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

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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 that 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 $\gg$ 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 that make up 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 4-hydroxy-2-nonenal (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).

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ABBREVIATIONS: HNE, 4-hydroxy-2-nonenal; DMEM, Dulbecco's minimal essential medium; hALDH3, human aldehyde dehydrogenase 3; PAGE, polyacrylamide gel electrophoresis; ALDH, aldehyde dehydrogenase.

Recently, HNE has been shown to induce apoptosis in certain cell lines (Li et al., 1996; Yildiz et al., 1996; Kruman et al., 1997), 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 C4 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 that 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 C1 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.

Materials and Methods

Cell Culture and Reagents. Mouse alveolar macrophage RAW 264.7 cells were grown at 37°C in a 5% CO_2 atmosphere in Dulbecco's minimal essential medium (DMEM; 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, Graz, Austria), or purchased from Cayman Chemical (Ann Arbor, MI). Analogous aldehydes *trans*-2-hexenal, *trans*-2-octenal, *trans*-2-nonenal, and nonanal were purchased from Aldrich (Milwaukee, WI); 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. Cells (1.2×10^6) were treated in suspension in 5 ml of PBS plus chemical agent for 30 min at 37°C. Cells were pelleted by centrifugation (1000 rpm for 5 min) and resuspended in DMEM + 10% fetal bovine serum. Cells (6×10^5) were plated in six-well dishes and allowed to grow for 2 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 to 20 h, cells were rinsed and treated with agents in serum-free DMEM. After a 1-h exposure, medium was removed and replaced with DMEM + 10% fetal bovine serum. Cells were allowed to incubate for an additional 9 h, at which time they were harvested in PBS, pH 7.4, and centrifuged at 4°C, 1000 rpm for 5 min. Cells were then lysed in 20 mM EDTA, 100 mM Tris, pH 8.0, and 0.8% sodium lauryl sarcosine and subjected to RNase treatment (0.5 mg/ml for 1 h at 37°C) followed by proteinase K treatment (5 mg/ml for 6–12 h at 55°C). Nonfragmented chromosomal DNA was removed by filtering the lysate through 0.45-μm syringe filters pretreated with 0.2 mg/ml BSA. Fragmented DNA were then precipitated with 0.1 volume of 3 M sodium acetate, pH 5.2, and 2.5 volumes of 100% ethanol. Redissolved DNA was electrophoresed on a 1.8% agarose gel, then stained with ethidium bromide; DNA fluorescence was recorded using a video imaging workstation (Alpha Innotech, San Leandro, CA).

Transfection of Human Aldehyde Dehydrogenase 3. The cDNA for human class 3 aldehyde dehydrogenase (hALDH3) was previously cloned by polymerase chain reaction amplification from human stomach cDNA and subcloned into the *Xho*I 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 to 80% confluency. Escort (30–50 μL) was incubated with DNA (15–25 μg) in 800 μL of Opti-MEM transfection medium (GIBCO) for 15 min. 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 h. After 24 h, cells were subcultured and selection medium (DMEM + 10% FBS) was added together with 0.7 mg/ml hygromycin. After 9 to 12 days, hygromycin-resistant colonies were cloned and expanded for aldehyde dehydrogenase (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 after 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 min. 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 of total protein was electrophoresed on a 10% SDS-PAGE 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, Vermillion, SD) that was cross-reactive with human ALDH-3. After probing with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Bio-Rad, 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 PBS + 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 1×10^6 cells were lysed in 2% sulfosalicylic acid on ice for 5 min, and centrifuged 12,000*g* for 10 min at 4°. Aliquots of the supernatant were assayed by determining the change in absorbance at 412 nm over a 2-min reaction. A standard curve for each assay was used to calculate nanomoles of GSH per reaction.

HNE Protein Adduct Detection. Cells were plated at 2.5×10^6 cells/60-mm dish; 16 to 20 h later, cells were exposed to agents for 1 h in serum-free medium. FBS was added to 10% at 1 h 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 phenylmethylsulfonyl fluoride. Lysates were centrifuged at 14,000*g* for 10 min at 4°C, and protein (50 μg/lane) was run on a 10% SDS-PAGE and transferred by semidry 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 (Bio-Rad) (1:3000) the protein was detected using Renaissance chemiluminescence reagent (NEN Life Science Products).

