The effect of malnutrition on insulin binding to rat erythrocytes

BY HAZEL M. PAYNE-ROBINSON

Tropical Metabolism Research Unit, University of the West Indies, Kingston 7, Jamaica

AND RICHARD BROWN

Department of Medical Biochemistry, Welsh National School of Medicine, Cardiff

(Received 23 August 1990 – Accepted 8 May 1991)

Insulin binding to erythrocyte receptors was compared in malnourished and control rats. Percentage specific insulin binding to malnourished rat erythrocytes was significantly lower than to control erythrocytes ($P < 0.001$). The low insulin binding in the malnourished rat erythrocytes was accompanied by low insulin receptor affinity ($P = 0.035$).

Malnutrition: Insulin binding: Erythrocytes: Rat

Abnormalities in cell membrane structure accompany many of the physiological changes characteristic of severe malnutrition. The evidence for abnormal structure and function has been obtained from studies with erythrocytes. In severely malnourished children, abnormalities in the lipid composition of erythrocyte membranes have been observed (Coward, 1971; Brown et al. 1978; Wolff et al. 1984; Vajreswari et al. 1990). Such alterations in membrane structure would be expected to affect membrane fluidity. Abnormal membrane fragility has also been observed (Ramanadham & Kaplay, 1982). Abnormal function of the sodium pump has been shown in the erythrocytes of malnourished children (Patrick & Golden, 1977; Narayanareddy & Kaplay, 1982). The fluidity of the membrane affects the activities of the membrane-bound enzymes, and membrane Na$^+$/K$^+$-transporting ATPase (EC 3.6.1.37) activity (Kaplay, 1978), acetylcholinesterase (EC 3.1.1.7) activity (Kaplay, 1975) and Ca$^{2+}$, Mg$^{2+}$-adenosinetriphosphatase (EC 3.6.1.3) activity (Ramanadham & Kaplay, 1982) have been shown to be altered in erythrocytes from kwashiorkor children. Another aspect of membrane function which may be disturbed by malnutrition is the ability of the receptors to bind insulin. Alterations of erythrocyte membrane structure which affect membrane fluidity have been shown to affect the properties of insulin receptors (Neufeld et al. 1986). The properties of erythrocyte insulin receptors have been studied in malnourished children and low-affinity binding has been found (Payne-Robinson et al. 1988).

The effect of nutrients on the erythrocyte insulin receptor has been extensively studied in adult human subjects, and dietary manipulations have been used to induce some of the changes observed (Spanheimer et al. 1982; Ward et al. 1984). However, although the effect of nutrient intake on insulin receptors in rat insulin target organs has been extensively studied (Ip et al. 1976; Sun et al. 1977; Grundlheger & Thenen, 1982), little is known of rat erythrocyte insulin receptors.

In the present study a convenient method was developed for the study of erythrocyte–insulin binding in a small-animal model, i.e. the weanling malnourished rat. It was considered important to investigate whether the properties of erythrocyte insulin
receptors in weanling malnourished rats were similar to those in weanling malnourished children, since an animal model lends itself to more comprehensive investigation of physiological abnormalities related to membrane function.

**MATERIALS AND METHODS**

**Animals**

Wistar rats were weaned to rat chow (Pillsbury rat diet (g/kg): crude protein (nitrogen × 6·25) 169, carbohydrate 486, oil 26, with added minerals and vitamins; metabolizable energy 13·5 kJ/kg). The rats had free access to drinking water. Five litters were required for the whole study. Each litter provided enough rats for two experiments. Four rats from each litter were used as controls, and the others (six to eight) were malnourished. The malnourished rats were individually housed. The control animals were offered the diet *ad lib.* The mean intake in the controls was 8–10 g rat chow/d, and the weight gain was 4–5 g/d. The other rats who were litter-mates of the controls were malnourished on a restricted diet which offered a daily ration of 3 g rat chow (average intake 2·6 g), and which did not promote weight gain (Fig. 1). At 18.00 hours on the day before the experiment, the control and the malnourished rats were meal-fed (75 mg rat chow/g body-weight). The experiment was performed on the next day at 10.00 hours. Malnourished and control rats of the same age (between 15 and 25 d after weaning) were compared.

**Experimental procedure**

The insulin-binding experiment was conducted with blood pooled from different rats of the same litter. Approximately 3–4 ml heparinized blood was obtained from three or four malnourished rats, or from one or two control rats. Blood was collected into heparin by cardiac puncture from animals lightly anaesthetized with diethyl ether. The blood from each animal was collected in a separate tube, and immediately after collection the tubes were placed in crushed ice. After centrifugation (600 g, 5 min at 4°), the plasma was aspirated and pooled for storage at −20° for determination of insulin and glucose concentrations. The sedimented blood cells were then pooled and washed twice in 10 ml HEPES–phosphate buffer (pH 8·2). After each wash, the upper 100 µl of cells were discarded with the supernatant fraction to remove residual mononuclear cells and reticulocytes. Finally, 6 ml HEPES–phosphate buffer with human serum albumin (10 g/l) was added to the sedimented erythrocytes. The leucocyte:erythrocyte in this final suspension was less than 1:7000000.

