Combining 2-iminobiotin and hypothermia for reducing hypoxia-induced neuronal damage: Implications from a human cell culture model of hypoxic-ischemic brain injury

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Background: Perinatal asphyxia represents one of the major causes of neonatal morbidity and mortality. Hypothermia is currently the only established treatment for hypoxic-ischemic encephalopathy, and no specific pharmacological therapy exists so far. We and others have shown that the biotin analogue 2-iminobiotin (2-IB) is able to reduce neuronal cell damage both in vitro as well as in vivo under normothermic conditions.

The aim of this study was to evaluate whether 2-IB has the potential to attenuate hypoxia-induced injury of neuronal cells under hypothermic conditions.

Materials and methods: In-vitro hypoxia was induced for 7h using IMR-32 cell cultures in combination with our described enzyme based hypoxia model. After the hypoxic insult, cultures were incubated at 33.5°C (hypothermia), with vehicle (citrate 1%) or different concentrations of 2-IB (10, 30, 50, 100 and 300ng/ml). A schematic representation of the applied protocol is shown in **Fig. 1A**. Cell morphology was evaluated by brightfield microscopy and cell damage was analyzed by lactate dehydrogenase (LDH) assays. Production of reactive oxygen species (ROS) was measured using fluorometric assays. Westernblotting for PARP, caspase-3 and phosphorylated as well as unphosphorylated akt and erk1/2 was performed.

Results: Hypoxia led to morphological signs of cell damage (**Fig. 1B**). Under hypothermia, LDH measurements revealed a significant LDH increase earliest at 25h after the hypoxic insult ($p < 0.01$ vs normoxia, **Fig. 2**), while under normothermic conditions comparable cell damage was already detectable after 17h (ESA 2015, poster presentation). The post-hypoxic application of 30ng/ml of 2-IB significantly reduced the hypoxia-induced LDH release to levels found under normoxia (0ng/ml, $p < 0.001$; 10ng/ml, $p < 0.001$; 30ng/ml, $p > 0.05$; 50ng/ml, $p < 0.01$; 100ng/ml, $p < 0.001$; 300ng/ml, $p < 0.01$ all vs normoxia; **Fig. 3 LDH**). Application of 2-IB (10 and 30ng/ml) after hypoxia reduced the hypoxia-induced ROS production under hypothermic conditions (hypoxia: $p < 0.001$ vs normoxia; hypoxia+2-IB: $p < 0.01$ vs normoxia for 10 and 30ng/ml of 2-IB). Higher concentrations of 2-IB were less effective in reducing hypoxia-induced ROS generation (**Fig. 3 ROS**). Culture medium concentrations of hydrogen peroxide were 9-fold increased by hypoxia under hypothermia ($p < 0.001$ vs normoxia) but not influenced by post-hypoxic application of 2-IB (data not shown). Cleavage of PARP, akt phosphorylation and caspase-3 expression were by trend increased after the hypoxic insult, while hypoxia significantly enhanced the phosphorylation of the pro-survival kinase erk1/2 ($p < 0.05$ vs normoxia). However, the addition of 2-IB under hypothermia did not influence cleavage, expression or phosphorylation of any of the mentioned molecules (**Fig. 4**).

Fig. 1 Experimental setting A-Human neuroblastoma cells, IMR-32, were subjected to hypoxia for 7h or normoxia (control). After the insult, fresh medium containing vehicle (citrate 1%) or 2-IB at clinically relevant concentrations (10, 30, 50, 100 and 300 ng/ml) were applied to the cultures, under hypothermic conditions (33.5°C). At different time points after the insult, cell protein or the respective cell culture media were used to evaluate cell protein expression by westernblotting (at 2h and 25h), cell viability by LDH release (at 25h) and ROS production (at 25h). B- Cell morphology under normoxia and hypoxia by optical microscopy.

Fig. 2 Hypoxia induced cell damage (LDH) at different time points under hypothermic conditions. Hypoxic damage was significantly apparent earliest at 25h after the end of insult under hypothermic conditions (**: $P < 0.01$; ***: $P < 0.001$).

LDH

ROS

Fig. 3 Cell damage (LDH) and production of reactive oxygen species (ROS). Vehicle (citrate 1%) as control or increasing concentrations of 2-IB (0 – 300ng/ml), were applied on IMR-32 after the insult (7h hypoxia) under hypothermic conditions (33.5°C). 2-IB showed protection against hypoxia at 30ng/ml and this protective effect was lost by increasing the concentration of 2-IB to 100ng/ml (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; #: $P < 0.05$; ##: $P < 0.01$; n=8; 2 way ANOVA). 10 and 30ng/ml of 2-IB lead to a diminution of the production of ROS (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ###: $P < 0.001$, n=9; 2 way ANOVA).

Fig. 4 Protein expression, cleavage and phosphorylation of pro-survival kinases. Phosphorylation of cell survival proteins (akt und erk) was evaluated by Westernblots using protein isolated 2h after hypoxia (A,B). Neither hypoxia nor 2-IB effected the phosphorylation of akt (A). The presence of 2-IB did not change the hypoxia induced protein phosphorylation of erk (B), and reduced by trend the upregulation of caspase-3 induced by hypoxia (C). The cleavage of PARP from 25h protein after the end of insult, was slightly upregulated by hypoxia but 2-IB does not exert any effect on the cleavage of PARP (D). (*: $P < 0.05$; n=4-5; 2 way ANOVA).

Conclusion

2-IB reduces hypoxia-induced neuronal cell damage in vitro under hypothermic conditions. Combining hypothermia with 2-IB might represent a promising treatment strategy for reducing hypoxia-induced neuronal injury.