Relationship between Insulin Binding and Glycogenesis in Cultured Fetal Hepatocytes

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Summary. Binding of ^{125}I-insulin and the stimulatory effect of insulin on ^{14}C-glucose incorporation into glycogen have been studied in cultured fetal rat hepatocytes. Measurement of both variables was possible at 37 °C because of the slow rate of insulin degradation in the medium. ^{125}I-insulin binding approached maximum after 10 min, thus largely preceding the insulin glycogenic effect which became significant after 45 min. Maximal effect was observed after 3 h with 10 nmol/l insulin when 16,000 specific sites per hepatocyte were occupied and half-maximal response was achieved with 0.3 nmol/l insulin (2,900 sites/hepatocyte). Dissociation of bound insulin was rapid ($t\frac{1}{2} = 3$ min) and accelerated by the availability of native insulin. In hepatocytes preincubated with insulin, binding was measured after 30 min incubation in the absence of hormone which allowed the liberation of most (95%) of the bound insulin. No modification of insulin binding was observed over extended periods (2–24 h) of exposure to 10 nmol/l insulin, when the glycogenic effect of insulin showed striking variations, notably a cessation of the effect between 4 and 12 h. Thus the time-dependence of the glycogenic effect of insulin cannot be related to a defect in insulin binding in cultured fetal hepatocytes.

Key words: Insulin receptors, glycogen, cultured hepatocytes, fetal rat liver, insulin.

It is interesting to study the relationship between binding and the biological effect of insulin as a function of the time of exposure to the hormone. Many studies have concentrated on the early cellular responses [1–5] but prolonged exposure may also induce modifications of insulin binding and hormone-mediated effects. Down regulation of insulin receptor concentration was first described in IM-9 lymphocytes [6] and a decrease in insulin sensitivity was found to be associated with an insulin-induced decrease in hormone binding in fat pad explants [7, 8]. These observations required long-term experiments with insulin-responsive cells. In cultured fetal rat hepatocytes grown for 2–3 days in the presence of cortisol, insulin produced a clear and rapid stimulation of glycogenesis at physiological concentrations of glucose [9, 10]. This stimulation was modified by the time of contact of the hepatocytes with insulin [10]. Since we are dealing with a homogeneous population of hepatocytes [11], measurements can also be made of their insulin binding characteristics. We have therefore used this cultured fetal hepatocyte model to investigate the relationship between insulin binding and hormonal response, not only after the initial glycogenic effect of insulin but also after prolonged exposure to the hormone.

