INSULIN STIMULATION OF AMINO ACID TRANSPORT IN PRIMARY CULTURED RAT HEPATOCYTES VARIES IN DIRECT PROPORTION TO INSULIN BINDING

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1. Introduction

We have shown that insulin stimulates amino acid transport in freshly isolated rat hepatocytes [1,2]. Using this effect of insulin, we could compare insulin binding to its receptor sites and insulin stimulation of amino acid transport in the liver [3]. However, there is no information concerning the relationship between receptor loss and changes in biological function, with regard to insulin action in the liver.

Here, monolayer cultures of adult rat hepatocytes were treated with trypsin to cause a loss of insulin receptors [4–7], and the effect of such a change on insulin binding and insulin stimulation of amino acid transport was analyzed.

2. Materials and methods

2.1. *Trypsin treatment of isolated hepatocytes and subsequent primary cultures*

Hepatocytes were isolated from adult male Wistar rats as in [2,8]. The cell suspension (6×10^6 cells/ml) was divided into two samples which were incubated without or with trypsin (Sigma, 5–50 μg/ml) 15 min at 30°C. Cells were then collected by centrifugation, resuspended in 30 ml Krebs-Ringer bicarbonate (KRb) buffer containing 1% bovine serum albumin (BSA) and soybean trypsin inhibitor (Sigma, 0.5–5 μg/ml). The washing procedure was repeated 3 times in Waymouth's medium (Gibco), and cells (1×10^6/ml) were allowed to plate on collagen-precoated culture dishes in Waymouth's medium and in the presence of 10% fetal calf serum (Eurobio, Paris). After the attachment period (4 h at 37°C), hepatocyte monolayers were incubated for 20 h in serum-free Waymouth's medium. Culture media contained 0.2% defatted BSA, 100 units/ml penicillin and 100 μg streptomycin/ml. All experiments were performed after 24 h culture had elapsed since the initial treatment with trypsin.

2.2. *Binding assays*

Hepatocyte monolayers were washed 3 times with 1 ml KRb buffer and incubated in 1 ml KRb containing 1% defatted BSA, 0.8 mg/ml bacitracin and 50 μg/ml gentamycin for 60 min at 37°C in the presence of 0.3 ng/ml (0.05 nM) ^{125}I-labelled insulin (250 μCi/μg) and unlabelled insulin (monocomponent, Novo) at 0.01 nM–1 μM. These conditions insure a steady-state binding of ^{125}I-insulin to hepatocyte monolayers (O. M. et al., unpublished). Non-specific binding was determined as in [3] and represented ~10% of the total binding. Following aspiration of the supernatant and washing (3 times with 1 ml chilled saline), the cells were digested with 1 ml 0.2 N NaOH and counted for ^{125}I radioactivity. The binding data were corrected for non-specific binding, and normalized per mg cell protein.

2.3. *Transport studies*

Monolayers were washed 3 times with 1 ml KRb buffer and incubated in 0.9 ml KRb buffer containing the same additions as above, in the presence or absence

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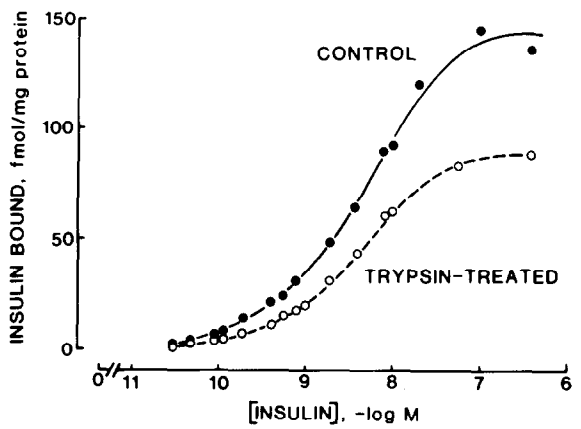
of varying concentrations of insulin. After 3 h of incubation at 37°C, transport assays were initiated by adding 0.1 ml of KRb buffer containing α-amino-[1-^{14}C]isobutyric acid (AIB, 0.2 μCi, 0.1 mM final conc.), and [^{3}H]inulin; the latter was used for determination of the extracellular space. After 15 min, the reaction was terminated by removing the medium and washing the monolayer 3 times with 1 ml chilled saline. The cells were digested with 0.5 ml 0.2 N NaOH and counted for ^{14}C and ^{3}H after neutralization. The transport data were corrected for extracellular trapping, and normalized per mg cell protein.

