Vol. 122, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 18, 1984 Pages 204-211

CONCANAVALIN A CAN TRAP INSULIN AND INCREASE INSULIN INTERNALIZATION INTO CELLS CULTURED IN MONOLAYER

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Received May 16, 1984

SUMMARY: When rat hepatoma cells (R-Y121B) were incubated with insulin at 37°C, concanavalin A increased insulin internalization into cells. When R-Y121B cells were first incubated with labeled insulin at 4°C then with concanavalin A at various concentrations at 37°C, the total cellular radioactivity was much higher at high lectin concentrations than at low lectin concentrations. This increase was not only due to an increase in insulin internalization into cells but also to an increase in insulin binding to cell surfaces. Concanavalin A can trap insulin on the insulin receptors - a "trapping" effect. It has been concluded that insulin and concanavalin A binding sites are very close to each other on the insulin receptors.

It is well known that the incubation of cells with concanavalin A (Con A) before insulin addition decreases insulin binding to cells (1-4). The same result was obtained with solubilized insulin receptors (5). When cells were first incubated with insulin then with insulin plus Con A (3) or wheat germ agglutinin (1), an increase in insulin binding to cells was observed at low lectin concentrations. Recently, the effects of Con A on insulin binding to cells were reinvestigated in rat hepatoma cells (R117-21B) cultured in a monolayer (6), and it was shown that the effects of Con A on insulin behaviour on the cell surface depended on the order of the addition of the ligands to the incubation medium in which the cells were cultured: The preincubation of cells with Con A decreases insulin binding, but the addition of Con A after preincubation with insulin increases the total cellular radioactivity due to labeled insulin even at high Con A concentrations. These different Con A effects on insulin behaviour on the cell surface were observed in various cell lines (7). The present study has been designed to clarify the reason why Con A

0006-291X/84 $1.50

increases the total cellular radioactivity, when cells are first incubated with labeled insulin and then with Con A.

MATERIALS AND METHODS

Chemicals. Con A was purchased from Calbiochem (San Diego, Calif., U.S.A.) and α-methyl-D-mannoside was from Nakarai Chemicals, Ltd. (Kyoto, Japan). Bovine insulin (24 I.U./mg) was purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Labeled insulin with ^{125}I (~100 μCi/μg) was from New England Nuclear (Boston, Mass., U.S.A.).

Cell culture. Rat hepatoma cells (R-Y121B) (8) were established from Reuber rat hepatoma cells (H4-II-E) (9). The culture conditions have been described in detail elsewhere (10). R-Y121B cells were cultured in plastic culture dishes with a 35-mm diameter (Corning, N.Y., U.S.A.). After the cells almost reached confluence, they were used for the binding assay of insulin.

Binding assay of insulin. Insulin binding was carried out according to a previously reported method developed for the cells cultured in a monolayer (11,12). R-Y121B cells were incubated with ^{125}I-labeled insulin in a modified Eagle's minimum essential medium (11) containing 0.5% bovine serum albumin.

Release of insulin bound to cells. After incubating the cells with labeled insulin under various conditions, the cells were washed twice with 0.5 ml of ice-cold phosphate-buffered saline, pH 7.4, and then they were exposed to fresh culture media containing a large excess of unlabeled insulin, α-methyl-D-mannoside or both materials. Our previous study showed that the release of Con A bound to cells was very slow at 4°C even in the presence of 50 mM of α-methyl-D-mannoside, and that Con A bound to cells was not released at 37°C by fresh medium without α-methyl-D-mannoside (12). As for insulin release by the addition of a large excess of unalbeled insulin, our study showed that 81% and 95% of the insulin bound to the cells at 4°C was released after 10 min and 20 min of incubation at 37°C, respectively, and insulin bound to cells was not released at 4°C by a large excess of unlabeled insulin (unpublished data). Therefore, in order to measure the amount of insulin bound to cell surfaces and internalized, after the cells had been incubated with labeled insulin under various conditions, the incubation media were replaced with fresh media containing unlabeled insulin or the mannoside at 25°C or 37°C. The radioactivity released by α-methyl-D-mannoside plus a large excess of unlabeled insulin was assumed to represent insulin bound to cell surfaces, and the unreleased radioactivity was assumed to represent internalized insulin.

