphorylation at Thr-14 and
Tyr-15 suppresses its kinase activity (29, 33). The effect of HNE on the phosphorylation and dephosphorylation status of CDK1 was therefore compared by Western blot analyses in lysates prepared from controls and HNE-treated cells. As compared with the respective controls, the phosphorylation of CDK1 at Thr-161 was significantly reduced (72% in HepG2 and 65% in Hep3B; Fig. 2, A and B) in cells treated with HNE for 24 h. On the other hand, phosphorylation of CDK1 at Tyr-15 increased by severalfold in HepG2 and Hep3B cells (Fig. 2, A and C) cells after 24 h of HNE treatment. Time course analysis indicated that HNE affected all of these parameters in a time-dependent manner. Taken together, these results indicated that the HNE-induced G2/M phase cell arrest in HepG2 and Hep3B cells was accompanied with a decline in CDK1 binding activity with cyclin B1 due to decreased phosphorylation of CDK1 at Thr-161 and a decreased CDK1 kinase activity due to increased phosphorylation of Tyr-15.

Cdc25C Plays a Role in HNE-mediated Inactivation of CDK1—It has been suggested that the phosphatase Cdc25C may be responsible for the dephosphorylation of CDK1 at residues Thr-14 and Tyr-15 and subsequent activation of CDK1 (26, 29, 33, 35). Therefore, we examined the effect of HNE on the expression of Cdc25C in both cell types. The results of these experiments showed that exposure to 40 μM HNE caused a consistent decrease in total Cdc25C protein in both cell types (Fig. 3, A and B) in a time-dependent manner. The levels of Cdc25C were reduced to about 97, 79, 68, and 46% of their respective controls in 40 μM HNE-treated HepG2 cells at the 4, 8, 12, and 24 h time points, respectively. Likewise, Cdc25C levels in HNE-treated Hep3B cells (Fig. 3, A and B) were reduced to about 96, 87, 79, and 62% at 4, 8, 12, and 24 h, respectively. These results suggested that HNE-induced CDK1 phosphorylation at Tyr-15 correlated with the low expression and decreased phosphatase activity of Cdc25C. It has been reported that the function of Cdc25C is negatively regulated by phosphorylation at Ser-216 that, by promoting the binding of Cdc25C with 14-3-3 protein, prevents nuclear localization of this dual specificity phosphatase (36, 37). Therefore, we examined the effect of HNE on Ser-216 phosphorylation of Cdc25C. As shown in Fig. 3, A and C, the phosphorylation of Cdc25C at Ser-216 was significantly increased at the early time points. An optimal induction in Ser-216 phosphorylation was seen at the 8
HNE-induced G₂/M Cell Cycle Arrest

FIGURE 2. Effect of HNE on phosphorylation of CDK1 protein. A, HepG2 and Hep3B cells were treated with 40 μM HNE for 0, 4, 8, 12, and 24 h at 37 °C. The protein lysates (30 μg of protein) were analyzed by Western blotting for p-CDK1 (Thr-161) and p-CDK1 (Tyr-15) expression. β-Actin was used as a loading control. Intensity of p-CDK1 (Thr-161) (A) and p-CDK1 (Tyr-15) (B) were determined by densitometry and normalized with internal loading control. The bar graph represents the -fold change in p-CDK1 (Thr-161) and p-CDK1 (Tyr-15) in HNE-treated cells compared with control cells. Each bar represents the mean ± S.E. (error bars) of three independent experiments.

and 12 h time points in HepG2 and Hep3B cells, respectively, and it significantly declined at 24 h. This reduction in Ser-216-phosphorylated Cdc25C at late time points was consistent with relatively more reduction in the expression of total Cdc25C protein at the 24 h time point.

Because the phosphorylation of Cdc25C at Ser-216 is required for its translocation from the nucleus to the cytoplasm for degradation by the ubiquitin-dependent proteasomal system, and accelerated proteasomal degradation of Cdc25C has been demonstrated during arsenic-induced G₂/M phase cell cycle arrest (38), the effect of HNE on the cytoplasmic accumulation of Cdc25C was therefore examined by Western blot analysis of the cytoplasmic and nuclear fractions prepared from the control and HNE-treated HepG2 cells using anti-Cdc25C antibody. For these experiments, cells treated with HNE for only 8 h were used to minimize the effect of the HNE-induced decline in Cdc25C protein levels at the late time points described above.

