Anandamide induces apoptosis in human endothelial cells: its regulation system and clinical implications

Kazuyo Yamaji, Krishna Pada Sarker, Koichi Kawahara, Satoshi lino, Munekazu Yamakuchi, Kazuhiro Abeyama, Teruto Hashiguchi, Ikuro Maruyama

Department of Laboratory and Molecular Medicine, Faculty of Medicine, Kagoshima University, Kagoshima, Japan

Summary
Anandamide (AEA), an endogenous cannabinoid, is generated by macrophages during shock conditions, and is thought to be a causative mediator of septic shock. Thus, we hypothesized that AEA plays a crucial role in endothelial cell (EC) injury. Here, we demonstrate that AEA induces apoptosis in a time- and dose-dependent manner in human umbilical vein endothelial cells (HUVECs). AEA triggered phosphorylation of c-Jun NH2-terminal kinase (JNK) and p38 mitogen activated protein kinase. AEA also showed a marked increase of interleukin 1β-converting enzyme (ICE)CED-3 family protease (caspase-3) activity. AEA-induced EC death was inhibited by a selective vanilloid receptor 1 (VR1) antagonist, capsazepine, and was enhanced by a VR1 agonist, capsaicin, indicating that AEA induces apoptosis in ECs via VR1. In conclusion, we propose that AEA may play a crucial role in EC injury under conditions of shock, and that the use of inhibitors of the AEA regulation system may have a therapeutic effect under these conditions.

Keywords
Anandamide, endothelial cell, apoptosis, vanilloid receptor 1, endotoxin shock

Introduction
The biologically active principal of marijuana, Δ⁹-tetrahydro-cannabinol (Δ⁹-THC), is a partial agonist of a G protein-coupled type 1 cannabinoid receptor (CB1). It has been demonstrated that N-arachidonyl-ethanolamide (AEA; anandamide), which was isolated from porcine brain lipid extracts, binds and activates CB1 and, to a lesser extent, type 2 cannabinoid receptor (CB2) subtypes; AEA acts as an endogenous agonist, and exerts diverse biological activities (1). In the cardiovascular system, AEA induces hypotension and bradycardia in rats (2) and produces endothelial cell (EC)-independent vasodilation in the rat hepatic artery (3).

The various effects of AEA are terminated by a rapid and selective carrier-mediated uptake of AEA into cells (4), followed by its degradation to ethanolamine and arachidonic acid by the enzyme fatty acid amide hydrolase (FAAH); it has been demonstrated that human umbilical vein endothelial cells (HUVECs) possess the tools to eliminate AEA (5).

Septic shock is a main cause of irreversible hypotension and multiple organ failure and remains a common, life-threatening event. It has been demonstrated that endotoxin was able to induce endothelial apoptosis both in vitro (6, 7) and in vivo (8), and it may play a role in hypotension during multiple organ failure. Recently, it has been suggested that AEA and 2-arachidonoxyglycerol (2-AG) were synthesized and released from
macrophages and platelets during shock induced by either hemorrhage (9) or lipopolysaccharide (10). In our previous study, we first identified that AEA levels are very high in the serum from endotoxin-shocked patients (11). Thus, it is suggested that AEA rises to a certain level in some pathological states and may play an important role in the pathomechanism of shock conditions. The challenge of managing septic patients is compounded by the development of key vascular complications (12), and a common denominator of these complications is EC injury and/or dysfunction. A micromolar concentration of AEA has been reported to trigger apoptosis in various types of cells (13, 14), and Maccarrone et al. reported that AEA induces apoptosis in human neuroblastoma and lymphoma cells via vanilloid receptors (VR1) (15). Initially, AEA was identified as a ligand of cannabinoid receptors; however, in later studies, it also was identified as an endovanilloid (16).

Mitogen-activated protein kinases (MAPK) play a central role in the signaling pathway of cell proliferation, differentiation, apoptosis, and survival (17). Recently, the relationship between JNK and interleukin 1β-converting enzyme (ICE)/CED-3 family proteases in apoptotic cell death has been investigated, and ICE/CED-3-like protease (caspase-3/CPP32) has been considered as a central component of the proteolytic cascade and plays a key role during apoptosis (18).

In the present study, we hypothesized that increased AEA may play a crucial role in cases involving EC injury. The goals of the present study were to determine the mechanism of AEA-induced apoptosis in human ECs, and to provide some clarification of the mechanism of the diverse complications associated with septicemia.

