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Cerebrolysin Inhibits Lipid Peroxidation Induced by Insulin Hypoglycemia in the Brain and Heart of Mice

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Summary

As a consequence of enhanced production of oxygen free radicals, lipid peroxidation leads to the degradation of membrane lipids and disturbances of membrane permeability. Lipid peroxidation increases under stress conditions such as hypoxia, ischemia or acidosis as well as in metabolic diseases, e.g. diabetes mellitus. We have shown that subcomatous doses of insulin (6.0 IU/kg) significantly increase thiobarbituric acid reactive substances (TBARs), especially malondialdehyde (MDA) – the endproduct of lipid peroxidation, in the brain and heart of mice. In our model of insulin-induced hypoglycemia, mice were treated with the neuroprotective, peptide-containing drug Cerebrolysin (100 mg/kg b.w.). Animals were sacrificed by decapitation two or three hours after the injection of tested substance and samples were taken to determine several serum parameters (glucose, total protein, triglycerides and lactic acid) and TBARs in the brain and heart. Although Cerebrolysin was not able to affect serum parameters after subcomatous insulin injection, the drug significantly influenced lipid peroxidation. A single injection of Cerebrolysin already decreased TBARs levels in the brain and heart tissue. Presuming that an increase of TBARs reflects disturbances of the cell membrane, we have documented a promising effect of Cerebrolysin on cell integrity.

Key words

Hypoglycemia • Lipid peroxidation • TBARs • Cerebrolysin

Introduction

Lipid peroxidation, a consequence of augmented production of oxygen free radicals, can lead to degradation of essential components of membrane lipids followed by disturbances of membrane permeability (Fridowich 1978). Under various stress conditions, such as ischemia (Agardh *et al.* 1991), acidosis (Bralet *et al.* 1991) and bacterial toxins (Lavický *et al.* 1992), lipid peroxidation is increased. Moreover, certain metabolic diseases are associated with growing lipid peroxidation, e.g. diabetes mellitus (Altomare *et al.* 1992). Although during hyperglycemia an enhancement of peroxidation was observed in various tissues (Wolf 1987, Selvam and Anuradha 1988, Altomare *et al.* 1992), hypoglycemia has attracted much less attention.

The generation of free radicals and peroxidation of lipids are extremely fast reactions, which are generally measured by their endproducts, mostly thiobarbituric acid reactive substances (TBARs), among which malondialdehyde (MDA) is the most usual. We have shown previously that subcomatous doses of insulin significantly increase MDA levels in the brain and heart of mice (Patočková *et al.* 1996). TBARs enhancement is thought to be a marker of cell damage which indicates an increased production of free radicals and lipid

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peroxidation. Hence, insulin induced-hypoglycemia is considered to be a further relevant model of brain injury, since it might reflect pathological changes in the brain of diabetic patients dependent on insulin. Since therapeutic interventions counteracting these neuronal lesions are of interest, the aim of this study was to investigate the possible effects of a neuroprotective drug (Cerebrolysin, EBEWE Arzneimittel, Austria) against the consequences of insulin-induced hypoglycemia. Cerebrolysin has been used in the treatment of different types of dementia (Vereschagin *et al.* 1991, Rüther *et al.* 1994), and in the therapy of stroke (Barolin *et al.* 1996) as well as against diabetic neuropathy (Biesenbach *et al.* 1997). The aim of the present study was to investigate the effects of Cerebrolysin under conditions of insulin induced hypoglycemia and to see whether the drug is able to counteract lipid peroxidation.

Methods

Animals

Male albino random-bred mice derived from the ICR strain (Velaz, Prague) weighing 17-20 g were used. All experiments were performed after one week of adaptation to laboratory conditions. All mice were kept under artificial lighting (with lights on from 7:30 h to 16.00 h) and a temperature ranging from 18 to 23 °C.

The proposed project of experiments in laboratory animals was consistent with the requirements of the Animal Protection Law 246/1992 Sb., its later amendments and the Regulation 311/1997 Sb. concerning the use of experimental animals.

Tested substances

Cerebrolysin (EBEWE Arzneimittel, Austria), is an injection solution of a porcine brain-derived peptide preparation produced by standardized enzymatic breakdown of lipid-free porcine brain proteins. It consists of approximately 25 % of low molecular weight peptides, based on the total nitrogen content. Cerebrolysin was administered in a dose 100 mg/kg body weight (b.w.), whereas control animals received saline. All substances were injected intraperitonealy (2.5 ml/kg b.w.).

