Hypoglycemic Effects of Hyaluronate-Endo-β-N-Acetylhexosaminidase Immobilized by Electron Beam Synthesis Nanotechnology

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Hypoglycemic effect of hyaluronate-endo-β-N-acetylhexosaminidase immobilized by electron-beam synthesis nanotechnology (imHEA-HA) was studied in experimental insulin-dependent and insulin-independent diabetes mellitus. The drug exhibited a hypoglycemic effect of its own and potentiated the pharmacological effect of exogenous insulin injected *in vivo*. Studies on liver cell culture demonstrated an increase of cell sensitivity to insulin after treatment with imHEA-HA.

Key Words: *diabetes mellitus; immobilized hyaluronate-endo-β-N-acetylhexosaminidase; hyaluronidase; insulin; electron-beam synthesis nanotechnology*

Diabetes mellitus (DM) is a highly prevalent severe disease, for which no radical treatment methods are known up to date [3,6]. An important mechanism in the pathogenesis of the insulin-independent variant of this disease and development of insulin resistance in insulin-dependent condition is reduction of specific insulin receptor sensitivity on target cells [3,6]. However, the relationship between the affinity of receptors for this ligand and cell sensitivity to glucose in DM, on the one hand, and the status of hyaluronic acid (HA; a component of the receptors and cell-cell matrix [9,13,14]), on the other hand, remains little studied. Experimental studies demonstrated potentiation of the specific effects of some bioactive substances by immobilized hyaluronate-endo-β-N-acetylhexosaminidase (imHEA-HA), an hyaluronidase analogue playing the key role in HA metabolism [2,4]. This drug created by electron-beam synthesis nanotechnology is a virtually nontoxic compound with manifest systemic effects (in contrast to unmodified enzyme), also effective in oral treatment [7,8,10], which is explained by its resistance to serum and tissue degradation factors [13,14] and proteolytic enzymes of the gastrointestinal tract [6,7].

We studied the hypoglycemic effects of imHEA-HA immobilized by electron-beam synthesis nanotechnology and mechanisms underlying these effects.

MATERIALS AND METHODS

Experiments were carried out on 2-month-old male CBA/CaLac mice (18-20 g; *n*=64) and 12-14-month-old male mice of the same strain (45-52 g; *n*=37). Certified animals were bred at Department of Experimental Biological Models of Institute of Pharmacology.

Insulin-dependent DM (type 1 DM, DM1) was induced by intraperitoneal injection of alloxan mono-

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hydrate in a dose of 300 mg/kg for 4 days [12]. Spontaneous age-associated hyperglycemia of CBA/CaLac mice aged 12-14 months served as the model of insulin-independent (type 2) DM [6]. Experimental animals received intragastrically imHEA-HA or intraperitoneally insulin (Actropid) or intragastrically imHEA-HA+intraperitoneal insulin. imHEA-HA was administered in a dose of 50 U/kg 2 h before insulin irrespective of the disease model. imHEA-HA was synthesized at Institute of Pharmacology in collaboration with Scientific Future Management Company. Immobilization of the enzyme molecules (Scientific Future Management) was realized on low molecular polyethylene oxide (1.5 kDa) by radiation synthesis nanotechnology using focused rapid electron flow [7,10]. Insulin was injected in a dose of 10 U/kg in DM1 and in a dose of 5 U/kg in DM2. In DM1 the preparations were administered on day 8 of experiment. Peripheral blood glucose was measured (SmartScan device, Johnson & Johnson) before drug injections and 2, 4, 6, and 24 h after insulin injection. Blood for analysis was collected from the tail vessels. Animals were were allowed a free access to water and food throughout experiment.

Cell sensitivity to insulin was evaluated *in vitro* on intact liver cells and liver cells preincubated (30 min) in culture medium with 0.5 U/kg imHEA-HA. The level of hepatic parenchymatous CFU (CFU-H) after addition of insulin (Sigma) in a concentration of 0.2 U/ml to culture medium was evaluated on day 10 of culturing in nutrient medium with high glucose concentration (6 mg/ml). Nonadherent round groups of at least 50 cells were regarded as CFU-H [11].

The results were processed by methods of variation statistical using the Student *t* test and nonparametric Mann–Whitney *U* test.

RESULTS

A course of alloxan injections led to the development of stable glycemia and animal death (16% by the end of experiment; Table 1). Treatment with the studied drugs was associated with a pronounced sugar-reducing effect: imHEA-HA reduced blood glucose in all periods of the study, the lowest level (42.7% of control, DM1) was recorded after 24 h. However, the parameter did not reach the basal level. In contrast to this treatment, parenteral short-acting insulin normalized glucose level in the peripheral blood as early as 2 h after injection; its corrective effects on hyperglycemia were recorded after 4, 6, and 24 h. Preliminary dose of imHEA-HA even more increased specific activity of the hormone. All animals developed hypoglycemic coma 2 h after insulin injection; immobilization lasted for 8-10 h, mortality after 24 h was 40%. Blood sugar concentration during all periods of the experiment was significantly lower than after insulin alone and was the least after 4 h (1.94 mmol/liter; Table 1).

