Physiol. Res. 48:51-58, 1999

Changes of Insulin Binding in Rat Tissues After Exposure to Stress

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Received October 31, 1997
Accepted October 1, 1998

Summary

The effects of various stressors on insulin receptors in adipose, liver and skeletal muscle tissues were studied in rats exposed to acute or repeated stress. Adult male rats were exposed to immobilization (IMO) for 2.5 hours daily for 1, 7 and 42 days, or to hypokinesia (HK) for 1, 7 and 21 days. We determined the values of specific insulin binding (SIB) and insulin receptor binding capacity (IR) of plasma cell membranes from adipose, liver and muscle tissue (IMO groups), or insulin binding to isolated adipocytes and hepatocytes (HK groups). A significant decrease of SIB and IR was observed in rats exposed to acute stress (1x IMO) in muscle, adipose and liver tissues. However, in animals exposed to repeated stress (7x and 42x IMO), SIB and IR were diminished in the muscle tissue, whereas no significant changes were noted in the liver and adipose tissue. When tissue samples were collected 3-24 hours after exposure to IMO stress, no changes of SIB and IR were found in liver and adipose tissue, but insulin binding was lowered in skeletal muscles. In animals exposed to HK for one day, a decrease of SIB and IR was found in isolated adipocytes, but no changes in insulin binding were noted in the liver tissue. In rats exposed to HK for 7 and 21 days, values of IR were similar as in control group. Our results indicate a) the different changes of IR in the liver, fat and muscle tissues after exposure to stress situations, b) a long-term decrease of insulin binding in muscles of rats exposed to repeated IMO stress, and c) the return of reduced SIB and IR (induced by acute stress) to control values in the liver and adipose tissue after a short recovery period.

Key words
Stress • Insulin binding • Muscle • Liver • Adipocytes

Introduction

Clinical studies in human subjects have shown that psychological stress may increase the metabolic instability of some patients with diabetes mellitus (Dutour *et al.* 1996, Viner *et al.* 1996). The exposure of animals or human subjects to various stressors is followed by activation of the hypothalamo-pituitary-adrenal cortex with increased plasma glucocorticoid concentrations (Mikulaj and Kvetňanský 1966, Vigaš 1985). It has repeatedly been demonstrated that high plasma levels of glucocorticoids, induced by

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administration of cortisol, corticosterone or dexamethasone, result in a lower response to insulin (Olefsky *et al.* 1975, DePirro *et al.* 1980, Yasuda and Kitabchi 1980, Kahn *et al.* 1981, Caro and Armatruda 1982).

The response of tissue metabolism to the regulatory action of insulin also depends on binding of the hormone to specific receptors and on postreceptor intracellular processes. It was reported that glucocorticoids and catecholamines affect insulin binding in several tissues (Olefsky *et al.* 1975, DePirro *et al.* 1980, Kahn *et al.* 1981, Fantus *et al.* 1981). However, their effects on the insulin receptors depend on the experimental conditions and on the specificity of tissues and cells studied (Montiel *et al.* 1987). In previous experiments we observed that glucocorticoids participate in the regulation of insulin receptors in adipocytes of adrenalectomized or dexamethasone-treated rats (Macho and Ficková 1992).

The observed influence of elevated plasma glucocorticoid levels after the administration of exogenous corticoids on the response to insulin and the presence of increased plasma levels of corticosterone in rats exposed to various stressors posed the following questions: 1) which changes of insulin binding or insulin receptors occur in target tissues after the exposure of animals to acute and repeated stress, 2) do the same changes occur in all insulin target tissues, 3) is repeated stress followed by a permanent decrease of insulin binding with diminished sensitivity of tissues to insulin. In the present study the effects of acute and repeated immobilization stress or hypokinetic stress were investigated on insulin binding in skeletal muscles, the liver and adipose tissues.

Methods

Adult male (SPF) Wistar rats (body mass 304±5 g) were obtained from Charles River (through Anlab, Prague, CR). The research protocol was approved by the Animal Care and Use Committee of Institute of Experimental Endocrinology, Slovak Academy of Sciences. The animals were fed a standard laboratory diet (ST1, Velaz, Prague, CR). Food and water were available ad libitum and animals did not fast before the examination.