Treatment of cells with HNE resulted in a remarkable increase of Cdc25C protein in the cytoplasm that was accompanied with a corresponding decrease in the nuclear fraction (Fig. 3, D and E). These results indicated that HNE promoted the translocation of Cdc25C from the nucleus to the cytoplasm even at an early time point of exposure. We then examined whether or not Cdc25C translocated by HNE treatment was degraded by ubiquitin-mediated proteasomal pathway by determining the effect of MG132 (a specific proteasomal inhibitor) on the HNE-induced decline in Cdc25C protein. The HNE-mediated decline in Cdc25C protein level in HepG2 cells was nearly completely blocked in the presence of MG132 (Fig. 3F). The blot was stripped and reprobed with anti-ubiquitin antibody to determine whether Cdc25C was ubiquitinated. Indeed, higher molecular weight polyubiquitin conjugates were abundant in the lane containing lysate from cells treated with HNE and MG132 and HNE alone but were remarkably reduced in lysates from control HepG2 cells (Fig. 3F). To further confirm HNE-mediated ubiquitination of Cdc25C, we immunoprecipitated Cdc25C or ubiquitin from the lysates of cells treated with HNE in the presence of MG132. Western blot analysis presented in Fig. 3G demonstrated the HNE-mediated binding of ubiquitin with Cdc25C. Together, these results suggest that the HNE-induced decline in the level of Cdc25C protein was due to its degradation in proteasomes and that it seems to play an important role in the mechanisms through which HNE decreases CDK1 activity, leading to G₂/M cell cycle arrest.

