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Do Statins Influence the Activity of *c-fos* Gene Following Transient Forebrain Ischaemia in the Adult Rat Hippocampus?

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Abstract

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The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) have been associated with stroke prevention. This stroke prevention appears to occur apart from cholesterol lowering effects. A number of mechanisms have been postulated for this prevention. The aim of our study was to investigate the effect of simvastatin on the *c-fos* gene activity and its relation to delayed neuronal death in CA1 region of hippocampus following transient forebrain ischemia in the adult rat hippocampus.

A total of 17 male Wistar albino rats were used in this study. The animals were divided into three groups: 5 sham-operated animals; 6 ischemised rats without statin pre-treatment and 6 ischemised rats with statin pre-treatment. We used simvastatin at the dose of 20 mg/kg during 14 days prior to the ischemic attack. Fifteen min long transient forebrain ischemia was induced by the four-vessel occlusion. Two and a half h reperfusion was used for the c-Fos activity detection using immunostaining and 72 h reperfusion was used for the determination of neurons surviving using haematoxylin/eosin staining.

The average neuronal density in the CA1 region of hippocampus in the sham-operated rats, in ischemised rats without pre-treatment and in ischemised rats with statin pre-treatment was 47.03 $\pm$ 3.09/0,025 mm^2, 9.05 $\pm$ 2.46/0,025 mm^2 and 16.45 $\pm$ 2.78/025 mm^2, respectively. A significant neuroprotective effect was observed in the pre-treated ischemic group ($P < 0.001$) in comparison to the ischemic group without pre-treatment.

The average of c-Fos positive nuclei density in the CA1 region of hippocampus in the sham-operated rats, in ischemised rats without pre-treatment and in ischemised rats with statin pre-treatment was 0.266 $\pm$ 0.074/025 mm^2, 28.2 $\pm$ 2.053/025 mm^2, 30.3 $\pm$ 4.816/025 mm^2, respectively. A highly significant difference in c-Fos positivity ($P < 0.001$) was found between the sham operated group and both ischemic groups (with and without pre-treatment). No significant difference in c-Fos positivity was observed between untreated ischemic and pre-treated ischemic groups ($P > 0.05$).

These findings indicate that simvastatin provides protection against CA1 hypoxic neuronal injury, which is independent of *c-fos* activation. We can conclude that simvastatin neuroprotection may be mediated by multiple mechanisms as can be expected based on its pleiotropic effects.

Simvastatin, cerebral ischemia, CA1 hippocampal region, c-Fos

Transient cerebral ischemia/recirculation induces alteration of the neurochemical conditions in the vulnerable regions of the brain such as the hippocampus and striatum. It was repeatedly documented that neurons located in CA1 region of the hippocampal formation respond to an ischemic injury in a characteristic way, whereas other hippocampal neurons are relatively resistant and can survive a longer ischemic period (Kirino et al. 1982; Mudrich and Baimbridge 1989; Bonnekoh et al. 1992; Burda and Chavko 1991b; Burda et al. 1994; Čížková et al. 1996).

Delayed neuronal death has attracted much interest in an effort to elucidate the pathogenetic mechanisms of brain function changes in patients suffering from cerebrovascular diseases.

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Hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase is the rate-limiting enzyme of the mevalonate pathway for cholesterol biosynthesis (Reinoso et al. 2002). Statins via their structural homology (Istvan and Dreisenhofer 2001) to HMG-CoA reductase can inhibit its activity. Recent studies have shown that statins, which are the most widely used cholesterol-lowering drugs, significantly reduce the incidence of ischemic stroke in patients with or without high serum cholesterol levels (Crouse et al. 1998; Van Mil et al. 2000). It is likely that statins have pleiotropic effects and they are beneficial in brain ischemia (Faggiotto et al. 1999). Simvastatin is a lipophilic statin which penetrates into endothelial cells and has high blood-brain barrier (BBB) permeability (Johnson-Anuna et al. 2005). Statin therapy may reduce stroke by ameliorating precerebral atherosclerosis in the carotid artery and the aorta (Vaughan and Delanty 1999; Hess et al. 2000; Takemoto et al. 2001). In addition to delaying atherosclerosis, there is emerging evidence indicating that statins have beneficial effects on cerebral vessels (Van Mil et al. 2000). These drugs possess anti-inflammatory and anti-thrombotic activity in blood and plaques (Delanty and Vaughan 1997; Kwak et al. 2000) and they reduce vascular inflammatory responses (Kwak et al. 2000), modulate cytokine production (Pahan et al. 1997), promote angiogenesis (Kureishi et al. 2000) and decrease oxidative stress (Aviram et al. 1998). Apart from the mentioned secondary preventive effects, there are many evidences of a neuroprotective effect, as well (Daimon et al. 2004; Berger et al. 2007; Jonson-Anuna et al. 2007). Until now, the mechanism underlying statin-induced neuroprotection has been poorly understood. One possibility of their action may be via *c-fos* inhibition.

