JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 1996, 47, 2, 311—319

T. TORLIŃSKA, P. MAĆKOWIAK*, L. NOGOWSKI*, J. KOŻLIK

EFFECT OF HYPOTHERMIA ON THE INSULIN — RECEPTOR INTERACTION IN SKELETAL MUSCLE PLASMA MEMBRANES

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

The aim of the study was to investigate the effect of hypothermia on (^{125}J)-insulin binding to rat skeletal muscle membranes and 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. Rat skeletal muscle membranes were prepared from control, normothermic rats ($T_r = 35.6 \pm 0.3$°C) and hypothermic rats ($T_r = 26.0 \pm 0.5$°C) and purified according to Havrankowa. In order to determine the kinetic parameters of the hormone-receptor interaction the data from the competition binding studies were analysed by the method of Scatchard using the LIGAND Pc.v.3.1. computer program of Munson and Rodbard. We have shown that under hypothermic conditions insulin receptors number is significantly increased in specific hindlimb skeletal muscles but the changes take place mainly in the low affinity receptors class. The phenomenon probably results from the lack of spare high affinity insulin receptors in skeletal muscle as shown recently by Camps et al.

Key words: *insulin receptor, plasma membranes, rat skeletal muscle, hypothermia*

INTRODUCTION

Hypothermia is defined as lowering of core body temperature to 35°C degrees or below. Severe hypothermia occurs when the body temperature falls below 28°C. It has been demonstrated that hypothermia may be advantageous in connexion with cardiac surgery (1, 2).

Impairment of metabolic substrate mobilization and utilization observed in hypothermia may be a factor limiting survival. Altered carbohydrate metabolism during hypothermia in the nonhibernators is accompanied by reduced secretion of insulin, hypoinsulinemia and reduction in the effectiveness

---

This work was presented at the First Polish-Czech International Symposium on Human Adaptability to Work and Thermal Environment held in Zakopane, March 28—29, 1994.

of administered insulin (3—6). As shown by Hoo-Paris and coworkers (7) plasma insulin decreases dramatically in rats during cooling. Because insulin is the main factor for glucose uptake by peripheral tissues, a lack of insulin release may limit the glucose uptake by skeletal muscles and therefore affect the thermogenetic capacity of the rat. It is unclear whether the decrease in serum insulin level during hypothermia is paralleled by an increased binding of insulin to its receptor sites in skeletal muscle in agreement with the theory of "down and up-regulation" (8).

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

METHODS

Animals

Male Wistar rats weighing 100—110 g were housed in individual cages and maintained with free access to laboratory standard chow (LSM) and water.

On the morning of the experiment they were randomly divided into a resting (control) and a hypothermic group.

The rats were anaesthetized with thiobutabarbital 10—30 mg/kg of body weight.

Two groups of rats were studied:

1. Acute hypothermia animals ($n = 18$) were surface — cooled with ice bags down to a rectal temperature of 26.0 ± 0.5°C over a period of 30 minutes.
2. Normothermic control animals ($n = 17$) were maintained at room temperature for the same period ($T_r = 35.6 \pm 0.3$°C).

The rats were sacrificed by cardiac puncture. Skeletal muscles were removed from hindquaters immediately after exsanguination of the animals.

Collection of muscle tissues

Specific hindlimb skeletal muscles were used in the experiment. The fiber composition of these muscles, as defined by Armstrong and Phelps (9) consists of tensor fascia latae (TFL: 10% fast-twitch oxidative glycolytic (FOG), 89% fast-twitch glycolytic (FG), 1% slow-twitch oxidative (SO) fibers), soleus (S: 13% FOG, O% FG, 87% SO) vastus intermedius (VI: 40% FOG, 1% FG, 59% SO) plantarius (P: 50% FOG, 41% FG, 9% SO). All muscle samples were quickly excised and frozen between blocks of solid CO_2 and stored at — 90°C until analyzed. The preparation of plasma membranes according to Havrankowa and coworkers (10) was performed.

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

Lactate was analysed on the basis of the enzymatic reaction with lactate dehydrogenase (Biochemia Test Combination Boehringer — Mannheim). Glycogen was isolated from the skeletal muscles by the method of Good, Kramer and Somogyi (11) and determined according to Sugar (12). Protein concentration was estimated by the method of Lowry et al (13).