In the first series of experiments, the animals were exposed to immobilization stress (IMO) for 2.5 hours as was described previously (Kvetňanský and Mikulaj 1970, Kvetňanský *et al.* 1993). Briefly, stress was induced by taping all four limbs to metal mounts attached to a plastic board (Kvetňanský *et al.* 1993). The rats were divided into the following eight groups, each comprising 10-12 animals: 1) rats exposed to IMO once and examined immediately after the exposure, 2) rats exposed to IMO for 2.5 hours daily for 7 days were examined immediately after the last IMO, 3) rats exposed to IMO for 2.5 hours daily for 42 days, examined immediately after the last IMO, 4) rats exposed to IMO for 6 days and examined on the 7th day (i.e. 24 hours after the last IMO), 5) rats exposed to IMO once and examined 3 hours after the exposure to stress, 6) rats exposed to IMO for 7 days and examined 3 hours after the last IMO, 7) rats exposed to IMO for 40 days and examined 3 hours after last IMO, 8) control animals without any stress manipulations.

The animals were sacrificed by decapitation, blood was collected for determination of insulin and corticosterone. Epididymal fat tissue, liver and quadriceps muscle from the right hind limb were collected, and immediately frozen in liquid nitrogen. Plasma cell membranes were isolated and insulin binding was determined according to Sakamoto *et al.* (1980). Briefly, the ^{125}I (Tyr_{A-14})-monoiodoinsulin, prepared by labeling monocomponent porcine insulin (NOVO, Nordisk, Denmark) according to the method described in detail in a previous paper (Zórad *et al.* 1985), was used for the binding studies. The specific activity of radioiodine-labeled insulin was 180-193 μCi/μg. The isolated fat cells (approx. $2x10^6$ cells in Krebs-Ringer bicarbonate solution, pH 7.4, total volume 0.5 ml) were incubated with ^{125}I-insulin in the absence or in the presence of 10^{-11} to 10^{-6} M of unlabeled insulin at 24 °C for 50 min and the cells were separated by centrifugation through silicon oil (Lukoil M, Synthesia, Pardubice, Czech Republic). The size of adipocytes was evaluated by optical measurement of their diameter under a light microscope after fixation of the cells in 2 % osmium tetroxide. The plasma cell membranes were incubated with the same amounts of insulin but at 4 °C for 21 hours and separated by centrifugation (Sakamoto *et al.* 1980). The insulin binding capacity was calculated by a computer-fitted multilinear regression curve according to DeMeyts and Roth (1985).

In the second series of experiments, adult male Wistar rats (body mass 250-310 g, 8 animals per group) were exposed to hypokinesia by restriction of movement (for 24h/day) in special adjustable plastic cages for 1, 7 or 21 days (Kovalenko 1977, Macho *et al.* 1984). Control rats were kept in controlled animal rooms for the same periods at 24 °C and under a 12:12 h light:dark regimen and housed in standard size cages, six animals

per cage (Macho *et al.* 1988). All rats were given food (standard laboratory diet ST1, Velaz, Prague, CR) and water *ad libitum*. The animals did not fast before the examination. At the end of the hypokinesia period, the animals were decapitated, the blood was collected for insulin and corticosterone determinations. The isolated adipocytes were prepared from epididymal fat pads according to Rodbel (1964). Isolated hepatocytes were obtained by perfusion of the liver with collagenase (Ficková and Macho 1988), while crude plasma membranes were prepared from the liver by the method described by Sakamoto *et al.* (1980). Serum insulin levels in serum were determined by a RIA kit (NOVO, Nordisk, Denmark), corticosterone by a modified protein binding method (Murphy 1967), glucose by the orthotoluidine method (Bio-Glu-test, Lachema, Brno, CR) and proteins in plasma membranes by the method described by Lowry *et al.* (1951). All values are expressed as means ± S.E.M. Differences between the two groups, control and stressed rats, were evaluated by the t-test, statistical significance was set at the 0.05 probability level.

