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ACUTE EFFECTS OF INSULIN ON SURFACE INSULIN RECEPTORS IN ISOLATED HEPATOCYTES. EVIDENCE FOR A RECYCLING PATHWAY

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SUMMARY: Isolated rat hepatocytes were incubated for 1 h at 37°C with 10 nM insulin. Following washout of insulin, cells were incubated with [^{125}I]mono-iodoinsulin at 15°C to assess surface insulin binding. Preincubation with 10 nM insulin did not cause a decrease in insulin binding. Scatchard analysis confirmed that insulin receptor number remained constant. In the presence of 200 μM chloroquine or 25 μM monensin, surface insulin binding after preincubation with 10 nM insulin fell to 81.1 ± 1.2% or 39.0 ± 2.7% of control, respectively. It is suggested that the maintenance of insulin receptor number following acute insulin treatment *in vitro* is due to an insulin receptor recycling pathway, possibly involving lysosomes and/or the Golgi apparatus.

INTRODUCTION: Following the binding of insulin to specific receptors on target cells, insulin-receptor complexes are internalized (1,2). Insulin is degraded intracellularly, possibly via a lysosomal pathway (3). The fate of the insulin receptor is less well-defined. Evidence reported with other ligand-receptor complexes which are internalized by potentially similar mechanisms as the insulin-receptor system has suggested that receptors might recycle back to the cell membrane (4,5). Several studies have provided evidence for recycling of insulin receptors (2,6,7). In contrast, it has also been proposed that insulin receptors are degraded after endocytosis (8,9).

If insulin receptors return to the cell surface following internalization, then in the presence of high concentrations of insulin which induce rapid internalization, surface receptor numbers should remain constant. This experimental approach has been employed in adipocytes and maintenance of surface receptors has been confirmed (7). The acute effect of insulin on surface insulin receptors has not been previously reported in isolated hepatocytes.

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The aim of the present study was to investigate the regulation of insulin receptors by insulin in isolated rat hepatocytes. This was accomplished by studying changes in [^{125}I]monoiodoinsulin binding after short-term exposure to high concentrations of insulin, and the effects of agents on these changes. The results might provide information concerning the existence of and possible mechanisms for recycling of insulin receptors in hepatocytes.

MATERIALS AND METHODS:

A14-[^{125}I]monoiodoinsulin and crystalline porcine monocomponent insulin were obtained from Novo Research Institute, Denmark. Bacitracin, chloroquine and cycloheximide were purchased from Sigma Chemical Co., USA. Monensin was from Calbiochem-Behring Corp., USA.

Hepatocytes were isolated by collagenase perfusion from normal 160-230 g male Wistar-Furth rats, fed *ad libitum*, as described previously (10). Cell number was adjusted to approx. 2 x 10^6/ml with Krebs-Ringer bicarbonate buffer (pH 7.4) containing 25 mM Hepes, 30 g/litre defatted bovine serum albumin and gassed with 95% O_2 : 5% CO_2.

PRETREATMENT ± INSULIN AND WASHOUT: Hepatocyte suspensions were incubated in 50 ml culture flasks, in the presence of porcine monocomponent insulin (final conc. 10 nM) or buffer (control) for 60 min at 37°C in a shaking water bath. Total incubation volume was 5.0 ml. The washout of insulin prior to measurement of [^{125}I]monoiodoinsulin binding was performed by a modification of the method of Marshall and Olefsky (7). Cells were transferred to 17 x 100 mm tubes and centrifuged (25 g, 90 s). Supernatant was removed and cells were resuspended in 5.0 ml buffer (pH 7.0). Tubes were centrifuged again and cells resuspended in 5.0 ml of the same buffer. Hepatocytes were incubated in fresh 50 ml flasks for 30 min at 37°C. The wash procedure was repeated, using buffer (pH 7.6) and cells were finally resuspended in 5.0 ml of this buffer. When the effects of an agent on preincubation ± insulin were studied, the agent was added to flasks at the same time as insulin or buffer. The agent was also included during the washout phase to prevent its effects being reversed during the second incubation.

BINDING OF [^{125}I]MONOIODOINSULIN: The specific association of [^{125}I]monoiodoinsulin with hepatocytes was performed as described previously (10). Incubations were for 120 min at 15°C. In competition studies, porcine monocomponent insulin was added to binding tubes in increasing amounts up to a final concentration of 87.1 nM.

EFFECT OF MONENSIN ON CELL-ASSOCIATED [^{125}I]MONOIODOINSULIN: Hepatocytes (300 µl) were preincubated with monensin (25 µM) or buffer (control) for 30 min at 37°C. [^{125}I]monoiodoinsulin was then added and incubation continued for the indicated times at 37°C. Cells were separated and radioactivity specifically associated with cells was determined.

