Enhancement of Endothelial Barrier Permeability by Mitragynine

Toshiyuki Matsunaga,*a Yoshifumi Morikawa,b Kyoko Kamase,a Misato Horinouchi,a Yasuhide Sasajima,b Koichi Suenami,b Kiyohito Sato,b Yuji Takekoshi,b Satoshi Endo,a Ossama El-Kabbani,c and Akira Ikari* 

Laboratory of Biochemistry, Gifu Pharmaceutical University; Gifu 501–1196, Japan; b Forensic Science Laboratory, Gifu Prefectural Police Headquarters; Gifu 500–8501, Japan; and c Nagoya University Graduate School of Medicine; Nagoya 466–8500, Japan.

Received February 7, 2017; accepted July 6, 2017.

Persistent inhalation of mitragynine (MG), a major alkaloid in the leaves of Mitragyna speciosa, causes various systemic adverse effects such as seizure, diarrhea and arthralgias, but its toxicity to endothelial cells and effects on barrier function of the cells are poorly understood. In this study, we compared toxicities of MG and mitraphylline, another constituent of the leaves, against human aortic endothelial (HAE), bronchial BEAS-2B, neuronal SK-N-SH, hepatic HepG2, kidney HEK293, gastric MKN45, colon DLD1, lung A549, breast MCF7 and prostate LNCaP cells, and found that MG, but not mitraphylline, shows higher toxicity to HAE cells compared to the other cells. Forty-eight-hours incubation of HAE cells with a high concentration of MG (60 µM) provoked apoptotic cell death, which was probably due to signaling through enhanced reactive oxygen species (ROS) generation and resultant caspase activation. Treatment of the cells with MG at sub-lethal concentrations less than 20 µM significantly lowered transendothelial electrical resistance and elevated paracellular permeability, without affecting the cell viability. In addition, the MG-elicited lowering of the resistance was abolished by a ROS inhibitor N-acetyl-L-cysteine and augmented by H2O2 and 9,10-phenanthrenequinone, which generates ROS through its redox cycle. These results suggest the contribution of ROS generation to the increase in endothelial barrier permeability.

Key words mitragynine; endothelial cell; permeability; oxidative stress

Materials and Methods

Materials MG and mitraphylline were purchased from Cayman Chemicals (Ann Arbor, MI, U.S.A.), and N-acetyl-l-cysteine (NAC), N-acetyl aspartic acid (Asp)-glutamic acid (Glu)-valine (Val)-Asp-7-amido-4-methylcoumarin and fluorescein isothiocyanate (FITC)-dextran were from Sigma-Aldrich (St. Louis, MO, U.S.A.). 6-Carboxy-2’,7’-dichlorodihydrofluorescein diacetate (DCFH-DA) and bicinchoninic acid protein assay reagent were obtained from Molecular Probes (Rockland, ME, U.S.A.) and Pierce (Rockford, IL, U.S.A.), respectively. Polyethylene glycol-conjugated catalase (PEG-cat) was generously gifted from Dr. Yoshito Kumagai (University of Tsukuba, Japan). All other chemicals were of the highest grade that could be obtained commercially.

Cell Culture and Cytotoxicity Assay HAE cells were...
obtained from Clonetics (Walkersville, MD, U.S.A.), and cultured in EBM2 medium in type-I collagen-coated dishes at 37°C in a humidified incubator containing 5% CO₂. In the majority of experiments, the cells were used at passage 4–8, and the endothelial cobblestone morphology was confirmed microscopically before use. For cytotoxicity assay, the cells were seeded at densities of 2×10⁴ cells/well into a type-I collagen-coated 96-well microplate and, after reaching a 90% confluence of the cells, the medium was replaced with EBM medium supplemented with 0.5% fetal bovine serum and antibiotics alone 2 h before treatment of agent. The culture and treatment of bronchial BEAS-2B (American Type Culture Collection, Manassas, VA, U.S.A.), neuronal SK-N-SH cells (RIKEN Cell Bank, Tsukuba, Japan), hepatic HepG2 (American Type Culture Collection) and kidney HEK293 cells (American Type Culture Collection), gastric MKN45 (Health Science Research Resources Bank, Osaka, Japan), colon DLD1 (American Type Culture Collection), lung A549 (American Type Culture Collection), breast MCF7 (American Type Culture Collection) and prostate LNCaP cells (American Type Culture Collection) were similarly performed, except that Dulbecco’s modified Eagle’s medium (DMEM)/F-12, Minimum Essential Medium alpha media and DMEM supplemented with the serum (10% for culture and 2% for assay) and antibiotics were used as culture media of BEAS-2B, SK-N-SH and other seven cells, respectively. Viable cell number was evaluated by a tetrazolium dye-based cytotoxicity assay using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Measurement of ROS, Caspase-3 Activity and DNA Fragmentation Level of intracellular H₂O₂ was monitored using the fluorogenic probe, DCFH-DA, as previously reported. After washing with Dulbecco’s phosphate-buffered saline (DPBS), the cells were incubated for 20 min in fresh serum-free medium containing 20 µM the fluorogenic probe, and then sufficiently washed with DPBS. DCF fluorescence-positive cells were counted using a Becton Dickinson FACSuite flow cytometer equipped with BD FACSuite software. The activity of caspase-3 in cell extracts was measured using acetyl Asp-Glu-Val-Asp-7-amido-4-methylcoumarin as the fluorogenic substrates. DNA fragmentation analysis was performed according to the method reported previously.

