Effect of the new pyridoindole antioxidant SMe1EC2 on functional deficits and oedema formation in rat hippocampus exposed to ischaemia in vitro

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Submitted: 2009-06-15 Accepted: 2009-10-11 Published online: 2009-11-11

Key words: hippocampus; pyridoindole antioxidant; neuronal transmission; oedema; ischaemia; hypoxia/hypoglycaemia; neuroprotection

Abstract

OBJECTIVES: The idea of neuroprotective therapy for ischaemic stroke is based on results from studies on experimental animal models of brain ischaemia demonstrating efficacy of many natural and synthetic agents. Contrary to positive conclusions with antioxidants from animal models, clinical experience failed to find neuroprotectants so efficient in human stroke, infarction, brain trauma, tissue preservation, etc. Thus new highly effective neuroprotective agents need to be discovered.

METHODS: Effects of 10-day oral treatment with the new pyridoindole derivative, code SMe1EC2, was analysed in the model of ischaemia in vitro measured five days after oral treatment, with focus on neuronal function recovery. The responses were determined by extracellular recording from rat hippocampal slices. Further, effect of SMe1EC2, applied into the incubation medium before and during 6-min hypoxia/hypoglycaemia in vitro, was studied on the oedema extent in neurons of the CA1 area.

RESULTS: Ten-day oral treatment of rats with SMe1EC2 at the doses 50 or 250 mg/kg resulted in improved resistance of hippocampal neurons to 6.5-min hypoxia/hypoglycaemia in vitro measured during reoxygenation, compared to untreated rats. Application of the drug tested into the incubation medium 30 min before and during 6-min hypoxia/hypoglycaemia resulted in reduction of oedema formation in the CA1 area compared to untreated slices exposed to ischaemia in vitro.

CONCLUSION: The current study confirmed the neuroprotective effect of the pyridoindole antioxidant SMe1EC2 on the level of recovery of neuronal function as well as on affection of morphological changes expressed by reduced oedema extent in the rat hippocampus under ischemic conditions in vitro.
INTRODUCTION

Recently it has been generally accepted that oxidative stress is one of the multiple mechanism participating in acute or chronic neuronal damage (Chan, 2001; Sugawara and Chan, 2003; Saito et al. 2005; Fatokun et al. 2006). Generation of reactive oxygen species is characteristic for hypoxia and especially for reoxygenation (Chan, 2001). Antioxidants and radical scavengers may protect the nervous system against toxic effects of reactive oxygen species and free oxygen radicals. Along with calcium channel blockers, glutamate antagonists, gamma-aminobutyric acid agonists, nitric oxide synthase inhibitors, etc., they exert neuroprotective activity (Levi and Brimble, 2004; Ginsberg, 2008). The idea of neuroprotective therapy for acute ischemic stroke is based on results from extensive studies on animal models of brain injury, demonstrating efficacy of many natural and synthetic agents. Contrary to positive conclusions with antioxidants from experimental animal models, clinical experience failed to find neuroprotectants so efficient in human stroke, infarction, brain trauma, tissue preservation, etc. (Chandak and Kasner, 2001; Gladstone et al. 2002; Wahlgren and Ahmed, 2004; Savitz and Fisher, 2007; Bacigaluppi and Hermann, 2008; Ginsberg 2008). Thus, new highly effective neuroprotective agents need to be discovered.