The *c-fos* and *c-jun* genes belong to the immediate early genes known to have rapid but brief responses. Products of these genes, proteins c-Fos and c-Jun form a dimer known as activator protein AP-1 which acts as transcriptional factor. The dimers bind to a specific DNA-TGAGTCA sequence of promoters and enhancers of many genes (Cox and Sinclair 1997). Examples of genes that contain AP-1 binding sites include neurotrophins, proenkephalin, glial fibrillary acidic protein, neuropeptide Y, vasoactive intestinal peptide, and tyrosine hydroxylase (Sharp et al. 1994). Some of them play an important role in neurodegenerative processes following brain ischemia.

The aim of our study was to determine the possible effect of simvastatin on the *c-fos* gene activity and its relation to delayed neuronal death following transient forebrain ischemia in the adult rat hippocampus.

Materials and Methods

Experimental animals

Male Wistar albino rats, with a body weight ranging from 240 to 280 g, were used in this study. The animals were divided into three groups. Group A (negative control) contained 5 sham-operated animals. Group B (positive control) consisted of 6 ischemised rats without statin pre-treatment and group C consisted of 6 ischemised rats with statin pre-treatment. We used simvastatin at the dose of 20 mg/kg during 14 days prior to the ischemic attack. The simvastatin was administered per os. The experiments were approved by the Animal Care Committee of the Slovak Republic and European Union.

After pre-treatment, the animals were anaesthetized with a mixture of 1.5% halotane, 30% oxygen, 70% nitrous oxide and subjected to 15 minutes of forebrain ischemia. Transient forebrain ischemia was induced by the four-vessel occlusion as reported in detail in the procedure of Pulsinelli (1988). The body temperature was maintained at 37 °C during the ischemia as well as during the early post-ischemic period. Only animals showing restless behaviour were involved in this study.

After the appropriate survival times 2.5 and 72 h (reperfusion period) rats were anaesthetized and transcardially perfused with saline, followed by 4% paraformaldehyde in phosphate buffer (pH = 7.4) as a fixative. Immediately after that the brains were removed from the skull and postfixed in the same fixative solution for 24 h and then prepared for immunostaining and haematoxylin/eosin staining.

Two and a half h reperfusion was used for c-Fos activity detection and 72 h reperfusion for the determination of surviving neurons.

Preparation for c-Fos immunostaining

After fixation, tissues were cryoprotected in solutions containing 10, 20, and 30% sucrose at 4 °C for the period of 48 - 72 h. Sagittal frozen sections (40 µm) were prepared and collected in PBS (0.9% NaCl in 0.1 M phosphate

buffer, pH 7.4). Immunohistochemistry was performed using standard procedures. In order to block peroxidase activity, free-floating sections were first incubated (30 min at room temperature) in PBS containing 0.3% H_2O_2. The sections were rinsed twice in PBS. To prevent non-specific binding sites, the sections were incubated for 3 h in 0.1 PBS containing 1% bovine albumin, 3% normal goat serum and 0.2% Triton X-100. The sections were then incubated in polyclonal Fos rabbit antibody (C-fos Ab-5 PC 38, Calbiochem, diluted 1 : 7000) for 24 h at 4 °C. This primary antibody recognises both Fos and Fos-related antigens. The sections were then rinsed twice in PBS and incubated for 2 h in biotinylated goat-anti rabbit antiserum (BA 100, Vector Laboratories, CA, diluted 1 : 600) in PBS and after incubation washed twice. Finally, sections were incubated in avidin-biotin-peroxidase complex (1 : 100, 1 h) (Peroxidase Vectastain Elite ABC kit VC-PK 6100, Vector Laboratories, CA). After two washes in PBS and one in Tris-HCl saline, the antigen-antibody complexes were visualised by 3, 3´-diaminobenzidine (DAB, Sigma, St. Louis, MO, USA) chromogene reaction. Finally, after two washes in distilled water, sections were mounted on gelatine-coated slides, then air-dried, dehydrated, cleared, and cover-slipped in Canadian balsam.