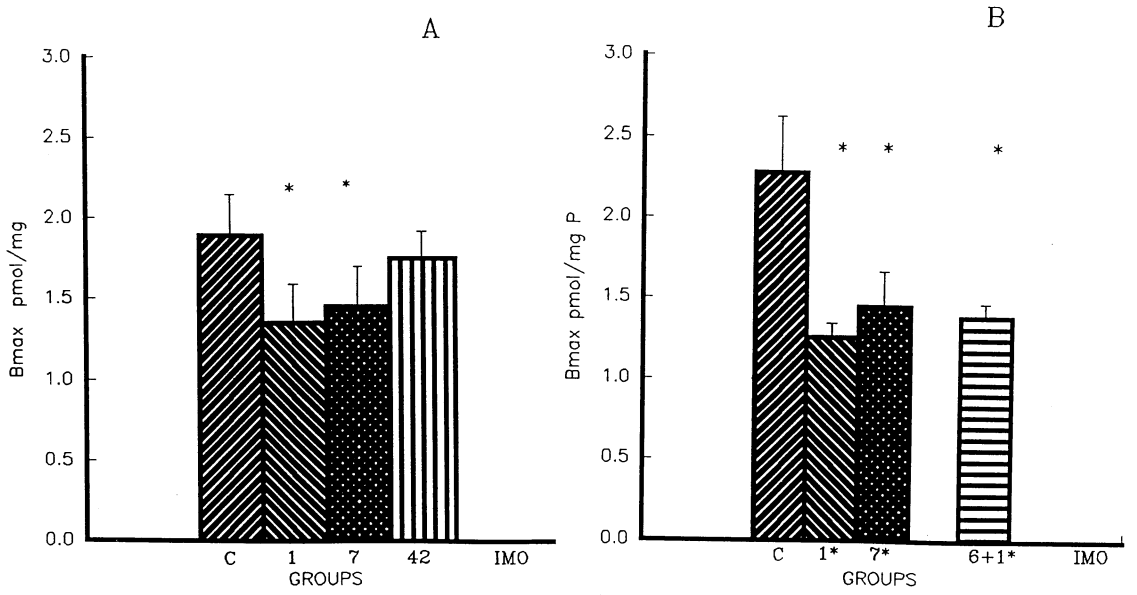

Fig. 1. A. *Insulin binding capacity (B_{max} in pmol per 1 mg of proteins) of insulin receptors in muscle membranes of rats exposed to acute and repeated immobilization stress and examined immediately after stress exposure.* **B.** *Insulin binding capacity of insulin receptors in muscle membranes of rats exposed to immobilization stress and examined 3 hours (1* and 7* days IMO) or 1 day (6 days IMO + 1*) after the last immobilization. C – controls, 1, 7 and 42 days of daily immobilization. Significant differences ($p<0.05$): * Controls vs IMO.*

Results

Decreased specific insulin binding (Table 1) and insulin receptor binding capacity (Fig. 1A) were found in muscles of rats exposed to acute immobilization stress and examined immediately after IMO. The specific insulin binding in muscles was also decreased in animals exposed to repeated stress (Table 1) in which the binding capacity of insulin receptors was slightly diminished (Fig. 1A). The lower values of specific insulin binding and insulin receptor binding capacity were observed 3 hours after exposure of animals to IMO stress either one or 7 times, and also in rats 24 hours after repeated IMO stress for 6 days (Table 1, Fig. 1B). The results showed that specific insulin binding and insulin receptor binding capacity in plasma membranes of muscle tissue are markedly decreased in rats exposed to acute and repeated stress. This attenuation of insulin binding could be observed even 3-24 hours after IMO stress had been discontinued.

The specific insulin binding and insulin receptor binding capacity were significantly decreased in the adipose tissue of rats exposed to acute IMO stress (for the first time) when examined immediately after immobilization (Table 1, Fig. 2A). In rats exposed to repeated immobilization stress (7 or 42 times), the changes of specific insulin binding and insulin receptor binding capacity in adipocytes were not significant.

Table 1. Insulin, glucose and corticosterone serum levels, the specific insulin binding (SIB) in tissues and body mass of rats exposed to immobilization stress.

