Antioxidant Capacity in Rat Brain After ICV Treatment with Streptozotocin and Alloxan - a preliminary study

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INTRODUCTION

Intracerebroventricular (icv) administration of betacytotoxic drug streptozotocin (STZ) produces long-term and progressive cognitive deficits in rats, as well as deficits in cerebral glucose and energy metabolism. These changes resemble those found in the brain of patients with sporadic Alzheimer's disease (sAD), and therefore, STZ-icv treated rats have been proposed as an experimental model of sAD. In this study the antioxidant capacity (AC), using manual oxygen radical absorbance capacity (ORAC) assay, was measured in the rat brain frontoparietal cortex (FC) and brainstem-cerebellum region (BS-CB) after administration of STZ and another betacytotoxic drug alloxan (AL). Region-specific differences of AC were found, which were more expressed when hydroxyl radical (ORAC-OH\(^a\)) generator was used in the assay. AC against ORAC-OH\(^a\) was significantly lower in BS-CB than in FC of the control rats. Furthermore, ORAC-OH\(^a\) significantly decreased in BS-CB 3-months following the icv administration of AL, but significantly increased following the TG+AL combined treatment in comparison with the controls. However, 3-months following the icv treatment of AL combination with a different glucose transport inhibitor, 3-O-methyl-D-glucose, ORAC-OH\(^a\) values in BS-CB and ORAC-ROO\(^a\) values in FC were significantly decreased in comparison to the controls. Our results suggest that betacytotoxic-icv treatment alters antioxidant defense systems in the brain, which particularly regarding the STZ-icv treatment, could be a useful tool in search for possible new antioxidant treatments of the neurodegenerative disorders such as sAD.

Keywords: Antioxidant capacity; Oxygen radical absorbance capacity (ORAC) assay; Peroxyl radical; Hydroxyl radical; Oxidative stress; Streptozotocin; Alloxan; Alzheimer's disease

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Lackovic and Salkovic, 1990; Salkovic-Petrisic and Lackovic, 2003). In addition to that, STZ icv administration produces also long-term and progressive deficits in learning and memory in rats, as well as deficits in cerebral glucose and energy metabolism that resemble those found in the brain of patients with sporadic Alzheimer’s disease (sAD) (Lannert and Hoyer, 1998; Prickaerts et al., 1999; Sharma and Gupta, 2001a,b). Because of that, STZ-icv treated rats have been proposed as an experimental model of sAD. Alterations of the enzymes downstream the insulin receptor signaling cascade in the brain at the level of glycogen synthase kinase (GSK-3α/β) have been recently reported in the STZ-icv treated rats (Salkovic-Petrisic et al., 2006). The changes were regionally specifically distributed between the frontoparietal cortex and hippocampus and dependent on the post icv-treatment time duration. Also, increased total and phosphorylated tau protein expression in the hippocampus of STZ-icv rats, and some indications of β-amyloid accumulation in the meningeal capillaries were found, suggesting the possibility of developing the sAD hallmarks in this experimental model, giving, thus, further support to the resemblance of this experimental model to human sAD (Salkovic-Petrisic et al., 2006; Grünblatt et al., 2007).

Oxidative stress in the brain has been widely recognized as playing an important role in the pathophysiology of sAD (Götz et al., 1992; Mariani et al., 2005). The brain is extremely vulnerable to oxidative stress, as concomitant low activity and capacity of antioxidant protection systems allow for increased exposure of target molecules to free radicals. Estimations of oxidative stress commonly utilize the measurement of malondialdehyde levels (MDA), a product of lipid peroxidation used as an indicator of free radical generation, and glutathione levels, an endogenous antioxidant that scavenges free radicals and protects against oxidative stress. Significant elevations of MDA levels and decreased glutathione levels have been found in the brain of STZ-icv treated rats (Sharma and Gupta, 2001a,b; Ishrat et al., 2006; Pathan et al., 2006). However, antioxidant capacity (AC), measured by oxygen radical absorbance capacity (ORAC) assay with two different free radical generators (Cao et al., 1993; 1995; 1996a,b,c; 1997; Sofic et al., 2002; Prior et al., 2003; Wang et al., 2004) modified by Sofic et al. (2006), is decreased in rat brain 3-months following the STZ-icv treatment (Tahirovic et al., 2007a,b). However, decrement was observed in non-cognitive related brain regions, brain stem and cerebellum (BS-CB), while no change was observed in hippocampus (Tahirovic et al., 2007a,b). The objective of this study was to investigate whether AC has been changed in another brain region important for the cognitive deficits, i.e., cerebral cortex, after the STZ-icv treatment, as well as following the icv treatment with another betacytotoxic drug alloxan, for which neither cognitive deficits nor brain oxidative stress development have been reported so far. Since the peripheral toxicity of both STZ and AL depends on their transport into the pancreatic beta cell via the glucose transporter (GLUT2) and consequently could be blocked by peripheral pre-administration of GLUT inhibitors (Szkudelski, 2001), our experiments were also aimed to show whether icv administration of these GLUT inhibitors would prevent possible STZ- and AL-icv induced effects on the brain AC.

