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SHORT COMMUNICATION

Oxidative Stress in the Brain Tissue of Laboratory Mice with Acute Post Insulin Hypoglycemia

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Summary

Malondialdehyde (MDA), Cu,Zn-superoxide dismutase (Cu,Zn-SOD) and selenium-dependent glutathione peroxidase (GSPHx) are currently considered to be basic markers of oxidative stress. MDA is one of the end-products of the peroxidation of membrane lipids, whereas enzymes Cu,Zn-SOD and GSHPx belong to the natural antioxidants. The role of oxygen free radicals in the pathogenesis of many diseases is well documented. The aim of this study was to ascertain the influence of insulin-induced acute hypoglycemia on oxidative stress in the brain tissue. Hypoglycemia was induced in ICR mice by intraperitoneal administration of insulin at a dose 24 IU/kg. There was a correlation between the severity of hypoglycemia and the levels of MDA, Cu,Zn-SOD and GSHPx. The results showed that in severe hypoglycemia (serum glucose concentration below 1.0 mmol/l) the lipoperoxidation in brain tissue expressed as the level of MDA was higher in comparison with normoglycemic controls (glycemia around 3.7 mmol/l) as well as in comparison with the levels of MDA during moderate hypoglycemia (glycemia ranging between 1-2 mmol/l). This indicates the enhancement of lipoperoxidation in the brain tissue during severe hypoglycemia. However, both enzymes – Cu,Zn-SOD or GSHPx – did not show a similar tendency.

Key words

Malondialdehyde • Cu,Zn-superoxide dismutase • Selenium-dependent glutathione peroxidase • Insulin-induced hypoglycemia

Oxygen-free radicals, especially superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$) and alkylperoxyl radical ($^{\cdot}OOCR$), are potent initiators of lipid peroxidation. A free radical overload damages many cellular components: cellular proteins, DNA and membrane phospholipids. Thus lipid peroxidation is the consequence of oxygen free radicals, the role of which is well established in the pathogenesis of a wide range of diseases. Some metabolic diseases are also associated with an enhanced level of lipoperoxidation. One of the

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most important is diabetes mellitus. Numerous publications describing the enhancement of lipid peroxidations in diabetes are concerned with hyperglycemia (Altomare *et al.* 1992, Selvam and Anuradha 1988). Only a few of them have recently described the oxidative stress in hypoglycemia (Bhardwaj *et al.* 1998). The central nervous system (CNS) is extremely sensitive to free radical damage because of a relatively small total antioxidant capacity. Furthermore, the central nervous system is extremely sensitive to hypoglycemic damage, because of the properties of the hematoencephalic barrier and because of the lack of other substrates in the CNS. Increased fatty acid metabolism in hypoglycemia may lead to acidosis, which enhances free radical aggressivity.

Among different markers of oxidative stress, malondialdehyde (MDA) and the natural antioxidants, metaloenzymes Cu,Zn-superoxide dismutase (Cu,Zn-SOD) and selenium-dependent glutathione peroxidase (GSHPx), are currently considered to be the most important. Malondialdehyde (MDA) is a three-carbon compound formed from peroxidized polyunsaturated fatty acids, mainly arachidonic acid. It is one of the end-products of membrane lipid peroxidation. Since MDA levels are increased in various diseases with excess of oxygen free radicals, many relationships with free radical damage were observed (Ohkawa *et al.* 1979, Guichardant *et al*. 1994). Cu,Zn-SOD is widespread in nature. It is present in all oxygen-metabolizing cells. Cu,Zn-SOD is an intracellular enzyme, which dismutates the extremely toxic superoxide radical into potentially less toxic hydrogen peroxide. GSHPx, an intracellular enzyme, belongs to several proteins in mammalian cells that can metabolize hydrogen peroxide and lipid hydroperoxides.

The aim of this study was to ascertain the influence of insulin-induced acute hypoglycemia on oxidative stress in the brain tissue.

Male albino random-bred mice of the ICR strain were used. All mice were housed under an artificial lighting (12 h light / 12 h dark), in an air-conditioned animal room with a temperature ranging between 21 °C and 22 °C. The diet was limited to two pieces of pellets per animal per day during one week, food was withdrawn 36 h before the experiment, while water was available *ad libitum* continuously. The experiments in laboratory animals were consistent with the requirements of the Animal Protection Law 246/1992 and the Regulation 311/1997 concerning the use of experimental animals.

