INHIBITION OF Na^+/H^+ EXCHANGER AND INSULIN-STIMULATED LIPOGENESIS IN RAT ADIPOCYTES

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ABSTRACT

Insulin-stimulated lipogenesis in rat adipocytes was inhibited by amiloride, an inhibitor of Na^+/H^+ exchange system. Amiloride at 0.1 mM inhibited the lipogenesis by 50%. The action of insulin on lipogenesis was shown to depend on the external Na^+ concentration. Other monovalent cations such as Li^+ , K^+ , Rb^+ and choline⁺ could not replace Na^+ . Although basal lipogenesis was stimulated 2-fold in the buffer containing Li^+ , insulin did not further stimulate the lipogenesis in Li^+ -buffer. Specific binding of insulin to adipocytes was increased by 1-3 mM amiloride in Na^+ -buffer, but remained unchanged in Na^+ -free buffers. In choline⁺-buffer, insulin binding was increased. Amiloride did not decrease ATP content in adipocytes under our experimental conditions. The results suggest that the Na^+/H^+ exchange system is involved in insulin-stimulated lipogenesis in rat adipocytes.

Insulin is known to accelerate the Na^+/H^+ exchange transport in muscle cells (9, 19-21, 24, 27), hepatocytes (2, 3) and fibroblasts (12, 22, 23, 29). Activation of the exchanger has been implicated not only in Na^+ entry but also in regulation of cytoplasmic pH (for reviews, see references 6, 18).

Moore demonstrated that insulin increased the intracellular Na^+ concentration and intracellular pH in frog skeletal muscle (19-21). Rosic *et al.* (27) reported that insulin induced a large increase in Na^+/H^+ exchange transport and that passive Na^+ influx was the mediator responsible for insulin-induced stimulation of membrane Na^+/K^+ -ATPase activity in cultured muscle cell line BC3H-1. Klip *et al.* (19) also reported that in cultured muscle cells, cytoplasmic alkalization by activation of the Na^+/H^+ antiport was one of the earliest responses to insulin, but this alkalization was not sufficient to stimulate glucose transport.

Letarte and Renold demonstrated that the presence of Na^+ in medium was essential for insulin-stimulated glucose uptake in isolated mouse adipocytes (13-15). However, Resh *et al.* (25) suggested that insulin did not increase the ^{22}Na uptake in rat adipocytes, but did affect Na^+/K^+ -ATPase.

In the present investigation, we examined the involvement of Na^+/H^+ exchanger in the lipogenic action of insulin in rat epididymal adipocytes.

MATERIALS AND METHODS

Materials

Collagenase (cell-disperse type) and bovine serum albumin (BSA, fraction V) were obtained from Wako Pure Chemicals. Amiloride and ouabain were from Sigma; D-[U-¹⁴C]glucose (specific activity 3.9 mCi/mmol) and ¹²⁵I-insulin (receptor grade; specific activity 2,200 Ci/mmol) were from NEN Research

Products.

Preparation of Adipocytes

Epididymal adipocytes were prepared from male Wistar rats (150–200 g). Collagenase digestion was carried out in Hanks' solution (pH 7.4) buffered with 25 mM *N*-2-hydroxy-piperazine-*N'*-2-ethanesulfonic acid (HEPES) containing 1 mg/ml collagenase, 4% BSA and 0.5 mg/ml glucose by the method of Rodbell (26). Cells were filtered through nylon mesh and washed twice with incubation buffer by centrifugation at 100 g for a few minutes.

Lipogenesis

A 50 μ l aliquot of the packed adipocytes was added to Hanks' buffer (pH 7.4) containing 4% BSA and 3 mM glucose in a final volume of 0.25 ml in a polypropylene tube and preincubated for 20 min at 37°C. Insulin and a test drug were then added. After 10 min incubation, [14 C]glucose was added and incubation was continued for 30 min. The reaction was stopped by adding 2.5 ml of Dole's extraction mixture (isopropyl alcohol/heptane/1 N H_2SO_4 =40/10/1; v/v) (1). The tube was shaken and 1.5 ml heptane and 1.25 ml water were added, and the mixture was again shaken and centrifuged. The heptane extract was washed with the same volume of alkaline ethanol (0.05 N NaOH in 50% ethanol) to remove free fatty acids. The radioactivity of upper heptane layer was counted. In each assay, lipogenesis was measured in triplicate and expressed as mean $\pm$ SE. A representative of at least three similar assays is shown.

In some experiments indicated, Na^+ in the medium buffered with 10 mM Tris at pH 7.4 was replaced by 140 mM Li^+ , K^+ or Rb^+ . Also, the concentration of Na^+ was reduced with the addition of equimolar choline chloride.

