Inhibition of endothelin A receptor protects brain microvascular endothelial cells against hypoxia-induced injury

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Abstract. Endothelin-1 (ET-1)-induced cell damage is commonly involved in ischemia/hypoxia-associated diseases. PD155080 [(sodium 2-benzo (1.3)dioxol-5-yl-3-benzyl-4-(4-methoxyphenyl)-4-oxobut-2-enoate] is a selective endothelin A receptor (ETAR) antagonist that inhibits ET-1-induced cell damage. The aim of this study was to investigate the effects of PD155080 on hypoxia-induced rat brain microvascular endothelial cell (BMEC) injury. BMECs were isolated from the cerebral cortex of Wistar rats and cultured in an anoxia chamber, containing 95% N₂ and 5% CO₂ for 12 h. BMEC injury was assessed by determining cellular ultrastructural changes and cell viability by MTT assay, trypsin blue (TB) staining and measuring the lactate dehydrogenase (LDH) levels. ET-1 mRNA expression was detected by in situ hybridization and reverse transcription PCR (RT-PCR); the ET-1 protein level was measured by radioimmunoassay. Following exposure to hypoxic conditions, the viability of the BMECs was markedly decreased and the ultrastructure of the BMECs was damaged, as demonstrated by chromat-in margination, chromatin agglutination, plasma edema, the increased number of intracellular liposomes and vacuoles, mitochondrial swelling and the expansion of a rough surfaced endoplasmic reticulum. The levels of ET-1 and ET-1 mRNA expression in the BMECs were increased following exposure to hypoxic conditions. Of note, the administration of PD155080 greatly enhanced the viability of the BMECs and ameliorated hypoxia-induced cellular injury. PD155080 also inhibited hypoxia-induced ET-1 production by the BMECs. In conclusion, PD155080 exerts protective effects against hypoxia-induced BMEC injury.

Introduction

Hypoxia is common in cerebral vascular diseases and leads to brain damage. Endothelial cells are particularly vulner-
ated with 0.1% collagenase VII (Sigma-Aldrich Co., LLC, St. Louis, MO, USA) at 37°C for 20 min, and centrifuged again at 800 rpm for 3 min. The liquid supernatant was discarded and the cell pellet was resuspended in M199 complete culture medium (HyClone Laboratories, Inc., Logan, UT, USA), which contained 15% fetal bovine serum. The cells were cultured in 35-mm plastic flasks, which were placed upside down immediately after cell implantation, and incubated at 37°C, 5% CO₂ for 2 h. The plastic flasks were then turned upward. The medium was replaced 1 day after the cells were plated, and again after 3 days and, thereafter, until the cells became unified and formed a monolayer. During the first 2-5 days of culture, the cultured cells were observed under an inverted microscope once a day. All the cells or cell clusters that were not typically triangular or shuttle-shaped with pale nuclei and a distinct cytomembrane were suspected as contaminated cells and were eliminated using a cell scraper. The cells were dissociated with 0.25% pancreatin (Sigma-Aldrich Co., LLC) for passage cultures, and the third generation of cultured cells was used in the experiments in this study.

Establishment of a hypoxic BMEC model in vitro. The cultured BMECs were plated in a hypoxic, hermetic container with 95% N₂ and 5% CO₂ (Chongqing Air Liquefaction Corporation, Ltd., Chongqing, China) and were cultured at 37°C for 12 h.

Experimental groups. The BMECs were divided into the normal controls, hypoxia and PD155080 groups. In the normal controls, the BMECs were conventionally cultured without any stimulatory factors. In the hypoxia group, the BMECs were cultured inside a container with 95% N₂ and 5% CO₂ for 12 h. In the PD155080 group, the BMECs were first treated with PD155080 (1 μM) (Pfizer Pharmaceutical Co., Ltd., New York, NY, USA) for 2 h, and were then cultured in the same hypoxic environment as the hypoxia group for 12 h in the presence of PD155080.

Detection of biomarkers

Identification of BMECs and their ultrastructures. In this study, the morphology of the endothelial cells was observed using an inverted microscope, a transmission electronic microscope and by immunocytochemical staining for factor VIII-related antigen (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China). To assess the ultrastructure of the cells, the BMECs were conventionally prepared for electron microscopic observation following fixation with 2% glutaric dialdehyde solution, and the changes to the BMEC ultrastructure were observed using a transmission electronic microscope.