MATERIALS AND METHODS

Chemicals

Fluorescein, Standard Fluka for fluorescence - free acid was obtained from Fluka Chemie GmbH, Steinheim, Germany. 2,2′-azobis (2 amidino-propane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), streptozotocin and alloxan monohydrate were purchased from Sigma Aldrich Chemie GmbH Germany. 5-thio-D-glucose (TG) and 3-O-methyl-D-glycopyranose (3-OMG) were purchased from BioChemika. Cupric sulphate pentahydrate and hydrogen peroxide were obtained from Kemika, Zagreb, Croatia.

Animals

Adult male 3 month old Wistar rats (Department of Pharmacology, School of Medicine, University of Zagreb, Croatia) were used throughout the study. All animals were kept on standardized food pellets and water ad libitum.
Treatments

Drug Administration, icv
Rats were randomly divided in groups and given general anaesthesia (chloral hydrate, 300 mg/kg, ip), followed by injection of different drugs icv bilaterally into the lateral ventricle (2 µl/ventricle), according to the procedure described by Noble et al. (1967). The following drug treatments were applied in a single dose: (I) STZ (1 mg/kg, dissolved in 0.05 M citrate buffer, pH 4.5); (II) AL (0.5 mg/kg dissolved in saline); (III) 5-thio-D-glucose (375 µg/kg, dissolved in either saline or 0.05 M citrate buffer); (IV) 3-OMG (1 mg/kg, dissolved in saline); (V) TG (375 µg/kg) + STZ (1 mg/kg); (VI) TG (375 µg/kg) + AL (0.5 mg/kg); (VII) 3-OMG (1 mg/kg) + AL (0.5 mg/kg); (VIII) an equal volume of respective vehicle (controls). Animals were sacrificed 4, 10 or 12 weeks after the drug icv treatment. Brains were quickly removed, frontoparietal cortex (FC) and brain stem-cerebellum (BS-CB) excised, immediately frozen and stored at -80°C. STZ-icv-treated animals had no symptoms of diabetes, and steady-state blood glucose level did not differ in comparison with control animals.

Sample Preparation
The crude tissue extracts from rat FC and BS-CB were prepared by homogenizing the tissues in 75 mM phosphate buffer, pH 7.3 (10 ml buffer per gram of tissue). The homogenates were centrifuged, and separation of the soluble fractions was performed by two-step centrifugation (15,000 rpm, 30 min; and 15,000 rpm, 10 min) at 4°C. The supernatant was ready for hydrophilic ORAC analysis after appropriate dilution with the buffer solution.

The Manual Oxygen Radical Absorbance Capacity (ORAC) Assay
Manual ORAC analyses were performed on a Perkin Elmer spectrometer LS 55 with a fluorescent filter (Ex: 485 nm; Em: 520 nm) (Sofic et al., 2006). In the final assay mixture (2 ml total volume) fluorescein (10.5 nM) was used as a target of free radical attack, with AAPH (32 mM) as a peroxyl radical generator (ORAC-ROO\(^{\cdot}\) assay), and \(\text{H}_2\text{O}_2\text{-Cu}^{2+}\) (H\(_2\)O\(_2\) 0.3%; CuSO\(_4\) x 5H\(_2\)O 0.9 mM) as mainly a hydroxyl radical generator (ORAC-OH\(^{\cdot}\) assay). Trolox (1 µM) was used as a control standard and prepared fresh daily. The spectrofluorometer was programmed to record the fluorescence of fluoresceine every 10 min after AAPH, or H\(_2\)O\(_2\)-Cu\(^{2+}\) was added for as long as 180 min and the samples were thermostated at 37°C (KP 20-D Lauda, Lauda Koenigshofen). All fluorescence measurements were expressed relative to the initial reading. Final results were calculated using the differences of areas under the fluoresceine decay curves between the blank and a sample, and expressed as µmol per g of fresh tissue.

