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Research article

(+)-Pentazocine attenuates SH-SY5Y cell death, oxidative stress and microglial migration induced by conditioned medium from activated microglia

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HIGHLIGHTS

- (+)-Pentazocin, a σ_1 agonist, inhibits microglial cell migration.
- (+)-Pentazocin protects SH-SY5Y cells from microglia activation.
- (+)-Pentazocin prevents oxidative stress and neuroinflammation following microglial activation.

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ABSTRACT

Background: Sigma receptors (σ_1R) are expressed both in neurons and microglia and can be considered as a promising target for developing pharmacological strategies for neuroprotection in various experimental models. The aim of the present study was to test the effect of (+)-pentazocine, a putative σ_1R agonist, in an in vitro model of neuron/microglia crosstalk following hypoxia/reoxygenation.
Methods: Microglia (BV2 cells) was exposed (3 h) to 1% oxygen and reoxygenation was allowed for 24 h. Conditioned media obtained from this experimental condition was used to treat neuroblast-like cell line (SH-SY5Y cells) in the presence or absence of (+)-pentazocine (25 μM). Cell viability was measured by cytofluorimetric analysis, whereas inflammation and oxidative stress were evaluated by the expression of Hsp70, GAD, SOD and p65. Microglial cell migration was also evaluated by Xcelligence technology.
Results: Our results showed that (+)-pentazocine was able to increase SH-SY5Y cell viability following exposure to microglial-conditioned medium. Furthermore, (+)-pentazocine was also able to inhibit microglial cell toward neuron treated with hypoxic conditioned medium. Finally, pharmacological treatment reduced the expression of inflammatory and oxidative stress markers (GAD, SOD and p65). Interestingly, hypoxic medium was able to reduce the expression of Hsp70 and such effect was prevented by (+)-pentazocine treatment.
Conclusions: (+)-Pentazocine exhibits significant neuroprotective effects in our in vitro model of SH-SY5Y/microglial crosstalk thus suggesting that σ_1R may represent a possible strategy for neuroprotection.

1. Introduction

Ischemic cerebrovascular diseases are among the most common disorders of the central nervous system. The cascade of events that underlie the mechanism of ischemia/reperfusion injury is very complex because it involves different cell types. Oxidative stress is considered to be one of the most important pathophysiological mechanisms of such damage since it would be at the base of the acti-

Abbreviations: DTG, 1,3-di-(2-tolyl) guanidine; DTNB, 2,2-dithio-bis-nitrobenzoic acid; CNS, central nervous system; Hsp70, heat shock protein 70; IL-10, interleukin 10; NO, nitric oxide; LPS, lipopolysaccharide; PBS, phosphate buffered saline solution; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α.

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vation of the crosstalk between the different cell types of the brain such as astrocytes, neurons and microglia [3]. In particular, this last cell type plays an important role in sensing and in the modulation of neuronal function. Following an inflammatory stimulus, microglia takes on an amoeboid phenotype and upregulates numerous cell surface receptors involved in the innate immune response.

Besides regulating neuronal apoptosis, microglia secretes various cytokines and growth factors controlling synapse formation and plasticity. Unarguably, microglial cells have to be kept in check under normal conditions. Furthermore, since excessive inflammation causes significant damage to the CNS, the microglia activation must be limited by a series of counter-regulatory mechanisms for a proper maintenance of CNS homeostasis [2,7]. Unfortunately, the mechanisms allowing the maintenance of a proper balance between these mechanisms remain to be fully elucidated. Various pharmacological strategies have been developed with the aim to regulate the crosstalk between neurons and microglia in order to obtain a significant neuroprotective effect. To this regard, σ_1R agonists are recently emerging as good candidates for neuroprotective strategies under various experimental models [11]. σ_1R are highly expressed in neurons as well as microglia [5]. The activated σ_1R can modulate the kinetics of Na^+ channels and the release of glutamate from presynaptic loci [4]. It also has been reported that σ_1R are involved in the modulation of neuroinflammation. σ_1R agonists, such as (+)-pentazocine and 1,3-Di-(2-tolyl) guanidine (DTG), inhibit lipopolysaccharide (LPS)-stimulated activation of microglia, and decrease the production of reactive oxygen species (ROS), the release of pro-inflammatory molecules [tumor necrosis factor- α (TNF-α), nitric oxide (NO), interleukin 10 (IL-10), etc.] as well as the expression of monocyte chemoattractant protein-1 [5,8,9,13]. Based on the findings described above, the present study aimed at evaluating the effect of (+)-pentazocine on the pathological crosstalk between neurons and microglia in an in vitro model using conditioned medium from hypoxia-activated microglial cells.

