Mitochondria undergo at least two types of structural alteration in response to various physiological and pathophysiological stimuli. One type is nonreversible and is associated with mitochondrial lysis. The second is reversible and appears to be associated with calcium-mediated activation of a specific inner mitochondrial membrane channel. The mechanisms underlying the induction of this second alteration, termed a mitochondrial permeability transition (PT), have been the subject of a great deal of recent research. Using rat liver mitochondria, our data demonstrate that calcium-mediated PT induction can be affected by the lipid peroxidation byproducts 4-hydroxynonenal and 4-hydroxyhexenal (HHE). 4-Hydroxynonenal appears inactive at concentrations <1 µM but displays both stimulatory and inhibitory effects as part of a biphasic dose response between approximately 1 and 200 µM. In contrast, HHE consistently enhances calcium-mediated induction of the PT, even at femtomolar concentrations. The exquisite specificity and sensitivity of HHE led to further studies to examine the nature of this induction. Studies showing that HHE-mediated induction could be prevented by cyclosporin A confirmed PT involvement. Further studies showed that induction was dependent on both calcium and electron transport chain function. Pretreatment of the HHE with glutathione also prevented PT induction, but simultaneous addition of the thiol reagents dithiothreitol or glutathione (GSH) (16, 17) prevented PT induction. The channel also appears regulated by membrane potential and matrix pH (7, 18, 19), and effectors of lipid structure may play roles in PT induction (e.g. phospholipase A2) (20) and its prevention (e.g. trifluoperazine) (21).

The potential lethality of PT induction, coupled with the apparent roles of phosphate, calcium, and oxidants in this induction, have suggested to many that activation of this pore may play roles in ischemia reperfusion injury (1, 22). Initial support for this concept comes from long standing observations of swollen mitochondria in ischemia reperfusion injury and the observation that free radical scavengers and anti-oxidants exhibit partial protective activity. More recently, direct support has come from studies of the protective activity of PT inhibitors in models of anoxia, hypoxia, and ischemia reperfusion injury (1, 22). Together, these studies suggest that the synergistic effects of oxidants and calcium on PT induction appear to represent a major way in which free radicals and uncontrolled calcium can damage cellular homeostasis.

Another major way in which calcium and oxidative stress impact on cellular physiology is through the induction of lipid peroxidation reactions. Lipid peroxidation, via its chain reaction processes, serves to propagate and amplify oxidant-mediated damage. Lipid peroxidation reactions have been associated with acute pathologic occurrences (e.g. ischemia reperfusion injury) (23) and chronic pathological conditions (e.g. diabetes) (24), as well as the aging process (26). It is currently believed that the byproducts of lipid peroxidation reactions, including malondialdehyde and the hydroxyalkenals, mediate many of the detrimental effects associated with lipid peroxidation reactions (27).

Although malondialdehyde has been considered as the major deleterious lipid peroxidation byproduct, much of the current work on the toxicity of lipid peroxidation byproducts focuses on to a potentially lethal efflux of mitochondrially sequestered calcium into the cytoplasm of the cell.

In vitro, induction of the channel may be observed in the presence of calcium. The addition of phosphate (3) or oxidants such as tert-butyl hydroperoxide (t-BuOOH) (4) greatly enhances the rate of induction. Induction may also be achieved by respiratory uncouplers (5), some fatty acids (e.g. palmitoyl CoA (6), and thiol cross-linkers such as phenylarsine oxide (7). Induction may be inhibited by cyclosporin A (8, 9), and this inhibition serves as a specific marker for PT induction. Channel opening by some inducers may also be inhibited by other compounds such as magnesium (believed to compete with calcium) (10), EGTA (by chelating calcium) (11), respiratory inhibitors (PT induction appears to require respiratory function) (12, 13), antioxidants (14, 15), as well as thiol reagents such as dithiothreitol or the presence of reduced glutathione (GSH) (16, 17). The channel also appears regulated by membrane potential and matrix pH (7, 18, 19), and effectors of lipid structure also appear to play roles in PT induction (e.g. phospholipase A2) (20) and its prevention (e.g. trifluoperazine) (21).