HNE Induces Phosphorylation of ATR and Chk1—Checkpoint kinases Chk1 and Chk2 are involved in Ser-216 phosphorylation of Cdc25C (26, 37, 39). Therefore, by examining the phosphorylation status of Chk1 and Chk2, we determined whether or not HNE activated these kinases. Results of the Western blot analysis presented in Fig. 4 showed that even at 5 μM concentrations, HNE caused a significant phosphorylation of Chk1 at Ser-296 within 8 h in HepG2 as well as Hep3B (Fig. 4, A and B) cells. Accordingly, a robust activation of Chk1 was seen in cells treated with 40 μM HNE in both cell types. HNE treatment did not induce the phosphorylation of Chk2 at Thr-68 in both cell types (data not presented), indicating that HNE specifically activated Chk1 and not Chk2. Chk1 phosphorylation at Ser-296 is mainly regulated by the activation of the upstream kinase ATR via its phosphorylation at Ser-428 (40, 41). Western blotting using an antibody specific for phospho-ATR (Ser-428) revealed remarkably increased phosphorylation of ATR in HNE-treated HepG2 and Hep3B (Fig. 4, A and B) cells. It has been suggested that, depending on the nature of the damage, in some cases, Chk1 phosphorylation may also be regulated by the upstream kinase ATM (42). Therefore, we also examined if HNE treatment induced the phosphorylation of ATM at Ser-1981. Results of these experiments showed that HNE did not affect the phosphorylation status of ATM in either cell line (data not presented). Together, these studies suggest that HNE-induced G₂/M arrest is specifically regulated by the activation of the ATR/Chk1 pathway. An early activation of ATR and Chk1 at relatively low HNE concentrations suggests that HNE may act as an early sensor of stress and DNA damage that may play an important role in the signaling events initiated by oxidative stress-induced DNA damage.
ATR/Chk1 Kinase Inhibitor (Caffeine) Mitigates HNE-induced G2/M Arrest and Inhibits Associated Signaling—Checkpoint kinase inhibitors or their analogues have been used to sensitize cells to killing by genotoxic agents because they override the drug-induced G2 checkpoint (43, 44). Therefore, we examined the effects of HNE on cell cycle distribution in the absence or presence of an ATR kinase inhibitor (caffeine) (45, 46). As expected, treatment with HNE led to a significant G2/M phase arrest in both cell types. In contrast, HepG2 cells pre-treated with caffeine were significantly protected from HNE-induced G2/M phase cell cycle arrest (Fig. 5A). The flow cytometric data also demonstrated that HNE-induced G2/M cell cycle arrest was partially bypassed by a concomitant treatment with caffeine. Intriguingly, the appearance of a sub-G0/G1 population was also remarkably reduced (Fig. 5A). The ultimate target of the G2/M checkpoint signaling pathway is the CDK1-cyclin B1 complex, whose activation depends on the dephosphorylation of Tyr-15 of CDK1, which, in turn, is regulated by Cdc25C, whose phosphatase activity is tightly regulated by the activation of the ATR-Chk1 kinase pathway. We therefore investigated whether activation of ATR-Chk1 kinase and its associated downstream signaling, which leads to the inactivation of CDK1-cyclin B1 complex, played a role in HNE-induced G2/M arrest. As shown in Fig. 5, B and C, HNE-induced activa-
tion of ATR and Chk1 kinases (via phosphorylation of Ser-428 in ATR and Ser-296 in Chk1) was significantly inhibited in the presence of ATR kinase inhibitor. Our results also demonstrated that HNE-induced degradation of Cdc25C was significantly attenuated in the presence of caffeine (Fig. 5, B and C), which was consistent with previous studies showing that the degradation of Cdc25C was mediated by the activation of Chk1. The HNE-induced decrease in the levels of cyclin B1 and CDK1 was also inhibited in the presence of ATR kinase inhibitor. HNE-induced phosphorylation of CDK1 at site Tyr-15 was also remarkably inhibited by ATR kinase inhibitor (Fig. 5, B and C). The results of co-immunoprecipitation studies presented in Fig. 5D further confirmed that the HNE-induced dissociation of CDK1-cyclin B1 complex, which plays an important role in G2/M arrest, was inhibited in the presence of caffeine. These results suggested that the inhibition of ATR/Chk1 activation by caffeine was accompanied with the attenuation of HNE-induced inhibition of CDK1-cyclin B1 complex, which was consistent with the observed partial inhibition of HNE-induced G2/M arrest in the presence of caffeine. Together, these data strongly suggest that similar to DNA damage-induced cell cycle arrest in G2/M phase, HNE also promotes G2/M phase cell cycle arrest via the activation of the ATR/Chk1/Cdc25C pathway, leading to the inactivation of the CDK1-cyclin B1 complex.

HNE Causes the p53-independent Activation of p21—After the addition of ATR/Chk1 kinase inhibitor, a significant number of HepG2 cells were still arrested in the G2/M phase of the cell cycle (Fig. 5A) upon HNE treatment. We hypothesized a role of p21 in this phenomenon, arguing that the association of p21 with CDK1-cyclin B1 complexes may also down-regulate the CDK1 activity, as shown previously (47, 48). The activation of p21 is mainly regulated by the activation of tumor suppressor
protein p53 (49, 50). A concentration-dependent activation and phosphorylation of p53 by HNE observed in WT p53 HepG2 cells (Fig. 6, A and B) was consistent with our previous studies with other cell types (51). However, HNE induced the activation of p21 independent of p53 because our results showed that in p53 wild type HepG2 as well as in p53 null Hep3B (Fig. 6, A and B) cells, the level of p21 was consistently increased in a concentration-dependent manner upon treatment with HNE. To rule out the requirement of p53 for the activation of p21 by HNE in WT p53 HepG2 cells, we suppressed the expression of p53 in these cells by specific siRNA before HNE treatment. Results of these experiments demonstrated that whereas the level of p53 was significantly down-regulated in HepG2 cells after 2 days of p53 siRNA transfection (Fig. 6, C and D), the suppression of p53 expression did not significantly affect HNE-induced activation of p21 (Fig. 6, C and D). Thus, HNE also seems to induce G2/M cell cycle arrest via p21 through a mechanism(s) that is independent of p53. Interestingly, although the addition of caffeine did not affect the HNE-induced activation of p21 expression in HepG2 cells, the expression and phosphor-