Study design

Mice had free access to food until 16.00 h on the day before each experiment, when food was withdrawn. Water was available permanently.

Mice were randomly assigned into four experimental treatments: 1) Saline - Saline (SS), 2) Insulin - Saline (IS), 3) Saline - Cerebrolysin (SC) or 4) Insulin - Cerebrolysin (IC). Initially, animals received an intraperitoneal injection (1st administration) of saline 10 ml/kg (SS, SC) or insulin 6.0 IU/kg diluted in saline (IS, IC). One hour later, mice were given intraperitoneal injection (2nd administration) of the tested substance Cerebrolysin (SC, IC) or saline (SS, IS) as control. Animals of all eight experimental groups (n=8 for each group) were sacrificed by decapitation 2 h (Experiment I) or 3 h (Experiment II) after the second administration and tissue samples for biochemical assays were taken.

Tissue samples

Following samples were withdrawn for biochemical assays: blood for determination of serum glucose, triglycerides, total protein, lactic acid, as well as the brain (the right hemisphere) and heart (the whole organ) for evaluation of the TBARs concentration.

Biochemical assays

Serum parameters (glucose, total protein and triglycerides) were measured using standard kits (Biotests Lachema – GLU 250.E, TP 300, TG 50). Serum lactic acid was determined according to Gay *et al.* (1968). The determination of TBARs in the brain and heart was done according to Ohkawa *et al.* (1979). Briefly, 0.2 ml 8.1 % sodium salt of lauric acid, 1.5 ml acetic acid solution (adjusted to pH 3.5 with NaOH) and 1.5 ml of 0.8 % of thiobarbituric acid were added to samples of 0.4 ml of 10 % tissue homogenates (w/v). The mixture was made up to 4.1 ml with distilled water and then heated in an oil bath at 95 °C for 60 min using a glass ball as a condenser. After cooling with tap water, 1.0 ml of distilled water and 5.0 ml of a n-butanol and pyridine mixture (15:1, v/v) were added and shaken vigorously. After centrifugation at 3500 rpm for 10 min, the organic layer was taken and its absorbency was measured at 532 nm. 1,1,3,3-tetra-methoxypropane was used as an external standard and the level of lipid peroxides was expressed as nmol of TBARs per g of tissue wet weight.

Statistical analysis

Analysis of the data was performed by one-way analysis of variance (ANOVA) and subsequent analysis was performed using the Tukey test. All statistical tests used two-tailed criteria, with alpha level of $p<0.05$.

Results

Effects of insulin and Cerebrolysin on serum parameters

The evaluation of serum glucose indicates that insulin (6.0 IU/kg) produced profound hypoglycemia (glucose/l<1.0 mmol; p<0.001) irrespective whether samples from IS animals were taken two or three hours (Experiments I and II, respectively) after the second injection (Table 1). Furthermore, a significant decrease of serum lactic acid from the control level was observed both two and three hours after insulin treatment (p<0.001, Table 1). Insulin moderately reduced serum total protein and also slightly decreased serum levels of triglycerides in samples taken two hours after the second injection (i.e. three hours after insulin) (Experiment I, Table 1). However, changes in total protein and triglyceride levels were not significant (Table 1). Cerebrolysin administered one hour following insulin administration did not antagonize any of these biochemical changes induced by insulin (Table 1).

Table 1. Serum parameters (mean ± S.E.M.) in particular experimental groups

Experiment	Group	Glucose mmol/l	Lactic acid mmol/l	Total protein g/l	Triglycerides mmol/l
Experiment I	*SS*	5.8 ± 0.6	5.7 ± 0.8	56.5 ± 4.4	0.9 ± 0.2
	IS	0.6 ± 0.1[a]	1.4 ± 0.2[a]	49.2 ± 5.1	0.4 ± 0.1
	IC	0.5 ± 0.1[b]	1.6 ± 0.1[b]	51.6 ± 5.6	0.6 ± 0.1
	SC	5.4 ± 0.5	4.3 ± 0.4	57.0 ± 5.9	0.8 ± 0.1
Experiment II.	*SS*	4.5 ± 0.3	7.4 ± 0.3	58.1 ± 3.0	0.4 ± 0.1
	IS	0.7 ± 0.2[a]	2.1 ± 0.2[a]	44.5 ± 1.1	0.4 ± 0.1
	IC	0.7 ± 0.2[b]	3.0 ± 0.3[b]	46.5 ± 1.9	0.3 ± 0.1
	SC	5.0 ± 0.4	7.4 ± 0.2	57.5 ± 5.3	0.5 ± 0.1

