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13. ABSTRACT *(Maximum 200 Words)*

4 hydroxy 2 nonenal is a highly reactive aldehyde byproduct of lipid peroxidation and is known to be highly cytotoxic, damaging both DNA and protein. HNE has a unique structure which includes a lipid tail, and a polar head with an C1 aldehyde, a C3-C4 trans double bond, and a C4 hydroxy. By using compounds analogous in structure to HNE but lacking one individual component we have examined the contribution of each structural feature to the overall toxicity of the compound and determined the rank order as; aldehyde>trans double bond>hydroxy. The length of the lipid tail is also important to the toxicity. These studies were done using growth inhibition and apoptosis induction as endpoints.

We have also examined the mechanism of HNE induced apoptosis and found that HNE induces a p53 independent apoptosis in RAW 264.7 mouse macrophage cells. Using RAW cells expressing anti-apoptotic protein BCL2 we have also found the HNE induced apoptosis is cytochrome c dependent. These studies not only contribute to the knowledge of HNE toxicity but also present a possible link between oxidant stress induced lipid peroxidation and oxidant stress induced apoptosis.

14. SUBJECT TERMS
Lipid peroxidation, apoptosis, hydroxynonenal, p53, mitochondria

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Foreword:

The original focus of this project was on the interaction between the MRP1 multidrug resistance protein, which has activity for efflux of glutathione-conjugated molecules out of cells, and the glutathione S-transferase (GST) family of detoxifying proteins, which catalyze conjugation of carcinogens with the thiol tripeptide glutathione. Early results in this project, together with technical problems with expression of MRP1 in transfected cell lines, led to a switch in emphasis to the mechanisms of induction of apoptosis induced by the toxic lipid peroxidation product 4-hydroxynonenal (HNE). In addition, we hace examined the relative contributions of GST and aldehyde dehydrogenase families of detoxifying enzymes to prevention of cytotoxicity, macromolecular damage, and apoptosis by HNE. This work has relevance to mechanisms of oxidative stress, inflammation and carcinogenesis, as well as pathways of induction of apoptosis in breast and other forms of cancer. This project is expected to be completed by December 2000.

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Introduction:

Oxidative stress, a subject of recent study, has been linked to many different disease states as well as aging and general cellular damage due to normal oxidative metabolism. It has been linked to breast cancer susceptibility primarily through the study of antioxidants such as isoflavins and vitamin E that have been shown to be successful anticarcinogenic agents in breast tissue. Because breast tissue has a high fat content, it is highly susceptible to oxidative damage via lipid peroxidation. The focus of this project centers on an extremely potent mediator of oxidant stress induced damage, 4 hydroxy 2 nonenal (HNE). HNE is one of the most reactive byproducts of lipid peroxidation which results from the interaction of reactive oxygen species with cellular membranes. The data presented focuses on the toxicity of HNE and the contributions of individual structural components to the overall reactivity. We also present data on mechanistic aspects of HNE induced apoptosis. Studying the cascades of events which lead to HNE induced cell suicide not only contributes to what is known about the cellular effects of HNE, but also provides evidence of a potential link between oxidant stress induced lipid peroxidation and oxidant stress induced apoptosis. The balance between oxidative damage and oxidant-induced apoptosis may have significance in controlling the death or progression of preneoplastic cells in breast, colon, and other types of cancer.

Summary of Year 2:

A. Structure-Activity studies: (manuscript submitted to *Molecular Pharmacology (*copy attached*)*)

The structure of HNE is very unique in that it contains both a nonpolar, lipid tail and a polar head consisting of 3 different functional groups: a C1 aldehyde, a trans 3-4 double bond, and a C4 hydroxy. Each of these functional groups contributes to the overall reactivity of the compound. Using compounds analogous to HNE but lacking a specific functional group, we were able to determine the relative contribution of each of the groups to the overall toxicity. For example, to determine the importance of the aldehyde moiety, we used nonenoic acid which lacks the aldehyde then compared the differences in toxicity between the acid and the complete compound. These differences were compared in general cytotoxicity/ growth inhibition as well as apoptosis induction. All the experiments described were done in RAW 264.7 mouse macrophage cells which were used because of the presence of wild type p53.

Key findings:

1) There is a dramatic decrease in toxicity when cells are exposed to nonenoic acid which lacks the aldehyde yet retains the double bond (IC50 = 1770 μM) as compared to trans-2 nonenal (IC50 =24 μM). This decrease in toxicity is also seen with the apoptotic endpoint DNA fragmentation. The loss of the aldehyde reduces the reactivity of the double bond, and also prevents the formation of HNE-protein crosslinks.

2) When cells are treated with nonanal, a 9- carbon alkyl analog which retains the aldehyde but lacks the double bond and C4 hydroxy, an intermediate toxicity (IC50=308 μM) between trans-2 nonenal (IC50= 24μM) and nonenoic acid (IC50= 1770μM) is seen. The loss of the aldehyde in nonenoic acid results in a 74-fold decrease in toxicity compared to trans-2-nonenal. The lack of a double bond in nonanal decreases toxicity 13-fold relative to trans-2-nonenal. The loss of the double bond prevents Michael additions at the C3 position, and also removes the potentiation of the reactivity of the aldehyde which alone can interact with proteins to form Schiff base adducts.

3) Both the aldehyde and C2=C3 double bond are essential for the full toxicity of HNE and induction of apoptosis. Their effects are additive and mutually interactive.

4) The third reactive group, the 4-hydroxy, contributes much less to the toxicity of HNE, as evidenced by the 2.7 fold decrease in the toxicity of trans-2-nonenal as compared to HNE (IC50 of 24uM vs. 9uM for HNE) and the parallel differences for induction of apoptotic DNA fragmentation for these two analogs.

5) The length of the alkenyl chain is important in the toxicity of the compound. Increasing the alkenal chain increases the toxicity, both in growth inhibition and in apoptosis induction.

6) Overexpression of human aldehyde dehydrogenase –3, which oxidizes the aldehyde of HNE into a carboxylic acid, completely protects the cells from apoptotic induction to at least 70μM HNE. It also protects the cell against HNE-protein adduct formation. Such a strong protection supports the conclusion that the aldehyde of HNE contributes a significant potency to the overall reactivity of the compound. It also confirms that the aldehyde group is exerting its toxicity intracellularly rather than at the plasma membrane.

B. Studies on the mechanism of HNE induced apoptosis: (Manuscript in preparation)

RAW cells overxpressing the anti-apoptotic protein BCL2 were used to determine the role of mitochondria and cytochrome c release in HNE induced apoptosis (Fig.1).

When parental and BCL2 cells were exposed to 50uM HNE, apoptotic laddering was detected as early as 3 hours after the exposure. BCL2 transfected cells showed no apoptotic laddering up to 24 hours after exposure (Fig 2). Although BCL2 cells at 50uM were completely protected against apoptotic death, cells did show a significant delay in growth when compared to untreated controls (data not shown). When HNE concentrations were increased to 70uM, BCL2 cells continued to show very little apoptosis. Beyond 70-80uM HNE, BCL2 was unable to protect against possible deleterious effects on cellular membrane integrity and necrotic damage (data not shown).

Because HNE is highly reactive to cellular proteins and readily forms protein adducts, we examined whether BCL2 overexpression protected the cell against adduct formation (Fig. 3). Interestingly BCL2 showed no detectable protection against adduct formation at any of the concentrations tested suggesting that BCL2's anti-apoptotic effects come later in the cascade of apoptotic events, beyond initial protein modification.

To examine the role of p53 and cytochrome c in HNE induced apoptosis, we dosed parental and BCL2 lines with 50uM HNE and examined both the release of cytochrome c into the cytosol and the stabilization of p53 protein as seen by an accumulation of p53 protein levels (Fig. 4). In RAW parental cells, both the appearance of cytochrome c in cytosolic fractions and p53 protein accumulation were detected at 3 hours after the initial exposure. Cytosolic cytrochrome c levels continued to increase through the 12 hours after exposure. p53 protein levels, however, peaked at 6 hours and began to decrease throughout the remaining 6 hours. Unlike parental cells, BCL2 cells showed no release of cytochrome c into the cytosol throughout the 12 hours after exposure. BCL2 cells did, however, show a slightly delayed increase in p53 when compared to parental cells. p53 protein levels, were however, sustained through the entire 12 hours and remained sustained at significantly high levels up to 96 hours when cells became confluent (Figure 5).

To better understand the dose dependence of HNE's effect, we expanded the dose range and examined the concentration effects on cytochrome c release and p53 stabilization (Figure 6). When RAW parental cells were dosed with an increasing amount of HNE, cytochrome c appeared in the cytosolic fractions of each concentration within 6 hours after the initial exposure. With increasing concentrations, a significant increase in cytochrome c release was observed. As before, BCL2 expressing cells showed no cytochrome c release throughout the concentration range tested. When we examined the dose dependence of p53 stabilization, interestingly, p53 accumulation was more prominent at lower concentrations. This was observed in the RAW parental cells as well as the BCL2 cells. It is possible that at concentrations as high as 75uM, the protein kinases necessary for the phosphorylation and resulting stabilization of p53 are inhibited. Figure 7 shows the corresponding DNA fragmentation assay for the concentrations tested in Figure 6. While parental cells show apoptotic laddering relatively early at 50 and 75uM, BCL2 cells show no detectable apoptotic ladders at any of the concentrations tested.

To further explore the role of p53 in the apoptotic induction we transfected the RAW parental cells with the simian virus large T-antigen (TAG). TAG is known to bind to p53 and form a TAG-p53 complex which is believed to abrogate p53 function. When empty vector control cells and TAG cells were exposed to HNE, control cells showed normal p53 patterns as seen before while TAG cells showed a significant and sustained increase in p53 accumulation (Fig. 8). This increase could be a result of stabilization of p53 due to the complexing of TAG to p53 as opposed to normal stabilizaiton of p53 due to phosphorylation of the protein. To show that TAG has an effect on p53 function we examined downstream targets of p53 transactivation function. When we examined p21 (known to be transactivated by p53) levels, we saw a significant increase in control lines upon exposure to HNE. TAG cells, however, showed very little increase in p21 protein. Levels increased only at 30uM after 6 hours then immediately dropped down to a level comparable to untreated control. We also examined BAX (also known to be transactivated by p53) levels (Fig. 9). Because there is such a high level of endogenous BAX in control cells, increases in BAX levels upon HNE treatment are not as notable. In TAG cells, relative levels of BAX were visibly decreased as compared to empty vector control cells. Only at 50uM, 12 hours did BAX levels increase to levels comparable to empty vector control cells

Although TAG expression showed significant effect on p53 transactivation function, TAG cells showed no detectable difference in apoptotic induction as determined by apoptotic DNA fragmentation. (Fig. 10). This suggest that total p53 function is not essential in HNE induced apoptosis and that although p53 is stabilized and accumulates in the cell, HNE may be inducing an apoptotic cascade of events that is p53 independent.