Materials and Methods

Culture Procedure

Primary cultures of hepatocytes were obtained from 18 day-old rat fetuses (Sprague-Dawley) by a method already described [11]. Dissociation of the cells was performed by mild trypsinization. The cells were then plated on a collagen substrate to which only the hepatocytes adhered, and after 18 h the non-adhering haemapoietic cells were removed. The hepatocytes were grown for up to 3 days. The composition of the medium was as follows: medium NCTC 109 [12], 90 parts; fetal calf serum, 10 parts; cortisol (10 µmol/l). Fetal calf serum was obtained from Sorga (batch 76–16). Glucose concentration in fresh medium was 1.05 mg/ml. The culture medium (2 ml/dish) was replaced after 18 h and supplemented with 20 µl of a 25 mg/ml glucose solution; the glucose concentration was thus maintained within a range of 0.8 to 1.05 mg/ml during the 3-day culture period. This procedure assured good reproducibility among the 300 culture dishes obtained from the same initial cell suspension prepared with 50 fetal livers. When measuring the protein content of the dishes, our coefficient of variation was 3%. All the experiments were performed after 3 days of culture in presence of cortisol (or after 2 days when stated), a stage when the glycogenic effect of insulin is fully expressed [9].
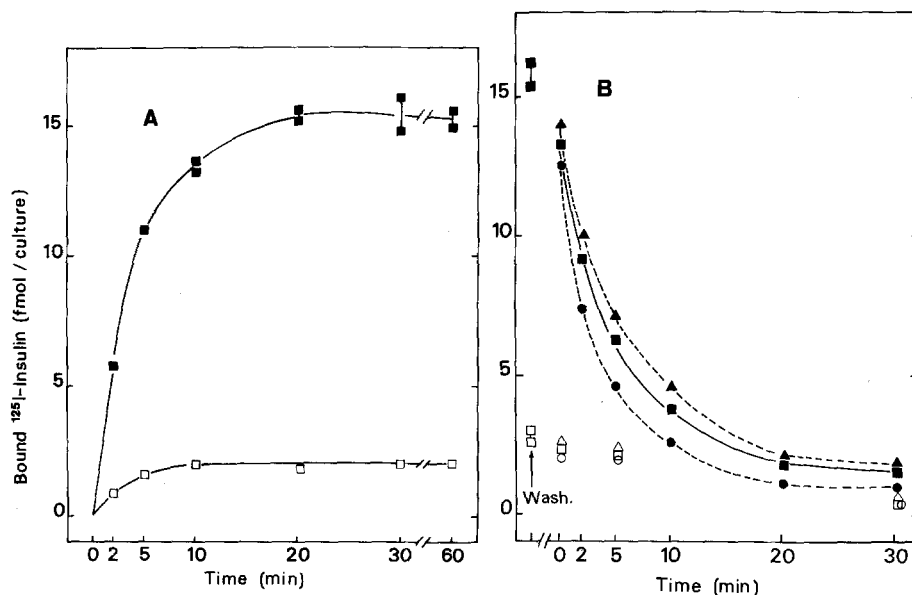
Fig. 1 A and B. Association and dissociation of ^{125}I-insulin in cultured fetal hepatocytes. **A** Hepatocytes were first incubated at 37 °C for the times indicated with ^{125}I-insulin in the absence (■, ▲, ● = total binding) or the presence of 10 μmol/l insulin (□, △, ○ = non-specific binding). **B** After 30 min, the hepatocytes were washed with unlabelled medium, then replaced at 37 °C (time zero of the experiment) in culture medium devoid of native insulin (▲, △) or containing 1 nmol/l (■, □) or 10 μmol/l (●, ○) insulin. After various incubation times indicated in the abscissa, the amount of bound insulin was determined as described in Methods. Each culture represents 1.1×10^6 hepatocytes in 2 ml of culture medium

Binding Studies

Porcine monocomponent insulin, both native and radioiodinated at 20–30 Ci/g was supplied by Novo Research Institute, Copenhagen. The degree of iodination was 0.2–0.4 atoms of iodine per insulin monomer. Hepatocytes (about 1.1×10^6 per culture) were incubated in 2 ml of culture medium (pH 7.3) at 37 °C in the presence of ^{125}I-insulin. Parallel cultures received ^{125}I-insulin plus an excess of native insulin (10^4 times excess). After a given incubation time, the medium was removed and the cultures washed four times with 2.5 ml of ice-cold Hanks solution. This washing step was achieved within 12 s. Cell material was extracted with 0.7 ml of 0.5 mol/l NaOH for 30 min at 75 °C. The dishes were then rinsed with 0.7 ml of 0.5 mol/l HCl. Both extracts were pooled and their radioactivity measured with a gamma counter at 75% efficiency. The amount of cell bound radioactivity in the presence of ^{125}I-insulin represents the "total binding". In the presence of excess native insulin, the residual cell bound radioactivity was referred to as "non-specific binding". "Specific binding" is the difference between total binding and non-specific binding. All are expressed as fmol bound ^{125}I-insulin per culture. In order to obtain the number of sites occupied per hepatocyte for a given insulin concentration, the amount of specifically bound insulin per hepatocyte (expressed in mol) was multiplied by 6.023×10^{23}. After various incubation times, the radioactivity in the culture medium was analysed for insulin degradation using 10% (w/v) trichloroacetic acid. The soluble radioactivity was expressed as percentage total radioactivity. The value of ^{125}I-insulin degradation when incubated in fresh medium without cells (3%) was subtracted from degradation values obtained in the presence of cells.