RESULTS

When R-Y121B cells were incubated with insulin at 37°C, both insulin bound to cell surfaces and that internalized reached a plateau after 20 min of incubation. When Con A and insulin were added together and incubated with the cells at various Con A concentrations, the highest cellular radioactivity due to insulin bound to cell surfaces and internalized was observed at 5-10 μg/ml of Con A (Fig. 1, upper panel). The highest radioactivity internalized was also observed at the same Con A concentration: The effect of Con A on insulin internalization rose with concentration at low Con A concentrations and fell at high, when Con A and

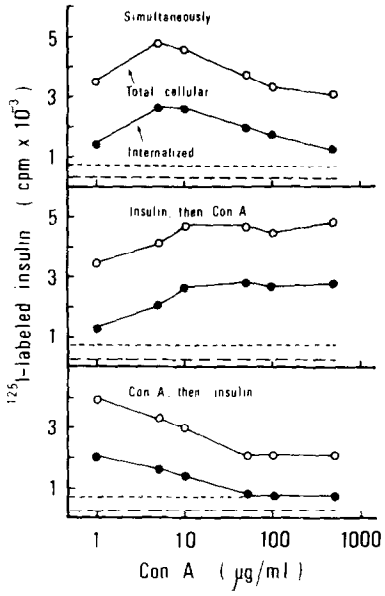

Fig. 1. Effects of Con A at various concentrations on insulin binding and insulin internalization by R-Y121B cells. Upper panel, cells (2.4 x 10^6 cells/dish) were incubated with insulin plus Con A for 1 h; middle panel, cells were first incubated with insulin for 30 min then with insulin plus Con A for 30 min; lower panel, cells were first incubated with Con A for 30 min then with Con A plus insulin for 1 h. The incubation medium consisted of 0.8 ml of culture medium, 0.1 ml of labeled insulin (71,000 cpm, 3.1 ng) and 0.1 ml of Con A. The release of insulin from the cells was carried out by addition of unlabeled insulin (20 μg/ml) plus α-methyl-D-mannoside (50 mM) at 25°C for 30 min of incubation. The dotted line represents the radioactivity released by unlabeled insulin plus the mannoside from the cells which had been incubated with labeled insulin in the presence of unlabeled insulin (20 μg/ml), and the broken line represents the radioactivity unreleased.

labeled insulin were incubated together with cells. The preincubation of the cells with labeled insulin before Con A addition increased the total cellular radioactivity with increasing concentration of Con A (Fig. 1, middle panel). This result shows that the increase in the total cellular radioactivity is due to the increase in the radioactivity internalized. On the other hand, the preincubation of the cells with Con A before insulin addition decreased the total cellular radioactivity with increasing concentration of Con A (Fig. 1, lower panel). It is clear that the decrease in the total cellular radioactivity is due to the decrease in the radioactivity internalized.

The radioactivity unreleased by α-methyl-D-mannoside plus a large excess of unlabeled insulin was very small, when the first incubation of

