Death receptors belong to the tumor necrosis factor receptor family. They can induce apoptosis following engagement with specific ligands and are known to play an important role in the regulation of the immune system. Here we report that epoxycyclohexenone (ECH) inhibits apoptosis induced by anti-Fas antibody, Fas ligand (FasL), or tumor necrosis factor-α but not by staurosporine, MG-132, C2-ceramide, or UV irradiation. These results suggest that ECH specifically blocks death receptor-mediated apoptosis. Neither the surface expression of Fas nor the Fas-FasL interaction was influenced by ECH. However, ECH did block the activation of pro-caspase-8 in the death-inducing signaling complex, although recruitment of Fas-associating death domain (FADD) and pro-caspase-8 was not affected. ECH inhibited the enzymatic activity of recombinant active caspase-8 at slightly lower concentrations than it did for active caspase-3 and active caspase-9 in vitro. However, in FasL-treated cells, ECH was only able to inhibit the activation of pro-caspase-8, and it had no effect on the already activated caspase-8 at a concentration that is effective at inhibiting Fas-induced apoptosis. ECH directly bound the large subunit of active caspase-8 that recognizes such as effector caspases and initiates the apoptotic cascade that ultimately leads to cell death.

In Fas-mediated apoptosis, two types of cells are proposed to transmit distinct death signals (9, 10). In type I cells, such as SKW6.4, the level of activation of pro-caspase-8 initiated at the DISC is sufficient to cleave pro-caspase-3 directly. However, in type II cells, such as Jurkat, less pro-caspase-8 is activated, and the mitochondrial pathway is required to amplify the weak death signal. The small amount of activated caspase-8 is able to cleave Bid efficiently, the truncated form of which translocates to mitochondria and induces the release of cytochrome c. Released cytochrome c then forms a complex called the "apoptosome" that contains Apaf-1 and pro-caspase-9, and it is this complex that generates active caspase-9 that subsequently cleaves and activates pro-caspase-3.

The Fas signaling pathway is a complex process that is regulated by cellular and viral proteins such as FLICE inhibitory proteins (FLIPs) (11, 12), cytokine response modifier A (CrmA) (13), and inhibitor of apoptosis proteins (IAPs) (14). Functional and structural analyses of these regulators have contributed to our understanding of the molecular basis of Fas-mediated apoptosis. However, several non-peptide small molecules that modulate apoptosis also have been reported (15–21). These molecules are useful for dissecting the apoptosis signal transduction pathway and also may be potential candidates for therapeutic use. To find specific inhibitors of Fas-mediated apoptosis, we have screened a library of microbial secondary metabolites and identified epoxycyclohexenone (ECH). Previously, it was reported that ECH inhibits Fas-mediated apoptosis, although the inhibitory mechanism of ECH was not characterized. We show here that ECH blocks the self-activation of pro-caspase-8 in the death-inducing signaling complex and thus selectively inhibits death receptor-mediated apoptosis.
Ephoxyclohexone Blocks Pro-caspase-8 activation in the DISC

EXPERIMENTAL PROCEDURES

Cells—Human T lymphoma Jurkat cells and Burkitt’s lymphoma SKW6.4 cells were cultured in RPMI 1640 medium (Sigma). Human embryonic kidney 293T cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) in 5% CO2 at 37 °C. Each medium was supplemented with 10% (v/v) heat-inactivated fetal calf serum (JRH Biosciences). Anti-FADD antibody was from Pharmingen. Anti-IκBα antibody was from Santa Cruz, CA. Anti-cytochrome C antibody was from Cell Signaling Technology (Beverly, MA). Purification of ECH and Synthesis of Biotinylated ECH—ECH was isolated from the culture broth of a producing fungal strain using bioassay-guided purification procedures. Biotinylated ECH was synthesized by a coupling reaction using an activated biotin reagent (Ficoll). The structures of ECH and biotinylated ECH were determined by their physico-chemical properties, detailed 1H- and 13C-NMR analyses including two-dimensional techniques, and mass spectroscopies. The details of these procedures and characterization will be reported elsewhere.