Key Findings:

1) Because BCL2 has been shown to be anti-apoptotic and is believed to act at the level of the mitochondria, preventing the release of cytochrome c, the fact that BCL2 overexpression protects RAW cells from HNE induced apoptosis implicates an essential role for mitochondria in this induction. This is supported by the cytochrome c data which show total protection by BCL2 against the release of cytochrome c into the cytosol.
2) BCL2 protection against apoptosis is complete but is concomitant with a significant growth delay. This is opposed to a temporal delay in apoptotic induction due to BCL2 expression.
3) Although p53 is shown to accumulate in parental cells and to accumulate to a significantly high and sustained level in cells. It is possible that HNE acts through an alternative pathway to induce a preliminary apoptotic event (ie. a direct interaction with mitochondria or through other mitochondria linked signalling pathway). It is also possible that although p53 in RAW cells appears functional, the p53 pathway of apoptotic induction may be nonfunctional with potential

defects in other signalling mediators necessary to produce the cascade from original insult to p53 to downstream effectors which initiate apoptotic cascades..

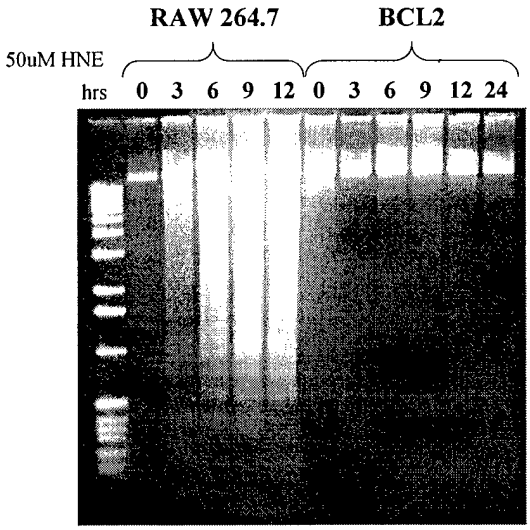
Figures:

1.

RAW BCL2
264.7 -14

— 25 kD

2.



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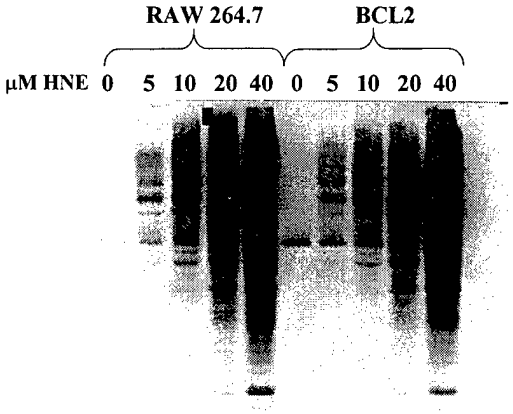
RAW 264.7
BCL2
μM HNE 0 5 10 20 40 0 5 10 20 40

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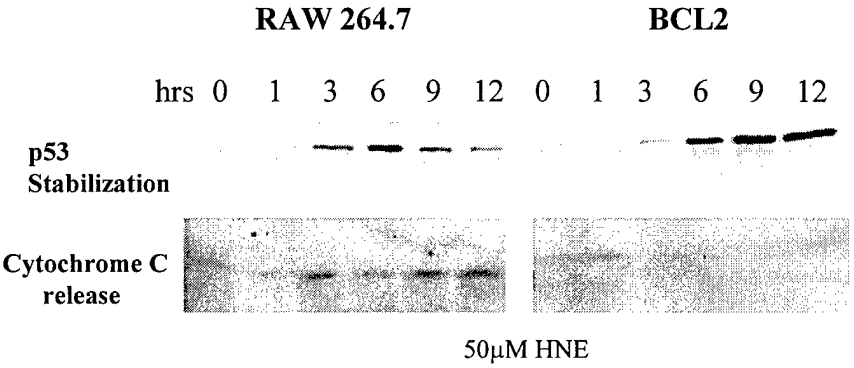
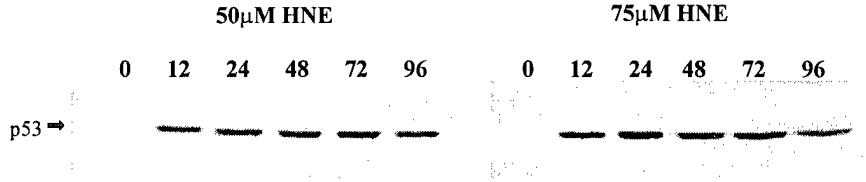
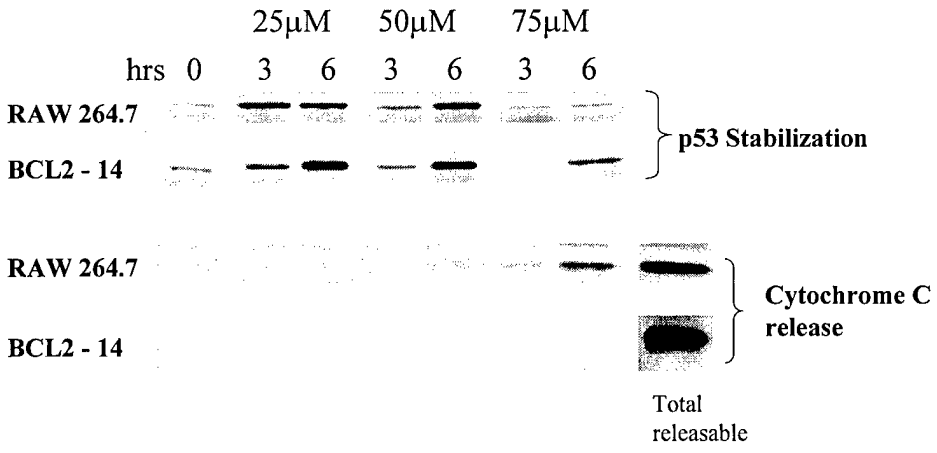

RAW 264.7
BCL2
hrs 0 1 3 6 9 12 0 1 3 6 9 12
p53 Stabilization
Cytochrome C release

50μM HNE

5.


50μM HNE
75μM HNE
0 12 24 48 72 96 0 12 24 48 72 96
p53

6.


25μM
50μM
75μM
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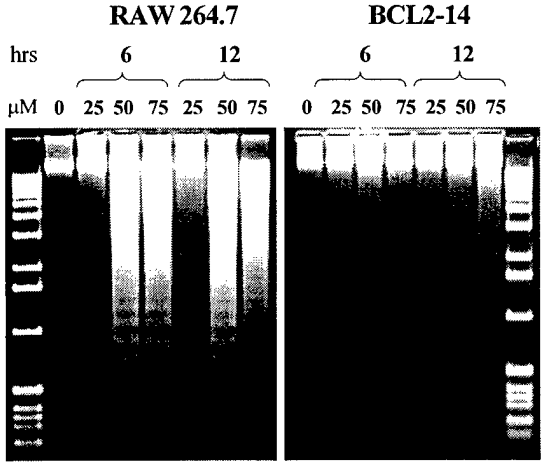
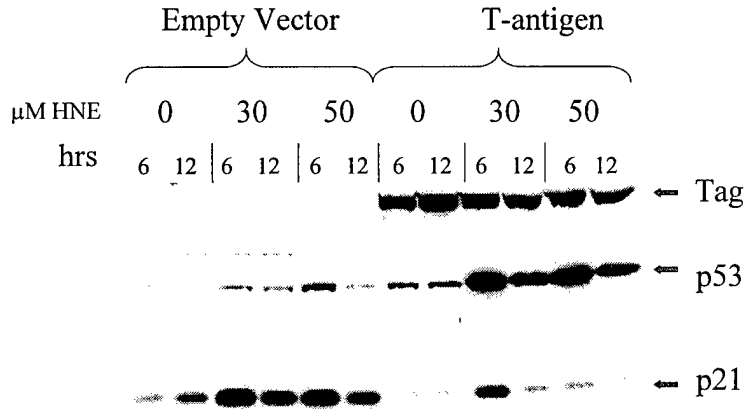
BCL2 - 14
p53 Stabilization
RAW 264.7
BCL2 - 14
Cytochrome C release
Total releasable

7.


RAW 264.7
BCL2-14
hrs
6
12
6
12
μM 0 25 50 75 25 50 75 0 25 50 75 25 50 75

8.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μM HNE
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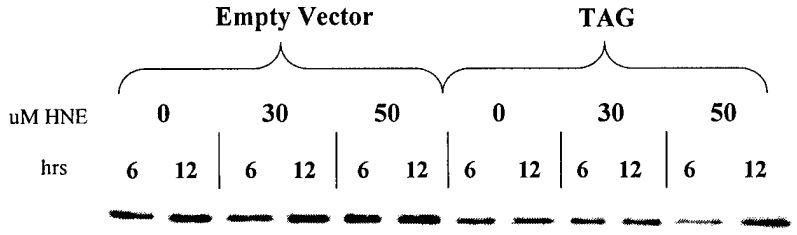
hrs
6 12 6 12 6 12 6 12 6 12 6 12
Tag
p53
p21

9.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uM HNE
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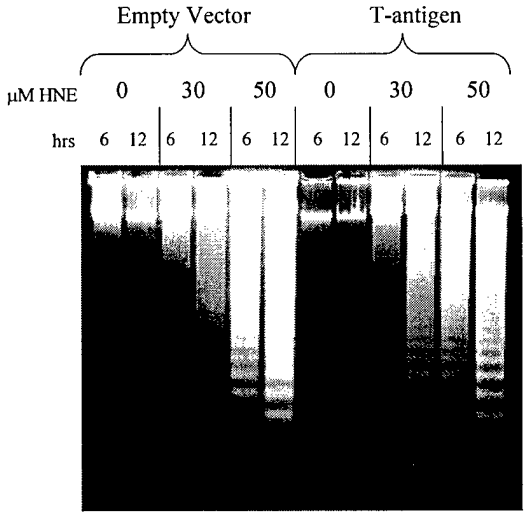
hrs
6 12 6 12 6 12 6 12 6 12 6 12

10.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T-antigen
μM HNE
0 30 50 0 30 50
hrs
6 12 6 12 6 12 6 12 6 12 6 12

Reportable Outcomes

Abstracts: (copies attached)

1. R. Haynes and A. Townsend . Lipid Peroxidation product 4-Hydroxy 2 Nonenal induces cytochrome c dependent, p53 independent apoptosis in murine macrophage line RAW 264.7. *American Association for Cancer Research,* Cell Death. Lake Tahoe, NV. Feb. 2000.
2. R. Haynes and A. Townsend. Structure-Activity and Mechanistic Studies on the Toxicity of 4-Hydroxy 2-Nonenal, byproduct of the peroxidation of Polyunsaturated Fatty Acids. *Dept. of Defense "Era of Hope" Breast Cancer Symposium.* Atlanta, Ga. June 2000.

Manuscripts: (copies of #1 attached)

1. Haynes, R.L Szweda, L, Pickin K, Welker M, Townsend A. Structure-Activity Relationships for Growth Inhibition and Induction of Apoptosis by 4- Hydroxy 2-nonenal in RAW 264.7 Cells. Submitted to *Molecular Pharmocology.* May 2000.
2. Haynes, R.L and Townsend A. Lipid Peroxidation product 4-Hydroxy 2 Nonenal induces cytochrome c dependent, p53 independent apoptosis in murine macrophage line RAW 264.7. In preparation.

Cell lines created:

1. Multiple clones of RAW 264.7 cells overexpressing SV40 Large T-antigen.

Structure-Activity Relationships for Growth Inhibition and Induction of Apoptosis by 4-Hydroxy 2-nonenal in Raw 264.7 Cells

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a) Running title : Structure requirements for hydroxynonenal-induced apoptosis.

