JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 1993, 44, 4, 399—408

T. TORLIŃSKA, K. W. NOWAK*, J. KOŻLIK, J. PALUSZAK

EFFECT OF HYPOTHERMIA ON THE INSULIN – RECEPTOR INTERACTION IN LIVER PLASMA MEMBRANES

Dept. of Physiology, University School of Medical Sciences, Poznań, Poland
*Dept. of Animal Physiology and Biochemistry, Academy Agriculture, Poznań, Poland

The aim of the study was to estimate the effect of hypothermia on (^{125}J) – iodoinsulin binding to liver plasma membranes. Rat liver membranes were prepared from control, normothermic rats ($T_r = 35.6 \pm 0.3°C$) and hypothermic rats ($T_r = 26.5 \pm 0.9°C$) and purified according to Havrankowa.
In addition, serum insulin and glucagon levels by means of RIA and glucose concentration using the glucose oxidase method were measured.
Scatchard analysis was used to determine the kinetic parameters of the hormone receptor interaction.
The data showed no significant differences in the affinity of the binding sites but indicated a decrease in receptor concentrations in liver plasma membranes from hypothermic rats. In contrast to changes in serum insulin level which was decreased by about 50% in hypothermic rats blood glucose concentrations did not significantly differ between the hypothermic and normothermic ones.
Our results show that in hypothermic rats the hormonal adaptation operates on the level of the number of liver receptors whereas the insulin receptor affinity remains unaffected.

Key words: *insulin receptor – plasma membranes – rat liver – hypothermia*

INTRODUCTION

It has been shown that hypothermia results in lowering of plasma insulin level with a concomitant increase in concentration of adrenalin and noradrenaline (1). Moreover, the activation of the sympathetic nervous system can also inhibit insulin release. As shown by Loubatieres-Mariani et al. (2) and more recently by Foreman and Taylor (3) lowering of temperature decreases insulin secretion from isolated perfused rat pancreas or rat islets of Langerhans, *in vitro*.

It has been suggested that the in vivo effect of hypothermia on carbohydrate metabolism is due to hyposecretion of insulin brought about both by a direct inhibition of insulin secretion at low temperatures, and secondarily by the activation of the adrenergic system.

It seems there are no reports of insulin binding studies regarding insulin-deficiency states like hypothermia.

The aim of the present investigation was to study the possible effect of hypothermia on insulin binding to liver plasma membranes from rats.

In order to determine whether the decrease in blood insulin concentration could be related to changes in the number or in the affinity of insulin receptor sites according to the down – regulation theory (4,5) the binding assay was performed along with the measurement of plasma immunoreactive insulin level.

METHODS

Animals

Male Wistar rats weighing 180–220 g were housed in individual cages and maintained with free access to LSM laboratory standard diet and water.

They were deprived of food for 24 h before the experiment.

Rats were anesthetized with thiobutabarbital 10–30 mg/kg of body weight.

Two groups of rats studied:

1. Acute hypothermia animals (n = 8) were surface – cooled with ice bags down to a rectal temperature of 26.5 ± 0.9°C lasting over 30 minutes.
2. Normothermic control animals (n = 6) were maintained at room temperature for the same period ($T_r = 35.6 \pm 0.3$°C).

   Rats were sacrificed by cardiac puncture. Livers were removed immediately after exsanguination of the animals and kept at-20°C until the preparation of plasma membranes according to Havrankowa and coworkers (6) was started.

Plasma insulin was measured by radioimmunoassay (RIA) using kits from IBJ Świerk, and glucose was determined by the glucose oxidase method (POCH Gliwice).

Glucagon concentration in blood plasma was determined with commercial radioimmunoassay kit "Radioimmunoassay of ^{125}J – Glucagon" produced by Cambridge Medical Diagnostics, Inc. Protein concentration was estimated by the method of Lowry et all. (7).

Isolation of crude membrane fraction

The livers were homogenised in 5 ml/g tissue of 0.001 M $NaHCO_3$ (1 mol/l) and centrifuged at 4°C for 30 min. at 600 × g. The resultant supernatant was centrifuged for 30 min. at 20000 × g.