Morris Water Maze Swimming Test
Cognitive functions were tested in the Morris Water Maze Swimming Test (Anger, 1991) during the last week before the sacrifice (week 11). Adaptation of rats to the experimental environment (pool size 150 x 60 cm, 50 cm deep, water temperature set at 25 ± 1°C) and behavioural activity (swimming in pool divided in four quadrants, I-IV) was done during two days before the experimental trials. In the experimental trials, performed from day 1 to day 4, rats were thought to escape from water by finding a hidden rigid platform submerged about 2 cm below the water surface in quadrant IV. One trial consisted of three starts, each from a different quadrant (I - III), separated by a 1-min rest period. Three consecutive trials were performed per day, separated by a 30-min rest period. After the third trial on day 4, the fourth trial was performed (starts from quadrants I-III) with a platform being removed from the pool, and the time spent in searching for the platform after entering quadrant IV was recorded. The cut off time was 1 min. Those rats which had no alterations in memory function (controls) were expected to remember that the platform had previously been there, and, in line with that, to spend a long time swimming within quadrant IV, looking for the platform. In case of drug-induced deterioration of memory functions, rats were expected to remember less intensively that the platform had been in quadrant IV, and thus to spend less time in searching for the platform within this quadrant, in comparison with the control rats, and make more mistakes by entering the other quadrants (I-III).

Data Collection and Statistical Analysis
Data was sent electronically from the Perkin-Elmer spectrofluorometer LS 55 to a PC system running...
FL WinLab software (Perkin-Elmer). The data were analysed with Microsoft Excel. Statistically significant differences between the control and the treated groups were calculated by the Student’s t-test. The significance of between-group differences in Morris Water Maze Swimming Test was tested by Kruskal-Wallis ANOVA median test, followed by Mann-Whitney U-test. *P < 0.05 considered statistically significant for all the tests.

Ethics
The animal treatments were carried out in Croatia, and were under the guidance of the Principles of Laboratory Animal Care (NIH Publication No. 86-23, revised in 1985), according to the Croatian Act on Animal Welfare (NN19; 1999), and were approved by The Ethics Committee of Zagreb University School of Medicine (No. 04-1343-2006).

RESULTS
Measurements of the AC against hydroxyl- and peroxyl-radical, in control rats treated icv with a vehicle only, demonstrated that ORAC-\(\text{OH}^\circ\) values were significantly lower in the brain stem-cerebellum than in the frontoparietal cortex while ORAC-ROO\(^\circ\) values did not differ between the regions (Table I).

The only change observed in the STZ-icv treated rats in which measurements of AC against hydroxyl and peroxyl radicals were recorded, 1 and 2 months following the drug treatment, was an increase against hydroxyl radical (ORAC-\(\text{OH}^\circ\)) found in the frontoparietal cortex 1-month following drug treatment (data not shown). Interestingly, this STZ-induced increment after 1-month was still present in the combined TG+STZ treatment group. The treatment of TG-icv alone, as well as the TG+STZ combined treatment measured 2 months after the drug treatment, did not influence AC against both free radicals in the investigated brain regions (Table I).

Three months following the icv treatment of another betacytotoxic drug, alloxan, a mild decrease of AC against hydroxyl radical was found in the frontoparietal cortex, while in the brain stem-cerebellum this was significant (Table II). AC against peroxyl radical remained unchanged in both regions (Table II). However, when the icv treatment of AL was combined with the icv treatment of 3-OMG, a decrement of AC against ORAC-ROO\(^\circ\) was found in the frontoparietal cortex (Table II). Furthermore, increased AC against ORAC-\(\text{OH}^\circ\) was found in the BS-CB when AL was combined with

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Data are expressed as mean ± SD; number of animals is given in parentheses; ORAC-\(\text{OH}^\circ\) oxygen radical absorbance capacity assay; FC- frontoparietal cortex; BS-CB- brain stem-cerebellum; C- control group; STZ- streptozotocin-intracerebroventricularly (icv) treated group; TG- 5-thio-D-glucose-icv treated group; TG+STZ-group with combined TG and STZ icv treatment. *P < 0.006 vs FC by Student t-test.

