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Impaired Insulin Binding to Isolated Adipocytes in Experimental Diabetic Ketoacidosis

J. Whittaker, C. Cuthbert, V. Hammond and K. G. M. M. Alberti

Department of Clinical Biochemistry and Metabolic Medicine, Royal Victoria Infirmary, Newcastle upon Tyne, UK

Summary. Insulin sensitivity in vivo and insulin binding in vitro to adipocytes have been studied in streptozotocin diabetic rats with ketoacidosis. Insulin sensitivity in vivo measured as the acute (20 min) fall in blood glucose in response to an insulin infusion of 1 U/kg body weight per hour correlated positively with arterial blood pH ($r = 0.92, p < 0.01 : n = 38$). At pH < 6.9 there was no fall in blood glucose. For studies of insulin binding to adipocytes ketoacidotic animals were divided into a group with moderate ketoacidosis (pH > 7.0) and a second group with severe ketoacidosis (pH < 6.9). Insulin binding to adipocytes was maximal in cells from both ketoacidotic and from normal rats at pH 7.6–7.8. Total binding was decreased in the diabetic rats ($p < 0.01$) and this was more marked in the severely diabetic group ($p < 0.001$) at all pHs studied. At pH 7.4, ^{125}I-insulin binding was decreased in diabetics compared with normal rats (0.89 ± 0.14 versus $2.0 \pm 0.24\%$ with 2×10^5 cells/ml: $n = 6; p < 0.01$) and also in the severe compared with the moderate ketoacidotic rats ($0.5 \pm 0.08\%/2 \times 10^5$ cells; $n = 6, p < 0.05$). Equilibrium binding studies showed that there was a small decrease in apparent affinity in adipocytes from both groups of diabetics ($K_D = 2.8 \pm 0.2 \times 10^{-9}$ mol/l, $n = 6$ in moderate ketoacidosis; $2.5 \pm 0.3 \times 10^{-9}$ mol/l, $n = 6$ in severe ketoacidosis) compared with control animals ($K_D = 1.8 \pm 0.15 \times 10^{-9}$ mol/l, $n = 6$). Scatchard analysis revealed that there was also a decrease in receptor concentration which was greater in the severely ketoacidotic group. These findings may explain in part the insulin resistance of severe ketoacidosis.

Key words: Diabetes, streptozotocin, rats, diabetic ketoacidosis, insulin receptors, insulin sensitivity, insulin resistance, Scatchard analysis.

Insulin resistance is found in severe ketoacidosis in the streptozotocin diabetic rat [1]. We have demonstrated that in this model the sensitivity of glucose metabolism to insulin in vivo is directly proportional to arterial pH [1]. The exact mechanism of this insulin resistance, however, remains obscure.

Recent studies on the insulin receptor have demonstrated that its affinity for insulin in vitro decreases with decreasing pH [2]. This has been proposed as a mechanism for the insulin resistance of diabetic ketoacidosis [3]. This is probably an over-simplification as increased binding to liver plasma membranes and to isolated adipocytes, two major targets of insulin action, have been reported in non-ketotic streptozotocin diabetic rats [4–6]. Thus in order to determine the role of insulin receptor status in the genesis of the insulin resistance of ketoacidosis, we have studied insulin binding to isolated adipocytes from severely ketoacidotic streptozotocin diabetic rats.

Materials and Methods

Materials

Collagenase (Worthington Type 1) was purchased from Cambrian Chemicals, Croydon, UK; Bovine Serum Albumin Fraction V, N-2-Hydroxyethyl piperazine-N'-2-ethanesulphonic acid (HEPES) from Sigma London Chemical Co., Poole, Dorset, UK, sodium 125 iodide (IMS 30) from the Radiochemical Centre, Amersham, Bucks, UK and lactoperoxidase (Calbiochem) from C. P. Laboratories, Bishops Stortford, UK. Crystalline porcine monocomponent insulin (Actrapid, Novo) was a gift from Dr. L. Heding, Novo Research Institute, Copenhagen, Denmark. Streptozotocin was a gift from Dr. W. Dulin, Upjohn Company, Kalamazoo, Michigan, USA.