The membrane preparation was washed twice at 4°C using 0.001 M $NaHCO_3$. The final pellet was resuspended in equal volume of 0.02 M Tris – HCl pH 7.4 containing 0.1% bovine serum albumin (BSA).

Binding assay

Insulin – binding activity was measured by incubating the membrane preparations (approximately 0.25 mg of protein) at 4°C for 16 h with 90 pg ^{125}J – labelled porcine insulin (specific activity 6.2 GBq/mg, IBJ Świerk) in a final volume of 0.5 ml 0.02 M Tris buffer pH 7.4 containing 0.1% bovine serum albumin.

Nonspecific binding was determined in the presence of 10 μmol/l unlabelled insulin. Bound and free fractions of insulin were separated by centrifugation at 20000 × g for 30 minutes and then the radioactivity of pellets was determined in a γ-counter.

For the competition binding assay, increasing amounts of unlabelled insulin were added to the reaction mixture to give a final concentration 0-700 nmol/l.

Data from competition binding studies were analysed by the method of Scatchard (8) using LIGAND Pc. v, 3.1. computer program (9). Student's t – test was used for statistical comparison and differences were considered to be significant if $p < 0.05$.

RESULTS

At the end of hypothermia the mean value of rectal temperature was 26.5±0.9°C. The corresponding rectal temperature in normothermic rats was 35.6±0.3°C (*Fig. 1* and *Tabl. 1*). Immunoreactive plasma insulin concentration (IRI) was significantly decreased in hypothermic rats (by about 50%) compared to the control normothermic group.

