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

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Abstract

Cerebrolysin (EVER Neuro Pharma, Austria) is a peptidergic drug indicated for clinical use in stroke, traumatic brain injury and dementias. 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(s) 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 $CoCl_2$-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 naïve PC12 cells and restored the cells' metabolic activity in naïve and differentiated PC12 cells. Cerebrolysin treatment also decreased the levels of superoxides 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 GSK3ß, resulting in the inhibition of GSK3ß. This might become clinically relevant for Alzheimer's disease, since GSK3ß 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.

Key words:

Neuroprotection

Neurotrophic factors

Cell death

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) [1] and brain-derived neurotrophic factor (BDNF) [2] triggered the search for druggable brain-derived neurotrophic activities. Cerebrolysin is produced by a standardized biochemical breakdown of brain lysates and consists of low molecular weight peptides and amino acids [3]. The resulting drug displays neurotrophic activity on neuronal cell lines [4], protects primary neurons from glutamate-induced excitotoxicity [5], and acts neuroprotective in animal models of neurodegeneration [6-10]. Furthermore, Cerebrolysin is used in human to treat dementia, stroke and traumatic brain injuries. In this respect, Cerebrolysin administration was reported to improve cognitive functions and activities of daily living in patients suffering of Alzheimer's disease [11-14]. 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 [15], prevention of lactose acidosis [16], and prevention of free radical formation [17]. Moreover, Cerebrolysin might also modulate neurotransmitter signaling since it was demonstrated to interact with adenosine [18] and with GABA b-receptors [19]. 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 [20], the production of reactive oxygen species (ROS), activation

of the pro-apoptotic gene APAF-1 [21], and activation of caspase-3 and p38 mitogen-activated protein kinase [22].

Materials and Methods

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 1x-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 x 10^4 cells per well, for AlphaScreen® Sure Fire® Assay Kits 5 x 10^4 cells per well, for CellTiter96® Aqueous One Solution Cell Proliferation Assay 5 x 10^4 cells per well (naïve PC12 assay) or 1 x 10^4 cells per well (PC12 differentiation assay) were seeded in coated 96-well-plate. For FACS, cell count and diameter analysis 1 x 10^6 cells per 10 ml were seeded in coated T-25 flasks. The next day (after >12h), the cells were either treated with Cerebrolysin, NGF or Prionex ± $CoCl_2$. The cobalt chloride ($CoCl_2$)-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 Natural Mouse (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 hours 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 oxidative species (ROS)/Superoxide production, and for phosphorylation of Akt and GSK3ß.

Differentiation of PC12 cells

$1x10^4$ PC12 cells per wells were seeded at 96-well-plate and then differentiated by NGF treatment for 6 days (day 0: seeding, day 1 - 5: 50ng/ml and day 6: 100 ng/ml NGF; adapted from Das *et al.* [23], see also figure 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).

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 1h 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).

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 2h 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.

Measurement of cell count / diameter of PC12 cells

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

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 1x 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.

Sure Fire Analysis of phosphorylation of GSK3β (p-Ser9), Akt (p-Ser473) and Akt (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.

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.

Results

Cerebrolysin and NGF protect PC12 cells from CoCl2-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 and significantly decreased the viable cell number suggesting the onset of dell death (Fig. 1A,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 significantly decreased (Fig. 1B). As expected from previous reports [23,24,25], 100 ng/ml of NGF induced neurite growth in naïve PC12 cells (Fig. 1C) which was confirmed by microscopy analysis as well as by a significant increase in cell diameter. 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 indicating the presence of a protective activity.

