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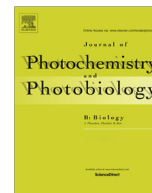

Opening of brain blood barrier induced by red light and central analgesic improvement of cobra neurotoxin

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A B S T R A C T

Cobra neurotoxin (NT) has central analgesic effects, but it is difficult to pass through brain blood barrier (BBB). A novel method of red light induction is designed to help NT across BBB, which is based on photosensitizer activation by red light to generate reactive oxygen species (ROS) to open BBB. The effects were evaluated on cell models and animals *in vivo* with illumination by semiconductor laser at 670 nm on photosensitizer pheophorbide isolated from silkworm excrement. Brain microvascular endothelial cells and astrocytes were co-cultured to build up BBB cell model. The radioactivity of ^{125}I-NT was measured in cells and tissues for NT permeation. Three ways of cranial irradiation, nasal cavity and intravascular irradiation were tested with combined injection of ^{125}I-NT 20 μg/kg and pheophorbide 100 μg/kg to rats, and organs of rats were separated and determined the radioactivity. Paw pressure test in rats, hot plate and writhing test in mice were applied to appraise the analgesic effects. NT across BBB cell model increased with time of illumination, and reached stable level after 60 min. So did ROS in cells. NT mainly distributed in liver and kidney of rats, significantly increased in brain after illumination, and improved analgesic effects. Excitation of pheophorbide at red light produces ROS to open BBB, help NT enter brain, and enhance its central action. This research provides a new method for drug across BBB to improve its central role.

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1. Introduction

More than 95% drugs cannot enter brain because of limitation by blood brain barrier (BBB) for brain protection from toxicity. Many medicines have been proved to be more effective with intracerebroventricular administration or intrathecal injection, but their large molecules impede the permeation of BBB [1,2]. Direct administration to central region is inconvenient and dangerous. Peripheral administration is safe but needs some measures to help these medicines cross BBB. Inflammation, mechanical and chemical injuries can open BBB [3,4], but they are harmful to central tissues. Less harmful ways of improving BBB permeation are prospective in clinical application, which is the aim of this research.

Photosensitizer can be activated by light at certain wavelength, and produces active oxygen species (ROS), which is found to open BBB in less harm [5]. Our former research disclosed that photosensitizer pheophorbide enhanced the permeation of some macromolecules to brain [6]. Natural pheophorbide, a porphyrin isolated from excrement of silkworm, has good photosensitizing effects. It produces ROS while excited by laser at 670 nm [7], acts on BBB and makes it open to expedite the permeation of other drugs. Red light of 670 nm has the property of weak tissue adsorption so that it has deeper penetration in tissues and little side effects [8]. Because opening of BBB induced by photosensitizer is temporary, perpetual damage will not happen [9], the combined administration of photosensitizer pheophorbide with some drugs is ideal method to improve their central effects.

Cobra neurotoxin (NT), a short-chain peptide isolated from snake venom of *Naja naja* atra with molecular weight of 8000 Da, has obvious central analgesic role, and is used for therapy of chronic pain such as cancer and neuropathic pain [10]. But it is difficult to pass through BBB so that clinical effect is not good enough with muscular injection. In order to increase the permeation of NT into brain, a method is designed to open BBB by means of pheophorbide excited with red light in this research. The scheme is illustrated in Fig. 1. The effects are evaluated by BBB cell model and animal *in vivo*.

There are two BBB models used in current researches: monolayer brain microvascular endothelial cells (BMEC) and co-culture of BMEC and astrocytes (AC). The former easily loses the characters of BBB such as little γ-glutamyl transferase (γ-GT) expression and low transendothelial electrical resistances (TEERs) [11]. Although tight junction may be induced by conditional medium of AC [12],

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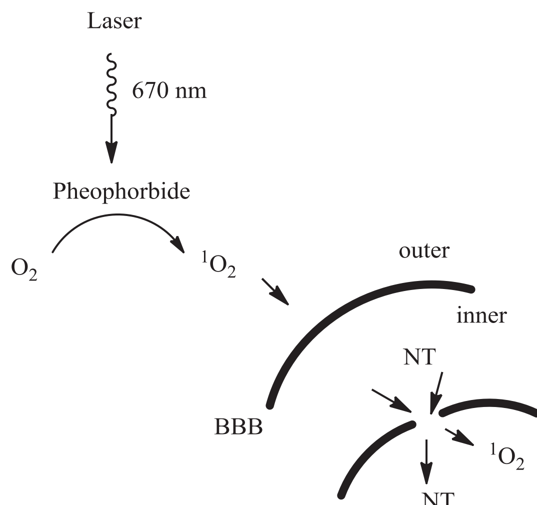

Fig. 1. Scheme of opening brain blood barrier (BBB) by red light and the permeation of cobra neurotoxin (NT) to brain.

the special enzymes of γ-GT and alkaline phosphatase (ALP) do not express [13]. It is controversy to use the monolayer BMEC as BBB model. The co-culture of BMEC and AC can up-regulate the expression of γ-GT, ALP, Na–K–ATP, transferrin, glucose transporter, P-glycoprotein, Na–K–Cl transporter etc., and maintains the characters of BBB [14]. Therefore, the co-culture of BMEC and AC is ideal BBB model as reported and testified in former studies [15,16].

