Cobalt chloride affects the death of SH-SY5Y cells induced by inhibition of ubiquitin proteasome system. Role of heat shock protein 70 and caspase 3

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Abstract. The aim our study was to investigate protective effect of cobalt chloride (CoCl₂) in the model of proteasome stress of neuroblastoma SH-SY5Y cells induced by bortezomib, an inhibitor of 26S proteasome. We have focused our interests on Hsp70 and activation of caspase 3. Finally, we have compared the effect of CoCl₂ with an effect of the pre-treatment of the cells with 17-AAG, an inhibitor of Hsp90 that is capable to induce expression of Hsp70, or with IOX2, an inhibitor of isoform 2 of prolyl hydroxylase that increases stability of hypoxia-inducible factor 1α (HIF1α). Pre-treatment of SH-SY5Y cells for 24 h with CoCl₂, at concentrations of 150 or 250 µmol/l, and with 17-AAG at concentration 1 µmol/l but not with IOX2 at concentration 100 µmol/l, was associated with significantly increased expression of Hsp70. We have shown that pre-treatment of SH-SY5Y cells with CoCl₂ but not with 17-AAG or IOX2 was associated with significant delay of the cell death induced by proteasome stress. CoCl₂-mediated effect was consistent with inhibition of bortezomib-induced caspase 3 activation in the cells pre-treated with CoCl₂. Despite established neuroprotective properties of Hsp70 our results do not provide strong evidence that the effect of CoCl₂ could be mainly attributed to the ability of CoCl₂ to induce expression of Hsp70 and other mechanisms have to be considered.

Key words: Neuroprotection — Ubiquitin proteasome system — Cobalt chloride — Heat shock proteins — Caspase 3

Abbreviations: 17-AAG, 17-N-allylamino-17-demethoxygeldanamycin; AD, Alzheimer’s disease; ALS, amyotrophic lateral sclerosis; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate hydrate; HD, Huntington’s disease; HIF1α, hypoxia-inducible factor 1α; Hsp, heat shock protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OGD, oxygen glucose deprivation; PD, Parkinson’s disease; UPS, ubiquitin proteasome system; VHL, von Hippel-Lindau protein.

Introduction

Ubiquitin proteasome system (UPS) represents an important intracellular pathway involved in the control of a wide range of cellular functions like protein stability, intracellular protein localization, protein-protein interactions, and transcriptional activity (Glickman and Ciechanover 2002). These effects are mediated by monomeric or poly-ubiquitinylation of specific proteins involved in the regulation of cell cycle, apoptosis, transcription and signal transduction (Schrader et al. 2009). In addition to regulatory functions, the most prominent function of UPS is elimination of aged and aberrant proteins, including misfolded or aggregated proteins. Proteinopathies that include diverse human pathologies including neurodegenerative disorders such as Alzheimer’s disease (AD),...
Parkinson's disease (PD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS) are characterised by accumulation of certain structurally abnormal proteins and protein aggregates with consequent disruption of cellular functions (Luheshi and Dobson 2009; Chiti and Dobson 2017; Hartl 2017). Proteasome stress resulting from either UPS overload or dysfunction is considered to be important common mechanism implicated in pathophysiology of mentioned neurodegenerative diseases (Ciechanover and Kwon 2015) as well as ischemic neurodegeneration (Luo et al. 2013; Caldeira et al. 2014). The previous research has documented that aggregates of proteins specific for particular neurodegeneration could itself cause strong inhibition of 26S proteasome. The inhibition of proteasome was documented for tau protein associated with AD (Miyeku et al. 2016), α-synuclein (Lindersson et al. 2004; Zhang et al. 2008) and parkin (Um et al. 2010) associated with PD, ALS specific proteins (Cheroni et al. 2009) as well as for ubiquitin-conjugated protein aggregates (Bence et al. 2001) produced in early reperfusion phase after global brain ischemia (Racay 2012).

Cobalt in the form of divalent cation is essential to human health since it plays a critical role in the synthesis of vitamin B12. In 1966, the toxic effect of Co²⁺ attributed to the addition of cobalt sulphate to the beer as a stabilizer (Sullivan et al. 1968) was first described among beer drinkers that developed a cardiomyopathy. In addition to heart, Co²⁺ exhibited toxic effects on the cells of other organs including cells of nervous tissue (Caltana et al. 2009; Yang et al. 2011; Guo et al. 2013). The mechanism of the detrimental action of Co²⁺ on the cells is not completely known. Previous studies have demonstrated mitochondrial dysfunction and transmembrane potential collapse caused by opening the mitochondrial transition pore and inhibition of the mitochondrial respiratory chain complexes (Battaglia et al. 2009). In addition, induction of apoptosis (Yang et al. 2004; Jung et al. 2007; Walls et al. 2009; Chang et al. 2016) autophagy (Yang et al. 2015; Fung et al. 2016) and oxidative stress (Chen et al. 2010; Stenger et al. 2011; Guan et al. 2015) has also been documented among the mechanisms of Co²⁺-induced cell death.