Fig. 1A

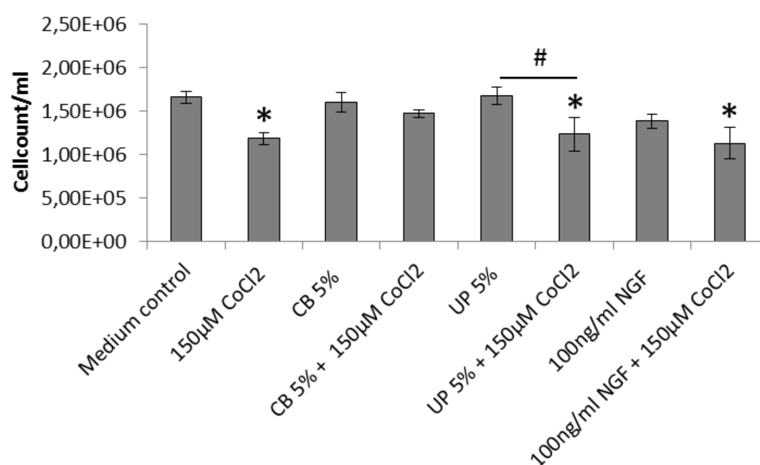

Fig. 1B

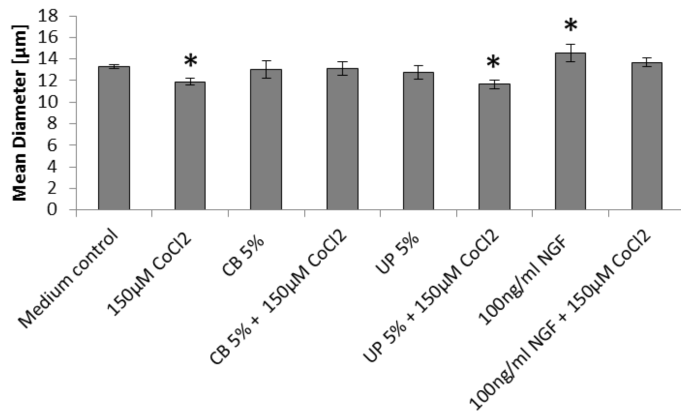

Fig. 1C

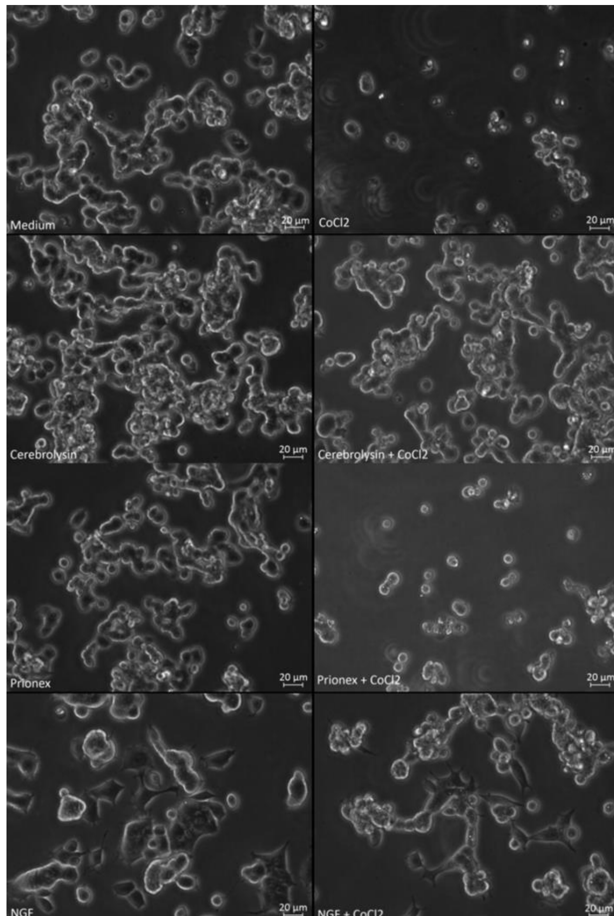

Figure 1: Cerebrolysin and NGF, protect PC12 cells from $CoCl_2$-induced cell death. The cells were stimulated for 24h with medium, 5% Cerebrolysin (CB), 5% Prionex (UP), 100 ng/ml NGF, 150 µM $CoCl_2$, 150 µM $CoCl_2$ + 5% Cerebrolysin (CB), 150 µM $CoCl_2$ + 5% Prionex

(UP), 150 µM $CoCl_2$ + 100 ng/ml NGF. A) shows the number of PC12 cells after treatment with the different substances. Note the significant decrease in cell number after 150 µM $CoCl_2$, 150 µM $CoCl_2$ + 5% Prionex and 150 µM $CoCl_2$ + 100 ng/ml NGF treatment. The cell death induced by these treatments is also seen in the microscopic pictures of C). B) shows the mean diameter of PC12 cells, which is significantly decreased after 150 µM $CoCl_2$, 150 µM $CoCl_2$ + 5% Prionex and increased after NGF treatment compared with medium control conditions. The NGF induced neurite growth is seen in C).

Asterix marks a p-value of <0.05 with ANOVA, Student-Newman-Keuls Method (*compared to medium control). The values are presented as mean +/- SD (N = 3).