**Insulin-binding experiment**

The method used was that of Gambhir *et al.* (1978). Approximately 6 ml erythrocyte suspension was available for each competitive-binding curve and for determination of cell counts. Portions (400 µl) of erythrocyte suspension (1·5–2·5 × 10⁸ erythrocytes/ml) in duplicate were added to a series of polypropylene microfuge tubes. Each tube contained 50 pg ¹²⁵I-labelled A14-porcine insulin (specific activity 250–275 Ci/g) in 50 µl buffer and, with the exception of one duplicate set, 50 µl of one of a series of solutions of unlabelled insulin (Sigma porcine, 25·5 U/mg) in HEPES–phosphate buffer with albumin (10 g/l). The concentration range of the unlabelled insulin solutions was 1·67 × 10⁻¹¹ M–1·67 × 10⁻⁶ M. There were seven tubes in duplicate for each binding curve, including one duplicate set without unlabelled insulin. The cells were vortex mixed (3 s) and incubated at 15° for 90 min. Then 400 µl of the incubated suspension were transferred to prechilled microfuge tubes containing 200 µl dibutylphthalate, and immediately centrifuged.
in an Eppendorf microfuge for 30 s. The supernatant fraction and dibutylphthalate were removed by suction. The microfuge tubes with sedimented erythrocytes were placed directly into counting tubes for determination of radioactivity in an autogamma counter. The percentage of radioactivity bound by $4 \times 10^9$ erythrocytes at an insulin concentration of $1.67 \times 10^{-9}$ M was used to measure non-specific binding (range 1.5–2.5 % of the total radioactivity added). The non-specific binding was subtracted from the percentage of total radioactivity bound in each tube to give the percentage of insulin specifically bound. The specific binding in the absence of unlabelled insulin was taken as the maximum specific insulin binding (SB). Iodinated insulin degradation in the incubation medium (tested by trichloroacetic acid precipitability) was < 10% of the total radioactivity, and did not differ between the malnourished and control experiments.

Analytical methods
Insulin was assayed by the double-antibody method of Hales & Randle (1963) modified for increased sensitivity (Robinson & Picou, 1977) using rat insulin standards (courtesy of Eli Lilly). Glucose was determined using glucose oxidase (EC 1.1.3.4) and peroxidase (EC 1.11.1.7) (Huggett & Nixon, 1957).

Data analysis
Insulin receptor binding to erythrocytes was quantitatively assessed as follows:

1. Competitive binding curves in which the percentage of total radioactivity specifically bound was plotted as a function of insulin concentration;
2. Scatchard analysis in which bound : free radioactive insulin was plotted as a function of bound hormone (Scatchard, 1949). This was a curvilinear plot, characteristic of insulin receptors (De Meyts & Roth, 1975). The number of receptor sites and the affinity constant were calculated from the plot for insulin concentrations in the ranges (1) $1.67 \times 10^{-11}$–$1.67 \times 10^{-9}$ M, and (2) $1.67 \times 10^{-9}$–$1.67 \times 10^{-7}$ M;
3. the significance of the difference between means was calculated by unpaired t test. To facilitate the use of parametric statistical analyses, the variables had to be transformed. Log transformations were used for specific insulin binding, receptor affinity and the numbers of receptor sites.

RESULTS
The change in body-weight (g) from weaning to 30 d post weaning in malnourished and control rats are compared in Fig. 1. In the malnourished rats, body-weight declined slightly during the first 5 d after weaning and did not change significantly during the following 25 d. In the control rats, body-weight increased slightly during the first 5 d after weaning, and then increased rapidly at the rate of 4–5 g/d. At 30 d post weaning, the control rats were still gaining weight at the same rate.

A comparison of the competitive-binding curves for erythrocytes from malnourished and control rats are shown in Fig. 2(a). The percentage specific insulin binding (SB) in the erythrocytes from the malnourished rats was significantly less than that in the control erythrocytes ($P < 0.001$). In the lower range of insulin concentrations (Fig. 2(a)) the low SB was accompanied by a low insulin receptor affinity in the malnourished rats compared with the control rats ($P < 0.05$), but no difference was shown in the number of receptor sites (Table 1). Affinity and sites in the higher range of insulin concentrations did not differ in the malnourished and control erythrocytes. Scatchard plots from which the affinity constants and the number of sites were calculated are shown in Fig. 2(b). A comparison of the affinity and the number of sites in the concentration ranges (1 and 2) of the Scatchard plot is shown in Fig. 3.
Fasting plasma insulin was approximately four times higher in the control rats than in the malnourished rats \((P < 0.001)\), but there was no difference in fasting plasma glucose levels (Table 1).