Isolation of the crude membrane fraction

The muscles were homogenised in 5 ml/g tissue of 0.01 M $NaHCO_3$ (1 mol/l) and centrifuged at 4°C for 30 min. at $600 \times g$. The resultant supernatant was centrifuged for 30 min. at $20000 \times g$. 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). Crude membranes were prepared from the muscles of 17 control and 18 hypothermic rats.

The pooled crude membranes were divided into five aliquots for each group.

Binding assay

Insulin — binding activity was measured by incubating the membrane preparations (approximately 0.5 mg of protein) at 4°C for 16 h with 80 pg ^{125}I-labelled porcine insulin (specific activity 8.0 GBq/mg, IBJ Świerk) in a final volume of 0.5 ml 0.04 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 the competition binding studies were analysed by the method of Scatchard (14) using the LIGAND Pc,v,3.1. computer program.

The Student's t — test was used for statistical comparison and differences were considered to be significant at $p < 0.01$.

RESULTS

Material was taken for further analysis at the end of the cooling period when the mean rectal temperature was 26.0±0.5°C. The corresponding rectal temperature for the control group was 35.6±0.3°C (*Fig. 1 and Table 1*).

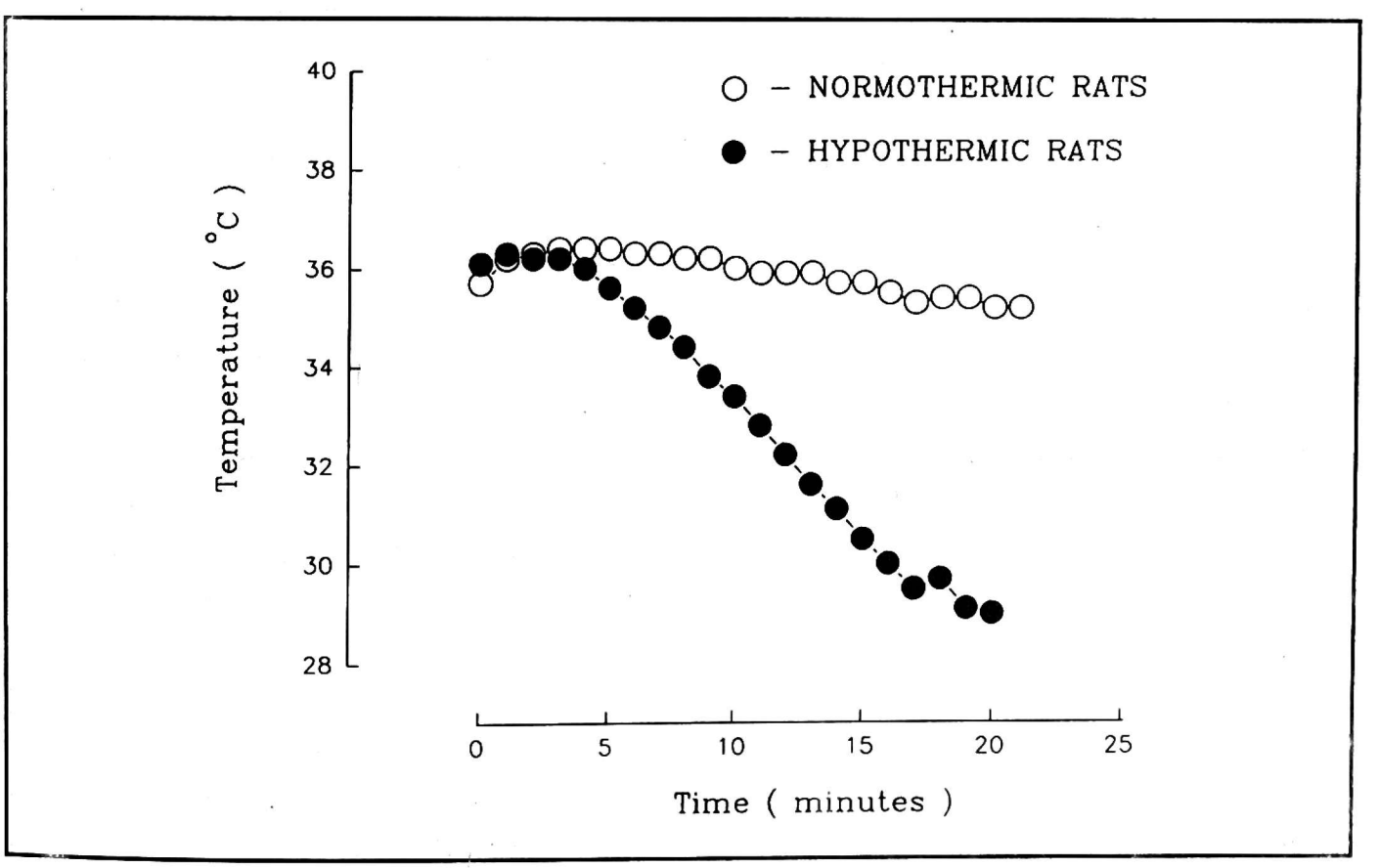


Fig. 1. Relationship of rectal temperature in normothermic (n = 17) and hypothermic (n = 18) rats and time of exposure.

Table 1. Serum glucose, lactate and insulin concentration, insulin/glucose ratios, skeletal muscle glycogen content and rectal temperature (T_{re}) of normothermic and hypothermic rats.

	Normothermia (n = 17)	Hypothermia (n = 18)
Glucose (mmol/l)	6.32±0.30	9.13±0.51 *
Lactate (mmol/l)	7.77±0.25	10.12±0.45 *
Insulin (IRI) (μU/ml)	50.2±0.7	25.0±0.8*
IRI/glucose (μU/mol)	7.9	2.7
Glycogen (μg/g of tissue)	5028±151	4094±325 *
T_{re} (°C)	35.6±0.3	26.0±0.5 *

