Variations in Insulin Responsiveness in Rat Fat Cells Are Due to Metabolic Differences Rather than Insulin Binding

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Summary. Insulin resistance was studied by comparing insulin response and insulin binding in four groups of rats. Glucose metabolism in isolated fat cells from male Wistar rats weighing 340 g was less responsive to a supramaximal dose of insulin than glucose metabolism in fat cells from rats weighing 200 g. Induction of streptozotocin-diabetes in rats weighing 200 g resulted in a marked decrease in the insulin responsiveness of fat cells. Ventromedial hypothalamic lesions of 340 g rats had the opposite effect and restored the insulin responsiveness of fat cells. The responsiveness in the four groups was correlated to the rate of glucose conversion to fatty acids in fat cells. The binding of ¹²⁵I-insulin was the same in both 340 and 200 g rats. The ventromedial hypothalamic lesioned rats and

the diabetic rats showed, in spite of their great difference in insulin responsiveness, the highest binding of ¹²⁵I-insulin to fat cells. Insulin binding was not correlated to the plasma insulin level which however was reflected in the lipoprotein lipase activity in the adipose tissue. In conclusion, these results indicate that variations in insulin responsiveness in fat cells are due to alterations in cellular metabolism rather than in insulin binding.

Key words: Insulin responsiveness, rat fat cells, glucose metabolism, insulin binding, VMH lesion, diabetes, obesity, insulin resistance.

Insulin resistance in human obesity can be defined as decreased glucose tolerance despite increased insulin secretion after a glucose load [1, 2]. A number of spontaneously obese animals show insulin resistance according to this criterion [3, 4]. When male Wistar rats fed ad libitum reach a body weight of about 300 g their fat cells are less responsive to insulin. This is a suitable model for studying the mechanism of this form of insulin resistance. The decrease in insulin response is associated with the degree of obesity but not with enlargement of the fat cells per se or the animals' age [5, 6].

A decrease in the number of receptors has been found in cells from obese animals and man [7–9]. A decreased number of receptors may theoretically lead to a decrease in sensitivity. A shift to the right of the insulin dose response curve with normal response to a supramaximal concentration of the hormone has failed to explain the low responsiveness of fat cells from obese rats [10, 11]. Fat cells from obese rats have a decreased capacity to incorporate glucose into fatty acids [11–14], suggesting that the low insulin responsiveness might be caused by a post-receptor defect due to changes in certain metabolic pathways. Low responsiveness to insulin without hyperinsulinaemia is found in experimental

diabetes [15–17] and a normal response to insulin in association with hyperinsulinaemia is described in ventromedial hypothalamic (VMH) lesioned rats [18, 19].

In order to characterize further the mechanism behind the insulin resistance on the cellular level, glucose and lipid metabolism and insulin binding were studied in fat cells obtained from obese and diabetic rats with low responsiveness and from lean and VMH lesioned rats with high insulin responsiveness.

Materials and Methods

Animals

Male Wistar rats (Møllegaard, Lille Skensved, Denmark) were fed standard laboratory chow and water ad libitum (Rostock-pellets containing 21% protein, 74% carbohydrate and 5% fat based on calorie content), caged individually and adapted to 12-h light-darkness cycle.

Two control groups consisted of rats weighing 200 g and 340 g, respectively. VMH lesions were performed 10–15 days before in rats weighing 320 g; the animals in this group weighed 340–370 g when sacrificed. The diabetic group, consisting of rats weighing 200 g, was treated with streptozotocin (80 mg/kg, IV; Upjohn, Kalamazoo, New Jersey, USA) 40 h before sacrifice. The resulting blood glucose was > 18 mmol/l in all these animals.

Fig. 1A and B. The effect of streptozotocin diabetes, body weight and VMH lesions on **A** plasma insulin and **B** lipoprotein lipase activity (LPL). Vertical bars indicate mean $\pm$ SEM. $n=7$ in each group

Surgical Procedure

Stereotactically-guided lesions (David Kopf Instruments, model 900, California, USA) were produced by electrolysis under pentobarbital anaesthesia. An anodal current (1.5 mA, 15 s) was passed through steel electrodes of even thickness (0.5 mm), insulated by varnish except for 0.5 mm at the tip. The stereotaxic coordinates used for destroying the ventromedial area of the hypothalamus were 6.5 mm anterior to the interaural line, 0.6 mm lateral to the midline and 1.0 mm above the ventral floor of the skull. The accuracy of the brain lesions was controlled histologically, and only results from animals which showed distinctly symmetrical, bilateral lesions in the VMH area were included [20].

