ORIGINAL ARTICLE

Maternal treatment of rats with the new pyridoindole antioxidant during pregnacy and lactation resulting in improved offspring hippocampal resistance to ischemia *in vitro*

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Abstract

OBJECTIVE: Damage to the developing brain may be caused by maternal environment, nutritional deficiencies, failure of protective mechanisms, etc. Further, the developing brain may be damaged by intrauterine ischemia or by ischemia in newborns complicated by perinatal asphyxia. There is an effort to find agents with neuroprotective effect on the developing brain. The aim was to study the effect of the new pyridoindole antioxidant SMe1EC2 on the resistance of offspring hippocampus exposed to ischemia *in vitro* after treatment of mothers.

MATERIALS AND METHODS: The electrically evoked responses were determined by extracellular recording from offspring hippocampal slices. The effect of oral treatment of rats with SMe1EC2 over 18 consecutive days, from day 15 of gestation to day 10 *post partum* (PP) was analyzed in the model of ischemia *in vitro* measured on the hippocampus of 21-day-old pups, with focus on neuronal function recovery in reoxygenation.

RESULTS: Increased recovery of neuronal response was found at the end of 20-min reoxygenation in offspring hippocampal slices exposed to 10-min hypoxia/hypoglycemia from rats whose mothers were treated with the dose of 50 and 250 mg/kg of SMe1EC2, compared to control offspring slices (mothers received vehicle over the same time).

CONCLUSIONS: The increased offspring hippocampus resistance to hypoxia/hypoglycemia due to 18-day maternal treatment with SMe1EC2 might have been obtained *via* the transplacental way as well as in the neonatal period *via* breast milk, skin and saliva. The manifested neuroprotective effect of SMe1EC2 on the developing brain might find exploitation during risk pregnancy and delivery.

Abbreviations:

ACSF - artificial cerebrospinal fluid
CA - *cornu ammonis*
EPSP - excitatory postsynaptic potential
FAP - field action potential
PP - *post partum*
PS - population spike
PV - presynaptic volley
SA - stimulatory artefact
SMe1EC2 - 2-ethoxycarbonyl-8-methoxy-2,3,4,4a,5,9b-hexahydro-1*H*-pyrido-[4,3b]indolinium chloride
STO - stobadine

INTRODUCTION

Currently, there is much evidence concerning structural and functional damage of the developing brain caused by maternal environment, involving nutritional factors, ethanol or caffeine intake, as well as the deleterious effects of tobacco, X-irradiation, dental amalgam and other agents (Tanaka 1997; Mitchell *et al.* 1999; Edwards *et al.* 2000; Laurberg *et al.* 2004; Mutter *et al.* 2005; Sharma & Mishra 2006). The developing brain may be damaged also by some undesirable events during pregnancy, as intrauterine ischemia, fever superimposed on the ischemic event, by ischemia/reperfusion brain injury in newborns complicated by perinatal asphyxia, etc. (Nakai *et al.* 2001; Laptook & Corbett 2002; Zhao & Zuo 2005; Gazzolo *et al.* 2009).

Damage to the developing brain as well as brain diseases during aging may be caused not only by dietary deficiency, for instance lack of trace elements, vitamins, especially vitamin B group, essential amino acids, essential fatty acids, including omega-3 polyunsaturated fatty acids, polyphenols, etc. but also by failure of protective mechanisms, for instance antioxidants (Bourre 2004). There is an increasing effort to find agents with neuroprotective effect on the brain. Antioxidants and radical scavengers may protect the nervous system against toxic effects of reactive oxygen species and free oxygen radicals and thus attract many researchers to study their effect also in the area concerning fetal and postnatal brain protection (La Grande *et al.* 1999; Mitchell *et al.* 1999; Viana *et al.* 1999; Edwards *et al.* 2000; Okatani *et al.* 2000; He *et al.* 2008).