Measurement of cell viability by methyl thiazolyl tetrazolium (MTT) assay. A total of 200 μl BMECs were plated in a 96-well culture plate at a density of 3x10⁴·ml⁻¹, and were cultured for the indicated periods of time. At the end of the experiments, the culture medium was discarded and 200 μl M199 + 20 μl of 5 mg/ml MTT (Sigma-Aldrich Co., LLC) were added to each well. The cells were then cultured for a further 4 h and centrifuged at 2,000 rpm for 5 min. The supernatant was discarded and 200 μl dimethyl sulfoxide were added to each well. The absorbance (A) in each well was measured with an automatic enzyme-labeled instrument at a wavelength of 570 nm.

Calculation of the mortality rate (%) of BMECs with trypan blue (TB) staining. The BMEC suspension of each group was stained with TB (0.4%), and 200 BMECs from each group (stained and unstained), were counted on blood cell counting plates under a light microscope. Mortality rate was calculated as follows: mortality = (number of dead BMECs/200) x 100%.

Measurement of lactate dehydrogenase (LDH) activity. A total of 100 μl of liquid supernatant from each group was collected at the indicated time points for each experiment. The LDH activity was measured using an LDH kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Determination of ET-1 concentration. The BMECs were plated in a 24-well plate at a density of 3x10³·ml⁻¹ (n=6 per group). At the end of each experiment, 100 μl of liquid supernatant from each group were collected to determine the concentration of ET-1 according to the instructions provided with the ET-1 radioimmunoassay kit (Dongya Immuno-technology Institute, Beijing, China).

Determination of ET-1 mRNA expression by in situ hybridization. An ET-1 in situ hybridization kit was purchased from Wuhan Boster Bio-Engineering Ltd., Co. (Wuhan, China). Digoxin-labeled oligonucleotides were used as the probe. The probe sequences were as follows: 5'-GATTATTTGGCCCATG ATCTTCTCTCTGCTGTTCGT-3' and 5'-CCACCTGGACAT CATCTGGGTCGCACTCCGGACG-3'. The cells were cultured on coverslips in 24-well plates. At the end of each experiment, the cells were fixed with 4% paraformaldehyde and washed with PBS. The cells were then immersed in pre-hybridization buffer at 37°C for 2 h, followed by incubation in ET-1 oligonucleotide probe hybridization buffer at 43°C for 30 h and 1:400 anti-digoxin antibody at 37°C for 4 h. Finally, the treated cells were washed using a 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) color-appearing system, using a hybridization buffer without a probe as the negative control. The results of in situ hybridization were analyzed using an image analysis system (Beihang CM2000B; Beihang University, Beijing, China) by measuring the absorbance (A) of each visual field under a microscope.

Determination of ET-1 mRNA expression by reverse transcription PCR (RT-PCR): a) extraction of total RNA from BMECs. Total RNA from the BMECs in each experimental group was extracted using TRIzol reagent (Sangon Biotech Co., Ltd., Shanghai, China) according to the manufacturer's instructions. RNA was dissolved with DEPC (Sigma-Aldrich Co. LLC) deionized water. The amount and purity of the RNA in each tube were measured using a UV-VIS spectrophotometer (Shanghai Precision Scientific Instrument Co., Ltd, Shanghai, China), and the samples with an optical density (OD) of ≥1.6 were reverse transcribed.

b) cDNA synthesis. cDNA was synthesized using the M-MuLV First-Strand cDNA Synthesis kit (Sangon Biotech Co., Ltd.). Briefly, 2 μl total RNA (1 μg/μl) from each group was mixed with 8 μl DEPC deionized water and 1 μl random hexamer primer (0.2 μg/μl), and then vortexed for 5 sec and incubated at 70°C for 5 min. Following incubation, the mixture was immersed in an ice-bath for 30 sec and vortexed again for 5 sec. Subsequently, 4 μl 5X reverse transcription buffer, 1 μl RNAase inhibitor (20 U/μl) and 2 μl deoxy-ribonucleoside triphosphate (10 mM) were mixed with the RNA, and...
the mixture was incubated at 70˚C for 5 min. Subsequently, 1 µl M-MuLV reverse transcriptase (200 U/µl) was added followed by sequential treatment in a water bath at 25˚C for 10 min, 37˚C for 60 min and 70˚C for 10 min; the mixture was then cooled on ice.