On contrary, pre-treatment of the cells or animals with non-toxic concentrations of CoCl₂ 24 h prior to lethal insult was documented to be protective in different models of brain ischemia in both neonatal (Bergeron et al. 2000; Jones et al. 2008; Dai et al. 2014) and adult animals (Valsecchi et al. 2011; Wacker et al. 2012) or brain hypoxia injury (Shrivastava et al. 2008). Hypoxia-inducible factor 1α (HIF1α) is considered to be essential molecule associated with protective effect of CoCl₂ (Sharp et al. 2001; Jones et al. 2013) since CoCl₂ is able to stabilize HIF1α, a key determinant of the cellular response to hypoxia. Stabilisation of HIF1α is mediated by inhibitory effect of Co²⁺ on the isoforms of prolyl hydroxylase, the enzyme responsible for hydroxylation of HIF1α protein on specific proline residue. Hydroxylation of HIF1α leads to binding of the von Hippel-Lindau protein (VHL), which recruits an ubiquitin protein-ligase that targets HIF1α for proteasome degradation (Semenza 2011). In addition to stabilisation of HIF1α, Co²⁺, as the other ions of transition metals, is able to induce expression of heat shock protein 70 (Hsp70) (Koizumi et al. 2013) that also exhibits protective effects in different models of neuronal cell injury (Kelly et al. 2001; Sinn et al. 2007) including brain ischemia (Lee et al. 2001; Matsumori et al. 2005) and other neurodegenerative conditions (Turturici et al. 2011).

In our previous studies, we have shown that proteasome stress is associated with death of SH-SY5Y cells despite induction of Hsp70 expression that was documented already 4 h after incubation of the cells with bortezomib, inhibitor of 26S proteasome (Klacanova et al. 2016; Pilchova et al. 2017). The aim of our study was to investigate impact of the proteasome stress on SH-SY5Y cells pre-treated with CoCl₂ 24 h prior to induction of proteasome stress with bortezomib. We have focused our interests on Hsp70 that was shown to be over-expressed after pre-treatment of the cells with CoCl₂ and activation of caspase 3. Finally, we have compared the effect of CoCl₂ with an effect of pre-treatment of the cells with 17-N-allylamino-17-demethoxygeldanamycin (17-AAG), an inhibitor of Hsp90 that is capable to induce expression of Hsp70, or with IOX2, an inhibitor of isoform 2 of prolyl hydroxylase that increases stability of HIF1α.

**Materials and Methods**

Sodium dodecylsulphate (SDS), bovine serum albumin (BSA), IOX2, 17-AAG, cobalt chloride and (3-((4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (all Sigma-Aldrich), 3-[((3-cholamidopropyl)methylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (all Sigma-Aldrich), 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS) (Aplichem), bortezomib (Santa Cruz Biotechnology), HALT³⁰⁰ protease inhibitor cocktail (ThermoFisher Scientific), prestained protein standards (BioRad, cat. no. 1610373), Mouse monoclonal antibodies against Hsp27 (SC-13132), Hsp70 (SC-66048), Hsp90 (SC-13119), caspase 3 (SC-271028), HIF1α (SC-71247) and β-actin (SC-47778) (all Santa Cruz Biotechnology). Goat anti-mouse (A0168) (all Sigma-Aldrich) secondary antibodies conjugated with horse radish peroxidase.

**Cell culture and treatment**

SH-SY5Y cells (ATCC) were maintained in DMEM:F12 (1:1) medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin (all PAA) at an optimal cell density of 0.5 × 10⁶ cells/ml at 37°C and 5% CO₂ humidified atmosphere. The media were changed every 3 days. Naïve
or SH-SY5Y cells pre-treated with CoCl₂, 17-AAG and IOX2 24 h prior to treatment with indicated concentrations of bortezomib (0, 10, 20, 50 nmol/l) for 24 and 48 hours at 37°C and 5% CO₂ humidified atmosphere were at the end of the treatment washed 3 times with ice cold PBS and then re-suspended in a lysis buffer (30 mmol/l Tris-HCl, 150 mmol/l NaCl, 1% CHAPS, 1× protease inhibitor cocktail, pH 7.6) for total protein extraction. Proteat concentrations were determined by protein Dc assay kit (Bio-Rad) using BSA as standard.

**Statistical analysis**

For the comparison of differences in protein expression non-treated and pre-treated cells, one-way ANOVA (GraphPad InStat V2.04a, GraphPad Software) was first carried out to test for differences among all experimental groups followed by the Tukey’s test to determine differences between individual groups. The value $p < 0.05$ was considered as being significant.