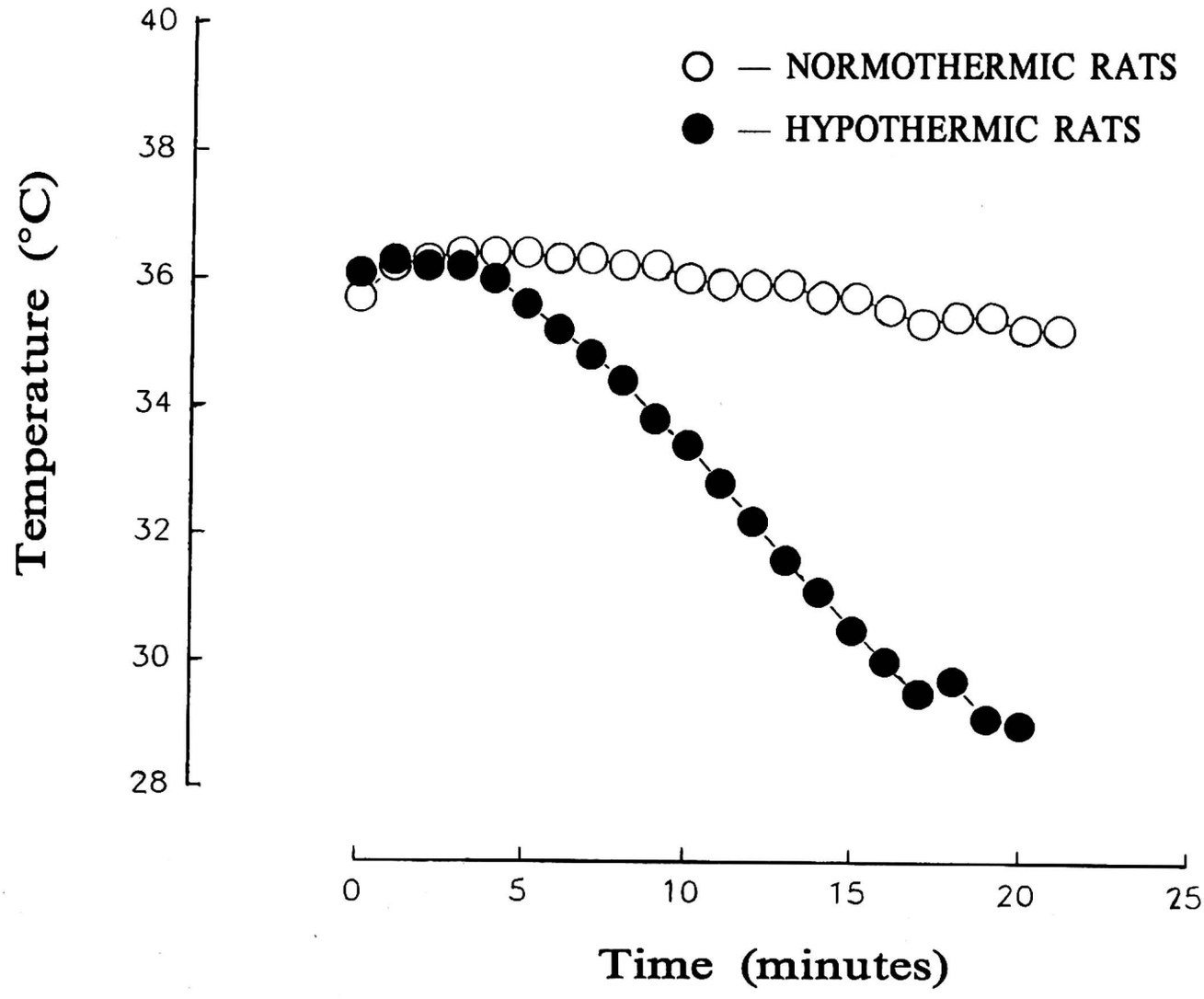

Fig. 1. Relationship of rectal temperature in normothermic (n = 6) and hypothermic (n = 8) rats and time of exposure.

On the other hand, immunoreactive plasma glucagon concentration was increased by about 70% in hypothermic rats in comparison with normothermic rats. As a result of these hormonal changes the glucagon – to – insulin ratio in the hypothermic rats was markedly increased as compared with the normothermic rats (*Tabl. 1*).

Table 1. Serum glucose and insulin concentrations, insulin/glucose ratios and rectal temperature (T_{re}) of normothermic and hypothermic rats.

	Normothermia (n = 6)	Hypothermia (n = 8)
Glucose (mmol/l)	5.22±0.25	5.39±0.83
Insulin (IRI) (μU/mol)	50.2±0.7	25.0±0.8*
IRI/glucose (μU/mol)	9.2	4.6
Glucagon (pg/ml)	286.6±30.1	487.5±75.0*
Glucagon/insulin (pg/μU)	5.7	19.5
T_{re} (°C)	35.6±0.3	26.5±0.4*

Values are expressed as means ± SEM of six and eight separate paired experiments for normothermic and hypothermic rats, respectively.

Despite the magnitude of hypoinsulinemia plasma glucose concentration was unchanged. Consequently the IRI – to – glucose ratio in the hypothermic rats was markedly decreased in comparison to those in control rats (*Tabl. 1*).

Scatchard analysis was used to determine the kinetic paramaters of the hormone receptor interaction. Curves shown in *Fig. 2* were obtained by fitting the binding data obtained by using the program described by Munson and Rodbard and they showed no significant differences in the rates of insulin binding to receptors between the livers of hypothermic and normothermic rats.

Computer analysis gave K_D values of 0.23±0.003 and 0.30±0.04 nmol/l (mean±SEM) for high – affinity insulin receptors from normothermic and hypothermic rats respectively. However the numbers of high – affinity binding sites expressed as binding capacities in normothermic (25.3±4.7 fmol of insuling/mg of protein) and hypothermic rats (12.6±3.0 fmol of insulin/mg