Preparation of FLAG-tagged FasL—FLAG-tagged FasL was prepared as reported previously (24) with the following modifications. The extracellular domain of human FasL (amino acids 128–291) was amplified by PCR from a Jurkat cDNA library using a 5’-forward primer containing a ClaI site (5’-ATCGTGGAGAAGAATAGGGCCACCC-3’) and a 3’-reverse primer containing an XbaI site (5’-TCTAGATATTACGAGGGAA-3’) and cloned into the pCI-neo vector (Promega, Madison, WI), designated pCI-FLAG-FasL. The extra-cellular and transmembrane domain of human Fas (amino acids 1–184) was amplified by PCR from a Jurkat cDNA library using a 5’-forward primer containing a Nhel site (5’-GCTAGCATGGACTTCAAGCAGAACATGC-3’) and a 3’-reverse primer containing an XbaI site (5’-GTCATATATTACGAGGGAA-3’) and cloned into pCI-neo vector digested with Nhel and EcoRI.

Measurement of Caspase Activity in Vitro—Recombinant human active caspase-3-8, or -9 were mixed with Ac-DEVD-methyl-coumaryl-7-amide (MCA), Ac-IETD-MCA, or Ac-LEHD-MCA, respectively (Peptide Institute Inc.) in reaction buffer (20 mM PIPES (pH 7.5), 100 mM NaCl, 0.1% CHAPS, 10% sucrose, 1 mM EDTA) for 60 min. The release of nitrile-tetrazolium acid (MTT; Sigma) for 2 h. MTT-formazan was solubilized in 5% SDS overnight, and the absorbance at 595 nm was measured using a plate reader (Wallac 1420 ARVOx; Amersham Biosciences). Cell viability (percent) was calculated as (experimental absorbance-background absorbance)/(control absorbance-background absorbance) × 100.

Western Blot Analysis—Cells were washed with phosphate-buffered saline (PBS) and lysed in lysis buffer (100 mM Tris-HCl (pH 7.5), 1% Triton X-100, 1 mM dithiothreitol, 1 mM EDTA, the protease inhibitor mixture (Complete; Roche Diagnostics)). The cytosolic fractions (50 μg/lane) were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked with dry milk in PBS-Tween 20 and probed with specific antibodies. The proteins were visualized using the ECL detection reagents (Amersham Biosciences).

Flow Cytometry—To measure the expression level of Fas on the cell surface, Jurkat cells were treated with anti-Fas antibody (B-10) for 45 min on ice, washed with PBS, and then treated with FITC-conjugated secondary antibody ( Molecular Probes, Eugene, OR) for 45 min on ice. The cells were then incubated with PBS, and surface expression of Fas was detected by flow cytometry (Profile II, Coulter Co., Hialeah, FL). The Fas-FasL interaction was measured as follows: Jurkat cells were treated with FLAG-tagged FasL and anti-FLAG M2 antibody for 60 min, washed with PBS, and chilled on ice. The cells were treated subsequently with FITC-conjugated secondary antibody for 45 min on ice. The cells were then washed with PBS, and surface binding of FasL was assessed by flow cytometry.

DISC Analysis—SKW6.4 cells were treated with 2 μg/ml FLAG-tagged FasL in the presence of anti-FLAG M2 antibody (2 μg/ml) for the indicated times. In the negative control, anti-FLAG M2 antibody was added after lysis. Following incubation, the cells were rapidly cooled down by the addition of 5 volumes of ice-cold PBS and then lysed in lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM sodium vanadate, 1 mM NaF, 10% glycerol, 0.5% Nonidet P-40, the protease inhibitor mixture). Cytosolic fractions were precleared with Sepharose 6B (Sigma) for 60 min and then incubated with protein A-Sepharose CL-4B (Amersham Biosciences) for 3 h. Sepharose beads were washed four times with lysis buffer. The proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes (Amersham Biosciences) followed by Western blotting using anti-caspase-8 and anti-FADD antibodies.

