Absence of down-regulation of the insulin receptor by insulin

A possible mechanism of insulin resistance in the rat

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Insulin resistance occurs in rat adipocytes during pregnancy and lactation despite increased or normal insulin binding respectively; this suggests that a post-receptor defect exists. The possibility has been examined that, although insulin binding occurs normally, internalization of insulin or its receptor may be impaired in these states. Insulin produced a dose-dependent reduction in the number of insulin receptors on adipocytes from virgin rats maintained in culture medium, probably due to internalization of the hormone–receptor complex. In contrast, adipocytes from pregnant and lactating rats did not exhibit this ‘down-regulation’ phenomenon. Down regulation was, however, apparent in all groups when the experiments were performed in Tris buffer (where receptor recycling is inhibited), suggesting that in pregnant and lactating rats insulin receptors are rapidly recycled back to the plasma membrane, whereas in virgin rats this recycling process is less effective. Internalization of insulin was also determined by using ¹²⁵I-labelled insulin. Adipocytes from pregnant and lactating rats appeared to internalize similar amounts of insulin to virgin rats. In the presence of the lysosomal inhibitor chloroquine, adipocytes from pregnant rats internalized more insulin than virgin or lactating rats. These results suggest that adipocytes from pregnant and lactating rats internalize insulin and its receptor normally, whereas intracellular processing of the insulin receptor may differ from that in virgin rats. In addition the rate of lysosomal degradation of insulin may be altered in adipocytes from pregnant rats.

Insulin resistance occurs during pregnancy in the rat (Knopp *et al.*, 1970, 1973; Leturque *et al.*, 1980; Sutter-Dub *et al.*, 1981) and man (Moore *et al.*, 1981; Puavilai *et al.*, 1982), gestational diabetes and an increased requirement for insulin in pregnant diabetics being apparent in the latter (McFarland, 1979). In the rat this resistance is particularly evident during late pregnancy, where, despite elevated concentrations of insulin in the circulation (Knopp *et al.*, 1973) and increased numbers of insulin receptors on the adipocyte, glucose incorporation into lipid is not enhanced (Flint *et al.*, 1979). Adipose tissue can also be considered insulin-resistant during early lactation in the rat, since circulating concentrations of insulin and the number of insulin receptors on the adipocyte are similar to those found in virgin rats (Flint *et al.*, 1979), whereas fatty acid synthesis is greatly reduced (Smith, 1973; Agius *et al.*, 1979; Flint *et al.*, 1979, 1980, 1981; Sinnett-Smith *et al.*, 1980).

Since insulin is internalized into cells by receptor-mediated endocytosis (for review see Jacobs & Cuatrecasas, 1981) leading to the so-called down-regulation phenomenon proposed by Gavin *et al.* (1974), we examined the possibility that during pregnancy and lactation internalization of insulin and its receptor may be impaired, thus leading to a decreased metabolic response.

Experimental

Materials

Bovine serum albumin (fraction V; essentially fatty acid-free), penicillin, streptomycin sulphate, amphotericin B and collagenase (C2139; 200 units/mg) were from Sigma (London) Chemical Co., London S.W.6, U.K.; medium 199 with Earle's salts and containing L-glutamine and 25 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]

was from Gibco-Biocult, Paisley, Scotland, U.K.: ^{125}I was obtained from Amersham International, Amersham, Bucks., U.K.; bovine insulin (23.6 units/mg) was donated by the Boots Pure Drug Co., Nottingham, U.K.

Animals

Wistar rats were from A. Tuck and Son, Rayleigh, Essex, U.K.: food (diet 41B: Oxoid, London E.C.4, U.K.) and water were supplied *ad libitum*. At the beginning of the experiment rats weighed between 200 and 250 g.