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Data are expressed as mean ± SD; number of animals is given in parentheses; ORAC-\(\text{OH}^\circ\) oxygen radical absorbance capacity assay; FC- frontoparietal cortex; BS-CB- brain stem-cerebellum; C- control group; AL- 5-thio-D-glucose-icv treated group; 3-OMG- 3-O-methyl-D-glucose intracerebroventricularly (icv) treated group; 3-OMG+AL group treated icv with 3-OMG + alloxan; TG+AL- 5-thio-D-glucose-icv + AL-icv treated group. *P < 0.02; **P < 0.003; ***P < 0.006 vs respective control by Student t-test.
The glucose transport inhibitor 3-OMG alone and in combination with AL induced a decrease in the AC against hydroxyl radical in the brain stem-cerebellum while there was a decrease of the AC using ORAC-OH° in the FC (Table II).

There were no changes in total protein and total cholesterol in rat brain 1-month following the icv treatment of streptozotocin and the combination of glucose transport inhibitor 5-thiogluucose + streptozotocin, and 3-months following the icv treatment of AL and the combination of AL with 5-thiogluucose (data not shown).

Preliminary results demonstrate that central administration of AL does not seem to alter rat memory and learning function, as measured at 3 months following AL icv- treatment, with neither number of errors nor time spent in platform search, significantly differ from control (FIG. 1). Both GLUT inhibitors, either alone or as pre-treatment to AL-icv administration, also did not induce significant cognitive deficits, although a large intra-group variation was recorded (FIG.1).

**DISCUSSION**

Free radical and oxidative stress involvement in the pathophysiology of AD has been in the focus of AD research (Götz et al., 1992; Mariani et al., 2005). Discovery of altered lipid composition (Crino et al., 1989), isoprostanes (Flirski and Sobow, 2005; Montine et al., 2005) and data from the recent studies performed in living patients (Calabrese et al., 2003; Pratico and Sung, 2003), make important contribution to better understanding and defining the role which oxygen radicals might play role in AD-pathogenesis. The amyloid protein precursor (APP) transgenic Tg2576 mice have been frequently exploited as an experimental AD model (Yamada and Nabeshima, 2000) in which oxidative stress development has been investigated (Pappoila et al., 1998). Frequently exploited parameters of oxidative stress have also been investigated in the STZ-icv rat model (Sharma and Gupta, 2001a,b; Ishrat et al., 2006; Pathan et al., 2006) which is not related to genetic manipulation, and because of that, more appropriate for experimental sAD research.

Since AC is dependent on the cell chemical composition and the free radical generator used in the assay, it is not surprising that different absolute values of AC against hydroxyl- (lower) and peroxyl (higher) radicals were found in the brain of control rats (Table I) (Tahirovic et al., 2007a,b). Within AC values against one radical, region specific differences were found with the brain stem-cerebellum part, demonstrating lower AC values against hydroxyl-radical in comparison to the frontoparietal cortex in controls (Table I). Similar uneven distribution of AC was demonstrated in the rat brain cortex and cerebellum by other authors (Cao et al., 1996b,c). In line with our previously published data (Tahirovic et al., 2007a,b), STZ-icv treatment induced changes of the antioxidant capacity, which
were free radical- and region- but also post-treatment time-dependent. Regarding our previously published data of decreased AC against hydroxyl-radical (~40%) in the brain stem and cerebellum observed 3 months following the STZ-icv treatment (Tahirovic et al., 2007a,b), no change of AC against this radical was observed in the same regions 2-months following the STZ-icv treatment. This suggests post-damage time-dependent development of changes. Data published by others has demonstrated changes of MDA and glutathione as parameters of oxidative stress measured up to 3-weeks following STZ-icv treatment (Sharma and Gupta, 2001a; 2002; Ishrat et al., 2006; Pathan et al., 2006), while the results presented here and recently published ones (Tahirovic et al., 2007a,b) are the first to demonstrate measurements of antioxidant capacity against particular free radicals in a period longer than 3-weeks and up to 3-months following STZ-icv administration, providing thus a kind of time course of AC changes (data for 1 month are not shown). It seems that changes of AC, measured by the ORAC assay, develop more slowly than changes of MDA and glutathione levels.