Glycogen studies

Glycogen labelling in the presence of ^{14}C-glucose was determined as previously described [11]. ^{14}C-glucose measurements in the presence and absence of insulin have been shown in agreement with the change of glycogen content [10]. To determine the insulin effect, ^{14}C-glucose was added to the culture medium at a final activity of 1 μCi/mg of glucose together with insulin at varying concentrations, or its solvent (10 μmol/l HCl). The radioactivity in glycogen was measured after various time intervals. Results are expressed per 10^6 hepatocytes, counting of hepatocytes being performed on parallel cultures [11].

Definitions

In order to express the insulin response, a "stimulation index" was used, defined as the following ratio: c.p.m./10^6 hepatocytes in treated cultures divided by c.p.m./10^6 hepatocytes in control cultures. For each protocol, at least three experiments were performed using different cell preparations. Data are presented as means ± SEM and the number of experiments (n). Except when stated every symbol (on graphs) or value (in Tables) corresponds to a different culture dish.

Results

Kinetics

Labelled insulin (1 nmol/l) was first allowed to associate with cultured fetal rat hepatocytes at 37 °C. Figure 1 A shows that the binding of the hormone occurred very rapidly and reached a steady state by 10–20 min. The proportion of labelled insulin bound to the hepatocytes was then 0.75%. In the presence of an excess of unlabelled insulin, ^{125}I-insulin binding (non-specific) represented 14% of the total binding and did not increase with time. After 30 min, when steady-state binding conditions were reached, the hepatocytes were washed, and the dissociation of previously bound ^{125}I-insulin determined in the absence and presence of unlabelled hormone. Figure 1 B shows that binding of ^{125}I-insulin was largely and rapidly reversible. When dissociation was allowed in the presence of 1 nmol/l unlabelled insulin, an insulin concentration identical to that used during the association phase, 50 ± 2% (n = 5) of insulin was liberated from its specific sites within 3 min and 94 ± 2% (n = 5) after 20 min. The dissociation curve levelled off after 20 min. The dissociation rate of insulin from hepatocytes was accelerated by availability of

unlabelled insulin. After 5 min of incubation, the percentage of dissociation was 64 ± 2% in the absence of insulin, 71 ± 3% and 83 ± 4% (n = 5) in the presence of 1 nmol/l and 10 μmol/l insulin, respectively. After 30 min, in the absence or presence of native insulin, this percentage was over 90%.

Insulin degradation during the incubation of hepatocytes (1.2 × 10⁶ hepatocytes in 2 ml of medium) at 37 °C was determined by trichloroacetic acid precipitation of intact ^{125}I-insulin in the medium. The percentage of insulin degradation increased linearly as a function of time and was lowered when the concentration of insulin increased (Table 1). After 30 min, a time chosen for the estimation of specific binding at steady-state in all experiments, the degradation of insulin, low for 1 nmol/l ^{125}I-insulin (1.5 ± 0.4%; n = 5), was not detectable for 10 nmol/l ^{125}I-insulin. Thus, specific binding will not take into account insulin degradation in this paper.

The stimulatory effect of insulin on glycogen synthesis (estimated by ^{14}C-glucose incorporation into glycogen) and ^{125}I-insulin binding were investigated in parallel cultures using 10 nmol/l insulin, a dose previously shown to have a maximal glycogenic effect [10]. The stimulatory effect of insulin was found to be significant after 45 min (stimulation index 1.81 ± 0.21; n = 5) and to increase gradually thereafter (Fig. 2). It reached a maximal value after 3 h (stimulation index 3.50 ± 0.43; n = 5). Figure 2 shows that steady-state binding conditions were obtained as early as after 10 min, which was largely before the glycogenic effect of insulin became significant.

Specificity

When binding of 1 nmol/l ^{125}I-insulin was measured in the presence of increasing concentrations of unlabelled peptide hormones, both insulin and proinsulin inhibited ^{125}I-insulin binding whereas glucagon was virtually without effect (Fig. 3). For 50% inhibition of ^{125}I-insulin binding, a proinsulin concentration of 0.15 μmol/l was needed, which was 30 times higher than the figure for insulin (0.005 μmol/l). High concentrations of both proinsulin and insulin (10 μmol/l) displaced 87% of insulin, the residual being referred to as non-specific binding. The maximal stimulation of glycogen synthesis was similar with insulin and proinsulin, and the half-maximal responses were obtained with 0.3 nmol/l insulin and 15 nmol/l proinsulin respectively.