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 of the cells (Fig. 2A). The $CoCl_2$ treatment (150 µM) significantly reduced the cell viability (Fig. 2B). While the metabolic breakdown of PC12 cells was only partly prevented by NGF and not at all by the unrelated peptidemix Prionex, Cerebrolysin had a dose-dependent protective effect, indicating a Cerebrolysin-specific activity (Fig. 2B). This was further supported by the fact that the protective effect of 2%, 3% and 5% Cerebrolysin was significantly higher compared with NGF and reached unstressed control conditions of proliferative activity.

Fig. 2A

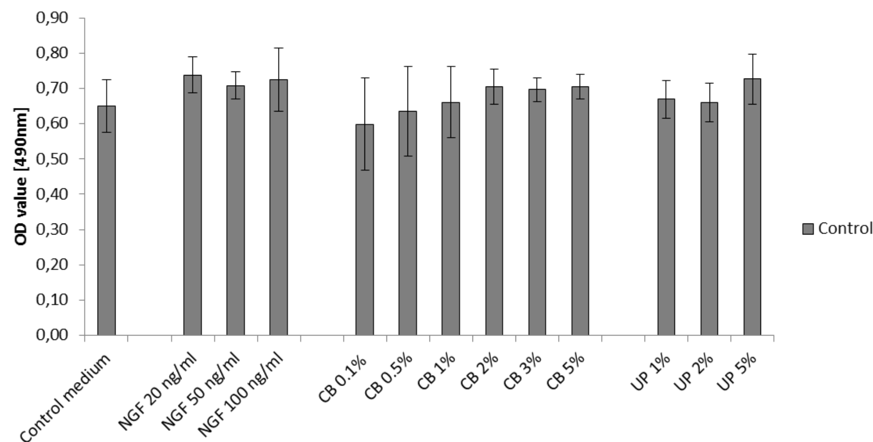

Fig. 2B

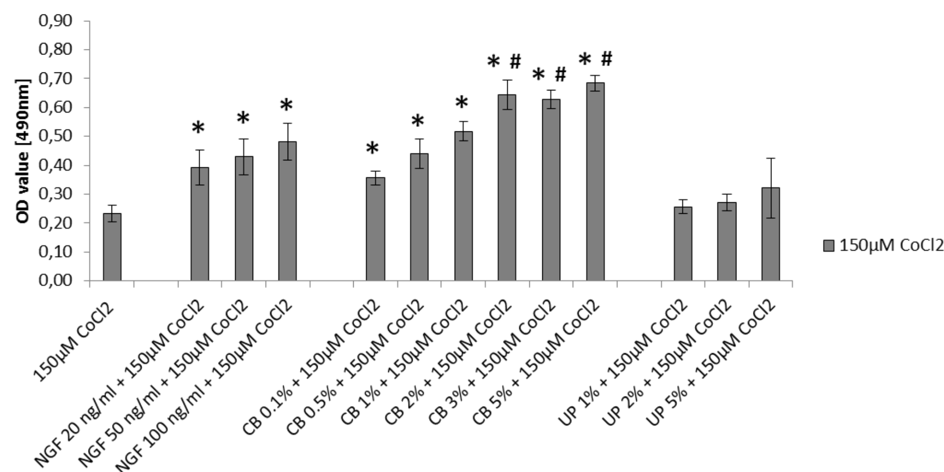

Figure 2: Cerebrolysin and to a lesser extent NGF, protect naïve PC12 cells from $CoCl_2$ induced-metabolic collapse. A) The metabolic activity of Cerebrolysin (CB), NGF and Prionex (UP) did not differ significantly from medium control conditions. B) Treatment with 150µM $CoCl_2$ compromised the metabolic activity. This toxic effect of $CoCl_2$ was significantly counteracted by Cerebrolysin and NGF (*). The protective effect of 2%, 3% and 5% Cerebrolysin (#) was significantly higher compared with NGF. Prionex had no protective effect.

