

## Effect of Tanakan on Postischemic Activity of Protein Synthesis Machinery in the Rat Brain

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**Abstract.** A growing body of evidence supports the role of free radicals in triggering the functional and metabolic disturbances following transient cerebral ischemia. This study was designed to evaluate whether the extent of reperfusion-induced inhibition of protein synthesis initiation as well as tissue injury can be reduced by Tanakan (Ginkgo biloba extract, EGb 761) (Beaufour-Ipsen Industrie). Rats received Tanakan in the dose of 40 mg/kg/day for 7 days before surgical intervention. Transient forebrain ischemia was induced by 4-vessel occlusion. Rats were subjected to 20 min of ischemia followed by 30 min, 4 h or 7 days of reperfusion. Protein synthesis rate, reinitiation ability and neurodegeneration in the frontal cortex and hippocampus were measured by the incorporation of radioactively labelled leucine into polypeptide chains in postmitochondrial supernatants and by Fluoro-Jade B staining.

The protective effect was observed, concerning both the protein synthesis and the number of surviving neurons, in the Tanakan-treated groups. Tanakan significantly reduced the ischemia/reperfusion-induced inhibition of translation in the neocortex as well as in the highly sensitive hippocampus. Our results indicate that free radicals play an important role in the development of reperfusion-induced injury, and the treatment of ischemic and reperfused brain with free radical scavengers may reduce the severity of reperfusion damage.

**Key words:** Ischemia — Protein synthesis — Ginkgo biloba — Hippocampus

### Introduction

Protein synthesis requires a number of precise steps as well as activity of many enzymes and, because of its complexity is extremely sensitive to the changes of cell energy charge and ionic concentrations. Although ischemia *per se* produces relatively moderate changes which are dependent on the model and duration of

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ischemia and intraschemic temperature, it generates background for profound protein synthesis alterations occurring during subsequent reperfusion. First minutes of postischemic reperfusion are characterized by nearly complete block of translation, focused mainly on the inhibition of initiation, which is manifested by disaggregation of polyribosomes to monosomes and ribosomal subunits (Kleihues et al. 1975; Cooper et al. 1977; Burda et al. 1980). After brain-wide transient inhibition of initiation, most brain regions recover their protein synthesis capability; however, in the selectively vulnerable regions, the inhibition of protein synthesis is persistent (Thilmann et al. 1986; Widmann et al. 1991; Kato et al. 1995). Deep inhibition of translation occurring immediately after the blood flow restoration to ischemic brain tissue seems to be caused mainly by the phosphorylation of  $\alpha$  subunit (38 kDa) of eukaryotic initiation factor 2 (eIF-2) (Burda et al. 1994; DeGracia et al. 1996).

The Tanakan (Ginkgo biloba extract, EGb 761) is a standardized mixture of active substances, including 24% flavonoid glycosides and 6% terpenoids (Drieu 1986), obtained from green leaves of the Ginkgo biloba tree. Therapeutic effects of Ginkgo biloba extracts are known from herbalists and its marked antioxidant and antiradical abilities have been described by modern science. Tanakan is a polyvalent agent capable of scavenging free radicals (Marcocci et al. 1994a,b), reducing  $\text{Ca}^{2+}$ -stimulated intracellular events (Oyama et al. 1993, 1994) and modulating intracellular signal transduction events.

In our experiment we tried to explore antioxidant capabilities of Tanakan to support our previous results indicating that acute postischemic inhibition of protein synthesis is caused by a burst of free oxygen radical production during the first minutes of reperfusion. The impact of this translational inhibition lies in the fact that this inhibition is practically irreversible in the selectively vulnerable neuronal populations of the brain (Thilmann et al. 1986; Widmann et al. 1991). This means that, in these neuronal populations, protein synthesis remains inhibited until the delayed death of neurons occurring three days after ischemic attack.

## Materials and Methods

Male Wistar rats weighing 300–400 g were used. Surgical intervention, approved by Ethics Committee of the Neurobiological Institute of the Slovak Academy of Sciences, was performed using the standard model of 4-vessel occlusion (Pulsinelli and Brierley 1979) in the Schmidt-Kastner's modification (Schmidt-Kastner et al. 1989). Briefly, on day 1, after anaesthesia with chloralhydrate (300 mg/kg i.p.), blood flow in both vertebral arteries was irreversibly interrupted by electrocauterization through the alar foramina of the first cervical vertebra. On day 2, both common carotid arteries were occluded for 20 min by atraumatic clips under anaesthesia with 2.5% of halothane. Two minutes before carotid occlusion, halothane was removed from the mixture. Normothermic conditions (37°C) were maintained using a homeothermic blanket. Sham control animals were prepared in the same way

without carotid occlusion. Tanakan was applied orally in the dose of 40 mg/kg/day for 7 days before surgical intervention.

