4-hydroxynonenal (HNE), an aldehyde product of membrane lipid peroxidation, has been suggested to be a key mediator of oxidative stress-induced cell death. In this study, we partially characterized the mechanism of HNE-mediated cytotoxicity. Incubation of human T lymphoma Jurkat cells with 20-50 μM HNE led to cell death accompanied by DNA fragmentation. Western blot analysis showed that HNE-treatment induced time- and dose-dependent activation of caspase-8, caspase-9 and caspase-3. HNE-induced caspase-3 processing was confirmed by a flow cytometric demonstration of increased catalytic activity on the substrate peptide. HNE treatment also led to remarkable cleavage of poly(ADP-ribose) polymerase (PARP), which was prevented by pretreatment of cells with DEVD-FMK as a caspase-3 inhibitor. The HNE-mediated activation of caspases, cleavage of PARP and DNA fragmentation were blocked by antioxidants cysteine, N-acety-L-cysteine and dithiothreitol, but not by two other HNE-reactive amino acids lysine and histidine, or by cystine, the oxidized form of cysteine. HNE rapidly decreased levels of intracellular reduced glutathione (GSH) and its oxidized form GSSG, and these were also attenuated by the reductants. Coincubation of Jurkat cells with a blocking anti-Fas antibody prevented Fas-induced but not HNE-induced activation of caspase-3. HNE also activated caspase-3 in K562 cells that do not express functional Fas. Our results thereby demonstrate that HNE triggers oxidative stress-linked apoptotic cell death through activation of the caspase cascade. The results also suggest a possible mechanism involving a direct scavenge of intracellular GSH by HNE.

Key words: HNE, Caspase, Redox, GSH, Fas

INTRODUCTION

4-hydroxynonenal (HNE), an aldehyde product of membrane lipid peroxidation, can be produced by oxidative stimuli and has been detected in several diseases such as atherosclerosis, diabetes and Parkinson’s disease (Jurgens et al., 1993; Traverso et al., 1998; Yoritaka et al., 1996). The formation of HNE and HNE-protein conjugate has become a marker of oxidative stress in tissues or cells. Oxidative stress-induced apoptotic cell death is believed to be involved in the pathological generation of those oxidative stress-related diseases, therefore HNE may be an important mediator of oxidative stress-induced apoptosis. It has been reported that HNE and HNE-protein adduct accumulate in neurons by oxidative insults and in lung cells by ozone exposure, and that they are associated with the apoptotic events in these cells (Kruman et al., 1997; Kirichenko et al., 1996; Keller et al., 1998). Exogenously administrated HNE has also been observed to form HNE-protein adduct and to induce apoptotic cell death in macrophages and neurons (Li et al., 1996; Kruman et al., 1997; Compton et al., 1998). Much attention has recently been paid to HNE-induced apoptotic cell death in the pathological development of neural and vascular degenerations, particularly because HNE is not only a mediator of amyloid β-peptide-induced damage (Mark et al., 1997) but also one effective component of oxidized low density lipoproteins (Uchida et al., 1995). Although studies have shown that HNE-mediated apoptotic cell death involves intracellular calcium uptake (Kruman and Mattson, 1999), and HNE directly interacts and activates c-Jun amino-terminal kinase (JNKs) (Parola et al., 1998), the mechanism of HNE-induced apoptosis has not been clearly delineated.

