Characterization of Insulin Binding to Isolated Ovine Adipocytes

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Abstract To study the mechanism of insulin resistance in ruminant adipose tissues, we measured the binding of insulin to isolated adipocytes obtained from sheep and rats. Our data demonstrated that insulin readily and specifically bound to ovine adipocytes as well as to rat adipocytes. The time course of this reaction indicated that steady state binding conditions remained constant during an incubation period between 1 and 2 hours. The specific binding of ^{125}I -insulin to ovine and rat adipocytes was 4.8% and 6.9% for 1×10^6 cells, respectively. At concentrations of unlabeled insulin between $3.9 \times 10^{-11}\text{M}$ and $4.2 \times 10^{-9}\text{M}$ ovine adipocytes showed significantly less specific binding than rat adipocytes. When analyzed by Scatchard plots based on negative cooperativity model, the total receptor concentration (R_0) in ovine adipocytes was identical to that in rat adipocytes, but the affinity of the empty receptor ($\bar{K}_e$) was reduced to about 70% of that in rat adipocytes. The average affinity profile at occupancy of 10% of insulin receptors on ovine adipocytes was considerably lower than that of rat adipocytes. These results indicated that a lower insulin binding to adipocytes in sheep was due to conspicuously lower affinity of the receptor for insulin, without any difference of receptor concentration. This may be related to insulin resistance in ruminants.

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Key words: insulin resistance, ovine adipocytes, receptor, insulin binding, affinity

It has been reported that adipose tissues from ruminants respond poorly to insulin *in vitro*¹⁻³⁾ suggesting that ruminant tissues are insulin resistant or insensitive. The mechanism of insulin resistance in ruminants is still unknown, although in non-ruminants it has been shown that insulin insensitivity in a number of different disease states may be due to an impaired ability of insulin binding to receptors⁴⁻⁶⁾.

It is thought that the initial step in insulin action involves binding of insulin to a specific plasma membrane receptors⁷⁾. In non-ruminants, insulin receptor binding has been well established by the investigations using adipocytes, hepatocytes, lymphocytes and erythrocytes^{7,8)}. It seems to be important to study insulin-receptor binding in adipocytes which is a major site of insulin action in ruminants as well as non-ruminants. However, insulin binding has been less thoroughly characterized in ruminant adipocytes, due to the difficulty in obtaining biologically active adipocytes⁹⁾. There are a few reports which indicate that ruminant adipocytes will be useful to study insulin binding^{10,11)}, although no quantitative information has been presented. More recently, VASILATOS *et al.*¹²⁾ have reported that the insensitivity to insulin in cattle was associated with a negligible capacity for insulin binding to adipocytes, but it has not

been clarified whether the lack of insulin-receptor binding might be due to decreased numbers of insulin receptor or decreased affinity for insulin to the receptors.

The aim of the present study is, therefore, to systematically characterize binding of insulin to the specific receptor located on isolated ovine adipocytes and to clarify the mechanism of insulin resistance in ruminants, according to compare insulin-receptor binding in ovine adipocytes with that in rat adipocytes.

Materials and Methods

Experimental animals

Four adult female Suffolk × Corriedale crossbred sheep weighing 33–43 kg were used. They were housed in individual cages at an environmental temperature of 19–21°C. They were fed 800 g of hay cube and 200 g of concentrate (New calf super pellet; crude protein 21%, crude fat 2%, crude fiber 5%, crude ash 7%, TDN 77%, DCP 19%, : Nihon Haigo Shiryō Co., Ltd.) once daily at 0800 h, but they were allowed free access to water. Food was generally consumed within 2 h. On the day of experiments, animals were not fed until the end of sampling of blood and adipose tissues. Blood samples were taken at 0800 h from the jugular vein with heparinized syringes. Plasma was separated by centrifugation and stored at –40°C until the assay of insulin and glucose.

Female Wistar strain rats weighing 190 g, 10 weeks of age, were used and fed Oriental rat chow (Oriental Yeast Co., Ltd.). Water was available continuously. Rats were decapitated immediately after a blow to the head between 0800–0900 h. Blood from the cervical stump was drained into a heparinized test tube.