Fresh cortex and hippocampus obtained under the different experimental conditions were dissected and homogenized 1:2 with following buffer: 50 mmol/l HEPES-Tris, pH 7.55; 140 mmol/l potassium acetate; 4 mmol/l magnesium acetate, 2.5 mmol/l dithiothreitol, 0.32 mol/l sucrose. Postmitochondrial supernatant (PMS) was obtained by centrifugation at  $11,000 \times g$  for 15 min. All procedures were performed at 4°C and the different subcellular fractions were stored at -70°C until used.

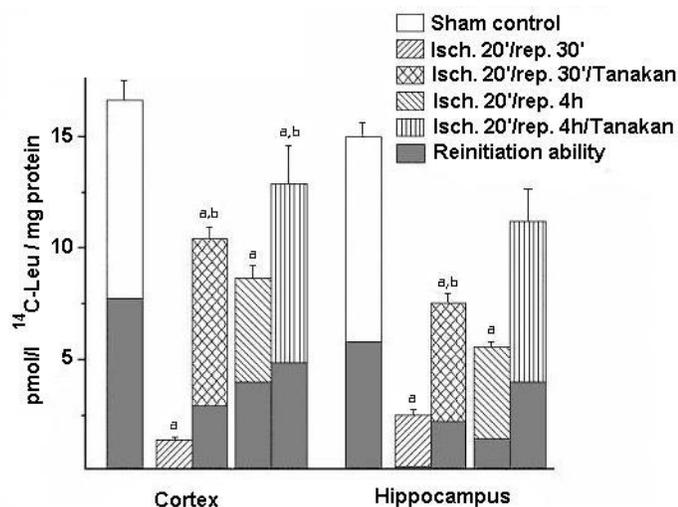
The complete reaction system contained, in the final volume of 50  $\mu$ l: 50 mmol/l HEPES-Tris, pH 7.55; 140 mmol/l potassium acetate; 4 mmol/l magnesium acetate; 2.5 mmol/l dithiothreitol; 0.32 mol/l sucrose; 1 mmol/l ATP; 0.75 mmol/l GTP; 20 mmol/l phosphocreatine; 150  $\mu$ g/ml creatine phosphokinase; 50  $\mu$ mol/l aminoacids; 100  $\mu$ g of PMS proteins and 5  $\mu$ Ci of L-[4,5-<sup>3</sup>H]-leucine (25  $\mu$ mol/l, 66 Ci/mmol). The reactions were carried out at 30°C for 45 min. TCA insoluble radioactivity was measured according to the method of Mans and Novelli (1961). Reinitiation ability was calculated as the difference between total leucine incorporated and value of elongation obtained by incubation of samples in the presence of 60  $\mu$ mol/l aurointricarboxylic acid.

For histological analysis, after recirculation periods, the rats were deeply anesthetized by chloralhydrate (300 mg *per* kg of body weight) and perfused transcardially with saline followed by freshly prepared 4% paraformaldehyde in 0.1 mol/l phosphate buffer, pH = 7.4. Following perfusion fixation, the brain was carefully dissected out and stored in same fixative for 3 h at 4°C. After postfixation, the brains were cut on a vibratome at 30  $\mu$ m and the sections were mounted onto gelatin-coated slides and air-dried overnight. To examine histological change in neurons, Fluoro-Jade B and cresyl-violet staining was performed. The slides were coverslipped with DPX. The slides were examined using an Olympus BX 51 fluorescent microscope with digital camera DP 50. Image analysis was performed by Image tool software (UTHSCSA, San Antonio, USA). Numbers of CA1 neurons was counted in the middle of the linear part of CA1 and expressed *per* 1 mm of the hippocampal CA1 region and 1 mm<sup>2</sup> of parietal cortex.

Statistical analysis of the differences between sham control animals and animals with Tanakan was performed using ANOVA followed by Dunnet's test.

## Results

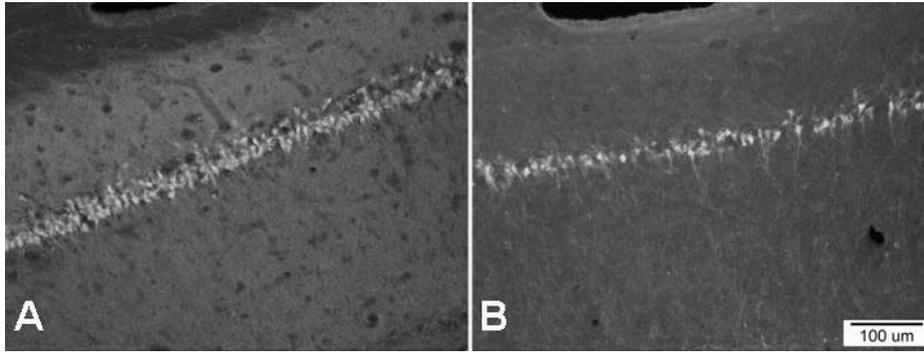
Protein synthesis rate measured *in vitro* in brain extracts (Fig. 1) clearly demonstrated known protein synthesis inhibition, which appeared 30 min after 20 min of ischemia. The extent of the ability of Tanakan to prevent this inhibition is surprising, especially at 20 min of ischemia with subsequent 30 min of reperfusion. Without Tanakan administration, these experimental conditions lead to 91% inhibition in the cortex and 83% inhibition in the hippocampus. With Tanakan, these values represent only 35 and 49.5% (both significantly improved,  $p < 0.05$ ). The