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c)

# of text pages : 12

# of figures : 6

# of tables : none

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# of words in introduction : 532

# of words in discussion : 1,659

(note : total for intro + discussion = 2191, less than the 2250 total allowed)

d) Abbreviations used : HNE, 4-Hydroxy-2-nonenal; ALDH, aldehyde dehydrogenase; hALDH3, human aldehyde dehydrogenase 3; DMEM, Dulbecco's Minimal Essential Medium; PMSF, phenyl methyl sulfonyl fluoride; GSH, glutathione; BSA, bovine serum albumin; PBS, phosphate buffered saline

Abstract

4-Hydroxy-2-nonenal (HNE) is a highly reactive lipid aldehyde byproduct of the peroxidation of cellular membranes. The structure of HNE features three functional groups, a C1 aldehyde, a C2=C3 double bond, and a C4- hydroxyl group; each of which may contribute to the toxicity of the compound. In addition, the length of the aliphatic chain may influence toxic potency by altering lipophilicity. Using analogous compounds which lacked one or more of the structural moieties, the role of each of these structural motifs in the cytotoxicity of HNE was examined in a mouse alveolar macrophage cell line (RAW 264.7) by a cell survival and growth assay. The importance of these functional groups in the potency of HNE for induction of apoptosis was also examined. The rank order of effects on toxicity was C1–aldehyde $\geq$ C2=C3 double bond >> C4–hydroxyl, with parallel results in both the survival / growth inhibition and apoptosis induction assays. The chain length also influenced toxicity in a series of α,β–unsaturated alkenyl aldehydes, with increasing chain length yielding increasing toxicity. To confirm the importance of the aldehyde moiety, and to examine the role of metabolic detoxification in cellular defenses against HNE toxicity, a RAW 264.7 cell line overexpressing human aldehyde dehydrogenase-3 (hALDH3) was generated. This cell line exhibited nearly complete protection against HNE-protein adduct formation as well as HNE–induced apoptosis. These results illustrate the comparative significance of key structural features of HNE in relation to its potent toxicity and induction of apoptosis.

Oxidative stress occurs in biological systems when prooxidant species are not adequately detoxified by antioxidant defenses, resulting in the accumulation of chemically altered macromolecules that may compromise function or cause the demise of the cell. Proteins and particularly the polyunsaturated fatty acids comprising biological membranes are susceptible to oxidative damage. When fatty acids such as arachidonic acid interact with free radicals in the presence of molecular oxygen, a self propagating lipid peroxidation reaction may be initiated that results in the formation of reactive byproducts such as lipid hydroperoxides and aldehydes. The α,β–unsaturated aldehyde 4-hydroxy-2-nonenal is the most reactive and cytotoxic of the aldehyde byproducts of lipid peroxidation (Benedetti et al., 1980). The C3 position of HNE is a highly reactive site for Michael addition reactions with cellular thiols (Witz, 1989), and hence readily forms adducts with glutathione or protein thiols. The terminal aldehyde head group can react with the amino group of lysine or the imidazole nitrogen in histidine, albeit more slowly than the Michael addition at C3. The C4 hydroxyl group can undergo a subsequent cyclization with the C1 aldehyde of the C3–thioether Michael adduct to form a relatively stable thiohemiacetal ring (Esterbauer et al., 1991). HNE has been shown to cause a number of deleterious effects in cells including glutathione depletion (Cadenas et al., 1983), DNA and RNA synthesis inhibition (Poot et al., 1988), calcium homeostasis disturbances (Benedetti et al., 1984), inhibition of mitochondrial respiration (Humphries et al., 1998), and morphological changes (Gadoni et al., 1993). HNE-induced protein damage has been associated with several pathological conditions such as ischemia–reperfusion injury (Siems et al., 1995), atherosclerosis (Yla-Herttuala et al., 1989), alcoholic liver disease (Li et al., 1997), Alzheimer's disease (Montine et al., 1997), and cellular aging (Lucas and Szweda, 1998).

Recently HNE has been shown to induce apoptosis in certain cell lines (Kruman et al., 1997; Li et al., 1996; Yildiz et al., 1996) suggesting a possible connection between oxidant stress–generated lipid peroxidation byproducts and oxidant stress–induced apoptosis. This observation presents questions regarding the mechanism of HNE initiation of apoptosis and the relative contributions of HNE structural components to the potency of induction of apoptosis. Unique structural features of HNE include the presence of two structural domains, a lipophilic tail and a polar head comprised of several functional groups. The polar head contains an aldehyde at the C1 position, a double bond between C2 and C3, and a

hydroxyl group at the C-4 position. These groups may participate independently or cooperatively to interact with cellular molecules. One way to evaluate the importance of each moiety in the toxicity of HNE is to compare the effects of HNE to analogous compounds which either vary in fatty acid chain length or lack a specific functional group. For example, compounds such as trans-2-hexenal, trans-2-octenal, and trans-2-nonenal lack the 4-hydroxyl group, and also vary in fatty acid chain length. Compounds such as nonanal and nonenoic acid lack the C2=C3 double bond or the C-1 aldehyde respectively, in addition to loss of the 4-OH group. The experiments described herein were designed to assess the contribution of HNE structural components to the toxicity of HNE and particularly to their ability to induce apoptosis.

Methods

Cell Culture and Reagents. Mouse alveolar macrophage RAW 264.7 cells were grown at 37°C in a 5% CO_2 atmosphere in DMEM medium (GIBCO Grand Island, NY) supplemented with 10% fetal bovine serum. 4-Hydroxynonenal was kindly provided by the lab of Dr. Herman Esterbauer (University of Graz, Austria), or purchased from Cayman Chemical (Ann Arbor, Michigan). Analogous aldehydes trans-2-hexenal, trans-2-octenal, trans-2-nonenal, and nonanal were purchased from Aldrich (Milwaukee, WI) and nonenoic acid was purchased from TCI (Portland, OR). Synthesis of 4-hydroxynonanal was via reduction of γ-nonanoic lactone by diisobutylaluminum hydride in toluene (Bloch and Gilbert, 1987). The product was purified by silica chromatography, solvent removed, and the oil characterized by NMR at 25°C in 1:1 CD_3OD:D_2O.

Growth Inhibition/ Cell Survival. 1.2×10^6 cells were treated in suspension in 5 ml PBS plus chemical agent for 30 minutes at 37°C. Cells were pelleted by centrifugation (1000 rpm for 5 minutes) and resuspended in DMEM + 10% fetal bovine serum. 6×10^5 cells were plated in 6-well dishes and allowed to grow for two days at which time cells were released by exposure to trypsin/EDTA and counted.

DNA Fragmentation Assay. Cells were plated at 2×10^6 cells per 60 mm petri dish. After 16 - 20 hours cells were rinsed and treated with agents in serum-free DMEM. After a one hour exposure, medium was removed and replaced with DMEM + 10% fetal bovine serum. Cells were allowed to incubate for an additional 9 hours at which time they were harvested in phosphate buffered saline (pH 7.4), and centrifuged at 4°C, 1000 RPM for 5 minutes. Cells were then lysed in 20 mM EDTA, 100 mM Tris (pH 8.0), 0.8% sodium lauryl sarcosine and subjected to RNase treatment (0.5mg/ml for 1 hour at 37°C) followed by proteinase K treatment (5 mg/ml for 6-12 hours at 55°C). Nonfragmented chromosomal DNA was removed by filtering the lysate through 0.45 μm syringe filters pretreated with .2 mg/ml bovine serum albumin (BSA). Fragmented DNA was then precipitated with 1/10 volume 3M sodium acetate (pH 5.2) and 2.5 volumes 100% ethanol. Redissolved DNA was electrophoresed on a 1.8% agarose gel, then stained with

ethidium bromide and DNA fluorescence was recorded using a video imaging workstation (Alpha Innotech (San Leandro, CA).

Transfection of hALDH3. The cDNA for human class 3 ALDH was previously cloned by polymerase chain reaction amplification from human stomach cDNA and subcloned into the XhoI site of the ΔpCEP4Δ mammalian expression vector, a derivative of the pCEP4 vector (Invitrogen Carlsbad, Ca.) modified to prevent episomal replication and favor host cell integration (Bunting et al., 1994). Both the ΔpCEP4Δ/hALDH3 vector and ΔpCEP4Δ (empty vector) were introduced into RAW 264.7 cells using the cationic liposome reagent Escort (Sigma St. Louis, MO). Briefly, cells were plated in 100 mm petri dishes and grown to 70-80% confluency. Escort (30-50 μL) was incubated with DNA (15-25 μg) in 800 μL of Opti-MEM transfection medium (GIBCO Grand Island, NY) for 15 minutes. Opti-MEM was added to Escort/DNA mixture to a total volume of 8 mL. Cells were then allowed to incubate in transfection medium for 6 hours. 24 hours later cells were subcultured and selection medium (DMEM + 10% FBS) was added together with 0.7 mg/ml hygromycin. After 9 – 12 days, hygromycin–resistant colonies were cloned and expanded for ALDH screening.

Analysis of ALDH Expression. Enzyme activity assays were performed using crude cytosol as previously described (Bunting et al., 1994) with 1 mM benzaldehyde as a substrate and 1 mM NAD+ as a cofactor. The product of HNE modification by hALDH3 was analyzed by electrospray mass spectrometry following incubation of purified hALDH3 with a similar reaction mixture containing 100 μM HNE as substrate and 200 μM NAD+ as oxidant cofactor. The reaction was followed by the change in absorbance at 340 nm, and was essentially complete after 3 minutes. Whereas the blank (no enzyme) reaction mix had only unreacted HNE (m/z = 154.98), the carboxyl product was almost all 4-hydroxynonenoic acid (m/z = 171.04). For hALDH3 protein detection, 50 μg total protein was electrophoresed on a 10% SDS-PAGE gel and transferred to nitrocellulose. The nitrocellulose was probed with a 1:3000 dilution of a rabbit anti-rat class 3 ALDH antisera (kindly provided by Dr. Ronald Lindahl, Univ. of South Dakota) that was cross-reactive with human ALDH-3. After probing with HRP–conjugated goat anti-rabbit secondary antibody (BioRad., Hercules, CA), protein was detected by chemiluminescence (NEN Life Science Products, Boston, MA).

Glutathione assay. Control or HNE-treated cells were placed on ice, pelleted by low-speed centrifugation, and washed with phosphate-buffered saline + 5 mM EDTA. Intracellular GSH content was assayed by the glutathione disulfide reductase method (Tietze, 1969). The assay buffer (0.1 M KPO_4, 1 mM EDTA, pH 7.5) included NADPH (0.4 mM), glutathione disulfide reductase (0.8 units), and 5,5'-dithiobis(2-nitrobenzoate) (0.44 mg/ml). Samples of 1 x 10^6 cells were lysed in 2% sulfosalicylic acid on ice for 5 minutes, and centrifuged 12,000 x g for 10 minutes at 4°. Aliquots of the supernatant were assayed by determining the change in absorbance at 412 nm over a 2 minute reaction. A standard curve for each assay was used to calculate the nmol of GSH per reaction.