Measurement of trans-Endothelial Electrical Resistance (TEER) and Paracellular Permeability Cells were seeded at a density of 5×10⁴ cells/well into a type-I collagen-coated Millicell Hanging Cell Culture Insert (8-µm pore) and then cultured for 5 d before treatment of the cells with MG and/or agents. TEER between apical and basal components of the insert was measured using a Millipore volt ohmmeter. To estimate the paracellular permeability, FITC-dextran (average molecular weight: 150000) was added into the insert and the fluorescence intensity in the basal side was measured 4 h after the addition.

Statistical Analysis Data are expressed as the means±standard deviation (S.D.) of at least three independent
experiments. Statistical evaluation of the data was performed by using Student’s t-test for comparison between two groups and one-way ANOVA followed by Dunnett’s test for multiple comparisons.

RESULTS AND DISCUSSION

Induction of ROS-Dependent Apoptosis in Aortic Endothelial Cell by MG Treatment When viability of HAE cells after 48-h treatment of MG was measured, it was remarkably lowered at MG concentrations higher than 40 µM (Fig. 2). As the treatment induced death of HAE, BEAS-2B and SK-N-SH cells with the 50% lethal concentration values of 43.1±6.7, 67.2±7.1 and 50.9±4.6 µM, respectively, the sensitivity to the drug toxicity of HAE cells is presumed to be higher than those of the bronchial and neuronal cells. It should be noted that no considerable alteration in viability by treatment of 60µM MG was observed for other seven cells (92.3% in HepG2, 86.7% in HEK293, 90.7% in MKN45, 85.9% in DLD1, 94.7% in A549, 84.4% in MCF7 and 87.1% in LNCaP). In contrast to MG, mitraphyline, an oxindole alkaloid of Kratom, at concentrations up to 60 µM did not affect viability of all the cells. The results suggest that exposure to MG exerts toxicity more potently against endothelial cells than against other cells.

To investigate whether production of ROS participates in the apoptotic mechanism triggered by MG, we measured effects of antioxidants, NAC and PEG-cat, on HAE cell toxicity elicited by a high concentration (60 µM) of the drug. Pretreatment with the antioxidants resulted in significant protection from the cell damage provoked by MG treatment (Fig. 3A). Additionally, flow cytometric analysis using the fluorogenic probe DCFH-DA revealed the elevation of intracellular concentration of H₂O₂ by MG treatment (Fig. 3B). Furthermore, the treatment showed typical apoptotic features, caspase-3 activation (Fig. 3C) and DNA fragmentation (Fig. 3D), which were almost completely abrogated by preincubation with NAC. These results clearly demonstrate that the treatment with MG elicits apoptosis of HAE cells through ROS

![Fig. 3. Induction of ROS-Dependent Apoptosis by MG Treatment](image-url)
production and resultant caspase-3 activation. We provide the first evidence that ROS production mediates the endothelial cell apoptosis elicited by MG. Because the MG-triggered cellular processes involved in ROS production are still unknown, biochemical and cell-based experiments to identify them are now ongoing in our laboratory.

Enhancement of Endothelial Barrier Permeability by MG Treatment

Postmortem screening of MG-related cases has shown that the drug concentrations in peripheral bloods were 0.23–1.06 mg/L (corresponding to 0.6–2.7 µM), which are much less than the 50% lethal concentration values mentioned above (43.1 µM for HAE cells). Considering that diverse effects such as seizure were observed even at the lower MG concentrations, it is assumed that at sublethal concentrations MG is capable of transmigrating itself across endothelial cell monolayer to extravascular tissues such as brain and muscle. To test this assumption, we monitored value of TEER between apical and basal compartments in the insert, where HAE cells grown to confluence were treated with MG. Intriguingly, at the low concentrations (less than 20 µM) that hardly affected the HAE cell viability as indicated in Fig. 4A, the TEER value was decreased in a manner dependent on concentration of the drug (Fig. 4B). By synchronizing decrease in TEER, it was evident that the endothelial monolayer permeability was increased, as the results of the paracellular permeability assay using the FITC-dextran are shown in Fig. 4C. Furthermore, the MG-decreased TEER was recovered and deteriorated by pretreating with NAC and ROS (H₂O₂ and 9,10-phenanthrenequinone that generates ROS via its redox cycling), respectively (Fig. 4B). These results strongly suggest the participation of ROS formation in the mechanism controlling endothelial barrier permeability. To our knowledge, this is the first report that MG elevates permeability of endothelial barrier via ROS production. The involvement of ROS in elevation of endothelial barrier permeability is consistent with previous reports.18–20) As evident from the TEER value in Fig. 4A, the endothelial permeability was significantly promoted by long-term (5 d) incubation with MG at its sublethal concentration (5 mM), which is relatively close to those in the postmortem screening, inferring the critical contribution of endothelial barrier dysfunction to the MG-elicited adverse effects. The permeability between endothelial cells is regulated by tight junction proteins, claudins and occludins, of which claudin-5 plays a key role in endothelial barrier permeability. Therefore, it would be of interest to elucidate alteration in expression, localization and function of claudin-5 as a mechanism underlying the endothelial barrier disruption by ROS.

Acknowledgment
We thank Dr. Akira Hara for insightful discussion and critical reading of the manuscript.

Conflict of Interest
The authors declare no conflict of interest.

REFERENCES