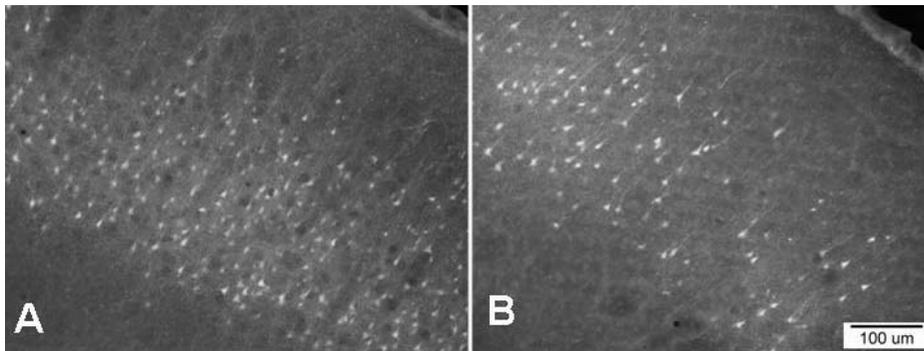
**Figure 1.** Incorporation of labelled leucine (Leu) into polypeptide chains in cell-free system from cortex and hippocampus of rats after ischemia and subsequent reperfusion with and without Tanakan administration. Expressed as pmol/l of Leu incorporated into TCA insoluble polypeptides  $\pm$  S.E.M. a, significant difference in comparison to sham control group ( $p < 0.05$ ); b, significant difference in comparison to Tanakan-treated group ( $p < 0.05$ ).

positive effect of Tanakan is even more obvious in the case of reinitiation capability which, without Tanakan pre-treatment, is practically lost after half an hour of postischemic reperfusion. Tanakan protects reinitiation ability; in our findings the values of reinitiation reach more than 30% in both monitored brain regions and stay improved during the subsequent progress of reperfusion. Very interesting is the recovery of translation in the hippocampus 4 h after ischemia. Without Tanakan pre-treatment, translation in the hippocampus is very poor at 34.25% of sham control animals (SHC) value but with Tanakan it reaches 74.63% of control value.

The protective effect of Tanakan, significantly shortening postischemic recovery of protein synthesis, leads to an increased number of surviving neurons in both monitored brain regions including the selectively vulnerable hippocampal CA1 after 7 days of reperfusion. Fig. 2 is a representative microphotography of the hippocampus. Quantification of degenerating neurons in the centre of the linear part of the CA1 region visualised by Fluoro-Jade B staining shows a significant ( $p < 0.05$ ) difference between groups with and without Tanakan administration. The number of degenerating CA1 neurons after 20 min of ischemia without Tanakan is  $364 \pm 9.9$  cells/mm (Fig. 2A) while in the group with Tanakan (Fig. 2B) it is  $129 \pm 33.8$  cells/mm. Similarly in the cortex, the administration of Tanakan reduced the count



**Figure 2.** Ischemia/reperfusion-induced neurodegeneration of hippocampal CA1 neurons, highly visible because of its white colour, 7 days after 20 min of ischemia without (A) and with Tanakan (B). Fluorescence staining by Fluoro-Jade B, magnification  $\times 200$ .



**Figure 3.** Ischemia/reperfusion-induced neurodegeneration of neurons in parietal cortex, highly visible because of its white colour, 7 days after 20 min of ischemia without (A) and with Tanakan (B). Fluorescence staining by Fluoro-Jade B, magnification  $\times 200$ .

of degenerating neurons in the group without treatment from  $251 \pm 13.1$  cells/mm<sup>2</sup> (Fig. 3A) to  $143 \pm 56.0$  neurons/mm<sup>2</sup> (Fig. 3B).