FIGURE 6. Effect of HNE on p53 and p21 activation. A, Hep3B and HepG2 cells were treated with 0, 5, 10, 20, and 40 μM HNE for 12 h at 37 °C. The protein lysates (30 μg of protein) were analyzed by Western blotting for the protein expression of p53, p-p53 (Ser-15), and p21. β-Actin was used as a loading control. B, intensity of p53, p-p53 (Ser-15), and p21 were determined by densitometry and normalized with loading control. The bar graph represents the mean ± S.E. (error bars) of three independent experiments. C, silencing of p53 was performed by the p53 siRNA as per the manufacturer’s instructions (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and control cells were transfected with control siRNA in a similar way. After transfection, the cells were harvested after 48 h, and the expression level of p53 was examined by Western blot analysis. For the analysis of p53-independent p21 expression, control and p53-depleted HepG2 cells were treated with 0 and 40 μM HNE for an additional 12 h, and the protein expression level of p21 was examined by Western blotting. β-Actin was used as a loading control. D, bar graph showing densitometric analysis of bands of p53 and p21, used in C. Data were collected from two independent experiments, and the mean ± S.E. was calculated (**, p < 0.01 versus control). E, inhibition of HNE-induced p-p53 (Ser-15), p53, and p21 was analyzed by pretreatment of HepG2 cells with caffeine. Control (without caffeine) and pretreated (with caffeine; 1 mM for 2 h) HepG2 cells were either left untreated or treated with 40 μM HNE for 12 h at 37 °C. The total cell lysates were subjected to Western blot analyses for the expression of p-p53 (Ser-15), p53, and p21 protein. Anti-β-actin antibody was used as a loading control. F, intensity of p53, p-p53 (Ser-15), and p21 were determined by densitometry and standardized with loading control. The bar graph represents the fold change in p53, p-p53 (Ser-15), and p21 expression in HNE-treated cells compared with untreated cells in the presence or absence of caffeine pretreatment. Each bar represents the mean ± S.E. of three independent experiments (***, p < 0.001 versus control).
ylation of p53 (Ser-15) were significantly inhibited (Fig. 6, E and F). Together these results suggest that Cdc25C and p21 cooperatively mediate G2/M checkpoint arrest caused by HNE. Further studies are required to explore this possibility.

HNE Treatment Induces Phosphorylation of Histone H2A.X at Ser-139—Because HNE causes DNA damage, we examined whether HNE treatment caused DNA double strand breaks by monitoring Ser-139 phosphorylation of H2A.X, which is a sensitive marker for the presence of DNA double strand breaks (52–54). Western blot studies clearly showed a concentration-dependent increase in p-H2A.X (Ser-139) in HNE-treated cells compared with control cells. Each bar represents the mean ± S.E. (error bars) of three independent experiments. When p-H2A.X (Ser-139) expression was analyzed by pretreatment of HepG2 cells with caffeine, intensity of p-H2A.X (Ser-139) expression was determined by densitometry and normalized with loading control. The bar graph represents the -fold increase in p-H2A.X (Ser-139) expression in HNE-treated cells compared with untreated cells in the presence or absence of caffeine pretreatment. Each bar represents the mean ± S.E. of three independent experiments (***, p < 0.001 versus control).