Preparation and Incubation of Isolated Fat Cells

All animals were killed by decapitation in the fed state between 10.00 and 11.00 h, the epididymal fat pads excised and fat cells prepared by treatment with collagenase 0.5 mg/ml (Sigma type I, St. Louis, Missouri, USA) [5]. Cells from each animal were incubated separately with and without insulin 10 U/l (Nordisk Gentofte, Denmark) in 20 ml polyethylene vials (B. N. Plastic, Copenhagen, Denmark), suspended in 1 ml HEPES buffer (N-2-hydroxyethylpiperazine-2'-ethane sulphonic acid 25 mmol/l, NaCl 124 mmol/l, KCl 4 mmol/l, KH_2PO_4 2 mmol/l, MgCl_2 1.5 mmol/l, CaCl_2 1 mmol/l) containing bovine serum albumin (10 mg/ml; Sigma fraction V, St. Louis, Missouri, USA) and glucose (4.7 mmol/l) labelled with $U\text{-}^{14}\text{C}$ -glucose (Radiochemical Centre, Amersham, Buckinghamshire, UK). The incubation time was 2 h at 37°C in a shaking water bath. The metabolism was stopped by addition of 0.2 ml 8 N H_2SO_4 and the CO_2 was collected as described by Gliemann [21]. The lipids were extracted with a heptane isopropanol mixture [22]. The radioactivity of triglycerides was measured in 1 ml heptane phase. The radioactivity of glyceride fatty acids in 1 ml heptane phase was determined after hydrolysis with 0.5 ml 0.5 N KOH in ethanol for 20 min at 60°C and acidification with 1 ml 1 N H_2SO_4 . The radioactivity in the heptane phase was then determined.

Lipolysis was studied by incubation of 1 ml fat cell suspension with or without noradrenaline (1 $\mu\text{g}/\text{ml}$) (Sigma) and noradrenaline (1 $\mu\text{g}/\text{ml}$) plus insulin (10 U/l) in HEPES buffer containing bovine serum albumin (20 mg/ml; Sigma fraction V). The incubation time was 1 h at 37°C in a shaking water bath. Metabolism was stopped by addition of 1 ml 10% (w/v) trichloroacetic acid, and glycerol release was determined colorimetrically as described by Korn [23].

Activity of lipoprotein lipase was determined in extracts of acetone powders of epididymal fat [24]. The powders were extracted in 0.05 mol/l Tris-HCl buffer (pH 8.0) containing 1 mol/l ethylene glycol [25] and centrifuged. Enzymatic activity of the clear supernatant was determined using as substrate a fatty acid-labelled trioleoylglycerol emulsion stabilized by phosphatidyl choline [26]. Released fatty acid was isolated by a liquid-liquid partition system described by Bel-frage and Vaughan [27]. One mU of enzymatic activity represents the release of 1 nmol fatty acid/min at 37°C. The protein concentration of the extracts was determined by a scaled-down version of the method of Lowry et al. [28]. The enzymatic activity of the extracts is expressed relative to the protein concentration. Essentially the same differences between the experimental groups were obtained using adipose tissue wet weight as reference [25].

Plasma Insulin Determination

Blood samples were collected from the neck at decapitation in heparinized tubes. Insulin was determined by radioimmunoassay using rat insulin as standard (Novo, Copenhagen, Denmark). Free and antibody-bound insulin was separated by ethanol precipitation [29].

Insulin Binding to Isolated Fat Cells

Incubation and separation of cells from the buffer were carried out as described previously [30]. In summary, a concentrated cell suspension was made up, and the cell concentration determined as a cytocrite. The cells (final concentration 5%, v/v) were incubated at 37°C in buffer containing 5% (w/v) albumin and 0.35 mmol/l bacitracin (in order to inhibit extracellular degradation of insulin) in the presence of 25 pmol/l $\text{Tyr}^{A14-125}\text{I}$ monoiodoinsulin [31] and different concentrations of unlabelled insulin. After 45 min the incubation was terminated by the addition of 10 ml chilled 0.15 mol/l NaCl and 1.2 ml silicone oil (0.99 kg/l) and centrifugation for 40 s at 1000 g. The cells concentrated on top of the oil were transferred with a piece of pipette cleaner to counting vials and assayed for radioactivity in a γ -spectrometer.

