

Differential binding of sulphated insulin to adipocytes and hepatocytes

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Summary. The polymerization and precipitation of highly purified insulins which causes major problems in portable infusion systems does not occur with sulphated insulin. To compare the biological behaviour of sulphated insulin with that of a neutral highly purified monocomponent insulin, insulin receptor studies were performed on human and rat adipocytes and rat hepatocytes. Sulphated insulin displayed a lower affinity for binding to both human and rat adipocytes compared with neutral insulin, approximately four times the concentration being required to achieve half-maximal displacement of monoiodoinsulin ($p < 0.05$ and 0.01 , respectively). A 20-fold

higher concentration of sulphated insulin was required for half-maximal displacement from rat hepatocytes ($p < 0.025$). However, sulphated insulin bound to liver membranes with an affinity more closely resembling that for adipocytes rather than hepatocytes. Differences in the intracellular processing of the negatively charged insulin could account for the observed lower affinity of binding to hepatocytes.

Key words: Sulphated insulin, adipocytes, hepatocytes, insulin receptors.

Highly purified insulins tend to polymerize and precipitate when shaken or exposed to various materials [1]. This property is most relevant when such insulins are used in portable infusion systems [2]. Infusion of partially aggregated insulin results in a loss of glycaemic control and necessitates frequent changes of the insulin reservoir. Long-term studies of intravenous insulin infusion in pancreatectomized dogs have shown that good glycaemic control was achieved only when the insulin in the reservoirs was changed every 3 days [3, 4]. Subsequent studies have shown that this is not necessary if a sulphated insulin is used [5]. This advantage is most likely to be a result of the stable monomeric nature of sulphated insulin (SI) which does not precipitate under conditions which render conventional insulins visibly turbid [6].

The binding of this modified insulin to insulin receptors has not been examined previously. SI is charged negatively due to the presence of four to six sulphate groups per molecule [1, 7, 8]. It can therefore be anticipated that changes in binding affinity may occur, such as the reduced affinity of SI for antibodies to bovine insulin [5, 9]. To examine this possibility, the binding-displacement activity of SI has been compared with that of a neutral monocomponent insulin, in human and rat adipocytes and in rat hepatocytes. In addition, binding to highly purified liver membrane and insulin degradation by hepatocytes have been examined.

Materials and methods

Insulin binding to human adipocytes

Subcutaneous adipose tissue (5 g) was obtained from the abdomen of patients, aged 20–65 years, undergoing gastroenterological surgery. None of the patients suffered from endocrine or metabolic disorders or were taking drugs known to affect metabolism. The study was approved by the Newcastle-upon-Tyne Ethical Committee and informed consent was obtained from the patients.

Adipocytes were isolated by a 90-min incubation with collagenase (0.5 mg/ml) in a Hepes buffer (pH 7.4) containing 25 mg/ml human serum albumin. The cells were subsequently washed four times in a HEPES buffer (10 mmol/l Hepes, 50 mg/ml human albumin, pH 7.4) [10]. Insulin binding to adipocytes (300 μ l cell suspension with an adipocyte volume fraction of 0.05, i.e. about 1×10^5 cells/ml cell suspension) was measured after incubation in the Hepes buffer at 37 °C for 60 min with [¹²⁵I]-A₁₄ monoiodoinsulin, with and without increasing amounts of unlabelled insulin. The reaction was stopped by adding 10 ml of chilled 0.154 mol/l saline and subsequent centrifugation through silicone oil [10]. Non-specific binding accounted for 6.8% of cell-bound insulin at tracer insulin concentration.

Insulin binding to rat adipocytes

Isolated fat cells were prepared from epididymal and retroperitoneal fat from fed rats of 190–240 g using collagenase (2 mg/ml) in a Hepes buffer containing bovine serum albumin (50 mg/ml). Binding studies were performed as described for human adipocytes, but using centrifugation through dinonylphthalate to separate cells from medium [11]. For all incubations a Hepes buffer (pH 7.4) containing bovine serum (25 mg/ml) albumin was used. Non-specific binding to rat adipocytes accounted for 10.6% of cell-bound insulin.