Since the 1990s, an extensive search has been made for new compounds with antioxidant and antiradical properties in the Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences. The research was based on stobadine, a pyridoindole drug derived from the gamma-carboline antidepressant and neuroleptic drug Carbidine (Barkov, 1973). The stobadine molecule reveals multiple pharmacological effects, including free radical scavenging, antioxidant, cardioprotective, alpha-adrenolytic, cholinolytic, histaminolytic, hypotensive and membrane stabilising, as well as other tissue protective effects (Horáková and Štolc, 1998). Sites in the pyridoindole stobadine molecule responsible for antioxidant and antiradical properties were identified and by an appropriate substitution more than 70 new stobadine derivatives have been prepared (Štolc et al.; 2006; Štolc et al. 2008). One of the new derivatives, substituted with methoxy-group on the aromatic cycle and ethoxycarbonyl-group substituted in position 2- of the piperidine nitrogen, is the compound 2-ethoxycarbonyl-8-methoxy-2,3,4a,5,9b-hexahydro-1H-pyrido-[4,3b]indolinium chloride, code SMe1EC2. The pyridoindole SMe1EC2 was found to have higher antioxidant capability than the parent molecule stobadine, at simultaneous elimination of the undesired alpha-adrenolytic activity (Štolc et al. 2006). Further, a prenatal developmental teratological study of SMe1EC2 showed its low toxicity and no embryotoxic and teratogenic effects on developing rats and no signs of maternal toxicity (Ujházy et al. 2008). This new pyridoindole with antioxidant properties might find use as a protective agent in acute or chronic diseases in which oxidative stress can be assumed.

To support promising findings concerning the new compound SMe1EC2, more positive experimental data need to be found. From this point of view, the aim of this study was to further analyse the effects of SMe1EC2 in brain injuries evoked by model ischaemic conditions (hypoxia with hypoglycaemia followed by reoxygenation) with focus on acute functional and morphologic disturbances. We studied 1) effect of 10-day oral treatment with SMe1EC2 on the recovery of neuronal transmission, and 2) effect of its application into incubation medium on oedema formation in vitro, both in rat hippocampal slices exposed to hypoxia/hypoglycaemia followed by reoxygenation.

MATERIAL AND METHODS

Animals

Male and female Wistar rats 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. 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 as well as by the State Veterinary and Food Administration of the Slovak Republic.

Female rats (weight 200–220 g, n=70) were used in the teratological study of the pyridoindole antioxidant SMe1EC2 (Ujházy et al. 2008) and hippocampal slices from nine of them were used for the present electrophysiological study. They were orally treated 10 days with SMe1EC2 at doses 50 and 250 mg/kg and then killed by cervical dislocation five days later on.

Six male rats (weight 200–240 g) were used in the histo-morphological study. They were anaesthetised by ether and then decapitated.

Drug

2-ethoxycarbonyl-8-methoxy-2,3,4a,5,9b-hexahydro-1H-pyrido[4,3-b]indolinium chloride, code SMe1EC2, was prepared in the Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences.

In oral treatment, the substance tested was dissolved in saline at a constant dose volume 0.5 ml/100 g body weight.
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}. Controls received vehicle over the same period.

In this histo-morphological study of in vitro SMelEC2 effect on oedema formation in the CA1 area of hippocampal slices exposed to ischaemic conditions, the substance tested was applied into the incubation medium (artificial cerebrospinal fluid – ACSF) at concentration 3 µmol/l. The concentration was determined according to the effect of the pyridoindole SMelEC2 on neuronal transmission recovery in rat hippocampal slices exposed to ischaemic conditions in our previous electrophysiological measurements (Štolc et al. 2006). The substance tested was present in ACSF 30 min before as well as during hypoxia/hypoglycaemia followed by reoxygenation. The stock solution of the pyridoindole SMelEC2 (1 mmol/l) was prepared in distilled water and then dissolved in ACSF.

Preparation of hippocampal slices

The brain was removed and the hippocampus was quickly dissected in ice-cold ACSF composed of (in mmol/l): NaCl 124, KCl 3.3, KH_{2}PO_{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. Coronal slices, 400 µm thick, were cut using the McIlwain Tissue Chopper. For electrophysiological or morphological measurements, each slice was placed into a gas/liquid interface chamber perfused with gas-saturated ACSF at a rate 0.6 ml.min^{-1}, the chamber volume being 0.6 ml. The gas mixture was used both to saturate the ACSF and to ventilate the interface chamber.