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 groups. 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 ± 1.1 μM, 24 ± 4.3 μM, 308 ± 34.9 μM, and 1770 ± 342 μM, respectively (Fig. 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 nontoxic 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 ($P < .0001$), whereas substitution of a carboxyl for the aldehyde group resulted in IC_{50} values that were 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 (Fig. 1B). These experiments showed increased toxicity with increased chain length as shown by IC_{50} values of 99 ± 20 μM, 30 ± 5 μM, and 24 ± 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 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 (Fig. 2). This response is relatively rapid, occurring as early as 8 h after a 1-h 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 GSH, a condition that could trigger apoptosis as a result of oxidative stress caused by 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 75 μM HNE resulted in only moderate depletion of total cellular GSH (84.0 ± 6.8, 71.7 ± 5.2, and 68.8 ± 4.5% 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 nmol/min/mg, 0.64 nmol/min/mg, and 0.79 nmol/min/mg were measured after exposure to 0 μM, 40 μM, and 70 μM HNE, respectively (data 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, more than 90% of the cells have formed apoptotic blebs, blistered, and lysed within 18 h after exposure to HNE (R. L. Haynes and M. Willingham, unpublished observations). These observations confirm that apoptosis is the primary mode of cell death in RAW 264.7 cells after 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 apopto-

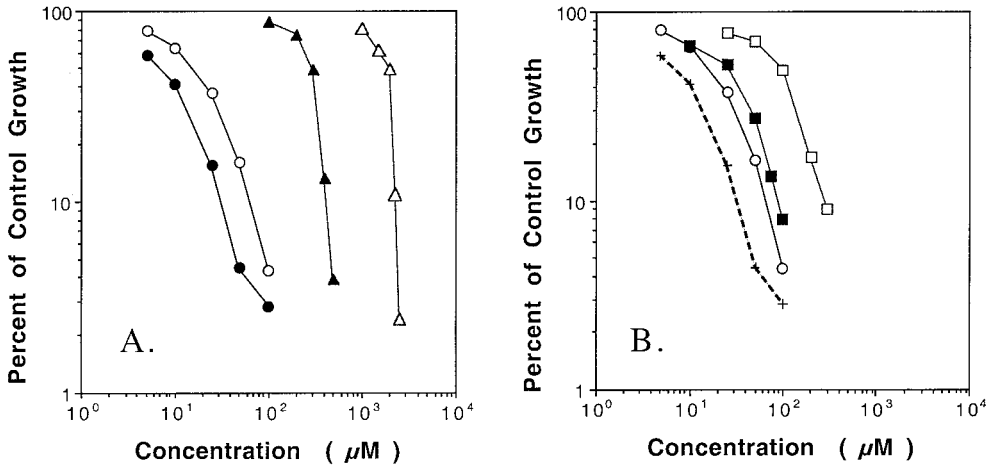

Fig. 1. A, growth inhibition/cell survival to determine the importance of individual functional groups. Cells were dosed with analogous compounds for 30 min as described under *Materials and Methods* and allowed to grow for 2 days, at which time they were trypsinized and counted. Analogous compounds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

sis compared 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 Fig. 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 to 75 μM (Fig. 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 (25–75 μM). 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 (data 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 two 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 (Fig. 4). These results parallel the growth inhibition data seen in Fig. 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; hence, it is not strictly analogous to HNE as a monofunctionally modified congener. Consequently, the role of the aldehyde in the toxicity of HNE was also examined by an indirect approach. We have previously found that expression of an hALDH3 conferred strong protection against HNE toxicity when expressed via stable transfection in V79 hamster lung fibroblast cells (K. D. Bunting, R. L. Haynes, L. Szweda, W. G. Jerome, and A. J. Townsend, unpublished results). 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 (K. D. Bunting, R. L. Haynes, L. Szweda, W. G. Jerome, and A. J. Townsend, unpublished results). The same expression vector was used to express hALDH3 by stable transfection in RAW 264.7 cells, as shown in Fig. 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 with 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 (Fig. 6A). The protection provided by hALDH3 expression was

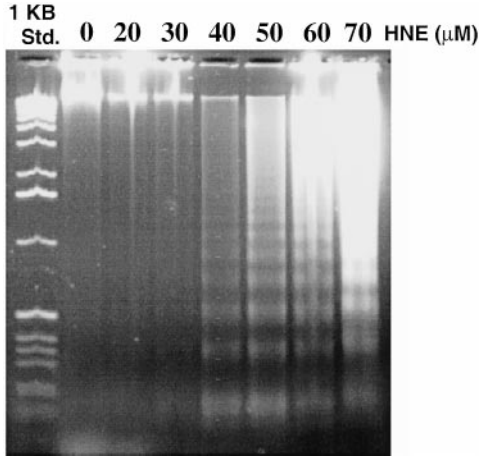

Fig. 2. Internucleosomal DNA fragmentation induced by HNE. RAW 264.7 cells were treated with increasing concentrations of HNE for 1 h in serum-free medium and then medium was replaced with fresh complete medium without HNE. Nine hours after removal of HNE, cells were harvested and DNA was isolated, electrophoresed,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