SS: Saline - Saline, IS: Insulin - Saline, IC: Insulin - Cerebrolysin, SC: Saline - Cerebrolysin, Significant differences (p<0.001), [a] IS vs. SS, [b] IC vs. SS

Effects of insulin and Cerebrolysin on TBARs level in the brain and heart

The levels of TBARs found in the brain and heart tissue of mice, which reflected the amount of lipid peroxidation, are shown in Figures 1 and 2. Brain levels of TBARs when collected two hours (Fig 1) after the last injection ranged from 52.3±4.0 nmol TBARs/g wet tissue for the Saline - Saline (SS) control group to 125.1±12.2 nmol TBARs/g wet tissue in the Insulin-Saline (IS) group. In animals treated with a combination of Insulin - Cerebrolysin (IC), the TBARs level was 77.6±10.5 nmol/g wet tissue and after treatment with Saline-Cerebrolysin (SC) the level reached 100.0±10.3 nmol TBARs /g wet tissue.

Levels of TBARs measured in the mouse brain three hours after the last injection (Fig. 1) ranged from 97.0±8.9 nmol TBARs/g wet tissue in the SS group to 125.4±10.6 nmol TBARs/g wet tissue in the IS animals. The values measured in IC animals were 90.2±4.0 nmol TBARs/g wet tissue and 129.7±8.9 nmol TBARs/g wet tissue after the SC combination.

Similar trends were also observed in the heart tissue of mice collected two hours (Experiment I) after the last injection (Fig. 2). In the hearts of SS controls, TBARs values were 108.3±5.0 nmol TBARs/g wet tissue, while the levels in IS treated animals reached 152.0±9.8 nmol TBARs/g wet tissue. The combined IC treatment produced TBARs level of 131.6±7.5 nmol/g and the corresponding SC levels were 151.3±7.6 nmol TBARs/g wet tissue. As in the brain, the heart TBARs levels of SS controls were significantly lower compared to IS-treated animals (p<0.001). The IC treated mice had significantly

lower values than IS-treated animals ($p<0.001$) and the levels of SS controls were lower than in the SC animals ($p<0.001$).

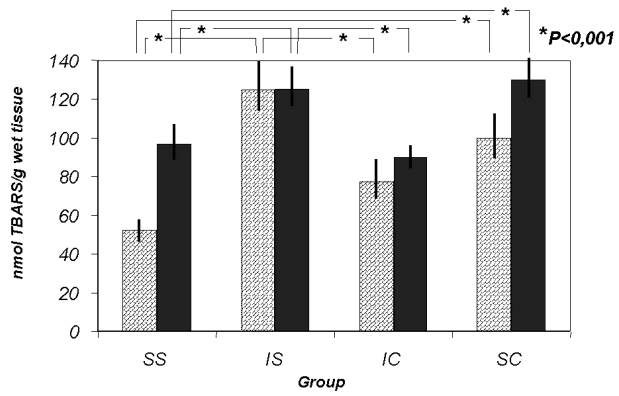

Fig. 1. *Lipid peroxidation in the brain (mean ± S.E.M.), asterisks indicate significant differences (P<0.001). Experiment I (dotted columns) - sampling 2 h after the second injection, Experiment II (full columns) - sampling 3 h after the second injection. SS: Saline - Saline, IS: Insulin - Saline, IC: Insulin - Cerebrolysin, SC: Saline - Cerebrolysin.*