Materials and methods

Cell culture
HUVECs were isolated from human umbilical cord veins and cultured in MEDIUM 199 (Gibco BRL, USA) supplemented with 20% fetal bovine serum, 2% penicillin/streptomycin, and 1% fungizon on collagen-coated dishes. Passages 2-3 were used for these experiments, and the cells were maintained in a humidified incubator at 37°C in a 5% CO2 atmosphere.

Evaluation of cell viability
A 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Dojindo, Japan) assay was performed to test cell viability (19). HUVECs were seeded at a density of 2.0 x 10⁴ cells in 96-well collagen-coated dishes (Iwaki, Japan) in MEDIUM 199 containing 0.1% bovine serum albumin (BSA, Sigma, USA). Cells were treated with AEA (Calbiochem, USA), Tumor necrosis factor-α (TNF-α, Genzyme, USA)/cycloheximide (Wako, Japan), and arachidonic acid (Sigma) for the indicated concentrations and periods. In some cases, HUVECs were pre-incubated for 2 h with the indicated agents. The VR1 agonist capsaicin ([N-(4-hydroxy-3-methoxy-phenyl)methyl]-8-methyl-6-nonenamide), VR1 antagonist capsazepine ([N-(2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothioamide), p38 MAP kinase inhibitor SB203580, and caspase inhibitors were purchased from Calbiochem (USA). MEK inhibitor U0126 was purchased from Promega (USA). The FAAH inhibitor, methylarachidonylfluorophosphonate (MAFP) was purchased from Cayman Chemical (USA). The CB1 antagonist, N-Piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazolecarboxamide (SR141716) and [the] CB2 antagonist, N-[1(S)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methyl-phenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR144528) were a kind gift from Sanofi Recherche (France). MTT (0.5 mg/ml) was added to each well and the wells were incubated for 3 h. As mitochondrial enzyme converts MTT to insoluble formazan crystals, 100 µl of 10% SDS containing 0.01 N HCl was added to dissolve the crystals. Absorbance was measured at 570 nm using an automatic microtiter plate reader (Immuno Mini NJ-2300, Nalge Nunc International, USA).

The CytoTox-96 assay (Promega) also was used to test cytotoxicity, which measured the release of lactose dehydrogenase (LDH) from dying cells, according to the manufacturer’s instructions. Total LDH release was achieved by adding Triton X-100 (100 µl/well of a 9% solution) to untreated control cells. Treatment values were then expressed as a percent of the total LDH release.

Nuclear morphology
Twenty-four hours after incubation with AEA, cells were collected, washed with phosphate-buffered saline (PBS), fixed with 10% paraformaldehyde for 30 min, and incubated in Hoechst 33258 (Wako) at room temperature for 30 min (5 µg/ml). Nuclear morphology was examined using fluorescence microscopy (Axioskop, Zeiss, Germany).

Analysis of DNA fragmentation
After the AEA treatment (10 µM) for 24 h, cells were washed with PBS, and permeabilized with 70% ethanol for 30 min, followed by incubation with PBS containing PI (250 µg/mL) for 15 min at room temperature in the dark. The PI fluorescence emission of individual nuclei was measured on a logarithmic scale by a FACS-can analyzer (Epics, Coulter, USA).

Determination of phosphatidyl serine (PS) externalization by FACS
To determine the externalization of PS, cells were plated onto 60 mm dishes at a density of 1 x 10⁶/well in MEDIUM 199 containing 0.1% BSA. Cells were treated with AEA (5, 10 µM) for 6 h, harvested, and incubated with annexin V-FITC (ImmunoTech, France) according to the manufacturer’s instruc-
Apoptosis in human endothelial cells induced by anandamide

Annexin V-positive cells were evaluated by the FACS-can analyzer.

Assays of MAPKs phosphorylation by immunoblotting

MAPKs were analyzed according to the manufacturer’s instructions (New England Biolabs Inc-Cell Signaling Technology, USA). Briefly, after the AEA treatment, cells were washed with ice-cold PBS, and cellular protein was extracted by the sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], and 1 mM Na2VO3). For immunoblotting, cell lysates were subjected to electrophoresis in 12% SDS-polyacrylamide gels. The separated proteins were transferred onto a nitrocellu-