Groups	MUSCLE SIB %/1 mg P	FAT TISSUE SIB %/0.1mg P	LIVER SIB %/0.1mg P	INSULIN μU/ ml	CORTICOSTERONE μg/100 ml	GLUCOSE mmol/l	BW Controls g	BW IMO g
Controls	8.5±0.3	5.9±0.3	4.2±0.3	51±4	8.1±1.1	4.9±0.1	304±5	–
1x IMO	5.8±0.1$^{\#\#}$	3.5±0.2$^{\#\#}$	2.5±0.1$^{\#\#}$	41±8	49.8±1.0$^{\#\#\#}$	6.9±0.3$^{\#\#}$	304±5	302±5
7x IMO	6.3±0.5$^{\#\#}$	5.5±0.3	3.9±0.2	37±2$^{\#}$	59.7±3.1$^{\#\#\#}$	5.1±0.5	320±4	302±4$^{\#}$
42x IMO	7.1±0.3$^{\#\#}$	5.8±0.3	3.8±0.2	19±6$^{\#}$	50.8±2.3$^{\#\#\#}$	4.9±0.2	462±8	382±9$^{\#\#}$
Controls	11.2±1.4	4.1±0.2	4.9±0.2	68±5	3.3±1.1	6.1±0.1	–	–
1x IMO + 3 h	7.3±0.4$^{\#}$	4.9±0.2	4.9±0.3	77±5	12.9±4.0$^{\#}$	5.9±0.1	–	–
7x IMO + 3 h	7.4±1.6$^{\#}$	5.6±0.3$^{\#\#}$	4.6±0.2	57±5	11.7±2.9$^{\#\#}$	5.7±0.1$^{\#}$	–	–
6x IMO+24 h	8.9±0.5$^{\#}$	6.3±0.2$^{\#\#\#}$	5.5±0.4	64±2	18.9±2.5$^{\#\#}$	6.2±0.2	–	–

SIB - specific insulin binding in % of added insulin per proteins (P) of membrane fractions, IMO – immobilization stress, IMO + 3h – animals examined 3 h after stress, IMO + 24h – animals examined 24 h after stress, C – non-stressed controls to IMO animals at the same period of immobilization. Significant differences (Controls vs IMO): $^{\#}$ *p<0.05 ,* $^{\#\#}$ *p<0.01,* $^{\#\#\#}$ *p<0.001.*

Table 2. Serum insulin, glucose and corticosterone levels, adipose tissue mass, adipocyte size and insulin binding in tissues of rats exposed to hypokinesia of different duration.

Groups	FAT CELLS SIB %/ 10^6 cells	LIVER MB SIB%/0.1mg P	LIVER CELLS SIB %/ 10^6 cells	INSULIN μU/ml	CORTICOSTERONE μg/100 ml	GLUCOSE mmol/l	IFC SIZE μm ∅	FAT MASS g	FOOD g/day
Controls	0.99±0.07	9.21±0.93	4.30±0.25	36±3	5.4±1.0	5.6±0.3	78±1	4.8±0.3	18.6±1.6
1 days HK	0.69±0.06$^{\#\#}$	9.72±0.87	3.75±0.31	37±3	10.2±2.1$^{\#}$	6.0±0.3	74±1$^{\#}$	4.4±0.1	–
7 days HK	1.01±0.12	9.41±0.75	4.55±0.43	31±2	7.5±1.5	6.2±0.4	63±2$^{\#\#}$	3.5±0.1$^{\#\#\#}$	17.4±1.3
21 days HK	1.16±0.02	–	–	28±1$^{\#}$	3.6±1.2	5.3±0.1	57±1$^{\#\#}$	3.4±0.9	15.3±1.0

LIVER MB – liver plasma membranes, SIB – specific insulin binding, IFC – isolated fat cells, FAT MASS – mass of epididymal fat pads, FOOD - food consumption per day, HK – hypokinesia. Significant differences (Controls vs Hypokinesia): $^{\#}$ *p<0.05 ,* $^{\#\#}$ *p<0.01,* $^{\#\#\#}$ *p<0.001.*

An unexpected increase of specific insulin binding and insulin receptor binding capacity in the adipose tissue was observed in rats exposed to acute or repeated IMO stress 3 and 24 hours after the exposure to stress (Table 1, Fig. 2B). These results showed that exposure of rats to acute IMO stress is followed by an immediate decrease of insulin binding in the adipose tissue. These changes of insulin binding and insulin receptor binding capacity are probably due to changes of insulin receptor affinity or lower translocation of insulin receptors to the cell surface, because insulin binding and receptor binding capacity were elevated even after a short recovery period (3 hours only).