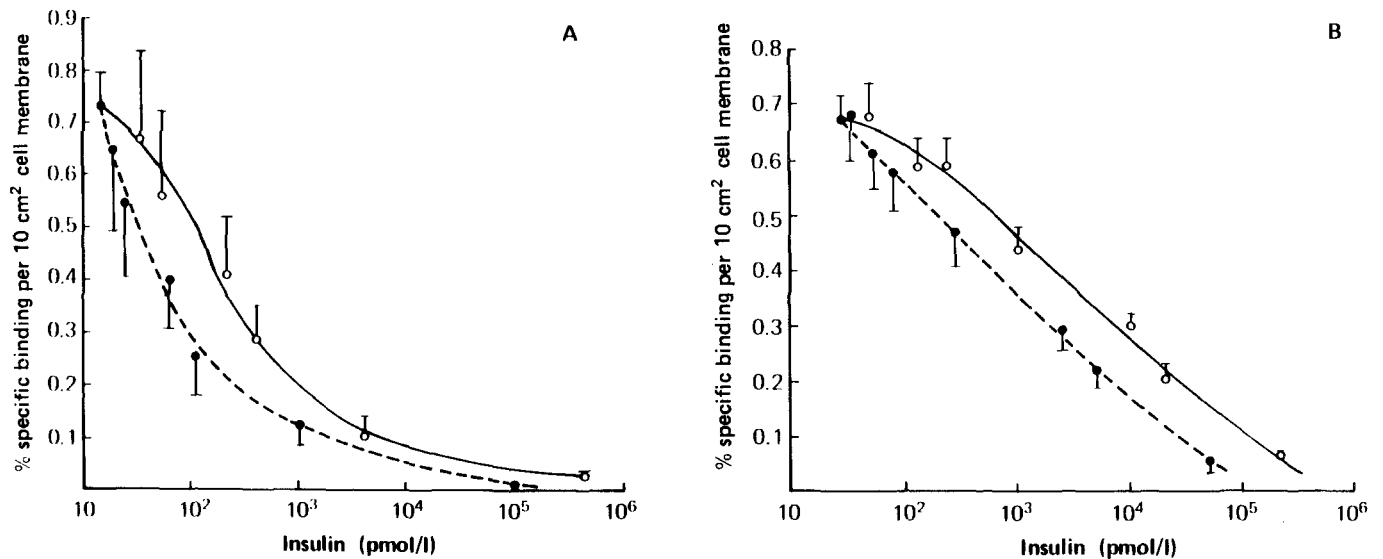


Fig. 1. Specific insulin binding to (A) human adipocytes incubated with 15 pmol/l monoiodoinsulin at 37 °C in the absence or presence of sulphated (○—○) or Actrapid (●---●) insulin in increasing concentrations ($n=7$); and (B) rat adipocytes incubated with 30 pmol/l monoiodoinsulin at 37 °C in the absence or presence of sulphate (○—○) or Actrapid (●---●) insulin in increasing concentrations ($n=5$).