Animals

Male Wistar rats 230–250 g (Charles River, Kent, UK) were maintained on a 12 h light cycle at 22° C and fed on standard laboratory

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meal (Oxoid, London, UK). Diabetes was produced by IV injection of streptozotocin (150 mg/kg) in 0.4 mol/l citrate buffer (pH 4.0) via the tail vein. Animals were maintained on once daily SC protamine zinc insulin according to a previously described protocol [7]. After 7 days insulin was withdrawn and 72 h later one femoral artery and one femoral vein were cannulated under light ether anaesthesia. The rats were then kept in restraining cages with free access to water but not food. Experiments were commenced 20 h later. Control rats received an IV injection of 0.4 mol/l citrate buffer (pH 4.0) and daily injections of 0.154 mol/l saline. Studies on insulin treated animals were carried out on day 7.

Insulin Sensitivity in Vivo

After an arterial blood sample (0.2 ml) was withdrawn for the determination of pH by a Corning EEL pH/blood gas analyser (Corning Scientific Instruments, Medfield, Massachusetts, USA) insulin made up in 0.154 mol/l saline containing 1 g/l of bovine serum albumin was infused at 1 U $kg^{-1}h^{-1}$ for 20 min using a Harvard infusion pump. At the end of this period 0.2 ml of arterial blood was withdrawn and deproteinized in 2 ml ice-cold 0.3 mol/l perchloric acid for subsequent analysis. Insulin sensitivity was determined as the fall in blood glucose at 20 min. Previous work from this laboratory [1] has shown that blood glucose falls linearly in normal rats until 20 min and then plateaus.

Isolation of Adipocytes

Adipocytes were isolated from epididymal fat pads in a second group of ketoacidotic and control rats by a modification of the method of Gliemann [8], after the determination of arterial pH. Krebs Ringer HEPES buffer [9] was used instead of Krebs Ringer bicarbonate buffer and the pH was adjusted to 7.7. This was found to be necessary for the maintenance of optimal pH for collagenase activity with the fat pads from the diabetic rats. Cells were quantitated by Kobayashi and Olefsky's modification [6] of method III of Hirsch and Gallian [10] and fat cell diameter was determined using a calibrated microscope by the method of Di Girolamo et al. [11].

Insulin Binding

Porcine monocomponent insulin was iodinated to a specific activity of 150–180 μCi/μg by a modification of the method of Thorell and Johansson [12]. To 5 μg insulin in 10 μl of 0.05 mol/l phosphate buffer (pH 7.0) were added 1 mCi Na 125 iodide, 10 μl 0.4 mol/l acetate buffer (pH 5.6) 20 μg lactoperoxidase in 10 μl 0.4 mol/l acetate buffer (pH 5.6) and two successive 10 μl aliquots containing 9 ng of hydrogen peroxide at 1 min intervals. The reaction was terminated 1 min after the addition of the second aliquot of hydrogen peroxide with 1 ml of 0.1 mol/l phosphate buffer (pH 7). The reaction mixture was then purified on a 1 × 30 cm Sephadex G-50 column eluted with 0.2 mol/l glycine buffer (pH 8.8) containing 2.5 g/l human serum albumin and 250 mg/l merthiolate. The fractions representing the central part of the insulin peak were pooled and stored at −20° C. ^{125}I insulin was repurified every 10 days.

For studies of pH effects on insulin binding 25 μl of concentrated cell suspension were added to 225 μl of Krebs Ringer HEPES buffer, containing 50 g/l bovine serum albumin, 0.8 g/l bacitracin and 33 pmol/l ^{125}I insulin at the indicated pHs giving a final cell concentration of 2–5 × 10^5 cells/ml. The cells were then incubated for 1 h at 37° C with gentle resuspension every 5 min. Incubations were terminated by the oil flotation method [13]. Time course studies showed that steady state was attained by 60 min at the lowest pH in all groups of animals studied. Non-specific binding was determined as the binding in the presence of 17 μmol/l unlabelled insulin. All incubations were performed in triplicate.

Competitive binding studies at pH 7.4 were performed in a similar manner except unlabelled insulin in concentrations of 6 × 10^{-11} to 10^{-7} mol/l was present and the duration of the incubation was reduced to 40 min.

Insulin Degradation

Insulin degradation was determined by trichloracetic acid precipitation [14]. ^{125}I insulin (33 pmol/l) was incubated for 40 min with adipocytes under the same conditions as for the competitive binding studies. After separation from the cells 50 μl of medium was added to 1 ml of ice cold 0.05 mol/l veronal buffer (pH 8.6) containing 2.5 g/l human serum albumin. Intact insulin was then precipitated by the addition of 1 ml 10% (v/v) trichloro-acetic acid. Control studies were performed incubating the ^{125}I insulin in the presence of buffer only.

