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Thiopental sodium preserves the responsiveness to glutamate but not acetylcholine in rat primary cultured neurons exposed to hypoxia

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ABSTRACT

Although many *in vitro* studies demonstrated that thiopental sodium (TPS) is a promising neuroprotective agent, clinical attempts to use TPS showed mainly unsatisfactory results. We investigated the neuroprotective effects of TPS against hypoxic insults (HI), and the responses of the neurons to L-glutamate and acetylcholine application. Neurons prepared from E17 Wistar rats were used after 2 weeks in culture. The neurons were exposed to 12-h HI with or without TPS. HI-induced neurotoxicity was evaluated morphologically. Moreover, we investigated the dynamics of the free intracellular calcium ($[Ca^{2+}]i$) in the surviving neurons after HI with or without TPS pretreatment following the application of neurotransmitters. TPS was neuroprotective against HI according to the morphological examinations (0.73 ± 0.06 vs. 0.52 ± 0.07, $P = 0.04$). While the response to L-glutamate was maintained (0.89 ± 0.08 vs. 1.02 ± 0.09, $P = 0.60$), the $[Ca^{2+}]i$ response to acetylcholine was notably impaired (0.59 ± 0.02 vs. 0.94 ± 0.04, $P < 0.01$). Though TPS to cortical cultures was neuroprotective against HI morphologically, the $[Ca^{2+}]i$ response not to L-glutamate but to acetylcholine was impaired. This may partially explain the inconsistent results regarding the neuroprotective effects of TPS between experimental studies and clinical settings.

1. Introduction

Thiopental sodium (TPS) is one of available intravenous anesthetics, which acts as a γ-aminobutyric acid A ($GABA_A$) receptor agonist [13]. Our previous studies [16,17,20,26] and those from other laboratories [5,14] have shown that TPS is neuroprotective against neurotoxic or hypoxic insults (HI) *in vitro*. Although several *in vivo* studies also demonstrated some beneficial effects of TPS [4,8,30], clinical attempts to use TPS for cerebral ischemia showed unsatisfactory results [12]. This inconsistency between the findings in experimental studies and the actual clinical outcomes is not fully understood.

Though our previous reports showed that primary cortical cultures were protected from HI with clinically relevant concentrations of TPS, to the best of our knowledge no reports that analyze the function of surviving neurons in terms of their responsiveness to neurotransmitters exist. Thus, here, we focused on the responses of the surviving neurons to neurotransmitters after HI. We investigated the neuroprotective effects of TPS against HI and the responses of the surviving neurons to two major neurotransmitters, L-glutamate (Glu) and acetylcholine (ACh), using calcium (Ca^{2+}) imaging and morphological examinations *in vitro*.

2. Methods

2.1. Ethical approval

All animals were treated in strict accordance with the National Institutes of Health guidelines and the institutional guidelines for the care and treatment of laboratory animals. The study protocol was approved by the Animal Care Committee at Osaka University Graduate School of Medicine (Ref: 21-061-2). We confirm that all efforts were made to minimize the number of animals used and their suffering.

2.2. Chemical reagents

The chemicals used in this study were obtained from the following sources: Dulbecco's modified Eagle's medium (DMEM), GABA, and KCl from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); 5-fluoro-2′-

Abbreviations: 5-FU, 5-fluoro-2′-deoxyuridine; ACh, acetylcholine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ANOVA, analysis of variance; Ca^{2+}, calcium; $[Ca^{2+}]i$, intracellular calcium; CCD, cooled charge-coupled device; DIV, days *in vitro*; DMEM, Dulbecco's modified Eagle's medium; E17, embryonic day 17; FCS, fetal calf serum; Fmax, the mean maximum change in fluorescence intensity; GABA, gamma-aminobutyric acid; GFAP, glial fibrillary acidic protein; Glu, L-glutamate; HI, hypoxic insult; HS, horse serum; HSD, honestly significance different; NBS, normal bath solution; NMDA, *N*-methyl-D-aspartate; PBS, phosphate buffer solution; SEM, standard error of the mean; TPS, thiopental sodium.