Insulin Binding

The binding of ^{125}I -insulin was measured by the oil centrifugation technique (4). Briefly, fat cells were incubated in the buffer (the same as for lipogenesis assay) at 15°C for 3 h in the presence of ^{125}I -insulin (33 pM) and 0–2,000 nM native insulin. The incubation was terminated by centrifuging 200 μ l-aliquot of

incubation mixture through 150 μ l dinonyl-phthalate. Nonspecific binding (^{125}I -insulin bound in the presence of 2,000 nM native insulin) was subtracted from the total binding to obtain the specific binding. Nonspecific binding was not more than 10% of the total binding. Fat cells were incubated at 37°C for 20 min before binding assay was initiated at 15°C. Binding assay was performed at 15°C because insulin internalization is negligible at this low temperature (17, 31). Also, binding assay was performed in the presence of 4% BSA because BSA at this concentration strongly prevents insulin degradation, without interfering with insulin binding or insulin effect of converting labeled glucose to lipid (5). Specific binding is expressed as mean $\pm$ SE of triplicate assay in each experiment, and each experiment is a representative of at least three similar experiments.

ATP Determination

Fat cells were incubated at 37°C for indicated periods in the presence or absence of amilo-

Fig. 1 Effects of amiloride and ouabain on basal (A) and insulin-stimulated (B) lipogenesis. Adipocytes were preincubated in drug-free buffer for 20 min at 37°C, and then were added amiloride (○) or ouabain (Δ), and insulin (1 nM; only in B). After 10 min, lipogenesis was initiated by addition of [14 C]glucose.

Fig. 2 Effects of K-buffer and amiloride on lipogenesis. After collagenase treatment of adipose tissue, adipocytes were washed with Na- (○, ●) or K-buffer (△, ▲) and then processed as described in Fig. 1 and Materials and Methods. Lipogenesis was initiated by addition of [¹⁴C]glucose in the absence (○, △) or presence (●, ▲) of amiloride (1 mM).

ride or KCN. At the end of incubation, ATP was determined in each sample with an ATP Bioluminescence CLS Kit (Boehringer Mannheim).

RESULTS

The effect of amiloride on lipogenesis from glucose was examined in rat adipocytes. As shown in Fig. 1, insulin-stimulated lipogenesis was 50% inhibited by 0.1 mM amiloride. Ouabain, which is known to influence cell Na⁺ concentration by inhibiting Na⁺, K⁺-ATPase, had no effect on insulin-stimulated lipogenesis. Basal lipogenesis was enhanced to 200% by 0.1–3 mM ouabain. Inhibition of insulin-stimulated lipogenesis by amiloride suggests an important role of the Na⁺/H⁺ exchange system in insulin-stimulated lipogenesis. In order to inhibit the Na⁺/H⁺ exchanger, equimolar KCl was substituted for NaCl (Fig. 2). In the K-buffer, one logarithmic rightward shift in the insulin dose-response curve was observed. Also, a decrease in the maximal insulin-stimulated lipogenesis was also observed (60% of that in Na-buffer) (Fig. 2). Amiloride inhibited the 100 nM insulin-stimulated lipogenesis by 76% in Na-buffer and by 100% in K-buffer.

Insulin-stimulated lipogenesis depended on

Fig. 3 Effects of Na⁺ concentrations on lipogenesis. After collagenase treatment of adipose tissue, adipocytes were washed with the buffer containing various concentrations of NaCl. Reduction in NaCl concentration was substituted by equimolar choline chloride. Adipocytes were preincubated and then incubated in the absence (shaded column) or presence (open column) of insulin (1 nM). After 10 min incubation, lipogenesis was initiated by addition of [¹⁴C]glucose.

the Na concentration in the buffer (Fig. 3). In Na-free buffer, insulin failed to stimulate lipogenesis; 50% stimulation of lipogenesis by insulin required about 50 mM Na.

In K-, Rb- or choline-buffer, insulin-stimulated lipogenesis was completely inhibited (Fig. 4). In Li-buffer, basal lipogenesis was considerably elevated, but insulin failed to further stimulate the basal lipogenesis.

In contrast to insulin-stimulated lipogenesis in Na-buffer which was inhibited by amiloride (Fig. 1), specific binding of insulin to adipocytes was markedly stimulated by amiloride at concentrations higher than 1 mM (Fig. 5). In Li- and K-buffer, insulin binding was not changed, whereas the binding was significantly increased in choline-buffer (Table 1).

Under our experimental conditions, amiloride failed to reduce the ATP content in adipocytes (Table 2). On the other hand, the ATP content was considerably decreased by KCN (Table 2).

DISCUSSION

In the present investigation, amiloride is found to inhibit insulin-stimulated lipogenesis from glucose in rat adipocytes. Amiloride is known to be an inhibitor of the Na⁺/H⁺

Fig. 4 Effects of cations on lipogenesis. After collagenase treatment of adipose tissue, adipocytes were washed with each cation buffer and preincubated for 20 min at 37°C. Then, adipocytes were incubated in the absence (shaded column) or presence (open column) of insulin (1 nM). After 10 min, lipogenesis was initiated by adding [¹⁴C]glucose.