Isolation of adipocytes

Ovine adipose tissues (3–4 g) were obtained by open biopsy from the rump region after an incisional area was locally anesthetized with 2% procaine hydrochloride. Subcutaneous adipose tissues were taken out and immediately placed in 0.154 M NaCl (38°C). Adipose tissues were then rinsed with fresh saline and dissected free of connective tissues and blood vessels. Isolated adipocytes were prepared according to modification of the method of RODBELL¹³⁾. Briefly, the tissue weighing approximately 1 g was placed in 3 ml Krebs-Ringer bicarbonate (KRB) buffer, pH 7.4, containing 1 mg/ml collagenase (Type II; Sigma Chemical Co.), 1.1 mM glucose and 3.5% BSA (Fraction V; Sigma Chemical Co.) in 20-ml siliconized vials. The tissues were then minced, gassed for 3 min with 95% O₂–5% CO₂, and incubated at 37°C for 2.5 hours, swirling gently every 30 min without continuous shaking. After collagenase digestion, isolated cells were filtered through 177 µm nylon mesh, centrifuged at 400 × g for 40 sec, and washed three times with warm KRB buffer containing 2% BSA, after which the cells were suspended in 35 mM Tris buffer (pH 7.6).

Rats were killed by decapitation and the parametrial adipose tissues were removed, and isolated adipocytes were prepared by the method of RODBELL¹³⁾.

The cell number was directly counted and the cell size was measured on Burker Turk haemocytometer with a calibrated microscope by the method of DI GIROLAMO

*et al.*¹⁴⁾ With this procedure, the yield of cells approximately 2.6×10^6 /g fresh tissue and 1×10^7 cells/g fresh tissue in sheep and rats, respectively. There was no significant difference in average diameter of isolated adipocytes (45–65 μ m) between sheep and rats.

Binding studies

Isolated fat cells were suspended in the buffer containing 35 mM Tris, 120 mM NaCl, 1.2 mM MgSO_4 , 2.5 mM KCl, 10 mM glucose, 2.0 mM CaCl_2 , and 2% bovine serum albumin, pH 7.6, and incubated with 0.05 nM ^{125}I -insulin (porcine insulin, specific activity; 200 $\mu\text{Ci}/\mu\text{g}$, Dainabot RI Lab. Ltd.) and various concentrations of unlabeled insulin (porcine mono-component insulin; Novo Industri). Incubations were performed in polyethylene tubes (Eiken RIA tube 2) at 24°C for 60 min in a shaking water bath. After the incubations were terminated, an aliquot of 200 μl of cell suspension was rapidly centrifuged in plastic microcentrifuge tubes (Eppendorf) containing 100 μl dinonyl phthalate as described by GAMMELTOFT and GLIEMANN¹⁵⁾. Dinonyl phthalate has a specific gravity intermediate between buffer and cells; therefore, after centrifugation, three layers were separated cells on the top, dinonyl phthalate in the middle, and buffer at the bottom. The tubes were cut with a knife through the dinonyl phthalate layer. The cells were transferred to the counting tubes and the radioactivity of the separated cell was determined with a well-type gamma counter (Aloka ARC-300). Nonspecific binding was assessed by incubating cells with ^{125}I -insulin and 50 $\mu\text{g}/\text{ml}$ unlabeled insulin and was subtracted from the total binding to determine the specific binding. Nonspecific binding was 17.2% of the total binding without any difference between sheep and rats.

Insulin and glucose determinations

Plasma insulin was measured with a RIA kit (Dainabot RI Lab. Ltd.) using rat (24.7 IU/mg, Eli Lilly Co.) and ovine insulin (24.1 IU/mg, Sigma Chemical Co.) as a standard. Blood glucose was determined by the glucose oxidase method¹⁶⁾.

Statistics

Statistical analysis of the data was preformed by Student's *t*-test. The level of significance was chosen at $P < 0.05$.

Results

Blood glucose levels of sheep were lower than that of the rats. There were no significant differences in plasma insulin levels between both species (Table 1).

Figure 1 depicts the time course of specific and nonspecific ^{125}I -insulin binding,

Table 1. General observations of the experimental animals

	Sheep	Rat
Body weight	37.1 $\pm$ 2.1 kg	192.5 $\pm$ 1.4 g
Blood glucose (mg/dl)	41.7 $\pm$ 3.0	95.4 $\pm$ 2.2
Plasma IRI (ng/ml)	0.95 $\pm$ 0.18	1.18 $\pm$ 0.06

Mean $\pm$ SE.

and the effect of cell concentration on ^{125}I -insulin binding to isolated ovine adipocytes. The specific binding of ^{125}I -insulin to ovine adipocytes occurred rapidly, reaching a peak at about one hour, followed by maintaining the maximal level until at least 2 hours of incubations. The nonspecific binding increased slightly with increasing incubation time (Fig. 1B). ^{125}I -insulin specifically bound to ovine adipocytes increased

Fig. 1. The effect of cell numbers (A) and incubation time (B) on specific and nonspecific binding of ^{125}I -insulin to ovine adipocytes.

Fig. 2. Specific binding of ^{125}I -insulin to ovine ($n=4$) and rat ($n=5$) adipocytes. Adipocytes were incubated at 24°C for 1 hour with 0.05 nM ^{125}I -insulin and different concentrations of native porcine insulin. The results represent the mean $\pm$ S.E.