Asterix marks p-value < 0.5 (* compared to control). The values are presented as mean +/- SD (N = 3). A) Kruskal-Wallis One Way Analysis of Variance on Ranks, Dunn's Method. B) ANOVA, Tukey Test

Cerebrolysin protects differentiated PC12 cells from $CoCl_2$-induced metabolic collapse

In the next experiments we tested if Cerebrolysin has protective effects on differentiated PC12 cells. NGF treatment for 6 days induced the differentiation of PC12 cells as indicated by process outgrowth (Fig. 3A). In differentiated PC12 cells no significant cell stress at 150 µM $CoCl_2$ was detected (OD values: medium control: 0.84 vs 150 µM $CoCl_2$: 0.79) whereas at elevated $CoCl_2$ concentrations (300 µM) metabolic activity was clearly abolished (OD value: 0.09). Cerebrolysin reversed the negative effect of 300 µM $CoCl_2$ in a dose dependent manner (Fig. 3B). Neither NGF nor Prionex exerted a significant effect on cell survival, which clearly supports a Cerebrolysin-specific effect also in differentiated PC12 cells.

Fig. 3A

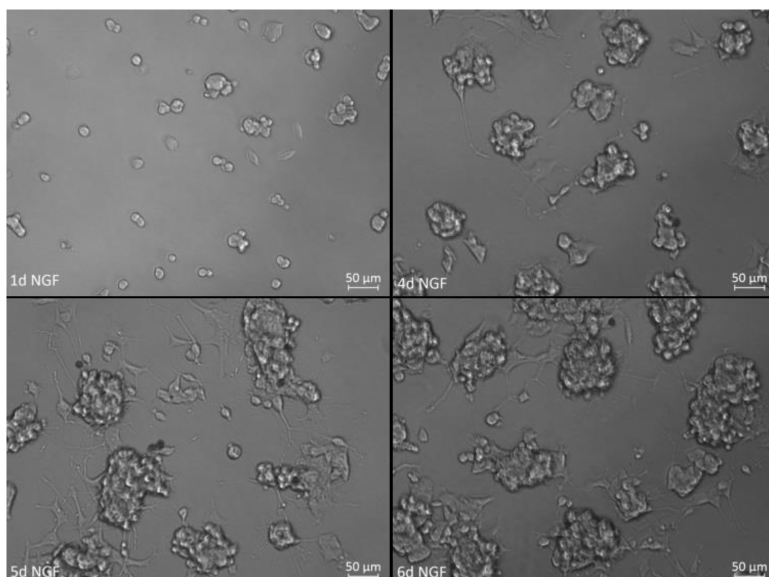

Fig. 3B

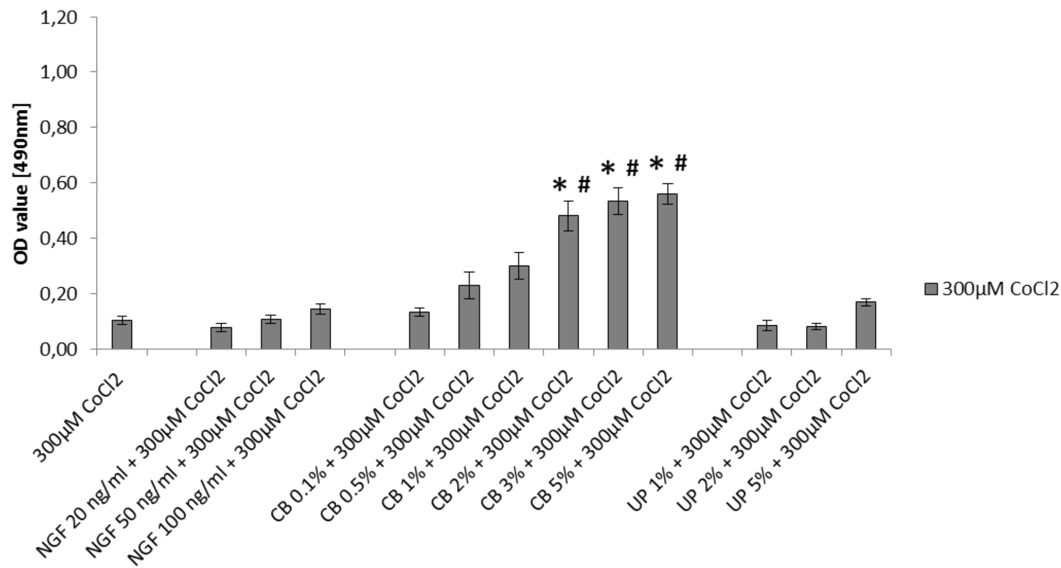

Figure 3: Cerebrolysin protects differentiated PC12 cells from $CoCl_2$-induced metabolic collaps. A) shows the differentiation process of PC12 cells by NGF. B) Cerebrolysin (CB 2% - 5%) reversed significantly (*) the $CoCl_2$ treatment-induced stress. The protective effect of Cerebrolysin (CB 2% - 5%) was significantly (#) higher compared with NGF.