The number of c-Fos positive nuclei was counted in a 0.025 mm^2 area of the stratum pyramidale of the CA1 hippocampus region using rectangular grids placed randomly on the investigated area under an Olympus microscope at a magnification of × 400. Data from six sections from both sides were averaged for each animal (mean ± S.E.M.).

Haematoxylin/eosin staining

The brains were embedded in paraffin. Ten µm thick sections were de-paraffined and processed by haematoxylin/eosin staining using Mayer haematoxylin for 10 min followed by eosin for 3 min. Finally, the sections were dehydrated and mounted in Canadian balsam.

The number of normal appearing neurons was counted in a 0.025 mm^2 area of the stratum pyramidale of the CA1 hippocampus region using an Olympus microscope at × 400 magnifications. Normal neurons were defined as showing a distinct nucleus and nucleolus.

Statistical analysis

The c-Fos positive cell density and surviving neuronal cell density stained by haematoxylin/eosin were expressed as the mean value ± the standard error of the mean (S.E.M.). Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by post hoc Dunnett’s test using the Instat software. The P value of < 0.05 was considered as statistically significant.

Results

The average neuronal density of the CA1 hippocampal region in the sham-operated rats was 47.03 ± 3.09/0.025 mm^2. In the untreated ischemic group, neuronal density was 9.05 ± 2.46/0.025 mm^2. This result showed that only less than 20% of neurons in the CA1 hippocampal region survived the ischemic insult in non-treated animals. However, in the pre-treated ischemic group, the average neuronal density was 16.45 ± 2.78/0.025 mm^2, which indicates that more than 35% of the neurons in the CA1 pyramidal cell layer survived the ischemic attack.

A significant neuroprotective effect was seen in the pre-treated ischemic group ($P < 0.001$) in comparison to the ischemic group without pre-treatment.

The average of c-Fos positive nuclei density of the CA1 region in the sham-operated rats was 0.27 ± 0.07/0.025 mm^2 (Plate IV, Fig. 1A). In the untreated ischemic group, neuronal density was 28.2 ± 2.05/0.025 mm^2 (Fig. 1B). However, in the pre-treated ischemic group, the average of c-Fos positive nuclei density was 30.3 ± 4.82/0.025 mm^2 (Plate V, Fig. 1C), which is slightly more than the number of activated neurons in the CA1 pyramidal cell layer after the ischemic attack without pre-treatment.

A highly significant difference ($P < 0.001$) was found between the sham-operated and both ischemic groups (with and without pre-treatment) in c-Fos positivity. No significant difference in c-Fos positivity was observed between untreated ischemic and pre-treated ischemic groups ($P > 0.05$).

Representative microphotographs of sections immunohistologically stained for c-Fos positivity of groups A, B, and C are shown in Figs 1A, 1B and 1C respectively.

Discussion

Our present study is one of the numerous works concerning the *c-fos* expression in response to cerebral ischemia. However, this is the first study focused on the influence of simvastatin on the *c-fos* expression in the CA1 hippocampal region.

Under normal conditions, the *c-fos* expression in the central nervous system is very low, especially in the hippocampus (Herdegen et al. 1995). The ischemic insult used in our study (four-vessel occlusion) represents one of the most common stressful stimuli. We found that this stimulus induced very high up-regulation of the *c-fos* expression in the CA1 region. This finding is in agreement with data published by Nyitrai et al. (2005). However, some studies reported only a moderate *c-fos* expression in the studied area (Takemoto et al. 1995; Némethová et al. 2005). Takemoto et al. (1995) and Némethová et al. (2005) used shorter time (10 min) of forebrain ischemia than was used in our study (15 min), and therefore they could observe a lower c-*fos* expression in the CA1 region.