Model of in vitro ischaemic conditions: glucose and oxygen deprivation

To retain ischaemic conditions, O_{2} in the original gas mixture was substituted by N_{2}. Simultaneously the perfusion medium was switched to ACSF saturated with 95% N_{2} + 5% CO_{2}, containing 4 mmol/l glucose. Reoxygenation was attained by restoring the former conditions. The rate of oxygen/nitrogen exchange (Figure 1) in the recording chamber and uniformity of ischaemic conditions was monitored by the miniature Clark oxygen electrode 733 (Diamond Micro Sensors, U.S.A.) placed approximately 10 mm next to the slice.

Electrophysiology

Neurons in the cornu ammonis CA1 region were trans-synaptically excited by stimulation of Schäffer collaterals. Supramaximal intensity of stimulation was set up for the first population spike (PoS). Neurons were stimulated in the region CA3 using bipolar stainless steel wire electrode by square wave pulses (0.01–0.05 ms × 0.1 Hz). PoS from the CA1 region were registered in the pyramidal stratum. The responses were recorded using a 3–5 MΩ glass electrode filled with ACSF. Recordings were amplified, recorded on DigiData 1322A (Molecular Devices, Axon Instruments, U.S.A.) with sampling rate 10 kHz and stored on personal computer for off-line analysis. Hippocampal slices were derived from animals divided into three experimental groups: 1) three control rats (received vehicle), 2) three rats treated 10 days orally with 50 mg/kg and 3) three rats treated 10 days orally with 250 mg/kg of the pyridoindole antioxidant SMelEC2. All hippocampal slices (n=34) were stabi-
lised 10–15 min in a measurement chamber and then exposed to 6.5-min hypoxia/hypoglycaemia followed by 20-min reoxygenation. The measure of synaptic transmission impairment was assessed by a decay of PoS and EPSP amplitude during 6.5-min ischaemia expressed as the half-time ($t_{0.5}$) of PoS and EPSP decay in minutes. Resistance of hippocampus to ischaemic conditions was evaluated according to the PoS and EPSP amplitude recovery during 20-min reoxygenation and expressed as the ratio of the normalised value 1, calculated as the mean value of responses recorded five min before onset of hypoxia/hypoglycaemia.

**Histo-morphological procedure of oedema determination**

Hippocampal slices (n=30) from six healthy untreated male rats were divided into three experimental groups: 1) control slices incubated in ACSF within three hours after cutting and then fixed in 4% formaldehyde (n=12), 2) slices stabilised, exposed to 6-min hypoxia/hypoglycaemia followed by 20-min reoxygenation, and fixed in 4% formaldehyde within three hours after cutting, (n=9), and 3) slices stabilised, incubated 30 min with SMe1EC2 (3 µmol/l), exposed to 6-min hypoxia with hypoglycaemia followed by 20-min reoxygenation with the antioxidant still present and then fixed in 4% formaldehyde within three hours after cutting (n=9). The slices were embedded in paraffin, routinely processed and stained by haematoxylin and eosin. The CA1 area (Figure 2) was selected and captured by optical microscope (Leica DM 2000, Wetzlar, Germany) with attached camera (S50, Canon, Japan). The tissue oedema was shown as optically empty spaces with low staining intensity around the cells and blood vessels. Images were transformed to the grey scale according to the staining intensity and morphometrically analysed by a two-dimensional image analyser (ImageJ 1.34n, National Institute of Health, U.S.A.). The cut-off value for tissue oedema was selected at the level 160 in the grey scale. The area of the tissue oedema, with the grey value between 160 and 255 was extracted from the image by digital colour subtraction and expressed as the percentage of the total field.

**Statistics**

The data were statistically evaluated using the GraphPad InStat and GraphPad Prism software. The values were expressed as means ± S.E.M. The half-time ($t_{0.5}$) of the PoS and EPSP decay during hypoxia with hypoglycaemia was expressed from the PoS and/or EPSP time-decay course respectively, using regression analysis. The differences between groups were tested using Students’ t-test. The significance limit of $p<0.05$ was considered statistically significant difference. The $n$ value expresses the number of hippocampal slices used in each experimental group.