**Results**

In order to mimic hypoxic preconditioning, SH-SY5Y cells were pre-treated for 24 h with CoCl₂ at concentrations 150 μmol/l and 250 μmol/l. Incubation of SH-SY5Y cells for 24 h with CoCl₂ at concentrations 150 μmol/l and 250 μmol/l was associated with significantly increased expression of Hsp70 to 829% ($p < 0.01$) and 1321% ($p < 0.001$) of control, respectively (Fig. 1A). Expression of Hsp27 was also significantly elevated to 337% of control ($p < 0.01$) in the cells incubated for 24 h with CoCl₂ at concentration 250 μmol/l while expression of Hsp90 was not significantly altered in the cells incubated for 24 h with CoCl₂ at concentrations 150 μmol/l and 250 μmol/l. The impact of bortezomib on the relative viability of the SH-SY5Y cells and effect of pre-treatment of the SH-SY5Y cells with CoCl₂ was assessed by MTT test (Fig. 1B, C). In agreement with our previous results (Klacanova et al. 2016; Pilchova et al. 2017), treatment of SH-SY5Y cells with bortezomib at concentrations 10 nmol/l and higher for 24 and 48 h was associated with decreased relative cell viability (Fig. 1B, C). Pre-treatment of the cells with CoCl₂ 24 h prior to application of bortezomib was associated with significantly higher relative viability of the cells treated 24 h with bortezomib at concentrations 20 nmol/l and 50 nmol/l (Fig. 1B). After 48 h of treatment, the relative viability of the cells pre-treated with CoCl₂ was higher than relative viability of the cells that were not pre-treated however, the difference was not statistically significant (Fig. 1C). Since activation of caspase 3 in SH-SY5Y cells treated with bortezomib for 48 h was clearly documented in our previous study (Pilchova et al. 2017), we have also investigated impact of pre-treatment of SH-SY5Y cells with CoCl₂ on bortezomib-induced activation of caspase 3. As shown on Fig. 1D, activation of caspase 3 in SH-SY5Y cells was documented by decrease of procaspase 3 and appearance of p17 fragment of active caspase 3. These changes were observed 48 h after the treatment of the cells with bortezomib at concentrations 10 and 20 and 50 nmol/l. The level of p17 fragment of active caspase 3 was decreased in the SH-SY5Y cells pre-treated with 150 μmol/l CoCl₂ and was not observed in the SH-SY5Y cells pre-treated with 250 μmol/l CoCl₂ and then treated with bortezomib at concentrations 10, 20 and

**Cell viability**

Cells were seeded in 96-well plates at concentrations of $0.4 \times 10^5$ SH-SY5Y cells per ml. Naïve or SH-SY5Y cells pre-treated with CoCl₂, 17-AAG and IOX2 24 h prior to treatment with bortezomib were incubated for 24 and 48 h with indicated concentrations of bortezomib (0, 5, 10, 20, 50 nmol/l) at 37°C in 5% CO₂ humidified atmosphere. At the end of treatment, 0.01 ml of MTT solution (5 mg/ml) was added to each well and the cells were further incubated for 4 hours at 37°C and 5% CO₂ humidified atmosphere. The insoluble formazan, which resulted from oxidation of added MTT by vital cells, was dissolved by addition of 0.1 ml of SDS solution (0.1 g/ml) and overnight incubation at 37°C and 5% CO₂ humidified atmosphere. The absorbance of formazan was determined spectrophotometrically using microplate reader Bio-Rad 2010. The relative viability of the cells was determined as ratio of optical density of formazan produced by treated cells to optical density of formazan produced by non-treated control cells and expressed as percent of control. For each treatment time, the optical density value of non-treated control cells was considered as 100% of viable cells.

**Western blotting**

Isolated proteins (30 μg of proteins loaded per lane) were separated on 12% SDS-polyacrylamide gels (PAGE) under reducing conditions. Separated proteins were transferred to nitrocellulose membranes using semidry transfer and membranes were probed with antibodies specific to Hsp90 (1:500), Hsp70 (1:500), Hsp27 (1:500), HIF1α (1:200), caspase 3 (1:200) and β-actin (1:1000). Further incubation of membranes with particular secondary antibodies (all 1:5000) was followed by visualization of immunopositive bands using the chemiluminescent substrate SuperSignal West Pico (Thermo Scientific) and Chemidoc XRS system (Bio-Rad). Intensities of specific bands were quantified by Quantity One software (Bio-Rad). The intensities of bands of interest were normalized to corresponding intensities of bands of β-actin and expressed as intensity of band of particular protein in treated cells relative to intensity of band in control non-treated cells.
Impact of CoCl$_2$ on expression of heat shock proteins, bortezomib-induced cell death and activation of caspase3. In order to assess the involvement of Hsp70 in the protection of SH-SY5Y cells against proteasome stress we have also used 17-AAG inhibitor of Hsp90 that is known to induce expression of Hsp70 (Powers and Workman 2007). In fact, incubation of SH-SY5Y cells for 24 h with 1 μmol/l 17-AAG was associated with significantly increased expression of Hsp70 to 632% ($p < 0.01$) of control (Fig. 2A). Expression of Hsp27 was also significantly elevated in the cells incubated with 1 μmol/l 17-AAG to 894% ($p < 0.001$) of control (Fig. 2A).

In order to assess the involvement of Hsp70 in the protection of SH-SY5Y cells against proteasome stress we have also used 17-AAG inhibitor of Hsp90 that is known to induce expression of Hsp70 (Powers and Workman 2007). In fact, incubation of SH-SY5Y cells for 24 h with 1 μmol/l 17-AAG was associated with significantly increased expression of Hsp70 to 632% ($p < 0.01$) of control (Fig. 2A). Expression of Hsp27 was also significantly elevated in the cells incubated with 1 μmol/l 17-AAG to 894% ($p < 0.001$) of control (Fig. 2A).

**Figure 1.** Impact of CoCl$_2$ on expression of heat shock proteins, bortezomib-induced cell death and activation of caspase3. A. Total cell extracts were prepared from SH-SY5Y cells after the pre-treatment with indicated concentrations of CoCl$_2$ for 24 h. The effect of CoCl$_2$ on the levels of Hsp70, Hsp27 and Hsp90 was evaluated by Western blot analysis of total cell extracts as described in Materials and Methods. Data are presented as means ± SD (n = 4). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (one-way ANOVA, followed by Tukey’s test to determine differences at the levels of particular proteins between control non-treated cells and cells pre-treated with CoCl$_2$).

