BASIC SCIENCE: OBSTETRICS

Nicotine restores endothelial dysfunction caused by excess sFlt1 and sEng in an in vitro model of preeclamptic vascular endothelium: a possible therapeutic role of nicotinic acetylcholine receptor (nAChR) agonists for preeclampsia

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OBJECTIVE: In this study we tested the hypothesis that nicotine restores proangiogenic functions to endothelial cells pretreated with soluble fms-like tyrosine kinase 1 and/or soluble endoglin.

STUDY DESIGN: Wound healing assay and tube formation assay were performed using human umbilical vein endothelial cells treated with nicotine (10^{-9} to 10^{-6} M), and with various combinations of soluble fms-like tyrosine kinase 1 (100 ng/mL), soluble endoglin (100 ng/mL), and nicotine (10^{-7} M). Enzyme-linked immunosorbent assay was performed to measure vascular endothelial growth factor, placental growth factor, and transforming growth factor-β1 concentrations in the conditioned media treated with nicotine (10^{-9} to 10^{-6} M).

RESULTS: Nicotine significantly facilitated endothelial migration and tube formation. By contrast, soluble fms-like tyrosine kinase 1 and/or soluble endoglin suppressed these endothelial functions. Nicotine restored these soluble fms-like tyrosine kinase 1 and/or soluble endoglin-reduced endothelial functions. Placental growth factor, but not transforming growth factor-β1, production was significantly stimulated by the presence of nicotine. Vascular endothelial growth factor was undetectable.

CONCLUSION: Our results suggest a possible mechanism for the protective effects of cigarette smoking against preeclampsia, thus proposing a therapeutic potential of nicotine or other nicotinic acetylcholine receptor agonists for preeclampsia.

Key words: nicotine, placental growth factor (PlGF), preeclampsia, soluble fms-like tyrosine kinase 1 (sFlt1), soluble endoglin (sEng)

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Preeclampsia, characterized by hypertension, proteinuria, and other systemic disturbances after 20 weeks of gestation, complicates 3-5% of pregnancies and results in substantial maternal and neonatal morbidity and mortality.[1] Preeclampsia cannot be prevented and, at present, the only effective therapy is delivery.

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Although the cause remains unclear, it has been postulated that the initiating event in preeclampsia is reduced uteroplacental perfusion caused by abnormal cytotrophoblast invasion of spiral arteries.[2-4] This results in the release of excessive quantities of placental soluble antiangiogenic factors that induce maternal systemic endothelial dysfunction.[5] Two key antiangiogenic circulating factors that give the highest strength of association with preeclamptic outcome are soluble fms-like tyrosine kinase 1 (sFlt1) (also known as soluble vascular endothelial growth factor [VEGF] receptor 1 [sVEGFR1]) and soluble endoglin (sEng).[6-8] Circulating levels of sFlt1 and sEng are increased weeks before the onset of clinical disease in the women with preeclampsia, and correlate with the severity of preeclampsia and proximity to the onset of clinical manifestations.[6-14] sFlt1 binds to the receptor-binding domains of VEGF and placental growth factor (PlGF), thereby preventing the interaction of these ligands with endothelial receptors on the cell surface.[15] sEng inhibits transforming growth factor-β1 (TGF-β1) signaling in endothelial cells and decreases angiogenesis and endothelial nitric oxide expression.[16,17] An imbalance in circulating pro- and antiangiogenic factors may be associated with maternal vascular endothelial dysfunction and the syndrome of preeclampsia.[18]

Although smoking during pregnancy is related to a variety of adverse pregnancy outcomes, such as low birthweight, prematurity, intrauterine growth restriction, and abruptio placenta, it is the only factor that has consistently been associated with a reduced risk of preeclampsia.[19,20] In addition, lower maternal sFlt1 concentrations during pregnancy has recently been reported in smokers.[21] There are more than 4000 different chemicals in cigarette smoke,

which makes it difficult to determine the individual effects of these agents on preeclampsia. Recently, nicotine, 1 of the major constituents of cigarette smoke, has been extensively studied for its proangiogenic properties[22,23] at concentrations found in the plasma of smokers (10^{-8} to 10^{-7} M).[24,25] Binding of nicotine to the nicotinic acetylcholine receptors (nAChRs) on endothelial cells is considered to stimulate secretion of several angiogenic molecules,[26] including VEGF[27,28] leading to endothelial proliferation, survival, and angiogenesis.[22,23]

Given these considerations, it seems reasonable to anticipate that nicotine or other nAChR agonists may have some therapeutic potential for preeclampsia through its proangiogenic properties. To test this hypothesis, we investigated whether nicotine restores damaged endothelial cell functions caused by excess sFlt1 and sEng, using human umbilical vein endothelial cells (HUVECs) as a model of the maternal vascular endothelium.