c) PCR reaction. PCR reactions were performed using the Gene/PCR System 2400 (PerkinElmer, Inc., Waltham, MA, USA). The sequences of ET-1 were as follows: sense primer, 5’-CGTTGCTCCTGCTCCTCCTTGATGG-3’ and antisense primer, 5’-AAGATCCCAGCCAGCATGGAGAGCG-3’. Following initial denaturation at 94˚C for 5 min, an additional denaturation, annealing and elongation were performed at 94˚C for 1 min, 60˚C for 1 min and 72˚C for 2.5 min for 35 cycles; the final elongation was performed at 72˚C for 7 min.

d) Identification and measurement of the ET-1 mRNA expression level. The mixture of 4 µl amplification product and 2 µl of sample loading buffer (6X) was loaded on a 1.5% agarose gel, which was run with a 0.5X TBE electrophoresis buffer (the buffers and the gel were produced by Sangon Biotech Co., Ltd). The gel was analyzed using an ultraviolet image analysis system (Gucun Electrical and Optical Instrument Factory, Shanghai, China), and the integral OD was measured using β-actin mRNA as an internal control. The ET-1 mRNA expression level was expressed as the ratio of the OD of the samples to β-actin.

Statistical analysis. The experimental results were analyzed using SPSS software, and are expressed as the means ± standard deviation (means ± SD). Analysis of variance was used for comparisons between the groups, and the differences between groups were considered significant at P<0.05. All figures were created using GraphPad Prism 6.01 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Identification of BMECs. To identify the characteristics of the isolated BMECs, observations were made under inverted and transmission electron microscopes. Under the inverted microscope, the endothelial cells were flaky, aggregated and closely arranged, and presented a typical pavestone-like structure (Fig. 1A). Typical claviform Weibel-Palada bodies were detected in the cytoplasm under the transmission electron microscope (Fig. 1B). We also performed immunocytochemical staining for factor VIII-related antigen, a marker for endothelial cells. As shown in Fig. 1C, the cytoplasm and the areas around the nuclear membrane were stained fuscous in immunocytochemical staining for factor VIII-related antigen under a light microscope (magnification, x200).

Ultrastructural changes of BMECs cultured under hypoxic conditions. In order to observe the changes in the ultrastructure of the BMECs cultured under different experimental conditions, a transmission electron microscope was employed. The normal BMECs (conventionally cultured) had an oblong-oval shape, as observed under an electron microscope, and were characterized by dense endonuclear chromatin, typical Golgi apparatus, endoplasmic reticulum and mitochondria, a high number of perinuclear plasma, a complete plasma membrane and a nuclear membrane (Fig. 2A). However, the BMECs cultured under hypoxic conditions for 12 h were characterized by chromatin margination, chromatin agglutination, plasma edema, an increased number of intracellular liposomes and vacuoles, mitochondrial swelling and the expansion of a rough surfaced endoplasmic reticulum (Fig. 2B). Of note, the BMECs cultured under hypoxic conditions with PD155080 showed only mild mitochondrial swelling and the expansion of a rough surfaced endoplasmic reticulum (Fig. 2C), suggesting that PD155080 alleviated hypoxia-induced ultrastructural BMEC injury.

Effects of PD155080 on the viability and mortality of BMECs. To assess the effects of PD155080 on BMEC viability and mortality, we calculated the viability and mortality of BMECs in the 3 experimental groups. Compared to the normal controls
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Effects of PD155080 on LDH release. In order to determine to what extent PD155080 protects BMECs from hypoxia, we measured the release of LDH from the BMECs. A significant increase in the release of LDH was detected in the BMECs from the hypoxic group (LDH concentration, 1,667.33±244.31) compared with the normal controls (LDH concentration, 191.71±34.26) (p<0.01), whereas the release of LDH in the PD155080 group (LDH concentration, 837.50±130.16) showed a marked reduction compared to the hypoxic group (p<0.05) (Fig. 5). These results further demonstrated that treatment with PD155080 reversed the hypoxia-induced cellular damage.

Effects of PD155080 on the secretion of ET-1 by BMECs. To clarify the effects of PD155080 on ET-1 secretion by BMECs, we measured the ET-1 levels in the BMECs. We found that hypoxia increased ET-1 secretion from the BMECs compared with the control group (p<0.01), whereas the administration of PD155080 significantly reduced hypoxia-induced ET-1 secretion from the BMECs compared to those in the hypoxic group (p<0.01) (normal controls, 160.19±32.34; hypoxic group, 426.13±64.80; PD155080 group, 266.40±50.45; p<0.01) (Fig. 6).