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

Serum glucose concentration was significantly increased in hypothermic rats (approx. 44%) as compared with the control normothermic rats. Lactate concentrations in the serum of the hypothermic rats increased approx. 30% in comparison with those observed in the control rats. (*Table 1*).

These changes were accompanied by a decrease in immunoreactive plasma insulin concentration (IRI) in the hypothermic rats by about 50% compared with the control normothermic group. As a result of these changes the insulin — to — glucose ratio in the hypothermic rats was greatly decreased as compared with the normothermic rats. (*Table 1*).

At the same time a slight but significant decrease in glycogen level was observed in the skeletal muscle of hypothermic rats (approx. 20% in comparison with control rats).

The kinetic parameters of the hormone-receptor interaction were determined by means of the Scatchard analysis. The curves shown in *Fig. 2* were produced by fitting the binding data obtained by using the Munson and Rodbard program. (15). Computer analysis gave K_D values of 0.82±0.21 nmol/l and 0.72±0.16 nmol/l (mean±SEM) for high — affinity insulin receptors from normothermic and hypothermic rats, respectively. The number of high — affinity binding sites expressed as maximum binding capacity in normothermic and hypothermic rats did not differ significantly (16.2±3.1 fmol/mg and 19.3±3.8 fmol/mg respectively).

On the other hand, the low — affinity component of the insulin binding showed K_D values of 282±29 nmol/l and 693±78 nmol/l for normothermic and hypothermic rats, respectively. The binding capacities of low — affinity receptors in normothermic rats (6651±1030 fmol/mg of protein) and hypothermic rats (16620±1760 fmol/mg of protein) were also very different showing the significant increase approximately 150% in the hypothermic rats. (*Table 2*).

