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Lysosomal Proteolysis: Effects of Aging and Insulin

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Abstract—Age-related characteristics of the effect of insulin on the activity of lysosomal proteolytic enzymes were studied. The relationship between the insulin effect on protein degradation and insulin degradation was analyzed. The effect of insulin on the activities of lysosomal enzymes was opposite in young and old rats (inhibitory in 3-month-old and stimulatory in 24-month-old animals). The activities of proteolytic enzymes were regulated by insulin in a glucose-independent manner: similar hypoglycemic effects of insulin in animals of different ages were accompanied by opposite changes in the activities of lysosomal enzymes. The inhibition of lysosomal enzymes by insulin in 3-month-old rats is consistent with a notion on the inhibitory effect of insulin on protein degradation. An opposite insulin effect in 24-month-old rats (i.e., stimulation of proteolytic activity by insulin) may be partly associated with attenuation of the degradation of insulin, resulting in disturbances in signaling that mediates the regulatory effects of insulin on protein degradation.

Key words: lysosomal proteolysis, aging, insulin, insulin degradation

Proteolytic enzymes are involved in the regulation of numerous cell processes. The activity of proteinases determines the rate of elimination of abnormal proteins (falsely translated, modified, or foreign), as well as the content of functionally active proteins, including enzymes and hormones. Proteolytic enzymes provide tissues with the substrates of numerous metabolic processes, such as gluconeogenesis, transmethylation, protein synthesis, etc. [1-3]. Some data published in recent years suggest that lysosomal enzymes may be involved in triggering the chain of reactions leading to apoptosis [4].

It is known that the activity of lysosomal enzymes (which account for approximately 70% of total protein degradation) is controlled by hormones. It was shown that insulin, glucagon, adrenalin, and thyroid hormones are involved in the regulation of the lysosomal pathway of protein degradation [5]. However, the age-related aspects of hormonal regulation of the activity of lysosomal proteinases are poorly understood. In view of this, the purpose of our work was to study the age-related characteristics of the insulin effect on the activity of lysosomal proteinases. Because the insulin effects are closely related to its transformation, the proteolytic activity of lysosomes was studied in connection with the estimation of insulin degradation.

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MATERIALS AND METHODS

The experiments were performed with approximately 200 male Wistar rats (3- and 24-month-old). The rats were kept under standard conditions in a vivarium.

Insulin (crystalline) was injected at the dose of 0.8 U per 100 g animal weight. The activity of lysosomal enzymes was determined 60 min after the hormone injection, and the glucose concentration in blood and liver was measured 10, 30, and 60 min after the injection.

The intensity of lysosomal proteolysis was evaluated by an increase in the Folin-positive products contained in the mitochondrial lysosomal fractions lysed with Triton X-100 [6]. The incubation medium contained 0.2 ml of mitochondrial lysosomal fraction (2 mg protein), 0.4 ml of 2.5% solution of egg albumin in 0.1 M acetate buffer (pH 5.3), and Triton X-100 (final concentration 0.2%). The experimental samples were incubated at 37°C for 1 h with constant stirring. The control samples were incubated in the cold. The reaction was stopped by addition of an equal volume of 5% TCA. To reduce sorption of the TCA-soluble products, the samples were allowed to incubate in a refrigerator for 12 h and then centrifuged in the cold at 3000 rpm. The supernatant was used for analysis. The enzyme activity was expressed in μmol tyrosine/h per mg protein.

The concentration of glucose was determined by the ortho-toluidine method.

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To estimate the intensity of insulin exchange, we studied the dynamics of [^{125}I]insulin injected into the rats [7]. [^{125}I]Insulin (with specific radioactivity of 5-7 GBq/mg protein; Institute of Nuclear Research, Poland) was injected into the rats under ether anesthesia into the tail vein at the dose of 12.5 kBq per 100 g of body weight. The animals were sacrificed in 30, 60, and 90 min after the injection. The liver was extracted, weighed, and homogenized on ice in a porcelain mortar with cooled 10% TCA. The homogenate was centrifuged at 2000*g* for 10 min. The supernatant was discarded, and the pellet was analyzed for radioactivity using a γ-counter. The concentration of TCA-precipitated radioactivity was expressed in percent of the amount of radioactivity injected. The latter was determined by radiometry of an aliquot of labeled insulin injected into the rat (with respect for the animal's weight).

The activity of the insulin-degrading enzymes was assessed by the ability of liver homogenates to degrade insulin added to the incubation medium [8]. Immediately after sacrifice of the animal, the liver was extracted and homogenized in 0.15 M phosphate buffer (pH 7.4). The homogenates were centrifuged at 2000*g* at 4°C. The insulin-degrading enzymes were released from the structural elements of the cell using the freeze–thaw method. The reaction mixture (0.9 ml) contained 0.1 ml of tissue homogenate (500 μg protein), 0.1 ml of insulin solution (3 ng) in 0.15 M phosphate buffer (pH 7.4) supplemented with 0.5% BSA, and 0.7 ml of buffer of the same composition. The samples were incubated at 37°C for 20 min. The reaction was stopped by addition of 0.1 ml of aqueous solution of 0.02 M N-ethylmaleimide. In the case of the control samples, N-ethylmaleimide was added prior to incubation. The insulin concentration was determined radioimmunologically using standard kits (Academy of Sciences of Belarus). The amount of insulin degraded was determined by the difference between the control and experimental values and expressed in percent of the added amount of the hormone.

