Effect of Dexamethasone on Activity of Alkaline Proteases and Thymocytic pH

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The effect of dexamethasone on activity of alkaline proteases and intracellular pH of rat thymocytes is studied. Dexamethasone increases the activity of alkaline proteases in the thymus. Intracellular pH changes under the effect of the hormone in two phases both *in vivo* and *in vitro*. Possible mechanism of rapid acidification of the cytoplasm followed by its alkalization is discussed.

Key Words: *dexamethasone; thymocytes; intracellular pH; alkaline proteases; phenylmethylsulfonyl fluoride*

Clinical use of glucocorticoid hormones and their synthetic analogs involves many complications. One of the effects of steroids with glucocorticoid activity is lymphoid tissue catabolism and involution of the thymus associated with it. The mechanism of this effect is not clear. Glucocorticoids in doses causing skeletal muscle atrophy increase the activity of proteolytic enzymes with optimum effect at alkaline pH [7].

Previously, we demonstrated that dexamethasone (DM), a synthetic glucocorticoid, increases thymic alkaline protease (AP) activity in animals [5,6]. Proteolytic activity of homogenate depended on pH of incubation medium. In this study we investigated the effect of DM on intracellular pH(pH_i) of thymocytes and AP activity.

MATERIALS AND METHODS

Thymus of male Wistar rats weighing 150-200 g was examined. After decapitation, the thymus was placed in the medium containing 0.14 M NaCl, 5 mM KCl, 1 mM Mg_2SO_4, 4 mM $NaHCO_3$, 0.5 mM KH_2PO_4, 0.4 mM Na_2HPO_2 $12\times H_2O$, 5 mM glucose, pH 7.2. Cells were obtained by careful disintegration of the thymus with Teflon brushes, and suspension was then filtered through a Kapron filter. Suspension of thymocytes (2×10^7 cell/ml) was stored as a thin (no more than 3 mm) layer at 37°C.

Viability of cells was assessed by Trypan Blue absorption [4]. The number of viable cells during incubation *in vitro* was 90-92%.

For assessing protease activity, 1 ml cell suspension was centrifuged at 1000*g* for 5 min. The precipitate was resuspended in 350 μl 0.05 M Tris-HCl buffer (pH 8.5) with 0.14 M NaCl. The cells were destroyed by homogenization. AP activity was estimated by azocasein (Sigma) hydrolysis [10]. 350 μl homogenate was incubated for 30 min at 37°C, then 50 μl fresh azocasein solution (2 mg/ml) in 0.05 M Tris-HCl buffer was added, and the mixture was incubated at 37°C for 60 min. The reaction was stopped by adding 400 μl cold 10% aqueous solution of trichloroacetic acid. The samples were kept on the cold (0-4°C) for 10 min and then centrifuged at 3000*g* for 15 min. Light absorbance of the supernatant was measured at 366 nm in a CPC-2CP photocolorimeter.

Intracellular (pH_i) was measured as described previously [1]. Cells were stained with fluorescein diacetate, which was added to a final concentration of 10^{-5} M, and cells were incubated at 37°C for 5 min.

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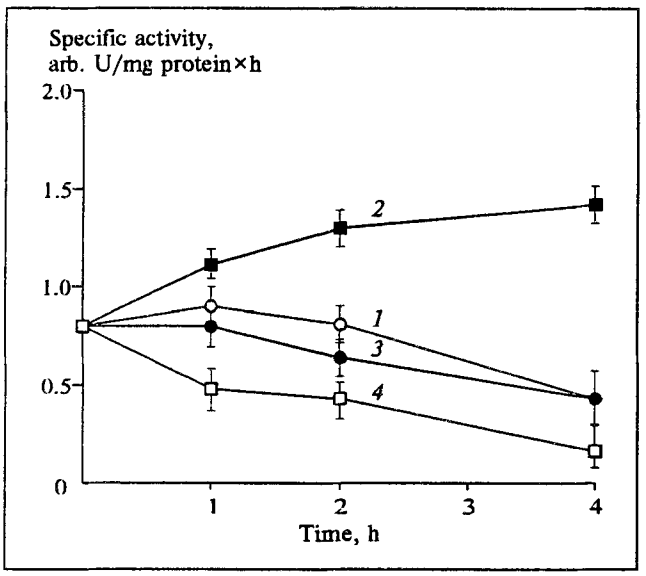

Fig. 1. Time course of thymus alkaline protease activity *in vitro: 1*) control; *2*) dexamethasone; 3×10^{-7} M; *3*) phenylmethylsulfonyl fluoride (PMSF), 5 mM; *4*) dexamethasone, 3×10^{-7} M and PMSF, 5 mM. Here and in Fig. 2 the values are the means of three determinations.

The ratio of fluorescence intensities at 530 and 570 nm minus background fluorescence of the medium adjacent to the cells was measured. Mean value pH_i for the population was calculated from individual pH_i of 40-60 cells. The error in the mean population pH_i was no more than 0.03 pH unit. Calibration curves were plotted by equilibration of medium pH and pH_i