Other Analyses

Blood glucose was measured by a hexokinase method [15]. Plasma insulin was assayed by a modification of the radioimmunoassay of Soeldner and Slone [16] using a rat insulin standard for the ketoacidotic and the fasted and fed control animals. Plasma insulin from the treated diabetics was assayed using a human insulin standard.

Statistical Analyses

All results are expressed as the mean ± SEM. Significance was determined by Student's t-test.

Results

Insulin Sensitivity in Vivo

Figure 1 shows the relationship between insulin sensitivity as assessed by the 20 min fall in blood glucose after infusion of insulin (1 U $kg^{-1}h^{-1}$) and the arterial pH in streptozotocin diabetic rats. There was a strong positive correlation between arterial pH and the fall in blood glucose concentration ($r = 0.92$, $p < 0.01$). However it should be noted that at pH values less than 6.9, there was no fall in blood glucose. For subsequent studies ketoacidotic rats were therefore divided into two groups depending on arterial pH: those with pH < 6.9 and those with pH > 7.0. The characteristics of the two groups of ketoacidotic rats and 24 h fasted control animals are shown in Table 1. Apart from the arterial pH (6.76 ± 0.02 versus 7.26 ± 0.02; $p < 0.001$) and blood glucose (15.8 ± 1.0 versus 20.1 ± 2.0 mol/l; $p < 0.001$) there were no significant differences between the two groups of diabetic animals.

Insulin Binding

Figure 2 shows the effects of altering the pH of the incubation medium on insulin binding by adipocytes

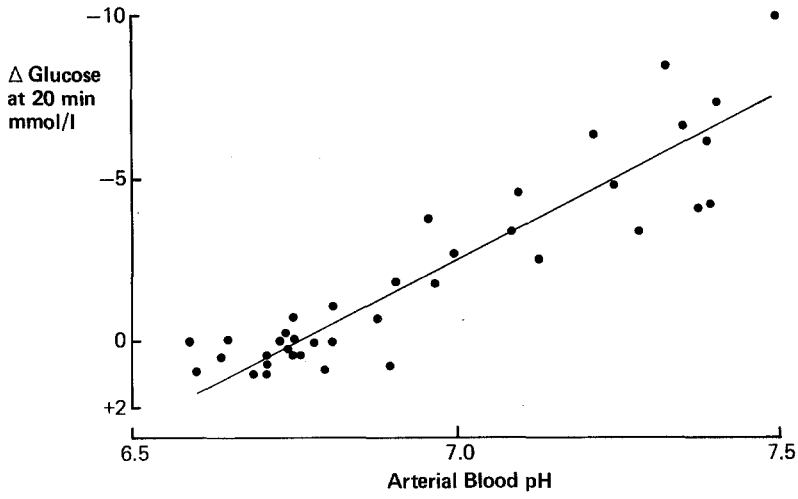

Fig. 1. Relationship between insulin sensitivity in vivo and arterial blood pH in diabetic rats. Glucose represents the 20 min fall in blood glucose in response to an insulin infusion of 1 U/kg body weight per hour. Initial blood glucose was 18.1 ± 0.75 mmol/l (n = 38)

Table 1. Details of control and diabetic rats

	Control rats (24 h fasted) (n = 12)	Moderate ketoacidosis (pH > 7) (n = 12)	Severe ketoacidosis (pH < 6.9) (n = 12)
Weight (g)	270 ± 8	256 ± 5[c]	260 ± 3
Insulin dose[a] (U/day)	–	3.3 ± 0.1	3.2 ± 0.1
Arterial blood pH	–	7.26 ± 0.26	6.76 ± 0.02
Blood glucose (mmol/l)	4.8 ± 0.1	15.8 ± 1.0[d]	20.1 ± 2.0[e]
Plasma insulin (mU/l)	16 ± 3	3 ± 1.7	4 ± 2.1
Fat cell volume (pl) (n = 6)	181 ± 22	110 ± 9[b]	110 ± 10
Fat cell surface area ($\times 10^3$ μm^2) (n = 6)	14.5 ± 1.2	10.4 ± 0.6[b]	10.3 ± 0.7

[a] Average daily dose of insulin given for the 7 days following induction of diabetes; [b] $p < 0.02$; [c] $p < 0.01$; [d] $p < 0.001$ versus controls; [e] $p < 0.01$ versus moderate ketoacidosis

from control and diabetic rats. In all groups binding was maximal between pH 7.6 and 7.8 with a rapid decrease in binding with decreasing pH. There was also a significant overall decrease in binding at all pH values in the diabetic animals. This was more marked in the group with pH < 6.9 . Non-specific binding did not change significantly with pH and there was no significant difference between the three groups (controls: 0.37 ± 0.04%; $p > 7.0$: 0.35 ± 0.03%; and pH < 6.9: 0.36 ± 0.06% of total radioactivity added: n = 4 for each group).