RESULTS AND DISCUSSION

The activity of lysosomal enzymes in the control animals decreased with age (Table 1), which is in good agreement with data on a decrease in the intensity of protein degradation on aging [9]. The insulin injection caused opposite changes in the activity of lysosomal enzymes in young (3-month-old) and old (24-month-old) animals. In young animals, insulin suppressed the protein-degrading activity of lysosomes. This is consistent with the notion that the main effect of insulin on protein metabolism is the inhibition of protein degradation [10]. It was assumed that the mechanism of the inhibitory effect of the hormone is largely based on the insulin-induced increase in the intracellular calcium concentration (which, similar to other divalent cations, exhibits a pronounced inhibitory effect on the activity of lysosomal enzymes). In addition, the insulin-induced increase in the amino acid transport also had an inhibitory effect on the activity of lysosomal enzymes [5]. It was found that amino acids not only suppress autophagic processes, but also may directly affect the activity of cathepsins [11].

In old rats, the insulin injection paradoxically increased the activity of the lysosomal enzymes. It can be assumed that, at this age, the balance between the inhibitory and stimulating factors is disturbed, with the latter being dominating. These factors may include disruption of "calcium response", recorded in old animals, due to an increase in the intracellular calcium concentration, which is caused mainly by a decrease in the activity of plasma membrane Ca^{2+}-ATPase with age [12]. In the absence of the inhibitory effect of Ca^{2+}, the effect of stimulators of enzymatic activity (particularly, thiols) may be manifested. Because thiols and nitric oxide compete for the binding sites on protein molecules, the ratio between these compounds is apparently important during realization of the stimulatory or inhibitory effects.

Although it has been reported that glucose has a stimulatory effect on the activity of lysosomal proteolytic enzymes [5], there is reason to believe that the enzyme activity is regulated in a glucose-independent way. As seen from our results (Tables 2 and 3), the insulin injection is accompanied by a decrease in glycemia in animals of both age groups, whereas the insulin-induced changes in the activity of lysosomal enzymes are oppositely directed. The results of the study on the relationship between the reception of insulin (or its analogs), on one hand, and glucose transport and protein degradation, on the other hand, also provide evidence in favor of a glucose-independent regulation of the studied process [13]. According to these data, the transport of glucose strongly depends on the receptor binding of insulin, whereas the hormonal regulation of protein degradation, besides the receptor binding of insulin, also includes postreceptor mechanisms. The latter includes intracellular pro-

Table 1. Activity of acid cathepsins (μmol tyrosine/h per mg protein) in the liver of rats of different age under insulin loading

Experimental conditions	Enzyme activity	
	3 months	24 months
Control	0.225 ± 0.024	0.132 ± 0.015*
After insulin injection	0.176 ± 0.018**	0.216 ± 0.023**

* $p_{3\text{-}24} < 0.05$.
** $p_{\text{control-experiment}} < 0.05$.

Table 2. Concentration of glucose in blood plasma of rats of different age under insulin loading (mmol/liter)

Duration of exposure, min	Glucose concentration, mM	
	3 months	24 months
Before insulin injection	4.44 ± 0.24	4.80 ± 0.24
10	3.36 ± 0.30*	2.94 ± 0.24**
30	3.00 ± 0.36*	2.40 ± 0.24**
60	2.82 ± 0.24**	2.64 ± 0.30**

* $p < 0.05$.
** $p < 0.01$.

Table 3. Concentration of glucose in the liver of rats of different age under insulin loading (mmol/kg)

Duration of exposure, min	Glucose concentration, mM	
	3 months	24 months
Before insulin injection	67.74 ± 7.74	51.36 ± 3.91 $p_{3\text{-}24} < 0.05$
10	61.50 ± 6.54	41.22 ± 3.06* $p_{3\text{-}24} < 0.01$
30	37.80 ± 4.56*	43.02 ± 3.78
60	36.60 ± 3.84*	41.46 ± 2.88*

* $p_{\text{control–experiment}} < 0.05$.

Table 4. Concentration of the TCA-precipitated radioactivity in the liver of rats of different age

Time after [^{125}I]insulin injection, min	Concentration of [^{125}I]insulin	
	3 months	24 months
30	6.8 ± 0.7	1.8 ± 0.1*
60	3.2 ± 0.2	3.3 ± 0.4
90	2.8 ± 0.3	3.2 ± 0.3

* $p_{3–24} < 0.01$.

cessing of insulin and its catabolism, which, being a necessary condition for the insulin effects on protein degradation [14, 15], may be associated with the age-related changes in the insulin-dependent processes of protein degradation.