Hypoglycemia was induced by intraperitoneal administration of insulin at a dose 24 IU/kg (10 mice). The control animals (5 mice), kept under identical conditions, were given saline instead of insulin. After two hours the animals were sacrificed by decapitation and samples for biochemical assays were taken. The animals treated with insulin were in a stage of hypotonia or atonia at this time. Blood (approximately 20 µl) for the determination of serum glucose and both brain hemispheres (approximately 120 mg per each hemisphere) were taken for the determination of MDA, Cu,Zn-SOD and GSHPx. Glucose strips and a standard glucometer (Glucotrend, Boehringer) were used for the determination of serum glucose.

The brain tissue was frozen immediately after the sampling, at first at –20 °C and than at –70 °C, and kept under these conditions (–70 °C) until chemical analysis. For the analysis of MDA-TBA complex (Fukunaga *et al.* 1995), the tissues were homogenized in ice-cold physiological solution (final concentration 15 %). At first, the homogenate was hydrolyzed by 3.4 mol/l NaOH solution to clear MDA, which was bound to membrane phospholipids (total levels of MDA were measured). Second, 3.4 mol/l $HClO_4$ (followed by centrifugation at 3000 rpm for 10 min) was used for removal of proteins. The supernatant was allowed to react with 2-thiobarbituric acid (TBA) solution (0.4 %) at 95 °C for 40 min. Before injection into HPLC system, MDA-TBA complex was passed through a 0.45 µm filter.

The elution of MDA-TBA complex was performed isocratically, using a Shimadzu high-pressure liquid chromatograph (Shimadzu Corporation, Kyoto, Japan), with a mixture of methanol and phosphate buffer consisting of 20 mM H_3PO_4, pH 6.0, adjusted with NaOH (40:60 v/v), at a flow rate of 0.4 ml/min at a temperature of 25 °C. The MDA-TBA complex was monitored by UV-VIS detector operating at a 532 nm and data station (CLASS VP 4.0). The column was SUPELCOSIL LC-18, 50 x 4.6 mm, 5 µm (Supelco, USA). The brain tissue levels of MDA were calculated using a calibration curve derived from 1,1,3,3- tetraethoxypropane as the external calibration standard. The calibration curve was linear in range from 0.5 µmol/l to 12.5 µmol/l ($r^2 = 0.997$).

The activity of Cu,Zn-superoxide dismutase was estimated spectrophotometrically at 540 nm by a method according to Sun *et al.* (1988), using the xanthine/xanthine oxidase (XOD) reaction as a source of substrate (superoxide) and reduced nitroblue tetrazolium as an indicator of superoxide. Inhibition of the XOD reaction was calibrated with commercial superoxide dismutase (Serva). Selenium-dependent glutathione peroxidase catalyses the oxidation of glutathione by

peroxide. The most suitable substrate for assay of the enzyme is t-butyl hydroperoxide. Glutathione reductase and NADPH reduce the resulting GSSG as a cofactor. The oxidation of NADPH is evaluated spectrophotometrically at 340 nm (Beutler 1975). UV-VIS spectrophotometer (Ultraspec III, LKB, England) equipped with our own special program that was developed in LabWindows version 2.3 (National Instruments) (Štípek *et al.* 1995).

Determination of protein concentration was carried out according to a method of Bradford (1976) using bovine serum albumin as a standard.

Statistical analysis was performed by one-way analysis of variance (ANOVA) and Duncan' s method for pairwise multiple comparison procedures was used. The results are expressed as the mean ± S.E.M**.**

Severe hypoglycemia was defined as a concentration of serum glucose below 1.0 mmol/l in our experiment. In the normoglycemic controls glycemia was ranging between 2.6-6.2 mmol/l and in the moderate hypoglycemia the concentration of serum glucose was in the range between 1-2 mmol/l.

Our results showed that in severe hypoglycemia the lipoperoxidation in brain tissue, expressed as the level of MDA (0.503±0.037 μmol/g of tissue protein), was higher in comparison with euglycemic controls (0.345± 0.027 μmol/g of tissue protein), as well as in comparison with the levels of MDA during moderate hypoglycemia (0.331±0.006 μmol/g of tissue protein) (Fig.1). Neither Cu,Zn-SOD nor GSHPx showed a similar tendency. Nevertheless, Cu,Zn-SOD tended to decrease in severe hypoglycemia, but this difference was not significant; GSHPx values were similar in all three groups (Table 1).