We focused on the newly synthetized pyridoindole compound 2-ethoxycarbonyl-8-methoxy-2,3,4,4a,5,9b-hexahydro-1*H*-pyrido-[4,3b]indolinium chloride, code SMe1EC2 (m.w. 312.79 Da, chemical purity < 99%) which was found to have higher antioxidant capability than the parent compound stobadine (STO) with neuroprotective and cardioprotective properties (Horáková & Štolc 1998; Štolc *et al.* 2006; Štolc *et al.* 2008). A toxicological and teratological study of SMe1EC2 showed its low toxicity (Štolc *et al.* 2008), no signs of maternal toxicity and no embryotoxic and teratogenic effects on developing rats (Ujházy *et al.* 2008). In our previous study, the neuroprotective effect of the pyridoindole antioxidant SMe1EC2 was shown in 2-month-old male rats by improved recovery of neuronal function after transient hypoxia/hypoglycemia in reoxygenation, as well as by morphological changes manifested as reduced edema extent in the treated hippocampus under ischemia *in vitro* (Gáspárová *et al.* 2009). The aim of this study was to assess a putative neuroprotective effect of this new compound on the offspring hippocampus exposed to ischemic conditions *in vitro*, mediated by 18-day treatment of mothers with SMe1EC2, eight last days of pregnancy up to day 10 *post partum* (PP).

MATERIALS AND METHODS

Animals

Female Wistar rats (weight 200–220 g, age 3–4 month, n=31) obtained from the breeding station Dobrá Voda (Slovak Republic, reg. No. SK CH 4004) had free access to water and food pellets and kept on a 12/12 h light/dark cycle. Their offspring aged 21 days (weight 40–50 g, n=15) were used for the present study. All procedures involving animals were performed in compliance with the Principles of Laboratory Animal Care issued by the Ethical Committee of the Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Bratislava as well as by the State Veterinary and Food Administration of the Slovak Republic.

Drug and its application

SMe1EC2, the drug tested, was prepared in the Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Bratislava, Slovak Republic. Female rats were once daily treated over 18 consecutive days, from day 15 of pregnancy to day 10 PP with SMe1EC2 by gavage at doses of 50 and 250 mg/kg and control mothers obtained vehiculum over the same time. In oral treatment, the substance tested was dissolved in saline at a constant dose volume of 0.5 ml/100 g body weight. The doses were determined according to LD_{50} of the drug tested (orally LD_{50} < 2 400 mg/kg), with the highest dose representing approximately 10% of LD_{50}. Control mothers received vehicle over the same period. Newborns were with their mothers and fed with breast milk. Offspring were weaned on day 21 PP and killed by cervical dislocation.

Preparation of hippocampal slices and model of in vitro ischemic conditions: glucose and oxygen deprivation

Rat hippocampal slices (400 μm) were prepared by conventional technique described in detail earlier (Vlkolinský & Štolc 1999; Gáspárová *et al.* 2006). Slices cut by McIlwain tissue chopper were positioned on a supporting monofile mesh separating water and gas phases in incubation chamber at 35 °C. The water phase consisted of artificial cerebrospinal fluid (ACSF) composed in mmol/l: NaCl 124, KCl 3.3, KH_2PO_4 1.25, $MgSO_4$ 2.4, $CaCl_2$ 2.5, $NaHCO_3$ 26, glucose 10 and saturated with 95% O_2 + 5% CO_2, at pH 7.4, while the gas phase consisted of 95% O_2 + 5% CO_2. Both phases were con-

