Propofol Protects against Anandamide-Induced Injury in Human Umbilical Vein Endothelial Cells

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Summary: Endocannabinoid anandamide, arachidonylethanolamine (AEA), is considered to be a causative mediator of hemorrhagic or septic shock, inducing death of several types of cells by producing free radicals such as reactive oxygen species (ROS). Propofol contains a phenolic hydroxyl group that donates electrons to the free radicals, and thus functions as an antioxidant. The purpose of this study was to investigate the protective effect of propofol against AEA-induced cell injury. After incubation with propofol at concentrations of 10, 50 or 100 μM, human umbilical vein endothelial cells (HUVECs) were stimulated with 10 μM of AEA for 24 h. ROS production, caspase-3 activity, and cell viability were evaluated 1, 8, and 24 h after the administration of 10 μM of AEA, respectively. Propofol (50 μM) significantly attenuated cell death induced by AEA, showing a protective effect against ROS production and caspase-3 activity. These results suggest that propofol at concentrations used during clinical anesthesia protects HUVECs against AEA-induced injury, in part by suppressing apoptosis.

Key words propofol, anandamide, apoptosis, reactive oxygen species, antioxidant, fatty amide acid hydrolase, human umbilical vein endothelial cell

INTRODUCTION

The endocannabinoid anandamide, (i.e., arachidonylethanolamine (AEA)) has been isolated from porcine brain lipid extract as an endogenous ligand for cannabinoid receptors in the central nervous and immune systems [1]. AEA is synthesized from N-arachidonoyl phosphatidylethanolamine in depolarized neurons, macrophages, endothelial cells and platelets [2-4], and quickly degraded by the fatty amide acid hydrolase (FAAH) into arachidonic acid and ethanolamine. In normal humans, AEA exists at low levels in blood and cerebrospinal fluid [5-7]. However, it has been demonstrated that the serum levels of AEA increase dramatically during the shock caused by either hemorrhage [8] or sepsis [9], and play a crucial role in the pathogenesis of hypotension [10,11]. Furthermore, in several types of cells, elevated levels of AEA can induce apoptosis by producing free radicals such as reactive oxygen species (ROS), and the production of ROS is exacerbated by the inhibition of FAAH [12-14].

Vascular endothelial cells have important physiologic functions as barriers, and in maintaining cardiovascular homeostasis and vascular stability. However, this function may be impaired in septic shock and ischemia-reperfusion injury, resulting in cellular necrosis and apoptosis [15,16]. On the other hand,
propofol (2,6-diisopropylphenol), an intravenous general anesthetic, possesses an antioxidant property because it contains a phenolic hydroxyl group that gives electrons to the free radicals generated during ischemia and reperfusion [17,18]. The purpose of the present study was to examine the protective effect of propofol against AEA-induced cell injury using human umbilical vein endothelial cells (HUVECs).

MATERIALS AND METHODS

Cell culture

The initial batch of HUVECs was purchased from Lonza, Inc. (Basel, Switzerland), and cultured in endothelial growth medium-2 (EGM-2), consisting of ascorbic acid, fibroblast growth factor, hydrocortisone, insulin-like growth factor-1, vascular endothelial growth factor, gentamicin, amphotericin-B, and 10% fetal bovine serum (FBS). The cells were grown in a humidified incubator at 37°C containing 95% air and 5% of carbon dioxide with media replenishment every 3 days. Following growth to 90% confluence, the cells were split (passage 2) and grown to confluence again. Before the experimental intervention, confluent HUVECs with 2-5 passages were incubated in a starved medium supplemented with 1% of FBS for 4 h. In the ROS production assay and the caspase-3 activity assay, HUVECs were divided into three experimental groups characterized by culture medium conditions as 1) control, 2) cultured with AEA alone, or 3) pretreatment with propofol for 30 min, then co-incubated with AEA.