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linearly with increasing the number of adipocytes (Fig. 1A). This linear relationship permits normalization of data among animals where different adipocyte concentrations were prepared.

The displacement curves for insulin binding in sheep and rats are shown in Figure 2. Ovine adipocytes showed a considerably greater decrease in the specific binding of ^{125}I -insulin tracer as compared with that in rat adipocytes ($4.82 \pm 0.41\%$ vs. $6.89 \pm 0.45\%$ for 1×10^6 cells; $P < 0.02$). At concentrations of unlabeled porcine insulin between 0.23 ng/ml and 25.2 ng/ml, the insulin binding capacity of ovine adipocytes was also significantly lower than that of rat adipocytes ($P < 0.01$ – $P < 0.02$), but no significant difference between sheep and rats was found at higher insulin concentrations than 50 ng/ml. On the other hand, the specific binding of ^{125}I -insulin to both sheep and rat adipocytes could be readily inhibited at physiological concentrations of unlabeled insulin.

The kinetics of insulin-receptor interaction was analyzed using Scatchard plots¹⁷⁾. As shown in Figure 3, Scatchard plots in both sheep and rats were curvilinear.

Fig. 3. Scatchard plot of the binding result shown in Figure 2. The values are corrected for the nonspecific binding.

Table 2. Affinity constants and total receptor concentration for adipocytes in sheep and rats

	Sheep	Rat
$\bar{K}_e$ ($\times 10^6 \text{M}^{-1}$)	1.29	1.76
$\bar{K}_f$ ($\times 10^6 \text{M}^{-1}$)	0.50	0.67
R_o ($\times 10^6$ sites/cell)	2.32	2.56

Scatchard plots shown in Figure 3 were analyzed by the negative cooperativity model according to De Meyts and Roth (1975). $\bar{K}_e$ is the affinity of the empty receptor, $\bar{K}_f$ is the affinity of the filled receptor, and R_o is the total receptor concentration.

Fig. 4. Average affinity profile for insulin binding to adipocytes estimated from Scatchard plot. The average affinity is calculated as $(B/F)/(R_0 - B)$ and is plotted as a function of the log of fractional occupancy (B/R_0). B ; insulin bound, F ; free insulin, R_0 ; total insulin receptor concentration.

Therefore, these plots were analyzed based on a negative cooperativity model according to DE MEYTS and ROTH^{18,19}. The affinity of the empty receptor ($\bar{K}_e$), where receptors are not occupied by insulin, was reduced in ovine adipocytes to about 70% of that in rat adipocytes. However, both the affinity of the filled receptor ($\bar{K}_f$) and the total number of binding sites per cell (R_0) were almost identical in sheep and rats (Table 2).

Since the affinity of adjacent receptors to insulin has been suggested to decrease as the receptors being combined with insulin in a stoichiometric (stepwise) manner, we tried to use "average affinity profile"¹⁸. Figure 4 illustrates the relationship between the fractional occupancy of receptor and the average affinity. When the comparison of average affinity was made at 10% occupancy between ovine adipocytes and rat adipocytes, since it has been suggested that maximal biological effect occurs at only 2–10% of occupancy of insulin receptors^{6,20,21}, the average affinity in sheep decreased to 50% of that in rats. The average affinity profiles for sheep and rats were also not parallel at all levels of occupancy.

Discussion

It has long been suggested that isolation of adipocytes from ruminant adipose tissues seemed rather difficult, because ruminant adipocytes are extremely fragile²², probably due to differences in the composition of fatty acids in adipose tissues²³ and

in the amount or hardness of connective tissues²⁴). The results of the present investigation clearly shows that ruminant adipocytes could be isolated by our careful modification in maintaining high temperature (38°C), avoiding the excess physical treatment such as high speed centrifugation and vigorous shaking during cell preparation. It was also important to pay attention to the selection of collagenase, since it is known that the collagenase containing high tryptic activity destroys the insulin receptor. It would be less difficult to isolate physiologically active adipocytes from ovine subcutaneous adipose tissues, using our improved techniques reported herein, which will be useful for further studies in examining hormone binding and action in ruminants.

Since it has been defined that insulin binding to a specific plasma membrane receptor is the first step in a sequence of reaction which leads to the biological responses⁷), the mechanism of insulin resistance in a large number of different disease states has been investigated in relation to the interaction between insulin and its receptor. Insulin resistance in target tissues is accounted by the decreased binding of insulin to its receptor and/or the post receptor coupling defect in insulin action^{6,26}). However, up to now, the mechanism of insulin resistance in ruminants is still unknown. The interaction between insulin binding and insulin action in non-ruminant adipocytes which is the essential target cell of insulin has been well documented; nevertheless, the characteristics of insulin binding to ruminant adipocytes have not been clearly established. In order to clarify the mechanism of insulin resistance in ruminants, we measured insulin binding to isolated ovine adipocytes in comparing with rats.