B, C. SH-SY5Y were first pre-treated with indicated concentrations of CoCl$_2$ and then treated with indicated concentrations of bortezomib for 24 h (B) and 48 h (C). After treatment, relative cell viability was determined by MTT test as described in Material and Methods. Data are presented as means ± SEM (4 independent experiments performed in triplicate). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (one-way ANOVA followed by Tukey’s test to determine differences between relative viability of control cells and cells treated with different concentrations of bortezomib as well as between relative viability of non-pre-treated cells and cells pre-treated with CoCl$_2$ and then treated with particular concentration of bortezomib). D. Total cell extracts were prepared from SH-SY5Y cells that were either non-pre-treated or pre-treated with indicated concentrations of CoCl$_2$ (150 and 250 μmol/l) and then treated with indicated concentrations of bortezomib 48 h. The activation of caspase 3 was evaluated by Western blot analysis of total cell extracts as described in Materials and Methods.
Expression of Hsp90 was not significantly altered in the cells incubated for 24 h with 17-AAG at concentrations 0.5 μmol/l and 1 μmol/l (Fig. 2A). Despite induction of Hsp70 expression, we did not observe significant differences of relative viability of the cells that were pre-treated with 17-AAG after the incubation with bortezomib in comparison to cells that were not pre-treated with 17-AAG but also incubated with bortezomib (Fig. 2B). In accord with the results of MTT test, pre-treatment of SH-SY5Y cells with 17-AAG did not suppress activation of caspase 3 since p17 fragment of active caspase 3 was observed in the cells treated with bortezomib irrespective of pre-treatment with 17-AAG (Fig. 2C). In addition, decrease of non-active pro-caspase 3 was observed in both experimental conditions.

Finally, we have pre-treated the cells with IOX2 inhibitor of prolyl hydroxylase 2 that is known to induce expression of HIF1a (Chan et al. 2015). Despite some opposing results, HIF1a is considered to be an essential molecule associated with neuroprotection in the model of hypoxic preconditioning induced by CoCl$_2$ (Jones et al. 2013). The level of HIF1a in the cell extracts from control un-treated SH-SY5Y cells was under Western blot detection limit (Fig. 3) but pre-treatment of the cells with IOX2 at concentration 100 μmol/l was associated with stabilization of HIF1a as documented by detection of proper signal on Western blot (Fig. 3A). Pre-treatment of the cells with IOX2 at concentration 100 μmol/l did not significantly alter expression of Hsp70 and Hsp90 (Fig. 3A) but was associated with significantly increased expression of Hsp27 to 186% of control (p < 0.05). Despite stabilization of HIF1a and increased expression of Hsp27, pre-treatment of the SH-SY5Y cells with IOX2 at concentration 100 μmol/l was not associated with significant differences of relative cell viability as documented by MTT test (Fig. 3B).

Discussion

We have shown in this study that pre-treatment of SH-SY5Y cells for 24 h with CoCl$_2$, at concentrations of 150 or 250 μmol/l, and with 17-AAG at concentration 1 μmol/l but not with IOX2 at concentration 100 μmol/l, was associated with significantly increased expression of Hsp70.
Pre-treatment of SH-SY5Y cells with CoCl\(_2\), but not with 17-AAG or IOX2, was associated with significant delay of the cell death induced by proteasome stress. CoCl\(_2\)-mediated effect was consistent with inhibition of bortezomib-induced caspase 3 activation in the cells pre-treated with CoCl\(_2\). Despite established neuroprotective properties of Hsp70 our results do not provide strong evidence that the effect of CoCl\(_2\) could be mainly attributed to the ability of CoCl\(_2\) to induce expression of Hsp70 since pre-treatment of the cells with 17-AGG.

Cobalt in the form of divalent cation is an essential microelement that at higher concentrations exhibits significant toxicity to the cells of different tissues including cells of nervous system. Detrimental effects of the Co\(^{2+}\) were among other mechanisms attributed to the induction of mitochondrial apoptosis (Lee et al. 2008) including activation of caspase 3 (Zou et al. 2002; Peng et al. 2015; Sun et al. 2015). On contrary, our results have shown that delay of bortezomib-induced cell death might be a result of inhibition of bortezomib-induced caspase 3 activation that was documented in the SH-SY5Y cells pre-treated with sub-toxic concentrations of CoCl\(_2\). Delay of the cell death after inhibition of caspases was documented in several previous studies. For example, fast apoptotic death of primary hippocampal and cortical neurones induced by ABT-737 that was shown to induce Bax/Bak-dependent mitochondrial apoptosis (Vogler et al. 2009) was not prevented but was significantly delayed by application of the broad spectrum caspase inhibitor zVADfmk (Young et al. 2010). In accord with our results, CoCl\(_2\) at concentrations 100 and 200 μmol/l blocked isocudrazanthone-induced apoptosis and activation of caspase 3 (Shin et al. 2014). Concentration of CoCl\(_2\) seems to be critical with respect to the impact of CoCl\(_2\) on the exposed cells. Pre-treatment of neuronal cells with CoCl\(_2\) at concentration 100 μmol/l for 20 h prior to exposure of the cells to oxygen glucose deprivation (OGD) attenuated OGD-induced neuronal death whereas treatment of the cells with CoCl\(_2\) at concentrations 300 or 500 μmol/l induced significant neuronal death, even without exposure to OGD (Jones et al. 2013). Similarly, activation of caspase 3 was documented in mouse cortical HT-22 neurones treated with CoCl\(_2\) at concentrations 300 μmol/l and higher (Peng et al. 2015).