3. Results

Fig.1 (top) shows that hepatocytes, exposed to trypsin (20 μg/ml) prior to plating, bound less insulin than control cells. The decrease in binding (40–50%) was observed over a broad range of insulin concentrations, including saturating levels of the hormone. Trypsin treatment caused a loss in receptor concentration without altering the apparent affinity of receptors for insulin, in agreement with studies with other cell types [4]: assuming a two-site model for the binding of insulin to its receptors in hepatocytes [3,9], the number of high-affinity binding sites in 3 separate experiments was 17 300 ± 3800 sites/cell (K_d = 0.44 ± 0.07 nM) in trypsin-treated cells, and 31 300 ± 3300 sites /cell (K_d = 0.41 ± 0.03 nM) in control hepatocytes. Low affinity binding (K_d ~ 10 nM) was 49 000 sites/cell and 90 000 sites/cell in trypsin-treated hepatocytes and in control cells, respectively.

As depicted in fig.1 (bottom), insulin enhanced the uptake of AIB in hepatocyte monolayers which had been exposed to the hormone for 3 h. The stimulatory effect of insulin was maximal at 10 nM and half-maximal stimulation (ED_{50}) occurred at 0.3 nM. In trypsin-treated cells, the basal rate of AIB transport (0.13 ± 0.03 nmol . mg protein^{-1} . 15 min^{-1}) was similar to that observed in control hepatocytes (0.14 ± 0.03 nmol . mg protein^{-1} . 15 min^{-1}) (means ± SE of 5 separate expt). In contrast, the stimulation of AIB uptake by insulin was reduced in hepatocytes pre-exposed to trypsin; this decrease (~40%) was observed at both maximally and sub-maximally stimulating insulin concentrations (fig.1, bottom).

Treatment of hepatocytes with increasing concentrations (5–50 μg/ml) of trypsin resulted in a progressive reduction (20–80%) in insulin binding, which



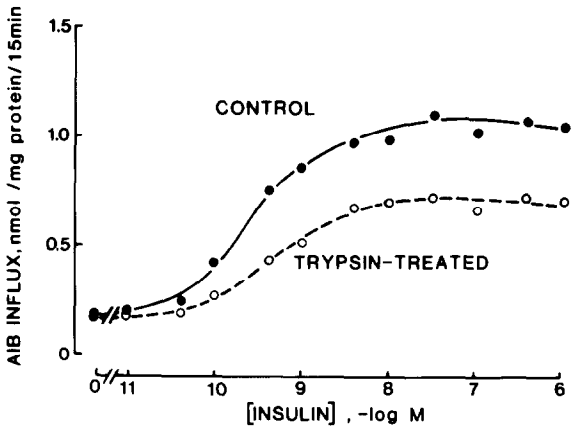


Fig.1. Effect of trypsin treatment of hepatocytes on insulin binding and insulin stimulation of AIB uptake. Top: monolayers were incubated with 0.3 ng/ml of [^{125}I]iodoinsulin and increasing concentrations of unlabelled insulin for 60 min at 37°C. The results are expressed as amounts of hormone specifically bound/mg protein. Bottom: transport of AIB was measured under initial velocity (15 min at 37°C) with 0.1 mM [^{14}C]AIB after a 3 h exposure to increasing concentrations of insulin. Each point is the mean of 3 determinations in a typical experiment.

was accompanied by a quantitatively similar decrease in the maximal biological response to insulin. In fig.2, the maximal response to insulin in trypsin-treated hepatocytes (expressed as % of that observed in control cells) has been plotted as a function of the binding of insulin (also expressed as % of control). Data yielded a straight line with a slope of 0.94. There was a highly significant correlation (r = 0.97, $p < 0.01$) between the decrease in insulin binding and the decrease in insulin responsiveness (fig.2).