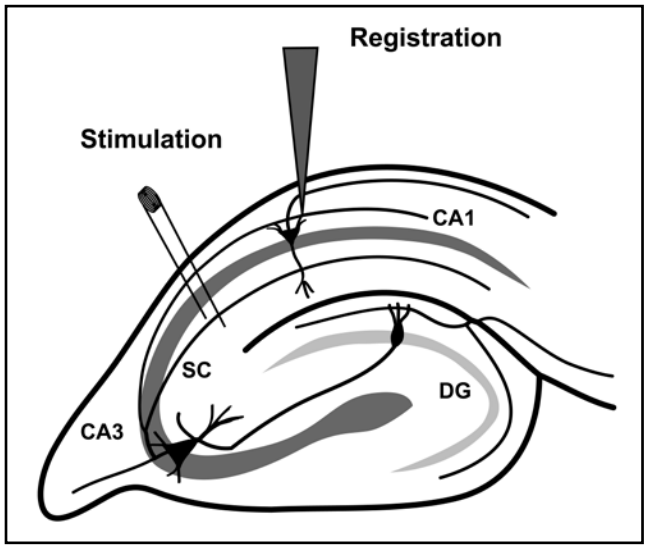

Fig. 1. Schema of hippocampal slice. Synaptic transmission close to the soma of hippocampal pyramidal neurons was assessed by electrical stimulation of Schäffer collaterals and by registration of transsynaptically evoked field action potential in the CA1 area. SC- Schäffer collaterals, DG - *gyrus dentatus*, CA - *cornu ammonis*.

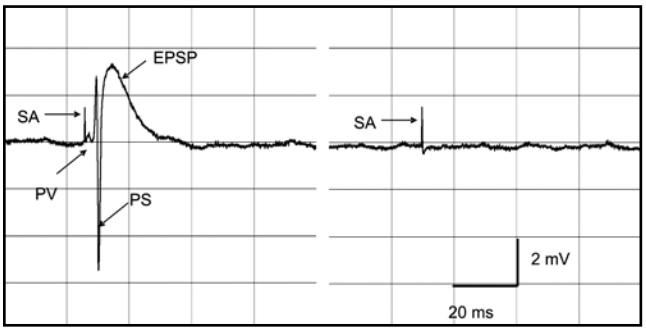

Fig. 2. Field action potential (FAP) composed of presynaptic volley (PV), population spike (PS) and excitatory postsynaptic potential (EPSP) and its cessation at the end of transient hypoxia/hypoglycemia. Stimulation artefact (SA) indicates the time of electrical stimulation. Calibration: horizontal (time) and vertical (magnitude of response) is marked.

tinuously flowing through the chamber. Bipolar wire electrodes were used to stimulate Schäffer collaterals by square wave pulses (0.01–0.05 ms × 0.1 Hz^{-1}) evoking trans-synaptically activity in CA1 pyramidal cell layer (Figure 1). Recordings of field action potential (FAP) were amplified, recorded on DigiData 1322A (Molecular Devices, Axon Instruments) with sampling rate 10 kHz and stored on personal computer for further analysis. After replacing the gas mixture with O_2 by the gas mixture with N_2 by switching the valves, along with superfusion of the slices with ACSF equilibrated with the oxygen free gas mixture and glucose diminished to 4 mmol/l, the FAP was quickly fading. The hippocampus of 21-day-old pups was less sensitive to ischemia compared to the 2-month-old rats tested in previous studies and thus exposure to hypoxia/hypoglycemia was prolonged from six to ten minutes. Recovery of FAP after hypoxia/hypoglycemia was monitored during 20-min reoxygenation and population spike (PS) amplitude was measured in later analysis. Ten to 12 offspring hippocampal slices were measured in each experimental group: 1) slices (n=12) where mothers were treated for 18 consecutive days orally with 50 mg/kg/day of SMe1EC2, 2) slices (n=10) where mothers were treated for 18 consecutive days orally with 250 mg/kg/day of SMe1EC2, and 3) control slices (n=10) where mothers received vehicle over the same time period. The hipocampal slices were stabilized 60–80 min in the incubatory chamber. Then each individual slice was stabilized 15–20 min in the measurement chamber and then exposed to 10-min hypoxia/hypoglycemia followed by 20-min reoxygenation. Resistance of the offspring hippocampus to ischemic conditions was evaluated according to the PS amplitude recovery in 20th min of reoxygenation compared to controls.