The isotherm for the binding at steady state can be described by the following equation:

$$\frac{\text{RI}}{\text{I}} = \frac{\text{R}_0}{\text{K}_d + \text{I}} + \left(\frac{\text{B}}{\text{F}}\right)_{\text{nonsp.}} \quad (1)$$

where RI is the concentration of receptor-insulin complex, I the concentration of unbound insulin, R_0 the total number of receptors, K_d the dissociation constant, and $\left(\frac{\text{B}}{\text{F}}\right)_{\text{nonsp.}}$ the bound/free ratio for non-specifically bound insulin, i.e. the binding tracer in the presence of 150 U/l insulin. For $\text{I} \ll \text{K}_d$, Equation 1 can be simplified to

$$\text{R}_0 = \left(\frac{\text{RI}}{\text{I}} - \left(\frac{\text{B}}{\text{F}}\right)_{\text{nonsp.}}\right) \times \text{K}_d \quad (2)$$

The number of cells per incubate was calculated from the measurement of the diameter distribution of 100 OsO_4 fixed cells [32].

Statistical Calculations

The differences between groups were tested for significance by the Wilcoxon test.

Results

The plasma insulin level at the time of sacrifice was similar in the rats weighing 200 g and 340 g while the diabetic animals showed reduced and the VMH lesioned rats elevated levels ($p < 0.05$; Fig. 1A). In the epididymal fat tissue the lipoprotein lipase activity

Fig. 2A–C. The effect of diabetes, body weight, and VMH lesions on the response to insulin on **A** glucose conversion to triglycerides and CO₂, **B** glucose incorporation in fatty acids and **C** lipolysis. Vertical bars indicate mean $\pm$ SEM. $n=7$ in each group except the relative values for the 340 g group in Fig. 2B where $n=3$. ▨ = absolute response; ■ = relative response

showed a similar pattern, i.e. no significant difference between the two control groups, but reduced after streptozotocin treatment and elevated levels after VMH lesions ($p < 0.01$; Fig. 1B).

The maximal response of total glucose utilization to insulin expressed in absolute values (i.e. the change in amount of metabolite from the basal level) or relative values (i.e. percentage increase over the basal level) was lower in 340 g rats than 200 g rats ($p < 0.01$; Fig. 2A). Streptozotocin treatment of the 200 g rats lowered the absolute response ($p < 0.01$), but did not affect the relative response primarily due to the low basal glucose metabolism (Fig. 2A). VMH lesions in the 340 g rats increased both the relative and absolute response ($p < 0.01$) to an even higher level than in the 200 g control rats. The absolute response to insulin on incorporation of glucose into fatty acids showed a similar pattern as on the glucose conversion to triglycerides and CO₂

Fig. 3A–C. The effect of diabetes, body weight and VMH lesions on **A** the maximal insulin stimulated U-[¹⁴C]-glucose conversion to CO₂ and triglycerides, **B** the maximal insulin stimulated glucose incorporation in fatty acids and **C** insulin binding to isolated fat cells. The insulin binding is indicated as the ratio between bound and free insulin B/F measured at insulin concentrations that are low compared to K_d . Vertical bars indicate mean $\pm$ SEM. $n=7$ in each group

(Fig. 2B). However, the relative response was the same in 200 g and 340 g and VMH-lesioned rats. Due to the low basal glucose incorporation into fatty acids in fat cells from the diabetic and the 340 g rats, the relative response in the former group could not be calculated and is based on only three animals in the latter group (Fig. 2B). The absolute antilipolytic response to insulin increased only significantly after VMH lesions in the 340 g rats ($p < 0.02$), while the relative response was the same in all groups (Fig. 2C).