HNE Protein Adduct Detection. Cells were plated at 2.5 x 10^6 cells / 60mm dish and 16 - 20 hours later cells were exposed to agents for 1 hour in serum-free medium. FBS was added to 10% at 1 hour and cells were allowed to incubate for an additional hour. Cells were harvested in PBS, centrifuged, and the pellets lysed in 50 mM Tris, 5 mM EDTA, and 1 mM PMSF. Lysates were centrifuged at 14,000 x g for 10 minutes at 4°C and protein (50μg/lane) was run on a 10% SDS-PAGE gel and transferred by semi-dry electrophoresis to nitrocellulose. Adducts were detected using an anti–HNE/protein adduct antibody (Cohn et al., 1996) at a dilution of 1:2500. After probing with goat anti–rabbit, horseradish peroxidase–conjugated secondary antibody (BioRAD, Hercules, CA.) (1:3000) the protein was detected using Renaissance chemiluminescence reagent (NEN Life Science Products Boston, MA).

Results

Cytotoxicity The contributions of the separate domains or functional groups of HNE to the inhibition of cell survival and growth were assessed using different congeners analogous to HNE but differing in one or more functional group. Cytotoxicity data for HNE, trans-2 nonenal (lacks the OH), nonanal (lacks the C2=C3 double bond), and nonenoic acid (lacks the aldehyde) yielded IC_{50} values of 9.0 $\pm$ 1.1 μM, 24 $\pm$ 4.3 μM, 308 $\pm$ 34.9 μM, and 1770 $\pm$ 342 μM respectively (Figure 1A). The IC_{50} values for HNE and trans-2-nonenal differed by 2.7-fold, reflecting a moderate but significant ($P < .001$) contribution of the hydroxyl group to the toxicity of HNE. Although 4-hydroxynonanal was successfully synthesized via reduction of γ-nonanoic lactone, the compound existed primarily (>98%) in the ring-closed hemiacetal form, and was non-toxic up to 2 mM (data not shown). Hence, in the presence of a 4-hydroxyl group, it was not possible to evaluate the mostly blocked aldehyde in the saturated alkanal, whereas the trans-double bond prevents this cyclization in the α,β-unsaturated aldehydes. The difference between the toxicity of trans-2-nonenal and its saturated analog nonanal was 13-fold ($p < .0001$), while substitution of a carboxyl for the aldehyde group resulted in IC_{50} values 5.7-fold higher than with nonanal (p , .0001), and 74-fold higher than with trans-2-nonenal ($p < .0001$). The effect of the lipophilicity on growth inhibition was examined by exposing cells to analogous α,β–unsaturated aldehydes of different alkenyl chain lengths (Figure 1B). These experiments showed increased toxicity with increased chain length as shown by IC_{50} values of 99 $\pm$ 20 μM, 30 $\pm$ 5 μM, and 24 $\pm$ 4.4 μM for trans-2 hexenal, trans-2 octenal, and trans-2 nonenal respectively.

Induction of Apoptosis Increasing concentrations of HNE were added to culture medium in order to determine the sensitivity of RAW 264.7 cells to induction of apoptosis, and cellular internucleosomal DNA fragmentation was monitored as an index of apoptosis. This HNE dose–response experiment showed the internucleosomal DNA fragmentation characteristic of apoptosis at HNE concentrations as low as 30 μM (Figure 2). This response is relatively rapid, occurring as early as 8 hours after a 1 hour exposure to HNE. As the HNE concentration was increased, the amount of fragmentation increased, indicating a greater fraction of cells undergoing apoptosis. In other cell lines HNE has been shown to rapidly deplete cellular

glutathione (GSH), a condition that could trigger apoptosis as a result of oxidative stress due to loss of the reducing potential of GSH. In the RAW 264.7 cell line used in these experiments we have found that exposure to 25, 50 and 75μM HNE resulted in only moderate depletion of total cellular GSH, to 84.0 +/- 6.8, 71.7 +/- 5.2, and 68.8 +/- 4.5 percent of GSH levels in control cells, respectively

Activation of the proapoptotic protease caspase 3 was also examined, as a biochemical index of apoptotic response to HNE exposure. Caspase activities of 0.31 nmole/min/mg, 0.64 nmole/min/mg, and 0.79 nmole/min/mg were measured following exposure to 0 μM, 40 μM, and 70 μM HNE respectively (not shown). This experiment confirms the dose–dependent nature of the response and also documents a second positive apoptotic endpoint in support of the conclusion that cells are dying by apoptotic rather than necrotic death. In addition, we have used time lapse video microscopy to show that at 50 μM HNE greater than 90% of the cells have formed apoptotic blebs, blistered, and lysed within 18 hours after exposure to HNE (R. Haynes and M. Willingham, unpublished observations). These observations confirm that apoptosis is the primary mode of cell death in RAW 264.7 cells following exposure to HNE.

Structure–Activity Correlation with Induction of Apoptosis. The growth inhibition studies provided an index of the relative overall toxicity of each of the compounds and the effect of modification of specific functional groups. A parallel series of experiments was performed to determine whether this relationship is explained by similar effects of these structurally distinct analogs on the degree of induction of apoptosis in comparison with HNE. To determine the extent to which the various functional groups influence this apoptotic induction, a dose–response experiment was carried out using the same compounds as in Figure 1A, but with DNA fragmentation as an index of apoptosis. As the HNE exposure was increased there was a progressive increase in apoptosis induction over the range of 25 μM to 75 μM (Figure 3). Trans–2–nonenal yields a dose–response relationship similar to that of HNE, confirming the similar toxicity of these two 9-carbon α,β–unsaturated aldehydes, with only modest loss of apoptotic efficacy in the absence of the hydroxyl group. Neither nonanal nor nonenoic acid induced any apoptotic DNA fragmentation within the concentration range tested (25 - 75μM). However, as shown in Fig. 1A, these concentrations may not be toxic enough to induce significant amounts of apoptosis. Indeed, cells treated

with nonanal or nonenoic acid concentrations in the IC_{50} to IC_{90} range exhibited significant DNA fragmentation (not shown), indicating an apoptotic mode of cell death with these compounds as well. The role of hydrophobicity in HNE-induced apoptosis was also examined for α,β–unsaturated aldehydes of different chain lengths, with DNA fragmentation as an endpoint. Trans-2-hexenal yielded very little apoptosis induction in the concentration range tested. Increasing the length of the chain by 2 carbons (trans-2-octenal) resulted in a significant increase in DNA fragmentation, and addition of a ninth carbon (trans-2-nonenal) further enhanced the apoptotic induction (Figure 4). These results parallel the growth inhibition data seen in figure 2B, with increased apoptotic induction in parallel with increasing chain length in the order trans-2-hexenal < trans-2-octenal < trans-2-nonenal.

The nonenoic acid used to examine the effect of loss of the aldehyde group also lacked a 4-hydroxyl group and hence is not strictly analogous to HNE as a monofunctionally modified congener. Hence the role of the aldehyde in the toxicity of HNE was also examined by an indirect approach. We have previously found that expression of a human class 3 aldehyde dehydrogenase conferred strong protection against HNE toxicity when expressed via stable transfection in V79 hamster lung fibroblast cells[1]. We also showed that crude cytosol from these cells supported oxidation of NAD+ with HNE as substrate, and that purified hALDH3 catalyzed a facile and essentially complete NAD-dependent oxidation of HNE to 4-hydroxynonenoic acid, as verifed by mass spectrometry[1]. The same expression vector was used to express hALDH3 by stable transfection in RAW 264.7 cells, as shown in Figure 5 by comparison of the clone hALDH3-109 to the empty vector-transfected control ΔpCEP4Δ-16. Activity assays yielded an ALDH activity of 100 ± 4 mU/mg in clone 109, compared to undetectable activity in the control line. When control and hALDH3–transfected cells were exposed to HNE, expression of hALDH3 protected against apoptosis induction throughout the concentration range tested (Figure 6A). The protection provided by hALDH3 expression was further characterized by measurement of HNE–protein adducts formed in each cell line. The HNE–protein adducts were detected by western blotting with an antibody specific for the products of reactions between HNE and protein thiols, amino groups, and histidine residues (Uchida et al., 1993). Figure 6B shows the dose–dependent nature of HNE- protein adduct formation in the empty vector-

transfected ΔpCEP4Δ-16 control line. Cells overexpressing hALDH3 were essentially completely protected as seen by extremely low levels of adduct formation throughout the concentration range tested. This protection is consistent with the demonstrated conversion of HNE to the carboxylic acid congener by hALDH3, in light of the earlier toxicity and apoptosis experiments that indicated far less toxicity with nonenal than nonenoic acid in the series of 9-carbon compounds tested.

Discussion

The chemical reactions with macromolecules such as protein and DNA that underlie the biological effects of many of the major lipid peroxidation products have been well-characterized (Esterbauer, 1993; Esterbauer et al., 1991; Witz, 1989). The α,β–unsaturated aldehydes such as HNE react with a range of macromolecules, but to widely variable extents depending on the reaction chemistry of the interacting functional groups and microenvironmental factors (e.g. accessibility, hydration, pH, proximity of other functional groups) (Esterbauer et al., 1991; Witz, 1989). The β-carbon (C3) and the carbonyl center (C1) readily undergo nucleophilic addition of thiols, and amino groups can also form adducts at the C1 or C3 carbon atoms via Schiff base or Michael addition reactions, respectively (Esterbauer et al., 1991; Witz, 1989). The interaction of HNE with proteins is complex due to the multiple reactive groups comprising the polar head of HNE, which allows for crosslinks between functional groups such as thiols, amino groups, and histidine residues. Structure-activity comparisons with compounds related to HNE have been used previously to assess the contribution of individual functional groups to the biological effects of HNE, with somewhat variable results, depending on the toxic endpoint examined (Kaneko et al., 1988; Hauptlorenz et al., 1985; Brambilla et al., 1986). Our present studies have focused on the structural contributions to induction of apoptosis, in comparison to the effects of these structural determinants on survival and subsequent growth in a murine macrophage cell line.

The cytotoxicity assay demonstrated that substitution of a carboxyl group for the aldehyde caused the greatest decrease in toxicity among the structural analogs studied. This is shown by the dramatic decrease in toxicity when cells are exposed to nonenoic acid which lacks the aldehyde yet retains the double bond (IC_{50} of 1770 μM) as compared to trans-2-nonenal (IC_{50} of 24 μM). Consistent with this observation, no apoptosis as evidenced by DNA fragmentation was induced by nonenoic acid up to 75 μM, which resulted in more than 90% apoptotic cells with HNE or trans-2-nonenal. Although a significant fraction of cells became apoptotic at millimolar concentrations near the IC_{50} (not shown), this could be due to nonspecific detergent-like effects of nonenoic acid on the integrity of the plasma membrane. Alternatively, the RAW 264.7 cell line may have a propensity toward the apoptotic mode of cell death. However, induction of apoptosis by HNE also occurs in several other cell types, including alveolar macrophages (Li et al., 1996),

neuronal cells (Kruman et al., 1997), and endothelial cells (Herbst et al., 1999), suggesting that a specific mechanism of apoptosis induction may be activated.