Competitive binding studies at pH 7.4 showed a similar pattern with a decrease in insulin binding at all insulin concentrations studied (Fig. 3). The apparent affinity of insulin receptors (50% inhibition of binding) was decreased in both diabetic groups (controls: $K_D = 1.8 \pm 0.15 \times 10^{-9}$; pH > 7.0: $K_D = 2.8 \pm 0.2 \times 10^{-9}$; pH < 6.9: $K_D = 2.5 \pm 0.3 \times 10^{-9}$ mol/l; n = 6 in all groups; $p < 0.05$ for controls versus both diabetic groups; p, NS between diabetic groups).

When these data were represented as a Scatchard plot [16] (Fig. 4), all three curves were approximately parallel suggesting that the decrease in binding was predominantly due to a decrease in the insulin receptor concentration of the cells. Also similar values for K_D were obtained by regression analysis of the initial, high affinity, almost linear part of the Scatchard plots, corresponding to insulin concentrations 6×10^{-11} to 10^{-9} mol/l (fasted controls: $K_D = 1.8 \pm 0.2 \times 10^{-9}$ mol/l, n = 6; high pH: $K_D = 2.7 \pm 0.4 \times 10^{-9}$ mol/l, n = 6; low pH: $K_D = 2.3 \pm 0.3 \times 10^{-9}$ mol/l, n = 6).

It can be seen from Table 1 that fat cell volume and surface area were significantly decreased from normal in the diabetic rats although not different in the two diabetic groups. When specific tracer binding was corrected for cell surface area (Table 2), the amount of insulin bound was significantly less in the diabetic animals and again the decrease was more marked in the severely ketoacidotic group.

Insulin Binding Before Insulin Withdrawal

In order to evaluate insulin binding in treated diabetic animals a group of insulin treated rats and fed controls were studied at 1000 h on the day following the cessation of insulin therapy. Details are given in Table 3. There was no significant difference in insulin binding per cell but plasma insulin levels were higher in

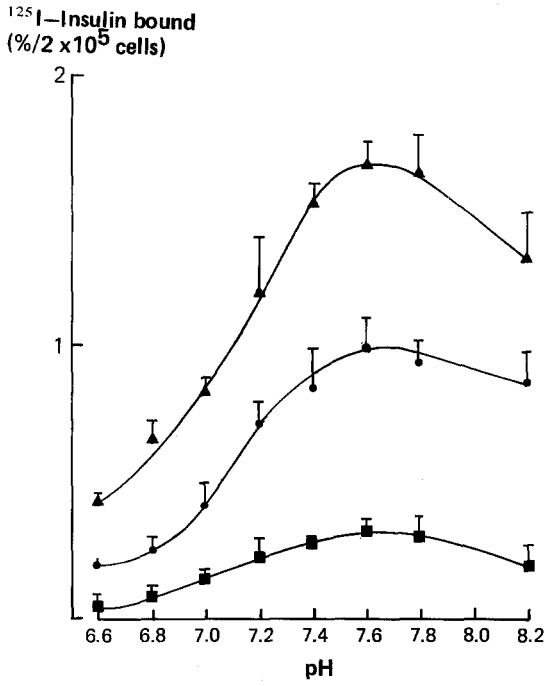

Fig. 2. Effect of pH on insulin binding to adipocytes. Percentage ^{125}I insulin (mean ± SEM) specifically bound to/2 × 10^5 cells/ml at 37° C from 25 h fasted control rats (–▲–, n =4), moderately ketoacidotic diabetic rats (–●– , n = 4) and severely ketoacidotic diabetic rats (–■–, n = 4) is plotted against pH in vitro. Binding was significantly reduced in the moderate diabetics compared with the controls ($p < 0.02$) and there was also a significant decrease in binding in the severely ketoacidotic group compared with the moderately ketoacidotic animals

treated diabetic rats although fat cell volume and surface area were decreased with respect to controls.

Insulin Degradation

Total insulin degradation was very low in all three groups studied probably due to inhibition of extracellular degradation by the bacitracin and high concentration of albumin in the incubation buffer.