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deoxyuridine (5-FU), poly-L-lysine, streptomycin, penicillin, pluronic acid F-127, L-glutamate acid, and acetylcholine chloride from Sigma-Aldrich (St Louis, MO, USA); trypsin from Difco Lab (Detroit, MI, USA); fetal calf serum (FCS) from MP Biochemicals (Solon, OH, USA); B-27 Supplement Minus antioxidants (AO) and horse serum (HS) from Gibco BRL (Carlsbad, CA, USA); TPS from Mitsubishi Tanabe Pharma Corp. (Osaka, Japan); and Fluo-4-AM from Dojindo (Kumamoto, Japan).

2.3. Cell culture

Primary cultures of cortical neurons were prepared as described previously [18,19,21,25,27]. 172 rat fetuses from 16 anaesthetized pregnant Wistar rats, which were obtained from Nihon SLC (Hamamatsu, Japan), were removed on embryonic day 17 (E17). Their brains were isolated under a microscope. Cerebral cortical neurons were treated with 0.25% trypsin in phosphate buffer solution (PBS) at 37 °C for 25 min and titrated with a Pasteur pipette. Dispersed cells were diluted to a concentration of 0.6–1.0 × 10^6 cells/mL in DMEM, which contained 8% FCS, 4% HS, 2% B-27 Supplement Minus AO, 50 μg/mL streptomycin, and 50 IU/mL penicillin. This suspension was placed in a poly-L-lysine-coated, 35-mm diameter, film-bottom dish (FD10300; Matsunami Glass Ltd, Osaka, Japan) for Ca^{2+} imaging experiments or a 2-mm grid tissue culture dish (1.5 mL per well) (Nunc, Naperville, IL, USA) for the cytotoxicity assay. We used the grid tissue culture dishes to observe the same neurons at multiple time points.

After 72 h in culture, the medium was fully replaced. The cells were treated with 5 μg/mL of 5-FU for 3 days to prevent non-neuronal cells from proliferating. The neurons were maintained in DMEM containing 8% FCS, 4% HS, and 2% B27 supplement in an atmosphere consisting of 5% CO_2 and 95% air at 100% humidity and a temperature of 37 °C. Half of the medium was changed twice weekly thereafter. All subsequent experiments were carried out after 13–14 days *in vitro* (DIV).

2.4. Cytotoxicity

Neurotoxicity was investigated using Shibuta's model with a photosystem (Axiovert25®, Carl Zeiss, Germany; Nikon D90, Japan), as described previously [21]. A hypoxic atmosphere was maintained in a special anoxic incubator (ASTEC water jacket type multi-gas incubator, APM-30D; Fukuoka, Japan). In this incubator, the ambient temperature and concentration of oxygen could be precisely controlled. Before performing the experiments, we used a gas analyzer (ABL 620, Radiometer Copenhagen Trading Co., Denmark) to measure changes in the partial pressure of oxygen in the medium. The oxygen concentration was then adjusted in the incubator to ensure that the medium was maintained in a hypoxic atmosphere. During the hypoxic periods, neurons were maintained in an atmosphere consisting of 5% CO_2, 1% O_2 and 93–94% nitrogen at 100% humidity. The viability of the neurons was evaluated shortly after HI without reoxygenation. As a result of our preliminary study (Fig. 1), we determined that the appropriate HI duration for the main experiment was 12 h.

We divided the culture dishes into four groups as follows: Control dishes that were not exposed to HI or TPS (Group C), culture dishes that were exposed to HI but not TPS (Group H), culture dishes that were exposed to HI and TPS (Group HT), and culture dishes that were exposed to TPS but not HI (Group T). TPS was applied at 100 μM after our previous study had demonstrated that this concentration provided enough neuroprotection for neurons to withstand the experimental conditions [22].