FIGURE 7. Effect of HNE on phosphorylation of histone H2A.X (Ser-139) and DNA damage. A, HepG2 and Hep3B cells were treated with 0, 5, 10, 20, 30, and 40 μM HNE for 12 h at 37 °C. Total protein lysates (30 μg of protein) were analyzed by Western blotting for the expression of p-H2A.X (Ser-139). β-Actin was used as a loading control. B, intensity of p-H2A.X (Ser-139) was determined by densitometry and normalized with loading control. The bar graph represents the -fold increase in p-H2A.X (Ser-139) in HNE-treated cells compared with control cells. Each bar represents the mean ± S.E. (error bars) of three independent experiments. C, immunofluorescence staining of γ-H2A.X in HepG2 cells showing concentration-dependent induction in γ-H2A.X foci in HNE-treated HepG2 cells. The cells were grown on glass coverslips, and untreated and treated (20 and 40 μM HNE for 8 h) cells were fixed, permeabilized, and incubated with anti-phospho-H2A.X (Ser-139) antibody, followed by FITC-conjugated secondary antibody (green). DNA was counterstained with DAPI (blue). Slides were analyzed using a Zeiss LSM 510 META laser-scanning fluorescence microscope. D, quantification of the average number of H2A.X foci per cell after HNE treatment in HepG2 cells. Data were collected from three independent experiments, and the mean ± S.D. was calculated (**, p < 0.01; ***, p < 0.001 versus control). E, HepG2 cells were incubated with 0, 10, 20, and 40 μM HNE for 8 h, and neutral single cell gel electrophoresis was performed to assess the DNA double strand breaks according to the manufacturer’s instructions. F, bar diagram shows tail intensity (percentage of DNA in tail) (mean ± S.E.) from three independent experiments. The results are given as mean ± S.E. The mean in each case was calculated from three parallel slides; 40 comets were evaluated per slide (**, p < 0.01; ***, p < 0.001 versus control). G, inhibition of HNE-induced H2A.X phosphorylation was analyzed by pretreatment of HepG2 cells with caffeine. Control (without caffeine) and pretreated (with caffeine; 1 mM for 2 h) HepG2 cells were either left untreated or treated with 40 μM HNE for 8 h at 37 °C. The total cell lysates were subjected to Western blot analyses for the expression of p-H2A.X (Ser-139) protein. Anti-β-actin antibody was used as a loading control. H, intensity of p-H2A.X (Ser-139) was determined by densitometry and standardized with loading control. The bar graph represents the -fold increase in p-H2A.X (Ser-139) expression in HNE-treated cells compared with untreated cells in the presence or absence of caffeine pretreatment. Each bar represents the mean ± S.E. of three independent experiments (***, p < 0.001 versus control).
ylation occurs at the sites adjacent to DNA double strand breaks. Shortly after induction of a double strand break, the appearance of γH2A.X in chromatin can be detected through immunofluorescence studies in the form of discrete nuclear foci, each of which presumably represent a single double strand break (52). As shown in Fig. 7, C and D, immunofluorescence analysis further confirmed Ser-139 phosphorylation of H2A.X and an increase in the formation of γH2A.X foci in a concentration-dependent manner after HNE treatment. In parallel, DNA damage was directly assessed by single cell gel electrophoresis (comet assay) under non-denaturing conditions, in which the presence of a comet tail is indicative of DNA double stand breaks. As shown in Fig. 7, E and F, HNE increased the comet tail movement in HepG2 cells in a concentration-dependent manner. These results suggest that HNE can induce significant DNA double strand breaks and caused the phosphorylation of H2A.X at Ser-139. Treatment of HepG2 cells with caffeine resulted in the inhibition of HNE-mediated phosphorylation of H2A.X at Ser-139 (Fig. 7, G and H).

Increased HNE Levels in the Liver of mGsta4 Null Mice Activate the Phosphorylation of Histone H2A.X at Ser-139 in Vivo—Earlier studies in our laboratory have shown that in the tissues of mGsta4 (-/-) null mice have remarkably elevated HNE levels as compared with wild-type (+/+) mice (57). The results of studies comparing the phosphorylation status of histone H2A.X at Ser-139 in the liver tissue of mGsta4 (-/-) and wild-type (+/+) mice presented in Fig. 8 showed significantly upregulated phosphorylation of histone H2A.X at Ser-139 in mGsta4 (-/-) mice as measured by Western blot analysis (Fig. 8, A and B) and immunohistochemistry (Fig. 8, C and D). These results show for the first time that in vivo increased phosphorylation of H2A.X at Ser-139 is determined by the intracellular levels of HNE, which causes DNA double strand breaks in tissues and that it is regulated by GSTs.