2. Materials and methods

2.1. Animals and chemicals

The experiments were performed on Wistar rats weighing 180–200 g and Kunming mice (male or female) weighing 18–22 g. The animals were housed under conditions of 25 ± 2 °C, 50 ± 10% humidity with a 12 h light/dark cycle. Food and water were accessible *ad libitum*. All animals used in this work were treated according to the guideline of animal handling, and the protocols of animal behavioral experiments were approved by Ethic Committee of South China University of Technology.

Cobra neurotoxin (NT) was purchased from Institute of Zoology, CAS (Kunming, China), ^{125}I-NT (1.5×10^8 Bq/mg, labeling yield 98%, radiochemical purity >99%) was labeled by Research Department of Nuclear Medicine, Sun Yat-sen University (Guangzhou, China). Other chemicals were pheophorbide made in our laboratory as reported [17], DMEM high glucose medium and D-Hank's buffer purchased from Gibco company (New York, USA), trypase, penicillin and streptomycin from Sigma company (California, USA), calf serum from Hyclone company (Utah, USA), endothelial cell growth supplement (ECGS) from ScienCell company (California, USA), 0.4 μm polyester multipore membrane (Transwell) from BD Falcon company (New Jersey, USA), ROS assay kit from Feibo Biotech company (Guangzhou, China), heprin, gelatin and L-glutamine from Qiyun Biotech company (Guangzhou, China).

2.2. Schemes of illumination

Illumination on cell cultures and animals *in vivo* was designed in following schemes. BBB cell model was built up by co-culture of BMEC and AC on transwell. Semiconductor laser (100 mW output) was fixed above the culture dish at the height of 30 cm, and illuminated on the surface of the transwell (diameter 15 mm) (Fig. 2a), the irradiance cells received was 0.5 mW/mm^2. 200 μm diameter of quartz optical fiber was inserted to fixed tube in cranial bone, nasal cavity and tail vein of animals, and performed cranial irradiation, nasal irradiation and vein irradiation (Fig. 2b), the optical irradiance at the fiber tip was 100 mW.

2.3. Buildup of BBB cell model

BMEC were separated according to the reference [18]. Brain was dissected from 2 to 3 weeks old rats, treated by trypase digestion and density gradient centrifugation. Middle white segment was collected and mixed with culture medium consisted of DMEM, 20% calf serum, 100 U/L penicillin, 100 U/L streptomycin, 2 mmol/L L-glutamine, 100 mg/L ECGS and 40,000 U/L heprin, then inoculated in 70 ml of fresh culture medium in bottle coated with 1% gelatin. The medium was changed 24 h later and two times a day from then on. The cells were subcultured when densely attached, and prepared for experiments after 5–6 passages.

Astrocytes were collected as reported [19]. Brain of new born rats was dissected and made into single cell suspension after removing meninges and superficial vessels, then adjusted the cell density to 1×10^6/ml, and cultured for 8–10 d. Attached cells were collected and cultured in fresh medium, and used for further experiments after 2–3 passages.

About 4×10^5 astrocytes were inoculated in outer pool of the inserts (Millicell-PET, Millniproe Company, Massachusetts, USA), and grew to 70% converging, then about 1×10^5 BMEC were inoculated on transwell pre-coated with 2% gelatin to form no touching co-culture. 3–4 d later, the medium was changed once a day for consecutive 5 d.

2.4. Measurement of TEERs

TEERs were the parameters for BBB integrity [20]. It was measured after monolayer cells covering the transwell on Millicell-ERS device (Millipore Company, Massachusetts, USA). The operating procedure was based on recommended protocol on the apparatus description. Transwells were washed by PBS for 3 times after removing the medium, then electric resistance (R) was determined in PBS. TEERs = (R–R_0)/transwell area. R_0 is the resistance of filter (transwell without cells).

2.5. Test of NT permeation in BBB model

Radiocounting is used for evaluation of drugs permeability in the BBB models [21]. Transwells were divided into 4 groups of 5 repetitions each, one with 0.25 ml of ^{125}I-NT (20 μg/ml) and the other with 0.25 ml of ^{125}I-NT (20 μg/ml) plus 0.25 ml of pheophorbide (20, 100 or 500 μg/ml) were added on apical side of the transwell, 1.5 ml of D-Hank's buffer was placed on the basolateral. The apical side of the tanswells was illuminated by 100 mW semiconductor laser (Sanding Photoelectric Company, Dongguan, China) at 670 nm for 10, 20, 30, 60, 90 and 120 min. 200 μl of the basolateral solution replaced by fresh buffer was collected to count the radioactivity at time intervals by SN-695B radioimmunoassay counter (Hesuo Rihuan Photoelectric Instrument Company, Shanghai, China). Radiocounting of basolateral solution in the total was recorded as percentage of permeation.

2.6. Determination of ROS in cells

ROS in cells was determined by fluorescence according to kit description and the reference [22]. The reagent of DCFH-DA (2′,7′-dichlorofluorescin diacetate) has no fluorescence, but easily enters the cell and transforms to DCFH, who is oxidized by ROS and emits fluorescence. The reagent of Rosup as the positive can produce ROS and judge the efficacy of DCFH-DA. The BBB cell

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