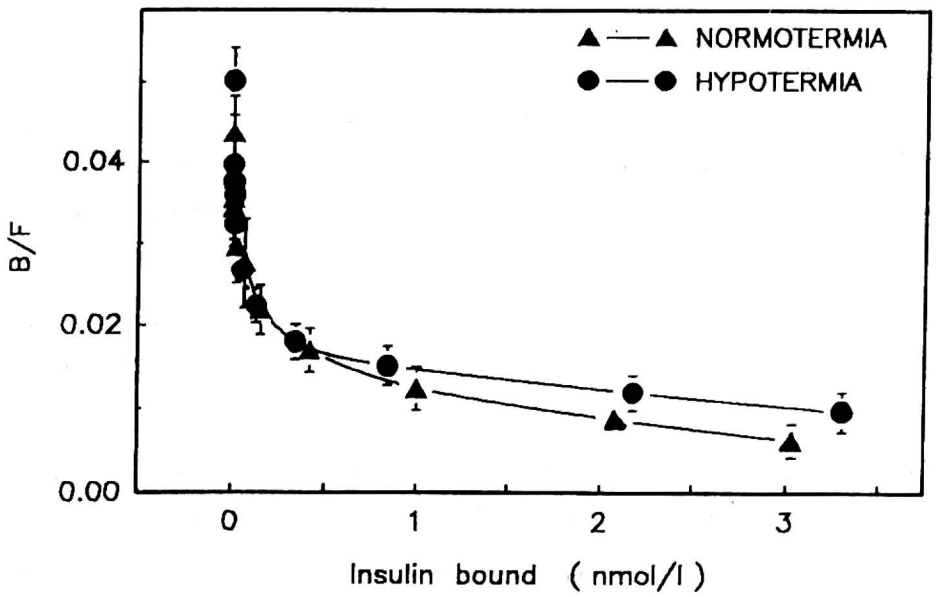


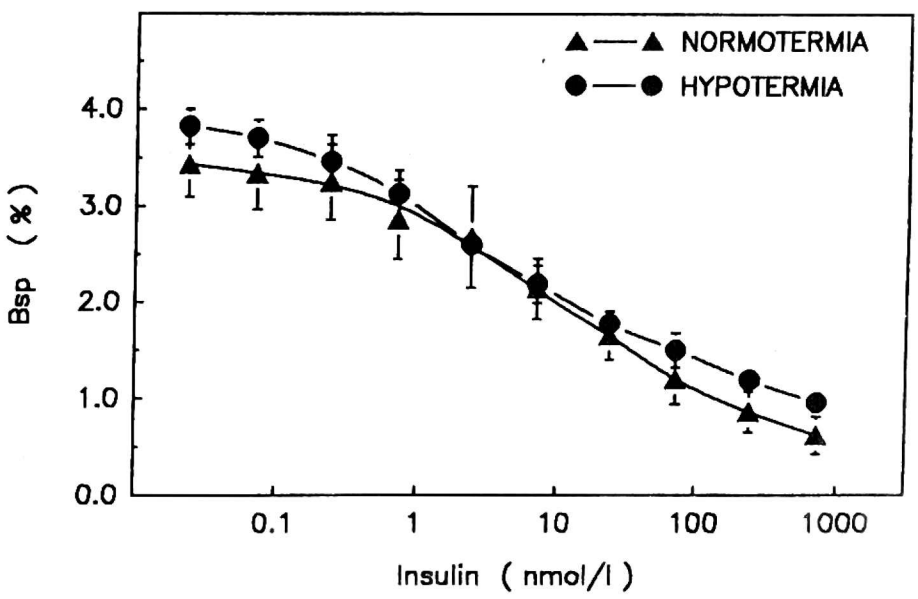
Fig. 2. Scatchard analysis (^{125}I)-iodoinsulin binding to skeletal muscle plasma membranes obtained from normothermic and hypothermic rats. (bound — free hormone versus hormone bound plotted).

Table 2. Affinities of insulin receptor sites and binding capacities in skeletal muscles plasma membranes of normothermic and hypothermic rats.

	Normothermia (n = 17)	Hypothermia (n = 18)
	High affinity receptor	
K_D (nmol/l)	0.82 ± 0.21	0.72 ± 0.16
B_{max} (fmol/mg protein)	16.2 ± 3.1	19.3 ± 3.8
	Low affinity receptor	
K_D (nmol/l)	282 ± 29	693 ± 78 *
B_{max} (fmol/mg protein)	6651 ± 1030	16620 ± 1760 *

K_D — dissociation constant B_{max} — binding capacity
Values are presented as means ± SEM.
* Significantly different from normothermic rats ($p < 0.01$)

Fig. 3. Displacement of (^{125}I)-insulin by native insulin from the skeletal muscle plasma membranes. The membranes (0.5 mg protein/ml) were incubated with 80 pg/ml of (^{125}I)-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.