Caspases are a group of cysteine proteinases responsible for the deliberate disassembly of a cell into apoptotic bodies. Caspases are present as inactive pro-enzymes, most of which are...
activated following cleavage at a specific aspartate cleavage site and assembly of their active subunit forms. Caspase-8, caspase-9 and caspase-3 are situated at pivotal junctions in apoptotic pathways. Caspase-8 initiates disassembly in response to extracellular apoptosis-inducing ligands and is activated in a complex associated with the receptor’s cytoplasmic death domain (Muzio et al., 1996; Boldin et al., 1996). Caspase-9 activates disassembly in response to agents or insults that trigger release of cytochrome c from the mitochondria and is activated when complexed with dATP, APAF-1 and extramitochondrial cytochrome c (Li et al., 1997; Hu et al., 1999). Caspase-3 appears to amplify caspase-8 and caspase-9 signals into fully fledged commitment to disassembly (Slee et al., 1999). Both caspase-8 and caspase-9 can activate caspase-3 by proteolytic cleavage, and caspase-3 may then cleave vital cellular proteins including the nuclear enzyme poly(ADP-ribose) polymerase (PARP) (Casciola-Rosen et al., 1996; Na et al., 1996). Caspase-3 is therefore one of the key executioners of apoptotic cell death, and activation of caspase-3 has become a biochemical verifier for cells undergoing apoptosis.

In this study, we investigated the mechanism of HNE-elicted cytotoxicity in human T lymphoma Jurkat cells. The results demonstrate the existence of an apoptotic pathway induced by HNE, which is dependent on cellular redox status-related activation of the caspase cascade.

MATERIALS AND METHODS

Cell culture
Jurkat and K562 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 i.u./ml penicillin and 100 mg/ml streptomycin. Cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. For experiments testing the ability of HNE to activate caspase-3, 2×10⁶ cells were incubated in serum-free RPMI medium in the absence or presence of HNE.

Reagents
HNE was obtained from Cayman Chemical (Ann Arbor, MI, USA). Anti-human PARP polyclonal antibody was from Upstate Biotechnology Incorporated (Lake Placid, NY, USA). Anti-caspase-8 monoclonal antibody (clone B9-2) and anti-caspase-9 polyclonal antibody were from Pharmingen (San Diego, CA, USA), and anti-caspase-3 monoclonal antibody (clone 19) was from Transduction Laboratories (Lexington, KY, USA). Cytotoxic monoclonal anti-Fas IgM (clone CH-11), Phosphilux G6D2 kit and peptide caspase-3 inhibitor Z-Asp-Glu-Val-Asp-Fluoromethyl ketone (DEVD-FMK) were obtained from Medical and Biological Laboratories (Nagoya, Japan). Non-cytotoxic blocking monoclonal anti-Fas antibody (clone SM1/23) was from Bender MedSystems (Vienna, Austria).

Electrophoretic analysis of DNA
DNA analysis by electrophoresis was performed by the method previously described (Kato et al., 1995). Briefly, Jurkat cells were collected and washed twice with PBS. After centrifugation, the pellet was suspended in DNA buffer (50 mM Tris, 0.5% SDS, 10 mM EDTA) and incubated at 55°C for 90 minutes. The sample was then treated with RNase and proteinase, and the DNA was collected and electrophoresed in a 1.0% agarose gel and visualized by UV illumination.

Western blot analysis
SDS-PAGE and immunoblot were performed as previously described (Nakashima et al., 1991). In brief, cells lysed with sample buffer (×2: 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol and 10% glycerol) were resolved on 10% SDS polyacrylamide gels. They were then transferred to a polyvinylidene difluoride membrane. After blocking with 2% BSA or 5% milk, the membrane was stained with the specific first antibody, followed by goat anti-rabbit or goat anti-mouse IgG conjugated to horseradish peroxidase (Tago, Burlingame, CA, USA), which was visualized using the Western Blot Chemiluminescence Reagent (DuPont NEN, Boston, MA, USA).

RESULTS

HNE induces cell death and DNA fragmentation of Jurkat cells
We first investigated the cytotoxic activity of HNE in Jurkat cells. As shown in Fig. 1A, HNE dose-dependently triggered cell death. After 9 hours incubation, 20 μM HNE reduced viable cell numbers by approximately 60% compared to the control, whereas 10 μM or lower concentrations of HNE had no effect. When the concentration was increased further to 50 μM or 100 μM, viable cell numbers decreased dramatically to below 10% of the control. Within 24 hours of incubation, untreated Jurkat cells proliferated continuously, whereas treatment with 20 μM HNE caused a reduction in the viable cell number from 6 hours after incubation (Fig. 1B). We then tested whether HNE-induced cell death was accompanied by DNA fragmentation. After treatment with or without HNE, the cells were analyzed by a DNA electrophoresis, and DNA fragmentation was detected in cells treated with 20 μM and 50 μM HNE (Fig. 1C).