Evaluation of HUVECs viability exposed to AEA

As shown in Figure 1A, the HUVECs were co-incubated with AEA (0, 0.01, 0.1, 1, 2.5, 5, 7.5 and 10 μM) at 37°C for 24 h. Then, the cell viability was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, 10 μL of MTT (Sigma Chemical, St. Louis, USA) solution (5 mg/L) was administered to each incubating well, and the wells were incubated at 37°C for 5 h. The formazan in each well, produced by the MTT assay, was dissolved in 100 μL of dimethyl sulfoxide. The absorbance of this colored solution was measured at 570 nm by a spectrophotometer. According to the results of this experiment, 10 μM of AEA was utilized as a positive control for AEA-induced cell injury in all subsequent experiments.

Effect of propofol on HUVEC viability

Propofol was prepared by diluting Diprivan (As- trazeneca, London, UK) with EGM-2 to a concentration of 10, 50, or 100 μM. After pretreatment of the culture medium with propofol for 30 min at the above concentrations, the HUVECs were stimulated without or with 10 μM of AEA and incubated at 37°C for 24 h. Thereafter, the cell viability was observed employing the MTT assay as mentioned above (Fig. 1B).

Analysis of ROS production

The Image-iT™ live green reactive oxygen species detection system (Molecular Probes, Eugene, OR), counterstained with Hoechst 33342 for nuclei, was used to visualize reactive oxygen species in live HUVECs under a microscope (Olympus FV1000, Tokyo, Japan), using fluorescein filter sets.

Intracellular ROS formation was detected using 5-(and-6)-carboxy-2’,7’-dichlorodihydrofluorescein diacetate (carboxyl H2DCFDA) as previously reported [19]. Briefly, starved HUVECs, seeded at a density of 2.0×10^4 cells /well, were loaded with the redox sensitive dye carboxyl H2DCFDA for 45 min, washed and pretreated with propofol (50 μM), and then stimulated with AEA alone (10 μM) or in combination with propofol (50 μM) at 37°C for 1 h (Fig. 1C). Then, ROS levels were measured with a multiwell fluorescence plate reader (Tecan, Männedorf, Switzerland), using excitation and emission filters of 485 nm and 535 nm, respectively.

![Fig. 1. Experimental protocol. (A) The HUVECs viability was examined using an MTT assay 24 h following the exposure to 0-10 μM of AEA. (B) The effect of propofol (0-100 μM) on the HUVECs viability was evaluated with the use of an MTT assay 24 h after the exposure to 0, 10 μM of AEA. (C) The intracellular ROS production in HUVECs was detected by spectrophotometry 1 h after the stimulation by AEA (10 μM) or coexistence with propofol (50 μM). (D) The caspase-3 activity in HUVECs was colorimetrically measured 8 h following the exposure to AEA (10 μM) or coexistence with propofol (50 μM).](image-url)
Caspase-3 activity assay

The caspase-3 activity in the incubated HUVECs was colorimetrically assayed 8 h following treatment with AEA alone (10 μM) or in combination with 50 μM of propofol (Fig. 1D). The cells were washed, lysed, and incubated with the caspase-3 specific labeled substrate, N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA), on ice for 15 min. The chromophore p-nitroanilide (pNA), which was released from Ac-DEVD-pNA upon cleavage by caspase-3, could be quantified using a microtiter plate reader at 405 nm. The relative increase in caspase-3 activity was determined by comparing the absorbance of pNA from an apoptotic sample to an uninduced control.

Statistical analysis

All data represent the mean of three independent experiments ± S.D. Statistical comparisons were made with a paired t-test or one sample test, followed by the Bonferroni test. Statistical analysis was performed with SAS ver.9.2 (SAS Institute Inc., Cary, NC, USA). A value of \( P<0.05 \) was considered to be statistically significant.

RESULTS

Reduction in HUVECs viability due to AEA

Evaluating cell viability by an MTT assay 24 h after the AEA exposure showed that 5 μM, 7.5 μM and 10 μM of AEA caused a significant deterioration of cultured HUVECs viability to 79.2±2.3%, 58.3±1.2% and 38.5±2.1%, respectively, compared with the control (Fig. 2).