Similar effects of the drugs were observed on the DM2 model. The development of this condition was confirmed by high initial blood glucose levels in CBA/CaLac mice aged 12-14 months (Table 2). Drug treatment of experimental animals led to a significant reduction of glucose levels in the peripheral blood. The effects of insulin were higher than of imHEA-HA early after injection (after 2 and 4 h). However, its normalizing effects on hyperglycemia were less pronounced (the maximum reduction, by 2.4 times, was recorded only 2 h postinjection) than in DM1 (maximum 5-fold reduction 2 h postinjection). Similarly as in DM1, the most significant correction was attained in case of combined use of the drugs. The changes were statistically significant only 2 h after injection (Table 2). This

TABLE 1. Dynamics of Peripheral Blood Glucose (mmol/liter) in 2-Month-Old Male CBA/CaLac Mice with Alloxan-Induced DM Treated with imHEA-HA, Insulin, or in HEA-HA in Combination with Insulin ($X \pm m$)

Group	Basal level	DM (before imHEA-HA) (7:45)	Before insulin (in groups 2 and 4 after imHEA-HA) (10:00)	2 h after insulin (12:00)	4 h after insulin (14:00)	6 h after insulin (16:00)	24 h after insulin (10:00)
1 (control; DM)			29.66±1.57*	24.36±2.51*	21.10±1.13*	32.88±0.42*	30.48±1.37*
2 (imHEA-HA)	7.78±0.23	28.54±1.07*	16.78±2.74*+	10.02±2.70+	13.42±2.43*+	31.20±1.08*	13.02±1.07*+
3 (insulin)			26.64±1.88*	4.91±1.25*+	3.96±0.66*+	17.71±3.97*+	17.64±1.94*+
4 (insulin+ imHEA-HA)			19.44±2.71*+o	2.08±0.12*+o	1.94±0.16*+o	3.90±0.68*+o	9.17±0.43*+o

Note. Protocol of drug administration and registration of results: 7:45: glucose measurements in all groups (DM); 8:00: imHEA-HA injections in groups 2 and 4; 10:00: glucose measurements in all groups; insulin injections in groups 3 and 4; 12:00, 14:00, 16:00, 10:00: glucose measurements in all groups. $p<0.05$ *vs.* *basal level, +control (group 1), °group 3. Basal level: intact animals.

TABLE 2. Dynamics of Glucose Levels in the Peripheral Blood of 12-14-Month-Old CBA/CaLac Mice with Insulin-Independent DM Treated with imHEA-HA, Insulin, and imHEA-HA in Combination with Insulin ($X \pm m$)

Group	Basal level	Before insulin (10:00)	2 h after insulin (12:00)	4 h after insulin (14:00)	6 h after insulin (16:00)	24 h after insulin (10:00)
imHEA-HA	16.07±0.60	13.76±0.31*	12.66±0.41*	15.18±0.42	13.54±1.01*	12.15±0.55*
Insulin		15.22±0.40	5.23±0.13*	10.07±0.71*	12.67±0.53*	11.52±0.22*
Insulin+ imHEA-HA		13.98±0.41*	4.22±0.16*+	9.56±0.65*	12.46±0.45*	12.16±0.23*

Note. $p \leq 0.05$ in comparison with: *control, +insulin alone.

fact can be explained by extremely severe disorders of the receptor system of insulin-sensitive cells to the ligand in old animals, which was in line with modern concepts on the key role of low sensitivity of insulin receptors in DM2 [3,6].

These results attest to significant potentiation of the specific effect of exogenous insulin and possible potentiation of the effects of endogenous hormone by imHEA-HA [9], this determining intrinsic hypoglycemic activity of the drugs.

In order to test the hypothesis on the relationship between these phenomena and the modulatory effect of imHEA-HA on target cell sensitivity to insulin, we carried out *in vitro* studies on hepatic cell culture, because glucose absorption by these cells is an insulin-dependent process [1]. Addition of the hormone to the culture medium increased significantly the CFU-H output (to 153.2% of control). Preincubation of cells with imHEA-HA more markedly stimulated colony formation under these conditions (Fig. 1): to 175.8 and 269.4% of the value in cultures without imHEA-HA in medium with insulin and in the control (without insulin), respectively.

Hence, imHEA-HA exhibited pronounced hypoglycemic activity in insulin-dependent DM and in spontaneous age-associated hypoglycemia (DM2). The mechanism of its effect consisted in modification of the properties of endogenous insulin receptors [6,9,13] paralleled by stimulation of target cell sensitivity to the ligand. On the other hand, stimulation of the therapeutic effects of exogenous insulin by imHEA-HA indicated good prospects for creation of a complex sugar-reducing drug. In addition, the phenomenon of imHEA-HA potentiation of progenitor cell stimulation by growth factors (for example, by insulin) suggested the involvement of this mechanism in the realization of the regenerative characteristics of modified enzyme [4,10].

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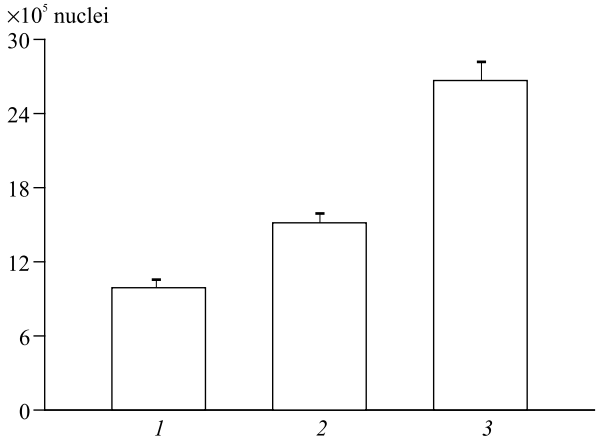

Fig. 1. Intensity of CFU-L growth from intact liver cells of CBA/CaLac mice in culture medium without insulin (1), with insulin (2), and from liver cells treated by imHEA-HA and insulin (3). Confidence intervals at $p \leq 0.05$.

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