In our work, the transformations of insulin in rats of different age were estimated judging by the distribution of [^{125}I]insulin injected into the animals. This methodology is based on the results of Bourgeois et al. [16] who used scintigraphic analysis of the distribution of radioactivity after intravenous injections of labeled insulin. They showed that the changes in the radioactivity of organs corresponded to the dynamics of specific [^{125}I]insulin binding (taking into account the negative cooperativity of the insulin receptors and the hormone clearance).

In our experiments, the concentration of labeled insulin in tissues was estimated by the level of the TCA-precipitated radioactivity and considered as the resultant of the intensity of the hormone binding and its degradation rate.

The distribution pattern of labeled insulin in the liver of young animals is suggestive of high insulin-binding activity of target tissues, rapid saturability of the hormone receptors, and intensive metabolism of insulin. As a result, the level of the TCA-precipitated radioactivity in the tissues markedly decreased by the end of the first hour after the labeled insulin injection (Table 4).

A low level of the TCA-precipitated radioactivity in the organs of old rats 30 min after the insulin injection and its subsequent increase 60 min after injection was apparently the result of a delayed insulin binding, on one hand, and disturbance of the processes of its catabolism, on the other hand. Therefore, the characteristics of kinetics of labeled insulin injected into the organism are indicative of the presence of age-related changes both in the hormone binding and in its degradation.

To date, it has been shown that the initiation of cell processing of insulin and its degradation are regulated by the insulin-degrading enzyme insulinase [17]. This enzyme catalyzes breaks of the insulin B-chain in two or more sites. Subsequent breaks of the disulfide bonds in the hormone molecule are catalyzed by protein-disulfide-isomerase (or a similar enzyme). These reactions yield an intact A-chain and several fragments of B-chain of insulin [18], which undergo further cleavage catalyzed by many proteolytic systems, including lysosomes. The degradation of insulin in cell homogenates is catalyzed predominantly by the insulin-degrading enzyme [19], with the cytosol fraction containing up to 95% of the total amount of the enzyme, whereas the plasma membranes, endosomes, and peroxisomes contain only a small portion of it [20-22].

The estimation of the insulin-degrading activity in the liver is of special interest because it is known that the insulin degradation in the liver and the insulin effects aimed at regulation of protein exchange in this organ are correlated [23].

Our data showed that, at the animal's age of 3 to 24 months, the insulin-degrading activity of liver homogenates significantly increased (Table 5). At first glance, these results are inconsistent with the conclusion

Table 5. Insulin-degrading activity (IDA) of liver homogenates of rats of different age (degraded insulin, %)

Activity	Degraded insulin, %	
	3 months	24 months
N-ethylmaleimide-inhibited IDA	47.1 ± 2.0	50.6 ± 4.2
N-ethylmaleimide-insensitive IDA	0	9.3 ± 1.3
Total IDA	47.1 ± 2.0	59.9 ± 5.3*

* $p_{3-24} < 0.05$.

on the moderation of the insulin metabolism on aging, which was made judging by the dynamics of [^{125}I]insulin injected. To explain the apparent inconsistence between the results obtained *in vivo* and *in vitro*, it should be taken into account that the degradation of insulin is catalyzed predominantly by the enzymes located inside the cell and, therefore, is mediated by receptor binding [17], i.e., binding of insulin is the factor that limits its degradation.

The activity of the insulin receptors decreases on aging [24], which results in a decrease in the hormone supply into the cell and, therefore, in limitation of the intracellular insulin-degrading activity.

It was traditionally believed that the biological function of insulin degradation is elimination and inactivation of the circulating hormone. Currently, there is decisive evidence that the interaction between insulin and insulin-degrading enzyme, as well as the hormone degradation mediated by this process, play a key role in the insulin signal generation [25]. In particular, it was shown that the inhibitors of insulin processing prevent the insulin effects on protein degradation and the transport of amino acids in the cell [14, 15]. The regulatory effect of insulin on protein metabolism is caused predominantly via direct intracellular interaction between insulin and the cytosolic insulinase–proteosomal complex. However, some authors assume that insulin is also involved in the regulation of lysosomal proteolysis [17]. With regard for this fact, it may be assumed that the age-related differences in the insulin regulation of the activity of proteolytic lysosomal enzymes are associated, to some extent, with the ontogenetic changes in the insulin metabolism.

The suppression of protein degradation by insulin in 3-month-old rats is consistent with the notion on the inhibitory effect of this hormone on protein catabolism [10]. An opposite response to insulin injection in 24-month-old rats (i.e., stimulation of the proteolytic activity) is apparently related to attenuation of the insulin degradation, which, according to the current view, leads to disturbances in the signal transduction system mediating the regulatory effects of insulin on protein degradation.

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