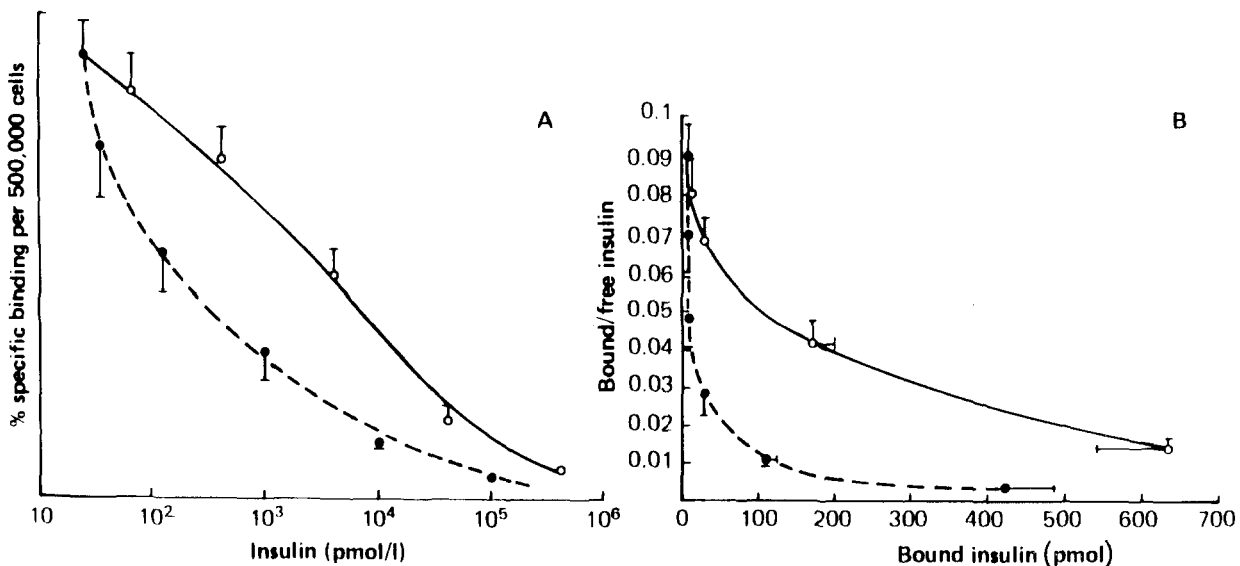


Fig. 2. A Specific insulin binding to rat hepatocytes incubated with 25 pmol/l monoiodoinsulin at 37 °C in absence or presence of sulphated (○—○) or Actrapid (●---●) insulin in increasing concentrations ($n=6$). B Scatchard analysis of the data.

The diameter of adipocytes ($n=150$) was measured at 200-fold magnification using an eyepiece micrometer (Graticules, Tonbridge, Kent, UK). Surface area and volume were calculated for every measured cell diameter [10]. Results of adipocyte insulin binding studies are expressed as percentage specific binding per 10 cm² cell membrane surface [12, 13].

Insulin binding to rat hepatocytes

Hepatocytes were prepared by collagenase perfusion of rat liver [14] and subsequently the isolated cells were suspended in a modified Krebs-Henseleit buffer (pH 7.4), containing Hepes (20 mmol/l), NaHCO₃ (10 mmol/l), bovine serum albumin (30 mg/ml), bacitracin (800 mg/ml) and gentamicin (50 mg/ml). Viability was estimated by exclusion of Trypan blue (0.4% w/v) and usually exceeded 90%. Insu-

lin binding to hepatocytes (about 1×10^6 cells/ml) was measured after a 40-min incubation at 37 °C with ¹²⁵I-A₁₄ monoiodoinsulin, with and without increasing amounts of unlabelled insulin. Non-specific binding accounted for 10.3% of cell-bound insulin at tracer insulin concentration.

Insulin degradation by rat hepatocytes

Total degradation (cellular and extracellular) of [¹²⁵I]-A₁₄ monoiodoinsulin was assessed after a 40-min incubation without and with increasing concentrations of insulin by the trichloroacetic acid precipitation method [15]. Data are presented as percentage total degradation, extracellular degradation (as assessed in the presence of 10⁻³ mol/l insulin) accounting for < 18% of total degradation for both sulphated and neutral insulin.

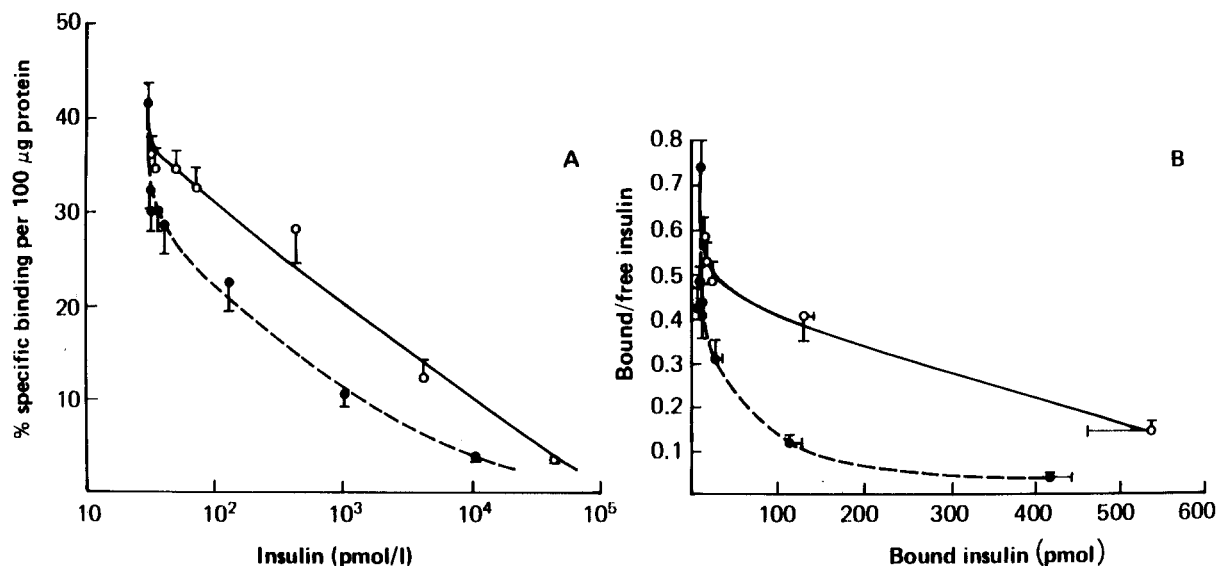


Fig. 3. A Specific insulin binding to rat liver plasma membrane incubated with 25 pmol/l monoiodoinsulin at 4°C in absence or presence of sulphated (○—○) or Actrapid (●---●) insulin in increasing concentrations (n=6). B Scatchard analysis of the data