Statistics

The data were statistically evaluated using the GraphPad InStat software. Magnitude of PS during stabilization period was expressed as normalized values, where value 1 was calculated as the mean value of amplitudes recorded five min before hypoxia/hypoglycemia onset. Then the ratio was established as the measure of functional recovery in reoxygenation. The values were expressed as means ± S.E.M. Individual data from normalized PS magnitudes were used in ANOVA Dunnett multiple comparison test. The limit of $p<0.05$ was considered statistically significant. The n-value expresses the number of hippocampal slices used in each experimental group.

RESULTS

All hippocampal slices (n=32) were exposed to 10-min hypoxia/hypoglycemia followed by 20-min reoxygenation. Extracellularly recorded response from the pyramidal layer of CA1 neurons, composed of presynaptic volley (PV), and excitatory postsynaptic potential (EPSP) with generated PS, disappeared during 10-min hypoxia/hypoglycemia in all offspring hippocampal slices tested (Figure 2). Recovery of electrically evoked neuronal response in reoxygenation was measured. Significantly increased recovery of the PS amplitude was found in reoxygenation in offspring hippocampal slices where mothers were over 18 consecutive days treated with the pyridoindole SMe1EC2 at the dose of 250 mg/kg/day, compared to control offspring hippocampal slices where mothers received only vehicle (Figure 3).

DISCUSSION

Perinatal hypoxia, occuring when oxygen availability drops below the normal level, is a major cause of hypoxic/ischemic brain injury (Zhao & Zuo 2005; Chen *et al.* 2009). Compared with the adult brain, the neo-

natal brain is different in physiological and structural changes related to its response to hypoxia. Neuropathological studies have shown major differences between postnatal and adult animal models in regard to cerebral vulnerability and resistance to ischemia. Towfighi and co-workers (1997) studied groups of rats from day 2 PP to day 30 PP exposed to unilateral *common carotid artery* occlusion followed by breathing 5–8% oxygen. They found that the hippocampus was remarkably resistant to hypoxic-ischemic insult in 2–3-day-old rats but later it became progressively vulnerable. By day 21 PP the adult pattern of CA1 vulnerability was reached in their experimental model. Similarly, Wise-Faberowski and co-workers (2009) found that cell death was evident due to 10-min oxygen/glucose deprivation in cultured organotypic hippocampal slices prepared from 21-day-old rat pups, while in slices prepared from 7-day-old rat pups neurodegeneration was not evident up to 20-min oxygen/glucose deprivation. In our previous electrophysiological studies, we used 2-month-old male Wistar rats to study the effect of different compounds in the rat hippocampus exposed to ischemia *in vitro* (Gáspárová *et al.* 2006; 2008; 2009). Six to 6.5-min lasting hypoxia/hypoglycemia was usually sufficient to elicit failure of neuronal transmission with poor recovery of neuronal function during 20-min reoxygenation in control hippocampal slices from these 2-month-old rats. Currently, in pilot measurements on control hippocampal slices from 21-day-old male pups, we observed very good resistance up to 6.5-min hypoxia/hypoglycemia and a still quite good over after prolonged eight- and nine-minute exposure. Thus 10-min hypoxia/hypoglycemia was used in this study. We consider hippocampal slices from 21-day-old pups to be more resistant to ischemic conditions than the hippocampus of 2-month-old adult rats. The age border between rat brain resistance/sensitivity to ischemia may be due to differences in experimental conditions of different laboratories and different strains of rats. Further discrepancies in rat resistance to ischemia were found by Marosi *et al.* (2006) who called attention to the important source of variability in the results of acute experiments on hippocampal ischemia due to different Wistar rat vendors.