Concentration Dependence

Insulin binding at steady state was also measured using concentrations of insulin varying from 0.1 to 30 nmol/l. The amount of ^{125}I-insulin specifically bound increased rapidly between 0.1 and 10 nmol/l

Table 1. Insulin degradation in the culture medium. Hepatocytes (1.1 million/culture) were incubated at 37 °C in 2 ml of culture medium with respectively 0.3, 1 or 10 nmol/l ^{125}I-insulin. At the times indicated, the amount of ^{125}I-insulin present in the medium and precipitable by 10% trichloroacetic acid was determined as described in Methods and is expressed as percentage of total labelled medium insulin

Time (min)		^{125}I-insulin concentration		
		(0.3 nmol/l)	(1 nmol/l)	(10 nmol/l)
		%	%	%
20	Exp. 1	–	1.2	not detectable
30	Exp. 1	2.2	1.6	not detectable
	Exp. 2	2.3	1.7	-
40	Exp. 1	–	3.1	0.2
240	Exp. 1	–	18.0	4.1
	Exp. 2	–	18.3	4.5

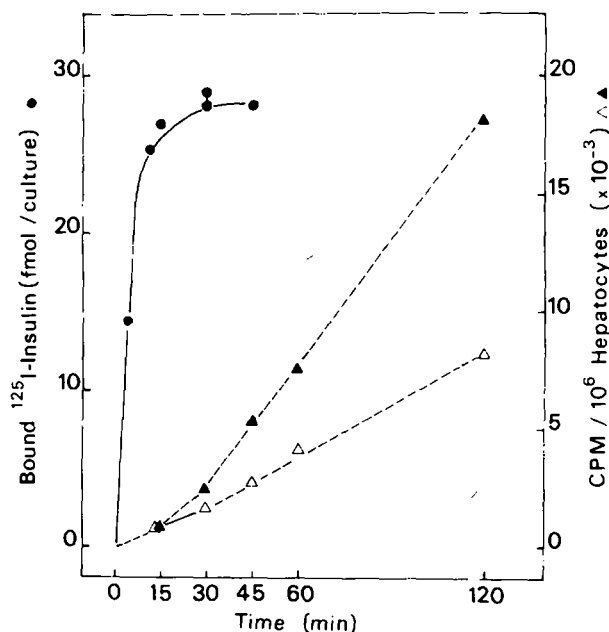**Fig. 2.** Early biological effect of insulin and insulin binding. At day 3 of the culture (time zero of the experiment), 10 nmol/l insulin (▲) or its solvent (△) was added together with ^{14}C-glucose (1 μCi/mg) and the radioactivity present in glycogen was followed for 2 h. In parallel cultures, the specific binding of insulin was determined using the same concentration of the hormone (●)

and apparent maximal binding occurred with a concentration of 30 nmol/l ^{125}I-insulin (Fig. 4). At this concentration, the amount of hormone bound (39.8 ± 2.0 fmol/culture; n = 6) represents about 24,000 bound molecules per hepatocyte. When the data, expressed as the ratio of bound (B) to free (F) insulin, were plotted against the concentration of bound insulin, according to the method of Scatchard, the resulting plot appeared to be curvilinear upwards (Fig. 4, inset). Both insulin binding and stimulation of glycogen synthesis by insulin were determined in parallel cultures using the same source of insulin at

various concentrations. Because of the different time courses of both processes, shown in Fig. 2, insulin binding and glycogen synthesis were respectively measured after 30 min and 4 h of contact with insulin. Half-maximal stimulation of glycogen synthesis was observed with 0.3 nmol/l insulin when 2,900 sites per hepatocyte were occupied and maximal response was achieved with 10 nmol/l insulin (16,000 sites/hepatocyte) (Fig. 5).