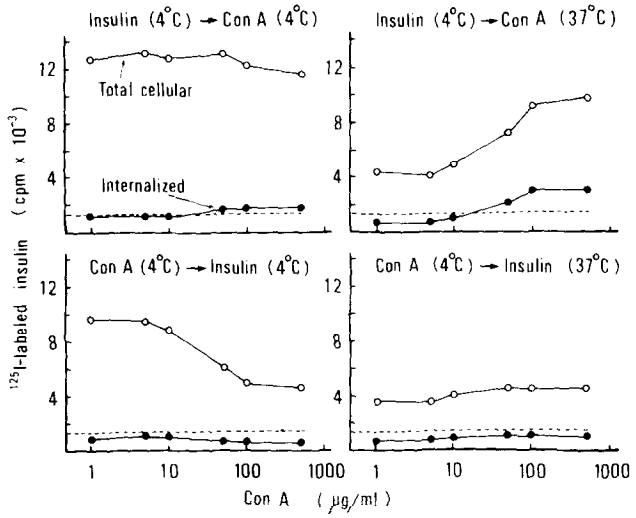

Fig. 2. Effects of various concentrations of Con A at different temperatures on insulin binding and insulin internalization by R-Y121B cells. The cells (2.1×10^6 cells/dish) were incubated with insulin at 4°C for 30 min then with insulin plus Con A at 4°C for 1 h (upper left panel) or at 37°C for 1 h (upper right panel). The cells were incubated with Con A at 4°C for 30 min then with Con A plus insulin at 4°C for 1 h (lower left panel) or at 37°C for 1 h (lower right panel). The incubation medium consisted of 0.8 ml of culture medium, 0.1 ml of labeled insulin (120,000 cpm, 1.5 ng) and 0.1 ml of Con A. The release of insulin from the cells was carried out by addition of unlabeled insulin (20 μg/ml) plus α-methyl-D-mannoside (50 mM) at 37°C for 30 min of incubation. The dotted line represents the total cellular radioactivity due to labeled insulin in the presence of unlabeled insulin (20 μg/ml).

cells with insulin was carried out at 4°C (Fig. 2, upper left panel). In this case, no effect of Con A on insulin binding was apparently observed. When the incubation of cells with insulin was carried out at 4°C then with insulin plus Con A at 37°C, the total cellular radioactivity increased with increasing concentration of Con A. The radioactivity internalized also increased at high Con A concentrations (Fig. 2, upper right panel).

When the incubation of cells with Con A then with labeled insulin plus Con A was carried out at 4°C, the total cellular radioactivity decreased with increasing concentration of Con A (Fig. 2, lower left panel). However, the second incubation of cells with Con A plus insulin at 37°C decreased the total cellular radioactivity at low Con A concentrations, although the total cellular radioactivity changed little at high Con A concentrations between 4°C and 37°C (Fig. 2, lower left and right panels). In the absence of Con A, insulin binding to cells at 4°C was much higher

than that at 25°C or 37°C (6,13). Even though insulin binding to cells reached a plateau at 37°C, the further incubation of the cells at 4°C increased insulin binding (data not shown). It seems, therefore, that lower insulin binding capacity at 37°C than at 4°C is due to changes in the characteristics of insulin receptors, rather than insulin degradation.

At a low Con A concentration (1 μg/ml), the effect of Con A on insulin binding and internalization was almost absent, when insulin bound to cells, preincubated with insulin at 4°C then with insulin plus Con A at 4°C and 37°C, was released under various conditions (Table 1); the released or the unreleased radioactivities from the cells were almost the same as those obtained from the incubation of cells with insulin at 37°C in the absence of Con A. On the other hand, when the cells preincubated with insulin at 4°C were treated with Con A at a concentration of 500 μg/ml at 4°C for 30 min then at 37°C for 1 h, a large amount of cellular radioactivity was released by α-methyl-D-mannoside in the presence or absence of a large excess of unlabeled insulin - a "trapping" effect of Con A on insulin binding to cells. However, only a large excess of unlabeled insulin released a small amount of cellular radioactivity from the insulin-, Con A- and cell-complexes.

DISCUSSION

The preincubation of cells with Con A reduces insulin binding to cells (1-4). Lectins mimic insulin in their enzyme regulation in cells (4,14-17). Judging from these results, it seems that Con A and insulin share the same binding sites, although Katzen et al. (18) have shown that these two ligands bind to insulin receptors without inhibiting each other. On the other hand, when cells were first incubated with labeled insulin, insulin binding increased with lectin concentration at low concentrations (<8 μg/ml) (1,3) and even at high Con A concentrations (6). Capeau and Picard (19) concluded that this phenomenon was nonspecific binding of Con A to cells. Recently, it has been speculated that the Con A, which is much larger than insulin, bound to insulin receptors interferes with the release

Table 1. Binding and internalization of insulin by R-Y121B cells under various conditions