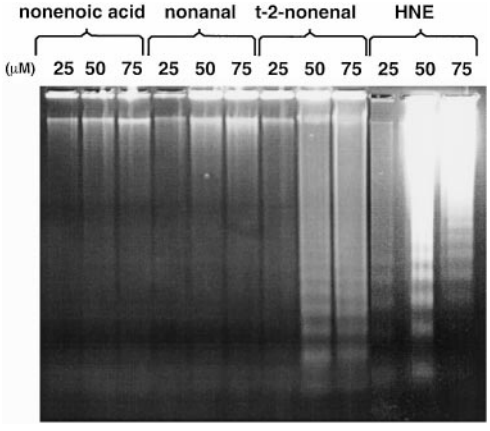

Fig. 3. Internucleosomal DNA fragmentation induced by 9-carbon analogs of HNE. Cells were exposed for 1 h in serum-free medium containing the indicated concentrations of one of the following compounds: nonenoic acid (lacks the C1 aldehyde and hydroxyl), nonanal (lacks the C2═C3 double bond and hydroxyl),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

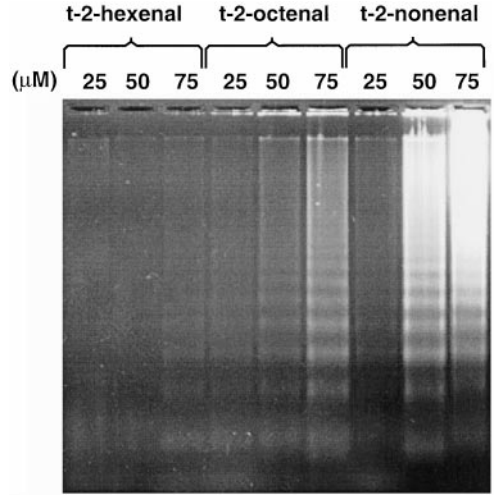

Fig. 4. Internucleosomal DNA fragmentation induced by lipid aldehydes of increasing chain length. Cells were exposed for 1 h in serum-free medium containing the indicated concentrations of one of the following lipid aldehydes: *trans*-2-hexenal (six carbons), *trans*-2-octenal (eight carbons), or *trans*-2-nonenal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

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 evidenced 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 (Witz, 1989; Esterbauer et al., 1991; Esterbauer, 1993). The α,β-unsaturated aldehydes such as HNE react with a range of macromolecules but to widely varying extents depending on the reaction chemistry of the interacting functional groups and microenvironmental factors (e.g., accessibility, hydration, pH, proximity of other functional groups) (Witz, 1989; Esterbauer et al., 1991). 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 because of 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 (Hauptlorenz et al., 1985; Brambilla et al., 1986; Kaneko et al., 1988). Our present studies have focused on the structural contributions to induction of apoptosis compared with 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) compared with *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} value (data not shown), this could have been caused by nonspecific detergent-like effects of nonenoic acid on the integrity of the plasma membrane. Alternatively, the RAW 264.7 cell line

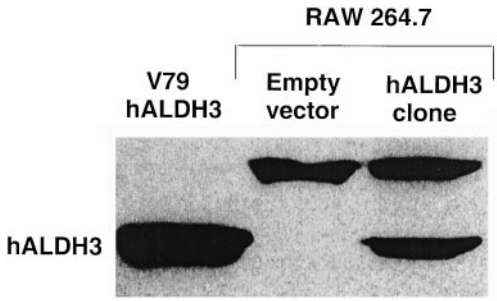

Fig. 5. Expression of hALDH3. Cytosolic protein (50 μg/lane) from control RAW 264.7 cells transfected with a ΔpCEP4Δ control (empty) vector (lane 2) or cells transfected with a ΔpCEP4Δ vector containing human ALDH3 cDNA (lane 3) was run on a 10% SDS-PAGE and transferred to nitrocellulose and probed with 1:3000 dilution of tALDH antisera. Enzyme activity was undetectable in control cells and 100 ± 4 mU/mg in the ALDH3 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 cross-reacting protein present in RAW 264.7 cells.