The binding displacement curve is shown in *Fig. 3.* Maximum specific insulin binding was 4.17 ± 0.43% for the normothermic group and 4.76 ± 0.38% for the hypothermic group.

DISCUSSION

Skeletal muscle is a major target organ for insulin but relatively few studies have examined insulin binding. Present methods for measuring binding in skeletal muscle are time consuming and demand large amounts of muscle (16). In addition, skeletal muscle is a tissue consisting of a heterogenous population of muscle fibers. These fibers differ in many characteristicts including the diameter of the fiber, capillarization of the fiber and in their metabolic capacities (17).

Previous reports have shown that the ability of insulin to stimulate glucose uptake varies as a function of rat skeletal muscle type, involving changes in both insulin sensitivity and responsiveness (18). On the other hand, as shown by Azhar and coworkers (19) insulin binding activity was similar in some rat muscle of different composition (m. tensor fascia latae, soleus and intermedius). No significant differences were noted in the affinity of insulin for its receptor from various muscle types. In order to obtain a sufficient amount of muscle for membrane preparation we used pooled hindlimb muscles (tensor fascia latae, soleus, vastus intermedius and plantarius) showing the same level of insulin binding activity. Olefsky et al. (20) have demonstrated that muscle plasma membranes from older, fatter rats have fewer receptors than those from younger, lean animals. The findings of Goodman and Rudeman (21) also indicate that the response of muscle to insulin diminishes with aging. For this reason we have carried out our experiments on young rats weighing 100—110 g.

The results obtained by Steffen (2) indicate repletion of skeletal and cardiac muscle glycogen during maintained hypothermia. Initial skeletal muscle glycogen concentration was reduced by 68% during hypothermic induction.

The marked decrease in skeletal muscle glycogen concentration during hypothermia in the rat is depicted in *Table 1* and supports these observations.

The significant reduction of glycogen concentration in skeletal muscle during the initial period of exposure to hypothermia may indicate an activation of shivering thermogenesis fueled in part by intramuscular carbohydrate stores. Muscle contraction has been shown in increase glucose uptake even in the absence of insulin (23) suggesting that blood glucose resulting from hepatic glycogenolysis could also provide a substrate for energy metabolism in muscle, despite decreased circulating insulin levels. The data of Steffen suggest that shivering activity decreases as body temperature falls below 27°C.

The decrease in skeletal muscle glycogen concentration found in our model of hypothermia is smaller than that observed previously by Steffen. Probably the general anaesthesia applied in our study significantly reduced shivering thermogenesis. This observation would appear to be consistent with the occurrence of shivering thermogenesis in rat muscle only at the beginning of

cooling. The present findings indicate a substantial contribution of muscle carbohydrate stores to metabolism during hypothermic induction which may result from an activation of the sympatho-adrenal system (24—26) and which is confirmed by the increase in serum glucose concentration in our hypothermic rats. We have also shown that concentration of plasma insulin declines during hypothermia in rats. These data suggest that hyperglycemia in the hypothermic rats can be accounted for by hypoinsulinemia and liver glycogenolysis as we have shown previously (27, 28).

Both a high physiological concentration of epinephrine and acute exercise have been shown to increase ^{125}I-insulin binding in skeletal muscle (16, 29). Probably epinephrine modulates the binding of insulin to its receptor by control of the degree of phosphorylation of the receptor. The effects of diabetes on the structure and function of insulin receptors was studied in rats, 7 days after streptozotocin injection, using solubilized, partially purified receptors from rat hindlimb muscles. Diabetes increased the number of insulin receptors per gram of muscle 60—70% without apparent change in insulin binding affinity. These receptors also showed diminished exogenous substrate kinase activity using histone H_2b and angiotensin II as phosphoreceptors (30).

Insulin receptor binding in different tissues was heterogenous which is due to multiple classes of receptors, to negatively cooperative interactions between receptors or to a combination of both (31, 32).

The role of ligand-induced regulation of receptor-binding affinity *in vivo* is uncertain. At low concentrations, insulin binds to high-affinity receptors, which are shifted to the low-affinity state as insulin levels rise.

