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Activity of some lysosomal enzymes in the adrenal cortex during experimental alloxan-induced diabetes mellitus in rabbits

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Summary

The aim of the study was the evaluation of changes in the adrenal cortex lysosomal enzymes activity during experimental alloxan-induced diabetes mellitus in rabbits. We checked the activity of acid phosphatase, β-D-galactosidase, N-acetyl-β-D-glucosaminidase (NAGL) and lipase. The study was performed on 124 rabbits divided into five groups: one control and four experimental. Diabetes mellitus was induced by a single injection of 10% alloxan solution into the auricular vein in a dose of 10 mg per kg body weight. Animals from experimental groups were killed in the 21st, 42nd, 90th and 180th days of the study. Adrenal glands were removed. Enzymes activity was assayed by spectrophotometric methods. Changes in free and bound fractions of examined lysosomal enzymes activity were noticed already in the 21st day of diabetes. The most escalated changes were observed in the 42nd day of the study. Performed statistical variance analysis demonstrated statistically highly significant differences for activity of both fractions of NAGL and lipase, as well as for free fraction activity of acid phosphatase and β-D-galactosidase. The obtained data confirmed the influence of diabetes mellitus on changes in the activity of examined lysosomal enzymes in the adrenal cortex.

Keywords: lysosomal enzymes, adrenal cortex

Diabetes mellitus is a group of metabolic abnormalities caused by a deficiency or diminished effectiveness of endogenous insulin. The most common diabetes symptom is a high glucose level (1, 2) Hyperglycemia is usually observed in many endocrinopathies, such as e.g.: hyperthyroidism, acromegaly, Cushing syndrome, glucagonoma, pheochromocytoma (1, 2, 4, 10, 13, 20-22); after glucocorticoids, administration, so called post-steroid diabetes (2, 23) or sometimes in pregnancy, so called gestational diabetes (2, 3). Also some viral infection can lead to abnormally high glucose level, especially Coxackie and cytomegalovirus infections (9, 11, 17). Diabetes mellitus causes vascular pathologies, divided into macro- and microvascular ones (2, 7, 24, 29), and by this means leads to the injury of different organs.

Lysosomal enzymes, which are known to be present in many tissues, are responsible for the intracellular degradation of macromolecules e.g.: glycoproteins, glycolipids and glycosaminoglycans (19), and play an important role in the pathology of cells (18). Kelly et al. (12) found an alteration in lysosomal enzymes activity in humans suffering from diabetes mellitus.

The aim of the study was to determinate changes in selected lysosomal enzymes activity in the adrenal cortex of rabbits with alloxan-induced diabetes mellitus. Changes were examined in the activity of acid phosphatase, β-D-galactosidase, N-acetyl-β-D--glucosaminidase (NAGL) and lipase.

Material and methods

The study was performed in accordance with international animal principles and approved by the Local Bioethical Committee on 124 New Zealand adult male rabbits, body weight about 2.6-3.2 kg (mean 2.88 kg). The animals were kept under standard laboratory conditions. Their regular

Tab. 1. Characterization of control and diabetic (alloxan--exposed) groups

Groups	Number of animals	Alloxan injection	Day of sacrifice
1	27	No	0
2	24	Yes	21
3	28	Yes	42
4	25	Yes	90
5	20	Yes	180

diet and water were provided *ad libitum*. The rabbits were randomly divided into 5 groups: one control and four experimental (Tab. 1).

The experimental diabetes mellitus was induced by a single injection of 10% alloxan solution (Sigma Chemical Company, St. Louis, MO, USA) into the auricular vein at a dose of 10 mg per kg body weight (8, 25). The physiological saline was administered in a control group. 10 days later the serum glucose level was measured using enzymatic method (Cormay GS 120L; Lublin, Poland). A level over 1.1 mmol/l (200 mg/dl) was considered as the primary biochemical criterion for selection to the experimental group and that day was designated as the first day of diabetes.

According to the study protocol animals from diabetic groups were sacrificed by decapitation on the 21st, 42nd, 90th and 180th day of diabetes. Non-diabetic animals were sacrificed ten days post saline injection. During autopsy the adrenal glands were removed and the adrenal cortex was separated. Samples of the organ were stored in a temperature of –20°C. Next the material was defrosted in 0.9% NaCl at a temperature of +4°C. Afterwards the adrenal cortex was placed in 5 ml of 0.3 M solution of sucrose and homogenized 3 times for 20 seconds with 15 second intervals. The obtained homogenate was centrifuged for 10 minutes at 700 g. The supernatant was decanted and centrifuged at 10 000 g for the next 20 minutes. Supernatant 1, which contained a free fraction of lysosomal enzymes and the precipitate, was placed into 5 ml of 0.3 M sucrose with 0.1% Triton X-100 and stored for 24 hours at +4°C. The precipitate was subsequently centrifuged for 20 minutes at 10 000 g. The obtained supernatant 2 contained a bound fraction of lysosomal enzymes.