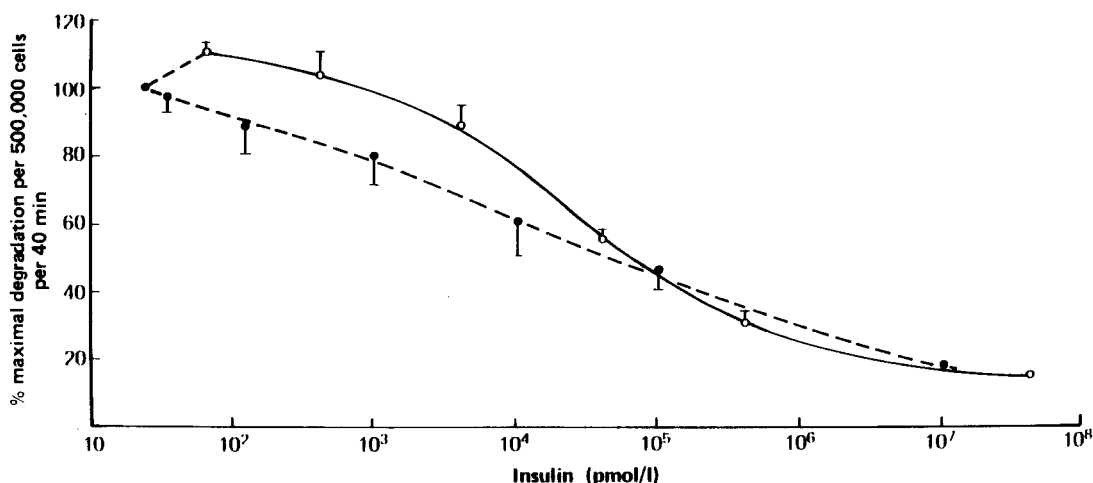


Fig. 4. Degradation of monoiodoinsulin by rat hepatocytes incubated at 37°C for 40 min in the absence or presence of sulphated (○—○) or Actrapid (●---●) insulin in increasing concentrations (n=5)

Insulin binding to highly purified liver membrane

Membranes were prepared by a two phase polymer method, using polyethyleneglycol 6000 and Dextran T500 [16], followed by suspension in a Tris-buffer (pH 7.8), containing bovine serum albumin (1 mg/ml) and bacitracin (1 mg/ml). Insulin binding was measured after a 20-h incubation at 4°C. Non-specific binding accounted for 0.96% of total bound insulin at tracer insulin concentration.

Chemicals

Sulphated insulin (6IU/mg, Connaught Laboratories, Willowdale, Ontario, Canada), Actrapid insulin (26.8 IU/mg, Novo, Copenhagen, Denmark), human serum albumin (Behringwerke, Marburg, FRG), bovine serum albumin, fraction V (Sigma, London) treated with activated charcoal and extensively dialysed before use, crude collagenase (Clostridium histolyticum, batch no.504/22, PL Biochemicals, Milwaukee, Wisconsin, USA), polyethyleneglycol 6000 (British Drug Houses Chemicals, Poole, Dorset, UK), Dextran T500 (Pharmacia, Uppsala, Sweden); bacitracin (Sigma, London); gentamicin (Sigma), [¹²⁵I]-A₁₄ monoiodoinsulin (sp. act. 231 µCi/mg; Novo); dinonyl-phthalate (British Drug Houses Chemicals); silicone oil (200/50 centistokes, Dow Corning, Midland, Michigan, USA).

Statistical analysis

All results are presented as mean ± SEM and statistical analysis was performed using Student's one-tailed t-test.

Results

Sulphated insulin displayed a lower affinity for binding to both human and rat adipocytes compared with neutral insulin (Fig. 1). The insulin concentrations at which half-maximal displacement of tracer occurred were: human adipocytes 234 ± 87 versus 59 ± 15 pmol/l (p < 0.05) and rat adipocytes 5560 ± 1230 versus 1216 ± 277 pmol/l (p < 0.01). Expressed as ratios, the relative half-maximal displacement concentrations were 4.0:1 for human adipocytes and 4.6:1 for rat adipocytes. SI bound to rat hepatocytes with a much lower affinity than did neutral insulin, half-maximal displacement of tracer being observed at 3975 ± 1426 versus 191 ±

55 pmol/l ($p < 0.025$; ratio 20.8:1). Scatchard analysis suggested that the difference was most marked for the high affinity receptors, the slopes of the high affinity portions of the plots being -1.78 ± 0.46 and -0.07 ± 0.02 , respectively ($p < 0.025$; Fig. 2).