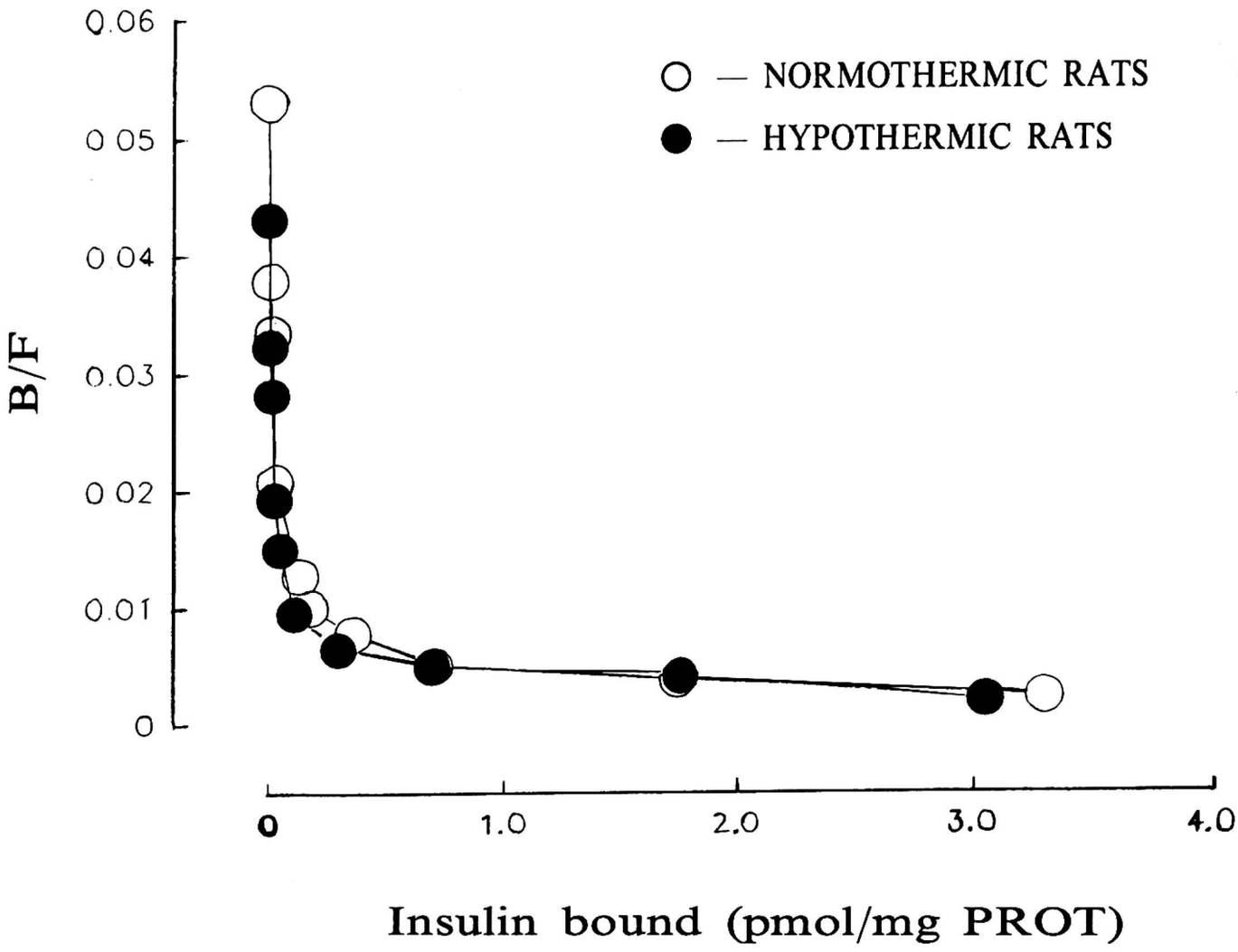

Fig. 2. Scatchard analysis of (^{125}J) — iodoinsulin binding to liver plasma membranes obtained from normothermic and hypothermic rats. (bound — free hormone versus hormone bound plotted). Each point represents the mean of six and eight seperate experiments with liver plasma membranes from normothermic and hypothermic rats, respectively.

of protein), were different showing a significant decrease by about 50% in the group of hypothermic rats.

The low — affinity component of the binding showed K_D values of 0.30 ± 0.06 μmol/l and 0.25 ± 0.02 μmol/l for normothermic and hypothermic rats. The binding capacities of low — affinity receptors in normothermic rats (5.63 ± 0.88 pmol/mg of protein) and hypothermic rats (4.39 ± 0.36 pmol/mg of protein) were also very similar (*Table 2*).

