Effect of Natural Polyphenols, Pycnogenol® on Superoxide Dismutase and Nitric Oxide Synthase in Diabetic Rats

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Abstract: The work is focused on clarifying the impact of diabetes and natural plant polyphenols contained in Pycnogenol® (PYC) on the activity and synthesis of Cu/Zn-SOD and synthesis of nNOS and eNOS in the cerebellum and cerebral cortex in rats with induced diabetes. Rats included in the study (n=38) were divided into three groups: the controls (C), (n=7), untreated diabetics (D) (n=19) and diabetic rats treated with PYC (DP) (n=12). Diabetes significantly decreased synthesis, as well as the activity of Cu/Zn-SOD in both studied parts of the brain. PYC significantly increased the synthesis of Cu/Zn-SOD but had no effect on its activity. Diabetes also reduced the synthesis of nNOS in cerebral cortex and administered PYC elevated its amount to the level of controls. In the cerebellum, diabetes does not affect the synthesis of nNOS and PYC reduces synthesis of

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nNOS. Diabetes as well as PYC had no influence on the synthesis of eNOS in both, the cerebellum and cerebral cortex. PYC modulated level of Cu/Zn-SOD and nNOS in cerebellum and cerebral cortex of diabetic rats, but in a different way.

Introduction

Diabetes mellitus (DM) is a genetically determined autoimmune disorder of glucose homeostasis. Higher blood concentration of glucose can cause many pathophysiological processes such as protein glycation and glycooxidation, free radicals and advanced glycation end-products (AGEs) formation, lipid peroxidation, protein oxidation, nitrosylation and fragmentation, DNA damage, etc. These processes can influence antioxidant enzymes. For example glycation can decrease activity of some enzymes.

Arai et al. (1987) demonstrated that human red blood cells contain glycosylated and unglycosylated form of Cu/Zn-superoxide dismutase (Cu/Zn-SOD), whereas the percentage of glycosylated forms, which have lower enzyme activity, was significantly increased in erythrocytes of patients with DM compared to healthy erythrocytes. This inactivation could be mainly caused by glycation of lysine units. Reduction of activity is explained by the fragmentation of Cu/Zn-SOD caused by glycation reactions together with participation of free radicals in this process (Oogawara et al., 1992; Yamakura and Kawasaki, 2009). Negative correlation between glycated haemoglobin (a marker of increased concentrations of glucose) in diabetics and activity of Cu/Zn-SOD confirmed the impact of glycation on the activity of this enzyme (Muchová et al., 1999).

In some pathological conditions change of Cu/Zn-SOD expression plays a role too. For example, significantly lower levels of Cu/Zn-SOD in plasma and erythrocytes were found in patients with DM type 2. Superoxide derived from NAD(P)H oxidase avidly reacts with NO and inactivates it in neurons and, thereby, modulates its bioavailability (Campese et al., 2007). On the other hand, superoxide and peroxynitrite anion formed from NO, damage proteins and other substances that can cause degenerative changes in neurons in the brain and spinal cord (Deng et al., 1993). In addition, impairment of tertiary and quaternary structure of the enzyme leads to the formation of so-called “wild” SOD, which exhibits 40–50% activity compared to the physiological form of Cu/Zn-SOD.

On the contrary, increased expression of Cu/Zn-SOD results in H_2O_2 accumulation. From this, the hydroxyl radical is formed and this can lead to the damage of various macromolecules. Increased expression of Cu/Zn-SOD was found, for example, in patients with Down syndrome (Sinet, 1982; Pueschel and Sustrova, 1997; Muchová et al., 2001).

In hippocampus, as a result of diabetes, expression of mRNA for neuronal NO-synthase (nNOS) is reduced and results in a deterioration of cognitive

function (Patil et al., 2006). This reduction of expression is mediated by advanced glycation products (Korenaga et al., 2006).