Our data demonstrated that insulin readily and specifically bound to ovine adipocytes as well as rat adipocytes, but the insulin binding in ovine adipocytes was significantly lower than that in rat adipocytes at physiological concentrations of insulin. Scatchard analysis indicated that the total receptor number in ovine adipocytes was identical to that in rat adipocytes, but $\bar{K}_d$ was reduced to about 70% of that in rat adipocytes. This result suggests that lower insulin binding to ovine adipocytes is due to lower affinity to the receptor rather than the number of receptor. Although precise interpretation of the curvilinear Scatchard plots is difficult, analyses of these curvilinear plots based on negative cooperativity model were used in this study solely for comparative purposes. An additional possible interpretation would be the presence of heterogeneity of receptors or a combination of negative cooperativity and heterogeneity. Specific differentiations of these latter possibilities could not be easily obtained. VASILATOS *et al*¹²). reported that the specific binding of insulin to bovine adipocytes was negligible, and nonspecific binding was 74% of the total binding. Their nonspecific binding is extremely higher than our results found in this studies (17% of the total binding). This high nonspecific binding suggests that separation of insulin bound from buffer was incomplete, leading to a relatively lower specific binding.

In this study, the average affinity in sheep was lower than that in rats. Furthermore, average affinity profile showed that a difference in average affinity between sheep and rats became larger in proportion to the decrease in occupancy of receptor.

It has been suggested that antireceptor antibodies, and counterregulatory hormone such as glucocorticoid and epinephrine are concerned in insulin binding^{26,27}. SASAKI *et al.*^{28,29} reported using intact and adrenalectomized sheep that glucocorticoid played an important role in ruminant insulin tolerance, but that epinephrine was less related to insulin tolerance than glucocorticoid. Although it is not known whether insulin resistance seen in ruminants is due to the direct effect of glucocorticoid on insulin binding site or not, insulin resistance in sheep may be due to, at least in part, a lower insulin binding caused by a lower affinity of the receptor for insulin.

In summary, the present studies have demonstrated and systematically characterized the binding of insulin to isolated ovine adipocytes, and clarified a part of the mechanism of insulin resistance in ruminants by comparing insulin-receptor binding in ovine adipocytes with that in rat adipocytes. It has been also shown that the binding of insulin to its receptor are not necessarily an index in regulation of insulin action in ruminants, as is known in non-ruminants. Further studies that clarify the relationship between insulin binding and the post receptor events are required to elucidate the detailed mechanism of insulin resistance or the role of insulin in ruminants.

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めん羊の単離脂肪細胞に対するインスリン結合の特性

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一般に反芻動物はインスリンに対して抵抗性あるいは低感受性であるとされているが, その耐性機構に関しては不明である. 今まで反芻動物において, 単離が困難であるとされていた脂肪細胞をめん羊の脂肪組織より, コラゲナーゼ処理にて単離することに成功した. そこで, この単離脂肪細胞を用いてインスリン受容体に対するインスリン結合の Kinetics を解析することにより, ラットのそれと比較し, 反芻動物におけるインスリン抵抗性の機構の一端を解明した.

めん羊の単離脂肪細胞に対する ^{125}I -insulin の特異結合は $4.82 \pm 0.41\%$ とラットのその 70% であった. なお, インスリン結合は $3.9 \times 10^{-11}\text{M}$ から $4.2 \times 10^{-9}\text{M}$ の生理的濃度範囲でラットに比し有意 ($P < 0.02$) に低かった. negative cooperativity model に従い Scatchard

plot を解析した結果, めん羊およびラットの $\bar{K}_d$ (受容体がインスリンによって全く占有されていない時の親和定数) は, それぞれ $1.29 \times 10^8 \text{M}^{-1}$, $1.76 \times 10^8 \text{M}^{-1}$, $\bar{K}_d'$ (全受容体がインスリンによって占有された時の親和定数) は $0.50 \times 10^8 \text{M}^{-1}$, $0.67 \times 10^8 \text{M}^{-1}$ であり, R_0 (全受容体数) は $2.32 \times 10^5 \text{ sites/cell}$, $2.56 \times 10^5 \text{ sites/cell}$ であった. さらに, average affinity profile は受容体占有率 10% 時での親和力がラットの 50% であることを示した. 以上の解析結果から, めん羊における低インスリン結合は, 受容体数の違いというよりはむしろ低親和力によるものであり, このことが反芻動物におけるインスリン抵抗性の一因をなすものと考えられる.

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