**RESULTS**

**Effect of 10-day oral treatment of rats with SMe1EC2 on resistance of hippocampal neurons exposed to ischaemic conditions expressed by functional recovery**

There were no significant differences in the mean magnitudes of PoS or EPSP at the beginning of hypoxia/hypoglycaemia between the three experimental groups in randomly selected slices (Figure 3). Exposition of hippocampal slices to ischaemic conditions elicited transient failure of neuronal excitability and synaptic transmission (Figure 4). Resistance of hippocampal neurons to ischaemia was expressed as the ratio of recovery of PoS and EPSP amplitudes, measured at the 20th min of reoxygenation (Figure 5). Hippocampal slices (n=12) from rats treated with the antioxidant at the dose 50 mg/kg of SMe1EC2 showed improved recovery of neuronal excitability and synaptic transmission compared to hippocampal slices (n=10) from untreated control rats. Hippocampal slices (n=12) from the rats treated with the dose 250 mg/kg of SMe1EC2 exhibited marked recovery of neuronal excitability and synaptic transmission after exposure to hypoxia/hypoglycaemia compared to hippocampal slices (n=10) from untreated control rats. Hippocampal slices (n=12) from the rats treated with the dose 250 mg/kg of SMe1EC2 exhibited marked recovery of neuronal excitability and synaptic transmission compared to the slices (n=10) from untreated animals (Figure 4; Figure 5 and 6). The time course of synaptic transmission cessation during 6.5-min hypoxia/hypoglycaemia was significantly delayed in slices from rats treated with the dose 250 mg/kg of SMe1EC2 compared to slices from control untreated rats. The differences in half time ($t_{0.5}$) of the PoS or EPSP decay are shown in Table 1.

**Effect of SMe1EC2 on oedema formation in the CA1 area of hippocampal slices exposed to ischaemic conditions**

The pericellular and diffuse oedema extent represented 22.52 ± 0.46% of total tested area in slices exposed to ischaemia in vitro. The oedema extent was significantly lower in ischaemic slices treated with 3 µmol/l SMe1EC2 ($p=0.0026$) and in control slices ($p=0.04$).
Fig. 3. Basic properties of responses recorded extracellularly in rat hippocampal neurons. The population spike (PoS) and excitatory postsynaptic potential (EPSP) amplitudes depict neuronal excitability and synaptic transmission in three experimental groups. There are no significant differences in the mean PoS and/or EPSP amplitude at the onset of hypoxia/hypoglycaemia in hippocampal slices from control untreated animals compared to treated rats. Values are means ± S.E.M. of responses from 10 to 12 slices in each experimental group.

Fig. 4. Effect of 10-day oral treatment with SMe1EC2 on resistance of hippocampal neurons to transient 6.5-min ischaemia in vitro. (A) Time course of neuronal excitability decay expressed by normalised population spike (PoS) amplitude during ischaemia and recovery in reoxygenation. (B) Time course of synaptic transmission decay expressed by normalised excitatory postsynaptic potential (EPSP) amplitude during ischaemia and recovery in reoxygenation. Duration of exposition of hippocampal slices to transient hypoxia/hypoglycaemia is marked by horizontal line. Values are means ± S.E.M. of responses from 10 to 12 hippocampal slices in each experimental group.

Fig. 5. Recovery of evoked responses in hippocampal slices exposed to 6.5-min ischaemia. To classify the degree of functional recovery in treated animals, the population spike (PoS) as well as excitatory postsynaptic potential (EPSP) amplitudes were compared at the end of 20-min reoxygenation with responses from control untreated animals. Hippocampal slices from rats treated with the dose of 250 mg/kg of SMe1EC2 manifested full recovery of evoked responses compared to their magnitude at the beginning of ischaemic insult (the normalised value 1). Significant difference was calculated from means ± S.E.M. using the Student t-test between responses from slices of treated animals compared to responses from slices of untreated animals, *p<0.05, and **p<0.001.

