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Cerebrolysin protects PC12 cells from $CoCl_2$-induced hypoxia employing GSK3β signaling

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ABSTRACT

Cerebrolysin (EVER Neuro Pharma GmbH, Austria) is a peptidergic drug indicated for clinical use in stroke, traumatic brain injury and dementia. The therapeutic effect of Cerebrolysin is thought to ensure from its neurotrophic activity, which shares some properties with naturally occurring neurotrophic factors. However, the exact mechanism of action of Cerebrolysin is yet to be fully deciphered. This study aimed to investigate the neuroprotective effect of Cerebrolysin in a widely used in vitro model of hypoxia-induced neuronal cytotoxicity, namely cobalt chloride ($CoCl_2$)-treatment of PC12 cells. $CoCl_2$-cytotoxicity was indicated by a reduced cell-diameter, cell shrinkage, increased pro-apoptotic Caspase-activities and a decreased metabolic activity. Cerebrolysin maintained the cell-diameter of $CoCl_2$-treated naïve PC12 cells, decreased the activation of Caspase 3/7 in $CoCl_2$-stressed naïve PC12 cells and restored the cells' metabolic activity in $CoCl_2$-impaired naïve and differentiated PC12 cells. Cerebrolysin treatment also decreased the levels of superoxide observed after exposure to $CoCl_2$. Investigating the mechanism of action, we could demonstrate that Cerebrolysin application to $CoCl_2$-stressed PC12 cells increased the phosphorylation of GSK3β, resulting in the inhibition of GSK3β. This might become clinically relevant for Alzheimer's disease, since GSK3β activity has been linked to the production of amyloid beta. Taken together, Cerebrolysin was found to have neuroprotective effects in $CoCl_2$-induced cytotoxicity in PC12 cells.

1. Introduction

A central hallmark of neurodegenerative diseases and acute CNS lesions is neuronal cell death, which constitutes the prime cause of associated functional deficits such as cognitive and motor disabilities. In consequence, strategies aiming to protect neurons from cell death have been under development over the past decades. The identification of neurotrophic factors such as nerve growth factor (NGF, Cohen, 1960) and brain-derived neurotrophic factor (BDNF, Lindsay et al., 1985) triggered the search for druggable brain-derived neurotrophic activities (Longo and Massa, 2013). Cerebrolysin is produced by a standardized biochemical breakdown of brain lysates and consists of low molecular weight peptides and amino acids (EBEWENeuroPharmaGmbH, 2009). The resulting drug displays neurotrophic activity (Mallory et al., 1999; Masliah and Diez-Tejedor, 2012), protects primary neurons from glutamate-induced excitotoxicity (Hutter-Paier et al., 1996), displays neuroprotective activity in animal models of neurodegeneration (Francis-Turner et al., 1996; Francis-Turner and Valouskova, 1996; Valouskova and Francis-Turmer, 1996; Masliah et al., 1999; Veinbergs et al., 2000) or ischemic and traumatic CNS lesions (Zhang et al., 2013a,b) and modulates endogenous neurotrophin levels (Ubhi et al., 2013). Furthermore, Cerebrolysin is used in human to treat dementia, stroke and traumatic brain injuries. In this respect, Cerebrolysin administration was reported

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to improve cognitive functions and activities of daily living in patients suffering of Alzheimer's disease (Ruether et al., 1994, 2000; Alvarez et al., 2006, 2011). Despite the strong preclinical data and accumulating evidence of Cerebrolysin's clinical efficacy, little is known about the mechanisms of action. There is evidence for Cerebrolysin-promoted metabolic stabilization of neurons via protein synthesis modulation (Piswanger et al., 1990), prevention of lactose acidosis (Windisch and Piswanger, 1985), and prevention of free radical formation (Sugita et al., 1993). Moreover, Cerebrolysin might also modulate neurotransmitter signaling such as adenosine (Xiong et al., 1995) or GABA (Wojtowicz et al., 1996). In the present study, we investigate the molecular mechanisms underlying Cerebrolysin neuroprotective activities following application of cobalt chloride ($CoCl_2$) on naïve and differentiated PC12 rat pheochromocytoma cells. This well-established and widely-used model constitutes an in vitro paradigm that mimics a hypoxic condition and induces cell death. It involves the induction of mitochondrial DNA damage (Wang et al., 2000), the production of reactive oxygen species (ROS), activation of the pro-apoptotic gene APAF-1 (Zou et al., 2001), and activation of Caspase 3 and p38 mitogen-activated protein kinase (Zou et al., 2002) and suppression of mTOR signaling (Zhong et al., 2014).