Interestingly, although cognitive deficits in the STZ-icv rat model have been seen, starting progressively already 2-weeks after the drug treatment, and were persistent up to 3-weeks post-treatment (Grünblatt et al., 2007), decreased AC against hydroxyl- and peroxyl-radical has not been found in the two regions involved in learning and memory functions (hippocampus) (Tahirovic et al., 2007a,b) and fronto-parietal cortex in these experiments). Surprisingly, increased AC against hydroxyl-radical has been found in the cortex but only as an early change seen 1-month after the STZ-icv treatment (data not shown), which returned to normal values 2-months after the drug treatment. It seems that cortical tissue is more prone to acutely react with free radicals than the brain stem-cerebellum region. It should also be kept in mind that STZ treatment generates reactive oxygen species and additionally releases nitric oxide (NO) (Szkudelski, 2001). Oxidative-nitrative stress has been found following a single STZ-icv dose without involvement of NO synthase-induced generation of NO (Prickaerts et al., 2000; Giasson et al., 2002).

Reduced AC against hydroxyl-radical found in the brain stem-cerebellum 3-months following a chemically different betacytotoxic drug, AL-icv (Table II), is in line with the one we found 3-months following STZ-icv treatment in this brain region (Tahirovic et al., 2007a,b). However, although STZ- and AL-icv treatment differ in inducing the cognitive deficits (their mechanism of action is actually different) (Szkudelsky, 2001), the former being capable and the latter incapable of inducing them, AL similarly to STZ did not reduce AC in the brain regions closely involved in learning and memory function (Table II) (Tahirovic et al., 2007a,b). It could not be excluded that large intra-group variability could account for not reaching the statistically significant difference in these regions, although the number of animals per group was not too low (N=7-8). Possible differences of drug-treated in comparison to the control animals are not likely to be related to the pooling of samples from the control animals (N=22) as all were of the same age, and the AC in brain was not significantly decreased during aging in the adult and old rats (Cao et al., 1996bc; Sapcanin et al., 2005).

The mechanism of STZ and AL action in the brain has not been explored so far. However, in line with the peripheral toxic effects of these drugs on the insulin-producing/secerning cells (Szkudelsky, 2001), and altered brain insulin receptor signaling in following the icv drug administration (Salkovic-Petriscic et al., 2006), similarity to the peripheral mechanism of action seems likely. Their betacytotoxicity at the periphery is related to their entering the beta cell through the glucose transporter GLUT2 (Szkudelsky, 2001), the expression of which has been found also in rat brain (McEwen and Reagan, 2004). STZ and AL transport by GLUT2, and consequently diabetes induction, is inhibited by glucose transport inhibitors TG and 3-OMG (Szkudelsky, 2001). In line with recently published data that pre-treatment with TG-icv and 3-OMG-icv did not abolish STZ-icv induced cognitive deficits (Grünblatt et al., 2007), icv pre-treatment with these GLUT inhibitors in the present experiments did not abolish the change of AC induced by STZ-icv or AL-icv treatment alone. On the contrary, it seemed that the betacytotoxic-icv induced effect had been strengthened; an additional increase in AC against hydroxyl-radical in FC 1-month after the STZ-icv treatment (155% over control /STZ/ vs 172% over control /TG+STZ/, data
not shown), additional decrease in AC against both radicals in FC (18% below control /AL/ vs 50% below control /3-OMG+AL/, Table II) and BS-CB (52% below control /AL/ vs 78% bellow control /3-OMG+AL/, Table II) 3-months after the AL-icv treatment. The increment of AC against hydroxyl-radical in BS-CB induced by the TG+AL treatment is difficult to explain (tissue of TG-icv treated group has unfortunately been lost during the analysis). It could not be excluded that TG and 3-OMG induce different alterations of AC against free radicals in the brain, since 3-OMG-icv treatment alone, cerebellum as well as AL and 3-OMG+AL treatments, decreased AC in the brain stem. The explanation of the possible synergistic effect of GLUT inhibitors and betacytotoxins could be a speculative one only. Icv administration of TG and 3-OMG could possibly inhibit the entrance of glucose widely within the brain, and by other GLUT types in addition to GLUT2, located on different cells/neurons and therefore could induce more extensive intraneuronal glucose loss and consequent damage, although the intracellular uptake of betacytotoxics by GLUT2 could actually be prevented.

Our results suggest that antioxidant defense systems in the brain of rats treated icv with betacytotoxic drugs STZ and AL, and with glucose transport inhibitors or the combination of these inhibitors and STZ/AL are time- and region-dependently disturbed. However, caution is needed in the interpretation of the results because of the possibility that these compounds themselves could mask the objective antioxidant status in the investigated tissue, and facilitation of AC upregulation can not be excluded. Further studies are necessary for the elucidation of this issue as the STZ-icv treated rat could be a useful model for searching also for possible new antioxidant treatments of neurodegenerative disorders (Mandel et al., 2006).

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