Figure 1:
Cytotoxic effects of AEA on HUVECs. Cells were incubated in the presence or absence of AEA (10 µM) for 24 h. A: Control cells (0.01% ethanol). B: AEA-treated cells. Representative photographs were taken at the same magnification. Original magnification, x100. C, D: For the determination of cell viability by MTT assay, cells plated onto 96-well collagen-coated dishes were subjected to AEA, TNF-α (10 ng/mL)/CHX (10 µg/mL) was used as a positive control. To determine the effects of AEA, we used arachidonic acid (10 µM) as a control. C: Concentration-dependent cytotoxicity of AEA (0-10 µM) at 24 h. D: Time-course of AEA (10 µM)-induced cell death. E: Measurement of LDH release of AEA (10 µM)-treated HUVECs. * p < 0.05, ** p < 0.01 compared with untreated control cells.
lose membrane (Schleicher & Schell, USA), and washed with Tris-buffered saline, pH 7.6, containing 0.02% Tween 20 (TBST). The membrane was then blocked with 5% nonfat milk plus 1% BSA for 1 h at room temperature. After washing with TBST, the membrane was incubated with the respective antibody at 4°C overnight. After washing with TBST, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody for 1 h at room temperature. The antigen-antibody complexes were visualized by chemiluminescence (ECL detection system; Amersham Pharmacia Biotech, UK).

**Assay of caspase-3/CPP32 enzyme activity**
Caspase-3/CPP32 enzyme activity was analyzed according to the manufacturer’s instructions. Briefly, after the treatment, HUVECs plated onto 10-cm collagen coated dishes were washed, and lysed with chilled lysis buffer (50 mM Tris- HCl, pH 7.4, 1 mM EDTA, 10 mM EGTA, and 10 µM digitonin), and incubated for 10 min on ice, followed by centrifugation (20000 g for 1 min). The supernatant was transferred, and the activity of caspases recognizing the sequence DEVD (Asp-Glu-Val-Asp) was assayed at 405 nm using DEVD-pNA (MBL, Japan) as a substrate.

**Detection of VR1 expression on HUVECs by flowcytometry**
Cells were incubated with anti-VR1 antibody (Santa Cruz Biotechnology, USA) or with control goat IgG for 1 h at 4°C, followed by FITC-conjugated anti-goat IgG antibody (ICN Pharmaceuticals, Inc., USA) for 30 min at 4°C. After washing the cells with PBS, immunofluorescence staining was analyzed by the FACS-can analyzer.

**Message analysis of VR1 by reverse transcriptase-polymerase chain reaction (RT-PCR)**
Analysis of the RNA from HUVECs for the presence of a VR1 transcript was carried by means of RT-PCR. Total RNA was extracted from the cells (2 × 10^6 cells) using TRizol- Reagent (Invitrogen, USA) according to the manufacturer’s instructions. After treatment with RNase-free DNase, the purified total RNA (2 µg) was converted to cDNA by reverse transcriptase (RT, Gibco BRL, USA), and then RT-PCR was performed. The reaction mixture (50 µl) consisted of 100 ng of cDNA, 1 µM of each primer, 200 µmol of each deoxynucleotide triphosphate, and 2.0 units of Taq polymerase (Takara Biomedicals, Japan). The amplification profile consisted of an initial denaturation of 3 min at 95°C and 30 cycles of 1 min at 95°C, 1 min at 58°C, and 2 min at 72°C. A final extension of 3 min was carried out at 73°C. The primers used were the VR1 sense primer 5’-GTTCACCGAGTGGGCTA-3’ and the VR1 antisense primer 5’-AGCCACATCTCCTTGAG-3’. The expected size of the amplicon was 511 bp. GAPDH was used as the housekeeping gene. PCR products (20 µl) were electrophoresed on 3% agarose gels and visualized by ethidium bromide fluorostaining. We used HEK cells, which lack VR1, as a negative control, and no PCR product was detected in the absence of cDNA or the primers.

**Statistical analysis**
All experiments were performed in triplicate. The results of multiple observations were presented as the means ± S.D. of at least three separate experiments, unless otherwise stated. Statistical significance was assessed by Student’s t-test.