HNE induces activation of caspases and cleavage of PARP in Jurkat cells
In order to investigate the possible mechanism of HNE-induced cell death and DNA fragmentation, we examined intracellular caspase activity in HNE-treated or untreated
HNE activates caspase-3

Jurkat cells. Firstly, by using an anti-caspase-8 antibody that specifically recognizes the inactive precursor caspase-8 we checked caspase-8, which mediates apoptotic signals from the cell surface death receptor. As shown in Fig. 2A (top), 20 μM HNE, which was shown to induce DNA fragmentation in Fig. 1C, cleaved the pre-caspase-8 in a time-dependent way, indicating activation of this proteinase. Both the 54-kDa and the 55-kDa isoforms of pre-caspase-8 showed sensitivity to the HNE stimulation. We next tested caspase-9, which is believed to transfer the death signal from mitochondria. Activation of caspase-9 was determined by using an anticaspase-9 antibody that recognizes both the pro-enzyme and its fragments. As shown in Fig. 2A (middle), procaspase-9 (approximately 48 kDa) was clearly cleaved by treatment with 20 μM HNE to generate a 37-kDa fragment, and the cleavage occurred mainly in the large pro-isoform. Finally, we investigated the processing of caspase-3, a crucial factor for apoptosis. As shown in Fig. 2A (bottom), the inactive 32-kDa caspase-3 precursor was cleaved by 20 μM HNE in a time-dependent way. The caspase-3 activity was also measured with the Phophilux G6D2 kit. Compared to the untreated cells, HNE-treated cells showed a marked increase in fluorescence, demonstrating an enhanced activity of caspase-3 (Fig. 2B).

To further characterize the HNE-induced processing of caspases, we measured the cleavage of PARP, a 116-kDa protein involved in DNA repair, which is specifically cleaved by caspases during apoptosis. As shown in Fig. 2C,D, PARP was cleaved into the characteristic 85-kDa fragment in the course of HNE treatment. This cleavage was strictly dose- and time-dependent. Such HNE-induced cleavage of PARP in Jurkat cells was completely blocked by pre-treatment of the cells with DEVD-FMK, a specific peptide inhibitor of caspase-3 (Fig. 2E), indicating that it is caspase-3-dependent.

Antioxidants block HNE-induced activation of caspases, cleavage of PARP and DNA fragmentation

It is known that HNE primarily reacts with the sulfhydryl group of cysteine, imidazole group of histidine and amino group of lysine (Brambilla et al., 1986; Esterbauer et al., 1991). These features of HNE prompted us to test the effect of pre-incubation of cells with cysteine, lysine or histidine on the action of HNE to induce caspases activation. We unexpectedly
found that cysteine, but not lysine or histidine, showed a powerful blocking effect on HNE-induced activation of caspase-8, caspase-9 and caspase-3 and cleavage of PARP (Fig. 3A). Since only cysteine is a reductant among the three amino acids, we next tested the effect of several other reducing reagents and an oxidized form of cysteine as a control on HNE-induced caspases activation. As shown in Fig. 3A, pre-incubation of Jurkat cells with N-acetyl-L-cysteine or dithiothreitol, but not cystine (oxidized cysteine), partially or completely inhibited HNE-induced cleavage of pro-caspases and PARP. Treatment of Jurkat cells with any of these reagents alone had no effect on caspase-3 activity (Fig. 3B). Finally, we confirmed that the blockage of the caspases activation by antioxidants was followed by inhibition of DNA fragmentation (Fig. 3C).