The difference in affinity of binding to liver membranes was not as pronounced as that observed with hepatocytes (Fig. 3), half-maximal displacement binding observed at 972 ± 150 versus 154 ± 12 pmol/l for SI and neutral insulin, respectively ($p < 0.001$; ratio 6.3:1).

The ability of SI to inhibit degradation of monoiodoinsulin was much less than that of neutral insulin at concentrations up to 10^5 pmol/l (Fig. 4). Inhibition of degradation by 10% was achieved at 3020 ± 1213 pmol/l for SI and 122 ± 43 pmol/l for neutral insulin ($p < 0.05$; Fig. 4). This measurement of degradation includes both cell-mediated and medium degradation. The contribution of the latter, as assessed by degradation in the presence of excess unlabelled insulin, was small ($1.3 \pm 0.3\%$ for SI and $1.6 \pm 0.5\%$ for neutral insulin).

Discussion

We have demonstrated that the binding of SI to the insulin receptor of both human and rat adipocytes is approximately four times less avid than that of a neutral insulin. For rat hepatocytes, the reduction in affinity of SI for the insulin receptor was more pronounced. This difference in binding behaviour between tissues provides a possible physiological basis for the recent observation that SI is metabolised in at least two different tissues at different rates (M. Nomura and A. M. Albisser, unpublished observations). The clearance rate of SI from the circulation is prolonged in diabetic dogs [5]. As the liver is the major organ responsible for insulin clearance, it is perhaps not surprising that SI demonstrates both reduced affinity for hepatic insulin receptors and a reduced clearance rate *in vivo*.

If the tissue differences were a consequence of differences in the characteristics of the insulin receptor of liver and fat, it could be anticipated that the binding interaction between SI and liver membranes would resemble that between SI and intact hepatocytes. This was not found to be the case, the ratio of half-maximal displacement concentrations for SI: neutral insulin for liver membranes (6.3:1) being closer to (although significantly different from) that for adipocytes (4.0:1 and 4.6:1). The lower measured affinity of SI binding to hepatocytes (ratio 20.8:1) is not accounted for by simple change in degradation of the insulin by hepatocytes as at low insulin concentrations SI decreases the rate of degradation of monoiodoinsulin less than does neutral insulin. If the effect of degradation upon the observed binding results was removed, the calculated specific insulin binding at any one SI concentration up to 10^5 pmol/l would be slightly higher and this would reduce

the apparent affinity still further. Therefore there must be differences in the dynamics of intra-hepatocyte handling of SI compared with neutral insulin. More detailed studies of intra-cellular processing of SI, using [35 S]-sulphated insulin, will be required to elucidate these differences.

The binding characteristics of other synthetic insulin analogues and some naturally occurring unusual insulins have been previously examined. A five fold difference in relative affinity of binding to liver and fat was also seen with hagfish insulin and some of the insulin dimers [17–20]. In the case of hagfish insulin, this difference was assumed to be intrinsic to the insulin receptors of each tissue [17], although studies with liver membranes were not performed to exclude variations in intracellular processing. Porcupine insulin shares with SI both the property of not aggregating in solution and a fourfold lower binding affinity for rat adipocytes than bovine insulin [21]. It has been suggested that the marked tendency to self-aggregation exhibited by native insulin is important in terms of cell surface receptor aggregation and hence insulin action [22, 23]. The study of SI action *in vitro* may be expected to explore this hypothesis, and such studies are currently underway. In order to consider fully the implications for insulin – insulin receptor interactions of the binding behaviour of insulin analogues, the molecular structure of the analogue must be defined. The disposition of the sulphate groups within the SI molecule is at present being investigated.

The finding of differential affinities of binding of SI for hepatocytes and adipocytes indicates possibilities for the independent manipulation of different tissues *in vivo*. Documentation of better glycaemic control during long-term infusion of SI in dogs [15] probably relates to the stability of this insulin in the infusion system, but additional *in vivo* effects of different tissue affinity are possible. The use of SI in portable infusion systems for diabetic subjects offers some theoretical advantages, particularly with respect to the development of implantable devices with long-term insulin reservoirs.

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