It was shown in numerous of the previous studies that heat shock proteins can suppress protein aggregation (Doyle et al. 2013) and toxicity in different models of neurodegenerative diseases (Stetler et al. 2010; Lindberg et al. 2015; Smith et al. 2015). With respect to apoptosis initiation, Hsp70 was shown to act at the level of extrinsic apoptosis (Clemons et al. 2005) as well as caspase-dependent (Beere et al. 2000; Saleh et al. 2000; Stankiewicz et al. 2005) or caspase-independent (Ravagnan et al. 2001) intrinsic (mitochondrial) apoptosis. With respect to neuronal cells, Hsp70 over-expression in rat primary cortical neurons and the SH-SY5Y cells protected the cells in four independent models of apoptosis: etoposide-, staurosporine-, C2-ceramide-, and β-amyloid-induced apoptosis (Sabirzhanov et al. 2012). It was demonstrated that Hsp70 binds and potentially inactivates apoptotic protease-activating factor 1 as well as apoptosis-inducing factor, key molecules involved in the development of caspase-dependent and caspase-independent cell death, respectively (Sabirzhanov et al. 2012). Over-expression of Hsp70 in dopaminergic
neurones of *Drosophila* protected them from paraquat-induced cell death via inhibition of caspase 3 activation (Shukla et al. 2014). In addition, Hsp70 is considered to be a key molecule conferring protection of CA1 pyramidal cells after preconditioned global brain ischemia (Burda et al. 2003; Tanaka et al. 2004) that is also associated with massive proteasome stress (Racay 2012). In our previous study, we have shown that the sensitivity to bortezomib and kinetics of death of leukemic cells correlated well with higher expression of Hsps in resistant and slowly responding K562 cells (Kliková et al. 2015). Thus, CoCl$_2$-induced massive expression of Hsp70 might represent plausible molecular mechanisms responsible for inhibition of caspase 3 activation and delay of bortezomib-induced death of SH-SY5Y cells.

To prove the involvement of Hsp70 in protection from proteasome stress, we have also pre-treated SH-SY5Y cells with 17-AAG, inhibitor of Hsp90 that was shown to induce of Hsp70 expression (Powers and Workman 2007) and to protect effectively the cells from different form of cellular stress including proteasome stress caused by proteasome inhibition (Bonner et al. 2010). In addition, 17-AAG was tested as neuroprotective molecule in several models of neurodegeneration and pre-clinical studies of neurodegenerative diseases. In our experiments, we did not observe protective effect of 17-AAG. The inability of 17-AAG to protect SH-SY5Y cells from proteasome stress observed in our experiments might be explained by lower extent of Hsp70 induction after pre-treatment of the cells with 17-AAG as compared to the levels of Hsp70 induced by CoCl$_2$. On contrary, despite induction of protective Hsp70 17-AAG exhibits also cytotoxic effects and was tested for treatment of different cancers (Chatterjee and Burns 2017). Although, we did not incubate cells simultaneously with 17-AAG and bortezomib, it is important to note that combination of 17-AAG with bortezomib was associated with synergistic cell death effect on U266 (Duus et al. 2006) and MCF-7 (Mimnaugh et al. 2004) cell lines.

Our results open the possibility that there are some other factors beyond Hsp70 that contributed to the protective effect of CoCl$_2$. For example, Hsp27 was considered to be more protective than Hsp70 in the model of $\alpha$-synuclein-induced neuronal cell death (Zourlidou et al. 2004). Hsp27 seems to exert its protective functions via inhibition of extrinsic mitochondria-independent pathway (Tan et al. 2009). Interestingly, the same study showed the protective effect of Hsp27 against CoCl$_2$-induced apoptosis (Tan et al. 2009) that depends predominantly on initiation of mitochondrial apoptosis. In our experiments, expression of Hsp27 was significantly increased after pre-treatment of cells with 250 μmol/l of CoCl$_2$ but not with 150 μmol/l CoCl$_2$. In addition, Hsp27 was shown to be over-expressed in the cells pre-treated with IOX2. Since pre-treatment of the cells with IOX2 was not associated with protective effect and pre-treatment with 150 μmol/l of CoCl$_2$ was associated with very similar results as pre-treatment with 250 μmol/l of CoCl$_2$, we consider the involvement of Hsp27 in the effects associated with CoCl$_2$ pre-treatment as unlikely.