Detection of Fas Clustering—Fas clustering was visualized as described previously (25). In brief, SKW6.4 cells were treated with 1 μg/ml FLAG-tagged FasL and 1 μg/ml anti-FLAG M2 antibody for 30 min on ice and washed with PBS to remove unbound FasL. The cells were stained with FITC-conjugated secondary antibody for 30 min on ice and washed with PBS. The cells were then warmed up and kept at 37 °C for 30 min to trigger Fas stimulation. After stimulation, the cells were adhered to glass slides precoated with poly-L-lysine (Sigma) and fixed in acetone-methanol (1:1). The gels were then incubated with PBS, washed, and observed under fluorescence microscopy (Olympus, Tokyo, Japan).
RESULTS

ECH Inhibits Fas-mediated Apoptosis in Both Type I and Type II Cells—Human Burkitt’s lymphoma SKW6.4 cells are classified as type I, in which Fas ligation is able to trigger sufficient activation of pro-caspase-8 to induce the direct activation of pro-caspase-3. In contrast, human T lymphoma Jurkat cells are classified as type II since, due to weak activation of pro-caspase-8, activation of pro-caspase-3 requires amplification via the mitochondrial pathway. To obtain specific inhibitors of Fas-mediated apoptosis, we screened a library of microbial metabolites and identified ECH as a compound that will inhibit Fas-mediated apoptosis in Jurkat cells. Agonistic anti-Fas antibody CH-11 and cross-linked FasL are both able to induce apoptosis in Jurkat cells (Fig. 1A). We found that ECH inhibited the apoptosis induced by CH-11 or FasL at equivalent concentrations and did not decrease cell viability under these conditions (Fig. 1B). Similar results were obtained with SKW6.4 cells that underwent apoptosis upon treatment with CH-11 or FasL (Fig. 1, C and D). Both Jurkat and SKW6.4 cells showed cell body shrinkage and chromatin condensation, and this was also reduced by ECH (Fig. 1E). Clonogenic assays revealed that ECH increases the survival rates of FasL-treated SKW6.4 cells, although ECH alone had little effect on cell proliferation in long term cultures (Fig. 1F).

ECH Selectively Inhibits Death Receptor-mediated Apoptosis—Jurkat cells are highly sensitive to a variety of apoptotic stimuli. In addition to Fas-mediated apoptosis, we examined the inhibitory effects of ECH on various apoptotic pathways induced by TNF-α, staurosporine (a protein kinase inhibitor), MG-132 (a proteasome inhibitor), C2-ceramide (a cell-permeable analogue of ceramide), and UV irradiation. All apoptosis inducers tested caused cell death in Jurkat cells within 8 h and reduced the cell viability. As observed with Fas-mediated apoptosis (Fig. 1B), ECH markedly inhibited apoptosis induced by TNF-α in a dose-dependent manner (Fig. 2A). In contrast, ECH did not significantly block apoptosis induced by staurosporine, MG-132, C2-ceramide, or UV irradiation (Fig. 2A). A pan-caspase inhibitor, zVAD-fmk, was able to prevent apoptosis irrespective of the inducer used (data not shown). These results suggest that in contrast to zVAD-fmk, ECH blocks death receptor-mediated apoptosis selectively.

Upon ligand binding, death receptors such as TNF receptor 1 initiate two distinct signals: the caspase-8-dependent death signal and the survival signal that activates transcription factor NF-κB (26). The degradation of IκB is a prerequisite event for NF-κB activation (27). Therefore, to examine whether ECH has an effect on the survival signal activated by TNF-α, we monitored the cellular levels of IκB. We observed degradation of IκB within 15 min of TNF-α stimulation in Jurkat cells (Fig. 2B). A proteasome inhibitor, MG-132, inhibited such TNF-α-induced IκB degradation; however, in ECH cells, degradation was still observed (Fig. 2B). These data indicate that ECH blocks the TNF-α-induced death signal but had no effect on the survival signal.

