Propofol and Aminophylline Antagonize Each Other During the Mobilization of Intracellular Calcium in Human Umbilical Vein Endothelial Cells

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INTRODUCTION

Propofol is a widely used, intravenous, general anesthetic that provides long-term sedation of patients in intensive-care units, with effects on the central nervous and cardiovascular systems as a modulator of intracellular calcium mobilization (1-3). Propofol suppresses the mobilization of intracellular calcium in myocytes, astrocytoma cells, pituitary cells, and endothelial cells (2-6).

We examined whether propofol affects the mobilization of intracellular calcium in human umbilical vein endothelial cells (HUVECs). Furthermore, we examined whether propofol gave influences on the mobilization of intracellular calcium by lysophosphatidic acid (LPA), which stimulates intracellular calcium mobilization (7, 8). We also tested whether other commonly used drugs in intensive-care units stimulated the mobilization of intracellular calcium. Aminophylline, a bronchodilator for asthmatics, stimulated the mobilization of intracellular calcium in HUVECs (9, 10). However, the interaction of propofol and aminophylline, which affects the mobilization of intracellular calcium, has never been studied in vitro. Therefore, we studied whether propofol and aminophylline affect the mobilization of intracellular calcium in HUVECs.

MATERIALS AND METHODS

Chemicals and reagents
Fetal bovine serum, bovine serum albumin, penicillin/streptomycin solution, and M199 medium were obtained from Gibco-BRL (Gaithersburg, MD, USA). Fluo-4-AM was obtained from Molecular Probes (Eugene, OR, USA). Propofol was obtained from Jeil Pharmaceutical Co. (Seoul, Korea). Aminophylline was obtained from Daewon Pharmaceutical Co. (Seoul, Korea), and LPA was obtained from Sigma (St. Louis, MO, USA).

Cell culture
HUVECs were obtained from Kangwon National University Hospital. HUVECs were prepared from human umbilical veins, which were aseptically removed from the placenta immediately after birth. HUVECs were isolated using a modified method by which the umbilical vein was washed with phosphate buffered saline (PBS) solution, followed by treatment with collagenase.
for 10 min at 37°C. The perfusate was centrifuged at 1,000 r.p.m. for 10 min and the resulting cells were cultured on 0.2% gelatin-coated dishes in M199 medium supplemented with 10% heat-inactivated fetal bovine serum, 5% heat-inactivated human serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 90 µg/mL heparin, and 20 µg/mL endothelial cell growth factor (ECGF) at 37°C in a 5% CO2 humid incubator until 80% confluent. Only adherent confluent cells originating from the same umbilical cord were used in each experimental group. Passages three to four were used for all experiments. The cells were analyzed in six multi-well plates (the area of each well was 9.6 cm2), with a mean value of 100,000 cells/cm2. Endothelial cells were identified by their typical morphological pattern (cobbledstone morphology), the presence of monolayers, and positive detection of von Willebrand factor. The quality of the monolayer was monitored by inverted microscopy (Diaphot 100; Nikon, Tokyo, Japan). Cells were starved for 6 hr in M199 medium supplemented with 1% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 90 µg/mL heparin and 20 µg/mL ECGF (starvation-medium) prior to any pharmacological treatment.

Measurement of intracellular Ca2+

Changes in [Ca2+] were monitored by laser scanning confocal microscopy, as described by Lee et al. (11). Briefly, cells were grown on round coverslips in multi-well culture plates, serum-starved for 6 hr and then incubated with 2 µM of Fluo-4-AM in starvation medium for 40 min. Round coverslips containing the stained cells were mounted on a perfusion chamber (MPS-1000, Seoul Engineering, Seoul, Korea) and examined by laser scanning confocal microscopy (Carl Zeiss LSM 410, Heidenheim, Germany). After this, coverslips were scanned with a 488 nm excitation argon laser and observed through a 515 nm longpass emission filter.

In addition, cells were incubated with propofol (0; 10; 30; 100; 300; 1,000 µM) for 30 min, followed by stimulation with LPA (0; 5 µg/mL) and aminophylline (0; 30; 100; 300; 1,000 µM) to measure the effects of propofol against stimulation-induced changes in [Ca2+]. Cells were also incubated with the indicated concentrations of propofol (0; 10; 30 µM) and aminophylline (0; 100; 1,000 µM) for 30 min, followed by stimulation with LPA (0; 5 µg/mL) and aminophylline (0; 100; 1,000 µM) to measure the interaction of propofol and aminophylline on the mobilization of [Ca2+].