## Discussion

Only a few experiments have been carried out with the intention of influencing postischemic protein synthesis by therapeutic intervention. Thilmann (1988) used cycloheximide to prevent the synthesis of proteins which are selectively expressed during the early postischemic recirculation period and which may trigger the pathological process leading to irreversible injury. As a result, protein synthesis uniformly recovered and morphological lesions were absent. However, the temperature drop,

which is induced by the drug, could have caused this effect and it cannot be excluded that ischemic injury did not reach the threshold of neuronal vulnerability. In the other study gerbils were treated after 5 min of global ischemia with barbiturates, which have previously been shown to prevent morphological lesion in hippocampal CA1 sector (Hallmayer et al. 1985). As a result, hippocampal protein synthesis was normal after two days of recirculation. Two hours after ischemia, however, the inhibition of protein synthesis was equal to or even more pronounced than in untreated animals. Barbiturate therapy, in summary, promoted the recovery of protein synthesis but did not prevent the initial postischemic disturbance (Hossmann and Paschen 1992).

The main reason of postischemic translation inhibition is  $\alpha$  subunit phosphorylation of eIF-2 (Burda et al. 1994). Phosphorylation is caused by transient decrease in activity of the protein phosphatases PP-1 and PP-2B (Martin de la Vega et al. 2001) as well as activation of the eukaryotic initiation factor 2alpha kinase, PERK (Kumar et al. 2001).

Antioxidant therapy has been shown to be beneficial in neurological disorders including Alzheimer's disease and cerebral ischemia. Our previous data document that postischemic changes in translation are mostly caused by severe accumulation of free oxygen radicals in the first minutes of reperfusion. A method of controlled postischemic reoxygenation used to prevent or decrease the formation of free oxygen radicals resulted in significant amelioration of the postischemic inhibition of protein synthesis (Burda et al. 1991). The same effect resulted from short postischemic hypoperfusion (Burda et al. 1995) and free oxygen radical scavenger stobadine administration (Burda and Némethová 1999).

The pharmacological study of Tanakan extract has required numerous experiments over several years. Different pathological models of cerebral ischemia were used to evaluate its effects, and measurements at both cellular and molecular levels to determine its mechanisms of action. In experimental models of ischemia, oedema and hypoxia, Tanakan reduced vascular, tissular and metabolic disturbances as well as their neurological behavioural consequences. Tanakan could prevent and treat acute cerebral ischemia. The effectiveness was more satisfactory when it was used preventively (Peng et al. 2003). Tanakan performs important neuroprotective actions, which depend on a direct effect on the neuronal cells and an indirect effect on the brain circulation. According to some authors, the neuroprotective effect of Tanakan may actually be correlated with its effect on glucocorticoid synthesis – ginkgolides A and B inhibit corticosteroid synthesis and restore the ability to adapt to stress (Amri et al. 2002). This extract is able to reduce lipid peroxide and phospholipids contents in the rat brain. These effects could be explained on the basis of antioxidant properties of Tanakan and could mediate its beneficial role in the protection against post-ischemic injury. (Seif-El-Nasr and El-Fattah 1995). Another mechanism of Tanakan neuroprotection involves keeping the balance of inhibitory/excitatory amino acids (Hu et al. 2002), platelet-activating factor receptor antagonism (Zagrean et al. 1998; Haines et al. 2000), ability to inhibit NO-stimulated protein kinase C activity (Bastianetto et al. 2000), protects against

ischemia induced changes of Na,K-ATPase activity (Pierr et al. 2002). Finally, Tanakan protects the neurons against glutamate excitotoxicity (Chandrasekaran et al. 2002), and against apoptosis (cell death) induced by  $\beta$ -amyloid protein, a known pathogenetic factor in pathological brain ageing (Yao et al. 2001).

As we hypothesized, on the basis of our results indicating that Tanakan can scavenge free radicals produced during ischemia/reperfusion and may reduce reperfusion damage in rabbit spinal cord (Mechírová and Domoráková 2002), Tanakan due to its antioxidant and antiradical ability significantly reduced the ischemia/reperfusion-induced inhibition of translation in the neocortex as well as in the highly sensitive hippocampus where, without protection, inhibition of protein synthesis persists up to the death of neurons. Both flavonoid and ginkgolide constituents are involved in the free radical – scavenging and antioxidant effects of Tanakan which decrease tissue levels of reactive oxygen species and inhibit membrane lipid peroxidation. Regarding Tanakan-induced regulation of cerebral glucose utilization, bilobalide increases the respiratory control ratio of mitochondria by protecting against uncoupling of oxidative phosphorylation, thereby increasing ATP levels, a result that is supported by the finding that bilobalide increases the expression of the mitochondrial DNA-encoded COX III subunit of cytochrome oxidase (DeFeudis and Drieu 2000). It is clear that irreversible block of protein synthesis in the selectively vulnerable CA1 field of hippocampus necessarily leads to the death of neurons. However, prevention of persistent inhibition of translation does not assure survival of CA1 neurons (Burda et al. 2003).

Mechanisms allowing neurons to survive, obviously including remodulation of gene expression, are not clear until now. Tanakan by its ability to protect translational machinery permits the newly synthesized mRNAs to be translated into functional proteins, thus allowing the altered gene expression to be effective.

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