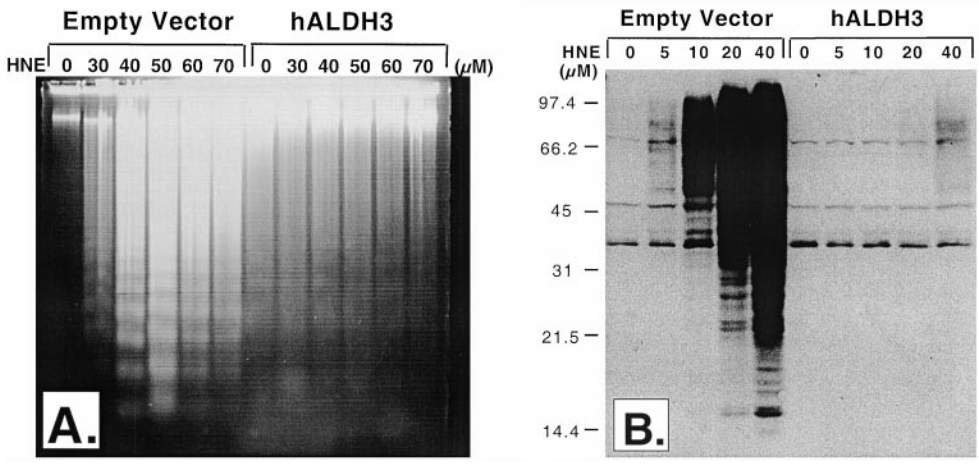

Fig. 6. A, ALDH protection against HNE-induced DNA fragmentation. Control empty vector-transfected RAW 264.7 cells or cells overexpressing hALDH3 were exposed to increasing concentrations of HNE for 1 h in serum-free medium. Cells were harvested 9 h after removal of HNE, and DNA was isolated, electrophoresed, and stained with ethidium bromide as described under *Materials and Methods*. 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 h in serum-free medium. Cells were lysed and cytosolic protein (50 μg/lane) was electrophoresed on a 10% SDS-PAGE and transferred to nitrocellulose. Protein adducts were probed using an antibody specific for HNE adducts, diluted 1:2500, as described under *Materials and Methods*.

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; Witz, 1989; Esterbauer et al., 1991). 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, because 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 after Michael addition of a thiol. This structure probably 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 that 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, although the loss of the aldehyde in nonenoic acid resulted in a 74-fold decrease in toxicity compared with *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 (data 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 the 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 compared with HNE (IC_{50} of 24 μM versus 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 endpoint (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; this may be less stable and more readily reversible (and therefore less toxic) than HNE. The effect of the 4-hydroxyl on the toxicity of the saturated 4-hydroxynonanal could not be evaluated because of cyclization of the chemically synthesized compound, but it would be expected to have minimal effect anyway, because 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 six to nine 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, although the alkenals are progressively correlated with hydrophobicity, the lack of correlation with HNE indicates that this factor contributes to rather than causes toxicity.

The observation that more 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 because of 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, whereas the exposure for the cytotoxicity assay was in suspension. Measured overall concentrations of HNE in cell cytosol of nonstressed 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 because of the nearness of 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., 1995, 1998). Changes in mitochondrial function have also been associated with exposure to lipid peroxidation products, including HNE, in mitochondria (Richter and Meier, 1990; Ullrich et al., 1996; Keller et al., 1997; Humphries et al., 1998), 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,b).

Exposures to the toxic compounds used in this study were via addition to the extracellular medium; thus, it was of interest to verify that the aldehyde group exerted its 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, compared with the nonexpressing control cells. This confirms the idea that the principal targets for the toxic effects are intracellular, because 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 because of 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 (Petronilli et al., 1994; Costantini et al., 1996; 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.

References

Benedetti A, Comporti M and Esterbauer H (1980) Identification of 4-hydroxynonenal as a cytotoxic product originating from the peroxidation of liver microsomal lipids. *Biochim Biophys Acta* **620:**281–296.

Benedetti A, Fulceri R and Comporti M (1984) Inhibition of calcium sequestration activity of liver microsomes by 4-hydroxyalkenals originating from the peroxidation of liver microsomal lipids. *Biochim Biophys Acta* **793:**489–493.

Bloch R and Gilbert L (1987) Synthesis of both enantiomers of γ-substituted α, β-unsaturated γ-lactones. *J Org Chem* **52:**4603–4605.