RESULTS:

Preincubation for 1 h at 37°C with 10 nM insulin did not decrease subsequently measured hepatocyte-associated [^{125}I]monoiodoinsulin (Fig. 1, treatment 1). Internalization is negligible at 15°C (10) so cell-associated

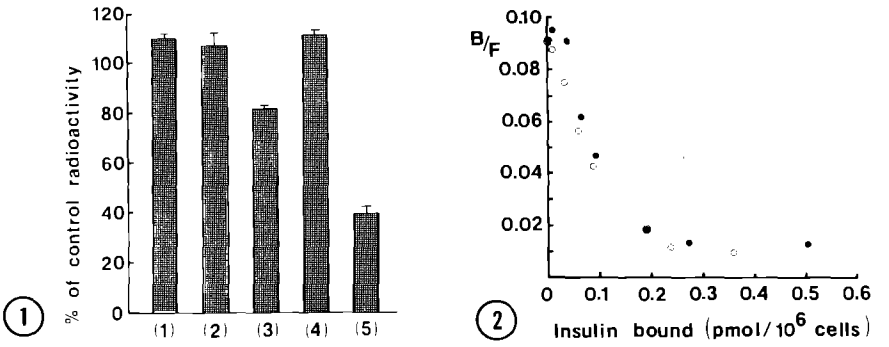

Fig. 1 Effects of preincubation with insulin and agents on hepatocyte-associated [^{125}I]monoiodoinsulin
Hepatocytes were preincubated ± insulin (10 nM) in the presence of the listed agents, as described in Methods. 1 = buffer; 2 = bacitracin (1.0 g/litre); 3 = chloroquine (200 μM); 4 = cycloheximide (100 μM); 5 = monensin (25 μM). After washout of insulin, cells were incubated with [^{125}I]monoiodoinsulin (34.6 - 43.8 pM) for 120 min at 15°C. Values are the mean (±SEM) specific cell-associated radioactivity after preincubation with insulin plus agents 1-5, expressed as a % of controls (that value obtained after preincubation with agents 1-5 alone). For treatment with buffer only, values are the means of quadruplicate determinations from eight separate experiments. For treatments 2-5, values are the means of quadruplicate determinations from three separate experiments.

Fig. 2 Scatchard plot of insulin binding ± preincubation with insulin
Isolated hepatocytes were preincubated in the presence (●) or absence (○) of insulin (10 nM) as described in Methods, then incubated with [^{125}I]monoiodoinsulin (34.6 pM) and increasing amounts of unlabelled insulin for 120 min at 15°C. Cell-associated radioactivity was then determined. B : bound ligand. F : free ligand.

radioactivity at this temperature reflects insulin binding to surface receptors. Scatchard analysis of data from competition studies confirmed that maintenance of receptor binding was due to maintenance of insulin receptor numbers (Fig. 2).

Chloroquine (200 μM) and monensin (25 μM) prevented the maintenance of surface binding of label, after treatment with 10 nM insulin (Fig. 1). In the absence of insulin, chloroquine and monensin did not affect [^{125}I]monoiodo-insulin binding at 15°C (data not shown). Thus the action of the agents was associated with an insulin-induced event and not due to a non-specific effect of the agents on [^{125}I]monoiodoinsulin binding. Scatchard analysis of binding data showed that the decreased binding capacity with chloroquine or monensin was due to a fall in insulin receptor number with little change in binding affinity (Fig. 3).

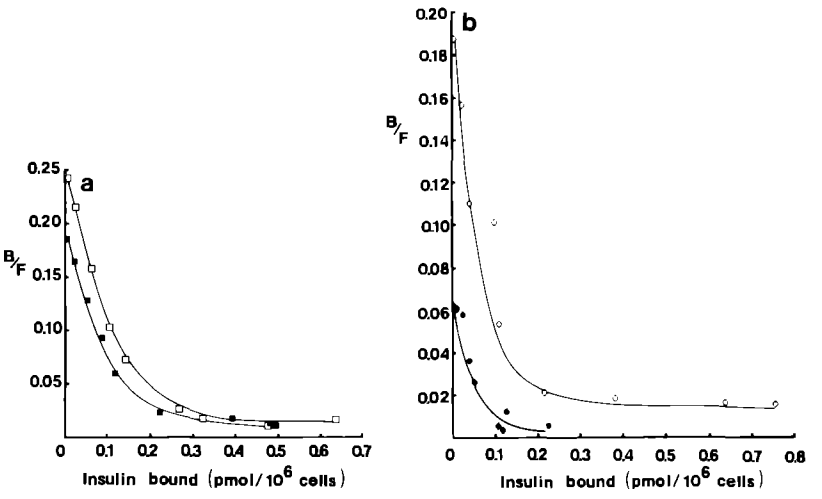

Fig. 3 (a) Insulin-induced receptor loss in the presence of chloroquine
Isolated hepatocytes were preincubated with chloroquine (200 μM) in the presence (■) or absence (□) of insulin (10 nM) as described in Methods, then incubated with labelled and unlabelled insulin for 120 min at 15°C.
(b) Insulin-induced receptor loss in the presence of monensin
(◆) denotes incubation with monensin (25 μM) plus insulin (10 nM).
(◇) denotes incubation with monensin alone.