The binding displacement curve is shown in *Fig 3*. Maximum specific insulin binding was $4.62 \pm 0.90\%$ for the hypothermic group and $5.01 + 0.60\%$ for the control group.

Half — maximum displacement of tracer insulin was observed at similar insulin concentrations (0.58 ± 0.13 nmol/l and 0.42 ± 0.03 nmol/l respectively), suggesting that the difference in binding was secondary to the reduced receptor number rather than the alteration of affinity.

Table 2. Affinities of receptor sites and binding capacities in liver plasma membranes of normothermic rats.

	Normothermia (n = 6)	Hypothermia (n = 8)
	High affinity receptor	
K_D (nmol/l)	0.23±0.03	0.30±0.04
B_{Max} (fmol/mg protein)	25.3±4.7	12.6±3.0*
	Low affinity receptor	
K_D (μmol/l)	0.30±0.06	0.25±0.02
B_{Max} (pmol/mg protein)	5.63±0.88	4.39±0.36

K_D – dissociation constant B_{Max} – binding capacity

Values are expressed as means±SEM of six and eight separate paired experiments for normothermic and hypothermic rats, respectively.

*Significantly different from normothermic ($p<0.05$)

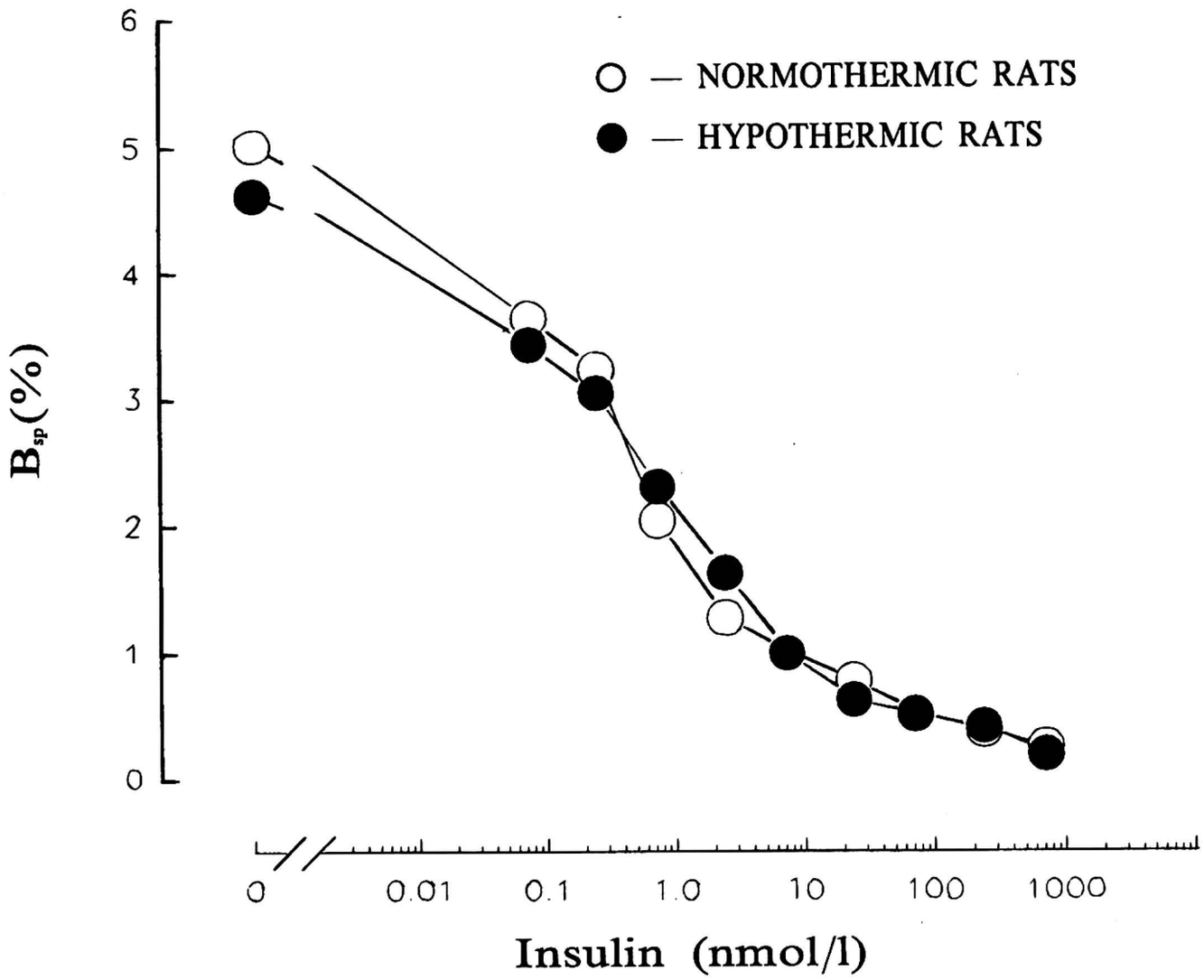

Fig. 3. Displacement of (^{125}J) – insulin by native insulin from the liver plasma membranes. Liver membranes (0.5 mg protein/ml) were incubated with 180 pg/ml of (^{125}J) iodoinsulin in the absence or presence of unlabelled insulin over a range of insulin concentration from 0–700 nmol/l. Data are expressed as the precentage of insulin bound per mg protein. Each point represents the mean of six and eight separate experiments with liver membranes from normothermic and hypothermic rats, respectively.

DISCUSSION

The specific insulin receptor has been demonstrated in a great number of species including man, monkey, rat, mouse, guinea pig, rabbit, sheep, pigeon and frog (10).

According to these authors, rat liver membranes tested in the binding assay exhibited a B_{max} of 1.4–1.6 pmol per mg protein. Previous studies by Kahn and coworkers (11) concerning quantitative aspects of the insulin – receptor interaction in liver plasma membranes have shown a heterogenous population of receptor sites. The data fit a model with a minimum of three classes of receptor sites: a high affinity – low capacity site with a K of $2.0 \times 10^9\,M^{-1}$ and a capacity of 5×10^{-14} moles per mg of membrane protein; a low affinity high capacity site with a K of $2.1 \times 10^8\,M^{-1}$ and a