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AGE DEPENDENT CHANGES OF INSULIN RECEPTORS IN RAT TISSUES

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Aging is associated with insulin resistance but the exact molecular mechanism is still unknown. Tissue insulin resistance can be evoked by the decreased sensitivity to insulin, the decreased responsiveness to hormone or both. As the first step in insulin action is its binding to alfa subunits of the receptor we, therefore, studied the insulin binding kinetics in plasma membranes of the liver, heart and skeletal muscle in order to establish whether their ability to bind the hormone is altered with aging. Plasma membranes were prepared and purified according to Havrankowa and binding assay was performed using (^{125}I)-iodoinsulin. The kinetic parameters of the hormone — receptor interaction were analysed by the method of Scatchard using the LIGAND-Pc v.3.1. computer program. The binding potency of insulin was calculated as IC_{50} using ALLFIT-Pc v.2.7. computer program. We have shown that there are striking differences in insulin binding kinetics in newborn and old rats, depending on kind of tissue tested. The liver plasma membranes ability for insulin binding, number of high (HAIR) and low (LAIR) affinity insulin receptors, values of the dissociation constants and products of association constants and number of insulin receptors, were almost the same, being not dependent on age of the rats. By contrast, there is less high affinity insulin receptors in skeletal muscle of the old animals. The most dramatic changes in insulin binding occur in the heart where both high and low affinity insulin receptors are greatly affected by aging. Our results indicate that the response of the three tissues tested to hyperglycemia and hyperinsulinemia, observed in the old rats, has not been identical and probably can be accounted for by the different distribution of insulin receptor isoforms in the liver, heart and skeletal muscles as shown recently by Vidal *et al.*

Key words: *aging, insulin receptors, rat tissues.*

INTRODUCTION

Despite intensive research, the mechanisms of aging are still unknown. Aging affects cells and the systems made up of them, as well as tissues components and their function. Current evidence favours the view that

decrease in glucose tolerance occurring with age is due to a reduction in glucose uptake rather than impaired insulin secretion. Insulin resistance in old, compared with young, humans and animals has been well documented (1, 2, 3, 4). According to the numerous data the resistance is due primarily to defects in skeletal muscle, but its molecular mechanism is not completely understood.

Insulin resistance may be caused by abnormalities of insulin signal transduction at any level of the signal transmitting chain in the target cell, i.e. at the level of insulin binding, activation of insulin receptor kinase, post-kinase signal transmitting events or directly at the level of major effector systems such as the glucose transport system or glycogen synthesis.

Insulin stimulates the tyrosine kinase activity of its receptor, resulting in the phosphorylation of its cytosolic substrate — IRS-1, which, in turn, associates with phosphatidylinositol 3-kinase (PI 3-kinase) thereby activing the latter (5). Both activity of insulin receptor kinase and its autophosphorylation are known to be a crucial event for the function of insulin receptor. As shown by Kono *et al.* basal and insulin — stimulated autophosphorylation of liver insulin receptor and its kinase activity did not alter with aging. On the other hand the rat skeletal muscle insulin receptor showed different results indicating that insulin — stimulated phosphorylation changed during process of aging (6).

Tissue insulin resistance can be evoked by the decreased sensitivity to insulin, the decreased responsiveness to hormone or both. It has been generally accepted that insulin resistance caused by the reduction in number of insulin receptors generates decreased tissue sensitivity whereas insulin resistance, connected with postreceptor resistance leads to decreased maximal responsiveness.

Recent findings suggest that alterations in expression of insulin receptor may be involved in the molecular mechanism of insulin resistance. According to Vidal and coworkers the liver expresses the mRNA variant with exon 11 predominantly and the hind limb skeletal muscle expresses the mRNA without exon 11. By contrast the heart and epididymal adipose tissue express both variants (7).

As the first step in insulin action is its specific binding to alfa-subunits we, therefore, studied the insulin binding kinetics in plasma membranes of the liver, heart and skeletal muscle in order to establish whether their ability to bind the hormone is altered with aging. Our review of the literature revealed that there is no study on insulin binding in aging, being carried out under identical conditions at the same time, in these three rat tissues which differ by the different insulin receptor isoform distribution.

MATERIAL AND METHODS

Male Wistar rats were housed in a temperature — controlled room at 20°C and maintained on a standard laboratory rat chow (LSM) with free access to food and water. All experiments were carried out between 9—11 a.m. throughout a whole year.

The rats were sacrificed by decapitation and after complete exsanguination hearts, livers and skeletal muscles were frozen in liquid nitrogen and stored at −70°C till taken for futher analysis. In the present study, liver, heart and skeletal muscle membranes were purified from two groups of rats: newborn (5 days old) and old (12 months) animals.