Prolonged Exposure to the Hormone

Experiments were performed to examine both insulin binding and the stimulatory effect of insulin on ^{14}C-glucose incorporation into glycogen in cultures grown up to 24 h in the presence of 10 nmol/l insulin. Insulin binding was determined using the following protocol. After a set preincubation period with 10 nmol/l insulin, the hepatocytes were washed twice and incubated in an insulin-free medium for 30 min; the specific binding was then measured. Control cultures, not preincubated with insulin, were given 10 nmol/l insulin just before the washing step in order to eliminate any possible effect of carry-over of insulin. Washings and short incubation in the absence of insulin allowed the release of most specifically bound insulin; in hepatocytes preincubated with 10 nmol/l insulin for 4 h, 96 ± 2% (n = 4) insulin was liberated from its specific sites after the dissociation step. These hepatocytes bound as much insulin as did controls at ^{125}I-insulin concentrations ranging from 0.1 to 1 nmol/l (Fig. 6). No significant modification of specific binding was observed after preincubation of hepatocytes with 10 nmol/l between 4 and 24 h
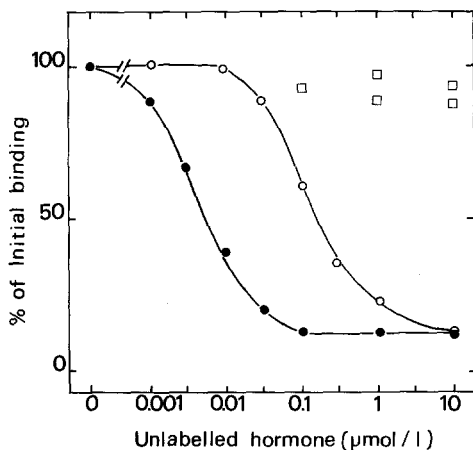

Fig. 4. Binding of insulin as a function of the hormone concentration. Hepatocytes (1.1 × 10⁶/culture) were incubated in 2 ml of culture medium at 37 °C for 30 min in the presence of increasing concentrations of ^{125}I-insulin (from 0.1 to 30 nmol/l). The specific binding was measured at each hormone concentration and used to calculate absolute amount of bound hormone. Each point is the mean of six separate experiments ± SEM. Inset: Scatchard plot of the binding data

Fig. 3. Inhibition of ^{125}I-insulin binding to cultured fetal hepatocytes by unlabelled peptide hormones. Hepatocytes (1.4 × 10⁶/culture) were incubated in 2 ml of culture medium at 37 °C for 30 min with 1 nmol/l ^{125}I-insulin alone (initial binding) and with 1 nmol/l ^{125}I-insulin plus native insulin (●), or proinsulin (○), or glucagon (□) at the concentrations indicated. Binding is expressed as percentage of ^{125}I-insulin initial binding
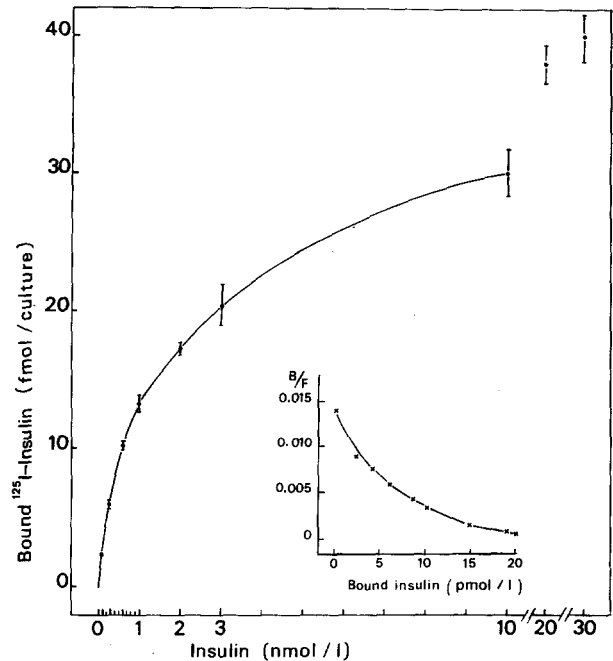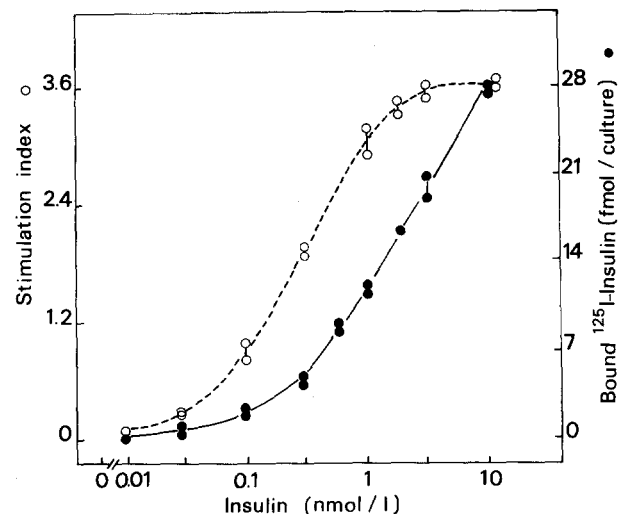