capacity of 15×10^{-12} moles per mg of membrane protein; and a very low affinity – high capacity site ("nonspecific site") representing about 5% of the total tracer binding. The affinity constans and the binding capacities are influenced by both temperature and ionic strength. Kinetic data for insulin receptor complex dissociation are also consistent with a heterogeneity of receptor sites.

The biological significance of the class of low affinity receptor sites has been questioned by Cuatrecasas (12) and Marinetti (13) who have reported that only the high affinity sites are associated with the physiological action of the hormone. More recently it appeared that there is no reason to consider some of the receptors as "active" and others as "inactive" even though a maximal response may be achieved with only a minority of receptor sites occupied.

As shown by Almira and Reddy (14) the insulin binding of isolated rat hepatocytes was different to that of purified plasma membranes prepared from livers of fed and fasted rats (72 h). Assuming two classes of specific receptors (a high affinity, low capacity receptor and a low affinity, high capacity receptor) the affinities of both types of receptors in hepatocytes were not significantly changed by fasting whereas the receptor concentrations were decreased. In contrast to intact hepatocytes, liver plasma membranes of fasted rats showed a significant increase in the receptor affinity but no change in the insulin receptor concentration.

There are several factors which might account for the difference in insulin binding between the results of Soll et all (15) and those of Broer an coworkers (16) among them, the duration of fasting, the experimental model utilizing isolated cells or purified plasma membranes, pathological states like obesity. Recent reviews on insulin binding to cells and plasma membranes indicate that insulin receptor concentration is negatively correlated with the plasma insulin level (17) but there are several reports which are not consistent with this hypothesis (18, 19).

Both fasting and hypothermia are accompanied by insulinopenia, reduced glucose metabolism, increased lipolysis and state of energy deficiency. Despite numerous investigations performed during artifical or accidental hypothermia, insulin receptor affinity or capacity in the liver has not been examined in hypothermic conditions.