Results obtained with cycloheximide show that *de novo* protein synthesis was unimportant in the maintenance of surface receptor binding.

We have proposed that bacitracin inhibits insulin internalization in rat hepatocytes (10). Since receptors are internalized along with insulin, it is likely that bacitracin also inhibits the internalization of insulin receptors. In the absence of internalization, insulin treatment would not be expected to alter surface receptor numbers. The finding that bacitracin did not affect surface binding of [^{125}I]monoiodoinsulin following treatment with 10 nM insulin supports this hypothesis.

Monensin (25 μM) caused a significant elevation of cell-associated radioactivity at 37°C, at all times studied (Table 1). This is the first report of an effect of monensin on insulin-receptor interactions.

Cell viability was not altered by any of the agents tested.

DISCUSSION

The present results suggest the existence of a recycling pathway for insulin receptors in isolated hepatocytes. Incubation of hepatocytes at

Table 1

Effect of Monensin on Hepatocyte-Associated [^{125}I]Monoiodoinsulin

	%Specific cell-associated [^{125}I]monoiodoinsulin		
Time of incubation (min)	Control	+ Monensin (25 μM)	P
10	6.94 (0.28)	7.91 (0.27)	< 0.025
20	5.19 (0.08)	8.83 (0.12)	< 0.0005
60	3.65 (0.27)	6.17 (0.22)	< 0.001

Values are the mean (SEM) of % specific cell-associated [^{125}I]monoiodoinsulin/ 5 x 10^5 cells after preincubation with monensin for 30 min at 37°C followed by addition of [^{125}I]monoiodoinsulin (37.5 pM) for the indicated times at 37°C. Significance of differences between means at each time point was assessed by Student's *t* test for unpaired observations.

37°C with 10 nM insulin should result in rapid internalization of insulin-receptor complexes. Without a means for replacing internalized receptors, surface receptor numbers would eventually decline, causing decreased binding of [^{125}I]monoiodoinsulin. Such a decrease was absent in the present study. The duration of incubation at 37°C with insulin was sufficient to have permitted receptor internalization and replacement (1,6).

In studies with adipocytes, Marshall and Olefsky reported that surface insulin receptor numbers remained constant following 2 h incubation with 100 ng/ml insulin at 37°C indicative of a receptor recycling pathway (7). The present study is the first time such investigations have been reported in isolated hepatocytes.

Based on the specific inhibitory effects of chloroquine and monensin on the insulin-induced maintenance of receptors, it is logical to conclude that these agents might interfere with the proposed receptor recycling pathway.

Chloroquine was originally regarded as a lysosomotropic agent (11), but recent studies suggest that it interferes with some Golgi functions (12). We have previously shown that chloroquine inhibits receptor-mediated insulin processing in rat hepatocytes (13). It has been proposed that chloroquine inhibits the recycling of certain receptors including insulin receptors in adipocytes (7), receptors for lysosomal enzymes in fibroblasts (14) and receptors for mannose glycoconjugates in macrophages (15).

Monensin is a monovalent carboxylic ionophore, which forms lipid-soluble complexes with alkali metal cations (16). Monensin has been shown to upset cytoplasmic Na^+/K^+ balance at the Golgi, and to interfere with the intracellular movement of secretory proteins from the Golgi to the cell membrane (17). Basu et al claimed that monensin inhibited the recycling of LDL receptors in fibroblasts by inhibiting some lysosomal function (18). Monensin might act in a manner similar to chloroquine. The increased cell-associated radioactivity with monensin might result from decreased intracellular insulin degradation.

The mechanisms by which chloroquine and monensin inhibit insulin receptor recycling are unknown. The dissociation of the ligand-receptor complex is an important step in receptor recycling (19). By inhibiting intracellular insulin processing, possibly at a lysosomal site, monensin and chloroquine might interfere with the dissociation step, and hence recycling. The Golgi apparatus may be important in recycling receptors to the cell membrane (20). Thus, the effects of monensin might also be explained by its inhibitory effects on Golgi functions.

It is noteworthy that insulin binding is unaltered after treatment with chloroquine or monensin alone. This indicates that in hepatocytes, insulin receptors do not internalize and recycle in the absence of insulin binding. Alternatively, the rate at which such internalization and recycling occurs is too slow to be detected by the methods used in this study.

In summary, it has been reported for the first time that acute insulin treatment *in vitro* results in the maintenance of surface insulin receptors in isolated rat hepatocytes. Studies with various agents support the existence of a pathway for recycling internalized receptors, possibly involving lysosomes or the Golgi apparatus.

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