Effects of PD155080 on the ET-1 mRNA level in BMECs. To further determine the effects of PD155080 on hypoxia-induced ET-1 gene expression in the BMECs, we measured the ET-1 mRNA expression in the BMECs by in situ hybridization and RT-PCR. In the image analysis of in situ hybridization,
These results suggested that the inhibition of the ETA receptor and death (13). In our study, we found that the LDH level in the changes, is a sensitive and convenient marker for cell injury LDH, which is in proportion to the membrane permeability higher and the mortality rate was reduced. The release of the BMECs in the PD155080 group was significantly to hypoxia. Compared with the hypoxic group, the viability of a rough surfaced endoplasmic reticulum following exposure only showed mild mitochondrial swelling and the expansion the BMECs with PD155080, the ultrastructure of the BMECs to the ultrastructural impairment of BMECs, characterized by rat BMEC model. Our results demonstrated that hypoxia leads brain microvascular endothelial cell injury using an in vitro -in vitro microcirculation and vascular remodeling. The overproduction of ET-1 is related to the damage of cerebrovascular endothelial cells, and plays an important role in the pathogenesis of atherosclerosis and ischemic cerebral vascular diseases (16-19). Hypoxia results in the deficiency of energy metabolism, intracellular acidosis and increased membrane permeability, all of which lead to the release of intracellular ET-1 (20). ET-1 mRNA levels in the hypoxic group was significantly higher in the hypoxic group compared to the normal controls, and the mRNA expression of ET-1, resulting in the increased synthesis of ET-1 (2). Similarly, our study revealed a higher level of ET-1 perivascular microapplication of PD155080 (30 µM) around pial vessels within the territory of occluded middle cerebral artery elicited an increase in the caliber of both dilated and constricted pial arterioles following middle cerebral artery occlusion (MCAO) (14). A similar effect of PD155080 has also been reported in studies on coronary arteries using isolated perfused rat hearts, in which the addition of PD155080 resulted in a significant increase in the recovery of coronary flow after 30 min of reperfusion (15). More importantly, it has also been reported that ‘increasing concentrations of PD155080 caused a progressive, parallel rightward shift of ET-1 concentration-response curve without detrimental effect on the maximal response to ET-1’ (8).

One of the most important vasoactive substances released by cerebral endothelial cells is ET-1, the most powerful vasoconstrictor involved in the regulation of cerebral capillary microcirculation and vascular remodeling. The overproduction of ET-1 is related to the damage of cerebrovascular endothelial cells, and plays an important role in the pathogenesis of atherosclerosis and ischemic cerebral vascular diseases (16-19). Hypoxia results in the deficiency of energy metabolism, intracellular acidosis and increased membrane permeability, all of which lead to the release of intracellular ET-1 (2). Similarly, our study revealed a higher level of ET-1 secretion in the BMECs in the hypoxic group, indicating that hypoxia can induce ET-1 secretion by BMECs. Of note, in the PD155080 group, ET-1 secretion was markedly reduced compared to the hypoxic group, suggesting that PD155080 inhibited hypoxia-induced ET-1 secretion from the BMECs.

The decrease in intracellular ET-1 and hypoxia activates the mRNA expression of ET-1, resulting in the increased synthesis of ET-1 (20). In our study, ET-1 mRNA levels in the hypoxic group were higher than those in the normal controls, and the ET-1 mRNA level in the PD155080 group was significantly reduced compared to that in the hypoxic group, indicating that PD155080 inhibits hypoxia-induced ET-1 gene overexpres-
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A previous study demonstrated that LU135252 (an ETAR antagonist) reduced the tissue concentration of ET-1, but increased the plasma levels of ET-1 in angiotensin II-treated animals, and suggested that this may be related to the displacement of ET-1 from tissues to the circulation or may be related to the direct interference of LU135252 with the production of ET in vascular tissue through its ETAR interaction (21). Another study using LU135252 suggested that ET-1 may act as an autocrine modulator of its own production in vivo through the activation of ETAR; thus, the blockade of ETAR prevented the increase in vascular ET-l (22). It has also been reported that the binding of ET-1 to endothelin B receptor (ETBR) inhibits endothelin-converting enzyme-1 expression in endothelial cells and ETBR mediates the reuptake of ET-1 by endothelial cells (23). Therefore, the blockade of ETAR may also increase the binding of ET-1 to ETBR, which results in a reduced ET-1 level and its production. In this study, a significant decrease in ET-1 mRNA expression in the BMECs was detected in the PD155080 group compared to the hypoxic group. It is possible that PD155080 directly interferes with the production of ET-1, but it remains unclear whether this interference is related to the blockade of ETAR or to the activation of ETBR. Further studies are required to determine whether the decrease in ET-1 expression in the PD155080 group was primarily due to the effects of PD155080 directly, or due to the reduced release of intracellular ET-1.