2. Materials and methods

2.1. PC12 cell cultures and $CoCl_2$-induction of cell death

For cell propagation, PC12 rat pheochromocytoma cells (ATCC CRL-1721™) were grown in RPMI 1640-medium (Gibco), supplemented with 10% heat inactivated horse serum (Gibco), 5% heat inactivated fetal bovine serum (Gibco), 100 U/mL penicillin/100 μg/mL streptomycin (Gibco) and 2 mM L-glutamine (Gibco) in a humified incubator with 5% CO_2 at 37 °C. The cells were grown as adherent cultures on poly-L-ornithine-hydrobromide coated (final concentration 100 μg/mL H_2O, Sigma Aldrich) T-75 flasks to a confluence of approximately 80%. For assay performance, the cells were harvested from the flasks using a 0.05% trypsin/EDTA 1×-solution (Gibco), were counted using a CASY cell counter (Roche) and were seeded after resuspension in cell culture medium at a defined concentration. For Caspase-Glo® 3/7 Assay 1×10^4 cells per well, for AlphaScreen® Sure Fire® Assay Kits 5×10^4 cells per well, for CellTiter96® Aqueous One Solution Cell Proliferation Assay 5×10^4 cells per well (naïve PC12 assay) or 1×10^4 cells per well (PC12 differentiation assay) were seeded in coated 96-well-plate. For FACS, cell count and diameter analysis 1×10^6 cells per 10 mL were seeded in coated T-25 flasks. The next day (after >12 h), the cells were either treated with Cerebrolysin, NGF or Prionex ± $CoCl_2$. The $CoCl_2$-solution (Sigma Aldrich) (stock: 10 mM-solution in sterile water stored at 4 °C) diluted with cell culture medium was added to a final concentration of 150 μM $CoCl_2$ for naïve PC12 cells and 300 μM $CoCl_2$ for differentiated PC12 cells. For medium control conditions, $CoCl_2$ was replaced by the corresponding volume of cell culture medium. Cerebrolysin (CB) was added at various final concentrations of 0.1%, 0.5%, 1%, 2%, 3% and 5%. The following batches were used: #237077, #201070 and #136086. Prionex® protein stabilizer solution from porcine collagen (1:10 diluted in H_2O, Sigma Aldrich) at final concentrations of 1%, 2% and 5% served as an unspecific peptide control (UP). Nerve growth factor (NGF) 2.5S Native Mouse Protein (Invitrogen) in concentrations of 20, 50 and 100 ng/mL was used as a positive control for neurotrophic activity. Cells were incubated in these various conditions for 22 ± 2 h in the incubator (37 °C, 5% CO_2). After the incubation they were further analyzed for caspase activation, metabolic activity/cell proliferation, cell diameter, cell count, reactive oxygen species (ROS)/superoxide production, and for phosphorylation of Akt and GSK3β.

2.2. Differentiation of PC12 cells

1×10^4 PC12 cells per well were seeded at 96-well-plate and then differentiated by NGF treatment for 6 days (day 0: seeding, day 1–5: 50 ng/mL and day 6: 100 ng/mL NGF); adapted from Das et al. (2004) (see also Fig. 3A). At the end of the treatment, the NGF medium solution was removed and 100 μL/well fresh medium was added to the cells before the specific treatment followed (procedure see previous chapter).

2.3. Caspase activation assay

The Caspase-Glo® 3/7 Assay Kit (Promega) was prepared according to the manufacturer's instruction and added to the wells (dilution 1:1). Cells were further incubated for 1 h at room temperature in the dark. Then, luminescence (RLU) was measured using a Tecan Infinite® M200 platereader. The background luminescence was excluded by performing blank corrections (subtraction of RLU values obtained for the same treatment without cells).