Two potentially interrelated factors could explain the major loss of potency in the absence of the aldehyde. First, a common effect of exposure of cells to HNE is facile alkylation of protein thiols, and also nonprotein thiols such as glutathione (Cadenas et al., 1983; Esterbauer et al., 1991; Witz, 1989). In the Michael addition reaction, the electron-withdrawing effect of the adjacent aldehyde facilitates the addition of a nucleophilic thiol or amino group across the C2=C3 double bond. Substitution of the more electron-rich carboxyl for the C1-aldehyde greatly reduces the reactivity of the double bond, resulting in decreased Michael addition reaction. Second, the reduced toxicity may in part reflect loss of the ability to form crosslinks, since the aldehyde is no longer available as a second site of adduction, leaving only the greatly weakened addition site at the double bond remaining. In the case of HNE, loss of the C1 aldehyde also prevents the intramolecular cyclization that can occur between the C1 carbon and the C4 hydroxyl following Michael addition of a thiol. This structure likely stabilizes the thioether linkage of the adduct.

The importance of the C2=C3 double bond was illustrated by exposure to nonanal, a 9-carbon alkyl analog which retains the aldehyde but lacks the double bond and C4 hydroxyl. The toxicity (IC_{50} of 308 μM) with the aldehyde alone was intermediate between trans-2-nonenal (IC_{50} of 24 μM) and nonenoic acid (IC_{50} of 1770 μM). Thus, while the loss of the aldehyde in nonenoic acid resulted in a 74-fold decrease in toxicity compared to trans-2-nonenal, the lack of a double bond in nonanal decreased toxicity by 13-fold relative to trans-2-nonenal. Again, similar results were observed for induction of apoptosis, with no internucleosomal DNA fragmentation at concentrations up to 75 μM nonanal or nonenoic acid, but significant apoptosis in the IC_{50} range (not shown). The loss of the double bond prevents Michael additions at the C3 position, and also removes its potentiation of the reactivity of the aldehyde. Saturated aldehydes can interact with proteins to form Schiff base adducts, but these reactions occur more slowly and are more readily reversible than the Michael additions, hence the intermediate toxicity of nonanal. The results with nonenoic acid, trans-2-nonenal, and nonanal indicated that both the aldehyde and C2=C3 double bond are essential for the full toxicity of HNE and induction of apoptosis, and also that their effects are additive and mutually interactive.

A third reactive group, the 4-hydroxyl, apparently contributes somewhat less to the toxicity of HNE, as evidenced by the 2.7-fold decrease in the toxicity of trans-2-nonenal as compared to HNE (IC_{50} of 24 μM vs. 9 μM for HNE) and the parallel difference for induction of apoptotic DNA fragmentation for these two analogs. Previously published results showed only a slight difference for inhibition of human umbilical vein endothelial cell growth by trans-2-nonenal and HNE (Kaneko et al., 1988), and about 2-fold greater toxicity of HNE when survival of human diploid fibroblasts was the measured toxicity end-point (Kaneko et al., 1987). In the mechanism of the HNE reaction with thiols, the 4-hydroxyl group contributes to the reactivity of the Michael addition site by acting as an electron-withdrawing group to increase the reactivity of the double bond for Michael additions at C3. Secondly, it may stabilize the resulting adduct by participating in an intramolecular cyclization with the aldehyde to yield a cyclic hemiacetal product that is in tautomeric equilibrium with the open chain aldehyde. With trans-2-nonenal the final product is a linear adduct at the C3 position, and this may be less stable and more readily reversible, and hence less toxic than HNE. The effect of the 4-hydroxyl on the toxicity of the saturated 4-hydroxynonanal could not be evaluated due to cyclization of the chemically synthesized compound, but it would be expected to have minimal effect anyway, since there is no adjacent double bond to be influenced by its electron-withdrawing effect, and the aldehyde is separated by two saturated carbon atoms.

The importance of the length of the alkenyl chain was apparent from the decreasing IC_{50} values in the toxicity assay as the length increased from 6 to 9 carbons, and the parallel effects on induction of apoptosis. Hydrophobicity constants for each of the lipid aldehydes used in this study are 0.85 for trans-2-hexenal, 1.89 for trans-2 octenal, 2.30 for trans-2-nonenal, and 1.01 for HNE (Bounds and Winston, 1991). Thus, while the alkenals are progressively correlated with hydrophobicity, the lack of correlation with HNE indicates that this is a contributory, rather than a causative component of toxicity.

The observation that greater than 90% of cells treated with 75 μM HNE ultimately undergo apoptosis is consistent with the parallel results of the cytotoxicity and apoptosis studies, with entirely analogous structure-activity relationships for the potency of the apoptotic induction. The concentration of HNE required to induce apoptosis was severalfold higher than the IC_{50} for the survival and growth assay, most likely due to a difference in the exposure conditions. The cells were at a higher density for the apoptosis

experiments, a variable that is known to affect the absolute toxicity of α,β-unsaturated aldehydes (Esterbauer et al., 1991; Norton et al., 1997). Another factor is that the cells were already attached in a monolayer for the apoptosis assay, with less surface area available and the potentially protective advantage of cell-cell interactions, while the exposure for the cytotoxicity assay was in suspension. Measured overall concentrations of HNE in cell cytosol of non-stressed cells or tissues are typically in the low micromolar range (Esterbauer et al., 1991). However, significantly higher concentrations were found in human monocytes, which generate large amounts of reactive oxygen species, and it is has been estimated that localized HNE concentrations can increase to as high as 4.5 mM within peroxidizing membrane bilayers (Esterbauer et al., 1991). Thus, mitochondrial membranes may accumulate high HNE concentrations due to the proximity to reactive oxygen species released during normal oxidative energy metabolism. During oxidant stress, key mitochondrial proteins such as cytochrome c oxidase and the adenine nucleotide transporter have been shown to be alkylated by HNE to a greater extent (Chen et al., 1998; Chen et al., 1995). Changes in mitochondrial function have also been associated with exposure to lipid peroxidation products including HNE in mitochondria (Humphries et al., 1998; Keller et al., 1997; Richter and Meier, 1990; Ullrich et al., 1996), and HNE has been shown to induce the mitochondrial permeability transition that is believed to be an irreversible event in the induction of apoptosis (Kristal et al., 1996; Marchetti et al., 1996a; Marchetti et al., 1996b).

Exposures to the toxic compounds used in this study were via addition to the extracellular medium, and thus it was of interest to verify that the aldehyde group exerted it's toxicity intracellularly, rather than at the plasma membrane. Stable expression of hALDH3, an HNE oxidizing enzyme, via transfection into RAW 264.7 cells completely protected the cells from apoptotic induction to at least 70 μM HNE, as compared to the nonexpressing control cells. This confirms that the principal targets for the toxic effects are intracellular, since ALDH-3 is cytosolic and would not be expected to protect surface membrane components or functions from extracellular HNE. Analysis of HNE-protein adduct formation showed potent protection against protein damage by hALDH3 expression, presumably due to oxidation of the aldehyde to the far less reactive carboxylic acid. This observation suggests that protein modification likely plays a direct causative role as a trigger mechanism for the apoptotic induction. Modification of key thiol groups in mitochondrial proteins

such as the permeability transition pore has been proposed as a trigger mechanism that initiates the role of this organelle in apoptosis (Costantini et al., 1996; Petronilli et al., 1994; Zamzami et al., 1998). Studies are currently in progress to investigate the role of acute damage to mitochondria in the mechanism of HNE induction of apoptosis.

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[1] Bunting, K.D., Haynes, R.L., Szweda, L, Jerome, W.G., and Townsend, A.J., unpublished results.

Figure Legends

Figure 1. *Panel A, Growth inhibition/ cell survival to determine the importance of individual functional groups.* Cells were dosed with analogous compounds for 30 minutes as described in Materials and Methods and allowed to grow for 2 days at which time they were trypsinized and counted. Analogous compounds used include HNE (●); trans 2- nonenal (○) (lacks the hydroxyl); nonanal (▲) (lacks the C2=C3 double bond and hydroxyl); and nonenoic acid (Δ) (lacks the C1 aldehyde and hydroxyl. IC_{50} values for each are 9.0 ± 1.1 μM, 24 ± 4.3 μM, 308 ± 34.9 μM, and 1770 ± 342 μM for HNE, trans 2-nonenal, nonanal, and nonenoic acid respectively. The results are mean ± standard error for four to seven separate experiments. *Panel B, Growth inhibition/ cell survival to determine the importance of chain length.* Cells were dosed for 30 minutes as described in Materials and Methods with analogous aldehydes trans-2 nonenal (○) (9 carbons), trans-2 octenal (■) (8 carbons), and trans-2 hexenal (❐) (6 carbons); the dashed line is the HNE data from Fig. 1A, for comparison. IC_{50} values for each are 24 ± 4.3μM, 30 ± 5μM, and 99 ± 20μM for trans-2 nonenal, trans-2 octenal, and trans-2 hexenal respectively. The results are mean ± standard error for five separate experiments.

Figure 2. *Internucleosomal DNA fragmentation induced by HNE.* RAW 264.7 cells were treated with increasing concentrations of HNE for 1 hour in serum-free medium and then medium was replaced with fresh complete medium without HNE. Nine hours after removal of HNE, cells were harvested and DNA was isolated, electrophoresed, and stained with ethidium bromide as described in Material and Methods.

Figure 3. *Internucleosomal DNA fragmentation induced by 9-carbon analogs of HNE.* Cells were exposed for 1 hour in serum-free medium containing the indicated concentrations of one of the following compounds: nonenoic acid (lacks the C1 aldehyde and hydroxyl), nonanal (lacks the C2=C3 double bond and hydroxyl), trans-2 nonenal (lacks the hydroxyl), or HNE. Cells were harvested and DNA was isolated, electrophoresed, and stained with ethidium bromide as described in Material and Methods.

Figure 4. *Internucleosomal DNA fragmentation induced by lipid aldehydes of increasing chain length.* Cells were exposed for 1 hour in serum-free medium containing the indicated concentrations of one of the following lipid aldehydes : trans-2 hexenal (6 carbon), trans-2 octenal (8 carbon), or trans-2 nonenal (9 carbon). Cells were harvested nine hours after removal of HNE and DNA was isolated, electrophoresed, and stained with ethidium bromide as described in Material and Methods.

Figure 5. Expression of human class 3 aldehyde dehydrogenase. Cytosolic protein (50 μg/lane) from control RAW 264.7 cells transfected with a ΔpCEP4Δ control (empty) vector (lane 2) or cells transfected with a ΔpCEP4Δ vector containing human ALDH3 cDNA (lane 3) was run on a 10% SDS-PAGE gel and transferred to nitrocellulose and probed with 1:3000 dilution of tALDH antisera. Enzyme activity was undetectable in control cells and 100 +/- 4 mU/mg in the ALDH-3 expressing clonal line. A V79 cell line overexpressing hALDH3 (Bunting and Townsend, 1996) was used as a positive control (lane 1). The upper band is a nonspecific crossreacting protein present in RAW 264.7 cells.