Another possibility how to explain effect of CoCl$_2$ consists in the ability of CoCl$_2$ to inhibit prolyl hydroxylases that is associated with stabilization HIF1α (Jones et al. 2013). To investigate possible involvement of HIF1α we have pre-treated cells with IOX2 inhibitor of prolyl hydroxylase 2. The ability of IOX2 to stabilize HIF1α depends on cell type (Chan et al. 2015) but we have clearly documented stabilization of HIF1α after pre-treatment the SH-SY5Y cells with IOX2 at concentration 100 μmol/l. HIF1α is considered to be critical molecule responsible for protective effect of CoCl$_2$-induced hypoxic preconditioning in different models of ischemic brain injury (Sharp et al. 2001; Jones et al. 2013). On contrary, previous studies indicated that CoCl$_2$ activates HIF1α, acting as a hypoxia-mimetic and inducing reactive oxygen species-mediated toxicity (Chen et al. 2010; Guan et al. 2015). Despite clear stabilisation of HIF1α level after pre-incubation of the cells with IOX2 at concentration 100 μmol/l we have observed only marginal non-significant effect of this pre-treatment. Thus involvement of HIF1α alone in neuroprotective mechanisms associated with CoCl$_2$ pre-treatment seems to be unlikely but the combined protective effect of both Hsp70 and HIF1α cannot be completely excluded. Finally, other mechanisms activated by CoCl$_2$, e.g. modulation of expression of Bcl2 family proteins (Shin et al. 2014), could to contribute to protective effects of CoCl$_2$.

In conclusion, we have shown in this study that pre-treatment of SH-SY5Y cells with CoCl$_2$ was associated with significant delay of the cells death induced by proteasome stress. In addition, we have shown that CoCl$_2$-mediated protective effect was most probably a result of inhibition of bortezomib-induced caspase 3 activation but the result presented in our study cannot be exclusively attributed to CoCl$_2$-dependent induction of Hsp70.

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**References**

Battaglia V, Compagnone A, Bbandino A, Bragadin M, Rossi CA, Zanetti F, Colombatto S, Grillo MA, Toninello A (2009): Cobalt induces oxidative stress in isolated liver mitochondria responsible for permeability transition and intrinsic apoptosis in hepatocyte primary cultures. Int. J. Biochem. Cell Biol. 41, 586–594

[https://doi.org/10.1016/j.biocel.2008.07.012](https://doi.org/10.1016/j.biocel.2008.07.012)
Beere HM, Wolf BB, Cain K, Mosser DD, Mahboubi A, Kuwana T, Tailor P, Morimoto RI, Cohen GM, Green DR (2000): Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. Nat. Cell Biol. 2, 469–475. https://doi.org/10.1038/35019501

Bence NF, Sampat RM, Kopito RR (2001): Impairment of the ubiquitin-proteasome system by protein aggregation. Science 292, 1552–1555. https://doi.org/10.1126/science.292.5521.1552

Bergeron M, Gidday JM, Yu AY, Semenza GL, Ferriero DM, Sharp FR (2000): Role of hypoxia-inducible factor-1 in hypoxia-induced ischemic tolerance in neonatal rat brain. Ann. Neurol. 48, 285–396. https://doi.org/10.1002/1531-8249(200009)48:3<285::AID-ANA2>3.0.CO;2-8

Bonner HP, Concannon CG, Bonner C, Woods I, Ward MW, Prehn JH (2010): Differential expression patterns of Puma and Hsp70 following proteasomal stress in the hippocampus are key determinants of neuronal vulnerability. J. Neurochem. 114, 606–616. https://doi.org/10.1111/j.1471-4159.2010.06790.x

Burda J, Hrehorovská M, Bonilla LG, Danielisová V, Cízková D, Bonner HP, Concannon CG, Bonner C, Woods I, Ward MW, Prehn JH (2010): Differential expression patterns of Puma and Hsp70 following proteasomal stress in the hippocampus are key determinants of neuronal vulnerability. J. Neurochem. 114, 606–616. https://doi.org/10.1111/j.1471-4159.2010.06790.x

Caldeira MV, Salazar IL, Curcio M, Canzoniero LM, Duarte CB (2016): Roles of microRNA-1 in hypoxia-induced apoptotic cell death in H9c2 cells. PLoS One 11, e0136990. https://doi.org/10.1371/journal.pone.0136990

Caltana L, Merelli A, Lazarowski A, Brucco A (2009): Neuronal and glial alterations due to focal cortical hypoxia induced by direct cobalt chloride (CoCl2) brain injection. Neurotox. Res. 15, 348–358. https://doi.org/10.1007/s12640-009-0038-9

Chan MC, Atasoyu O, Hodson E, Tumber A, Leung I, K. Chowdhury R, Gómez-Pérez V, Demetriaides M, Rydzik AM, Holt-Martyr, et al. (2015): Potent and selective triazole-based inhibitors of the hypoxia-inducible factor prolyl-hydroxylases with activity in the murine brain. PLoS One 10, e0132004. https://doi.org/10.1371/journal.pone.0132004

Chang CY, Lui TN, Lin JW, Lin YL, Hsing CH, Wang JJ, Chen RM (2016): Roles of microRNA-1 in hypoxia-induced apoptotic insults to neuronal cells. Arch. Toxicol. 90, 191–202. https://doi.org/10.1007/s00204-014-1364-x

Chatterjee S, Burns TF (2017): Targeting heat shock proteins in cancer: A promising therapeutic approach. Int. J. Mol. Sci. 8, E1978. https://doi.org/10.3390/ijms18091978