Bounds PL and Winston GW (1991) The reaction of xanthine oxidase with aldehydic products of lipid peroxidation. *Free Radic Biol Med* **11:**447–453.

Brambilla G, Sciaba L, Faggin P, Maura A, Marinari UM, Ferro M and Esterbauer H (1986) Cytotoxicity, DNA fragmentation and sister-chromatid exchange in Chinese Hamster Ovary cells exposed to the lipid peroxidation product 4-hydroxynonenal and homologous aldehydes. *Mutat Res* **171:**169–176.

Bunting KD, Lindahl R and Townsend AJ (1994) Oxazaphosphorine-specific resistance in human MCF-7 breast carcinoma cell lines expressing transfected rat class 3 aldehyde dehydrogenase. *J Biol Chem* **269:**23197–23203.

Bunting KD and Townsend AJ (1996) Protection by transfected rat or human class 3 aldehyde dehydrogenases against the cytotoxic effects of oxazaphosphorine alkylating agents in hamster V79 cell lines—Demonstration of aldophosphamide metabolism by the human cytosolic class 3 isozyme. *J Biol Chem* **271:**11891–11896.

Cadenas E, Muller A, Brigelius R, Esterbauer H and Sies H (1983) Effects of 4-hydroxynonenal on isolated hepatocytes. *Biochem J* **214:**479–487.

Chen JJ, Bertrand H and Yu BP (1995) Inhibition of adenine nucleotide translocator by lipid peroxidation products. *Free Radic Biol Med* **19:**583–590.

Chen J, Schenker S, Frosto TA and Henderson GI (1998) Inhibition of cytochrome c oxidase activity by 4-hydroxynonenal (HNE). Role of HNE adduct formation with the enzyme subunits. *Biochim Biophys Acta* **1380:**336–344.

Cohn JA, Tsai L, Friguet B and Szweda LI (1996) Chemical characterization of a protein-4-hydroxy-2-nonenal cross-link: Immunochemical detection in mitochondria exposed to oxidative stress. *Arch Biochem Biophys* **328:**158–164.

Costantini P, Chernyak B, Petronilli V and Bernardi P (1996) Modulation of the mitochondrial permeability transition pore by pyridine nucleotides and dithiol oxidation at 2 separate sites. *J Biol Chem* **271:**6747–6751.

Esterbauer H (1993) Cytotoxicity and genotoxicity of lipid-oxidation products. *Am J Clin Nutr* **57 (5 Suppl):**779S–786S.

Esterbauer H, Schaur RJ and Zollner H (1991) Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med* **11:**81–128.

Gadoni E, Olivero A, Miglietta A, Bocca C and Gabriel L (1993) Cytoskeletal modifications induced by 4-hydroxynonenal. *Cytotechnology* **11:**S62–S64.

Hauptlorenz S, Esterbauer H, Moll W, Pumpel R, Schauenstein E and Puschendorf B (1985) Effects of the lipid peroxidation product 4-hydroxynonenal and related aldehydes on proliferation and viability of cultured Ehrlich ascites tumor cells. *Biochem Pharmacol* **34:**3803–3809.

Herbst U, Toborek M, Kaiser S, Mattson MP and Hennig B (1999) 4-hydroxynonenal induces dysfunction and apoptosis of cultured endothelial cells. *J Cell Physiol* **181:**295–303.

Humphries KM, Yoo Y and Szweda LI (1998) Inhibition of NADH-linked mitochondrial respiration by 4-hydroxy-2-nonenal. *Biochemistry* **37:**552–557.

Kaneko T, Honda S, Nakano S-I and Matsuo M (1987) Lethal effects of linoleic acid hydroperoxide and its autoxidation products, unsaturated aliphatic aldehydes, on human diploid fibroblasts. *Chem-Biol Interact* **63:**127–137.

Kaneko T, Kaji K and Matsuo M (1988) Cytotoxicities of a linoleic acid hydroperoxide and its related aliphatic aldehydes toward cultured human umbilical vein endothelial cells. *Chem-Biol Interact* **67:**295–304.

Keller JN, Pang Z, Geddes JW, Begley JG, Germeyer A, Waeg G and Mattson MP (1997) Impairment of glucose and glutamate transport and induction of mitochondrial oxidative stress and dysfunction in synaptosomes by amyloid beta-peptide: Role of the lipid peroxidation product 4-hydroxynonenal. *J Neurochem* **69:**273–284.

Kristal BS, Park BK and Yu BP (1996) 4-Hydroxyhexenal is a potent inducer of the mitochondrial permeability transition. *J Biol Chem* **271:**6033–6038.