Acid phosphatase activity was assayed using substrate 45 mg of sodium 4-methylumbelliferyl phosphate dissolved in 100 ml of 0.1 M acetate buffer (pH 5.0) (Sigma Chemical Co., St. Louis, MO, USA), which releases 4-methylumbelliferol when it reacts with the enzyme.

β-galactosidase activity was determined based on the degradation of 51 mg of 4-methylumbelliferyl-β-D-galactopyranoside dissolved in 100 ml of 0.1 M citrate buffer (pH 3.6).

β-N-acetyl-glucosaminidase (NAGL) activity was determined based on the degradation of 57.2 mg of 4-methylumbelliferyl-N-acetyl-β-D-glucosaminidine dissolved in 100 ml of 0.1 M citrate buffer (pH 4.3) with 0.3 M NaCl.

Lipase activity was determined based on the degradation of 52.7 mg of 4-methylumbelliferyl-estolate in 10 ml of acetone, 100 times diluted in 0.1 M acetate buffer (pH 5.0) with 0.1% Triton X-100 supplementation.

100 µl of each of the free and bound fractions were incubated with 500 µl of the above-mentioned substratum for 18 hours at 37°C. The reaction was inhibited by the addition of 600 µl of alkaline buffer, and after 5 minutes the extinction was measured at 360 nm using a spectrophotometer (Spekol 221, Carl Zeiss-Jena, Germany).

The level of protein was determined by the Lowry method (14).

The values of free and bound fractions of lysosomal acid phosphatase, β-galactosidase, β-N-acetyl-glucosaminidase and lipase are given in pmol/mg of protein/hour of incubation.

The homogeneity of the obtained data was evaluated by the Kolmogoroff-Smirnoff test. Due to the normal distribution of numerical data ANOVA followed by the Duncan test was employed to check the differences between examined groups. The effect was statistically significant when $p < 0.05$.

Results and discussion

The activity of free and bound fractions of examined lysosomal enzymes of the adrenal cortex during experimental alloxan-induced diabetes mellitus is shown in detail in tables 2 and 3, respectively. On the other hand, the results of variance analysis of lysosomal enzymes activity are shown in Tab. 4.

The activity of the free fraction of acid phosphatase decreased during the study, and reached its minimal value on the 42nd day of the disease, after which it increased again in the 90th and 180th days of diabetes, but did not attaint the value as observed in control group. The activity of the bound fraction of acid phosphatase was higher than the free fraction with a maximal value on the 90th day of the disease and it was statistically highly significant.

The activity of the free fraction of β-D-galactosidase after its decrease in the early stage (on 21st day) increased significantly to a maximal value on the 180th day of diabetes. On the other hand the activity of the bound fraction of the enzyme decreased on the 42nd day of the study and increased significantly again to maximal value on the 90th day.

The activity of the free fraction of N-acetyl-β-D--glucosaminidase (NAGL) was time-dependent. It decreased in an early stage of the study, and reached minimal value at the day 42nd. Among animals sacrificed on the 90th and 180th days of diabetes the enzyme activity increased up to the maximal value on the 180th day. The statistically significant increase of the bound fraction was found on day 21st day, whilst on the 42nd day only trace activity was observed. The highest activity was noted on the 180th day.

A time-dependent decrease was also determined for the activity of the free fraction of lipase until the 90th day. On the day 180 a significant increase was noted. The bound fraction also decreased during the first 6 weeks but significantly increased on the 90th and 180th days.

Tab. 2. The activity of free fractions of examined lysosomal enzymes of the adrenal cortex during experimental alloxan--induced diabetes mellitus expressed as pmol/1 mg of protein/1 h of incubation, mean ± SD. Values signed to the same litter do not differ statistically

Enzyme	Control	Diabetes mellitus 21-days	Diabetes mellitus 42-days	Diabetes mellitus 90-days	Diabetes mellitus 180-days
Acid phosphatase	2.415 ± 1.297[a]	1.536 ± 0.187[b]	1.351 ± 0.203[b]	1.405 ± 0.449[b]	1.625 ± 0.262[b]
β-d-galactosidase	3.177 ± 1.696[a]	2.113 ± 0.264[b]	4.001 ± 0.597[c]	7.648 ± 1.957[d]	9.581 ± 1.883[d]
Nagl	3.192 ± 1.703[a]	1.859 ± 0,859[b]	0.149 ± 0.064[c]	11.765 ± 3.548[d]	12.883 ± 2.113[d]
Lipase	2.931 ± 1.503[a]	1.963 ± 0.228[a]	0.957 ± 0.136[b]	2.4 ± 1.616[a]	11.889 ± 1.963[c]