The level of nitric oxide (NO) plays an important role in physiological as well as some pathological processes of central nervous system (CNS), such as learning and memory formation, diabetic neuropathy, cerebral ischemia and neurodegenerative diseases (Patil et al., 2006). While NO produced by endothelial NO-synthase (eNOS) is involved in the regulation of cerebral circulation, NO produced nNOS has an important role in regulation of brain function (Campese et al., 2007). DM may lead to functional and structural changes in the CNS, which may be caused by microvascular dysfunction, but also by oxidative stress (Patil et al., 2006). In an experimental model of diabetes induced by streptozotocin (STZ) it was found that the nNOS in the brain decreases independently on glucose increase, but this decrease is associated with the absence of insulin and with elevated concentration of advanced glycation end-products (AGEs) (Yu et al., 1999). In consequence, this decreases also bioavailability of NO needed for mentioned processes.

Among substances that could have an influence on the level and activity of these enzymes flavonoids are included. Flavonoids exhibit a wide range of biological effects, such as elevation of the antioxidant capacity via stimulation of antioxidant enzymes (Stefanescu et al., 2001; Chovanová et al., 2006). In addition, they also exert vasodilatation (Ďuračková et al., 2003), antitrombic (Golański et al., 2006), anti-inflammatory (Schäfer et al., 2006) and antiapoptotic effects. Their antimutagenic ability results from the inhibition of the binding of carcinogens to DNA (Križková et al., 2008).

Natural polyphenols may participate in signalling pathways that affect cell function (Dekermedjian et al., 1999; Williams et al., 2004; Spencer, 2005). Flavonoids can selectively interact inside the MAP kinase signalling pathways, which are included in signalling of neuronal survival, regeneration, development and death (Spencer, 2005; Ďuračková, 2010). One of the important substances containing natural polyphenols is Pycnogenol® (PYC). PYC has strong free radical-scavenging activity against reactive oxygen and nitrogen species and a wide range of positive effects *in vitro* and *in vivo* (Rohdewald, 2005).

Our work had two objectives. The first one was to clarify the impact of diabetes on the synthesis and activity of Cu/Zn-SOD and synthesis of both nNOS as well as eNOS in the cerebellum and cerebral cortex, as a representative of insulin independent tissue of diabetic rats. The second objective was to verify the possible effect of natural plant polyphenols contained in PYC on above mentioned parameters in diabetic rats.

Material and Methods

Induction of diabetes in rats

Adult male Wistar rats with a body weight of 312 ± 48 g were housed and cared for in accordance with the guidelines of the International Guiding Principles

for Biomedical Research Involving Animals of the Council for International Organizations of Medical Sciences (CIOMS, 1983), which concurred with the principles of respect for life. Rats were allowed a standard laboratory pellet chow and free access to water. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication number 85-23, revised 1985), as well as with the guidelines formulated by the European Community for the Use of Experimental Animals (L358-86/609/EEC). Diabetes was induced by an intravenous injection of streptozotocin (STZ) (Sigma-Aldrich, Germany) at a dose of 45 mg/kg body weight. STZ was dissolved in 0.1 mol/l citrate buffer (pH 4.5) and injected into the tail vein. The control animals received an equal amount of a citrate buffer. Diagnosis of diabetes was made by monitoring glucose concentration in blood from tail vein. The rats were classified as diabetic and included in further study when their blood glucose was higher than 15 mmol/l. Body weight was determined prior to and then every 7 days after STZ administration until the termination of the experiment. Rats were not treated with insulin or any other hypoglycaemic agent.

Study protocol

The animals (38 subjects) we randomly divided into three groups: controls (C) (7 subjects), untreated diabetics (D) (12 subjects) and diabetics treated with PYC at a dose of 5 mg/kg/day (DP) (12 subjects). The duration of animal treatment was 7 weeks. After that time the animals we sacrificed by the intramuscular administration of ketamine (100 mg/kg of body weight) and xylazine (15 mg/kg of body weight). Consequently we took away tissues from cerebellum a cerebral cortex and immediately we froze them by liquid nitrogen and stored them at –80 °C.