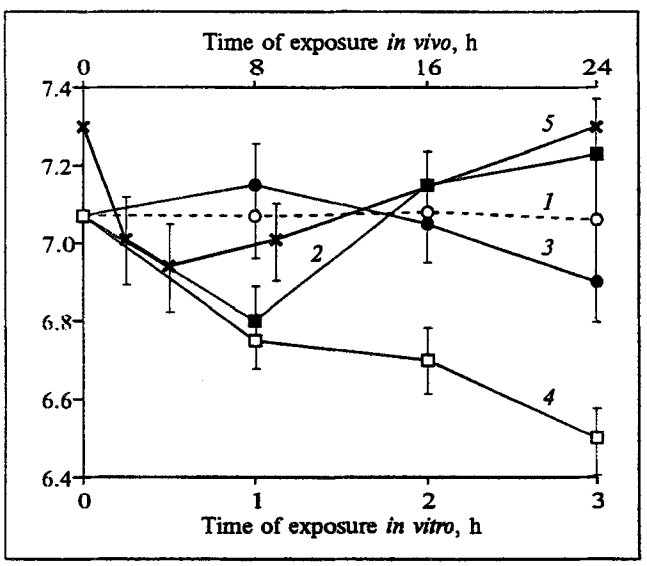

Fig. 2. Time course of intracellular pH *in vitro: 1*) control; *2*) dexamethasone; 3×10^{-7} M; *3*) phenylmethylsulfonyl fluoride (PMSF), 5 mM; *4*) dexamethasone, 3×10^{-7} M and PMSF, 5 mM; *5*) time course of intracellular pH *in vivo* (dexamethasone dose 2 mg/kg). Ordinate: intracellular pH, U.

with nigericine (Calbiochem) [11]. Fluorescence of cells and solutions was measured at 37°C.

DM phosphate (Dexasone, Galenica) was used in a final concentration of 3×10^{-7} M *in vitro;* the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF, Sigma) was added to a final concentration of 5 mM 10 min before the hormone.

For assessing changes in intracellular pH *in vivo,* DM was injected intraperitoneally in 0.9% NaCl solution (0.5 ml) in a dose of 2 mg/kg. To controls, 0.5 ml 0.9% NaCl was injected. The animals were sacrificed 2, 4, 9, and 24 h after injection.

Protein was measured by the method of Lowry [9].

Results were processed by standard mathematical statistics methods [3].

RESULTS

The chosen experimental conditions allowed us to study the effect of hormone on thymocytes in the presence of an inhibitor.

Figure 1 shows changes in the activity of thymocytic AP *in vitro.* Figure 1, *1* shows that the activity in the control suspension decreases negligibly during 4 h. After addition of DM (curve *2*), AP activity increases.

In order to identify the class of DM-activated proteases, PMSF was added to suspension. The inhibitor virtually does not decrease the basic enzymatic activity (Fig. 1, *3*). The hormone-inhibitor combination (curve *4*) suppresses hormone-dependent activation of enzymes. The hormone activated thymic AP. Activated enzyme may be more sensitive to the inhibitor, or other serine hydrolases may participate in the protease activation.

Alteration of pH_i is a possible mechanism regulating enzyme activity.

Figure 2 presents data on pH_i measurements in cell suspension. The pH_i changed negligibly after incubation of control suspension of thymocytes (curve *1*) at 37°C, indicating that incubation conditions are favorable. Alteration of pH_i in the presence of hormone (curve *2*) is biphasic. During the first stage (the first hour) pH_i drops and then starts to grow, its value surpassing the control after 2 h.

During the first minutes after addition of the hormone, pH_i (curve *2*) drops below 6.5, i. e., below the lower pH_i threshold value determined by this method. Therefore, the first pH_i value after addition of the hormone is the value for 1 h (Fig. 2). It means that the maximum drop in pH_i is observed during the first minutes after hormone addition and anticipates the increase in enzyme activity. Figure 2, *2,* shows that pH_i during exposure to hormone and pH_i (curve *4*) during combined exposure to hormone and

inhibitor virtually coincide 1 h after hormone addition. The presence of an inhibitor modifies the time course of pH_i changes in principle, preventing its recovery.

The concentration of intracellular Ca^{2+} in competent cells increases under the action of glucocorticoids [2]. After increase of intracellular concentration of Ca^{2+}, both the production and uptake of H^+ are stimulated in the cells, leading to a decrease in intracellular pH [9]. This causes activation of Na^+/H^+-antiport, expressed in alkalization of the cytoplasm. Hence, the two-phase pH_i response to hormonal exposure *in vitro* can be explained by alteration of ionic currents Ca^{2+}, H^+, and Na^+.

Addition of serine protease inhibitor to thymocyte suspension caused no changes in pH_i (curve *3*). A monotonous decrease of pH_i was observed in suspension to which PMSF was added before the hormone (curve *4*). Suppression of pH_i recovery by serine protease inhibitor and contribution of AP to this process are still to be investigated.

Effect of inhibitor on enzyme activity and pH_i is stronger in the presence of the hormone than during exposure to the inhibitor alone.

For study of DM effect on pH_i *in vivo*, pH_i was measured 2, 4, 9, and 24 h after hormone injection. Figure 2, *5*, shows that, like *in vitro*, the pattern of pH_i changes *in vivo* was biphasic. After hormone injection, pH_i decreased at the first stage (up to 4th h) and then was gradually normalized. It is noteworthy that DM cytotoxicity in fact did not manifest itself during 4 h. Involution of the thymus was observed 24 h after injection of DM. By response to stress exposure and glucocorticoids, thymocytes are divided into 2 subpopulations: hormone-resistant and hormone-sensitive (dying during 24 h after hormone exposure). The resultant pH_i after 24 h appears to correspond to the response of hormone-resistant cells. The two-phase pattern of pH_i changes *in vivo* can be explained by the effect of hormone on the ratio of thymocyte subpopulations.

From our results it can be concluded that dexamethasone increases the activity of thymic AP *in vitro*. Injection of dexamethasone to animals and its addition to thymocyte suspension brought about changes in pH_i, running through two phases. Presumably, this change is not responsible for increased AP activity, although the probability of this mechanism at some stages of hormone response should not be ruled out. Serine hydrolases affect changes in pH_i after dexamethasone injection.

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