Explanation: a, b, c, d means with different superscript letters differ significantly at $p \leq 0.05$

Tab. 3. The activity of bound fractions of examined lysosomal enzymes of adrenal cortex during experimental alloxan-induced diabetes mellitus expressed as pmol/1 mg of protein/1 h of incubation, mean ± SD. Values signed the same litter don't differ statistically

Enzyme	Control	Diabetes mellitus 21-days	Diabetes mellitus 42-days	Diabetes mellitus 90-days	Diabetes mellitus 180-days
Acid phosphatase	4.515 ± 1.19[a]	4.124 ± 1.262[a]	3.603 ± 0.655[a]	5.764 ± 4.187[a]	3.38 ± 0.637[a]
β-d-galactosidase	3.062 ± 1.478[a/b]	3.761 ± 3.044[a/b]	2.37 ± 0.37[a]	6.829 ± 5.474[b]	5.896 ± 4.131[b]
Nagl	5.904 ± 1.72[a]	6.059 ± 1.835[a]	0.065 ± 0.017[b]	44.751 ± 34.989[c]	27.286 ± 5.834[c]
Lipase	5.48 ± 1.441[a]	5.371 ± 1.642[a]	2.448 ± 0.443[b]	7.239 ± 5.369[a]	25.27 ± 4.954[c]

Explanation: a, b, c, d means with different superscript letters differ significantly at $p \leq 0.05$

Lysosomal enzymes activity is a matter of interest to a number of scientists. There are some studies focused on lysosomal enzyme activity in many organs after different drugs are administered (5, 6). For example, Burdan et al. (5) revealed that the administration of high doses of omeprazol caused a decrease in the bound fraction of β-galactosidase connected with the increase in the free fraction of sulphatase in the rat's liver. Also observed was a temporary elevation of the activity of some pancreatic lysosomal enzymes as a result of omeprazol administration (6).

Waters et al. (24) observed a significant increase in plasma level of β-D-glucuronidase, β-D-Nacetylo-glucosaminidase and β-D-galactosidase in patients suffering from diabetes mellitus type 1, compared with non-diabetic patients.

As was described in previous studies performed on rabbits with experimental alloxan-induced diabetes mellitus, diabetes led to changes in the activity of some lysosomal enzymes in numerous examined endocrine glands: thymus, pancreas, testis, (16, 22, 26, 27), blood vessels (28), or internal organs such as salivary glands (15).

Despite the studies mentioned above, there are no publications on adrenal cortex lysosomal enzymes activity. The current study demonstrated significant differences for the examined lysosomal enzyme in the adrenal cortex among rabbits with experimentally induced diabetes mellitus.

As described above, we revealed significant differences for the activity of both fractions of NAGL and lipase, as well as for free fraction activity of acid phosphatase and β-D-galactosidase. The activity of the bound fraction of β-D-galactosidase was also affected, whilst bound fraction activity of acid phosphatase was insignificantly changed.

In our study the changes of enzymes activity were already noticed on the 21st day, but the most spectacular changes took place on the 42nd day. Maciejewski et al. also observed in rabbits' pancreas with alloxan--induced diabetes mellitus the most statistically specific changes in enzyme activity in the 42nd day: a decrease of N-acetyl-β-D-glucosaminidase activity and an increase of both lipase fractions. That result is in opposition to our findings. Although in the 42nd day group the authors also found a statistically significant decrease of the bound fraction of NAGL, the activity of both fractions of lipase were decreased and reached minimal values when compared to the control.

When Wójtowicz et al. examined the activity of testicular enzymes in alloxan-induced diabetic rabbits they also found the most spectacular changes on the 42nd day. There were significant decreases of the bound fraction of NAGL, as well as a significant elevation of

Tab. 4. Variance analysis of activities of lysosomal enzymes in adrenal cortex during alloxan-induced diabetes mellitus

Enzyme		P value
Acid phosphatase	ff	0.003219**
β-d-galactosidase	ff	0.000000**
β-d-galactosidase	bf	0.040286*
Nagl	ff	0.000000**
Nagl	bf	0.000000**
Lipase	ff	0.000000**
Lipase	bf	0.000000** p < 0.0001

Explanation: FF – free fraction, BF – bound fraction

the free fraction of acid phosphatase (27). On the 42nd day the authors also noticed only a trace activity of the bound fraction of NAGL. Furthermore, in our study the free fractions of acid phosphatase, NAGL and lipase reached minimal values on the 42nd day.

The obtained results revealed that time-dependent lysosomal enzyme activity changes in the adrenal cortex during experimentally-induced diabetes mellitus in rabbits. That is consistent with previous dates, and confirms the influence of diabetes mellitus on endocrine glands.

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