Determination of down-regulation of the insulin receptor (net internalization)

In culture medium. Virgin, 20-day pregnant and 10-day lactating rats were killed by cervical dislocation. Parametrial adipose tissue was removed under aseptic conditions and isolated adipocytes were prepared as described previously (Flint *et al.*, 1979). The isolated fat cells [approx. $(1-5) \times 10^6$ cells] were maintained in 20 ml of medium 199, pH 7.4, containing 10 mM-glucose, 20 mM-Tricine {N - [2 - hydroxy - 1,1 - bis(hydroxymethyl)ethyl]glycine}, 1% bovine serum albumin, 0.2 mM-penicillin, 60 μM -streptomycin, 11 μM -neomycin and 5 μM -amphotericin B at 37°C in an atmosphere of O_2/CO_2 (19:1) in the absence or presence of various concentrations of insulin for a period of 1-3 days. At the end of the culture period medium was saved and stored at -20°C for determination of insulin content by radioimmunoassay (Flint *et al.*, 1979). The cells were washed twice with Krebs Ringer phosphate, pH 7.4, containing 1% bovine serum albumin and twice with the same buffer at pH 7.8. The cells were incubated at 37°C for 10 min between each wash to ensure that any insulin bound to the cells or remaining in the medium was removed completely. Insulin binding to the cells was then determined by using ^{125}I -labelled insulin as described previously (Flint *et al.*, 1981). Since the number of receptors on the cell surface is a function of the rate at which receptors leave the plasma membrane and the rate at which they are inserted, this method detects the net rate of receptor internalization.

Determination of receptor internalization in Tris buffer (total internalization). The procedures used were identical with those used for determining down-regulation in culture medium except that 35 mM-Tris buffer containing 120 μM -NaCl, 1.2 mM-MgSO₄, 2.0 μM -CaCl₂, 2.5 mM-KCl, 10 mM-glucose and 12% bovine serum albumin was used throughout and incubations were performed for 3 h. This method measures the absolute rate of receptor internalization since Tris prevents reinsertion of receptors back into the plasma membrane (Olefsky *et al.*, 1982).

Determination of insulin internalization

Internalization of insulin was determined by the method of Kahn & Baird (1978). Briefly, 600 μl of adipocytes (approx. 10^6 cells) was incubated at 37°C in triplicate with 2 ng of ^{125}I -labelled insulin/ml in a final volume of 1 ml of culture medium. One of the replicates also received excess unlabelled insulin (5 $\mu\text{g}/\text{ml}$) to determine non-specific binding. At the times indicated in the Results section 250 μl portions were removed from two of the replicates (one being the non-specific binding tube) and spun through dinonyl phthalate oil to determine the amount of insulin specifically bound to the cells. The proportion of the cell-associated radioactivity that had been internalized was determined by adding excess unlabelled insulin to the third tube and incubating for a further 5 min before spinning 250 μl portions through oil. The unlabelled hormone should compete with the ^{125}I -labelled insulin bound to the cell surface but not with any hormone that had been internalized. The amount of radioactivity still associated with the cells after this attempt to dissociate was considered to be internalized hormone.

Since insulin is rapidly degraded and released from adipocytes, such measurements indicate the net amount of insulin internalized. For this reason identical experiments were performed with the exception that 0.1% bacitracin (to inhibit insulin degradation) and 0.5 mM-chloroquine (to inhibit lysosomal activity) were included throughout the binding studies. Since insulin is released from cells via lysosomes, the inclusion of chloroquine leads to an accumulation of radioactivity in the cells, which should then represent the absolute amount of insulin internalized.

Statistical comparisons were made by using Student's paired or unpaired *t* test where appropriate.