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the more reactive hydroxyalkenals, such as 4-hydroxynonenal (HNE) and 4-hydroxyhexenal (HHE) (27). These products are readily generated in vivo following application of various biological/chemical insults. These compounds are highly reactive to amino acids, especially those containing thiol groups, and highly toxic to many cellular systems, such as mitochondria (27). For example, HNE has been shown to impair both mitochondrial transcription (28) and respiration (29), decrease mitochondrial membrane fluidity (30), and impair function of the adenine nucleotide translocase (31).

Consideration of the biochemical natures of HNE in conjunction with the current body of understanding of PT inducers (e.g. thiol cross-linkers, calcium, a possible role for lipid peroxidation in irreversible induction), led to the hypothesis that the hydroxyalkenals would be potent inducers of the PT. This possibility was further supported by the observed ability of HNE to inhibit the adenine nucleotide translocator, a protein whose functional status is believed to interact with the PT. In the current work, we therefore directly tested the prediction that hydroxyalkenals would induce the PT.

MATERIALS AND METHODS
HHE and HNE—The HHE and HNE used in these experiments were generous gifts of Dr. Herman Esterbauer. Both chemicals are now commercially obtainable from Bioxytech.

Animal Husbandry—Male Fischer 344 rats were obtained from Charles River at 4 weeks of age and maintained under specific pathogen-free conditions. Animals were allowed ad libitum access to water and a soy protein-based semi-synthetic diet (21% RP101 soy protein isolate, 15% sucrose, 3% solka floc, 3.33% RP vitamin mix, 5% mineral and a soy protein-based semi-synthetic diet (21% RP101 soy protein 6034 3.33% RP vitamin mix, 5% mineral mix with reduced sodium, 0.35% NaF, 0.33% chloride, 6% corn oil, and 45.99% dextrin, (Purina)). Animals used in the studies described were between 4 and 10 months of age.

Isolation of Mitochondria—Following decapitation, livers were rapidly dissected out and placed in ice-cold buffer 1 (250 mM mannitol, 75 mM sucrose, 100 mM EDTA, and 10 mM HEPES, pH 7.4) supplemented with 500 uM EGTA. Livers were homogenized with a motor-driven teflon pestle. Following homogenization, samples were centrifuged at 1000 × g for 10 min. This and all other centrifugation steps used a Beckman JA-20 rotor and Beckman J21B centrifuge. Supernatants were removed and centrifuged at 10,000 × g for 15 min. Pellets were washed twice in buffer 1 supplemented with 0.5% fatty acid free bovine serum albumin (Sigma A-6003) and resuspended at 10,000 rpm. Following the final wash, mitochondria were resuspended in buffer 1. Protein was estimated spectrophotometrically as described previously (32).

Mitochondrial Permeability Transition Assays—Changes in the status of the PT pore were assessed spectrophotometrically as described previously (31). Mitochondria were resuspended at room temperature in 1 ml of 215 mM mannitol, 71 mM sucrose, 3 mM HEPES, pH 7.4, and 5 mM succinate (3). Induction of a permeability transition was monitored following exposure to calcium alone or in combination with other inducers as described in specific figure legends. Changes in absorbances were followed until they dropped and restarted, and the time at which one-half of the absorbance was lost was determined.

Note for All Figures—Unless otherwise noted, samples were processed as follows. 1) Calcium was added to the buffer, and the solution was mixed. 1.3 mg of mitochondria was added, and the solution was mixed. 3) The remaining solutions were added, and the solution was mixed. To maximize clarity throughout this paper, all traces presented within a given figure are redrawn to start at exactly the same point, so absolute absorbances are not given. Adjustments for a given figure were always less than 3%. Absorbances for 1 mg/ml mitochondria were generally in the range of 1.25–1.5, and the total loss of absorbance was approximately 40–50% of the initial value. All traces within a given figure were done on the same day with identical mitochondria.