The decrease of specific insulin binding (Table 1) and insulin receptor binding capacity was found in liver plasma membrane from rats exposed to acute IMO stress (B_{max} – IMO 1.98±0.28, controls 6.55±1.30 pmol/mg of proteins). Repeated stress was not followed by significant changes of these parameters in liver. In animals examined 3 hours after immobilization the binding of insulin and insulin receptor number were not significantly different from control.

The marked elevation of serum corticosterone levels was found in rats immediately after acute or repeated IMO stress (Table 1). The small augmentation of serum corticosterone was also observed in animals 3 and 24 hours after the exposure to stressor. The small decrease of serum insulin levels was noted in rats exposed to IMO stress when examined immediately after stress exposure. No significant changes of serum insulin values were observed 3 and 24 hours after IMO stress. The blood glucose levels were increased in rats exposed to stress for the first time, however, the changes of glucose concentration were not significant in animals exposed to repeated stress. A lower body mass increase was noted in repeatedly stressed rats (Table 1). The food intake and the weight of fat tissues were not recorded in this experiment.

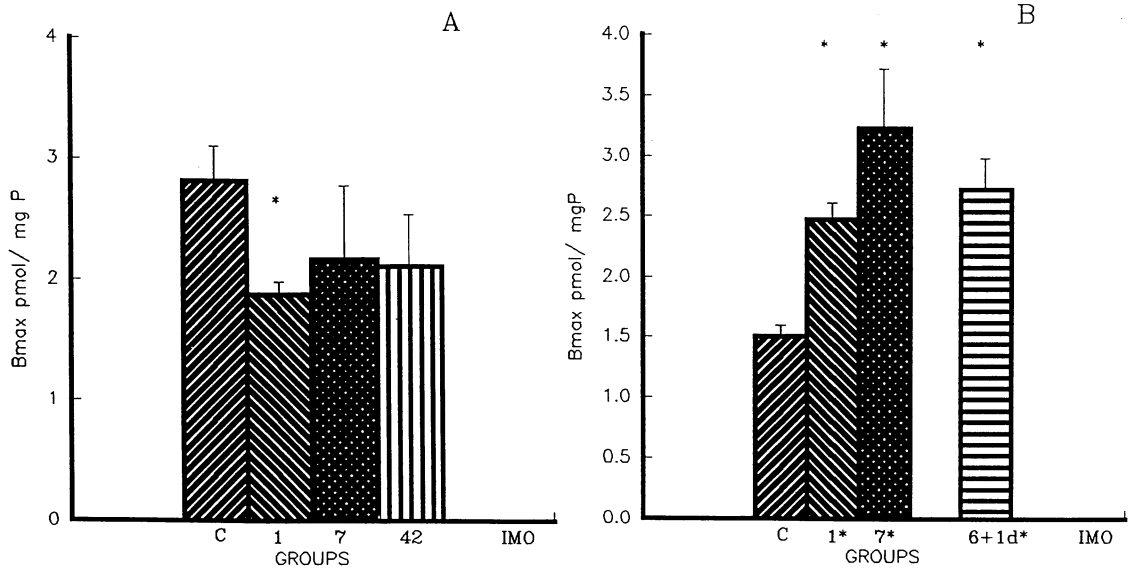

Fig. 2. A. *Insulin binding capacity (B_{max} in pmol per 1 mg of proteins) in plasma cell membranes from adipose tissue of rats exposed to immobilization stress for 1, 7 and 42 days and examined immediately after immobilization.* **B.** *Insulin binding capacity of plasma cell membranes from adipose tissue of rats exposed to immobilization stress and examined 3 hours (1* and 7* days IMO) or 1 day (6 days IMO + 1*) after the last immobilization. Data are means ± S.E.M, C -controls, 1, 7 or 42 days of IMO. Significant differences ($p<0.05$): * Controls vs IMO.*