Figure 6. *Panel A, ALDH protection against HNE–induced DNA fragmentation.* Control empty vector-transfected RAW 264.7 cells or cells overexpressing hALDH3 were exposed to increasing concentrations of HNE for 1 hour in serum-free medium. Cells were harvested nine hours after removal of HNE, and DNA was isolated, electrophoresed, and stained with ethidium bromide as described in Material and Methods.
Panel B, ALDH protection against HNE–induced protein adduct formation. Control empty vector-transfected RAW 264.7 cells or cells overexpressing hALDH3 were exposed to increasing concentrations of HNE for 1 hour in serum-free medium. Cells were lysed and cytosolic protein (50 μg/lane) was electrophoresed on a 10% SDS-PAGE gel and transferred to nitrocellulose. Protein adducts were probed using an antibody specific for HNE adducts, diluted 1:2500, as described in Materials and Methods.

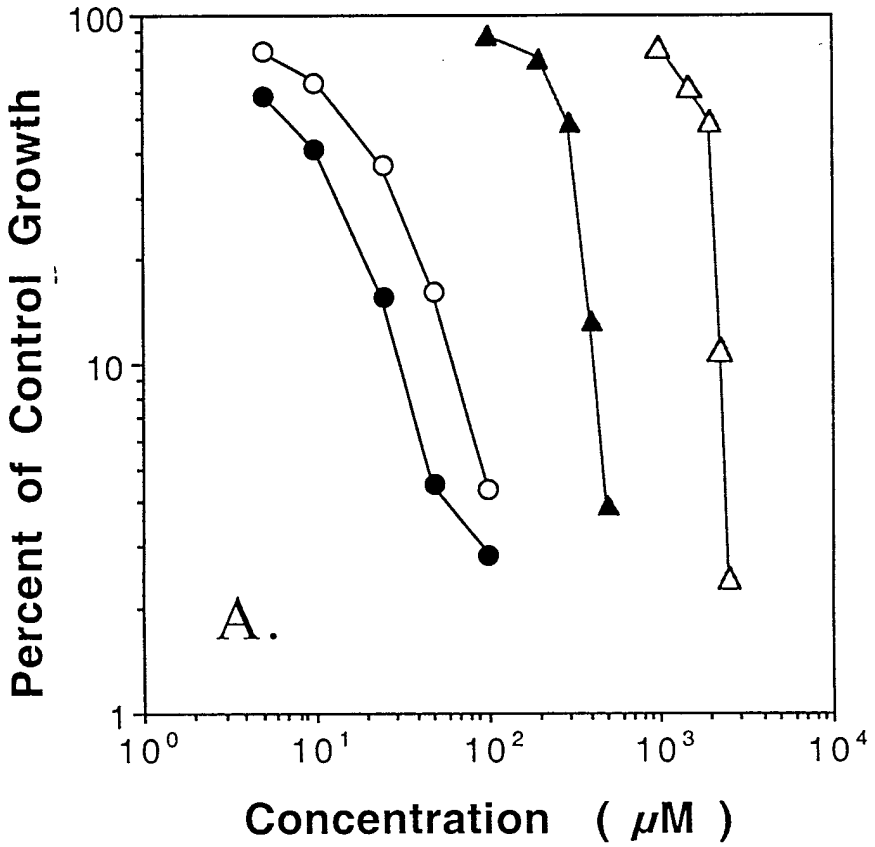

Figure 1A

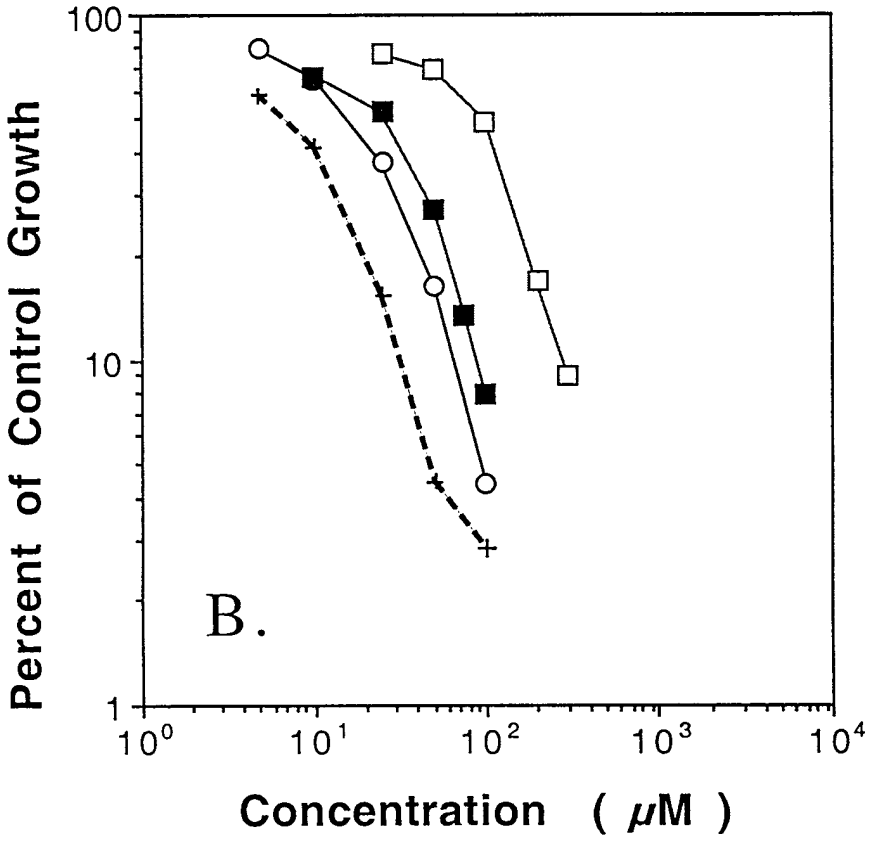

Figure 1B

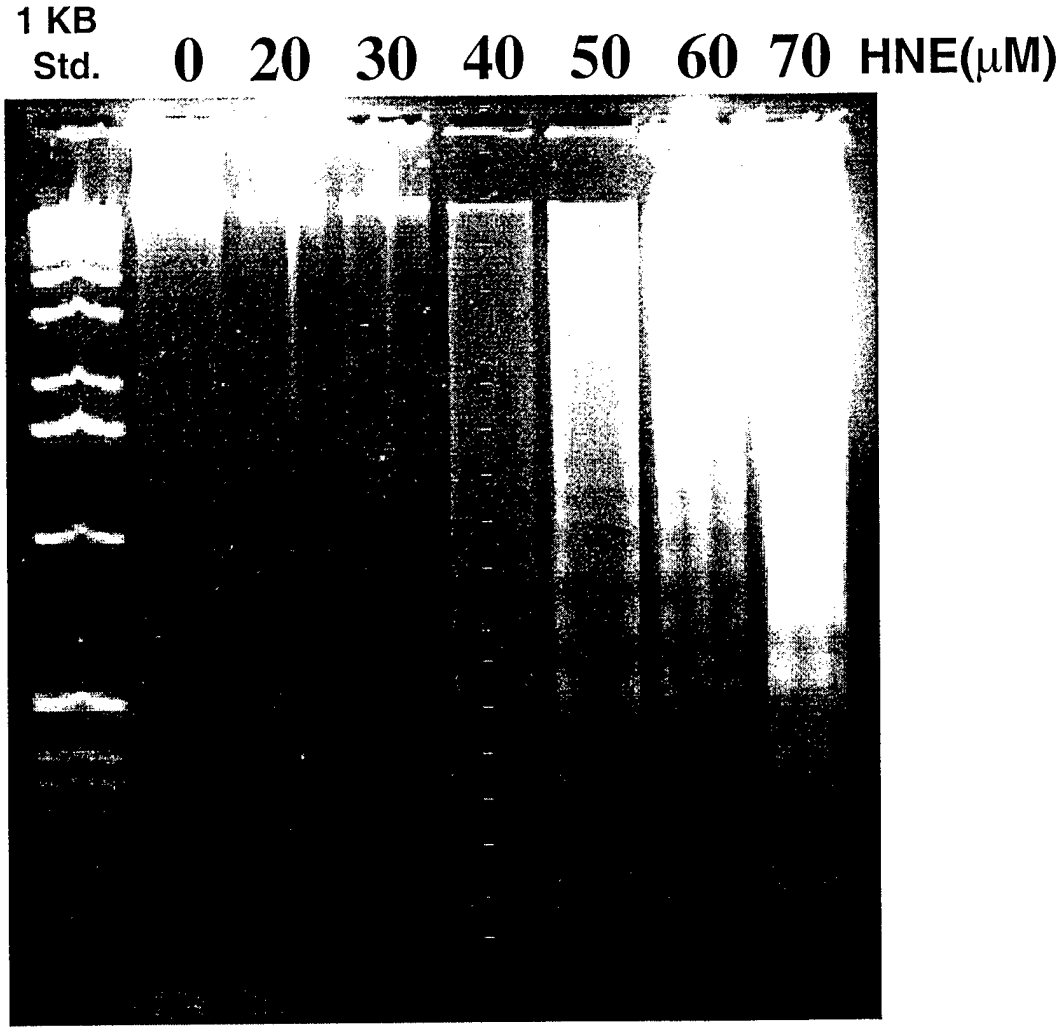

FIGURE 2

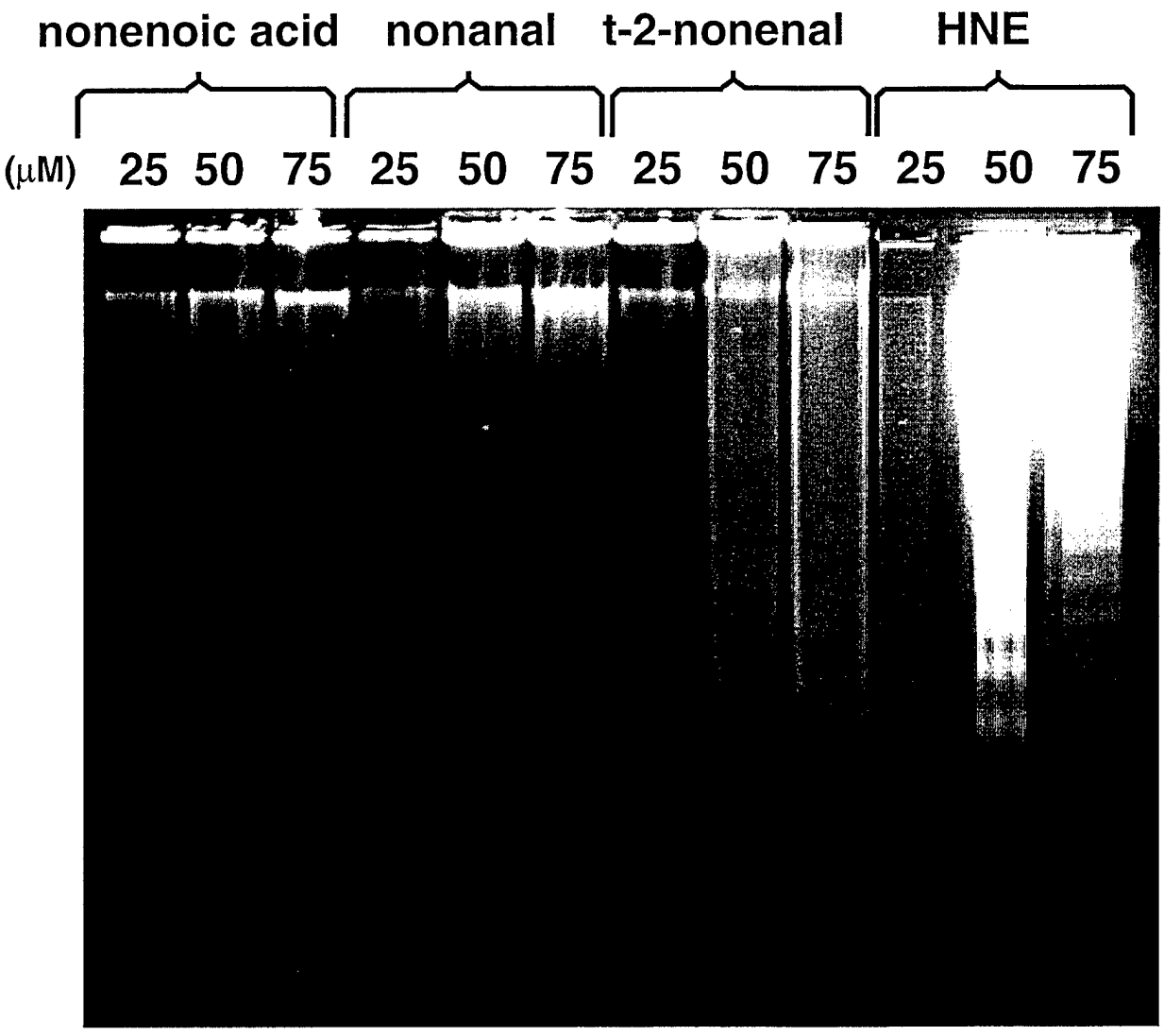
nonenoic acid
nonanal
t-2-nonenal
HNE
(μM) 25 50 75 25 50 75 25 50 75 25 50 75