2.4. Metabolic activity/cell proliferation – MTS

MTS-solution (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega) was added to each well (dilution 1:6) and cells were further incubated for 2 h at 37 °C and 5% CO_2, before absorbance (OD) was measured at 490 nm using a Tecan Infinite® M200 platereader. Background absorbance was excluded by performing blank corrections.

2.5. Measurement of cell count/diameter of PC12 cells

After treatment the cells were detached by 0.05% trypsin/EDTA 1×-solution. Then, the diameter of the cells and the cell count were measured using the CASY cell counter (Roche).

2.6. FACS analysis of reactive oxygen species (ROS)/superoxide levels

For the analysis of the generation of ROS and superoxides the total ROS/Superoxide Detection Kit (Enzo) was performed according to the manufacturer's instruction. Briefly, after treatment of the cells the medium was removed and cells were detached, washed with 1× wash buffer and then treated with the ROS/Superoxide Detection Mix for 30 min at 37 °C. Positive control (Pyocyanin) was applied 30 min before treatment with the ROS/Superoxide Detection Mix.

2.7. Sure fire® analysis of phosphorylation of GSK3β (p-Ser9), Akt (p-Ser473) and Akt1 (p-Thr308)

After treatment the medium was removed, the cells were lysed and then treated with the assay reagents according to the manufacturer's instructions (AlphaScreen® Sure Fire® GSK3β (p-Ser9) Assay Kit; AlphaScreen® Sure Fire® Akt (p-Ser473) Assay Kit, AlphaScreen® Sure Fire® Akt1 (p-Thr308) Assay Kit, Perkin Elmer). The signal was detected by using the Alpha Technology on Perkin Elmer, EnSpire® 2300 platereader.

2.8. Statistics

Data were expressed as mean ± standard deviation (SD). All analyzed assay data resulted from at least three independent experiments. Multiple comparisons were analyzed by ANOVA (parametric) or Kruskal–Wallis One Way Analysis of Variance on Ranks (non-parametric) and specific Post hoc Tests, as Tukey Test, Student–Newman–Keuls Method, or Dunn's Method. A p value of $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Cerebrolysin and NGF protect PC12 cells from $CoCl_2$-induced cell death

In the first set of experiments, the effects of $CoCl_2$ treatment on cell viability and morphology of naïve PC12 cells and the protective activities of the neurotrophic compounds NGF and Cerebrolysin were studied. Treatment of PC12 cells with 150 μM of $CoCl_2$ induced shrinkage of cells (medium control: 13.3 ± 0.2 μm vs. $CoCl_2$: 11.9 ± 0.3 μm) and significantly decreased the viable cell number suggesting the onset of cell death (Fig. 1A and C). This effect of $CoCl_2$ on PC12 cells was not influenced by the unspecific peptide mix Prionex (5%) or 100 ng/mL NGF. Furthermore, shrinkage of PC12 cells was observed as the cell diameter of $CoCl_2$ and $CoCl_2$ + Prionex treated cells (11.7 ± 0.4 μm) significantly decreased (Fig. 1B). As expected from previous reports (Das et al., 2004; Greene, 1978; Young et al., 1983), 100 ng/mL of NGF induced neurite outgrowth in naïve PC12 cells (Fig. 1C) which was confirmed by microscopy analysis as well as by a significant increase in cell diameter (14.6 ± 0.8 μm). Cerebrolysin (5%), in contrast to NGF, did not induce process outgrowth in naïve PC12 cells, but protected from $CoCl_2$-induced loss of viability and cell shrinkage (13.1 ± 0.6 μm) indicating the presence of a protective activity.

3.2. Cerebrolysin and to a lesser extent NGF protect naïve PC12 cells from $CoCl_2$-induced metabolic collapse

The MTS assay was used as readout for cell viability/proliferation and metabolic activity. In naïve unstressed PC12 cells, neither Cerebrolysin, nor NGF or Prionex had any effect on metabolic activity