Characterisation of tested substance Pycnogenol®

Pycnogenol® (Horphag Research Ltd., UK) is standardized extract from the bark of the French maritime pine (*Pinus pinaster*), which grows in Les Landes in southwest France. It is a concentrate of polyphenols consisting of procyanidins, catechin, taxifoline and phenolic acids (Rohdewald, 2005).

Tissue homogenisation

Thawed tissue samples we divided into aliquots with the weight of cca 0.3 g. We added homogenization medium (0.01 mol/l phosphate buffer with 0.2 mmol/l EDTA, 0.14 mol/l NaCl, pH 7.4) to the samples. We homogenised the tissue on the ice by electric blender 6 times. Subsequently we centrifuged homogenates 20 min at 4 °C and 2,000 *g*. Removed supernatant we centrifuged an hour at 4 °C and 12,000 *g*. We stored homogenates at –80 °C until analysis.

Determination of proteins concentration
We determined protein concentration spectrophotometrically at wavelength 280 nm (PharmaSpec UV-1700 Shimadzu). Bovine Serum Albumine (20 mg/ml) we used as a standard. Concentration of protein we expressed as mg/ml.

Western blot analysis
Samples, standards and molecular weight marker we mixed with the loading buffer (1:1) and loaded into the SDS-PAGE gel. We conducted electrophoresis at a constant voltage of 80 V. After about 15 min when the sample reached the separation gel, we increased the voltage to 100 V and we conducted electrophoresis while the loading buffer reached the end of the gel (1 to 1.5 hour). After electrophoretic division of proteins, we passed "blotting sandwich". We transferred proteins to the membrane by wet transfer at a constant voltage of 60 V and we let the transmission run 3 h in the cold. After transfer we blocked membrane overnight in blocking solution at 4 °C. Next we incubated membrane with primary anti-Cu/Zn-SOD (anti-eNOS or anti-nNOS) (Sigma-Aldrich, Germany) rabbit antibody for 1 hour. Subsequently, we incubated membrane with the secondary mouse antirabbit antibody conjugated with horseradish peroxidase (Sigma-Aldrich, Germany) for 1 hour. We visualized the bends by chemiluminescence and their density was measured densitometrically using program FotoCaptMw. The amount of antioxidant enzyme protein was expressed as optical density.

Determination of Cu/Zn-SOD activity
To determine the activity of Cu/Zn-SOD, we used the commercial kit for SOD (Fluka, Germany), using bovine Cu/Zn-SOD (Sigma, Germany) as a standard. This method employs xanthine oxidase to generate superoxide radical from xanthine and oxygen, which reduce Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfofenyl)-2H-tetrazolium, monosodium salt) to form a yellow water-soluble formazan dye absorbing at 505 nm. At the presence of superoxide dismutase the reduction of WST-1 is decreased. 1 U of SOD activity is defined as the enzyme activity causing 50% inhibition of reduction of WST-1 under the assay conditions. SOD activity was expressed in U SOD/mg proteins.

Statistic evaluation of results
For statistical evaluation of the results we used the program StatsDirect® 2.3.7 (Stats Direct Sales, Sale, Cheshire M33 3UY, UK). Nonparametric results from Western blot analysis, we evaluated by Wilcoxon's test. We evaluated results of measurements of Cu/Zn-SOD activity using Student's unpaired *t*-test.

Results

In the cerebellum of diabetic rats, we observed reduction in levels of expressed protein Cu/Zn-SOD by about 30% compared to control rats ($p<0.01$). In the group of diabetic rats treated with PYC, we have seen an increase in levels of protein by about 40% compared to rats without PYC treatment ($p<0.01$).

In the cerebral cortex of diabetic rats, we observed similar results. The level of expressed protein Cu/Zn-SOD in the cerebral cortex of diabetic rats is reduced by about 20%. PYC administration significantly increased the level of Cu/Zn-SOD by about 30% compared to rats, which were not treated with PYC (Figure 1).

In the case of activity of Cu/Zn-SOD, we also monitored a significant reduction in diabetic rats compared with controls and not significant increase of Cu/Zn-SOD activity after PYC administration compared with rats without PYC treatment in the cerebellum, as well as in the cerebral cortex.