Fig. 2. Cytotoxicity of AEA in HUVECs. The HUVECs viability was significantly attenuated at 5 μM, 7.5 μM and 10 μM of AEA to 79.2±2.3%, 58.3±1.2% and 38.5±2.1% of the control, respectively. *P<0.05 vs. non-treated cells.

Protective effect of propofol on HUVECs viability

Propofol itself did not affect the cell viability at the concentrations of 10-100 μM. Of great importance was that pretreatment with 50 and 100 μMs of propofol significantly increased viability in cells exposed to 10 μM of AEA from 29.7±3.2%, to 67.9±11.0% and 78.4±6.5%, respectively, although 10 μM of propofol had no protective effect (Fig. 3).

Decrease in ROS production by propofol

HUVECs stimulated with AEA alone were ROS-positive (green) (Fig. 4B), whereas the pretreatment of cells with propofol significantly attenuated the green ROS signal (Fig. 4C), and almost no green ROS signal was observed in controls (Fig. 4A).

ROS production in the cultured HUVECs 1 h after exposure to 10 μM of AEA was significantly attenuated by 50 μM of propofol, falling from 113.8±2.0% in the AEA-exposed cells to 103.7±1.0% in the propofol treated cells. (Fig. 4D)

Protected caspase-3 activity by propofol

Caspase-3 activity 8 h after the exposure to 10 μM of AEA in the cultured HUVECs was significantly ameliorated by 50 μM of propofol, dropping from 277.6±83.3% in the AEA-exposed cells, to 132.1±12.0% in the propofol-treated cells (Fig. 5).

DISCUSSION

This study demonstrated the protective effects of propofol against AEA-induced cell injury. One of the crucial mechanisms of cell injury due to anandamide is surmised to be ROS production followed by apopto-
Sieg mund et al. [20] reported that glutathione, an antioxidant, attenuated the AEA-derived ROS formation and effectively suppressed the death of primary hepatic stellate cells. Propofol is also known to have antioxidant activity in scavenging ROS and suppressing apoptosis, coinciding with our results [17,18,21-25].

Lipid rafts, localized in gamma-aminobutyric acid A receptors, are supposed to play an important role in AEA-induced cell death [12,26]. One possible protective mechanism of propofol might be its action on these receptors [27].

As mentioned above, AEA is metabolized by FAAH and the inhibition of FAAH could enhance AEA-induced cellular toxicity. Meanwhile, propofol is thought to be an FAAH inhibitor [28]. However, Schelling et al. [29] observed no remarkable increase in blood level of AEA during general anesthesia using propofol. It is not clear whether the mechanism by which propofol protected HUVECs against AEA-induced injury in this study involved the inhibition of FAAH.

Fig. 4. Attenuation of intracellular ROS production by propofol. The representative photographs of visualized ROS formation in the control (A), 10 μM of AEA-exposed (B) and 50 μM of propofol-treated (C) HUVECs were shown. (D) The ROS production was significantly increased by 10 μM of AEA (113.8±2.0%). Propofol significantly attenuated the AEA-derived ROS production to 103.7±1.0%. *P<0.05 vs. control, **P<0.05 vs. propofol treated.
It is notable that in the present study propofol exerted a protective effect at a blood concentration level used clinically to achieve general anesthesia, (i.e., 10-60 μM). This finding suggests the clinical usefulness of propofol as a general anesthetic for patients with shock caused by hemorrhage, endotoxin, and so forth.

There have been a number of reports on the blood concentration of AEA in normal humans, most of which were conducted on the nM-level [3,9,12]. However, this could increase to the μM-level in endotoxic shock, and a level of over 10 μM, at which the death of cells was observed, could be considered a pathologic state. Concerning anandamide-induced cell death, the involvement of nitric oxide and several receptors of cannabinoid and capsaicin, and the differences in pathway leading to cell death depending on the types of cell have been suggested [2,13,30,31]. Further investigations are needed to clarify both the mechanism of and the therapeutic strategy for anandamide-induced cell death.

In conclusion, our results suggest that propofol at clinically used blood concentrations protects HUVECs against AEA-induced injury, in part by suppressing ROS production and subsequent apoptosis.

REFERENCES