Confirmation of intracellular calcium by illumination was demonstrated by the appearance of ‘hot spots’ in both the cytoplasmic and nuclear regions following the addition of LPA, propofol, or aminophylline. The increased density of hot spots was analyzed by automated recording of the same region of a cell and then plotted with time. The average density of hot spots within a cell was an average of 10 areas. Continuous recordings of the cell were made by the automatic photography system by scanning every 10 sec. The results are expressed as relative fluorescence intensity (RFI) for changes of [Ca2+], at the single cell level and expressed as fold stimulation (mean±SD) determined by comparing RFIs before stimulation from three separate determinations. Each determination represents the mean of at least 10 cells.

Data analysis

The data are presented as the mean±standard deviation (SD). Data were analyzed by performing repeated measurements of one-way analysis of variance (ANOVA) for the serial comparisons before and after stimulation by LPA, propofol, or aminophylline within the cell and ANOVA between the cells, followed by the Scheffe test. In all comparisons, a P value less than 0.05 was considered statistically significant. The statistical analyses were performed using the SPSS program (version 12.0; SPSS Inc., Chicago, IL, USA).

RESULTS

The fluorescence intensity in untreated HUVECs loaded with Fluo-4 was very low and distributed homogeneously throughout the cells (Fig. 1A). Fluo-4 fluorescence in cells treated with 5 µg/mL of LPA or 1,000 µM of aminophylline increased rapidly (Fig. 1C, D). Propofol (300 µM) blocked the increase induced by

Fig. 1. Fluorescence of intracellular calcium in human umbilical-vein endothelial cells (HUVECs) incubated with Fluo-4 and detected by a fluorescence spectrophotometer (confocal microscope). (A) Control group (resting cells) before treatment. (B) Preincubation with propofol (300 µM) blocks lysophosphatidic acid (LPA) signals. (C) Aminophylline (1,000 µM) treatment increases Fluo-4 fluorescence. (D) LPA (5 µg/mL) increases Fluo-4 fluorescence.
Aminophylline induced a very rapid, dose-dependent increase in [Ca\(^{2+}\)]\(_i\) (Fig. 2A), but propofol decreased the concentration of [Ca\(^{2+}\)]\(_i\). Aminophylline (1,000 μM) rapidly increased Fluo-4 fluorescence in cells to a maximum three to four-fold higher than the control (Fig. 3). Propofol (10 μM) treatment for 30 min decreased [Ca\(^{2+}\)]\(_i\), induced by LPA (5 μg/mL) and aminophylline (100 μM) (Fig. 4A). Propofol (30 μM) showed similar activity (Fig. 4B). Following incubation with propofol (30 μM) and aminophylline (100; 1,000 μM) for 30 min, the peak level of [Ca\(^{2+}\)]\(_i\), following LPA (5 μg/mL) treatment was higher than propofol (30 μM) only (Fig. 5). Furthermore, HUVECs incubated with propofol (30 μM) and aminophylline (1,000 μM) for 30 min had peak [Ca\(^{2+}\)] levels higher than propofol (30 μM) and aminophylline (100 μM) (Fig. 5).

**DISCUSSION**

In this study, propofol decreased the concentration of [Ca\(^{2+}\)]\(_i\) in HUVECs, whereas aminophylline (30-1,000 μM) increased mobilization of [Ca\(^{2+}\)]\(_i\), in a concentration-dependent manner. Propofol (10-1,000 μM) suppressed the LPA-induced mobilization of [Ca\(^{2+}\)]\(_i\), in a concentration-dependent manner. Propofol further prevented the aminophylline-induced increase of [Ca\(^{2+}\)]\(_i\) at clinically relevant concentrations (10 and 30 μM). However, aminophylline reversed the inhibitory effect of propofol on the elevation of [Ca\(^{2+}\)]\(_i\), by LPA.

Cells employ various mechanisms for regulating the concentration of free Ca\(^{2+}\) in the cytosol, which usually is maintained...
In this study, we determined changes of [Ca\textsuperscript{2+}]i that are mediated by that specific substance, rather than directly measuring propofol (30 µM) and aminophylline (100 µM)-incubated cells. Each determination represents the mean of at least 10 cells. Values represent the mean peak intracellular calcium response. Results are expressed as fold-stimulation, determined by comparing relative fluorescence intensities (RFI) before stimulation and expressed as mean ± SD from three separate determinations.

Below 0.2 µM, therefore, most investigators measure the effect of a substance on the increase and/or decrease of [Ca\textsuperscript{2+}], mediated by that specific substance, rather than directly measuring [Ca\textsuperscript{2+}] (5). In this study, we determined changes of [Ca\textsuperscript{2+}], that occurred in response to LPA.