Tab. 1. Neuronal excitability and synaptic transmission decay during 6.5-min ischaemia of rat hippocampal slices expressed by half time (t0.5) in minutes.

<table>
<thead>
<tr>
<th>Group</th>
<th>PoS (min)</th>
<th>EPSP (min)</th>
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<tbody>
<tr>
<td>Control</td>
<td>1.96 ± 0.22</td>
<td>2.25 ± 0.19</td>
</tr>
<tr>
<td>50 mg/kg SMe1EC2</td>
<td>2.20 ± 0.24 ns</td>
<td>2.41 ± 0.34 ns</td>
</tr>
<tr>
<td>250 mg/kg SMe1EC2</td>
<td>3.24 ± 0.15 ***</td>
<td>3.46 ± 0.15 ***</td>
</tr>
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Values represent means ± S.E.M., n-values represent the number of slices in each experimental group. PoS - population spike, EPSP - excitatory postsynaptic potential, t0.5 - half time of decay during ischaemia, ns - non significant difference, *** - significant difference where p<0.0001, 50 mg/kg SMe1EC2 and 250 mg/kg SMe1EC2 – oral doses of the pyridoindole antioxidant tested.
Fig. 6. Representative electrically evoked responses from hippocampal slices of control and treated rats.
Ten-day oral treatment with the substance SMe1EC2 in the dose of 50 or 250 mg/kg was terminated five days before the animals were sacrificed. On the left: action potential at the beginning of ischaemia in vitro. On the right: action potential at the end of 20-min reoxygenation. Insert shows horizontal and vertical calibration. AxoScope 10.2 software was used to record and analyse data.
DISCUSSION

Recently many experimental data have been provided showing that antioxidants can improve histological, biochemical, neurobehavioural and electrophysiological outcomes after brain ischaemia/reperfusion, hypoxia/reoxygenation or neurotrauma (Hall et al. 1992; Hall et al. 1996; Vlkolinský and Štolc, 1999; Gáspárová et al. 2006; Reiter et al. 2007; Ek et al. 2007; Reiter et al. 2008; Štolc et al. 2008; etc.). On the other hand, neuroprotective activity trials have not been so successful in human clinical practice. Therefore highly effective new neuroprotective agents need to be discovered and combination therapies should be assessed (Wahrgren and Ahmed, 2004).

Based on the well-known antioxidant effect of s tobadine, an effort was made to design and synthesise new, more efficient drugs with even more favourable properties. The compound SMé1EC2 has proved to be one of the prospective neuroprotective pyridoindole derivatives. In this article two different experimental methodological approaches were used to provide further data on its effects. Previously, improved resistance of CA1 hippocampal neurons was found after in vitro application of SMé1EC2 in concentrations from 0.03 to 10 µmol/l into the perfusion medium in our model of transient ischaemia in vitro (Štolc et al. 2006). On the basis of these previous results, now we tried to confirm the neuroprotective effect of SMé1EC2 after its 10-day oral treatment, to show ameliorated neuronal endurance to ischaemia. Similarly as its maternal compound stobadine, which showed central neuroprotective effect after intravenous administration during three consecutive days prior ischaemia (Horáková et al. 1991), we expected the pyridoindole derivative studied to present neuroprotective efficiency in the brain after oral treatment. The results showed that 10-day oral treatment of rats with SMé1EC2, at the dose of 50 mg/kg and especially 250 mg/kg was sufficient to increase the resistance of hippocampal neurons to transient ischaemia even five days after treatment. Pharmacokinetic and pharmacodynamic data of SMé1EC2 are not available yet, but it may be retained and effective in brain thanks to its high lipophilicity. Ten-day treatment resulted in significantly high recovery of neuronal excitability and synaptic transmission at the end of 20-min reoxygenation of hippocampal slices after their exposure to 6.5-min hypoxia/hypoglycaemia. Thus the neuroprotective effect of SMé1EC2 was corroborated after its application into the incubation medium, as found previously (Štolc et al. 2006) and the resistance of hippocampal neurons to ischaemia was also improved after its oral treatment.