Discussion

The presence of severe insulin resistance in diabetic ketoacidotic rats has been confirmed. We have shown that it is highly dependent on blood pH and in addition have confirmed our earlier finding [1] that there is a marked heterogeneity of responsiveness. At pH < 6.9 there is no response to insulin, whereas at pH > 7.0 a response to insulin is present.

In examining the role of the insulin receptor status of a major target tissue for insulin, we have demonstrated several defects in insulin binding which could explain in part the insulin insensitivity observed in experimental diabetic ketoacidosis. Firstly there is the effect of decreasing pH on the binding of insulin to its receptor. In all groups of animals studied maximum binding was found between pH 7.6 to 7.8, decreasing rapidly with decreasing pH, consistent with the behaviour of the solubilized receptor of turkey erythro-

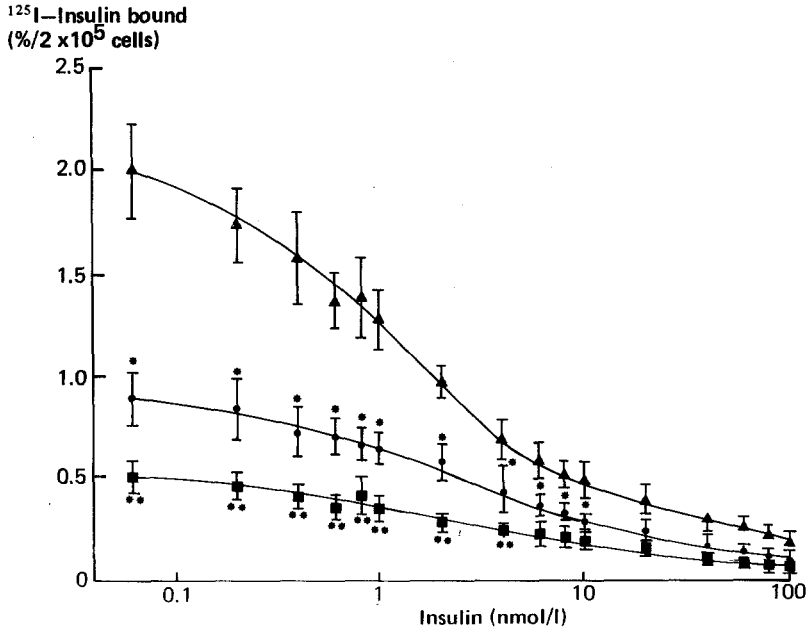

Fig. 3. Equilibrium binding studies. Percentage ^{125}I insulin specifically bound to 2 × 10^5 adipocytes/ml at 37° C from control 24 h fasted rats (–▲–, n = 6), moderately ketoacidotic (–●– , n = 6) and severely ketoacidotic rats (–■–, n = 6). Values (mean ± SEM) marked * for the moderate ketoacidotics are significantly different from the controls ($p < 0.05$) and those ** for the severe ketoacidotic rats are significantly different from the moderate ketoacidotic rats

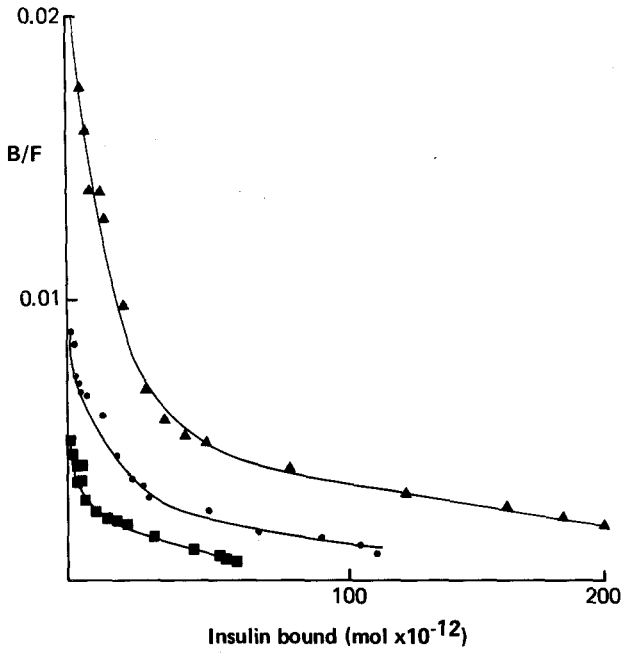

Fig. 4. Scatchard plots derived from the equilibrium binding studies for 24 h fasted controls (-▲-), moderately ketoacidotic (-●-) and severely ketoacidotic rats (-■-)