LPA has multiple cellular effects, including the stimulation of phospholipases, the mobilization of [Ca\textsuperscript{2+}], and the inhibition of adenylate cyclase (12). The extracellular addition of LPA increased [Ca\textsuperscript{2+}] through mobilization of intracellular calcium stores as well as through the influx of extracellular Ca\textsuperscript{2+} (6). However, the ability of LPA to stimulate increases in [Ca\textsuperscript{2+}] is mediated predominantly via the mobilization of calcium stores (13). This action is transduced by the production of inositol trisphosphate (IP\textsubscript{3}) through the G-protein-mediated activation of phospholipase C (12). In this study, propofol decreased the concentration of [Ca\textsuperscript{2+}], in HUVECs as in other cells (2-6). Also, propofol suppressed the increase of [Ca\textsuperscript{2+}] by LPA in a concentration-dependent manner.

Propofol is a brief-acting, intravenous, anesthetic agent possessing significant cardiovascular effects (14). Systemic hypotension caused by a marked decrease in systemic vascular resistance is often observed in the clinical use of propofol, probably via a decrease in [Ca\textsuperscript{2+}]. The negative inotropic and vasodilatory effects associated with propofol are likely due the voltage-gated influx of extracellular calcium being blocked, and to the decrease in availability of intracellular calcium (1, 4). Propofol affects [Ca\textsuperscript{2+}], by inhibiting the mobilization of intracellular-calcium stores (6). It also blocks the voltage-gated influx of extracellular Ca\textsuperscript{2+} by acting as a calcium channel blocker (6). In endothelial cells, the activation of adenosine-trisphosphate-sensitive potassium channels (K\textsuperscript{ATP} channels) is dependent upon L-type, voltage-gated calcium channels, and causes an increase in K\textsuperscript{+} efflux, membrane hyperpolarization and calcium influx (15). Propofol attenuates the effect of K\textsuperscript{ATP} channels by inhibiting the nitric oxide pathway (16). The mobilization of calcium from intracellular stores is related to the hydrolysis of inositol-4,5-bisphosphate (PIP\textsubscript{2}) to diacylglycerol and IP\textsubscript{3} (17). IP\textsubscript{3} releases internal calcium in endothelial cells, therefore triggering the calcium cascade and its associated biological process, such as vasocon-

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**Fig. 4.** Levels of mean peak intracellular calcium ([Ca\textsuperscript{2+}]i) generated by lysophosphatic acid (LPA) or aminophylline treatment after incubation with propofol (10, 30 µM) for 30 min. (A) Levels of mean peak intracellular calcium ([Ca\textsuperscript{2+}]i) generated by lysophosphatic acid (LPA) or aminophylline treatment after incubation with propofol (10 µM) and for 30 min. LPA only: treatment with LPA (5 µg/mL); P10→LPA: incubation with propofol (10 µM) and treatment with LPA (5 µg/mL); P30→A100: incubation with propofol (10 µM) and treatment with aminophylline (100 µM); P10→A100: incubation with propofol (10 µM) and treatment with aminophylline (100 µM) Values represent the mean peak intracellular calcium response. Results are expressed as fold-stimulation, determined by comparing relative fluorescence intensities (RFI) before stimulation and expressed as mean ± SD from three separate determinations. Each determination represents the mean of at least 10 cells. *P<0.05 compared with propofol (30 µM)-incubated cells; †P<0.05 compared with treatment with LPA (5 µg/mL).

**Fig. 5.** Levels of mean peak intracellular calcium ([Ca\textsuperscript{2+}]i) generated by lysophosphatic acid (LPA) treatment after incubation with propofol (30 µM) and aminophylline for 30 min. LPA (s): treatment with LPA (5 µg/mL); P30→LPA (s): incubation with propofol (30 µM) and treatment with LPA (5 µg/mL); P30→A100→LPA (s): incubation with propofol (30 µM) and aminophylline (100 µM) and treatment with LPA (5 µg/mL); P30→A1000→LPA (s): incubation with propofol (30 µM) and aminophylline (1,000 µM) and treatment with LPA (5 µg/mL).

Values represent the mean peak intracellular calcium response. Results are expressed as fold-stimulation, determined by comparing relative fluorescence intensities (RFI) before stimulation and expressed as mean ± SD from three separate determinations. Each determination represents the mean of at least 10 cells.