Asterix marks p-value < 0.5 (*compared to control). The values are presented as mean +/- SD (N = 3). A) ANOVA, Tukey Test. B) Kruskal-Wallis One Way Analysis of Variance on Ranks, Dunn's Method

Cerebrolysin protects PC12 cells from $CoCl_2$-induced apoptosis

Since the observed cell shrinkage, the reduction in cell viability and the reduced metabolic activity were indicative for an apoptotic cell death, the activation of Caspases 3 and 7 in response to $CoCl_2$ was analyzed. Cerebrolysin induced a small increase in Caspases 3/7 in unstressed naïve PC12 cells compared to NGF treatment (Fig. 4A). However, these small

changes did not correspond to reduced cell viability as demonstrated by the MTS Readout (Fig. 2A). The treatment with 150 μM $CoCl_2$ induced a significant increase (approximately 7.4 fold) of Caspase 3/7 activity compared to control conditions (Figs. 4A,B). Cerebrolysin exerted a strong protective effect by inducing a significant, dose-dependent decrease of Caspase 3/7-activity already at 0.5% final concentration (Fig. 4B) and completely abolished Caspase 3/7 activation at higher concentrations. NGF also induced a significant protective effect, which however showed no dose-dependency in the range from 20 to 100 ng/mL and was significantly smaller than the effect of >1% Cerebrolysin. Since the unspecific peptidmix Prionex even increased the amount of Caspases 3/7 in $CoCl_2$-treated PC12 cells, this anti-apoptotic effect of Cerebrolysin was found to be specific.

PC12 cell differentiation caused a time-dependent massive increase of Caspase 3/7 activity in PC12 cells. After 6 days of differentiation neither 150 μM nor 300 μM $CoCl_2$ were able to increase the Caspase 3/7 activity. Therefore the apoptotic potential of $CoCl_2$ could not be studied with the Caspase-Glo 3/7 Assay Kit. This increase of Caspase3 activity in differentiated cells has been previously described as well as the fact that the neuronal Caspase 3 has not only apoptotic effects but mediates also non-apoptotic, physiological functions, such as synaptic plasticity [26].