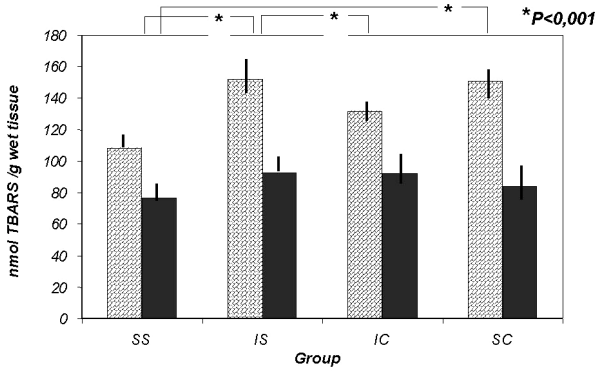

Fig. 2. *Lipid peroxidation in the heart (mean ± S.E.M.). Experiment I (dotted columns) - sampling 2 h after the second injection, Experiment II (full columns) - sampling 3 h after the second injection. SS: Saline - Saline, IS: Insulin - Saline, IC: Insulin - Cerebrolysin, SC: Saline - Cerebrolysin.*

A similar but less pronounced tendency, which was not statistically significant, was observed in the heart tissue collected one hour later (Experiment II). In the case of SS controls, the values were 76.5±7.2 nmol TBARs/g wet tissue while those in the IS group reached 93.0±3.7 nmol TBARs/g wet tissue. After IC treatment, TBARs in the heart were 92.3±7.8 nmol/g and after SC treatment the values were 84.2±8.5 nmol TBARs/g wet tissue.

Discussion

The present study shows that insulin-induced profound hypoglycemia can increase lipid peroxidation (as determined by TBARs) in the brain. Similar findings in the heart indicate that tissue damage during profound hypoglycemia is not limited only to the brain. These results suggest that lesions in diabetic patients treated with very high doses of insulin may be extensive.

Glucose metabolism is very closely regulated. It has been reported that free radical production is increased in patients with insulin-dependent diabetes mellitus. This might be a consequence of insulin treatment rather than of a lack of insulin (Rybka *et al.* 1990). Subcomatous insulin doses might be the cause of brain damage following severe hypoglycemia. Furthermore, we have recently shown that this TBARs increase is dependent on the severity of hypoglycemia but not on the insulin dosage (Patočková *et al.* 2003).

The present study shows that the neuroprotective peptide-containing drug Cerebrolysin significantly attenuated lipid peroxidation in a model of subcomatous insulin treatment, because a pronounced decrease of TBARs levels in the brain and heart was observed after Cerebrolysin treatment. Cerebrolysin seems to act in a multimodal way on neuronal metabolism and synaptic transmission, similarly to naturally occurring growth factors (Windisch *et al.* 1998). A variety of neuroprotective effects of this peptide-containing drug have been described under different experimental conditions such as ischemia (Hutter-Paier *et al.* 1996, Schwab *et al.* 1997), fornix transsections (Akai *et al.* 1992) and apolipoprotein deficiency (Masliah *et al.* 1999).

Furthermore, preventive effects of Cerebrolysin against the generation of free radicals and the resulting consequences have been reported (Sugita *et al.* 1993, Hutter-Paier *et al.* 1998). Cerebrolysin increases the amount of blood-brain-barrier glucose transporter GLUT-1 *in vitro* and *in vivo* (Boado 1995, 1996, 1998, Gschanes *et al.* 2000). These facts could be of special interest in the model of insulin-induced hypoglycemia used in the present study. The ability of the drug to inhibit lipid peroxidation and resulting TBARs generation

as reported here may reflect its ability to protect neuronal (as well as heart) tissue from free radical damage. This could probably be a scavenger/antioxidant effect (under the conditions of enhanced TBARs levels) or a substrate-like effect (under conditions of substrate "hunger" in profound hypoglycemia). As antioxidants could have a pro-oxidative effect (mostly in overdosage), this could explain the TBARs increase after Cerebrolysin without insulin pretreatment. This increase of TBARs after Cerebrolysin could indicate the dose-dependent scavenger/antioxidant effect of this drug. A similar positive effect of Cerebrolysin on the enhanced levels of TBARs in the heart suggests its possible use in the experimental treatment of heart disturbances.

Although Cerebrolysin did not antagonize the serum changes produced by the insulin-induced hypoglycemia or by insulin itself, it was able to decrease TBARs levels in the brain and the heart. Supposing that the increase of TBARs reflects the disturbances of the cell membrane, we have documented a promising effect of Cerebrolysin on cell integrity.

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Reprint requests

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