Fig. 5. Relationship between dose-response curves of insulin binding and insulin effect in cultured fetal hepatocytes. At day 3 of the culture, varying concentrations of ^{125}I-insulin indicated in the abscissa or its solvent were added together with ^{14}C-glucose (1 μCi/mg) and the radioactivity present in glycogen determined after 4 h. The insulin response is expressed using a "stimulation index" defined as the following ratio: cpm/10⁶ hepatocytes in treated cultures divided by cpm/10⁶ hepatocytes in control cultures (○). Using identical concentrations of the hormone, the specific binding was determined in parallel cultures as described in Figure 4 (●)
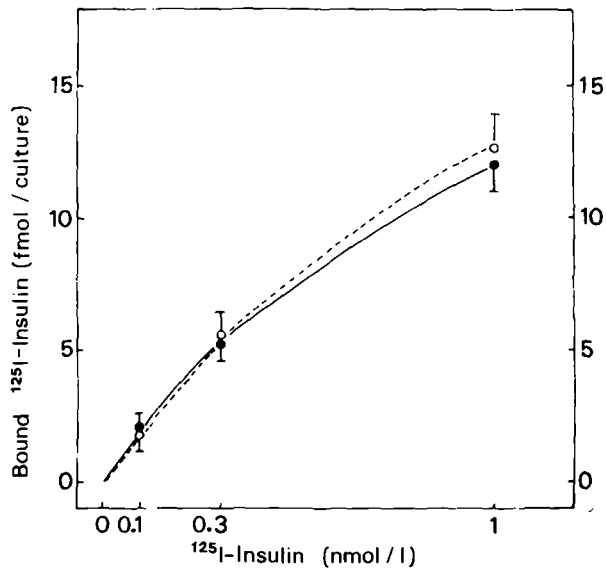
Fig. 6. Binding of insulin to hepatocytes preincubated with insulin for 4 h. Hepatocytes were preincubated for 4 h in the presence of 10 nmol/l insulin or its solvent. Then the hepatocytes were washed twice with insulin-free medium and incubated under these conditions for 30 min. Measurements of hormone binding were then performed as described in Fig. 4, in the presence of 0.1, 0.3 and 1 nmol/l ^{125}I-insulin respectively, in hepatocytes which had been preincubated with 10 nmol/l insulin (●) or not (○). In these control cultures, 10 nmol/l insulin was added just before the washing step. Each point is the mean of four separate experiments ± SEM
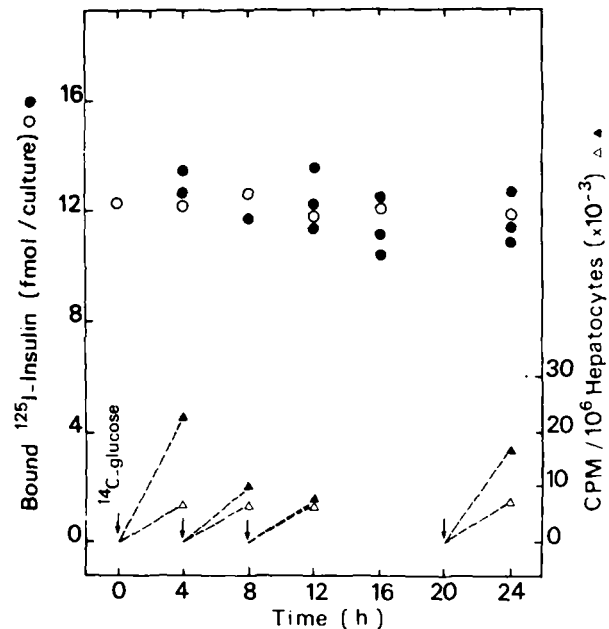