The maximal insulin stimulated capacity of the glucose metabolism in fat cells is shown in Figure 3. Fat cells from rats weighing > 340 g showed a lower capacity to metabolize glucose ($p < 0.01$; Fig. 3A) mainly due to reduced incorporation into fatty acids compared with fat cells from 200 g rats ($p < 0.01$; Fig. 3B). Streptozotocin treatment of 200 g rats resulted in a marked decrease in the ability to metabolize glucose ($p < 0.01$) and espe-

cially a decrease in incorporation of glucose in fatty acid ($p < 0.01$; Fig. 3 A, B). The opposite effect on glucose metabolism was observed after VMH lesions in the 320 g rats resulting in reappearance of the ability to incorporate glucose in fatty acids. Insulin binding expressed as insulin distribution between medium and fat cells was similar in the two control groups, but tended to be higher in VMH lesioned ($p < 0.075$) and diabetic rats ($p < 0.15$; Fig. 3 C).

Discussion

This study deals with the cellular mechanism behind the insulin resistance, i.e. impaired glucose tolerance in spite of increased insulin secretion as seen in obese animals and man [1, 2]. The finding of reduced number of insulin receptors in obese man and animals [for review, 7–9] has suggested that insulin resistance in obesity might be caused by a decrease in number of insulin binding sites which theoretically leads to a decrease in sensitivity (i.e. shift to the right of the insulin dose response curve with a normal response to a supramaximal concentration of the hormone) [33]. In agreement with normal insulin binding described in obese Zucker rats [34] and increased binding found in diabetic rats [15–17], insulin-resistant fat cells from 340 g and diabetic rats in the present study showed normal and increased insulin binding, respectively (Fig. 3). Increased binding of insulin to fat cells from VMH-lesioned rats (Fig. 3) confirms the results in a recent report [35].

Insulin responses can be expressed in both absolute and relative terms as shown in Figure 2. However, as the relative values depend strongly on the ability to detect base-line levels and as relative values 'rather obscure the nature of the change' [10], the conclusions in this study are based on absolute responses.

The lack of correlation between the number of binding sites and responsiveness (i.e. response to a supra-maximal insulin dose) imply that changes at the receptor level do not contribute to insulin resistance in 340 g and diabetic rats and that regulation occurs at the post-receptor level.

In agreement with earlier studies in obese [11–14] and diabetic rats [15–17], the results in Figures 2 and 3 demonstrate a close association between responsiveness to insulin and the ^{14}C -glucose incorporation in fatty acids indicating that there is a post-receptor metabolic defect. While VMH lesions in female and weanling rats did not affect the maximal response to insulin on lipogenesis [18, 19], a transient increase in responsiveness to insulin after VMH lesion in 160 g male rats has recently been reported [35]. The improvement of the diabetic state in *db/db* mice after VMH lesion also indicates an increased responsiveness to insulin [36]. These apparent discrepancies might be explained by the metabolic state before the lesion. VMH lesions lead to an enhancement of insulin responsiveness only in animals with a severe

reduction in responsiveness to insulin on glucose metabolism, as were used in the present study.

The pronounced insulin resistance of fatty acid synthesis in diabetic and 340 g rats was not reflected in the antilipolytic response (Fig. 2). This is consistent with the study of Östman et al. [37] who found a normal antilipolytic response to insulin in adipose tissue from obese subjects with decreased glucose tolerance.

These results indicate that insulin resistance does not imply reduced responsiveness of all insulin sensitive pathways. This concept is further supported by normal lipoprotein lipase activity in the 340 g insulin resistant rats (Fig. 1 B). The low enzyme activity in the streptozotocin treated rats agrees with earlier studies [38, 39] and the marked increase in lipoprotein lipase activity after VMH lesion, in parallel with the elevation of plasma insulin levels, further supports the prominent role of insulin in the regulation of lipoprotein lipase activity [25, 40, 41]. Thus the VMH syndrome is an example of a condition with hyperinsulinaemia in conjunction with increased responsiveness on several pathways.

Furthermore, in spite of hyperinsulinaemia no reduction in insulin binding was found in the VMH lesioned rats. This is in contrast to current hypothesis of an inverse relationship between insulin binding and insulin level [for review, 2].

From these results it is concluded that (a) insulin resistance may occur independently of circulating insulin concentration and the number of insulin binding sites; (b) insulin resistance does not imply reduced responsiveness of all insulin sensitive pathways; and (c) insulin resistance is reflected in a reduced responsiveness of glucose metabolism due to a limited capacity to incorporate glucose in fatty acids.

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