**Results**

**AEA causes a loss of cell viability among HUVECs**
To determine the possible role of AEA in the fate of HUVECs, we first examined the effects of AEA on HUVEC cell death. When exposed to AEA (10 µM) for 24 h, the HUVECs shrank and retracted from the neighboring cells, and floating cells appeared in the culture medium (Fig. 1B). AEA induced cell death in a concentration (Fig. 1C)- and time-dependent (Fig. 1D) manner such that approximately 40% of the cells were killed within 24 h at a concentration of 10 µM; however, arachidonic acid (10 µM) failed to affect the cells under the same experimental conditions. As shown in figure 1D, cell death was occurred within 4h of exposure to AEA (10 µM) and remained elevated at 24 h. To assess necrosis in AEA-treated HUVECs, we measured LDH release into the extracellular medium. Necrotic cells damage plasma membranes and therefore contribute to the release of LDH, and necrosis did not significantly take place in AEA-treated HUVECs (Fig. 1E).

**AEA induces apoptosis in HUVECs**
Following an apoptotic trigger, the subsequent chain of events leads to cell shrinkage, membrane blebbing, nuclear condensation, loss of membrane integrity, and enzymatic DNA degradation. We assessed DNA fragmentation by nuclear staining using a Hoechst 33258. In AEA-treated HUVECs, condensed chromatin or fragmented nuclei were visualized (Fig. 2B), which demonstrated that apoptosis was taking place. Next, AEA-induced apoptosis in HUVECs was examined by measuring the decrease in the cellular DNA content, as revealed by PI staining and flowcytometry. As shown in Fig. 2C, AEA (10 µM) induced a 2.34±0.07-fold increase in the number of cells that underwent apoptosis at 24 h.

Another important hallmark of apoptosis is the loss of aminophospholipid asymmetry of the plasma membrane (20). PS, which is confined to the inner membrane leaflet in normal healthy cells, becomes exposed to the outer plasma membrane of apoptotic cells. Exposure of PS is an early event, as it pre-
Apoptosis in human endothelial cells induced by anandamide

cedes cell membrane permeabilization, cell shrinkage, and nuclear condensation (21). To confirm further AEA-induced apoptosis in HUVECs, we next determined the exposure of PS. It is well known that growth factor deprivation of HUVECs induces apoptosis (22); approximately 18% of the control cells were annexin V-positive. As shown in Fig. 2D, the number of annexin V-positive cells increased by 21.5 and 45.9% with 5-µM and 10-µM addition of AEA, respectively, at 6 h. Taken together, our results strongly suggest that AEA induces apoptosis in HUVECs.

Figure 2:
Apoptotic features in AEA-treated HUVECs. A, B: AEA (10 µM)-induced apoptosis was determined by Hoechst staining, as described in Methods. A: Control cells. B: AEA-treated cells. Representative photographs were taken at the same magnification. Original magnification, × 400. C: Quantification of DNA fragmentation in HUVECs induced by AEA. Data were plotted on log histograms as fluorescence intensity (x axis) vs. relative cell number (y axis). *p=0.011, vs. untreated cells. D: Dose-dependent effects of AEA on PS externalization. Cells were treated with AEA (5, 10 µM) for 6 h and PS externalization was determined by an annexin V-FITC binding assay. Data were plotted as the annexin V FITC intensity (x axis) vs. the relative number of PI-positive cells (y axis).

For personal or educational use only. No other uses without permission. All rights reserved.
AEA triggers the phosphorylation of MAP kinases and Caspase-3/CPP32 in HUVECs

In our previous report, we demonstrated that AEA-induced cell death is accompanied by the generation of superoxide in PC-12 cells (14). It has also been reported that increased reactive oxygen species (ROS) trigger MAPK phosphorylation and cell death (23). To examine the effects of AEA on the activity of MAP kinases during apoptosis, the phosphorylation of MAP kinases was determined as described in Methods. A: phosphorylation of p38 kinase (upper panel); B: phosphorylation of JNK (upper panel); C: phosphorylation of p44/42 (upper panel) in AEA-treated cells; D: AEA-induced CPP-32-like protease activation in HUVECs. CPP-32-like protease activity was assayed as described in Methods. TNF-α (10 ng/mL)/CHX (10 µg/mL) was used as a positive control.