MATERIALS AND METHODS

Reagents

Recombinant human sFlt1 and human sEng and enzyme-linked immunosorbent assay (ELISA) kits for human VEGF, human PlGF, and human TGF-β1 were purchased from R&D Systems (Minneapolis, MN). Nicotine-free base was obtained from Sigma-Aldrich (St. Louis, MO). Growth factor reduced Matrigel was obtained from BD Biosciences (Bedford, MA).

Cells and culture conditions

After approval by the Institutional Internal Review Board of Osaka University, informed consent was obtained from each patient. HUVECs were isolated by trypsin digestion of umbilical veins from undamaged sections of fresh cords after uncomplicated deliveries at term. The umbilical vein was cannulated, washed with phosphate-buffered saline solution (PBS), and perfused with trypsin for 20 minutes at room temperature. After perfusion, the detached cells were collected and resuspended in HuMedia-EG2 medium (Kurabo, Osaka, Japan) and then plated on plastic culture dishes. HuMedia-EG2 medium consists of the base medium (HuMedia-EB2) supplemented with 2% fetal bovine serum (FBS), 10 ng/mL human epidermal growth factor, 5 ng/mL human fibroblast growth factor-B, 1 μg/mL hydrocortisone, 50 μg/mL gentamicin, 50 ng/mL amphotericin B, and 10 μg/mL heparin. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Subcultures were obtained by trypsinization and were used for experiments at passages 3 to 5.

Wound healing assay

HUVECs were cultured to subconfluent in collagen-coated 24-well culture plates. Before experiments, cells were cultured under serum-free conditions in M199 medium for 8 hours. For wound healing assay, cells were treated with nicotine (1×10^{-9} to 1×10^{-6} M), and with various combinations of sFlt1 (100 ng/mL), sEng (100 ng/mL), and nicotine (1×10^{-7} M) in M199 medium containing 0.1% FBS. Then, 1 wound per well was made with a plastic tip, and the starved cells were further cultured in the preincubated media. After 16 hours of incubation, migration was assessed by examining photographs of the cells that had migrated inside of the wound area through an inverted phase contrast microscope at 4× (Nikon Corporation, Tokyo, Japan) and quantitatively analyzed (percent wound area filled) using Image J Imaging System Software Version 1.3 (National Institutes of Health, Bethesda, MD). The experiments were carried out in triplicate.

Tube formation assay

Before experiments, cells were cultured under serum-free conditions in M199 medium for 8 hours. The surface of the prechilled 96-well plates was coated with 30 μL growth factor reduced Matrigel, which was allowed to polymerize at 37°C for 1 hour. For tube formation assay, cells were treated with nicotine (1×10^{-9} to 1×10^{-6} M), and with various combinations of sFlt1 (100 ng/mL), sEng (100 ng/mL), and nicotine (1×10^{-7} M) in M199 medium containing 0.1% FBS. The starved cells were harvested with the preincubated media and then plated into the Matrigel-coated wells (2×10^4/well) in triplicate. After 8 hours of incubation, tube formation was assessed through an inverted phase contrast microscope at 4× and quantitatively analyzed (total tube length) using Image J Imaging System Software.

ELISA

Commercially available ELISA kits were used to measure human VEGF, PlGF, and TGF-β1 according to the manufacturer's instructions. In brief, cells were cultured to subconfluent in 96-well plates. Before experiments, cells were cultured under serum-free conditions in M199 medium for 8 hours. The starved cells were treated with nicotine (1×10^{-9} to 1×10^{-6} M) in M199 medium containing 0.1% FBS for 24 hours. The conditioned media was collected, centrifuged, and stored at −80°C. Human VEGF, PlGF, and TGF-β1 in the conditioned media were measured according to the protocol provided by the manufacturer. The minimum detectable doses of VEGF, PlGF, and TGF-β1 were less than 5.0, 7.0, 1.7 pg/mL, respectively. The experiments were carried out in duplicate.

Statistical analyses

The data are presented as means ± SEM and statistical comparisons between groups were performed using 1-way analysis of variation (ANOVA), followed by Newman-Keuls post hoc test using GraphPad Prism (GraphPad Software, San Diego, CA). Significant differences are defined as $P < .05$.

RESULTS

Wound healing assay

Endothelial migration is an essential component of angiogenesis. In this study, we first examined the direct angiogenic effect of nicotine against endothelial cells. Excess circulating sFlt1 and sEng in patients with preeclampsia are considered to cause endothelial dysfunction and lead to an anti-angiogenic state. We therefore investigated whether sFlt1 and sEng suppress endothelial migration, and whether nicotine counteracts this function. Figure 1, A shows that nicotine stimulated the cell migration of HUVECs in a dose-dependent manner (1×10^{-9} to 1×10^{-6} M) as assessed by