It is well known that different types of statins differ in their properties and in their ability to reduce brain injury caused by an ischemic insult (Endres 2005). Most studies analysed the effect of pravastatin (Plehn et al. 1999; White et al. 2000; Sterzer et al. 2001; Daimon et al. 2004; ten Dam et al. 2005; Tseng et al. 2005; Trinkl et al. 2006), although hydrophilic pravastatin penetrates only weakly into endothelial cells and has low blood-brain barrier permeability. We focused on simvastatin, one of the lipophilic statins with higher permeability and found that two weeks of pre-treatment with simvastatin followed by 15 min long forebrain ischemia in rats significantly reduced delayed cell death in the CA1 hippocampal region. These results are in agreement with a recent study that reported an effective neuroprotective action of statins after an acute brain injury (Cimino et al. 2005). Our present study provides further experimental support to the hypothesis that statins have a significant neuroprotective effect. Our results are consistent with those of Endres et al. (1998), demonstrating that simvastatin has a neuroprotective effect in adult mice after middle cerebral artery occlusion and reperfusion. It suggests that a prophylactic treatment with this drug might also be useful in the prevention of ischemic strokes in general.

Surprisingly, we found a non-significant increase in the *c-fos* expression among simvastatin pre-treated rats in comparison with non-treated ischemic animals; we expected a reduction of the *c-fos* expression. Our expectation was based on studies dealing with neuroprotection trough preconditioning (a short ischemia preceding a long one), that reported quite a high decrease of the *c-fos* expression after preconditioning (Yoneda et al. 1998; Truettner et al. 2002; Nyitrai et al. 2005). Johnson-Anuna et al. (2005) also published that simvastatin treatment reduced the *c-fos* expression in the mouse brain.

However, the function of c-Fos in neurodegeneration or neuroprotection is controversial. According to some articles, c-Fos plays an important role in the stimulation of neuroprotective mechanisms (Cho et al. 2001; Jiang et al. 2001; Kawahara et al. 2004), whereas other studies emphasize its role in neurodegenerative processes (Zablocka et al. 2003). The dentate gyrus is quite a resistant area to the transient brain ischemia, although the *c-fos* expression following ischemia is very high. On the other hand, the vulnerable CA1 region expresses high *c-fos* activation as well. In spite of these findings, most cells of the dentate gyrus survive the ischemic insult and pyramidal neurons of CA1 hippocampal region die after ischemia. Based on these data, we assume that the *c-fos* expression may have different repercussion in different cell lines, or the effect of final AP 1 protein can be modulated in different cell lines in different ways.

Our results support previous evidence that simvastatin reduces neuronal death after an acute brain insult and has a neuroprotective effect. Moreover, they suggest that simvastatin probably can not reduce neuronal death in the CA1 hippocampus region through the pathway involving the *c-fos* gene. Based on literature data and our findings, we can conclude that simvastatin neuroprotection may be mediated by multiple mechanisms as can be expected from its pleiotropic effects. More studies are needed for a better understanding of possible mechanisms of the role of simvastatin during brain ischemia.

Ovplyvňujú statíny aktivitu génu *c-fos* v hipokampe u dospelých potkanov po prechodnej mozgovej ischémii?

Inhibítory 3-hydroxy-3-metylglutaryl koenzým A reduktázy (statíny) sú asociované s prevenciou mozgovej príhody. Zdá sa, že táto prevencia nesúvisí s ich schopnosťou znižovať hladinu cholesterolu. Doteraz bolo postulovaných mnoho mechanizmov na vysvetlenie prevenčného účinoku statínov. Cieľom našej práce bolo skúmať vplyv simvastatínu na aktivitu génu *c-fos* a jeho vzťah ku predĺženej neurónovej smrti v CA1 oblasti hipokampu u dospelých potkanov po prechodnej mozgovej ischémii.