ECH Inhibits Death Receptor-mediated Apoptosis Upstream of Pro-caspase-8 Activation—To investigate the molecular target of ECH during the inhibition of Fas-mediated apoptosis, we first examined whether ECH treatment decreases the expression level of cell surface Fas. Jurkat cells were pretreated with ECH and then treated with anti-Fas antibody (B-10). Following assessment by flow cytometry, we concluded that ECH did not produce observable changes in the level of cell surface Fas (Fig. 3A). We then examined whether ECH disrupts the Fas-FasL interaction. For this, Jurkat cells were pretreated with ECH and incubated with cross-linked FasL followed by staining with FITC-conjugated secondary antibody. Equal amounts of cell surface FasL binding were detected in both the control and ECH-treated cells, suggesting that ECH did not affect the interaction between Fas and FasL (Fig. 3B).

Therefore, to determine which step of the Fas-mediated
ECH selectively inhibits Fas- and TNF receptor-mediated apoptosis. As shown in A, Jurkat cells were pretreated with various concentrations of ECH for 30 min and then incubated without apoptosis inducers (open circles) or with the following apoptosis inducers for 8 h: 100 ng/ml TNF-α plus 10 nM cycloheximide (closed circles), 300 nM staurosporine (open triangles), 3 μM MG-132 (closed triangles), 100 μM C2-ceramide (open squares), 10 μM CH-11, and 10 μM UV irradiation (closed squares). Cell viability was measured by using MTT reagent. Data points represent the mean ± S.D. of triplicate determinations. As shown in B, Jurkat cells were pretreated with 20 μM ECH or 10 μM MG-132 for 30 min. The cells were then incubated with 10 ng/ml TNF-α for 15 min. Cytosolic fractions were separated by SDS-PAGE and analyzed by Western blotting. α-tubulin (upper panel) and α-tubulin (lower panel) were detected by specific antibodies.

Apoptosis is blocked by ECH, caspase-dependent cleavage of proapoptotic molecules was assessed by Western blot analysis. CH-11, FasL, and TNF-α induced the cleavage of pro-caspase-8 into intermediate cleaved forms, which leads to triggering of the downstream cascade such as the cleavage of Bid, the release of cytochrome c into the cytosol, the cleavage of pro-caspase-3, and the cleavage of poly (ADP-ribose) polymerase, a substrate of active caspase-3 (Fig. 3C). ECH blocked the self-cleavage of pro-caspase-8, and thus, the downstream events were all suppressed. Treatment with staurosporine, MG-132, C2-ceramide, and UV irradiation also triggered the apoptotic cascade; however, ECH did not affect all events under these conditions. These results were consistent with the observations made above (Fig. 2A), which demonstrated that ECH was not able to inhibit death receptor-independent apoptosis. In contrast, the pan-caspase inhibitor, zVAD-fmk, was able to block all of the steps initiated by any of the apoptotic stimuli tested. These results suggest that ECH targets an early signaling event(s) upstream of pro-caspase-8 activation in the apoptosis pathways via the Fas and TNF receptor.

**ECH Blocks the Activation of Pro-caspase-8 in the DISC**

The engagement of FasL with Fas initiates the recruitment of FADD and pro-caspase-8 to Fas, enabling formation of the DISC. Immediately following DISC formation, pro-caspase-8 is placed in a configuration that facilitates self-processing, resulting in the generation of its active form. In SKW6.4 cells, both FADD and pro-caspase-8 were rapidly recruited to Fas upon treatment with FasL, and pro-caspase-8 was processed to yield the intermediate cleaved form, p43 (Fig. 4A). Although the recruitment of FADD and pro-caspase-8 to Fas was observed to be unaffected in ECH-treated cells, the self-processing of pro-caspase-8 was markedly blocked in a dose-dependent manner (Fig. 4A). DISC formation reached a maximum at 15 min and was reduced after 60 min (Fig. 4B). However, in ECH-treated cells, the DISC remained stable for 60 min without any processing of pro-caspase-8 (Fig. 4B). These results suggest that ECH blocks self-processing of pro-caspase-8 after its recruitment to FADD.