Figure 3:
Time-course effects of AEA on MAPKs and CPP-32-like protease activation. HUVECs were exposed to AEA (10 µM) for the indicated period of time. A-C: The phosphorylation of MAP kinases was determined as described in Methods. A: phosphorylation of p38 kinase (upper panel); B: phosphorylation of JNK (upper panel); C: phosphorylation of p44/42 (upper panel) in AEA-treated cells; D: AEA-induced CPP-32-like protease activation in HUVECs. CPP-32-like protease activity was assayed as described in Methods. TNF-α (10 ng/mL)/CHX (10 µg/mL) was used as a positive control. * p < 0.05, ** p < 0.01 compared with untreated control cells. E: Effects of MAPKs and Caspase inhibitors on AEA-induced cell death in HUVECs. Values are expressed as % cell death induced by AEA (10 µM) for 6 h after the pre-incubation of each compound for 2 h (MTT assay). * p < 0.05 compared with AEA-treated cells.
Since caspase-3 plays an important role in apoptosis, we investigated whether or not caspase-3 was involved in AEA-induced apoptosis, and we confirmed that AEA induced CPP32-like protease activation in a time-dependent manner in HUVECs. As shown in Figure 3D, AEA stimulated a 1.86 ± 0.10-fold increase in caspase-3-like activities at 6 h and a 4.9 ± 0.15-fold increase at 12 h, indicating that CPP32-like protease is involved in AEA-induced HUVEC death. The apoptosis induced by AEA could also be effectively inhibited by pretreatment with a caspase-3/CPP32-specific inhibitor (100 µM), and cell viability increased by 47.6% in comparison with that of the control (Fig. 3E). To determine whether or not p38 MAPK phosphorylation and caspase activation are required for the AEA-induced death of HUVECs, we pretreated cells with MAPK inhibitors and caspase inhibitors. Cell death caused by AEA was significantly attenuated in cells pretreated with SB203580, caspase inhibitor-1 (zVAD-fmk), and caspase-9 inhibitor, whereas U0126 and caspase-8 inhibitor had no protective effects (Fig. 3E). These data suggest that activation of p38 MAP kinase and the caspase-9 pathway is involved in AEA-induced cell death in HUVECs.

Regulation of AEA-induced cell death in HUVECs by VR1 and the AEA degradation system

In order to investigate the possible role of cannabinoid receptors on AEA-induced cell death in HUVECs, the two following specific CB receptor antagonists were used: SR141716 and SR144528, which bind to CB1 and CB2, respectively (24, 25). Figure 4A shows that the CB2 antagonist SR144528 (0.1 µM) was ineffective; however, CB1 antagonist SR141716 (0.1 µM) significantly increased AEA-induced cell death in HUVECs, suggesting that CB1 may protect cells against the cytotoxicity of AEA. The FAAH inhibitor MAFP (1 µM) significantly increased the AEA toxicity (to approximately 1.74-fold). We next tested the ability of capsazone, a selective inhibitor of VR1 (26), to inhibit AEA-induced apoptosis in HUVECs, and 1 µM of capsazone reduced cell death to 12% of the AEA-treated cells. Moreover, the physiological agonist of vanilloid receptor1, capsaicin (16), increased AEA-induced cell death to approximately 10%, suggesting that VR1, at least in part, mediates AEA-induced apoptosis in HUVECs.

Expression of VR1 in HUVECs

VR1 expression on the surface of HUVECs was examined by using an antibody specific for human VR1. Figure 4B illustrates the expression of VR1 on the cell surface, as revealed by a FACS analyzer. Next, we examined the expression of VR1 mRNA in HUVECs by RT-PCR (Fig. 4C). The amplified fragment was analyzed by agarose gel electrophoresis and showed a single band of the molecular size (511 bp) that was expected from the human VR1-encoding cDNA fragment.

Discussion

In the present study, we demonstrated that AEA caused time- and dose-dependent cell death in HUVECs; we also investigated whether AEA would increase MAPK activity, because MAPKs are the most well-studied protein kinases that play a central role in cell survival and apoptosis signaling pathways. We demonstrated that AEA activated p38 MAPK and JNK, but not ERK, in early the phase of exposure in HUVECs. Furthermore, we also evaluated that SB203580, a p38 MAPK inhibitor, protected cells from AEA cytotoxicity, whereas U0126 did not affect the degree of cell death. Recently, we investigated the role of MAPKs in AEA-treated PC12 cells using inhibitors or dominant negative mutants of MAPKs, and we found that activated p38 MAPK and JNK, but not ERK1/2, mediated AEA-induced cell death (27). Thus, the activation of JNK/p38 MAPK in AEA-treated endothelial cells may be positively related to cell death.

We also assessed the effect of caspase inhibitors on AEA-induced endothelial cell death. It is believed that death protease caspase activation may be occurred in a mitochondrial-dependent and –independent manner, and it has already been established that caspase-9 and caspase-8 are activated before caspase-3 in mitochondrial-dependent and receptor-dependent pathways, respectively. We found that an inhibitor of caspase-9, but not of caspase-8, inhibited anandamide-induced cell death, in a result consistent with our previous results (27); these findings suggested that AEA induces a cell death, mitochondria-dependent pathway.