Chen SL, Yang CT, Yang ZL, Guo RX, Meng JL, Cui Y, Lan AP, Chen PX, Feng JQ (2010): Hydrogen sulphide protects H9c2 cells against chemical hypoxia-induced injury. Clin. Exp. Pharmacol. Physiol. 37, 316–321. https://doi.org/10.1111/j.1440-1618.2009.05289.x

Cheronti C, Marino M, Tortarolo M, Vegliantese P, De Biasi S, Fontana E, Zuccarello L, V, Maynard CJ, Dantuma NP, Bendotti C (2009): Functional alterations of the ubiquitin-proteasome system in motor neurons of a mouse model of familial amyotrophic lateral sclerosis. Hum. Mol. Genet. 18, 82–96. https://doi.org/10.1093/hmg/ddn319

Chiti F, Dobson CM (2017): Protein misfolding, amyloid formation, and human disease: a summary of progress over the last decade. Annu. Rev. Biochem. 86, 27–68. https://doi.org/10.1146/annurev-biochem-061516-045115

Ciechanover A, Kwon YT (2015): Degradation of misfolded proteins in neurodegenerative diseases: therapeutic targets and strategies. Exp. Mol. Med. 47, e147. https://doi.org/10.1038/emm.2014.117

Clemons NJ, Buzzard K, Steel R, Anderson RL (2005): Hsp72 inhibits Fas-mediated apoptosis upstream of the mitochondria in type II cells. J. Biol. Chem. 280, 9005–9012. https://doi.org/10.1074/jbc.M414165200

Dai Y, Li W, Zhong M, Chen J, Liu Y, Cheng Q, Li T (2014): Preconditioning and post-treatment with cobalt chloride in rat model of perinatal hypoxic-ischemic encephalopathy. Brain Dev. 36, 228–240. https://doi.org/10.1016/j.braindev.2013.04.007

Doyle SM, Genest O, Wickner S (2013): Protein rescue from aggregates by powerful molecular chaperone machines. Nat. Rev. Mol. Cell Biol. 14, 617–629. https://doi.org/10.1038/nrm3660

Duus J, Bahar HI, Venkataraman G, Ozpuyan F, Izban KF, Al-Masri H, Maududi T, Toor A, Alkan S (2006): Analysis of expression of heat shock protein-90 (HSP90) and the effects of HSP90 inhibitor (17-AG) in multiple myeloma. Leuk. Lymphoma 47, 1369–1378. https://doi.org/10.1080/10428190500472123

Fung FK, Law BY, Lo AC (2016): Lutein attenuates both apoptosis and autophagy upon cobalt (ii) chloride-induced hypoxia in rat Müller cells. PLoS One 11, e0167828. https://doi.org/10.1371/journal.pone.0167828

Glickman MH, Ciechanover A (2002): The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol. Rev. 82, 373–428. https://doi.org/10.1152/physrev.00027.2001

Guo L, Lan J, Lin Y, Guo P, Nie Q, Mao Q, Ren L, Qiu Y (2013): Hypoxia/ischemia up-regulates Id2 expression in neuronal cells in vivo and in vitro. Neurosci. Lett. 554, 88–93. https://doi.org/10.1016/j.neulet.2013.08.044

Hartl FU (2017): Protein misfolding diseases. Annu. Rev. Biochem. 86, 21–26. https://doi.org/10.1146/annurev-biochem-061516-044518

Jones NM, Kardashtyan L, Callaway JK, Lee EM, Beart PM (2008): Long-term functional and protective actions of preconditioning with hypoxia, cobalt chloride, and desferrioxamine against hypoxic-ischemic injury in neonatal rats. Pediatr. Res. 63, 620–624. https://doi.org/10.1203/PDR.0b013e3181ed9117

Jones SM, Novak AE, Elliott JP (2013): The role of HIF in coxyl-induced ischemic tolerance. Neuroscience 252, 420–430.
Shrivastava K, Shukla D, Bansal A, Sairam M, Banerjee PK, Ilavazhagan G (2008): Neuroprotective effect of cobalt chloride on hypobaric hypoxia-induced oxidative stress. Neurochem. Int. 52, 368–375
https://doi.org/10.1016/j.neuint.2007.07.005

Shukla AK, Pragya P, Chauhan HS, Tiwari AK, Patel DK, Abdin MZ, Chowdhuri DK (2014): Heat shock protein-70 (Hsp-70) suppresses parautaparaduced neurodegeneration by inhibiting JNK and caspase-3 activation in Drosophila model of Parkinson's disease. PLoS One 9, e98886
https://doi.org/10.1371/journal.pone.0098886

Sinn DJ, Chu K, Lee ST, Song EC, Jung KH, Kim EH, Park DK, Kang KM, Kim M, Roh JK (2007): Pharmacological induction of heat shock protein exerts neuroprotective effects in experimental intracerebral hemorrhage. Brain Res. 1135, 167–176
https://doi.org/10.1016/j.brainres.2006.11.098

Smith HL, Li W, Cheetham ME (2015): Molecular chaperones and neuronal proteostasis. Semin. Cell. Dev. Biol. 40, 142–152
https://doi.org/10.1016/j.semcdb.2015.03.003

Stankiewicz AR, Lachapelle G, Foo CP, Radicioni SM, Mosser DD (2010): Hsp27 operates predominantly by blocking the mitochondrial inner membrane. J. Biol. Chem. 285, 38729–38739
https://doi.org/10.1074/jbc.M509497200