In the present study, a relationship between the severity of hypoglycemia and oxidative stress markers (MDA, Cu,Zn-SOD, GSHPx) was investigated. Hypoglycemia and oxidative stress are mostly studied as two independent stressors. The papers concerning their relationship are very rare. Most published papers are based on the studies of tissue cultures or brain slices (Saransaari and Oja 1999a,b, Mitchell *et al.* 1999).

The severity of hypoglycemia depends on the duration of the starvation, the dosage of insulin, the mode of administration of insulin and on the interval between the injection of insulin and tissue sampling.

In severe hypoglycemia (serum glucose below 1.0 mmol/l), we have demonstrated the enhancement of lipid peroxidation in the brain expressed in μmol MDA/g of tissue protein. Similar changes were already observed in our pilot study (Patočková *et al.* 1996) as well as by other authors (Bhardwaj *et al*. 1998). It is evident that MDA is a sufficiently precise marker of oxidative stress in the brain following acute hypoglycemia. In severe hypoglycemia, the brain tissue suffers from "substrate hunger", which decreases anabolic processes including enzyme constitution. In our experiment, the balance between production and inactivation of oxygen free radicals seems to be impaired by the lack of endogenous antioxidant enzyme synthesis, because neither Cu,Zn-SOD nor GSHPx activity was increased significantly.

As to the changes in the activities of both enzymes, the findings in literature vary from a significant increase to a significant decrease of enzyme levels during different forms of oxidative stress (Nielsen *et al* 1999, De La Fourniere *et al.* 2000). The character of observed changes depends mainly on the experimental design, where the time interval seems to be a crucial factor determining the enzyme activity (Yatin *et al*. 1998).

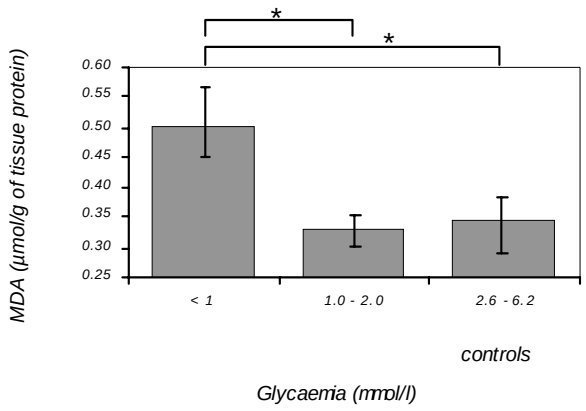

Fig. 1. *MDA levels in dependence on glycemia; MDA – malondialdehyde, The values are means ± S.E.M (n=5), * p<0.05 significantly different from hypoglycemia.*

Table 1. Enzymes activity in dependence on glycemia.

Glucose (mmol/l)	Cu,Zn-SOD activity U/g of tissue protein	GSHPx activity U/g of tissue protein
2.6-6.2	2046.0±123.6	30.52±1.76
1.0-2.0	2294.2±282.0	30.09±0.73
< 1	1922.8±152.2	31.57±1.57

The values are means ± S.E.M., n = 5; Cu,Zn-SOD – superoxide dismutase, GSHPx – glutathione peroxidase.

It is highly probable that the time interval between the beginning of the exposure to the stress (administration of insulin) and the tissue sampling was not large enough to induce measurable changes in the enzyme activities in our experiment. Since this time interval refers to the relationship between intraperitoneally applied insulin and consequential hypoglycemia, it could not be changed a lot. The peak effect of insulin *in vivo* is demonstrated by hypotonia or atonia of the tested animals. In our study, shorter period does not allow insulin to reach the maximal effect and the longer one enables the physiological biofeedback to enhance the level of glycemia again.

We can conclude that severe hypoglycemia leads to a significant increase of the MDA levels in the brain, which provides the evidence of oxidative stress. This finding would have further consequences both for the understanding of the mechanism of brain tissue damage in the condition of hypoglycemia and for the possible prevention of this event.

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