Fig. 4A

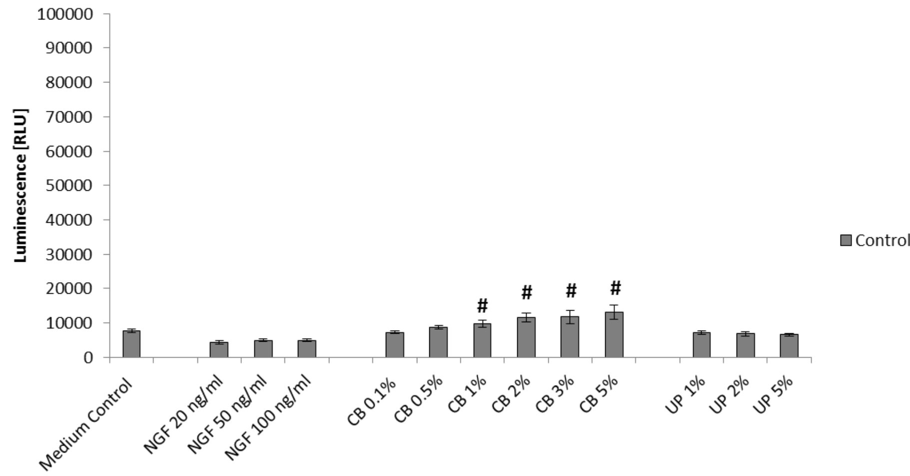

Fig. 4B

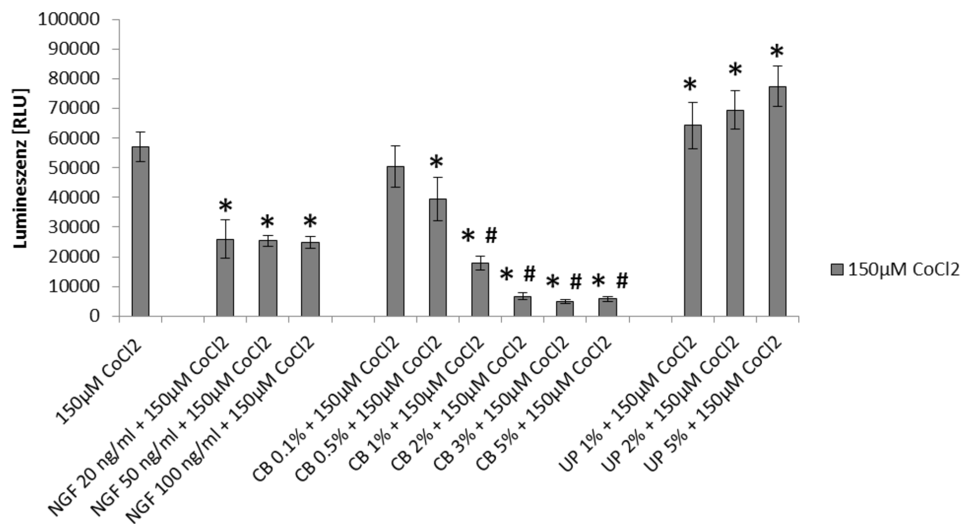

Figure 4: Cerebrolysin protects naïve PC12 cells from $CoCl_2$-induced apoptosis. A) Cerebrolysin (CB 1% - 5%) induced slightly significant (#) higher RLU values compared to NGF. B) Cerebrolysin (CB 0.5% - 5%) reversed significantly (*) the $CoCl_2$ treatment-induced

Caspase 3/7 activation. The protective effect of Cerebrolysin (CB 1% - 5%) was significantly (#) higher when compared to NGF. Prionex (UP) even elevated the effect of $CoCl_2$ on caspase activation.

Asterix marks p-value < 0.5 (*compared to control). The values are presented as mean +/- SD (N = 3). A) Kruskal-Wallis One Way Analysis of Variance on Ranks, Tukey Test. B) ANOVA, Tukey Test

Decreased superoxide levels in $CoCl_2$-stressed PC12 cells co-treated with Cerebrolysin

One of the central hallmarks of neuronal, in particular of hypoxia-induced neuronal cell death is the production of ROS. To analyze the generation of ROS upon treatment with $CoCl_2$, we used as FACS based assay kit that allows to separately address the formation of superoxide anions and other ROS species. Treatment with $CoCl_2$ (150 µM) induced a clear and specific production of superoxides, which was completely reversed by Cerebrolysin, whereas NGF only partly reversed superoxide production and Prionex did not display any protective effect at all (Fig. 5). $CoCl_2$ induced superoxide production in 70% of the cells. This effect was in accordance with the previous observations, where the majority of PC12 cells underwent apoptotic cell death upon treatment with $CoCl_2$.