Stenger C, Naves T, Verdier M, Ratinaud MH (2011): The cell death independent/extrinsic pathway of cellular apoptosis. Mol. Cells 30, 1326–1338
https://doi.org/10.1016/j.celldeath.2011.08.008

Sullivan J, Parker M, Carson SB (1968): Tissue cobalt content in 'beer drinkers' myocardioopathy.' J. Lab. Clin. Med. 71, 893–911

Sun L, Liu N, Liu SS, Xia WY, Liu MY, Li LF, Gao JX (2015): Beclin-1-independent autophagy mediates programmed cancer cell death through interplays with endoplasmic reticulum and/or mitochondria in colbat chloride-induced hypoxia. Am. J. Cancer Res. 5, 2626–2642

Tan CY, Ban H, Kim YH, Lee SK (2009): The heat shock protein 27 (Hsp27) operates predominantly by blocking the mitochondrial-independent/extrinsic pathway of cellular apoptosis. Mol. Cells 27, 533–538
https://doi.org/10.1007/s10059-009-0079-y

Tanaka H, Yokota H, Jover T, Cappuccio I, Calderone A, Simionescu M, Bennett MV, Zukin RS (2004): Ischemic preconditioning: neuronal survival in the face of caspase-3 activation. J. Neurosci. 24, 2750–2759
https://doi.org/10.1523/JNEUROSCI.5475-03.2004

Turturici G, Sconoza G, Geracci F (2011): Hsp70 and its molecular role in nervous system diseases. Biochem. Res. Int. 2011, 618127
https://doi.org/10.1155/2011/618127

Um JW, Im E, Lee HJ, Min B, Yoo L, Yoo J, Lübbert H, Stichel-Gunkel C, Cho HS, Yoon JB, Chung KC (2016): Parkin directly modulates 26S proteasome activity. J. Neurosci. 36, 11805–11814
https://doi.org/10.1523/JNEUROSCI.2862-09.2010

Valsecchi V, Pignataro G, Del Prete A, Sirabella R, Matrone C, Boscia F, Scorzziello A, Sisalli MJ, Esposito E, Zambrano N, Di Renzo G, Annunziato L (2011): NCX1 is a novel target gene for hypoxia-inducible factor-1 in ischemic brain preconditioning. Stroke 42, 754–763
https://doi.org/10.1161/STROKEAHA.110.597583

Vogler M, Weber K, Dinsdale S, Schmitz I, Schulze-Osthoff K, Dyer M, Cohen GM (2009): Different forms of cell death induced by putative BCL2 inhibitors. Cell Death Differ. 16, 1030–1039
https://doi.org/10.1038/cdd.2009.48

Wacker BK, Perfater JL, Gidday JM (2012): Hypoxic preconditioning induces stroke tolerance in mice via a cascading HIF, sphingosine kinase, and CCL2 signaling pathway. J. Neurochem. 123, 945–962
https://doi.org/10.1111/jnc.12047

Walls KC, Ghosh AP, Ballestas ME, Klocke BJ, Roth KA (2009): Bcl-2/Adenovirus E1B 19-kd interacting protein 3 (BNI3P) regulates hypoxia-induced neural precursor cell death. J. Neuropathol. Exp. Neurol. 68, 1326–1338
https://doi.org/10.1097/NEN.0b013e3181c3b9be

Yang SJ, Pyen Y, Lee I, Lee H, Kim Y, Kim T (2004): Cobalt chloride-induced apoptosis and extracellular signal-regulated protein kinase 1/2 activation in rat C6 glioma cells. J. Biochem. Mol. Biol. 37, 480–486

Yang T, Li D, Liu F, Qi L, Yan G, Wang M (2015): Regulation on Beclin-1 expression by miTOR in CoG2-induced HT22 cell ischemia-reperfusion injury. Brain Res. 1614, 60–66
https://doi.org/10.1016/j.brainres.2015.04.016

Yang YF, Chen Z, Hu SL, Hu J, Li B, Li JT, Wei LJ, Qian ZM, Lin JK, Feng H, Zhu B (2011): Interleukin-1 receptor associated kinases-1/4 inhibition protects against acute hypoxia/ischemia-induced neuronal injury in vivo and in vitro. Neuroscience 196, 25–34
https://doi.org/10.1016/j.neuroscience.2011.08.059

Young KW, Pi-on LG, Dhiraj D, Twiddy D, Macfarlane M, Hickman J, Nicotera P (2010): Mitochondrial fragmentation and neuronal cell death in response to the Bcl-2/Bcl-xL(Bcl-w) antagonist ABT-737. Neuropharmacology 58, 1258–1267
https://doi.org/10.1016/j.neuropharm.2010.03.008

Zhang NY, Tang Z, Liu CW (2008): alpha-Synuclein protofibrils inhibit S proteasome-mediated protein degradation: understanding the cytotoxicity of protein protifibrils in neurodegenerative disease pathogenesis. J. Biol. Chem. 283, 20288–20298
https://doi.org/10.1074/jbc.M710506200

Zou W, Zeng J, Zhuo M, Xu W, Sun L, Wang J, Liu X. (2002): JNK and caspase-3 activation in Drosophila model of Parkin's disease. PLoS One 7, e98886

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