Fig. 7. Insulin binding and insulin effect as a function of time of preincubation of hepatocytes with insulin. Hepatocytes were preincubated in the presence of 10 nmol/l insulin (●, ▲) or its solvent (○, △) for various times as indicated on the abscissa. Measurements of hormone binding were then performed as described in Fig. 6 in the presence of 1 nmol/l ^{125}I-insulin (●, ○). The radioactivity present in glycogen after a 4-h incubation period in the presence of ^{14}C-glucose (1 μCi/mg) was determined in parallel cultures (▲, △)

(Fig. 7). After 24 h, for example, the amount of ^{125}I-insulin specifically bound at a concentration of 1 nmol/l was 12.7 ± 0.6 and 13.1 ± 0.5 fmol/culture in preincubated and control hepatocytes respectively (n = 6). In parallel cultures incubated in the continued presence of 10 nmol/l insulin, a clear stimulatory effect on ^{14}C-glucose incorporation was observed during the first 4 h; this effect was then diminished over the period from 4 to 8 h and ceased between 8 and 12 h. It reappeared, but to a lesser extent, after 20 h (Fig. 7). This transitory cessation of the insulin effect has previously been shown not to be due to hormone inactivation in the medium, since a second 10 nmol/l dose of insulin did not significantly modify glycogen synthesis [10]. No modification in specific binding could be correlated with these time variations in the glycogenic effect of insulin.

Discussion

Insulin Binding and the Early Hormonal Response

Insulin binding and glycogenesis were both measured in hepatocytes from 18-day old rats under physiological conditions (37 °C, pH 7.3). When these cells were grown for 3 days in the presence of cortisol they

showed a clear biological response to insulin (glycogenesis was stimulated 3.5 times after 3 h), which was already significant after 45 min [10]. The cortisol-dependent cell maturation is fundamental if one is to obtain this glycogenic effect of insulin in explants [13], as well as in cultured hepatocytes [9] from fetal rat liver. Insulin binding could be assessed without an insulin degradation inhibitor because fetal hepatocytes only caused minimal insulin degradation in the medium (Table 1). The reason why this should be lower than in cultured adult hepatocytes [14] is not known. It may be related to the low proportion of damaged cells [15] and/or is a characteristic of the fetal stage of the cells. This might be due to the presence of protease activities in fetal liver membranes which could be different from those found in the adult [16]. The insulin binding is hormone specific and the reduced affinity of proinsulin for the receptor of insulin corresponds closely to the lower potency of proinsulin (Fig. 3). Also the data presented in this study, as in other studies performed on adult hepatocytes [14, 15, 17–20] concern "specific binding", which represents specific cell associated radioactivity. Apparent maximal binding occurred with a hormone concentration of 30 nmol/l (Fig. 4), which corresponds to 24,000 insulin bound molecules per

hepatocyte. This number of specific receptor sites per cell is close to [15], or lower than [21], that found in isolated adult hepatocytes. It must be pointed that partially purified fetal liver membranes bind less well than those obtained from the adult [22]; this would suggest an increase in the number of insulin receptors during development.

Maximal stimulation of glycogenesis occurred with 10 nmol/l insulin while ^{125}I-insulin binding corresponded to 70% of the apparent maximal binding (measured at 30 nmol/l insulin). Half-maximal response was obtained with 0.3 nmol/l insulin when 13% of the "total" number receptor sites were occupied (Figs. 4 and 5). In isolated adipocytes, a maximal response to insulin for several parameters was achieved with only a minority of hormone-filled receptor sites (5–10%) [1, 3]. By contrast, a close correlation has been seen between insulin binding and stimulation of aminoacid uptake in both isolated adult hepatocytes [4] and thymocytes [2]. In isolated soleus muscle, a maximal stimulation of 2-deoxyglucose uptake by insulin has been found to occur when only 20% of the total receptors were occupied. Moreover, the concentration dependence of several biological effects was different [5]. In particular, the relationship between insulin binding and glycogenesis, an effect rather distal to insulin-receptor interaction, appeared to be similar to that described in cultured fetal hepatocytes.