Next we investigated whether or not ECH inhibited Fas clustering. Under non-stimulatory conditions, Fas was distributed equally on the cell surface (Fig. 4C, panel d). FasL engagement triggered the formation of Fas clustering at the cell surface (Fig. 4C, panel d). However, ECH markedly reduced this clustering (Fig. 4C, panel f), as did the pan-caspase inhibitor, zVAD-fmk (Fig. 4C, panel h). Therefore, these observations support the idea that ECH blocks the self-processing of pro-caspase-8 during Fas-mediated apoptosis. **ECH Inhibits Self-activation of Pro-caspase-8**—The above observations suggest that the primary target of ECH in Fas-mediated apoptosis may be the self-processing of pro-caspase-8 following its binding to FADD. To support this hypothesis, we constructed a Fas-caspase-8 fusion protein (Fas-casp8) consisting of the caspase-8 catalytic domain (amino acids 180–479) and the Fas extracellular and transmembrane domains (amino acids 1–184) (Fig. 5A). The assay system can detect directly that cross-linked FasL induces FADD-independent activation of pro-caspase-8. Although transfection of Fas-casp8 in HEK293T cells slightly induced caspase-8 activity, exogenous addition of FasL strongly enhanced the activity. ECH significantly suppressed the proteolytic activity of caspase-8 in Fas-casp8-transfected cells (Fig. 5B). Under the same conditions, self-processing of Fas-casp8 was monitored by Western blot
Fig. 4. ECH inhibits self-processing of pro-caspase-8 in the DISC. As shown in A, SKW6.4 cells were pretreated with the indicated concentrations of ECH for 30 min and incubated with 2 μg/ml FLAG-tagged Fasl in the presence or the absence of 2 μg/ml anti-FLAG M2 antibody for 15 min. As shown in B, SKW6.4 cells were pretreated with or without 40 μM ECH for 30 min and then incubated with 2 μg/ml FLAG-tagged Fasl in the presence or the absence of 2 μg/ml anti-FLAG M2 antibody for the indicated time periods. Postnuclear lysates were precleared with Sepharose 6B for 1 h, and the DISC was immunoprecipitated (IP) using protein A-Sepharose CL-4B for 3 h. Proteins were separated by SDS-PAGE. The DISC and whole lysates were analyzed by Western blotting using anti-caspase-8 and anti-FADD antibodies. The nonspecific band is indicated with an asterisk.

ECH Inhibits the Activation of Pro-caspase-8 but Does Not Inhibit Active Caspase-8 in Intact Cells—We examined the direct effect of ECH on recombinant active caspase-8. ECH inhibited the enzymatic activity of active caspase-8 in a dose-dependent manner (Fig. 6A). Unexpectedly, ECH also inhibited recombinant active caspase-3 and recombinant active caspase-9 (Fig. 6, B and C). The IC_{50} value of active caspase-8 (24 μM) was slightly lower than that of active caspase-3 (50 μM) and active caspase-9 (62 μM); however, the IC_{50} difference might be insufficient to explain the selectivity of ECH in the cell-based assay. Thus, we examined the effect of ECH on pro-caspase-8 and active caspase-8 in intact cells. Jurkat cells were preincubated with Fasl for 60 min (during which time caspase-8 activity steadily increases) and then treated with ECH or zVAD-fmk. ECH inhibited further activation of pro-caspase-8 but did not affect the already activated caspase-8 (Fig. 6D). In contrast, zVAD-fmk reduced caspase-8 activity to background levels, thus demonstrating that zVAD-fmk is able to inhibit both pro-caspase-8 and active caspase-8. Therefore, these results suggest that ECH inhibits the activation of procaspase-8 but does not affect active caspase-8 in intact cells.

ECH Preferentially Binds to Pro-caspase-8 in Intact Cells—To compare the binding capacity of ECH with pro-caspase-8 and active caspase-8, we synthesized a biotin-labeled form of ECH (Fig. 7A). The biotinylated ECH bound to both the recombinant pro-caspase-8 and the recombinant caspase-8 large subunit (p18) that contains the active site cysteine. However, pro-caspase-8 had a relatively higher affinity to ECH than did caspase-8 p18 (Fig. 7B). A competition assay using unlabeled ECH showed that the binding of ECH to pro-caspase-8 and caspase-8 p18 is specific. We then examined whether ECH is able to bind to pro-caspase-8 in crude cell extracts. As shown in Fig. 7C, the ECH bound to pro-caspase-8 and caspase-8 p18 in the presence of active caspase-8 but not in the absence of active caspase-8. These results suggest that ECH preferentially binds to pro-caspase-8 in intact cells.