Oedema is one of the symptoms of acute brain ischaemia and injury (Nukada and Dyck, 1987; Kreisman and LaManna, 1999) and may be connected to acidosis resulting from accumulation of lactate and disturbance of ionic homeostasis resulting from energy depletion (Siesjo, 1988). In ischaemic cells synaptic potential changes associated with oedema were reported (Payne et al. 1996; Kreisman and LaManna, 1999; Zhang et al. 1999) and a novel method for direct real-time observation and quantification of cell oedema formation was prepared in acute ischaemic brain slices (Nakajima et al. 2004). Focal swelling can serve as a marker of impaired function, since both electrophysiological and metabolic recovery are related spatially and temporally to recovery of normal volume (Hossmann et al. 1994). Nakajima and co-workers (2004) found evident swelling in the cell soma and dendrites of pyramidal hippocampal neurons approximately 300 sec after the start of the ischaemic insult caused by oxygen and glucose deprivation. Increased transmittance of image was observed 120 sec after the start of the ischaemic insult. The correlation between the degree of recovery of cell volume and the degree of recovery of synaptic transmission during reoxygenation was established (Kreisman and LaManna, 1999). In this article, histomorphometric image analysis of stained rat hippocampal slices showed increased volume of oedema in slices exposed to 6-min hypoxia/hypoglycaemia and immediately fixed in formol at the end of 20-min reoxygenation. An inhibitory effect of SMé1EC2 on oedema forma-

**Fig. 7.** Tissue oedema in the CA1 area of the rat hippocampal slices.

Histological evaluation of the CA1 area of the hippocampus showed increased tissue oedema in the slices affected by hypoxia/hypoglycaemia and reperfusion injury (B) when compared to the control slices (A). The treatment by SMé1EC2 3 µmol/l, (C) decreased this oedema extent in ischaemia exposed slices. Staining by haematoxylin and eosin; magnification 630 ×.
tion in the CA1 area of hippocampal slices exposed to transient ischaemia in vitro has been reported. This effect of SMe1EC2 may be explained by its high anti-lipoperoxidation activity (Štolc et al. 2006), which could contribute to the preservation of the neuronal cell membrane and of its permeability, and thus might protect neuronal vitality. These findings of the inhibitory effect of SMe1EC2 on oedema formation in ischaemic hippocampal slices are in good agreement with results obtained in the model of acute head trauma (AHT) in mice, where a single dose of SMe1EC1 (1.137 mg/kg i.v. within 1 min after AHT) eliminated the increase in brain wet weight ascribed to acute brain oedema as confirmed by routine histopathology (Štolc et al. 2008).

CONCLUSIONS

The presented findings provide further supportive evidence on the neuroprotective effect of the new pyridoindole antioxidant SMe1EC2 under ischaemic conditions. The study showed 1) improved resistance of hippocampal neurons to transient ischaemia expressed by increased recovery of neuronal excitability and transmission at reoxygenation measured five days after 10-day oral treatment with SMe1EC2, and 2) inhibitory effect of SMe1EC2 on oedema formation in the CA1 area after exposure of hippocampal slices to ischemic conditions. In conclusion, there is further evidence of successful modification in the stobadine molecule resulting in remarkable enhancement of new derivative capacity to protect the brain, as expressed by increased degree of functional and morphological recovery after ischaemic insult. The complex mechanisms involved in the neuroprotective action of SMe1EC2 have to be investigated further.

ACKNOWLEDGMENT

The study was supported by the Slovak Grant Agency for Science VEGA (2/0083/08; 2/0093/08) and APVV Grant (51-017905).

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