Fig. 5 Effect of amiloride on specific insulin binding. Adipocytes (10%, v/v) were preincubated in the buffer containing indicated concentration of amiloride for 20 min at 37°C and then were incubated in the presence of 33 pM ¹²⁵I-insulin for 3 h at 15°C. After incubation, the reaction was stopped as described in Materials and Methods. Specific ¹²⁵I-insulin binding was obtained by subtracting nonspecific binding (¹²⁵I-insulin bound in the presence of 2,000 nM native insulin) from the total binding. Nonspecific binding did not vary at each concentration of amiloride and was not more than 10% of the total binding.

Table 1 Effects of Cations on Specific Insulin Binding

Buffer	B/F × 100
Li ⁺	4.13 ± 0.28
Na ⁺	3.53 ± 0.09
K ⁺	3.22 ± 0.04
Choline ⁺	5.35 ± 0.03*

After collagenase treatment, adipocytes were washed with each cation buffer and preincubated for 20 min at 37°C, and then incubated in the presence of 33 pM ¹²⁵I-insulin for 3 h at 15°C, and processed as described in Materials and Methods. Nonspecific binding (¹²⁵I-insulin bound in the presence of 2,000 nM native insulin) was subtracted from the total binding to obtain specific binding. Nonspecific binding did not vary in each cation buffer, and was not more than 10% of the total binding. *Specific binding in the choline-buffer was significantly increased compared with that in Na-buffer.

exchanger (6). Besides this action, amiloride is reported to reduce cellular ATP and inhibit protein synthesis (3, 11). Therefore, there is a possibility that amiloride inhibits insulin-stimulated lipogenesis by actions other than inhibition of the Na⁺/H⁺ exchange system in adipocytes. In fact, KCN, which reduces the cellular ATP (Table 2), was found to inhibit both basal and insulin-stimulated lipogenesis

(our unpublished observations; 10). In the present experiment, however, amiloride did not reduce the ATP content in the adipocytes (Table 2), and cycloheximide, an inhibitor of protein synthesis, did not affect basal and insulin-stimulated lipogenesis (unpublished data). These results suggest that the inhibitory action of amiloride on insulin-stimulated lipogenesis is mediated through the suppression

Table 2 *Effects of Amiloride and KCN on the Cellular ATP Content in Rat Adipocytes*

Incubation (min)	Control	Amiloride (1 mM)	KCN (1 mM)
ATP (10^{-10} mol/50 μ l packed adipocytes)			
0	2.6	—	—
15	2.6	2.6	0.4
45	2.5	3.1	—

Adipocytes were preincubated in the Hanks buffer (pH 7.4) containing 4% BSA and 3 mM glucose for 20 min at 37°C, and then incubated in the absence or presence of amiloride or KCN for indicated periods at 37°C. The reaction was stopped by addition of 10 vol of boiled water. The mixture was vigorously shaken and centrifuged at 1,500 g for 10 min, and the supernatant solution was used for ATP assay. Each value represents the mean of duplicate assay; a representative of three similar experiments is given.

of the Na^+/H^+ exchanger.

Furthermore, we have demonstrated that Na^+ is required for the lipogenic action of insulin, and replacement of Na^+ with Li^+ , K^+ , Rb^+ or choline $^+$ considerably reduces the insulin action. The fact that Na^+ is required also affords additional evidence that the Na^+/H^+ exchanger participates in the insulin action. In Li-buffer, the basal lipogenesis was increased as compared to that in Na-buffer. Li^+ is reported to have a higher affinity toward the Na^+/H^+ exchanger than Na^+ (28, 8, 7). Therefore, it seems likely that Li^+ stimulates lipogenesis and the Na^+/H^+ exchanger through the same mechanism as Na^+ .

Sonne *et al.* (30) reported that an acidic shift of medium pH (pH 7.8 to 7.2) caused marked reductions in both the lipogenic action of insulin and its specific binding to rat isolated adipocytes, and concluded that reduction in the insulin specific binding induced the decrease in the lipogenic action of this hormone. In our present investigation, however, insulin-stimulated lipogenesis was reduced in the presence of amiloride or in K-, Rb- and choline-buffers without reduction in specific insulin binding to adipocytes. Further, amiloride and choline-buffer enhanced the specific insulin binding. All these conditions, including the acidic shift of medium pH, were known to induce the inhibition of the Na^+/H^+ exchanger. Therefore, it can be alternatively proposed that the inhibition of insulin-stimu-

lated lipogenesis under all these conditions is due to inhibition of the Na^+/H^+ exchanger.

In conclusion, we propose that the amiloride-sensitive Na^+/H^+ exchanger is involved in part in the mechanism of insulin-stimulated lipogenesis in adipocytes.

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