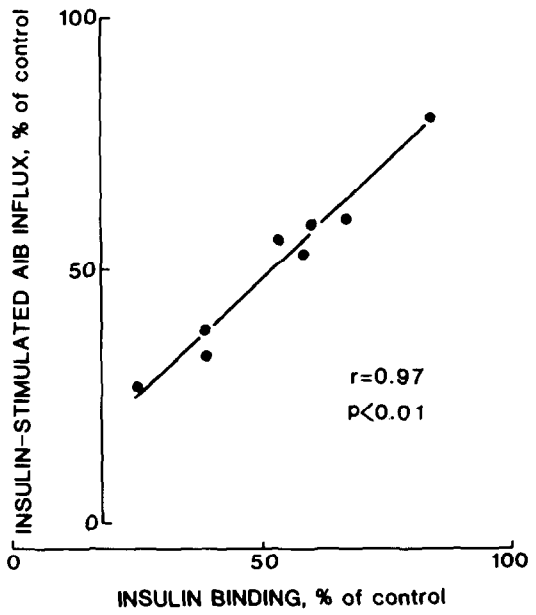


Fig.2. Correlation between insulin binding and insulin stimulation of AIB uptake. Freshly isolated hepatocytes were incubated without or with varying concentrations (5–50 μg/ml) of trypsin for 15 min at 30°C prior to plating, as in section 2. After 24 h culture, the corresponding monolayers were used for measurement of insulin binding and insulin stimulation of AIB uptake. For binding, hepatocyte monolayers were incubated with 0.3 ng/ml (0.05 nM) ^{125}I-labelled insulin for 1 h at 37°C in the absence (total binding) or presence (non-specific) of unlabelled insulin at 6 μg/ml (1 μM). Binding data have been expressed as:

$$\frac{\text{Specific [}^{125}\text{I]iodoinsulin binding in trypsin-treated cells}}{\text{Specific [}^{125}\text{I]iodoinsulin binding in control cells}} \times 100\%$$

For insulin-stimulated AIB uptake, monolayers were incubated without or with 100 nM insulin for 3 h at 37°C. Results have been expressed as:

$$\frac{\text{Insulin-stimulated uptake} - \text{basal uptake in trypsin treated cells}}{\text{Insulin-stimulated uptake} - \text{basal uptake in control cells}} \times 100\%$$

Each point is the mean of triplicate determinations.

4. Discussion

Exposure of isolated hepatocytes to trypsin results in a decreased concentration of insulin receptors in primary cultured monolayers obtained from these cells. This decreased insulin binding is accompanied by diminished sub-maximal and maximal insulin effects on AIB uptake. These data also show that the stimulation by insulin of AIB uptake varies in direct proportion to the binding of the hormone, thus indicating that there are no 'spare' receptors for this type of insulin effect in hepatocytes. This contradicts observations made for the effect of insulin on glucose transport and metabolism in isolated adipocytes (review [6]) and in soleus muscle (review [10]). However, insulin stimulation of AIB uptake closely paralleled insulin binding to its receptors in thymocytes [11] and in freshly isolated hepatocytes [1]. Studies with various cell types have provided evidence that the stimulation of AIB uptake correlates with the hormone's binding to a high affinity site ([2,12–14], J. D.-K., in preparation) or to a low affinity site [15], in agreement with this report.

Here, we used trypsin to evaluate the consequence of a receptor loss on a biological response. The data clearly indicate that the dose–response relationship of insulin stimulation of AIB transport is altered in direct proportion to the receptor loss in hepatocytes.

These findings have two implications:

(1) The mechanism(s) of insulin action: the stimulation of amino acid transport varies in direct proportion to the signal generated by the binding of insulin to its receptors on the plasma membrane, although this bioeffect is relatively distant to the insulin–receptor interaction and involves a number of intermediate steps such as new protein synthesis [2].

(2) Pathophysiological situations with decreased insulin receptor concentrations are likely to be accompanied by diminished insulin effects on the stimulation of amino acid uptake by the hepatocyte.

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