FIGURE 3

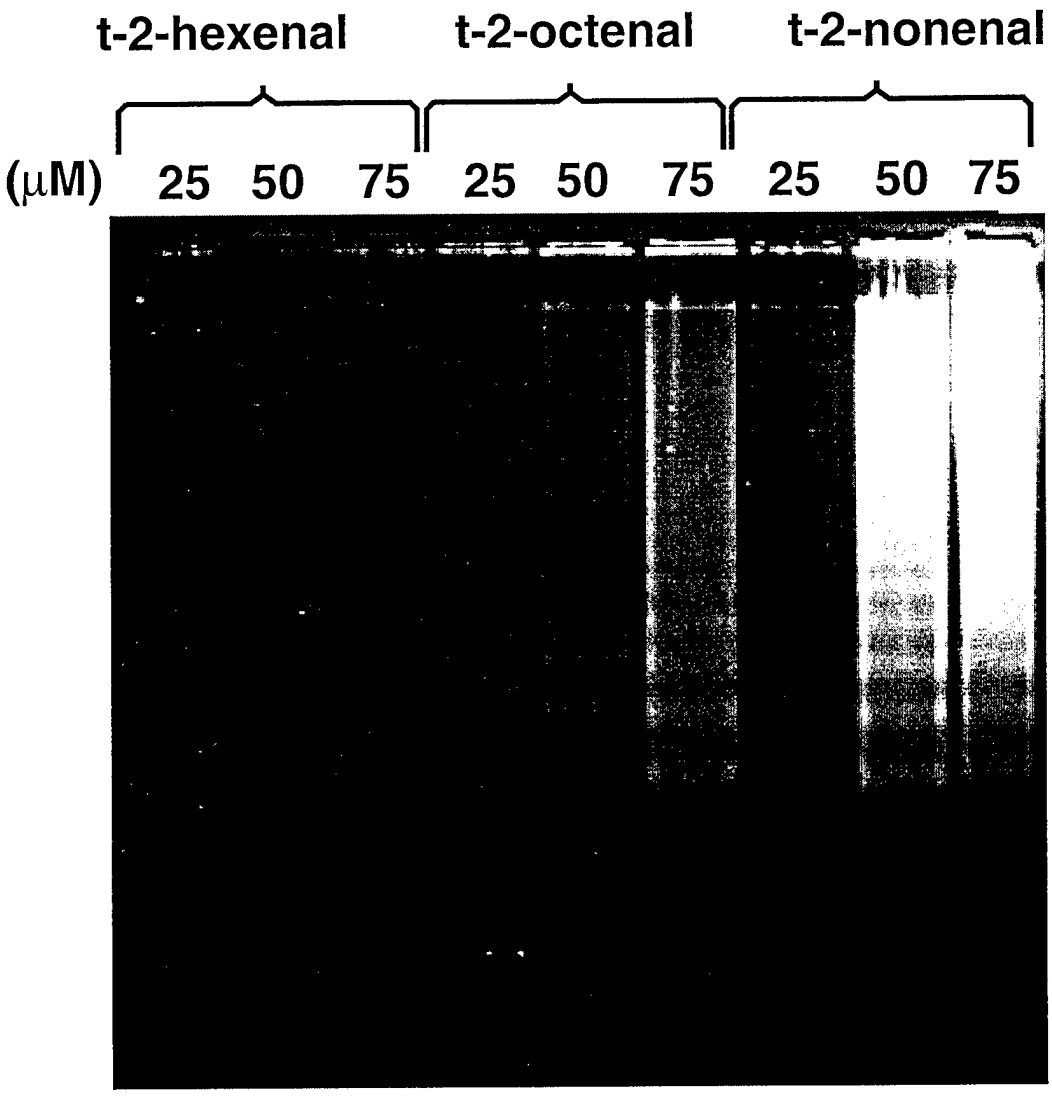

FIGURE 4

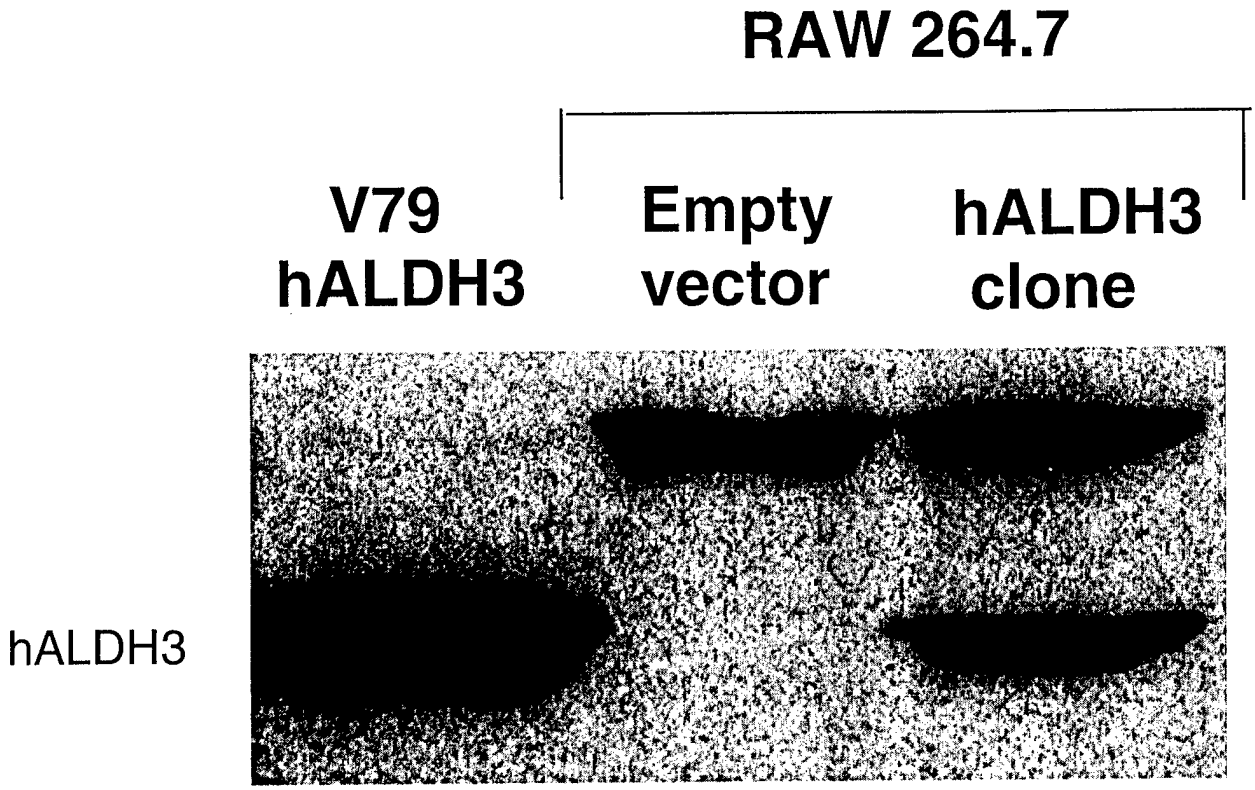

FIGURE 5

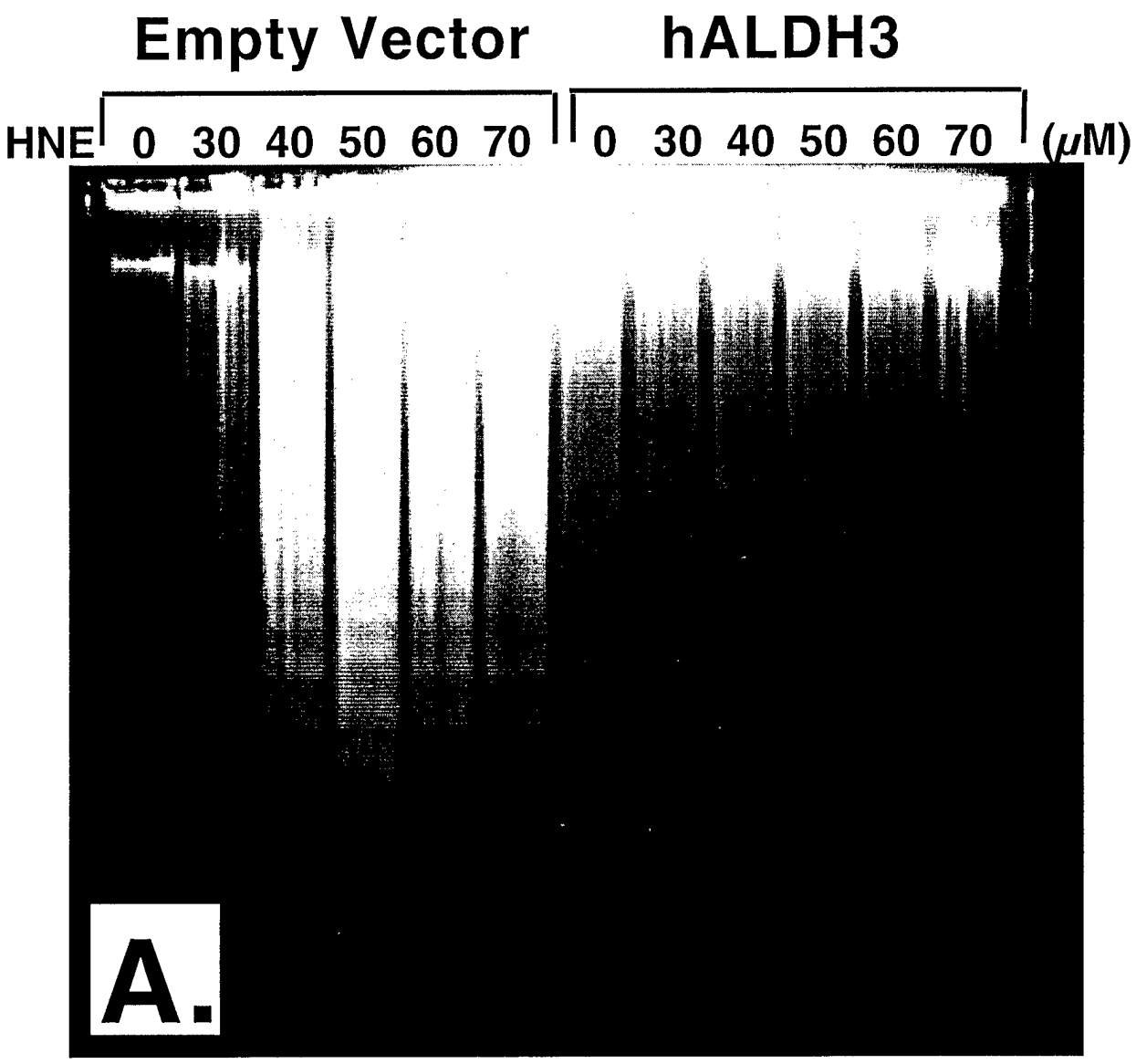

FIGURE 6A

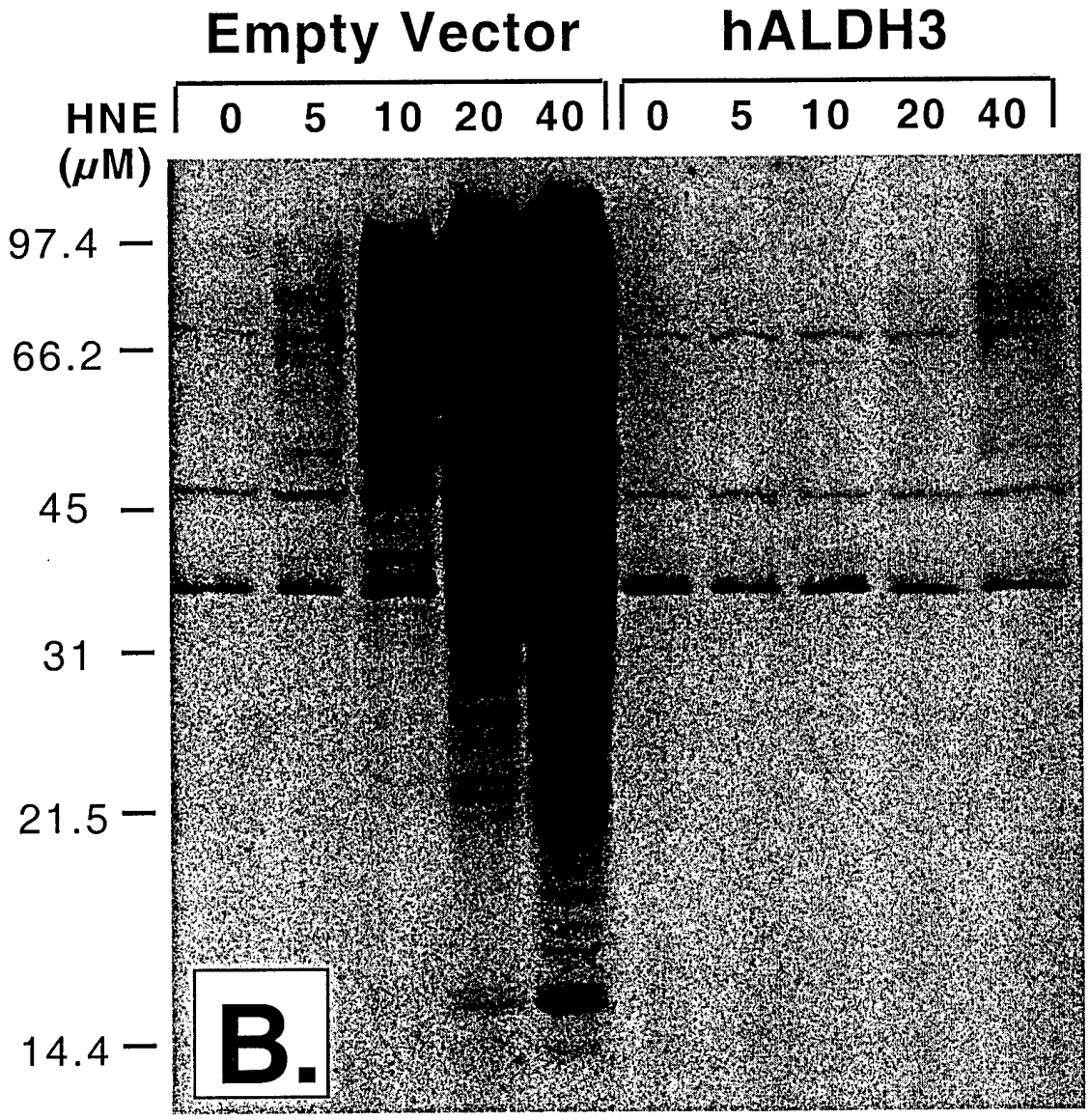
Empty Vector
hALDH3
HNE (μM)
0 5 10 20 40
0 5 10 20 40
97.4
66.2
45
31
21.5
14.4
B.

FIGURE 6B

Lipid Peroxidation product 4-Hydroxy 2 Nonenal induces cytochrome c dependent, p53 independent apoptosis in murine macrophage line RAW 264.7

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4- Hydroxy 2 nonenal (HNE) is a highly reactive lipid aldehyde byproduct of oxidative stress induced lipid peroxidation. HNE is known to damage both DNA and protein and has been shown to play a role in the cellular deterioration associated with oxidant stress mediated injury. HNE has recently been shown to induce apoptosis in certain cell lines. Here we examine two potential mediators of apoptosis, mitochondrial cytochrome c and p53 and present our findings on their roles in HNE induced apoptosis in the murine macrophage cell line RAW 264.7.

When RAW 264.7 parental cells and RAW 264.7 cells overexpressing anti-apoptotic protein BCL-2 were exposed to HNE, apoptotic DNA laddering was seen in parental cells only. This laddering was induced in a dose dependent manner and was observed as early as 6-8 hours after exposure. While HNE-treated BCL2 overexpressing cells showed protection against apoptotic laddering at all concentrations tested, they did show a temporary (24-48 hr) growth delay compared to nontreated controls. When we examined the release of mitochondrial cytochrome c into the cytosol we found that BCL2 cells were protected against the release of cytochrome c while parental cells showed detectable release as early as 3 hours after exposure. Protection by BCL-2 against both cytochrome c release and apoptotic laddering, a downstream effect of apoptosis, suggests that cytochrome c plays an essential role in HNE induced apoptosis in RAW 264.7 cells.

When paremtal and BCL-2 overexpressing cells were examined for the stabilization and subsequent accumulation of p53 protein, we found that p53 levels increased substantially in both cell lines. In parental cells, p53 levels peaked at 6-9 hours after exposure then began to decrease. In BCL-2 cells, levels peaked at 6-9 hours but remained at significantly high levels up to 96 hours after exposure at which time cells became confluent. In both cell lines the level of p53 accumulation was HNE concentration dependent with higher accumulation levels appearing at lower HNE concentrations.

To further examine the role of p53, we overexpressed SV40 large T-antigen in RAW 264.7 parental cells. Large T-antigen is known to complex p53 protein and to affect its transactivation function. When we examined the effects of T-antigen expression on p53 transactivtion of downstream effectors, p21 and BAX, we found that the p53 transactivation was impaired. Endogenous p21 and BAX levels were lower in T-antigen cells. Increases in p21 and BAX upon HNE exposure were also significantly less in T-antigen cells. When we examined p53 in parental and T-antigen cells, parental cells showed normal p53 patterns while T-antigen cells showed an increased and sustained p53 accumulation. When we examined HNE induced apoptosis as determined by apoptotic DNA laddering in both control and T-antigen cells we saw no detectable difference in apoptotic induction. The fact that T-antigen significantly affects p53 levels as well as p53 function yet has no effect on the level of HNE induced apoptosis suggest that total p53 function is not essential in HNE induced apoptosis. Although p53 is stabilized and accumulates in the cell, HNE may be inducing an apoptotic cascade of events that is p53 independent.