RESULTS
The ability of lipid peroxidation byproducts such as HHE and HNE to induce a mitochondrial permeability transition was directly addressed through comparison with well characterized systems. As most systems that induce the PT require calcium, we tested HHE and HNE in the presence of 25 µM Ca2+. As can be seen in Fig. 1, 180 µM HNE appears to reduce the induction time of the PT compared with induction by calcium alone, but a decreased rate of propagation leads to an overall slowing of the time required for half-maximal induction. In contrast, 60 µM HHE speeds up calcium-mediated induction by about 30%. We addressed each of these products separately.

HNE dose response analysis (Fig. 2) revealed that HHE displays a complex biphasic dose response curve. It is inactive at concentrations <1 µM. In the preparation shown in Fig. 2, we noted that 1.8 µM and 180 µM HNE enhanced calcium-mediated PT induction, whereas 18 µM and 60 µM inhibited it. The general form of this dose response appears consistent, but the absolute values at which the noted effects occur is variable (e.g. compare Fig. 1, trace a, with Fig. 2A, trace A). The remainder of Fig. 2 shows that the effect of HNE is mediated through effects on the rate of propagation. Fig. 2B shows how the slope (rate of propagation) relative to that obtained with calcium alone is modulated by dose. Fig. 2C shows how the time of half-maximal induction relative to that obtained with calcium alone is modulated by dose. Fig. 2D shows the time inverse relationship between these two parameters. The regression analysis in Fig. 2E shows that the inverse of the slope is indeed a strong predictor of the overall time to half-maximal induction. In the dose response curve shown in Fig. 2, lag also appears correlated, but note that Fig. 1 (trace B) shows that this is not always the case. Thus, although alterations in lag may be associated with overall response to HNE, they appear to play minor roles in its effects on PT induction kinetics.

HHE dose response analysis (Fig. 3) revealed that HHE enhanced calcium-mediated PT induction at concentrations as low as 60 fm. Enhancement was regularly seen at femtomolar concentrations (n = 9 of 9). Attomolar concentrations were also occasionally seen to increase PT induction (not shown), but this result was not consistent. Analysis of HHE-mediated PT induction showed two distinct phases. At concentrations in the micromolar range, HHE consistently reduces lag time (Fig. 1, trace c, and Fig. 3, trace B) but has a variable effect on slope (compare Fig. 1, trace c, with Fig. 3, trace B), which leads to a marked variability in the time until maximal induction. This variability at high doses makes determination of a true EC50 for HHE both impractical and uninformative. In contrast to both this data and the data on HNE presented above, submicromolar concentrations of HHE act predominantly or exclusively by decreasing lag time (Fig. 3, B and C).}

HHE-mediated induction was next compared with other effectors of PTP opening. As shown in Fig. 4A, HHE-mediated effects on PT induction by nanomolar and femtomolar HHE, although consistently more rapid than calcium alone, are...
slower than induction by either 2 mM inorganic phosphate or 75 
μM t-BuOOH. As pointed out above, however, induction by 
higher HHE concentrations (e.g. 60 μM) is extremely sample-
specific and may be nearly as rapid as phosphate normally is 
(e.g. Fig. 3, trace B) or roughly the level of enhancement seen 
with t-BuOOH (e.g. Figs. 1 and 4B). Note the different effects 
on lag and slope in Figs. 1 and 4B, suggesting that the effects 
of micromolar concentrations of HHE may also differ from 
sample to sample.

We next began characterization of the HHE-mediated re-
sponse through the use of compounds known to slow or prevent 
PT induction. To confirm that response to HHE represented PT 
induction, we examined the ability to block induction with 
cyclosporin A, a well characterized specific inhibitor of the PT. 
As shown in Fig. 5, cyclosporin prevents the loss of absorbance 
induced by HHE. To further evaluate factors involved in HHE-
mediated PT induction, we examined the ability of several 
compounds to affect HHE-mediated induction. EGTA, myx-
othiazol, and antimycin A (data not shown, identical to myx-
othiazol) were all able to inhibit PT induction by Ca^{2+} and 
HHE (Fig. 5), but glutathione (Fig. 6) and dithiothreitol (2 mM, 
data not shown, identical to glutathione) were not. In contrast, 
pretreatment of HHE with glutathione did prevent induction 
(Fig. 6). Because of the variability observed with micromolar 
concentrations of HHE, EGTA, cyclosporin, and myxothiazol 
were tested against 60 nM HHE. Because these inhibitors were 
effective against this concentration, lower HHE doses were not 
tested. In the glutathione experiment, the use of femtomolar 
HHE allowed us to determine if GSH could block induction by 
even the lowest effective HHE dose, which, as seen in Fig. 6, it 
could not.