ET-1 leads to vasoconstriction, by activating ETAR, which is expressed in endothelial cells (6,12,24), indicating that the

Figure 7. Effect of PD155080 on endothelin-1 (ET-1) mRNA expression in brain microvascular endothelial cells (BMECs) measured by in situ hybridization. (A) Normal controls: expression of ET-1 mRNA in BMECs. (B) Expression of ET-1 mRNA in BMECs cultured under hypoxic conditions (indicated by the arrows). (C) Expression of ET-1 mRNA in BMECs cultured with PD155080 (magnification, x200). (D) Comparison of expression of ET-1 mRNA expression in BMECs in the 3 groups. (A and C) Only weak staining was observed. (D) ET-1 mRNA expression was calculated as the mean ± SD, ***P<0.01 vs. hypoxic group; n=6 per group and the data shown are the average of 2 individual experiments. PD, PD155080.

Figure 8. Effect of PD155080 on endothelin-1 (ET-1) mRNA expression in brain microvascular endothelial cells (BMECs) measured by reverse transcription PCR (RT-PCR). (A) Comparison of ET-1 mRNA expression in the BMECs in the 3 groups. (B) Agarose gel electrophoresis of the 3 groups; The ET-1 mRNA expression was calculated as the mean ± SD, ***P<0.05 vs. hypoxic group; n=6 per group and the data shown are the average of 2 individual experiments. PD, PD155080.
effects of ET-1 on BMECs are mainly mediated by ETAR. ETAR antagonists can compete against ET-1 to bind specifically to ETAR and block the biological interaction between ET-1 and ETAR. A recent study on the effects of selective ETAR antagonists in an ischemic animal model reported that ETAR antagonists appear to offer multiple neuroprotective mechanisms, including the prevention of the blood-brain barrier disruption and leukocyte infiltration (25). BQ-485, an ETAR antagonist, has been shown to prevent the oxygen saturation decrease in ischemic cerebral tissue, and increase the cerebral oxygen utilization coefficient (26). ABT-627 and A-147627 are both ETAR antagonists that partially normalize the neurological deficits and infarct volume of MCAO mice, whilst the ETBR antagonist has no such effect (4,27). In addition, rats treated with Clazosentan® (an ETAR antagonist) have shown a reduction of encephalic edema at 72 h and at day 7, and a decreased serum ET-1 level at 72 h and at day 7 (7). SB 234551 (an ETAR antagonist) protected MCAO rats by enhancing collateral blood flow and the salvage of penumbra (28). However, other studies have reported that ABT-627 had no effect on cerebral edema, infarct volume and neural function of ischemic rats (29). There are only limited in vitro studies that have reported that the effects of selective ETAR antagonists, such as BQ-123, may improve the survival of human fetal astroglial and neuronal cells upon hypoxic injury (30). To date, there are no studies on their effects on ischemic cerebral vascular endothelial cells.

PD155080 is a non-peptide specific ETAR antagonist, its oral bioavailability is 87%, and it has up to 1,000-fold selectivity for the human ETA receptor than for ETBR (8,31). PD155080 exerts a variety of effects on ET-1-related physiological and pathophysiological processes by blocking ETA receptors. Long-term ETAR antagonism can normalize myocardial cytosolic Ca²⁺ modulation, which may contribute to the anti-hypertrophic and cardioprotective effects of ETAR therapy (32). In a previous study on isolated rabbit atrial cardiomyocytes, PD155080 (1 μM) was found to prevent the ET-1-induced inhibition of IK (ACh) (33). A combination of A-192621 (an ETBR antagonist) and PD155080 abolished endotoxin-induced pulmonary hypertension, enhanced cardiac performance and improved systemic oxygen delivery and acid-base balance (34). In experimental chronic renal failure rats, PD155080 reduced their high blood pressure without affecting renal function (35). Our study demonstrated that PD155080 significantly inhibited the hypoxia-induced release of ET-1 and ET-1 mRNA expression and protected BMECs from hypoxic injury. Therefore, PD155080 and other ETAR antagonists may provide therapeutic strategies for many ET-1-related diseases. Further studies on the anti-hypoxic mechanisms of PD155080 are required in order to develop novel drugs for the treatment and prevention of ischemic vascular endothelial injury.

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