The main question of this work was to study the effect of maternal treatment with SMe1EC2 on the resistance of offspring brain to acute hypoxia/hypoglycemia *in vitro*. Antioxidant therapy of pregnant rats from current literary data concerned especially reduced carcinogenesis, decreased risk of developing cardiovascular disease in adult life, mediators in diabetic embryopathy, etc. (Kinalski *et al.* 1999; Cederberg *et al.* 2001; Castro *et al.* 2008; Chen *et al.* 2009), yet it was not studied in connection with exposure of the offspring brain to ischemia and subsequent improved neuronal function recovery. We found that the neuroprotective effect due to 18-consecutive-day oral treatment of mothers with the dose of 250 mg/kg/day of SMe1EC2 on offspring hippocampal neurons exposed to 10-min ischemia *in vitro* was significant. The effect of the drug found at the low dose of 50 mg/kg/day was less expressive. It is to be stressed that this effect was obtained not by direct animal treatment but *via* treatment of mothers. To date, there is evidence on the pharmacokinetics of only the parent drug STO (Bezek *et al.* 1990; Trnovec *et al.* 1990; Šoltés *et al.* 2000; Ujházy *et al.* 2000). Pretreatment of pregnant rats with STO prevented to a certain extent reproductive and fetal development alterations caused by chronic intrauterine hypoxia induced by phenytoin (Mach *et al.* 2009). The new derivative of stobadine, as a small molecule with a similar structure, may cross the placenta and the blood-brain barrier during PP administration to mothers, get into breast milk and pass other barriers, such as mother skin and saliva. These results suggest that SMe1EC2 may become a potential protectant in pregnancies with high risk of hypoxic/ischemic brain damage, perinatal asphyxia or pre-term delivery, in which oxidative stress plays a crucial role.

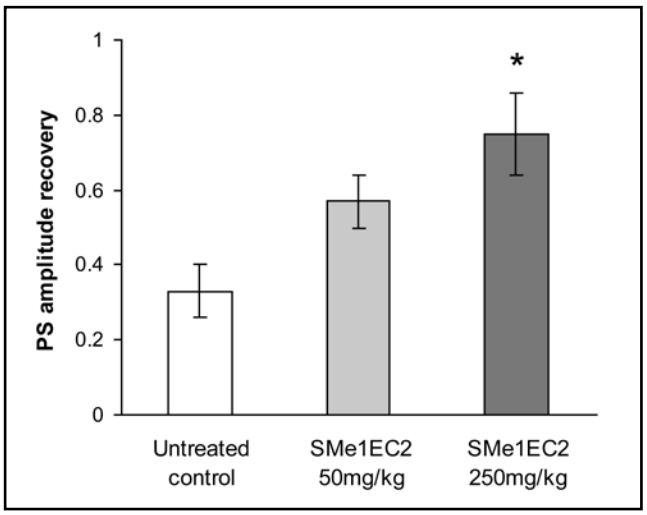

Fig. 3. Resistance of 21-day-old rat pup hippocampal neurons in the CA1 area to transient 10-min hypoxia/hypoglycemia followed by 20-min reoxygenation: effect of 18-consecutive-day treatment of mothers with SMe1EC2 (once daily 50 or 250 mg/kg/day orally). Normalized value 1 represents the PS amplitude at the onset of ischemic conditions *in vitro*. Significantly increased recovery of population spike (PS) amplitude was found at the end of 20-min reoxygenation in hippocampal slices of offsprings from mothers treated 18-consecutive days with the dose of 250 mg/kg of SMe1EC2, compared to recovery of PS in hippocampal slices of offspring from untreated mothers. Mean ± SEM is shown. Significant difference was calculated by ANOVA Dunnett multiple comparisons test,*$p<0.05$. Ten to 12 slices were used in each experimental group.

CONCLUSION

This study demonstrates the neuroprotective effect of maternal treatment with SMe1EC2 on improved resistance of the offspring hippocampus against transient ischemia *in vitro*, documented by improved recovery of electrically evoked neuronal response in reoxygenation. The manifested neuroprotective effect of SMe1EC2 on

the developing brain might find exploitation during risk pregnancy and delivery.

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