*P<0.05 compared with propofol (30 µM)-incubated cells; †P<0.05 compared with aminophylline (100 µM)-incubated cells.
striction (18). Propofol reduces the production of IP3 and can thus act as a calcium blocker preventing the entry of calcium into the vasodilatation pathway (18). It further inhibits the entry of capacitative calcium and modulates Na+-Ca2+ exchange activity via the protein kinase C-signaling pathway (19, 20). The activation of protein kinase C by phorbol esters can be inhibited by propofol (20). In the present study, propofol suppressed the LPA-induced mobilization of [Ca2+]i in HUVECs in a dose-dependent manner, presumably via one of these mechanisms.

Aminophylline is a bronchodilating drug widely used for the treatment of asthma and chronic airway-obstructing diseases (21). Aminophylline exerts multiple pharmacological effects either through phosphodiesterase inhibition or via adenosine receptor blockage (22, 23). It furthermore activates cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) (24) and enhances calcium currents. Adenosine inhibits calcium channels through a pertussis-toxin-sensitive G protein (24). In the present study, aminophylline increased intracellular calcium in a concentration-dependent manner in HUVECs. Specifically, aminophylline probably enhanced calcium release from the sarcoplasmic reticulum, along with the influx of extracellular calcium.

In the present study, there are two possible cellular mechanisms of the opposing effects of propofol and aminophylline on intracellular calcium mobilization. First, propofol blocks the voltage-gated influx of extracellular Ca2+ by acting as a calcium channel blocker (6), but aminophylline induced an influx of Ca2+ by activating calcium channels (23, 24). Second, propofol reduces the production of IP3 and calcium release from intracellular stores (16, 17), but aminophylline induced Ca2+ release from intracellular stores, occurs via increased production of IP3 through G-protein-mediated activation (21, 25).

Aminophylline has a narrow therapeutic index (9), with side effects of nausea, headache, and diuresis. The misuse or abuse of aminophylline can cause life-threatening cardiovascular conditions, such as cardiac arrhythmias and seizures (9, 10). Overdoses of aminophylline are the most common cause of death due to hospital-related asthma (10). Serious toxicity has been reported when high, prolonged doses of aminophylline are administered through continuous intravenous infusion (26). Aminophylline changes intracellular calcium concentrations (27), and calcium plays a major role in aminophylline-induced toxicity and death (28).

Propofol is widely used in intensive care medicine, especially for the long-term sedation of critically ill patients (29). Side effects, such as hypotension, vasodilation, and propofol infusion syndrome, are possible when used clinically. These side effects act as calcium channel blockers that cause cardiovascular depression (1). Aminophylline reverses propofol-induced postoperative sedation (22).

In this study, propofol and aminophylline showed opposing effects on intracellular calcium mobilization in HUVECs. Although this may decrease drug-related side effects, it may also decrease therapeutic effects. Therefore, clinicians should give adequate doses when administered together. Further work is required to understand the mechanism of interaction of propofol and aminophylline on intracellular calcium mobilization in HUVECs and its effects in the other cells such as bronchial and neuronal cells.

In conclusion, propofol and aminophylline antagonize [Ca2+]i mobilization at clinically relevant concentrations in HUVECs. Serious consideration should be given to drug interactions on intracellular calcium mobilization when used together.