The exposure of animals to hypokinesia for 24 h (one day period) was followed by marked decrease of specific insulin binding and insulin receptors in adipocytes (Table 2, Fig. 3). In rats exposed to hypokinesia for a longer period, 7 or 21 days, no changes or even a slight increase of insulin receptors was noted in adipocytes. There were no changes of insulin specific binding and insulin receptor binding capacity in isolated hepatocytes of rats exposed to hypokinesia for one, 7 or 21 days (Table 2). Serum corticosterone levels were elevated in rats exposed to hypokinesia for one day (Table 2). No significant changes of serum corticosterone levels were found in rats with permanent hypokinesia for 7 or 21 days. A slight decrease of insulin levels was

observed in rats exposed to hypokinesia for 21 days (Table 2). The changes of glucose levels were not significant in animals exposed to hypokinesia as compared to the controls. A reduction of body mass was observed in rats exposed to hypokinesia for 7 and 21 days (controls 322±5 g vs 7 days HK 301±4 g, controls 379±8 g vs 21 days HK 324±13 g). The mass of epididymal fat pads and the size of adipocytes were lower in rats after 7 and 21 days of hypokinesia (Table 2). The measurement of daily food intake showed a slight decrease in rats exposed to hypokinesia for 21 days (Table 2).

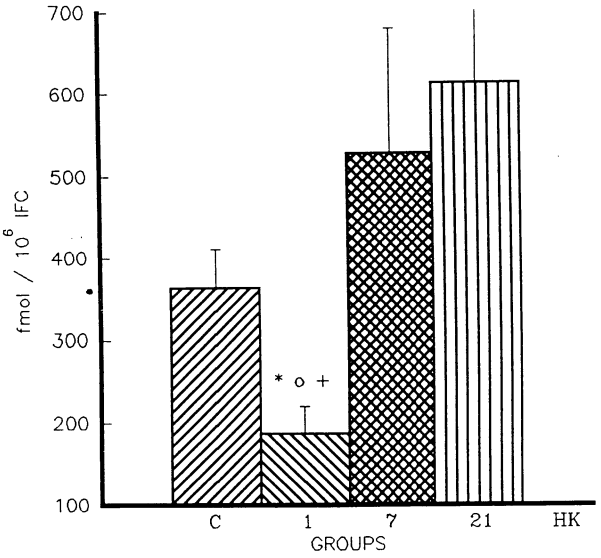

Fig. 3. *Number of insulin receptors of rat adipocytes exposed to hypokinesia (HK) for 1, 7 or 21 days. Data are means ± S.E.M., C – controls. Significant differences ($p<0.05$): * Controls vs 1 day HK, ° 1 day vs 7 days HK, + 1 day vs 21 days HK.*

Discussion

The results of these experiments showed that there are differences in the changes of insulin binding and insulin receptor binding capacity in muscles, the liver and adipose tissue after exposure of rats to various stressors. The insulin receptors of cell membranes in target tissues of insulin action are predominantly regulated by plasma insulin levels. However, glucocorticoids and catecholamines have been shown to exert effects on insulin binding and insulin action (Olefsky *et al.* 1975, Fantus *et al.* 1981). The elevated plasma insulin levels are associated with a decreased number of insulin receptors by the mechanism of down-regulation (Lane 1981). Since no marked changes of plasma insulin levels were noted in rats exposed to short-term hypokinesia, it is not possible to explain the decreased number of insulin receptors in adipocytes of these hypokinetic animals by a down-regulation mechanism. Significant activation of the sympathoadrenal system with a high elevation of epinephrine and norepinephrine plasma levels was found immediately after the beginning of hypokinesia and the high plasma catecholamine levels were maintained during the whole period of hypokinesia (Macho *et al.* 1989). It is unlikely that plasma catecholamine changes are involved in the reduction of insulin receptors in adipocytes during short-term hypokinesia, because the same changes of insulin receptors as in non-treated rats were also observed in adipocytes from hypokinetic animals after removal of the adrenal medulla and suppression of sympathetic system activity by guanethidine (Ficková *et al.* 1988). The decrease of insulin binding and reduced number of insulin receptors in adipocytes observed after the first exposure to hypokinesia are probably related to the elevation of plasma corticosterone levels, because the decrease of insulin receptors in this stress situation can be prevented by adrenalectomy. The negative correlation between plasma corticosterone and insulin binding capacity in adipocytes was previously observed (Macho and Ficková 1992). The decreased insulin binding in adipocytes from rats exposed to short-term hypokinesia was not related to changes in the cell size, because marked diminution of cell size was noted after long- term hypokinesia which was not followed by any changes in insulin binding. This lower insulin binding in adipocytes from rats after short-term hypokinesia is probably related to the attenuation of insulin stimulatory effect on lipogenesis (Macho *et al.* 1988).