HNE induces alternations in intracellular GSH and GSSG

Inhibition of HNE-induced activation of caspase-3 by reducing reagents prompted us to investigate whether an exogenously added HNE could influence the cellular redox status. We therefore checked intracellular GSH and GSSG levels. As shown in Fig. 4A, intracellular GSH concentration in HNE-treated cells fell rapidly, and the peak of reduction was observed at 2 hours after incubation. Then the GSH level began to recover, showing a cellular response to the GSH loss. Coincubation of the cells with cysteine, NAC or DTT clearly compensated the HNE-induced GSH depletion (Fig. 4B). On the other hand, the intracellular GSSG level was also unexpectedly reduced by HNE addition. This reduction lasted for 6 hours, and then GSSG remained at a low level (Fig. 4C). Pretreatment of cells with the antioxidants inhibited intracellular GSSG reduction (Fig. 4D).

**HNE-induced caspase-3 activation is independent of cell surface Fas**

Our earlier study demonstrated that in A431 cells HNE can bind to the cell surface EGF receptor and activate this protein kinase (Liu et al., 1999). This raised the question of whether HNE-induced activation of caspase-3 could be through an action on the cell surface Fas protein. First, we compared the levels of induction of PARP cleavage by incubation of Jurkat cells with HNE in the presence or absence of an anti-Fas blocking antibody (SM1/23). As shown in Fig. 5A, both HNE and cytotoxic monoclonal anti-human Fas antibody (CH-11) induced cleavage of the PARP protein in Jurkat cells. However, CH-11-induced cleavage was completely prevented by the blocking antibody, whereas there was no remarkable inhibition of HNE-induced PARP cleavage. We next determined that HNE can also induce PARP cleavage in K562 cells that do not express functional Fas (Hampton et al., 1997; Boggs et al., 1998) (Fig. 5B). Finally, we made comparative measurements of the expressions of the Fas receptor and Fas ligand on normal and HNE-treated cells. Both the Fas receptor and Fas ligand were constitutionally expressed on Jurkat cells, but their expression was not changed by HNE treatment (data not shown).

**DISCUSSION**

Our results demonstrate for the first time that HNE induces apoptotic death of Jurkat cells through a cellular redox status-related activation of the caspase cascade. We investigated the action of HNE on caspase-3, caspase-8 and caspase-9, and we found that all of the three caspses can be activated by HNE. Caspase-8 and caspase-9 are considered to be two initiator caspses for mediating apoptotic signals from death receptors or from cytochrome c translocation, leading to activation of caspase-3 as a key factor in apoptotic cell death. However, it has recently been proposed that cytochrome c release is also mediated after Fas-triggered activation of caspase-8 by Bid (Luo et al., 1998), a member of the proapoptotic Bcl-2 family, and that caspase-8 can be activated not only by death receptor signaling but also by cytochrome c translocation (Bantel et al., 1999). Because the activation of caspase-8 and caspase-9 occurred with a roughly similar time course, these data by themselves did not unequivocally predict the initiating events for caspase activation.

Reduced glutathione is a critical component of the cellular antioxidant network, being directly involved in scavenging ROS and in maintaining intracellular thiol proteins in their reduced...
HNE activates caspase-3