Results

Fig. 1 illustrates the effects of culturing adipocytes in medium 199 in the presence of insulin on the subsequent ability to bind ^{125}I -labelled insulin. Insulin binding to adipocytes cultured in the absence of insulin was stable for at least 2 days, but tended to decline slightly on day 3. Culture in the presence of insulin led to a dose-dependent decrease in insulin binding, which was accounted for by a decrease in number but not affinity of insulin receptors (Fig. 2). Fig. 1 also shows that even in the presence of high concentrations of insulin for 3 days, approx. 25% of the receptors were still present. Insulin was as effective in reducing binding when the cells were cultured 24 h before addition of the insulin and if cells were cultured for 24 h in the presence of insulin and subsequently washed and cultured in the absence of insulin they regained completely their

Fig. 1. ^{125}I -labelled insulin binding (performed as described in the Experimental section) after culture in the absence (■) or presence of insulin (1 nM, □; 10 nM, ▲; 100 nM, ●).

Also shown is the effect of washing cells cultured in the presence of insulin and subsequently culturing in the absence of insulin (○).

Fig. 2. Scatchard analysis of insulin binding to adipocytes from virgin rats cultured in the absence (●) or presence of 100 nM-insulin for 1 (△) or 2 (▲) days. ^{125}I -labelled insulin binding was determined and corrected for non-specific binding as described in the text. Each point is the mean value obtained from three separate determinations with pooled adipocytes from three to five rats for each determination. B/F is the ratio of insulin bound to adipocytes to free unbound insulin.

ability to bind insulin (Fig. 1). The decreased insulin binding was not due to the presence of insulin in the culture medium masking receptor sites, since when insulin was added 30 min before the end of the culture period (a period that allows maximal insulin binding but is too short for detectable down-regulation to occur) no reduction in ^{125}I -labelled insulin binding was observed (results not shown). This shows that the washing procedure removed completely any insulin bound to the cells or present in the medium.

Table 1 illustrates the effect of culture in medium 199 in the absence or presence of insulin on subsequent insulin binding to isolated adipocytes from virgin, pregnant or lactating rats. Specific insulin binding in control cultures was higher in pregnant than in virgin or lactating adipocytes ($P < 0.05$). Although culture in the presence of $0.1 \mu\text{M}$ -insulin significantly reduced insulin binding to the adipocytes of virgin rats, no such effect was apparent in pregnant or lactating rats. If the cells were precultured for 24 h before addition of insulin then adipocytes from pregnant rats also exhibited the 'down-regulation' phenomenon, whereas adipocytes from lactating rats did not (Table 1).

The absence of down-regulation did not seem to be due to different rates of insulin degradation in the three conditions, since there were no significant differences in the disappearance of insulin from the culture medium or in the precipitability of ^{125}I -labelled insulin in 10% trichloroacetic acid at the completion of the binding studies. Approx. 50–80% of the insulin present in the culture medium disappeared during a 24 h culture period and approx. 15% of the ^{125}I -labelled insulin added for the binding studies was degraded during a 60 min incubation period. These results show that down-regulation of the insulin receptor is impaired in the adipocyte during pregnancy and lactation.

The extent of down-regulation is a function of the rate at which insulin receptors leave the cell surface and the rate at which they are inserted into the plasma membrane, i.e. the net rate of receptor internalization. If such experiments are repeated in Tris buffer, then re-insertion of receptors into the plasma membrane is prevented and hence the method estimates the absolute rate of receptor internalization. In contrast with experiments performed in culture medium, down-regulation was evident in all three conditions when performed in Tris buffer for 3 h (Table 1). These results suggest that adipocytes from virgin, pregnant and lactating

Table 1. *Effects of culture in the presence of insulin on the subsequent ability of isolated adipocytes to bind ^{125}I -labelled insulin*

Adipocytes were cultured at 37°C in medium 199 or Tris for the stated periods, washed and their ability to bind ^{125}I -labelled insulin determined as described in the text. All values are quoted as specific binding after subtraction of the non-specific binding. All values are means $\pm$ S.E.M. for at least five separate observations using cells pooled from two animals for each observation. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared with the respective control (Student's paired t test).