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lysates. For this, HEK293T cells were transiently transfected with FLAG-tagged pro-caspase-8. The cell lysates were treated with 20 μM biotinylated ECH and immunoprecipitated with anti-FLAG M2 antibody. The immunoprecipitated complexes were separated by SDS-PAGE and analyzed by Western blotting using anti-FLAG M2 antibody and avidin-conjugated HRP. As shown in D, the cell lysates (500 μg of protein) of SKW6.4 cells were treated with 20 μM biotinylated ECH and immunoprecipitated with anti-caspase-8 antibody. The immunoprecipitated complexes were separated by SDS-PAGE and analyzed using anti-caspase-8 antibody and avidin-conjugated HRP. As shown in E, the cell lysates (500 μg of protein) of SKW6.4 cells were treated with various concentrations of biotinylated ECH for 1 h, and biotinylated ECH was depleted with avidin-agarose for 4 h at 4 °C. Then, the supernatants were separated by SDS-PAGE and analyzed by anti-caspase-3, caspase-8, caspase-9, and α-tubulin antibodies. As shown in F, recombinant pro-caspase-8 was pretreated with various concentrations of glutathione (left panel) or cysteine (middle panel) or serine (right panel) for 1 h on ice and then incubated with 30 μM ECH for 2 h on ice. Protein samples were separated by SDS-PAGE and analyzed by Western blotting using anti-caspase-8 antibody (upper panel) and avidin-conjugated HRP (lower panel).

**DISCUSSION**

Bioprobes are low molecular weight chemical inhibitors that can be used for the functional analysis of complex cellular processes (28). To date, various bioprobes that are able to modulate apoptosis have been reported (21, 29). Caspase substrate-mimicking peptide inhibitors have been most frequently used to block apoptosis. Radical scavengers such as N-acetylcysteine and metalloporphyrin can protect against apoptosis induced by reactive oxygen species (15–17). Isatin sulfonamide derivatives directly inhibit effector caspases such as active caspase-3 and active caspase-7 and are able to block apoptosis induced by cycloheximide or camptothecin (18). It has been reported that protein kinase C activators, such as phorbol esters, suppress Fas-mediated apoptosis via blocking the oligomerization of Fas and recruitment of FADD and pro-
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caspase-8 into the DISC (19, 20). In this report, we show that ECH inhibits Fas-mediated apoptosis by blocking the activation of pro-caspase-8. Since ECH is structurally different from the apoptosis modulators thus far reported, ECH is a novel type of non-peptide apoptosis inhibitor.

ECH inhibited Fas-mediated apoptosis in all of the cell lines tested, such as Jurkat, SKW6.4, Raji, U937, and HepG2 cells (Fig. 1 and data not shown). The inhibitory effect of ECH was highly selective for death receptor-mediated apoptosis (Fig. 2). Consistent with these observations, ECH blocked the activation of pro-caspase-8, which is the specific initiator caspase for death receptor-mediated apoptosis (6). Activation of pro-caspase-8 is known to be the first step in the cascade of apoptosis events induced by Fas stimulation. Upon FasL binding, pro-caspase-8 is recruited to the DISC multiprotein complex. The recruited pro-caspase-8 associates with the cytoplasmic portion of the Fas receptor via the adaptor protein FADD. In the DISC, pro-caspase-8 is proteolytically auto-processed to the active form (7, 8). In Fas-treated cells, although recruitment of FADD and pro-caspase-8 was unaffected, the self-processing of pro-caspase-8 was blocked (Fig. 4, A and B), suggesting that the molecular target of ECH is part of the DISC component. As shown in Fig. 3, A and B, ECH affected neither Fas expression nor Fas-FasL interaction. Therefore, FADD and pro-caspase-8 were both possible candidates as target molecules for the action of ECH. To clarify this issue, we constructed a Fas-casp8 fusion protein, which allows caspase-8 to be activated without the involvement of FADD, by the addition of cross-linked FasL as described previously (30). ECH blocked the FADD-independent caspase-8 activation (Fig. 5), indicating that the inhibitory target of ECH is pro-caspase-8 rather than FADD. This is consistent with the observation that ECH suppressed Fas clustering, which was reported previously to be a caspase-8-dependent event (25).