2. Materials and methods

2.1. Cell culture and pharmacological treatments

Human neuroblast-like cell line (SH-SY5Y) and microglia (BV2) cells were purchased from ATCC Company (Milan, Italy). Cells were suspended in culture medium (Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin). At 80% confluence, cells were passaged using trypsin-EDTA solution (0.05% trypsin and 0.02% EDTA). To obtain a conditioned media from activated microglia, BV2 cells were washed 3 times with Phosphate Buffered Saline solution (PBS), replaced with serum-free DMEM medium, and placed in an incubator at 37 °C containing 1.0% O_2 for 3 h to initiate hypoxia, followed by 24 h reoxygenation in an incubator at 37 °C containing 5% CO_2. In order to test the neuroprotective effects of (+)-pentazocine, SH-SY5Y cells were also treated with (+)-pentazocine (Sigma–Aldrich, Milan, Italy) at a final concentration of 25 μM. This concentration was used on the basis of our previous experiments in BV2 cells and by our preliminary data [6] assessing the toxicity of this compound under our experimental conditions (data not shown).

2.2. Cell viability evaluation cytofluorimetric analysis

Cell viability was assessed by Muse™ Count & Viability Kit (Catalog No. MCH100102, Millipore, Milan, Italy) according to the manufacture's guidelines. Briefly, 50 μL of cell suspension containing 1×10^6 cells/mL were mixed with 450 μL of Count & Viability Reagent. Cells were allowed to stain for 10 min at room temperature and samples were read by Muse™ Cell Analyzer (Millipore).

2.3. Microglia migration assay

Real-time monitoring of BV2 cell migration was performed using the xCELLigence system with the CIM-Plate 16 (Roche). The upper chamber was seeded with 50,000 BV2. When BV2 cells migrated through the membrane into the bottom chamber seeded with SH-SY5Y cells treated or untreated with pentazocine and cultured in the presence or absence of activated microglia conditioned medium, they contacted and adhered to the electronic sensors, resulting in an increase in impedance. The cell-index values, reflecting impedance changes and cell migration, were automatically and continuously recorded every 15 min for 37 h.

2.4. Immunocytochemistry

Immunocytochemistry for oxidative and endoplasmic reticulum (ER) stress was performed following mouse anti-Hsp70 (Catalog number A-400), mouse anti-SOD, p65 (Catalog number MAB3419) and mouse anti-GAD (Catalog number MAB2086) specific antibody (1:200) (R&D system, Milan, Italy), and then incubated with a species-specific FITC-conjugated secondary antibody (1:400) (Chemicon, Milan, Italy). Specimens were washed thoroughly in between incubations and counterstained with DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride) (Sigma–Aldrich). The sections were mounted with polyvinyl alcohol mounting medium with DABCO (Sigma–Aldrich) and visualized under fluorescence microscope. As a control, the specificity of immunostaining was verified by omitting incubation with the primary or secondary antibody. Digital images were acquired using a Leica fluorescence microscope connected to a digital camera (Spot, Diagnostic Instruments; Sterling Heights, USA). Immunoreactivity was evaluated taking into account the signal-to-noise ratio of immunofluorescence.

2.5. Statistical analysis

The data were expressed as the means ± SD. Statistical analysis was performed via one-way analysis of variance (ANOVA) using SPSS11.0 software. $P < 0.05$ was considered to be significant.

3. Results

Our data showed that conditioned medium from hypoxia-activated microglia significantly reduced SH-SY5Y cells viability (Fig. 1B and D) when compared to non-hypoxic conditioned medium (Fig. 1A and D). Interestingly, (+)-pentazocine treatment resulted in a significant protection following treatment with hypoxic conditioned medium (Fig. 1C and D) when compared to untreated hypoxic group (Fig. 1B).

Furthermore, we showed that hypoxic medium results in a significant increase of microglia migration (Fig. 2) and this effect is prevented by treatment with (+)-pentazocine. To our knowledge, this is the first report showing the effect of (+)-pentazocine in microglia migration and thus suggesting a role in the crosstalk between SH-SY5Y cells and microglia.

In order to further confirm the positive effects of (+)-pentazocine on the crosstalk between SH-SY5Y cells and microglia we evaluated the expression of proteins involved in neuroinflammation and oxidative stress. In particular, this set of experiments showed that hypoxic medium resulted in a significant increase in the expression of proteins involved in inflammation and ER stress SOD, GAD and p65 (Fig. 3). Consistently with the above mentioned results, (+)-pentazocine resulted in a significant decrease in the expression of