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Research article

The effects of poloxamer 188 on the autophagy induced by traumatic brain injury

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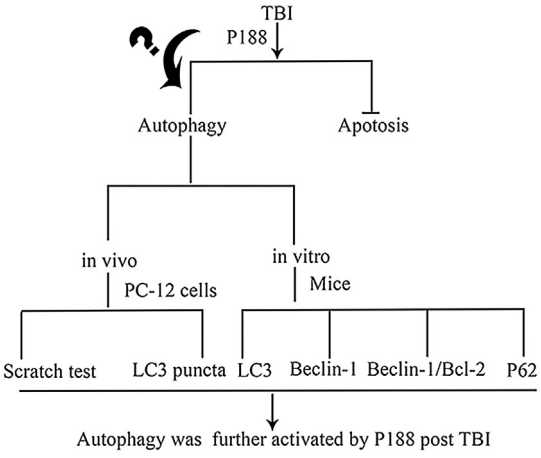
HIGHLIGHTS

- Autophagy was activated both in vivo or in vitro post-TBI.
- P188 further enhanced the autophagy activity after TBI both in vivo or in vitro.
- P188 exhibited neuroprotection against TBI maybe via the enhanced autophagy.

GRAPHICAL ABSTRACT



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ABSTRACT

Poloxamer 188 (P188) has been reported to reseal plasma membranes and attenuate TBI-induced neuronal death by suppressing apoptosis. Recent studies also confirm increased autophagy after traumatic brain injury (TBI). The present study aimed to investigate the effects of plasmalemmal resealing by P188 on neuronal autophagy in TBI. Scratch test was performed in rat cell line PC-12 in vitro, followed by immunofluorescence analysis of LC3 24 h after PC-12 cell stretch-injury in vitro. CD1 mice were randomized into saline and P188-treatment groups (both undergoing intravenous injection of 4 mg/ml, 100 μl via the caudal vein 30 min after TBI) as well as sham group. To analyze the effect of P188 on autophagy, the LC3 protein levels were assessed by western blotting 1 h, 6 h, 12 h, 24 h, and 48 h after TBI. The autophagy-associated protein levels of Beclin-1, Bcl-2, and p62 were likewise determined. In vitro, the scratch test showed that the wound healing rate was significantly improved at 12 h and 24 h in P188 groups, and LC3 immunofluorescence analysis indicated that P188 induced extensive formation of LC3 puncta in PC-12

Abbreviations: TBI, traumatic brain injury; P188, poloxamer 188; CCI, controlled cortical impact.

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cells. In vivo, western blotting analyses revealed elevations of the LC3-II/LC3-I and Beclin-1/bcl-2 ratios as well as downregulation of p62 in the saline group, in contrast with the more significant increases of LC3-II/LC3-I and Beclin-1/bcl-2 ratios and the further downregulation of p62 in P188-treated group. These results revealed that plasma membranes were resealed after TBI, in which P188 aggravated autophagy in vivo.

1. Introduction

Traumatic brain injury (TBI) typically contributes to the disintegrity of cell membrane and threatens neuronal survival [9,18]. However, poloxamer 188 (P188) is an amphiphilic polyethylene-polypropylene-polyethylene copolymer, characterized by its properties in sealing membranes and restoring the plasma membrane integrity of neurons following membrane injury [8,13,16].

Autophagy leads to degradations of long-lived proteins, aggregated proteins and damaged organelles, and ultimately the maintenance of material recycling and homeostasis [3,10]. There have been controversies regarding the genesis of autophagy, such as the formation of double-membrane structures that engulf cytoplasmic materials and fuse with lysosomes, or consequence of hypoxia, nutritional deprivation, or exposure to radiation, chemicals and other stimulants [3,10]. The microtubule-associated protein 1 light chain 3 (LC3) is a biomarker in biochemical assays at autophagosomal levels, with the conversion of endogenous LC3-I to LC3-II indicating the extent of autophagy [3,10]. Bcl-2 can reportedly regulate autophagy via its interaction with Beclin-1. As a component of the main signal-initiating complex (class III PI3 kinase, Beclin-1 and p150 protein), Beclin-1 is one of the key proteins regulating autophagy and triggering a cascade of proteins involved in the formation of autophagolysosomes [3,10]. Autophagy plays multifunctional roles in the maintenance of cellular homeostasis. Autophagy is an evolutionarily conserved procedure that leads to the degradation of proteins or entire organelles in cells subjected to stress [3,10].