REFERENCES

1. Cook DJ, Housmans PR. Mechanism of the negative inotropic effect of propofol in isolated ferret ventricular myocardium. Anaesthesia 1994; 80: 859-71.
2. Zhou W, Fontenot HJ, Liu S, Kennedy RH. Modulation of cardiac calcium channels by propofol. Anaesthesiology 1997; 86: 670-5.
3. Barhoumi R, Burghardt RC, Qian Y, Tiffany-Castigioni E. Effects of propofol on intracellular Ca2+ homeostasis in human astrocytoma cells. Brain Res 2007; 1145: 11-8.
4. Ya Deau JT, Morelli CM, Desravines S. Inhibition by propofol of intracellular calcium mobilization in cultured mouse pituitary cells. Anesth Analg 2003; 97: 1325-30.
5. Ryu TG, Kim NS, Min YD, Ha KS, Kong MH, Lim SH. Preventive effects of propofol against the elevation of intracellular Ca2+ and reactive oxygen species induced by lysophosphatidic acid in endothelial cells. Korean J Anesthesiol 2004; 46: 54-9.
6. Chang HC, Tsai SY, Wu GI, Lin YH, Chen RM, Chen TL. Effects of propofol on mitochondrial function and intracellular calcium shift in bovine aortic endothelial model. Acta Anaesthesiol Sin 2001; 39: 115-22.
7. Lee H, Goetzl EJ, An S. Lysophosphatidic acid and sphingosine 1-phosphate stimulate endothelial cell wound healing. Am J Physiol Cell Physiol 2000; 278: C612-8.
8. Panetti TS. Differential effects of sphingosine 1-phosphate and lysophosphatidic acid on endothelial cells. Biochim Biophys Acta 2002; 1582: 190-6.
9. Barnes PJ. Drugs for asthma. Br J Pharmacol 2006; 147 Suppl 1: S297-303.
10. Fanta CH. Asthma. N Engl J Med 2009; 360: 1002-14.
11. Lee ZW, Kweon SM, Kim BC, Leem SH, Shin IC, Kim JH, Ha KS. Phosphatidic acid-induced elevation of intracellular Ca2+ is mediated by RhoA and H2O2 in Rat-2 fibroblasts. J Biol Chem 1998; 273: 12710-5.
12. An S, Bleu T, Zheng Y, Goetzl EJ. Recombinant human G protein-coupled lysophosphatidic acid receptors mediate intracellular calcium mobilization. Mol Pharmacol 1998; 54: 881-8.
13. Tokumura A, Okuno M, Fukuzawa K, Houchi H, Oka M. Two effects of lysophosphatidic acid on Ca(2+)-movement in cultured bovine adrenal chromaffin cells. J Lipid Mediat Cell Signal 1996; 14: 127-35.
14. Coates DP, Monk CR, Prys-Roberts C, Turtle M. Hemodynamic effects of infusions of the emulsion formulation of propofol during nitrous oxide anesthesia in humans. Anesth Analg 1987; 66: 64-70.
15. Lückhoff A, Busse R. Activators of potassium channels enhance calcium influx into endothelial cells as a consequence of potassium currents. Nau-
Son H-J, et al. • Propofol and Aminophylline Antagonize [Ca\textsuperscript{2+}]. Mobilization

16. Roh WS, Ding X, Murray PA. Propofol and thiopental attenuate adenosine triphosphate-sensitive potassium channel relaxation in pulmonary veins. Am J Physiol Lung Cell Mol Physiol 2006; 291: L636-43.
17. Berridge MJ, Irvine RF. Inositol phosphates and cell signalling. Nature 1988; 341: 197-205.
18. Xuan YT, Glass PS. Propofol regulation of calcium entry pathways in cultured A10 and rat aortic smooth muscle cells. Br J Pharmacol 1996; 117: 5-12.
19. Yang M, Ding X, Murray PA. Differential effects of intravenous anesthetics on capacitative calcium entry in human pulmonary artery smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 2006; 291: L1007-12.
20. Wickley PJ, Shiga T, Murray PA, Damron DS. Propofol modulates Na+-Ca\textsuperscript{2+} exchange activity via activation of protein kinase C in diabetic cardiomyocytes. Anesthesiology 2007; 106: 302-11.
21. Stirt JA, Sullivan SF. Aminophylline. Anesth Analg 1981; 60: 587-602.
22. Niemand D, Martinell S, Arvidsson E, Ekström-Jodal B, Svedmyr N. Adenosine in the inhibition of diazepam sedation by aminophylline. Acta Anaesthesiol Scand 1986; 30: 493-5.
23. Fratacci MD, Shimahara T, Bournaud R, Atlan G. cAMP-dependent modulation of L-type calcium currents in mouse diaphragmatic cells. Respir Physiol 1996; 104: 1-9.
24. Mei YA, Le Foll F, Vaudry H, Cazin L. Adenosine inhibits L- and N-type calcium channels in pituitary melanotrophs. Evidence for the involvement of a G protein in calcium channel gating. J Neuroendocrinol 1996; 8: 85-91.
25. Ridings JW, Barry SR, Faulkner JA. Aminophylline enhances contractility of frog skeletal muscle: an effect dependent on extracellular calcium. J Appl Physiol 1989; 67: 671-6.
26. Mitra A. The current role of intravenous aminophylline in acute paediatric asthma. Minerva Pediatr 2003; 55: 369-75.
27. Delbono O, Kotsias BA. Effect of aminophylline-Ca\textsuperscript{2+} blocker interaction on membrane potential of rat diaphragm fibers. J Appl Physiol 1993; 74: 2745-9.
28. Whitehurst VE, Joseph X, Vick JA, Alleva FR, Zhang J, Balazs T. Reversal of acute theophylline toxicity by calcium channel blockers in dogs and rats. Toxicology 1996; 110: 113-21.
29. Ho KM, Ng JY. The use of propofol for medium and long-term sedation in critically ill adult patients: a meta-analysis. Intensive Care Med 2008; 34: 1969-79.