Depletion of GSH can be an early event in the apoptotic process, and can lead to or increase sensitivity to apoptosis in different systems (Merad-Boudia et al., 1998; Celli et al., 1998). In our study, HNE was shown to reduce intracellular GSH levels in a time-dependent manner. Although this reduction was not so intense, the result was quite stable and was clearly associated with caspase-3 activation. Pre-raised GSH level by thiol antioxidants rescued the GSH decrease and caspase-3-dependent DNA fragmentation, suggesting that HNE initiated apoptotic signals dependent on GSH depletion. The HNE-induced decrease in cellular GSH level could be through one or more of several different pathways. As an end product and remnant of lipid peroxidative events, HNE itself may be a potential source of intracellular pro-oxidant (Uchida et al., 1999). By binding with proteins, HNE can activate cellular enzymes through conformational changes or molecular aggregations (Liu et al., 1999), which then evoke intracellular signaling involved in the possible generation of ROS. As a small diffusible molecule, exogenous HNE can also easily pass through the cytoplasmic membrane, thereby directly scavenging intracellular GSH. It is also possible that diffused HNE could decrease intracellular GSH through inhibition of GSH synthetase. In the present study, HNE-induced reduction of intracellular GSH was quite quick and was accompanied by a marked decline rather than elevation of intracellular GSSG levels, suggesting a direct reaction of HNE with the GSH pool. By interfering with the intracellular redox status, HNE possibly triggers impairment of mitochondria, thereby initiating the activation of caspases. Our results also suggest that the intracellular GSH level may be a critical parameter for HNE-provoked signaling for the caspase-3-dependent apoptosis.

A rapid and specific efflux of reduced GSH was observed recently in Jurkat cells during anti-Fas antibody-induced apoptosis (van den Dobbelsteen et al., 1996). In that system,
the release of GSH to the extracellular medium, which was rapidly followed by DNA fragmentation, was prevented by a caspase inhibitor, demonstrating that it is an event downstream of caspase activation. In the present study, we were not able to detect a significant increase in GSH in the extracellular medium (data not shown). Moreover, the intracellular GSH loss in our experiment occurred much earlier than the caspase activation and was correlated to the decreased intracellular GSSG level, suggesting that it resulted from the depletions by intracellular scavenger rather than the activation of a transmembrane channel which contributes to the later GSH efflux in Fas-apoptosis.

The Fas receptor system has been extensively studied as a model of apoptosis, since cross-linking of the Fas receptor with Fas ligand or specific agonist antibodies results in rapid programmed cell death (Lynch et al., 1995; O’Connell et al., 1996). Various oxidative stresses can evoke ligand-dependent or -independent Fas-mediated apoptosis (Caricchio et al., 1998; Kohno et al., 1996). As a mediator of oxidative stress-triggered intracellular signal transduction, HNE was recently reported to bind directly to cell surface receptors and cause their activation (Suc et al., 1998; Liu et al., 1999). These findings tempted us to examine whether HNE-induced apoptosis involves cell surface Fas or not. Although Fas-mediated signaling for apoptosis may involve ROS production and its downstream generation of HNE, which might account, in part, for the effects of Fas-mediated apoptosis, our results presented here demonstrated that HNE can induce caspase-mediated apoptosis without participation of Fas. Since HNE-induced apoptosis has been considered to be responsible for several oxidative stress-related neurodegenerative conditions such as Alzheimer’s, Parkinson’s and cerebral ischemia (Pappolla et al., 1992; Selley et al., 1996), in which there is no Fas expression but caspase-3 is detectable, our data may be helpful for elucidating the mechanisms of these diseases. These results, together with the alternations in intracellular GSH and GSSG levels in association with caspase activation and their inhibition by GSH precursors further imply the possibility that HNE triggers activation of caspase-3 through a cellular redox state-linked pathway without involvement of the interaction of HNE with cell surface death receptors, although direct evidence showing that there is no involvement of other death receptors is needed.

It has recently been reported that arginine-glycine-aspartate containing peptides directly induce auto-processing and activation of caspase-3 (Buckley et al., 1999), suggesting that caspase-3 may also be activated without signals from cell surface death receptors or from mitochondria. In this study, we did not obtain any clear evidence of direct binding of HNE with caspase-3 (data not shown). However, this does not rule out the possibility that intracellular HNE or HNE-triggered alteration of the intracellular redox has some direct impact on caspases activity through their self-processing, because common to the caspase family is the existence of a reactive cysteine in the active site, and this reactive cysteine is sensitive to the redox status of the cell.

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REFERENCES


Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M.,