The rate of dissociation of insulin from its specific sites is rapid and accelerated by the presence of unlabelled hormone in the medium at 37 °C (Fig. 1 B). In isolated adult hepatocytes, the rate of dissociation of insulin has been found to be accelerated by the addition of native insulin at 12 °C, but not at 37 °C [15]. Accelerated dissociation of cell-bound insulin by the availability of free insulin is particularly marked below 20 °C and has been interpreted in terms of "negative cooperativity" among insulin receptors [23]. This means that as the fractional receptor occupancy increases, the affinity of the receptors decreases (faster dissociation rate) owing to site-site interactions. The insulin binding is largely reversible (Fig. 1 B), just as in isolated adult hepatocytes [15, 17]. Whatever the time of the previous exposure most of insulin was liberated from its specific receptor sites after a 30 min incubation period in hormone-free medium. This allowed insulin binding in insulin-treated hepatocytes to be measured.

Insulin Binding and the Time Dependent Hormonal Response

A negative regulation of insulin receptor concentration on the cell after prolonged exposure to the hor-

mone has been observed in IM-9 lymphocytes [6] and found to be hormone-specific [24]. This receptor down regulation process has not been observed in 3T3-L1 fibroblasts [25, 26] which are target cells for insulin, but it has been described in cultured adult hepatocytes [14] and RL-PR-C hepatocytes [27]. It was not found in this study carried out with cultured fetal hepatocytes, where no modification of insulin binding could be observed after prolonged preincubation of cells with 10 nmol/l insulin (Figs. 6 and 7). The down regulating effect of insulin might result from an imbalance between the rate of internalisation of the insulin receptor complex and that of reinsertion of newly synthesised, and possibly recycled receptors into the plasma membrane [28]. In the fetal hepatocyte, receptor down regulation may not be seen because of some characteristic of the fate of receptors in the cell, e. g. rapid turnover of the insulin receptor sites. More work will be needed to resolve these differences.

In genetically obese-hyperglycaemic rodents, hyperinsulinaemia has been found to be associated with decreased concentration of insulin receptors in several types of cells and loss of sensitivity to exogenous insulin [21]. However when hyperinsulinaemia in ob/ob mice was normalised by fasting or by streptozotocin treatment, there was an apparent lack of direct relationship between insulin binding and insulin effects in liver and adipose tissue, but in muscle both variables changed in parallel [29]. Besides, in fa/fa rats, the insulin resistance of muscle, whose insulin binding is barely altered, can be ascribed principally to defects that are located beyond the insulin-receptor interaction [30]. In IM-9 lymphocytes, the physiological significance of the decrease in insulin binding is questionable because these cells have not been shown to give specific insulin biological responses [31]. In explants from epididymal fat pads, a decrease in insulin sensitivity has been observed after prolonged exposure to insulin [7], which can be related to a decrease in specific binding at low concentrations of the labelled hormone [8]. Prolonged exposure of cultured fetal hepatocytes to 10 nmol/l insulin modifies the stimulatory effect of insulin on net glycogen synthesis with, in particular, cessation of the early insulin effect between 4 and 12 h [10]. This transient cessation of the insulin effect is not linked to any modification of insulin binding (Figs. 6 and 7). It could be caused by a defect located somewhere along the insulin-stimulated glycogenic pathway and/ or by the occurrence of an antagonistic regulation via stimulation of glycogenolysis [10]. In conclusion, cultured fetal hepatocytes allow direct correlation studies to be made between changes in insulin-mediated functions and in receptor binding, and ultimately on the fate of insulin in the cell. This study

shows a lack of relationship between the time dependence of the glycogenic effect of insulin and specifically cell-associated insulin.

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