The data presented not only contributes to our knowledge of the cellular effects of HNE but also provides evidence of a possible connection between oxidative stress induced apoptosis and oxidative stress generated lipid peroxidation products.

Manuscript in progress.

STRUCTURE-ACTIVITY AND MECHANISTIC STUDIES ON THE TOXICITY OF 4-HYDROXY 2-NONENAL, BYPRODUCT OF THE PEROXIDATION OF POLYUNSATURATED FATTY ACIDS

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The role of cellular oxidant stress in carcinogenesis has recently become the focus of intense study. Oxidative stress is generated through the accumulation of reactive oxidative species (ROS) which interact with cellular molecules and lead to the initiation of downstream reactions and an accumulation of reaction byproducts. Lipid peroxidation, a series of self propagating reactions, is initiated upon the interaction of ROS with the polyunsaturated fatty acids of cellular membranes. This reaction, prominent in tissue with high fat content such as breast tissue, results in a number of byproducts including the most toxic 4 hydroxy-2 nonenal (HNE) which is the focus of the research presented.

HNE is a highly reactive lipid molecule which has been shown to damage both DNA and proteins. HNE contains several structural moieties including an aldehyde, a trans- double, a hydroxyl group, and a 9-carbon lipid chain. To better understand the mechanism of HNEs toxicity we have examined the contribution of each of these structural components to the overall reactivity using analogous compounds which lack one of more components. Using growth inhibition/ cell survival and apoptosis induction as endpoints we find that the order of toxicity of each component is as follows; C_1-aldehyde>>3,4-double bond>fattty acid chain length> C_4-hydroxyl. The potency of the aldehyde and its importance to the toxicity of the compound is supported by studies in which cells overexpressing the lipid aldehyde-metabolizing enzyme aldehyde dehydrogenase-3 (ALDH-3) show complete protection against HNE induced apoptosis as well as HNE-protein adduct formation.

In addition to structure-activity correlations we are also studying mechanistic aspects of HNE-induced toxicity. Because HNE is known to damage DNA, we examined the possibility that HNE induced a stabilization/activation of tumor supressor protein, p53. These studies were done in a RAW 264.7 mouse macrophage cell line because of the presence of wildtype p53. We find that p53 levels increase substantially at HNE concentrations between 25 and 50μM but show no increase at the higher concentration of 75 μM. When apoptosis induction is examined as well as p53 stabilization, we find that apoptosis increases with increasing concentrations suggesting the presence of multiple, concentration dependent, pathways of apoptosis induction. Cells stably expressing the anti-apoptotic protein BCL-2 showed a greater increase in p53 protein than parental cells yet showed protection against apoptotic DNA fragmentation. The effect of elevated p53 on the cell cycle of BCL-2 transfectants is currently being studied.

These findings on HNE cellular toxicity not only contribute to the increasing knowledge of the potential role of oxidative stress in carcinogenesis but also present possible opportunities for modulation of cellular defense levels, i.e. modulation of phase 2 defense enzymes, in order to regulate the degree of cell death in either normal or tumor cells.