Fig. 5

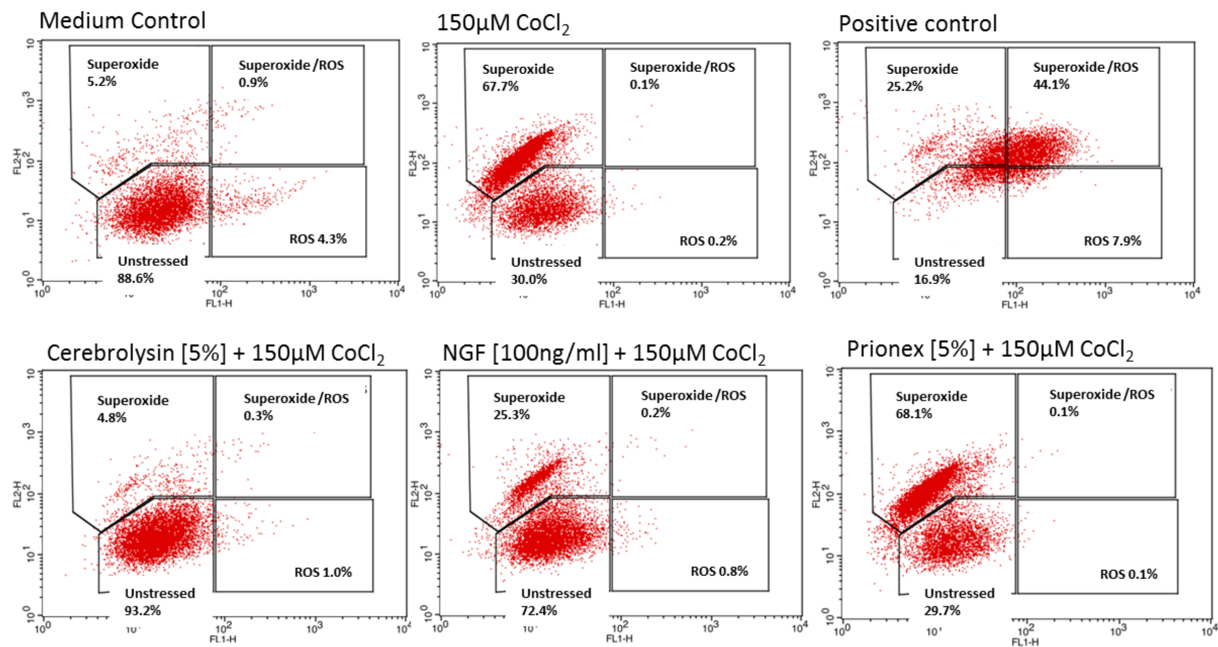

Figure 5: Cerebrolysin decreases superoxide levels in $CoCl_2$-stressed PC12 cells. Figure shows a representative example of FACS based analysis of ROS/superoxide generation. Pyocyanin (positive control) induced ROS and superoxide production. $CoCl_2$ (150 µM) induced only superoxide production in 68% of the PC12 cells. An effect, which was completely reversed by 5% Cerebrolysin (<5% superoxide production).

The neuroprotective effect of Cerebrolysin and NGF correlates with phosphorylation of GSK3β

Finally, we analyzed in $CoCl_2$-treated PC12 cells one of most relevant signaling cascade for cell survival, namely the Akt/GSK3β signaling. Treatment of $CoCl_2$-challenged PC12 cells with Cerebrolysin (5%) led to a strong increase in the levels of phosphorylated GSK3β Serine 9 (S9) (Fig. 6), but had neither effect on phosphorylation of Ser473 of Akt nor on Thr308 of

Akt (data not shown). NGF (100 ng/ml) also induced GSK3β phosphorylation independent of Akt-signaling pathway. Prionex (5%) was not able to target this pathway in $CoCl_2$-stressed cells and showed therefore comparable low phospho-GSK3β levels as 150µM $CoCl_2$ only (Fig.6).

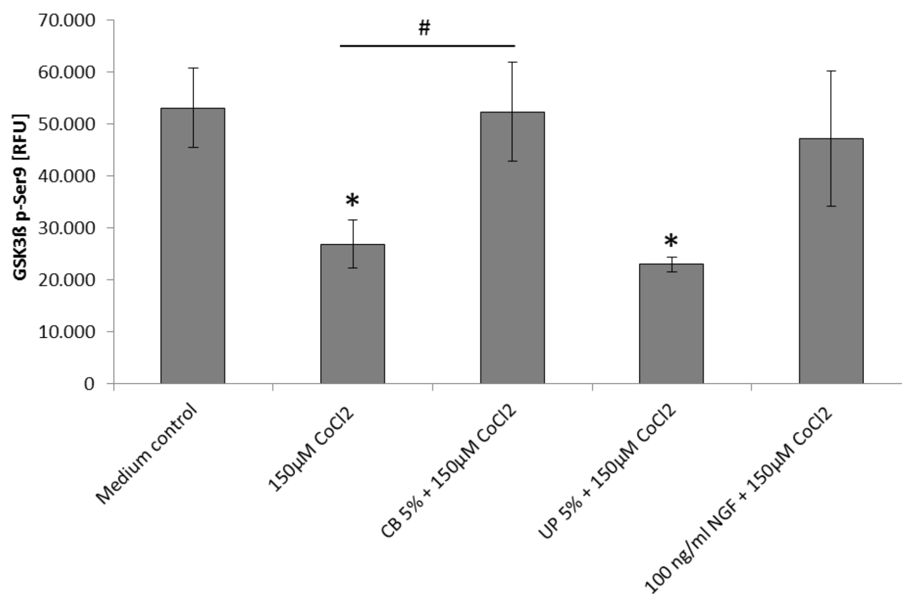

Figure 6: The neuroprotective effect of Cerebrolysin and NGF correlate with phosphorylation of GSK3β. In $CoCl_2$-stressed PC12 cells Cerebrolysin (5%) and NGF (100ng/ml) induced phosphorylation of GSK3βS9 independent of Akt signaling pathway (Ser473 and Thr308, data not shown). Prionex (5%) could not induce GSK3β phosphorylation. Asterix marks p-value < 0.5 (*compared to control). The values are presented as mean +/- SD (N = 3: medium control, 150 µM $CoCl_2$, CB 5% + 150µM $CoCl_2$; N = 2: 100 ng/ml NGF + 150 µM $CoCl_2$, UP 5% + 150 µM $CoCl_2$). Kruskal-Wallis One Way Analysis of Variance on Ranks, Dunn's Method.