Three photomicrographs were taken of each well shortly before the experiment. The grid arrangement of the dish helped us to determine the specific location of each well. TPS was administered 15 min before the last 12 h in Group T, or the 12-h HI exposure in Group HT (final 1.5 mL and 100 μM TPS). After 12 h of normoxia or HI, the cells were exposed to 0.4% trypan blue with PBS to stain nonviable cells, and three more photomicrographs of each well identical to those above were taken. The nonviable neurons were either stained with trypan blue or washed from the culture dish, whereas the viable neurons remained unstained and adherent. A second observer, who was blinded to the arrangement of the photomicrographs, study design, and treatment protocol, replicated all manual counts to ensure count accuracy and minimal inter-observer variability. Survival rates were calculated using the following formula: The number of unstained cells at the end of the experiment divided by the number of total cells shortly before the experiment. The survival ratio was calculated as the survival rate of a specific dish divided by the survival rate of the control dish. Therefore, the survival ratio of control neurons was defined as 1.

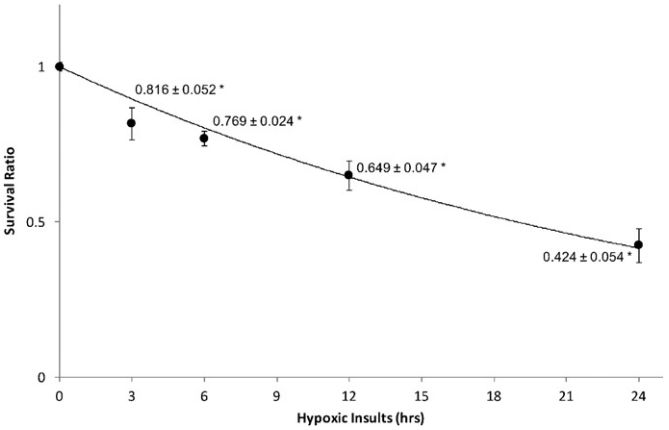

Fig. 1. Relationship between the duration of hypoxic insults (HI) and the survival ratios of the neurons. Black points and error bars show the means and standard errors of the mean of the survival ratios from all of the experimental groups. N = 14 dishes for all groups. The regression for the graph is $y = e^{-0.037\times}$. The differences between the means were assessed using ANOVAs followed by the Tukey-Kramer honestly significant difference test. The survival ratio of the group exposed to 3 h hypoxia was significantly lower than the survival ratio of the control group. Note that as the HI duration increased, the survival ratio of the neurons decreased. *$P < 0.05$ indicates a significant difference from the control.

2.5. Ca^{2+} imaging

Ca^{2+} imaging was performed according to methods published previously [15,22]. Free intracellular calcium ($[Ca^{2+}]i$) was measured using a fluorescence measurement system (Aquacosmos®; Hamamatsu Photonics, Hamamatsu, Japan) and an inverted phase contrast microscope (Axiovert 200®, Zeiss). Neurons were transferred to a normal bath solution (NBS; 137 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.3, and 22 mM glucose) containing a Ca^{2+}-sensitive indicator, 10 μM Fluo-4-AM, and 0.025% pluronic acid F-127 for 30 min at room temperature. Cultures were rinsed twice with fresh NBS and placed onto a microscope stage. A 150-W xenon lamp was used for fluorescence excitation (450–490 nm) of neurons, and 16-bit fluorescence images (512 × 512 pixels; 2 × 2 binned) were obtained using a cooled charge-coupled device camera (Orca ER®; Hamamatsu Photonics) that was connected to the microscope with an oil-immersion objective lens (Fluor® × 40, oil, numerical aperture 1.30; Zeiss). A series of images was acquired at 2-s intervals for 120 s. During the exposures, excitation light was blocked using a filter exchanger (C8214; Hamamatsu Photonics) to avoid potential cell damage. Images were analyzed using Aquacosmos®. Enhancement of the fluorescence intensity indicated an increase in $[Ca^{2+}]i$. In the present study, data are expressed as the mean relative fluorescence within a defined region of each neuron.

To evaluate the effects of neurotransmitters on primary cultured neurons, the mean maximum change in fluorescence intensity (Fmax) upon addition of the neurotransmitter (Glu or ACh) was measured, then normalized to the baseline fluorescence obtained before neurotransmitter application (F0).