A physiological role of negative cooperativity may be to buffer against high insulin secretions and to reduce oscillations in insulin action. The regulation applies primarily to the liver, which is exposed to greater fluctuations in portal plasma insulin but may not be physiologically important when the insulin concentrations of peripheral plasma are lower and less variable. The results of Parrizas (33) showed that fish can respond to both acute and maintained increasses in insulinemia by increasing the number of insulin receptors. According to Kobayashi (34) high glucose level in the diabetic state may suppress *in vivo* the up-regulation of high affinity insulin receptors in skeletal muscles of mice.

As shown by Balage (35) and his coworkers, insulin binding capacity was increase in fasting rats liver and skeletal muscle as determined by Scatchard analysis. On the other hand the affinity of the receptors was not modified by fasting. Many reports have shown that both fasting and hypothermia are accompanied by insulinopenia and reduced glucose metabolism. Both are regarded as a state of energy deficiency but insulin receptor capacity or affinity in rat skeletal muscles has not yet been examined in hypothermia. In our present study we have shown that under hypothermic conditions insulin

receptors number, expressed as binding capacity, is significantly increased in skeletal muscle. These improvements in insulin binding in the skeletal muscle sites of hypothermic rats probably result from an "up-regulation" process that is induced by the low concentration of circulating insulin.

In conclusion, hypothermia produces increased insulin binding in rat skeletal muscle but it seems to be unclear why the changes take place mainly in the low — affinity receptors. This phenomenon probably results from the lack of spare high affinity insulin receptors in skeletal muscle as shown recently by Camps et al (36).

REFERENCES

1. Reitz BA, Ream AK. Uses of hypothermia in cardiovascular surgery. Acute Cardiovascular Management, AK Ream AK, RP Fogdall (eds), Philadelphia, JB Lippincott 1982; 830—851.
2. Morray JP, Pavlin EG. Oxygen delivery and consumption during hypothermia and rewarming in the dog. *Anesthesiology* 1990; 72: 510—516.
3. Curry D, Curry KP. Hypothermia and insulin secretion. *Endocrinology* 1970; 87: 750—754.
4. Helman A, Gilbert M, Pfister-Jemaire N, Reach G, Assan R. Glucagon and insulin secretion and their biological activities in hypothermia rats. *Endocrinology* 1984; 115: 1722—1728.
5. Benzing G, Francis PD, Kaplan S, Helmsworth JA, Sperling MA. Glucose and insulin changes in infants and children undergoing hypothermic open-heart surgery. *Amer J Cardiol* 1983; 52: 133—136.
6. Steffen JM. Blood glucose, serum insulin and tissue glycogen concentrations in the hypothermic rat. *Fed Proc* 1986; 45: 535—540.
7. Hoo-Paris R, Sutter BChJ. Role of glucose and catecholamines in the regulation of insulin secretion in the hibernating hedgehog (Erinaceus europeans) during arousal. *Gen Comp Endocrinol* 1980; 41: 62—65.
8. Geiger D, Carpentier JL, Gordon P, Orci L. Down-regulation of insulin receptors is related to insulin internalization. *Exp Cell Res* 1989; 185: (1) 33—40.
9. Armstrong RB, Phelps RO. Muscle fiber type composition of the rat hindlimb. *Am J Anat* 1984; 171: 259—272.
10. Havrankova J, Roth J, Browstein MJ. Insulin receptors are widely distributed in the central nervous system of the rat. *Nature* 1987; 272: 827—829.
11. Good C, Kramer H, Somogyi. The determination of glycogen. *J Biol Chem* 1933; 100: 485.
12. Sugar EA. The carbohydrate content of serum mucoprotein. Comparative study of normal and silicotic sera. *Clin Chim Acta* 1963; 8: 347.
13. Lowry OH, Rosenbrought NJ, Narr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193: 265—275.
14. Scatchard G. The attractions of proteins for small molecules and ions. *Ann NY Acad Sci* 1949; 51: 600—672.
15. Munson PJ, Rodbard D. Ligand: a versatile computerized approach for characterization of ligand — binding systems. *Anal Biochem* 1980; 107: 220—239.
16. Webster BA, Vigna SR, Paquette T. Acute exercise, epinephrine, and diabetes enhance insulin binding to skeletal muscle. *Am J Physiol* 1986; 250: (13) E186—E197.
17. Close RJ. Dynamic properties of mammalian skeletal muscle. *Physiol Rev* 1972; 52: 129—197.
18. Bonen A, Tan MH, Watson-Wright WM. Insulin binding and glucose uptake diffrences in rodent skeletal muscles. *Diabetes* 1981; 30: 702—704.