Table 2. Insulin binding by adipocytes from control and diabetic rats

	Control ($n = 6$)	Moderate ketoacidosis ($n = 6$)	Severe ketoacidosis ($n = 6$)
Insulin bound[a] (mol/μm² × 10^{15})	0.0451 ± 0.0055	0.0301 ± 0.0041[b]	0.0173 ± 0.0035[c]

[a] Insulin specifically bound per μm² surface area per cell in the presence of 33 pmol/l ^{125}I insulin; [b] $p < 0.02$ versus controls; [c] $p < 0.05$ versus moderate ketoacidosis

Table 3. Details of fed normal and insulin treated diabetic rats

	Fed normal rats ($n = 6$)	Insulin treated diabetic rats ($n = 6$)	p
Weight (g)	283 ±8	281 ± 6	NS
Blood glucose (mol/l)	7.5 ± 0.1	5.0 ± 1.0	NS
Plasma insulin (mU/l)	26 ± 8	117 ± 24	0.01
Cell volume (pl)	241 ± 20	135 ± 25	0.01
Cell surface area (× 10^3 μm²)	17.8 ± 1.0	11.9 ± 0.8	0.01
Insulin bound[a] (%/2 × 10^5 cells)	1.40 ± 0.6	1.29 ± 0.4	NS

Animals were sacrificed at 1000 h. Diabetic animals had been treated with 3.1 ± 0.2 U insulin/day for 7 days. The last dose of insulin was given 18 h before sacrifice.

[a] percentage ^{125}I-insulin (33 pmol/l) bound 2 × 10^5 adipocytes/ml

cytes [18] and the receptor of cultured human lymphocytes [2]. More surprising, however, there was also a decrease in tracer binding in both groups of diabetic animals. In addition there was a decrease in insulin receptor concentration which was greatest in the severely acidotic group of animals. Previous studies of insulin binding to adipocytes from streptozotocin diabetic rats have demonstrated either an increase in affinity [5] or an increase in both affinity and concentration of receptor [6]. However it should be pointed out that there are marked differences in the models used in these studies compared with ours. The diabetes in the previous studies was much less severe and none of the animals were noted to be ketoacidotic. In addition the period of insulinopaenia was longer than in our study protocol.

A further difference is that our animals were treated with insulin before the production of ketoacidosis. It is therefore possible that insulin therapy 'down regulated' the receptors [19, 20]. This is, however, untenable as an explanation of the differences we have observed as insulin binding before insulin withdrawal was comparable with that of control animals and, in addition, if this mechanism were acting in this context, one would perhaps anticipate a higher requirement for insulin in the low pH group, whereas requirements were in fact identical in both groups.

It is possible that for the comparative quantitation of metabolic effects in isolated cell systems, the unit surface area should be considered as the functional unit rather than the individual cell. When this approach was applied to the present results for insulin binding (Table 2), there was still a significant decrease in binding to adipocytes from the diabetic rats, with a significantly greater decrease in the animals with pH < 6.9. Interestingly when binding is expressed on the basis of cell surface area, the decrease in binding is almost entirely attributable to the change in affinity in the high pH group whereas it is not in the low pH group, suggesting that in the latter a decrease in receptor concentration is the major factor involved in the decreased binding.

At present we can offer no explanation for these findings. It is perhaps possible that failure of the affinity of the receptor to change with fasting in diabetes relates to a change in the membrane composition. In the case of the decrease in receptor concentration in the severely acidotic animals, this could be due to the severe cellular metabolic disturbance present in the cells, resulting in impairment of receptor synthesis. Studies are at present in progress to evaluate the roles of acidaemia and severe diabetes in producing these phenomena.

Although it is possible that these receptor changes are contributory to the insulin resistance of diabetic

ketoacidosis, caution should be exercised in extrapolating to the in vivo situation. These binding studies in vitro were carried out in a medium free from ketone bodies and non-esterified fatty acids and the former have been reported to increase receptor concentrations in cultured human lymphocytes sufficiently to compensate for the decrease in binding with decreased pH [21]. In addition, before altered receptor status can be confirmed as a major cause of insulin insensitivity in ketoacidosis, these studies should be extended to other tissues.

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J. Whittaker
Department of Clinical Biochemistry and Metabolic Medicine
Royal Victoria Infirmary
Newcastle upon Tyne NE1 4LP, UK