**DISCUSSION**

The polyunsaturated fatty acids that comprise biological 
macromolecules are extremely sensitive to oxidant-mediated 
damage. Although much early work focused on the damage 
done to the lipids themselves, more recent work has also ad-
dressed the roles that the byproducts of these peroxidation 
reactions play in ongoing cellular function and dysfunction.

Because of its prevalence and its ability to cross-link biomol-
ecules, malondialdehyde was the focus of initial studies on the 
effects of lipid peroxide byproducts. As understanding of the 
field grew, however, more emphasis began to be placed on the 
hydroxyalkenals, especially HNE (27). HNE is produced in 
during lipid peroxidation reactions from the breakdown of lin-
oleic acid, arachidonic acid, and 15-hydroperoxyarachidonic 
acid (27, 33, 34); it is far more reactive than malondialdehyde, 
often impairing biological function in the low micromolar 
range. HHE is similarly reactive and can be formed through 
both lipid peroxidation (by the degradation of omega 3 polyun-
saturated fatty acids, e.g. 22:6) and through nonperoxidative mechanisms, such as the metabolism of the alkaloid senecionine (27, 35–37). Interestingly, the mechanism underlying senecionine poisoning has been shown to be HHE-mediated disruption in calcium homeostasis (38).

It is also interesting to note the comparison between our observations and the commonly held belief that the chemical similarity between HHE and HNE means that they are also biologically similar. HHE, which is only recently beginning to be studied in detail, enhanced calcium-mediated induction of the PT at femtomolar concentrations. Higher levels of HHE (micromolar) can give extremely rapid induction, but for reasons we have been unable to determine, effects are highly variable. These observations are especially interesting given the known effect of HHE to disrupt calcium homeostasis (38).

In contrast to HHE, the effects of HNE are extremely dose-dependent, thus indicating a reactivity distinct from that evidenced by HHE. For example, HNE can slow induction at ∼10–100 μM, whereas no inhibitory effects have ever been observed with HHE at micromolar concentrations (data not shown). Notably, Richter and Meier (25) have shown that concentrations of HNE that we find slow PT induction (∼10–100 μM) can prevent pro-oxidant-induced calcium release from mitochondria, apparently by interfering with pyridine nucleotide hydrolysis (25, 27), which can favor PT induction (1).

Inhibitors of the PT were used to further probe the mitochondrial alterations induced by HHE and calcium. The cyclosporin A studies confirmed that we were indeed studying induction of PT, as opposed to nonspecific swelling. The EGTA studies demonstrate that calcium is required for PT induction by HHE, as it is for most other inducers. The studies with respiratory inhibitors (e.g. myxothiazol, antimycin A) confirmed the role of respiration in PT induction (probably through a free radical-mediated mechanism).

The failure of both GSH and dithiothreitol to protect when added just prior to addition of HHE was unexpected, as the major known chemical activity of HHE is as a thiol-oxidant, and both GSH and dithiothreitol have been previously demon-
defenses become compromised due to progressive age or disease status.

In conclusion, it is important to note that the potent effects of HHE on the PT suggest that the time has come to focus more attention on the lipids that comprise the inner mitochondrial membrane and how these lipids may play roles in PT induction. Free radical-mediated lipid peroxidation and the associated production of both HHE and HNE may affect PT function during aging, chronic disease, and acute events such as ischemia reperfusion injury. Membranes may also affect PT function in other ways, because alterations in membrane structure and function, such as those that occur with age, may alter calcium balance or mitochondrial potential, both of which can affect the PT.

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Bruce S. Kristal, Brian K. Park and Byung P. Yu

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