Our results show that the population of insulin receptors in the purified liver membrane can indeed be considered heterogenous with respect to affinity for insulin. The Scatchard plot analysis of the binding data shows nonlinear curves in both normothermic and hypothermic rats. Different interpretations of these plots are possible: either the presence of a receptor population with high and low affinity or, a negative cooperativity as suggested by De Meyts et all (20). According to this evidence insulin receptors might constitute a single set of homogenous binding sites that undergo negative cooperative site interactions.

Non – specific binding, measured in the presence of unlabelled insulin at the concentration of $10\,\mu mol \times l^{-1}$ represents only about 5% of the total insulin binding.

Our results indicate that hypothermia causes a significant change only in the class of high affinity receptors from rat liver membranes. The values of dissociation constants do not differ significantly in both classes of liver receptors. These findings may indicate that hypothermia does not affect the insulin receptor afffinity, neither in the high affinity nor low affinity class. The analysis of these data indicates only a significant decrease (by about 50%) in the binding capacity of the high affinity liver receptors of plasma membranes from hypothermic rats. It thus seems that the decrease in the specific insulin binding in hypothermia is due to a decrease in the insulin receptor number in liver plasma membranes, rather than to a change a in the receptor affinity of both receptor classes.

However a possibility that the differences in binding capacities were due to a faster or slower degradation of insulin at the receptor level should also be taken into consideration. According to Williams and coworkers (21) working on a isolated perfused liver, (^{125}J) – insulin is rapidly degraded in vivo and the process is temperature – dependent. At 15°C both the internalization and degradation of labelled hormone were inhibited. Only 11.4% of unbound insulin and 17.4% of surface – bound insulin were degraded at 35°C. Probably during hypothermia in our experiment (26°C) the degradation of insulin could be decreased and this might have influenced the binding capacities of liver insulin receptors.

The findings reported here also indicate that the serum insulin concentration is decreased in hypothermic rats. Thus the present data does not confirm the results of others indicating the existence of down regulation at the peripheral insulin receptor level, in response to decreased plasma insulin

concentrations. On the other hand, the results of Flint (22) working on isolated rat hepatocytes have shown that during lactation the number of insulin receptors declined to values similiar to those of virgin rats in comparison to pregnant rats. Serum insulin concentrations determined by radioimmunoassay, elevated during pregnancy, were significantly reduced as compared with virgin rats by day 15 of lactation.

These results including ours, illustrate that the number of insulin receptors may decrease, despite reduced serum insulin concentrations, in apparent conflict with the "down-receptor" hypothesis.

It has been shown that during lactation, both serum insulin concentration and the number of insulin receptors on the hepatocytes were reduced whilst serum glucagon was elevated (23). The same results can be observed during hypothermia where a situation develops which should favour glucose production, inhibit glucose utilization and inhibit lipogenesis.

In view of the current concept of the bihormonal (insulin/glucagon) control of glucose metabolism (24) it seems to be probable that in the states like lactation or hypothermia in which the need for energy substrates is apparently increased, the normal regulatory mechanism according to the "down" or "up theory" doesn't work.

In contrast to changes in serum insulin level, the blood glucose concentration in the group of hypothermic rats does not significantly differ from that of normothermic rats which may indicate that glucose production is equal to its utilization. In conclusion, our results show that in hypothermic rats adaptation operates by way of adjusting liver receptor numbers whereas the insulin receptor affinity remains unaffacted.

The present studies could answer the question of how different tissue receptors adapted to endogenous insulin concentration during hypothermia may be counterbalanced, at least in part, by the receptor concentration of the respective target tissue. Thus, blood "insulin deficiency" described by many authors during hypothermia is only a relative phenomenon compensated quickly at the liver receptors level.