Overexpression of hGSTA4-4 Inhibits the Activation of G2/M Cell Arrest—Previous studies have shown that the glutathione S-transferase isozyme GSTA4-4 is one of the major regulators of HNE concentration in cells (21, 25, 58, 59). Therefore, we examined the effect of GSTA4-4 overexpression on HNE-induced cell cycle arrest and associated signaling. Results presented in Fig. 9 showed that HNE-induced G2/M cell cycle arrest and associated signaling events could be attenuated by the forced overexpression of GSTA4-4. For these experiments, we treated the cells transfected with empty vector and hGSTA4-4 with 40 μM HNE for 24 h and quantified the percentage of cells in the different phases of cell cycle by flow cytometry. The results of these studies presented in Fig. 9A demonstrated that HNE caused a significant accumulation of the empty vector-transfected cells in G2/M phase, whereas the overexpression of hGSTA4-4 rescued these cells from G2/M cell cycle arrest. Overexpression of GSTA4-4 isozyme in these cells also resulted in the inhibition of ATR and Chk1 phosphorylation, abrogation of HNE-mediated degradation of Cdc25C, and resulting downregulation of CDK1 (Fig. 9, B and C). Likewise, HNE-induced phosphorylation of H2A.X (Ser-139) was also inhibited in GSTA4-4-transfected cells. Collectively, these results suggest that the overexpression of GSTA4-4 attenuates HNE-mediated down-regulation of CDK1-cyclin B complex activity and indicate that G2/M cell cycle arrest observed in these cells can be attributed specifically to HNE. More importantly, these results
suggest a role of GSTA4-4 in the regulation of stress-induced cell cycle arrest.

**DISCUSSION**

Although HNE is known to cause DNA base modifications, its effects on cell cycle signaling are poorly understood. The results of the present studies provide insight into the mechanisms involved in HNE-induced cell cycle arrest. It was previously shown that HNE effectively inhibits the proliferation of human hepatocellular carcinoma HepG2 cells (32). The results of the present studies probing into the mechanisms through which HNE affects cell cycle signaling indicate that HNE-mediated inhibition of proliferation of these cells may result from the arrest of cells in G2/M phase, which is followed by an increase in programmed cell death at relatively high concentrations of HNE. Results of the present study demonstrate that HNE down-regulates the activity of CDK1-cyclin B1 kinase complex by reducing levels of these proteins. HNE inhibited CDK1-cyclin B1 complex formation by suppressing the phosphorylation of CDK1 at Thr-161. In addition, HNE also inhibits CDK1-cyclin B1 kinase activity by increasing the accumulation of the inactive form of CDK1 phosphorylated at Tyr-15 along with a decline in the level of Cdc25C caused by HNE-induced proteasomal degradation of this protein. The induction of cell cycle arrest by HNE has been reported in other cellular models, but the involved mechanisms are not clear. For example, an HNE-induced increase in the percentage of G0/G1 phase cells in HL-60 human leukemic and SK-N-Be neuroblastoma cells has been reported (23, 60). HNE causes G0/G1 arrest in these cells by decreasing the levels of cyclin D1 and D2 and up-regulation of p21 (60, 61). The reasons for this differential effect of HNE in these cell lines and HepG2 and Hep3B cell lines used in the present studies should be investigated.

The phosphorylation of Cdc25C at Ser-216 represents an important regulatory mechanism by which cells delay or block mitotic entry under normal conditions as well as in response to DNA damage (39). In previous studies on the role of Cdc25C in DNA damage-induced cell cycle arrest, the phosphorylation at Ser-216 and the subsequent subcellular localization of Cdc25C in cytoplasm has been shown (36–39). The present studies demonstrate that HNE induces the phosphorylation of Cdc25C at Ser-216 as early as 4 h and persists at least until 24 h and that it also induces the translocation of Cdc25C to cytoplasm, where its degradation occurs via the ubiquitin-proteasome system. Thus, the translocation of Cdc25C to cytoplasm and ubiquitin-mediated proteasomal degradation of Cdc25C appear to be the main mechanism of cell cycle arrest by HNE. Here we demonstrate that increased Ser-216 phosphorylation of Cdc25C in HNE-treated cells is linked with ATR-dependent activation of Chk1. Because the ATR/Chk1 pathway is activated by the bulky DNA lesions and replication fork collapse (26), it is possible that HNE may also cause bulky DNA lesions in a concentration-dependent manner in cells, leading to the activation of ATR-dependent Chk1 phosphorylation. This idea is consistent with the effect on HepG2 cells of caffeine, which inhibits the HNE-induced G2/M phase arrest and associated signaling.