Kruman I, Bruce-Keller AJ, Bredesen D, Waeg G and Mattson MP (1997) Evidence that 4-hydroxynonenal mediates oxidative stress-induced neuronal apoptosis. *J Neurosci* **17:**5089–5100.

Li L, Hamilton RF Jr, Kirichenko A and Holian A (1996) 4-Hydroxynonenal-induced cell death in murine alveolar macrophages. *Toxicol Appl Pharmacol* **139:**135–143.

Li CJ, Nanji AA, Siakotos AN and Lin RC (1997) Acetaldehyde-modified and 4-hydroxynonenal-modified proteins in the livers of rats with alcoholic liver disease. *Hepatology* **26:**650–657.

Lucas DT and Szweda LI (1998) Cardiac reperfusion injury: Aging, lipid peroxidation, and mitochondrial dysfunction. *Proc Natl Acad Sci USA* **95:**510–514.

Marchetti P, Castedo M, Susin S, Zamzami N, Hirsch T, Macho A, Haeffner A, Hirsch F, Geusekns M and Kroemer G (1996a) Mitochondrial permeability transition is a central coordinating event of apoptosis. *J Exp Med* **184:**1155–1160.

Marchetti P, Hirsch T, Zamzami N, Castedo M, Decaudin D, Susin S, Masse B and Kroemer G (1996b) Mitochondrial permeability transition triggers lymphocyte apoptosis. *J Immunol* **157:**4830–4836.

Montine KS, Olson SJ, Amarnath V, Whetsell WO Jr, Graham DG and Montine TJ (1997) Immunohistochemical detection of 4-hydroxy-2-nonenal adducts in Alzheimer's disease is associated with inheritance of APOE4. *Am J Pathol* **150:**437–443.

Norton ND, Mamiya BM and Kehrer JP (1997) Relationships between cell density, glutathione and proliferation of A549 human lung adenocarcinoma cells treated with acrolein. *Toxicology* **122:**111–122.

Petronilli V, Costantini P, Scorrano L, Colonna R, Passamonti S and Bernardi P (1994) The voltage sensor of the mitochondrial permeability transition pore is tuned by the oxidation-reduction state of vicinal thiols. Increase of the gating potential by oxidants and its reversal by reducing agents. *J Biol Chem* **269:**16638–16642.

Poot M, Verkerk A, Koster J, Esterbauer H and Jongkind J (1988) Reversible inhibition of DNA and protein synthesis by cumene hydroperoxide and 4-hydroxynonenal. *Mech Ageing Dev* **43:**1–9.

Richter C and Meier P (1990) Inhibition of pro-oxidant-induced mitochondrial pyridine nucleotide hydrolysis and calcium release by 4-hydroxynonenal. *Biochem J* **269:**735–737.

Siems WG, Grune T and Esterbauer H (1995) 4-Hydroxynonenal formation during ischemia and reperfusion of rat small intestine. *Life Sci* **57:**785–789.

Tietze F (1969) Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione. *Anal Biochem* **27:**502–522.

Uchida K, Szweda LI, Chae HZ and Stadtman ER (1993) Immunochemical detection of 4-hydroxynonenal protein adducts in oxidized hepatocytes. *Proc Natl Acad Sci USA* **90:**8742–8746.

Ullrich O, Henke W, Grune T and Siems WG (1996) The effect of the lipid peroxidation product 4-hydroxynonenal and of its metabolite 4-hydroxynonenoic acid on respiration of rat kidney cortex mitochondria. *Free Radic Res* **24:**421–427.

Witz G (1989) Biological interactions of alpha, beta-unsaturated aldehydes. *Free Radic Biol Med* **7:**333–349.

Yildiz D, Ercal N, Frank RL and Matthews RH (1996) Effects of 4-hydroxynonenal and *N*-acetyl-L-cysteine on Myc-induced apoptosis. *Toxicol Lett* **89:**215–221.

Yla-Herttuala S, Palinski W, Rosenfeld ME, Parthasarathy S, Carew TE, Butler S, Witztum JL and Steinberg D (1989) Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J Clin Invest* **84:**1086–1095.

Zamzami N, Marzo I, Susin SA, Brenner C, Larochette N, Marchetti P, Reed J, Kofler R and Kroemer G (1998) The thiol crosslinking agent diamide overcomes the apoptosis-inhibitory effect of Bcl-2 by enforcing mitochondrial permeability transition. *Oncogene* **16:**1055–1063.

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