In this study, P188 was used as a cell membrane sealing agent to determine whether plasmalemmal resealing could affect autophagy in neurons after TBI. The previous study and our experiment have found that P188 restores the intactness of the plasma membrane and attenuates TBI-induced BBB disruption, brain edema and neural cell apoptosis in vivo [1,18]. At present, cell line PC12 has been used as an in vitro model to mimic in vivo studies such as neuronal apoptosis, necrosis and autophagy. Cell line PC12 is considered a valid in vitro neuronal model to study the mechanisms of neuronal injury as depicted in TBI [2,5].

2. Methods

2.1. TBI model and drug administration

Adult male CD1 mice (20–25 g, $n = 66$) from the Experimental Animal Center, Xuzhou Medical University were anesthetized under 4% chloral hydrate (0.4 mg/g) and mounted in a Kopf stereotactic apparatus. All experimental procedures were in compliance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Xuzhou Medical University. Mice were randomly assigned to saline, P188 treated, and sham groups after TBI ($n = 6$). The protein expression was detected at 1 h, 6 h, 12 h, 24 h, 48 h post TBI. Mice were subjected to TBI in the left hemisphere of brain using a drop-weight apparatus, in which a 40 g counterpoise was dropped from 2-mm-diameter footplate 20 cm above onto the dura mater with a controlled depth of 1.0 mm, as previously described [9,14]. The reproducibility and consistency of this TBI model were ensured by the accurate location and controlled strength, depth and duration of the impact. The subsequent craniotomy and immediate scalp closure did not significantly affect physiological parameters. In addition, 100 μl P188 (4 mg/ml, ScienceLab, USA; Pluronic® F68, Av. M.W. 8400) dissolved in normal saline was intravenously injected via the caudal vein.

Sham-operated mice, which underwent the craniotomy without the impact injury, also received the administration of 100 μl P188 or normal saline (vehicle) by the caudal vein. For western blotting, mice were likewise injected with P188 (4 mg/ml, 100 μl) or saline (100 μl) 30 min after TBI and sacrificed 1 h, 6 h, 12 h, 24 h and 48 h after TBI. Tissue samples were obtained from injured cortex ($2 \times 2 \times 2$ mm tissue block including the impact site and surrounding tissue) and hippocampus (the entire ipsilateral hippocampus including the impact site and surrounding tissue) for western blotting analyses.

2.2. Western blotting analyses

The LC3 and p62 levels, and Beclin-1/Bcl-2 ratios were determined by western blotting as from 1 h till 48 h after TBI to detect the levels of autophagy in vivo. For western blotting analysis, the injured cortical and hippocampal samples were isolated and handled as previously described [1]. 30 μg protein from each sample underwent electrophoresis on a 10% SDS-PAGE gel on a constant direct current. Proteins were transferred to polyvinylidene fluoride membranes on a semidry electrotransfer unit (Bio-Rad) and incubated with primary antibodies against LC3 (Abcam, ab51520, 1:6000) Beclin-1 (Santa Cruz Biotechnology, sc-11427, 1:1000), Bcl-2 (Millipore, 04-436, 1:1000), and p62 (Abcam, ab51416, 1:8000) in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% skim milk overnight at 4 °C. Thereafter, the membranes were rinsed thrice and incubated with horseradish peroxidase-conjugated secondary antibody in TBST for 2 h. The PageRulerTM Prestained Protein Ladder (Thermo, 26617, 5 μl) was applied according to the manufacturer's instructions. The immunoreactivity was detected using enhanced chemoluminescent autoradiography (ECL kit, Amersham), according to the manufacturer's instructions. The membranes were stripped and reprobed between the different primary antibodies, as well as with GAPDH (Santa Cruz Biotechnology, sc-166545, 1:1000). The signal intensity of the primary antibody binding was quantified using Quantity One (Bio-Rad) and normalized to the loading control, GAPDH.

2.3. Cell culture

To further confirm the effects of P188 and the underlying protective mechanism, we performed the scratch test with PC-12 in vitro. PC-12 cells (ATCC, CRL-1721) were cultured at 37 °C in DMEM at 5% CO2 containing 5% fetal calf serum, and 1% penicillin/streptomycin (Gibco, Carlsbad, CA). PC-12 cells (4×106 cells) were seeded on 14 cm petri dishes coated with 50 μg/ml poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA) and cultured in 20 ml DMEM containing