19. Azhar S, Butte JC, Santos RF, Mondon CE, Reaven GM. Characterization of insulin receptor kinase activity and autophosphorylation in different skeletal muscle types. *Am J Physiol* 1991; 260: (23) 1—7.
20. Olefsky J, Bacon VC, Baur S. Insulin receptors of skeletal muscle: specific insulin binding sites and demonstration of decreased numbers of sites in obese rats. *Metabolism* 1976; 25: (2) 179—191.
21. Godman MN, Rudeman NB. Insulin sensitivity of rat skeletal muscle: effects of starvation and aging. *Am J Physiol* 1979; 236 (5): E519—E523.
22. Steffen JM. Glucose, glycogen, and insulin responses in the hypothermic rat. *Cryobiology* 1988; 25: 94—101.
23. Ploug T, Galbo H, Richter EA. Increased muscle glucose uptake during contractions: No need for insulin: *Amer J Physiol* 1984; 247: E726—E731.
24. Chernow B, Lake CR, Zaritsky A et al. Sympathetic nervous system "switch off with" severe hypothermia. *Crit Care Med* 1983; 11: 677—680.
25. Johnson DG, Hayward JS, Jacobs T, Collins ML. Eckerson JD, Williams RH. Plasma norepinephrine response of man in cold water. *J Appl Physiol* 1977; 43: 216—220.
26. Jones SB, Depocas F, Chan CC. Plasma catecholamines in rats during rewarming from induced hypothermia. *J Appl Physiol* 1984; 57: 808—814.
27. Torlińska T, Paluszak J, Gryczka AZ, Torliński L, Walkowiak K, Kożlik J. Effects of insulin and beta-adrenergic blockade on certain indicators of carbohydrate metabolism in the blood and tissues of rats during short-lasting hypothermia. *Acta Physiol Pol* 1982; 33: 3—4.
28. Torlińska T, Nowak KW, Kożlik J, Paluszak J. Effect of hypothermia on the insulin-receptor interaction in liver plasma membranes. *J Physiol Pharmacol* 1993; (44) 4: 399—408.
29. Santos RF, Mondon CE, Reaven GM, Azhar S. Effects of exercise training on the relationship between insulin binding and insulin-stimulated tyrosine kinase activity in rat skeletal muscle. *Metab Clin Exp* 1989; 38: 376—386.
30. Burant CF, Treutelaar MK, Buse MG. Diabetes-induced functional and structural changes in insulin receptors from rat skeletal muscle. *J Clin Invest* 1986; 77: 260—270.
31. Gammeltoft S. Insulin receptors: binding kinetics and structure-function relationship of insulin. *Physiol Rev* 1984; 64: (4) 1321—1379.
32. Brillom DJ, Freidenberg GR, Henry RR, Olefsky JM. Mechanism of defective insulin receptor kinase activity in NIDDM. Evidence of two receptor populations. *Diabetes* 1989; 38: 397—403.
33. Parrizas M, Banos M, Baro J, Planas J, Gutierrez J. Up-regulation of insulin binding in fish skeletal muscle by high insulin levels. *Regul Pept* 1994; 53: 211—222.
34. Kobayashi S, Naitoh T, Nakadate T, Kimura J, Kimura M. Suppresion by high glucose concentration of insulin receptor up-regulation in diaphragm and flexor digitorum brevis muscles from diabetic KK-Cay and streptozotocin — diabetic mice. *J Pharmacobiodyn* 1992; 15: 175—180.
35. Balage M, Grizard J, Sornet C, Simon J, Dardevet D, Marin M. Insulin binding and receptor tyrosine kinase activity in rat liver and skeletal muscle: effect of starvation. *Metabolism* 1990; 39: (4) 366—373.
36. Camps M, Gumf A, Vinals F, Testar X, Palcin M, Zorzano A. Evidence for the lack of spare high-affinity insulin receptors in skeletal muscle. *Biochem J* 1992; 285: 993—999.

Received: December 22, 1994
Accepted: September 2, 1995

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