	^{125}I -labelled insulin bound (pg/ 10^5 cells)					
	Medium 199				Tris/HCl buffer	
	Control (24 h)	Insulin (0–24 h)	Control (48 h)	Insulin (24–48 h)	Control (3 h)	Insulin (0–3 h)
Virgin	12.9 $\pm$ 2.2	9.0 $\pm$ 1.4*	10.4 $\pm$ 1.6	6.1 $\pm$ 0.8*	13.4 $\pm$ 1.8	7.8 $\pm$ 1.0***
Pregnant	20.9 $\pm$ 2.1	22.7 $\pm$ 2.9	15.2 $\pm$ 1.7	10.4 $\pm$ 1.9**	16.7 $\pm$ 1.8	9.5 $\pm$ 1.6**
Lactating	11.5 $\pm$ 1.7	12.0 $\pm$ 2.4	7.1 $\pm$ 0.9	6.6 $\pm$ 1.1	11.7 $\pm$ 0.8	8.4 $\pm$ 0.7***

Fig. 3. *Effect of incubation time on the non-dissociable cell-bound radioactivity (internalized hormone) for virgin (●), pregnant (■) and lactating (▲) rats*

^{125}I -labelled insulin bound to the cells was determined after attempting to dissociate the hormone by using excess unlabelled insulin. All values were corrected for non-specific binding as described in the text. Each point is the mean value obtained from four to five separate determinations with pooled adipocytes from two rats for each determination.

rats internalize insulin receptors at similar rates. The absence of down-regulation in culture medium must therefore be due to different rates at which receptors are inserted into the plasma membrane. This in turn suggests that almost all receptors in adipocytes from pregnant and lactating rats are recycled in physiological medium (hence no down-regulation), whereas in adipocytes from virgin rats a proportion of the receptors are not recycled and are presumably degraded.

The ability of adipocytes to internalize insulin is shown in Fig. 3. Internalization of the hormone appears linear only during the first 2–5 min, presumably since after this time degradation products of the insulin are released from the cells (Kahn & Baird, 1978) and eventually equilibrium will be reached between the rate at which ^{125}I -labelled insulin enters and leaves the cell. In contrast with the results for receptor down-regulation, insulin internalization was not impaired in adipocytes from pregnant or lactating rats (Fig. 3) and at equilibrium approx. 30–40% of the cell-associated radioactivity was internalized in all three groups (results not shown).

When these experiments were repeated in the presence of chloroquine and bacitracin, an increase in the amount of insulin internalized was observed at all time points for all treatments (compare Figs. 3 and 4). Preliminary experiments revealed that bacitracin, although effectively inhibiting degradation, did not affect the proportion of insulin that was internalized, whereas chloroquine, although not producing any consistent decrease in total insulin degradation, increased hormone internalization (results not shown). Under these conditions, significantly greater amounts of insulin were internalized in adipocytes from pregnant rats than in those from virgin or lactating rats (Fig. 4). Since the total amount of insulin internalized in the pregnant animal was greater in the presence of a lysosomal inhibitor but not in its absence, the rate of lysosomal processing and removal of insulin from the cell must be greater in adipocytes from pregnant rats.

Discussion

Recent studies have illustrated that insulin, after binding to its receptor, is internalized by receptor-mediated endocytosis (for review see Jacobs & Cuatrecasas, 1981), which can lead to a decrease in

Fig. 4. Effect of incubation time on the non-dissociable cell-bound radioactivity (internalized hormone) for virgin (●), pregnant (■) and lactating (▲) rats determined in the presence of bacitracin and chloroquine

Other details are as described in the legend to Fig. 3.

Each point is the mean $\pm$ S.E.M. for five to six observations. * $P < 0.05$; ** $P < 0.001$, compared with values for virgin rat.

the number of insulin receptors on the cell surface – so called ‘down-regulation’ (Gavin *et al.*, 1974). The rate of insulin entry, however, greatly exceeds the rate at which down-regulation of the receptor occurs (Terris & Steiner, 1980), which suggests that the majority of the receptors are recycled back into the plasma membrane, with only a small proportion being retained within the cell or degraded. Thus internalization of insulin can take place without a requirement for receptor loss from the plasma membrane.