Condition	Radioactivity (cpm/dish)		
	Released	Unreleased	Total
Insulin, 4°C → Con A, 4°C → Mannoside + Cold insulin (1 μg/ml)	7,300 ± 400	1,280 ± 50	8,600 ± 500
→ → 37°C → Cold insulin	1,850 ± 90	740 ± 50	2,600 ± 100
→ → → Mannoside	2,100 ± 200	650 ± 40	2,700 ± 200
→ → → Mannoside + Cold insulin	1,980 ± 30	690 ± 10	2,670 ± 30
+ Cold insulin, 4°C → Con A, 4°C → 37°C → Mannoside + Cold insulin	630 ± 60	280 ± 50	900 ± 40
Insulin, 4°C → Con A, 4°C → Mannoside + Cold insulin (500 μg/ml)	4,870 ± 70	3,100 ± 200	8,000 ± 200
→ → 37°C → Cold insulin	1,520 ± 80	7,500 ± 300	9,000 ± 300
→ → → Mannoside	5,600 ± 90	2,800 ± 100	8,400 ± 200
→ → → Mannoside + Cold insulin	5,500 ± 200	3,000 ± 200	8,500 ± 300
+ Cold insulin, 4°C → Con A, 4°C → 37°C → Mannoside + Cold insulin	600 ± 80	220 ± 30	820 ± 60
Insulin, 37°C → Mannoside + Cold insulin	2,200 ± 100	530 ± 40	2,700 ± 100
+ Cold insulin, 37°C → Mannoside + Cold insulin	800 ± 100	230 ± 10	1,000 ± 100

R-Y121B cells (2.6×10^6 cells/dish) were incubated with labeled insulin (70,000 cpm, 1.2 ng). After the cells were first incubated with insulin for 1 h and then with insulin plus Con A for 30 min, they were kept at 37°C for 1 h. In some cases, the second and third steps were omitted. The release of insulin was carried out by a large excess of cold insulin (20 μg/ml) plus α-methyl-D-mannoside (50 mM) at 37°C for 30 min of incubation. The incubation medium with labeled insulin consisted of 0.7 ml of culture medium, 0.1 ml of labeled insulin, 0.1 ml of Con A and 0.1 ml of cold insulin or 0.1 ml of culture medium. The final volume was 1 ml. The value given is the mean ± S.D. for the values of three culture dishes.

of insulin from the insulin-, Con A- and cell-complexes or with the subsequent binding of insulin to insulin receptors (6). Since the effects of Con A on insulin binding to cells were different at 4°C depending on the order of addition to the incubation medium, they might be due to the interaction of insulin molecules bound to insulin receptors and Con A (6).

The present experiments showed that Con A increased insulin internalization. Since Con A bound to insulin receptors inhibits subsequent insulin binding, eventually insulin internalization is apparently inhibited by Con A (Fig. 1, lower panel). Therefore, when cells were incubated together with insulin plus Con A, the effect of Con A on insulin internalization rose with concentration at low concentrations and fell at high (Fig. 1, upper panel).

The decrease in insulin binding to cells as the temperature changes to 37°C from 4°C in the presence of Con A was observed only at low Con A concentrations (<10 μg/ml), but not at high Con A concentrations (>50 μg/ml) (Fig. 2, lower left and right panels). Our previous data (6) and other data (2,20,21) showed that insulin receptors are classified into two types of insulin binding sites for insulin: In one, insulin binding is independent of Con A; in the other, binding is affected by Con A. In addition, when the cells were first incubated with insulin at 4°C then with Con A (500 μg/ml) at 37°C, only 17% of cellular radioactivity was released by a large excess of unlabeled insulin in the absence of α-methyl-D-mannoside, but 65-68% was released by α-methyl-D-mannoside alone or α-methyl-D-mannoside plus unlabeled insulin (Table 1). It appears that insulin receptors which are affected by Con A are also affected by the temperature.

Insulin binding to cells at 4°C was much higher than that at 25°C or 37°C (6,13). In addition, the Con A added after insulin might interfere with the release of insulin from the insulin-receptor complexes which have Con A binding sites - the "trapping" effect. Eventually, insulin internalization is accelerated at high Con A concentrations (Fig. 2, upper right panel and Table 1). These results suggest that Con A and insulin binding

sites are different and very close to each other on the insulin receptors on the cells.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the help of Dr. Seymour Wollman of the National Cancer Institute, U.S.A. in preparing this manuscript.

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