Isolation of crude membrane fraction

The plasma membranes were prepared according to the method of Havrankowa *et al.* (8). Briefly, the tissues were homogenized in 20 ml of 0.001 M $NaHCO_3$ and centrifuged at 600 × g for 30 min. The resultant supernatant was centrifuged for 30 min. at 20000 × g.

The membrane preparations were washed twice with 0.001 M $NaHCO_3$. The final pellets were resuspended in 0.04 M Tris-HCl buffer (pH 7.4) containing 0.1% bovine serum albumin (BSA). All the procedures mentioned above were carried out at 4°C. All the chemical reagents were produced by Sigma Chemicals Co., USA.

Binding assay

Insulin binding activity was measured by incubating the membrane preparations (0.25 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% BSA. For the competition binding assay, increasing amounts of unlabelled insulin (Novo, Denmark) were added to the reaction mixture to give a final concentration of 0—700 nmol/l.

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 8 minutes and then the radioactivity of the pellets was determined by means of a gamma-counter.

The dissociation constant (K_d) and the number of receptors measured as a binding capacity were determined by the Scatchard method (9) using the LIGAND-PC v.3.1. computer program (10). The binding potency of insulin was calculated as IC_{50} using the ALLFIT-PC v.2.7. computer program.

Other analyses

Serum insulin was measured by a standard radioimmunoassay (RIA) using kits for rat insulin (Linco, Research Inc. USA). Glucose was determined by the glucose oxidase method (Sigma). Protein concentration was estimated by the method of Lowry *et al.* (11). Student's t-test was used for statistical comparison and differences were considered to be significant if $p < 0.05$.

RESULTS

As shown in *Table 1* serum glucose concentration was significantly increased in old, 12 month rats (by about 136%) as compared with the newborn rats. These changes were accompanied by an increase in immunoreactive serum insulin concentration in the old rats by about 233%

compared with the newborn rats. As a result of these changes the insulin — to — glucose ratio in the old rats was greatly increased in comparison with the newborns.

Table 1. Serum glucose and insulin concentrations and the insulin/glucose ratio in newborn (5 days) and old (12 months) rats.

	Newborn rats	Old rats
Glucose (mmol/l)	4.35 ± 0.50	10.26 ± 0.30 *
Insulin (IRI) (μU/ml)	14.88 ± 3.71	114.06 ± 5.59 *
IRI/glucose (μU/mol)	3.35 ± 0.66	11.16 ± 0.56 *

Results are presented as mean ± SEM ($n = 10$).
* Significantly different from newborn rats ($p < 0.01$).

Despite these evidences showing obviously insulin resistance, the data presented below reveal that there are striking differences in insulin bindings kinetics in newborn and old (12 months) rats, depending on kind of tissue tested. Because the procedure of insulin labelling and purification may modify its binding to receptors, each change may in consequence influence Scatchard's plot shape and the final estimation of insulin receptor number and the affinity to insulin. Considering these observations all our experiments were carried out using the same set of labelled insulin. As depicted in *Table 2* there was no significant changes in any parameter tested in case of the livers taken from newborn and old rats. Suprisingly, the liver plasma membrane ability for insulin binding, expressed as the maximum specific insulin binding, number of high (HAIR) and low (LAIR) affinity insulin receptors, value of the dissociation constant (K_{d1} and K_{d2} respectively for HAIR and LAIR), product of association constant and number of insulin receptor ($K_{a1} \times R_1$ and $K_{a2} \times R_2$ respectively for HAIR and LAIR), were almost the same, being not dependent on age of the rats (5 days newborns versus 12 month old rats). Half-maximum displacement of tracer insulin (IC50) was observed at similar insulin concentrations in both groups of rats (3.33 ± 0.82 nmol/l and 3.59 ± 0.14 nmol/l). By contrast, age-related changes have been observed both for skeletal muscles and hearts. The data on the kinetics of the insulin binding are presented in the form of a Scatchard plot showing significant differences in the I^{125}-insulin binding to the skeletal muscle and heart receptors between newborn and old rats (*Fig. 1* and *Fig. 2*). The characteristic features of insulin receptors (binding capacities of HAIR and LAIR, dissociation constants K_d and products of association constants and number of HAIR and LAIR) as well as IC50 are summarized in *Table 2*. There is less high affinity insulin receptors (HAIR) in skeletal muscles of the old animals, but the dissociation constant of

Table 2. Values characterizing insulin receptors in liver, heart and skeletal muscle membranes of 5 day old rats and 12 month old rats.