ECH inhibited the enzymatic activity of recombinant active caspase-8 at a slightly lower concentration than its inhibition of recombinant active caspase-3 and caspase-9 (Fig. 6, A–C). However, ECH was not able to block apoptosis mediated by active caspase-3 and active caspase-9 in cells treated with chemical drugs and UV irradiation (Figs. 2A and 3C). Similar results have been reported in the case of zVAD-fmk (31, 32). To resolve this discrepancy, we examined whether ECH inhibits active caspase-8 in FasL-treated cells. In contrast to the caspase inhibitor zVAD-fmk, ECH inhibited activation of pro-caspase-8 but did not inhibit the activity of already active caspase-8 (Fig. 6D). Binding analysis revealed that ECH preferentially bound recombinant pro-caspase-8 rather than the large subunit of recombinant active caspase-8 (Fig. 7B). Moreover, ECH bound pro-caspase-8 in the cell lysates (Fig. 7, C and D), and pro-caspase-8 was more efficiently depleted from these lysates as compared with pro-caspase-3 and pro-caspase-9 (Fig. 7E). These results indicate that ECH possesses a higher affinity to pro-caspase-8 than to active caspase-8, pro-caspase-3, or pro-caspase-9 and that it inactivates the intrinsic proteolytic activity of pro-caspase-8. It may also be possible that the cellular amount of pro-caspase-8 is less than that of pro-caspase-3 and pro-caspase-9 and that the total pool of pro-caspase-8 in the cell is predominantly inactivated by ECH. ECH has a molecular structure of α, β-unsaturated ketone and epoxide that are highly reactive to addition of nucleophiles. Therefore, ECH may act by binding to the thiol group of cysteine residues within the active site of pro-caspase-8. Consistent with this hypothesis, the addition of either glutathione or cysteine was able to attenuate the binding of ECH on pro-caspase-8 (Fig. 7F). Thus, we conclude that ECH is able to bind to cysteine residues possibly within the active center of pro-caspase-8 and thereby inhibits the self-activation of pro-caspase-8 in the DISC.

Death receptor-mediated apoptosis has important functions for the homeostasis of the immune system and immune surveillance. Such functions include the elimination of harmful cells by cytotoxic T-lymphocytes and the maturational selection of T- and B-lymphocytes. Consequently, derailment of death receptor-mediated apoptosis is seen in severe diseases. For therapeutic use, the peptide-based caspase inhibitors including zVAD-fmk are first generation drugs, and a number of studies describe their potency in reducing cell death in acute situations of fulminant hepatitis, ischemia, and bacterial meningitis (33–35). These caspase inhibitors may also be useful in reducing cell death in organs awaiting transplant (36) and in reducing apoptosis of normal cells caused by chemotherapeutic drugs for cancer treatment. As a potential pharmacological drug, ECH has important characteristics. It is highly selective for death receptor-mediated apoptosis, it is a membrane-permeable non-peptide compound, and it is a small molecule that promises easy modification. Recently, death receptor-independent activation of pro-caspase-8 in neuronal diseases such as Huntington disease has been reported (37, 38). In such diseases, expansion of polyglutamine repeats induces unfavorable protein aggregation that eventually recruits pro-caspase-8 and activates apoptosis independently of death receptors, resulting in neuronal cell death. Consistent with this model, the caspase-8 inhibitory proteins, CrmA and e-FLIP, as well as the dominant-negative mutant of FADD, prevent neuronal cell death (37, 38). Therefore, ECH, a novel non-peptide inhibitor preventing the activation of pro-caspase-8, could be a good candidate for therapeutic use to treat such neuronal diseases.

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