V našej práci sme sledovali 17 potkaních samcov kmeňa Wistar albino. Zvieratá boli rozdelené do troch skupín: prvú skupinu tvorilo 5 slepo operovaných zvierat; druhú 6 zvierat ischemizovaných bez predchádzajúceho podávania statínov a tretiu 6 ischemizovaných zvierat, ktorým bol podávaný statín. Poslednej skupine sme v priebehu 14 dní pred ischemickým atakom podávali simvastatín v dávke 20 mg/kg. Prechodnú mozgovú ischémiu trvajúcu 15 minút sme uskutočnili oklúziou štyroch ciev. Imunohistologickú detekciou c-Fos proteínu sme robili po 2,5 hodinovej reperfúzii a po 72 hodinovej reperfúzii sme stanovovali počet prežívajúcich neurónov použitím hematoxylín/eozínového farbenia.

Zistili sme, že priemerný počet neurónov v CA1 oblasti hipokampu bol u slepo operovaných zvierat $47.03 \pm 3.09/0{,}025\ mm^2$, u zvierat ischemizovaných bez predchádzajúceho podávania statínov $9.05 \pm 2.46/0{,}025\ mm^2$ a u ischemizovaných zvierat, ktorým bol podávaný statín $16.45 \pm 2.78/025\ mm^2$. Štatisticky významný neuroprotektívny účinok bol zistený v skupine ischemizovaných zvierat, ktorým bol podávaný statín ($P < 0{,}001$) v porovnaní so skupinou slepo operovaných zvierat a ischemizovanou skupinou bez predchádzajúceho podávania statínu. Ďalej sme zistili, že priemerný počet c-Fos pozitívnych jadier v CA1 oblasti hipokampu bol u slepo operovaných potkanov $0.266 \pm 0.074/025\ mm^2$, u zvierat ischemizovaných bez predchádzajúceho podávania statínov $28.2 \pm 2.053/025\ mm^2$ a u ischemizovaných zvierat, ktorým bol podávaný statín $30.3 \pm 4.816/025\ mm^2$. Významný rozdiel v c-Fos pozitivite ($P < 0{,}001$) bol zistený medzi skupinou slepo operovaných zvierat a oboma ischemickými skupinami (s podávaním aj bez podávania simvastatínu). Významný rozdiel v c-Fos pozitivite medzi skupinou zvierat ischemizovaných bez predchádzajúceho podávania statínov a skupinou ischemizovaných zvierat, ktorým bol podávaný statín nebol zistený ($P > 0{,}05$).

Tieto nálezy naznačujú, že simvastatín chráni CA1 oblasť pred hypoxickým neurónovým poškodením, nezávisle na aktivácii *c-fos* génu. Môžeme usudzovať, že neuroprotekcia simvastatínu môže byť sprostredkovaná viacerými mechanizmami ako sa predpokladá na základe jeho pleiotropického účinku.

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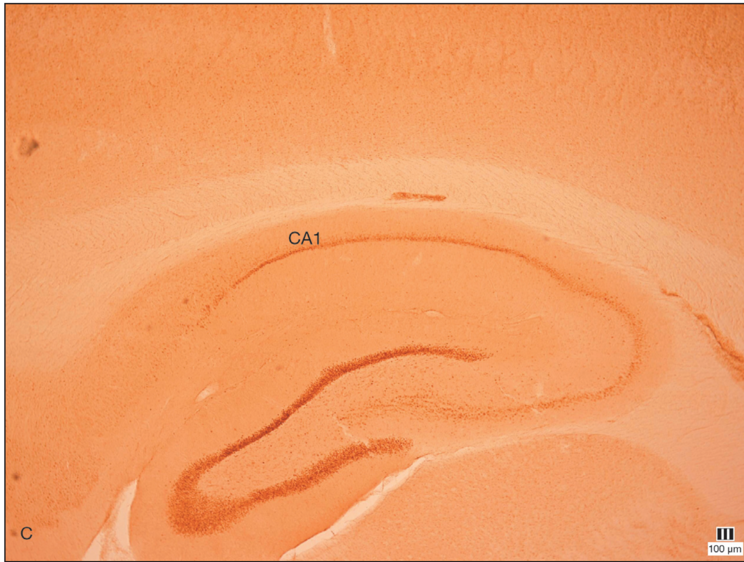
Plate V



C- ischemised group with simvastatin pre-treatment at the dose of 20 mg/kg for 14 days