REFERENCES

1. Hirvonen J, Huttinen P, Nuutinen L, Pekkarinen A. Catecholamines and free fatty acids in plasma of patients undergoing cardiac operations with hypothermia and bypass. *J. Clin Pathol* 1978; 31: 949–953.
2. Loubatieres-Mariani MM, Chapal J, Puech R, Manteghetti M. Different action of hypothermia on insulin release from the isolated, perfused rat pancreas, depending on the stimulating agent. *Diabetes* 1980; 29: 895–898.
3. Foreman J, Taylor MJ. The effect of rapid cooling and culture on in vitro insulin release in cryopreserved rat islets of Langerhans. *Diabetes Res* 1989; 11: (3) 149–154.

4. Almira EC, Reddy WJ. Effect of fasting on insulin binding to hepatocytes and liver plasma membranes from rats. *Endocrinology* 1979; 104: 205–211.
5. Geiger D, Carpentier JL, Gorden P, Orci L. Down-regulation of insulin receptors is related to insulin internalization. *Exp Cell Res* 1989; 185: (1) 33–40.
6. Havrankova J, Roth J, Browstein MJ. Insulin receptors are widely distributed in the central nervous system of the rat. *Nature* 1987; 272: 827–829.
7. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193: 265–275.
8. Scatchard G. The attractions of proteins for small molecules and ions. *Ann N Y Acad Sci* 1949; 600–672.
9. Munson PJ, Rodbard D. Ligand: a versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* 1980; 107: 220–239.
10. Kemmler W, Renner R, Zynamon A, Hepp KD. Interactions between insulins and liver membrane receptors of guinea pig, calf and chicken. *Bioch Biophys Acta* 1978; 543: 349–356.
11. Kahn CR, Flier JS, Bar RS, et al. The syndromes of insulin resistance and acanthosis nigricans. Insulin receptor disordes in man. *N Eng J Med* 1976; 294: 739.
12. Cuatrecasas P. Insulin Action In: Fritz I B ed. New York, Academic Press 1972; 137–170.
13. Marinetti GV, Schlatz L, Reilly K. Insulin Action. In: Fritz I B ed. New York, Academic Press 1972; 207–296.
14. Almira EC, Reddy WJ. Effect of fasting on insulin binding to hepatocytes and liver plasma membranes from rats. *Endocrinology* 1979; 104: 205–211.
15. Soll AH, Kahn CR, Neville DM, Roth J. Insulin receptor deficiency in genetic and acquired obesity. *J Clin Invest* 1975; 56: 769.
16. Broer Y, Freychet P, Rosselin G. Insulin and glucagon receptor interactions in genetically obese Zucker rat: studies of hormone binding and glucagon – stimulated cyclic AMP levels in isolated hepatocytes. *Endocrinology* 1977; 101: 236.
17. Bar RS, Roth J. Insulin receptor status in disease states of man. *Arch Intern Med* 1977; 137: 474.
18. Amatruda JM, Livingston JN, Lockwood DH. Insulin receptor: role in the resistance of human obesity to insulin. *Science* 1975; 188: 264.
19. Kahn CR, Freychet P, Roth J. Quantitative aspects of the insulin receptor interaction in liver plasma membranes. *J Biol Chem* 1974; 249: 249.
20. De Meyts P, Roth J. Cooperativity in ligand binding: a new graphic analysis. *Biochem Biophys Res Commun* 1975; 66: 1118.
21. Williams FG, Johnson DE, Bauer GE. (^{125}J) – insulin metabolism by rat liver in vivo, evidence that a neutral thiolprotease mediates rapid intracellular insulin degradation. *Metabolism* 1990; 39: (3) 231–241.
22. Flint DJ. Changes in the number of insulin receptors of isolated rat hepatocytes during pregnancy and lactation *Biochim Acta* 1980; 628: 332–327.
23. Kuhn NJ. In Comparative Aspects of Lactation Peaker M, (ed.) Academic Press, London, 1977, pp. 165–192.
24. Unger RH. Glucagon and the insulin glucagon ratio in diabetes and other catabolic illnesses. *Diabetes* 1971; 20: 834–838.

Received: April 16, 1992
Accepted: August 30, 1993

Author's address: T. Torlińska, Department of Physiology, University School of Medical Sciences, ul. Święcickiego 6, 60-781 Poznań, Poland.