Concerning the nNOS exprimed protein levels in the cerebellum, we observed no significant change between diabetic and control group. PYC administration significantly increased levels of nNOS in cerebral cortex compared to both diabetic ($p<0.001$) and control ($p<0.001$) group. PYC significantly reduced levels of nNOS

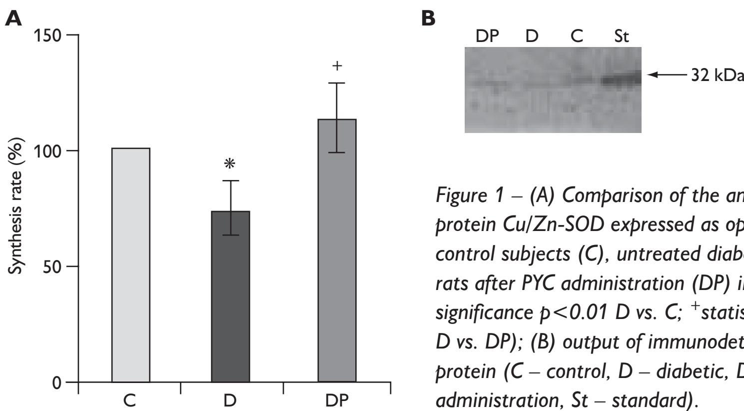

*Figure 1 – (A) Comparison of the amount of expressed protein Cu/Zn-SOD expressed as optical density (%) among control subjects (C), untreated diabetic rats (D) and diabetic rats after PYC administration (DP) in cerebellum (*statistical significance $p<0.01$ D vs. C; $^{+}$statistical significance $p<0.01$ D vs. DP); (B) output of immunodetection of Cu/Zn-SOD protein (C – control, D – diabetic, DP – diabetics after PYC administration, St – standard).*

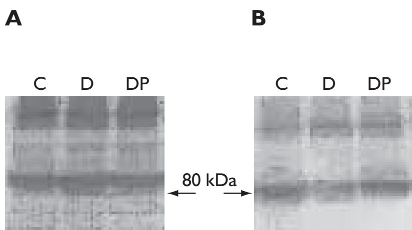

Figure 2 – Output of immunodetection of nNOS protein in the cerebellum (A) and cerebral cortex (B). Proteins were divided by SDS-PAGE in 12% gel, transferred to the membrane by wet transfer and nNOS was detected by primary polyclonal antibody. Bound antibody was detected by secondary antirabbit antibody conjugated with horseradish peroxidase and visualized by chemiluminescence (C – control, D – diabetics, DP – diabetics after administration of PYC).

in the cerebellum by about 10% compared to the control and diabetic groups ($p<0.01$ resp. $p<0.05$). However, we observed a significant reduction (10%) in the cerebral cortex of diabetic rats ($p<0.001$) compared to the control group (Figures 2 and 3).

In eNOS expressed protein levels not significant changes of impact of diabetes or PYC administration in both parts of brain, cerebral cortex and cerebellum were seen.

Discussion

Diabetes mellitus is one of the most common "free radical diseases". Free radicals are involved in the interaction of diabetes with immunogenetic destruction of pancreatic β-cells. Increased oxidative stress as a result of hyperglycaemia, leads to subsequent complications, which are the main cause of reduction of life span and quality of life of diabetics. Since the brain is the insuline independent tissue, the powerful proteins glycation occurs there due to hyperglycaemia. Other brain damages result from the high fatty acids content suitable for lipid peroxidation. Since superoxide dismutase is the most important intracellular antioxidant enzyme it is essential to monitor the impact of diabetes on its synthesis and activity.

To determine the activity and amount of expressed protein Cu/Zn-SOD and amount of expressed protein eNOS and nNOS in different parts of rat brain tissue, homogenates of three groups of rats were used: controls, untreated diabetics and diabetics supplemented with PYC.