Discussion

Here we demonstrate that Cerebrolysin protects PC12 cells from $CoCl_2$-induced cell death. Our findings are consistent with previous reports illustrating neuroprotective properties of Cerebrolysin in different neuronal *in vitro* systems [5,28]. For example, pre-treatment with Cerebrolysin enhanced the survival of chick embryo telencephalic neurons that were exposed to toxic levels of glutamate [5]. Similarly, Cerebrolysin protected primary embryonic chick cortical neurons from apoptosis induced by serum-deprivation [29].

It is noteworthy that NGF and Cerebrolysin had different effects on $CoCl_2$-challenged PC12 cells suggesting diverse underlying mechanisms. NGF promoted neurite growth in naïve PC12 cells and displayed only partial protection from $CoCl_2$-induced cell death. In differentiated PC12 cells, NGF showed no protective activity at all; suggesting, that the positive effect of NGF is related to its' cell differentiation stimulating activity. Cerebrolysin, in contrast, did not induce neurite outgrowth, but strongly promoted cell survival of $CoCl_2$-treated PC12 cells, independent of the final $CoCl_2$ concentration (150 µM for naïve and 300 µM for differentiated PC12 cells. The higher $CoCl_2$ concentration, which was necessary to impair differentiated PC12 cells, indicates a higher stress resistance of these cells. An effect which has been previously described by Ekshyyan *et al.*, who demonstrated a decreased vulnerability of differentiated PC12 cells due to enhanced peroxide reduction and increased redox cycle enzyme activities [27].

The $CoCl_2$-induced neurotoxicity of PC12 cells involves a number of different pathways that might be targeted by Cerebrolysin. For example, $CoCl_2$ exposure releases cytochrome c from mitochondria into the cytosol resulting in the activation of pro-apoptotic caspases and pro-apoptotic proteins such as Bax [30, 31]. In addition, it involves the death receptor pathway since $CoCl_2$ upregulates expression of Fas and Fas-L in PC12 cells [30]. Finally, $CoCl_2$

induces the generation of ROS and the transcriptional change of hypoxia-related genes such as hypoxia inducible factor (HIF-1), p53, and p21, thereby mimicking hypoxia [20,32].

In the present study, Cerebrolysin completely and specifically abolished the $CoCl_2$-induced production of superoxides, prevented the $CoCl_2$-triggered activation of Caspases 3 and 7 and maintained the metabolic activity of naïve PC12 cells, supporting a neuroprotective effect of Cerebrolysin. In addition, Cerebrolysin led to the phosphorylation of GSK3ß at Serine 9 (S9) a modification associated with inhibition of the kinase [33], which among others consequently reduced the apoptosis rate. The observed GSK3βS9 phosphorylation was not mediated via the Akt-signaling pathway in the $CoCl_2$-stressed PC12 cells, as Cerebrolysin did not induce Akt phosphorylation at Ser473 or Thr308. This missing Akt activation suggests that the GSK3β phosphorylation at S9 is mediated by different kinases, such as serum and glucocorticoid inducible kinase (SGK1) or proteinkinase A [33], which can phosphorylate GSK3β at S9 [34]. The relevant pathways for GSK3βS9 phosphorylation upon treatment with Cerebrolysin have not been identified, but might involve IGF1 and insulin signaling. Moreover, this finding might be clinically relevant for Alzheimer's disease, since GSK3ß activity has been involved in the production of amyloid beta [35].

To summarize, Cerebrolysin is able to protect PC12 cells from $CoCl_2$-induced apoptosis as shown by the reduction of Caspase 3 and 7-activity, inhibition of cell shrinkage and reduction of cell diameter. Furthermore, Cerebrolysin is able to restore the metabolic activity in $CoCl_2$-stressed naïve and differentiated PC12 cells. These protective effects might be associated with a decreased production of superoxides and the involvement of GSK3ß pathway.

Acknowledgements

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