**DISCUSSION**

Our results demonstrated that food restriction in weanling malnourished rats induced changes in the erythrocyte membrane which led to decreased insulin binding and receptor affinity. However, severe malnutrition is apparently a different physiological state from acute starvation in which the opposite effect has been shown. Acute food deprivation is known to induce increased insulin binding to its receptor, and this has been demonstrated in circulating cells (DeFronzo et al. 1978; Wachslicht-Rodbard et al. 1979).

Specific insulin binding was approximately 44% less in the malnourished rat erythrocyte than in the control. The decreased insulin binding was associated with a 40% decrease in receptor affinity, with no apparent decrease in the number of receptor sites. However, the method used in the present study did not allow accurate analysis of receptor concentration. Nevertheless the results are in agreement with those from studies in malnourished children in which low erythrocyte insulin receptor affinity was also found (Payne-Robinson et al. 1988). In the malnourished rat hepatocyte, low receptor affinity was also found, but this was associated with an increase in receptor concentration (Thakur et al. 1988). In healthy men with marginal energy deficiency, erythrocyte insulin binding was 16% decreased but receptor affinity was not altered (Garrel et al. 1984).

Whether the erythrocyte abnormalities in malnutrition are reflected in the typical insulin target cells is unknown. In some physiological conditions erythrocyte insulin receptors have similar binding properties to typical insulin target cells (Olevsky & Reaven, 1977; De Pirro et al. 1980). However, there are problems in extrapolating from insulin binding in
MALNUTRITION AND ERYTHROCYTE INSULIN BINDING

malnourished (●) and control (○) rats. For details of dietary treatments, see p. 280. Points are means with their standard errors represented by vertical bars. (b) Scatchard plots derived from the first four points of the binding curve. The insulin receptor affinity and the number of receptor sites were calculated from this plot (Table 1).

**Table 1. Comparison of the properties of erythrocyte insulin receptors from malnourished and control rats** (Mean values and standard deviations)

<table>
<thead>
<tr>
<th>No. of experiments...</th>
<th>Malnourished</th>
<th>Control</th>
<th>Statistical significance of difference: P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>sd</td>
<td>Mean</td>
</tr>
<tr>
<td>Specific $^{125}$I-labelled A14 insulin bound (%)</td>
<td>4.2</td>
<td>3.2</td>
<td>7.6</td>
</tr>
<tr>
<td>Affinity constant* (× 10⁹/m)</td>
<td>2.5</td>
<td>1.9</td>
<td>7.0</td>
</tr>
<tr>
<td>No. of receptor sites per cell*</td>
<td>46</td>
<td>53</td>
<td>29</td>
</tr>
<tr>
<td>Plasma insulin (µM)</td>
<td>42.2</td>
<td>12.7</td>
<td>161.3</td>
</tr>
<tr>
<td>Plasma glucose (mm)</td>
<td>3.9</td>
<td>0.56</td>
<td>3.7</td>
</tr>
</tbody>
</table>

* The affinity constants and the numbers of sites were calculated from Scatchard plots derived from the percentage radioactivity bound by $4 \times 10^9$ erythrocytes/ml in the insulin concentration range 16.7–16700 PM.

erythrocytes to that in other tissues. For instance, there are differences in the mechanism by which the receptors of erythrocytes and monocytes are regulated (Dons et al. 1981), although they have the same structure (Ward & Harrison, 1986). In addition, erythrocyte insulin binding is closely related to cell age (Kosmakos et al. 1980), and a preponderance...
of old erythrocytes and few newly synthesized erythrocytes were found in malnutrition suggesting that erythropoiesis is impaired (Ramdath et al. 1985).

There is impaired glucose tolerance, using either an oral or intravenous glucose load, in both children and experimental animals with severe malnutrition (Baig & Edozien, 1965; James & Coore, 1970; Weinkove et al. 1976), and insulin insensitivity has been demonstrated in vivo (Alleyne et al. 1972). Comparison of the erythrocyte and insulin target-cell receptors in malnutrition would help to resolve this problem.

Low fasting plasma insulin was found in the malnourished rats. This confirms the findings of others (Weinkove et al. 1976). However, in the present study there was no difference in the fasting plasma glucose in the malnourished and control rats.

In conclusion, the sensitivity of the rat erythrocyte to insulin was decreased during malnutrition. This may be related to the structural abnormality of the erythrocyte membrane characteristic of severe malnutrition. Whether the erythrocyte receptor faithfully mimics the receptor in typical insulin target tissues can only be ascertained by studies on such tissues. These studies may be conveniently carried out in an animal model such as the rat. Low insulin receptor binding or affinity, or both, may be related to the carbohydrate intolerance of malnutrition.

H. M. P.-R. gratefully acknowledges the facilities for this work while on study leave provided by Dr Stuart Woodhead, Department of Medical Biochemistry, Welsh National School of Medicine, and the kind hospitality of the staff at this institution. The authors wish to thank Dr D. T. Simeon for advice on statistical analysis.

This work was jointly supported by the British Council and the Welsh National School of Medicine.

REFERENCES
MALNUTRITION AND ERYTHROCYTE INSULIN BINDING