Little is known about the requirement for internalization of insulin or its receptor to produce a biological effect. Rapid effects of insulin such as stimulation of hexose transport do not appear to require entry of insulin into the cell (Marshall & Olefsky, 1980), whereas the requirement of insulin internalization for long-term effects, such as the stimulation of enzyme synthesis, has not been studied. Insulin receptors have been identified on a

number of intracellular organelles including Golgi vesicles, endoplasmic reticulum and the nucleus, and the possibility that insulin acts directly on these structures has been proposed (Goldfine, 1981). It has also been suggested that degradative fragments of insulin may be involved in producing some of its biological effects (Goldstein & Livingston, 1981). The results of the present study indicated that internalization of insulin is not impaired in adipocytes from pregnant and lactating rats. Indeed, in the presence of the lysosomal inhibitor, chloroquine, adipocytes from pregnant rats internalized greater amounts of insulin than did adipocytes from virgin rats. This enhanced internalization was not apparent, however, when lysosomal activity was unimpaired. This suggests that lysosomal processing of insulin (which ultimately leads to the release of insulin degradation products from the cell) may be more rapid in the pregnant rat. Lysosomal degradation of insulin could therefore be involved in the insulin resistance of pregnancy. Such a proposal is in apparent conflict with the findings of Hammons & Jarett (1979) and Marshall & Olefsky (1980), who showed identical insulin-stimulated glucose transport in the absence or presence of insulin degradation. The insulin resistance of adipocytes from lactating rats does not appear to be due to abnormal internalization or processing of insulin, since both of these processes occurred at similar rates to those observed in virgin rats.

The results of the present study revealed a decreased ability of insulin to down-regulate the insulin receptor in culture medium in adipocytes from pregnant and lactating rats. It may be that the insulin receptor, along with insulin, since insulin accelerates the entry rate of the receptor (Reed & Lane, 1980), is required for at least some of the long-term actions of insulin.

The absence of down-regulation was not due to an inability to internalize the insulin receptor since down-regulation was evident in adipocytes from pregnant and lactating rats when the experiments were performed in Tris buffer rather than in culture medium. Tris inhibits receptor recycling (Olefsky *et al.*, 1982) and hence measures the absolute rate of receptor internalization. Thus it would seem that insulin receptors are internalized at similar rates in virgin, pregnant and lactating rats but in the pregnant and lactating rat most of the receptors are recycled back to the plasma membrane (Terris & Steiner, 1980), whereas in adipocytes from virgin rats some receptors are not recycled and presumably undergo degradation (Pilch & Czech, 1980), ultimately leading to down-regulation of the receptor. Pilch & Czech (1980) have described a proteolytic cleavage of the insulin receptor that is enhanced by insulin binding and results in the removal of a small fragment of the β -subunit of the

receptor, a process apparently mediated by lysosomes and which may be the first step in receptor down-regulation (degradation). Coupled with the report that insulin stimulates the release from the plasma membrane of a peptide that has been proposed as a second messenger (Larner *et al.*, 1979; Seals & Czech, 1981) and which is itself released by proteolytic activity (Seals & Czech, 1981), it is possible that cleavage of the insulin receptor gives rise to the putative second messenger of insulin action and ultimately leads to loss of the receptor. Taken together, these findings link some of the biological responses elicited by insulin with down-regulation of the insulin receptor rather than with internalization of insulin, since the latter can take place without a requirement for receptor down-regulation essentially by means of receptor recycling.

The altered responsiveness of adipose tissue to insulin during pregnancy and lactation does indeed coincide with the apparent inability to down-regulate the insulin receptor despite normal rates of insulin internalization. Enhanced rates of lysosomal processing of insulin may also be involved in the insulin resistance of adipocytes from pregnant rats.

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