Only partial inhibition of HNE-induced G2/M arrest by caffeine suggests that ATR/Chk1-independent mechanisms may also be contributing to HNE-induced cell cycle arrest through

**FIGURE 9. Effect of HNE on cell cycle arrest and its associated signaling in hGSTA4-overexpressing HepG2 cells.** A, bar chart showing the effect of HNE on the cell cycle of HepG2 (VT- and hGSTA4-Tr-transfected) cells. VT- and hGSTA4-Tr HepG2 cells were treated with 0 or 40 μM HNE for 24 h at 37 °C. Both floating and attached cells were collected and processed for analysis of cell cycle distribution by FACS as described under “Experimental Procedures.” Data were collected from three independent experiments, and the mean ± S.E. (error bars) was calculated (**, p < 0.01 versus control). B, expression of p-ATR, p-CHK1, p-H2AX, Cdc25C, p21, cyclin B1, and CDK1 in VT (empty vector-transfected) and hGSTA4-Tr HepG2 cells treated with HNE (40 μM) for 24 h was monitored by Western blot analysis. Cell extracts (30 μg of protein) were resolved on 4–20% SDS-PAGE and immunoblotted using the anti-phospho-ATR (Ser-428), anti-phospho-CHK1 (Ser-296), anti-phospho-H2AX (Ser-139), anti-Cdc25C, anti-p-CDK1 (Tyr-15), anti-CDK1, anti-cyclin B1, anti-p21, and anti-hGSTA4-4 antibodies. β-Actin was used as the loading control. The blot was developed using chemiluminescence (Supersignal West Pico, Pierce) reagents. C, a bar graph showing densitometric analysis of bands used in B. Data were collected from three independent experiments, and the mean ± S.E. was calculated.
activation of p21 because the treatment of these cells with caffeine did not block the induction of p21 by HNE. It is known that p53 protein plays an important role in regulating cell cycle progression after DNA damage. The mechanism by which it mediates cell cycle arrest at the G$_2$ checkpoint involves the transactivation of the cyclin-dependent kinase inhibitor, p21 (48–50). Activated p21 is known to associate with the activated Tyr-15-dephosphorylated form of CDK1, and this complex is devoid of kinase activity (49, 50). Although p53 independent p21 expression has also been observed in HNE-treated HL60 cells (23), it is generally believed that p21 expression is rarely p53-independent because its expression has been shown to be blocked in cells from p53 knock-out mice (62). In the present studies, the activation of p21 by HNE was seen in HepG2 as well as in p53 null Hep3B cells. Also, there was no effect on HNE-induced activation of p21 upon suppression of p53 in HepG2 cells. Together, these results indicate that activation of p21 does not necessarily require p53. Because p21 expression is also regulated by other p53 family proteins (e.g. p73 and p63) (63) and previous studies have shown that p73 and p63 are activated by HNE in SK-N-BE neuroblastoma cells (60), it is possible that these proteins contribute to p21-mediated G$_2$/M arrest caused by HNE.

In response to DNA damage, checkpoint kinases are activated, which leads to cell cycle arrest, and in the case of severe DNA damage, the cell cycle arrest leads to apoptotic cell death. The effects of HNE on these parameters seem to be similar, whereas sublethal concentrations of HNE induce DNA damage and inhibit cell proliferation along with cell cycle arrest, and at high concentrations, HNE leads to cell death. It is widely accepted that phosphorylation of H2A.X in response to DNA double strand breaks initiates the signal for the recruitment of DNA repair machinery, and several repair proteins (53BP1, pNBS1, MDC1, and Brca1) co-localize with γH2A.X at the sites of double strand breaks (53, 64 – 66). The present studies demonstrate that HNE-induced DNA double strand breaks are accompanied with increased phosphorylation of H2A.X at Ser-139. The increased phosphorylation of H2A.X was also observed in the liver tissues of mice that have higher basal levels of HNE due to the disruption of the mGsta4 gene (57). This finding is of significant importance because it may enhance our understanding of the mechanism involved in the manifestation of the toxicity of reactive oxygen species and oxidants and the role of relevant defense mechanisms in vivo. Although mGsta4 (–/–) mice are more sensitive to tumorigenesis (67), they seem to have no apparent toxic manifestations in stress-free conditions. The answer to this question may lie in our results showing that, in parallel, HNE invokes signaling for the defense mechanisms against its own toxicity by activating the phosphorylation of H2A.X at Ser-139, which initiates the signal for the recruitment of DNA repair machinery. HNE-induced phosphorylation of H2A.X is regulated by the ATR/Chk1 pathway because the addition of ATR kinase inhibitor (caffeine) significantly inhibited this phosphorylation in HNE-treated HepG2 cells.