In the present study, we investigated the role of cannabinoid receptors using antagonists of CB1, and CB2. Consistent with the results of Maccarrone et al, it was observed that inhibition of CB1 did not inhibit AEA-induced cell death, rather enhanced the cell death. These data extend to previous observations of HUVECs as regards the ability of CB1 receptors to protect human cells against the pro-apoptotic activity of AEA (15), suggesting that cannabinoid receptors may not be involved in AEA-induced cell death. We then investigated whether or not a selective VR1 antagonist, capsazone, and a VR1 agonist, capsaicin (16), exert influence on AEA-induced cell death in HUVECs. It has previously been suggested that AEA behaves as a full agonist at human vanilloid receptors (16), the activation of which can induce apoptosis in various cells (16, 28, 29). This receptor, referred to as the VR1 receptor, is a ligand-gated, nonselective cation channel, and is most abundant in sensory neurons, although it is also found in select areas of the central nervous system of rats and humans (30). We demonstrat-
Yamaji, et al. ed that capsazepine was able to inhibit AEA-induced cell death significantly in HUVECs. In agreement with this result, we observed that capsaicin mimicked the pro-apoptotic activity of AEA in HUVECs, which express VR1. These data suggest that AEA-induced cell death in HUVECs is mediated by VR1.

AEA has been suggested to activate VR1 by acting from the cytosolic site of the cell, as the capsaicin binding domain on this

Figure 4:
Regulation of AEA-induced cell death in HUVECs by VR1 and the AEA degradation system and VR1 expression in HUVECs. A: Values are expressed as % cell death induced by AEA (10 µM) for 6 h after the pre-incubation of each compound for 2 h (MTT assay). Treatment of each cell with any of the compounds listed, in the absence of AEA, did not significantly affect cell death under the same experimental conditions. * p < 0.05 compared with AEA-treated cells. B, C: VR1 expression in HUVECs. B: Flowcytometric analysis of HUVECs stained with anti-VR1 antibody. The x-axis indicates fluorescence intensity and the y-axis indicates the number of cells. To determine VR1 expression, cells were incubated with anti-VR1 antibody (filled curve) or with control goat IgG (open curve). C: RT-PCR analysis of human VR1 and GAPDH gene expression in HUVECs and HEK cells. Amplification was carried out by using specific primers for the human VR1 receptor and human GAPDH.
Apoptosis in human endothelial cells induced by anandamide

site is intracellular (31). Petrocellis et al. have also suggested that the degradation of AEA by FAAH limits AEA activity on VR1 (32). The inhibitor of FAAH, MAFP, greatly increased AEA-induced EC death, appearing that AEA degradation system may be involved in HUVECs death. These results altogether suggest that AEA could be transported into the intracellular level via anandamide membrane transporter, and binds to VR1, which, in turn, induces apoptosis in HUVECs, and inhibition of AEA degrading system may enhance binding of anandamide to VR1 and accelerate AEA-induced cell death in HUVECs.

However, capsazepine was unable to block AEA-induced cell death completely in HUVECs; we therefore concluded that VR1, at least in part, mediates AEA-induced cell death in HUVECs.

It has been demonstrated that AEA increases during shock conditions. The fundamental pathologic lesion in idiopathic thrombocytopenic purpura (TTP) and adult/sporadic hemolytic uremic syndrome (HUS) is thrombotic microangiopathy, which is accompanied by localized endothelial injury, in the absence of an inflammatory response (33). Laurence and co-workers have documented the ability of plasmas from TTP and sporadic HUS patients to induce apoptosis of cultured microvascular endothelial cells (MVECs) (34); however, the mechanism of endothelial cell apoptosis in HUS is still unknown. Very recently, we obtained data suggesting that endogenous AEA is extremely elevated in the serum from HUS patients (unpublished data, 2002). These data also suggest that increased levels of endogenous cannabinoids may play a crucial role in endothelial injury. Our present study supports the pathophysiological significance of AEA-induced EC apoptosis during shock conditions, and suggests the appropriateness of considering apoptosis inhibitors for the experimental therapeutics applied under these conditions.

Acknowledgment
We thank N. Uto for her technical assistance. This work was supported by the Toray Pharmaceutical Co (Tokyo, Japan).

References