In the cerebellum as well as in the cerebral cortex, we recorded a significant reduction of expressed protein Cu/Zn-SOD due to diabetes and its significant increase after PYC administration. We found a significant reduction of Cu/Zn-SOD

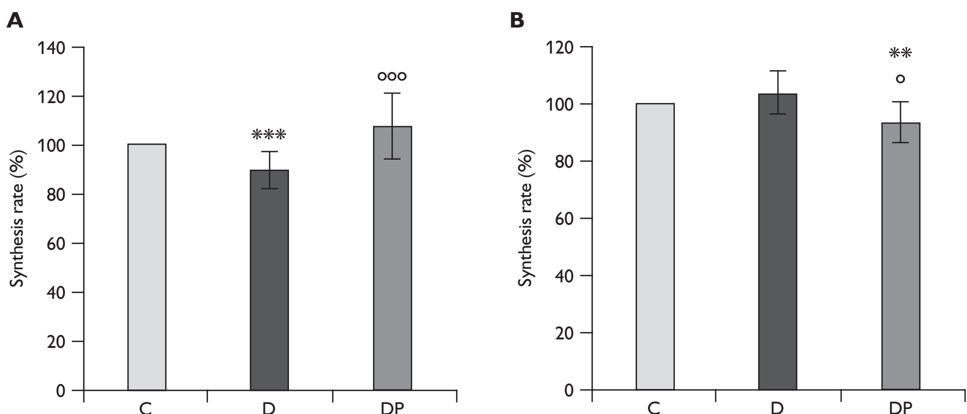

*Figure 3 – Comparison of the amount of expressed protein neuronal NOS expressed as optical density in the cerebral cortex (A) and cerebellum (B) in control (C) and diabetic rats (D) and in diabetic rats supplemented with PYC (DP). Statistical significance for graph A: ***p<0.001 compared to C, °°°p<0.001 compared to D; and for graph B: **p<0.01 compared to C, °p<0.05 compared to D.*

activity in diabetic rats, whereas this fact is consistent with results of Oogawara et al. (1992) and Arai et al. (1987). These authors report glycation of Cu/Zn-SOD in diabetes leading to the inhibition of the enzyme activity. We observed a not significant increase in Cu/Zn-SOD activity after PYC administration. This fact can be explained by glycation and following inhibition of Cu/Zn-SOD in diabetes, although its synthesis is enhanced. Another cause of inhibition of Cu/Zn-SOD activity may be nitrosylation of Cu/Zn-SOD (Yamakura and Kawasaki, 2009). Increased synthesis of Cu/Zn-SOD confirms biomodulative effects of natural polyphenols (Williams et al., 2004; Spencer, 2005; Chovanová et al., 2006). We also conducted the *in vitro* experiment of Pyc effect on Cu/Zn-SOD activity. The pure 1 U Cu/Zn-SOD was incubated with PYC at a dose of PYC which is expected to be in blood plasma. Our preliminary results confirm the assumption that PYC increases the activity of Cu/Zn-SOD (unpublished results). But *in vivo* in diabetic rats, PYC has no impact on Cu/Zn-SOD activity, because DM inhibits it.

The recorded reduction of nNOS synthesis is probably related to the reduction of its mRNA expression (Xu et al., 2005; Patil et al., 2006). From our results we can assume that PYC modulates the level of neuronal NOS in the cerebral cortex and cerebellum during experimentally induced diabetes in rats, but in a different way. Our results also advert to regional differences in the levels of NOS in the brain of diabetic rats. These differences are caused by the fact, that different brain compartments are separated tissues and have different levels of enzyme synthesis and activity (Campese et al., 2007).

Conclusion

Based on our results we can conclude that diabetes causes reduction of the level and activity of Cu/Zn-SOD in the cerebral cortex as well as in the cerebellum. PYC increases the synthesis of Cu/Zn-SOD, what can be explained by its interference with signalling pathways. Diabetes also reduced the synthesis of nNOS in cerebral cortex and PYC elevated it to the level of controls. In the cerebellum diabetes had no effect on the synthesis of nNOS and PYC reduced its synthesis. Neither diabetes nor PYC had effects on the synthesis of eNOS in the cerebral cortex, as well as in cerebellum.

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