Hypokinesia had no significant effect on insulin binding in liver and red blood cells suggesting the different changes of insulin receptors in various tissues after short-term hypokinesia (Macho *et al.* 1993).

The changes of insulin binding in muscle, liver and adipose tissues in rats exposed to IMO stress can not be explained by a down regulatory mechanism due to alterations of insulin levels in the blood, because there were no significant changes of insulinemia during exposure to acute IMO stress. In animals exposed to repeated IMO, even a small decrease of serum insulin levels was noted as compared to the controls. In spite of this fact, a significant decrease in the number of insulin receptors was found in muscles of rats exposed to IMO. It is also probable that, besides other factors, the elevation of serum corticosterone concentrations in IMO

rats is involved in the reduction of insulin receptors in muscles of IMO rats. In the liver and in adipose tissue, insulin binding was lowered only after acute IMO stress, but no changes were found after repeated stress in spite of elevated serum corticosterone levels. These results indicate that other regulatory mechanisms of insulin binding and insulin receptors could play a role during repeated IMO stress. There are differences in the changes of insulin receptors in muscle, liver and fat tissues after exposure to acute or repeated IMO stress. The tissue differences of insulin binding during IMO stress could be due to structural variations and different membrane distribution of insulin receptors in adipocytes and muscles (Joost 1995). The differences in the changes of insulin receptors and insulin signaling pathway at postreceptor levels in muscles and the liver were also observed after an excess of glucocorticoid (induced by daily injection of cortisone acetate or dexamethasone for 5 days) (Saad *et al.* 1993, Giorgino *et al.* 1993). It was found that the expression and/or phosphorylation of the insulin receptor and insulin receptor substrate-1 protein are decreased in skeletal muscles of rats treated by glucocorticoids. It will therefore be important to investigate the pathophysiological significance of these alterations. However, a very high glucocorticoid-induced hyperinsulinemia was present in the experiments of Saad *et al.* (1993) and Giorgino *et al.* (1993), suggesting that the chronic increase of plasma insulin levels appears to be essential for the induction of changes in insulin action. During exposure of rats to IMO stress, no increase of serum insulin levels was observed, so that reduced number of insulin receptors in skeletal muscles is probably related to other regulatory factors including the elevation of glucocorticoids. The decrease of insulin receptors in skeletal muscle of rats exposed to IMO stress is probably responsible for the lowered oxidation of glucose in this tissue during the acute and repeated stress (Macho *et al.* 1968). Nevertheless, it has no effect in rats exposed to immobilization stress for a longer period (40 days).

The results of these experiments showed that 1) there are differences in the changes of insulin receptors in the liver, fat and muscle tissues after exposure to various stressors (IMO, hypokinesia), 2) the stress-induced diminution of specific insulin binding and insulin receptor binding capacity in the liver and fat tissues returns to normal values after a short recovery period, 3) the acute or repeated IMO stress decreased insulin binding in muscles suggesting that high levels of corticosterone in animals exposed to repeated stress could have modulatory effects on insulin receptors and insulin effects in the muscle tissue.

Acknowledgments

The competent technical assistance of Mrs. E. Visztová and M. Škultetyová is gratefully acknowledged. This work was supported by grants from the Slovak Academy of Sciences, No. 2/541/93 and VEGA-2/4130/97.

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