GSTA4-4, which has high specificity and exceptionally high catalytic efficiency for the conjugation of HNE to GSH, is one of the major enzymes involved in detoxification of HNE (58, 59). The inhibition of HNE-induced cell cycle arrest and the associated signaling events in GSTA4-4-overexpressing cells strongly suggest that GSTA4-4 plays a crucial role in protecting cells from DNA damage, as presented in the model shown in Fig. 10. Perhaps this model can be extrapolated to the overall protective role of GSTs during oxidative/electrophilic stress and the role of HNE in the regulation of cell cycle signaling. In the absence of insufficient levels of defense mechanisms, HNE can not only damage DNA but also impair DNA repair mechanisms. It may be argued that the 40 μM concentration of HNE used in the present studies is too high and physiologically irrelevant. However, HNE is formed in membranes where its local concentration may be very high. Furthermore, based on the partition coefficient of HNE between water and lipophilic solvents (68), a 40 μM membrane concentration of HNE would correspond to only about 1 μM in aqueous cytosol. Consistent with this idea, in vitro HNE activates membrane tyrosine kinase receptors (like EGFR, VEGFR, and PDGFR) at low concentrations of <1 μM (69–71), but cytosolic components (e.g. p53, HSF1, and DAXX) require much higher HNE concentrations for their activation (32, 51). Interestingly, activation of JNK, p53, and Nrf2 has also been observed in tissues of mGsta4 knock-out mice (51, 72, 73). Taken together with the results of the present study showing the activation of H2A.X in mGsta4 null mice, these findings strongly suggest that the in vitro effects of HNE presented here are physiologically relevant.

Similar to that reported for DNA damage-induced G$_2$/M cell cycle arrest, HNE promotes G$_2$/M cell cycle arrest via inactivation of CDK1-cyclin B1 complex through the ATR, Chk1, and Cdc25C pathway or p53-independent activation of p21. How-

![Proposed model for activation of the ATR/Chk1 signaling pathway and G$_2$/M phase cell cycle arrest induced by HNE.](image)
HNE-induced G₂/M Cell Cycle Arrest

However, these similarities in HNE- and DNA damage-induced cell cycle arrest do not provide information as to whether both of these signaling pathways operate in parallel or one of these is preceded by the other. Oxidative stress causes both DNA damage and lipid peroxidation, leading to the formation of HNE. Thus, both DNA damage and HNE are likely to activate a checkpoint-mediated defense mechanism to protect genome stability. However, based on our previous findings, we suggest that the low levels of HNE formed at the onset of oxidative stress act as an initial stress sensor to activate the defense mechanisms, including cell cycle arrest. We have previously shown that even a small transient rise in HNE levels upon exposure to stress as mild as 50 μM H₂O₂, 5 min UVA exposure, or 42 °C for 15 min leads to induction of defense mechanisms against oxidative stress to protect from the toxicity of electrophiles generated (6,7). These defense mechanisms include the induction of HNE-metabolizing GST isozymes, synthesis of GSH, increased transport of GSH conjugates by RLIP76 (6, 21), activation of HSF1 (32) and Nrf2 (73), and EGFR- and VEGFR (69, 70)-mediated proliferative mechanisms. This seems to be consistent with the role of HNE in the initial signaling for protection mechanisms. Sustained stress leading to accumulation of HNE also contributes to DNA damage, leading to the induction of additional defense mechanisms, such as cell cycle arrest and induction of additional antioxidant enzymes, but beyond a certain threshold, it induces apoptosis to protect the organism from the genome and organ toxicity.

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