	B_{sp} (%)	HAIRs (fmol/mg)	LAIRs (pmol/mg)	Kd_1 (nmol/l)	Kd_2 (nmol/l)	$Ka_1 \times R_1$	$Ka_2 \times R_2$	IC_{50} (nmol/l)
Liver (5 days)	14.6±2.5	284.9±53.3	9.36±0.12	0.926±3.65	125.0±29.6	0.335±0.060	0.065±0.014	3.33±0.82
Liver (12 months)	16.4±1.5	316.8±37.8	10.65±1.59	1.001±0.09	157.3±17.6	0.319±0.028	0.071±0.011	3.59±0.14
	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Skeletal muscle (5 days)	9.5±0.7	143.2±35.3	15.01±3.51	1.152±0.23	182.0±19.7	0.116±0.013	0.080±0.013	10.96±2.38
Skeletal muscle (12 months)	4.5±0.4	24.6±7.2	14.8±3.3	0.499±0.12	313.6±71.5	0.048±0.003	0.049±0.005	13.17±2.65
	$p < 0.01$	$p < 0.01$	N.S.	$p < 0.05$	N.S.	$p < 0.01$	$p < 0.05$	N.S.
Heart (5 days)	12.5±0.9	22.6±6.9	14.7±2.6	0.111±0.02	82.8±15.6	0.189±0.020	0.178±0.013	7.92±1.71
Heart (12 months)	4.7±0.4	18.1±2.4	7.6±1.6	0.297±0.05	175.2±26.2	0.068±0.008	0.043±0.007	5.10±1.74
	$p < 0.01$	N.S.	$p < 0.05$	$p < 0.01$	N.S.	$p < 0.01$	$p < 0.01$	N.S.

Results are presented as mean±SEM (n = 6).
B_{sp}(%) — (%) of specific binding of (^{125}I) — insulin to membrane protein in the absence of unlabelled insulin.
HAIRs — binding capacity of high affinity insulin receptors.
LAIRs — binding capacity of low affinity insulin receptors.
Kd_1, Kd_2 — dissociation constants for HAIRs and LAIRs, respectively.
$Ka_1 \times R_1$ — association constant for HAIRs multiplied by binding capacity of HAIRs.
$Ka_2 \times R_2$ — association constant for LAIRs multiplied by binding capacity of LAIRs.
IC_{50} — concentration of unlabelled insulin which reduces maximum specific binding by 50%.

the complex hormon — receptor for HAIR is lower as compared to the newborn rats, showing that high affinity insulin receptors of old rats exhibit higher affinity for the hormone. We have also found that the products of $K_a \times R$ (affinity constant multiplied by binding capacity) for HAIR and LAIR are lower in the group of the old rats. Therefore, we may conclude that the total ability for insulin binding in skeletal muscle of old rats is lower as compared with the newborns, being not compensated by higher affinity of HAIRS observed in the case of old rats. Even worse situation exists in the hearts of old rats comparing to the newborns. Both number of high affinity (HAIR) and low affinity (LAIR) insulin receptors was significantly reduced, by aprox 42% and 48.3% respectively. In addition the value of K_{d1} for HAIR was

higher, showing that the affinity of these receptors for insulin is lower. As the result, products of $K_{a1} \times R_1$ and $K_{a2} \times R_2$ were lower, indicating that the entire ability to bind insulin is much more worse in the old rats. This findings were in good agreement with the results of the maximum specific insulin binding which was significantly lower (by about 62.4%) in the case of hearts from old animals.

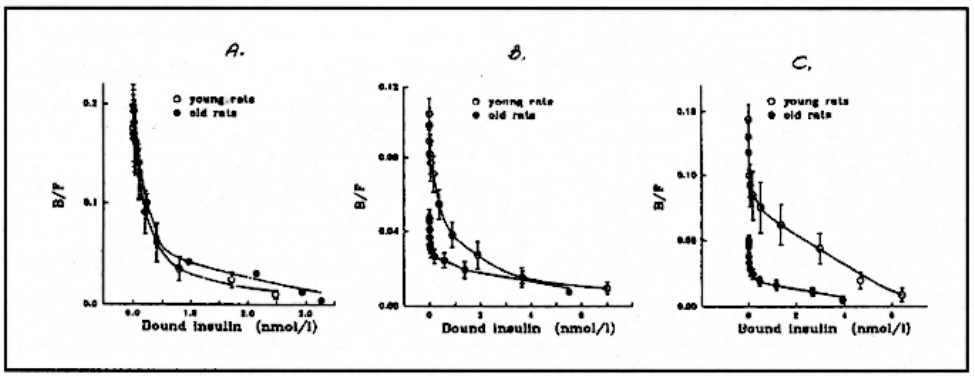

Fig. 1. Scatchard analysis of (^{125}I) — iodoinsulin binding to liver plasma membranes (A), skeletal plasma membranes (B) and heart plasma membranes (C) obtained from newborn and old rats. (bound — free hormone versus hormone bound plotted).

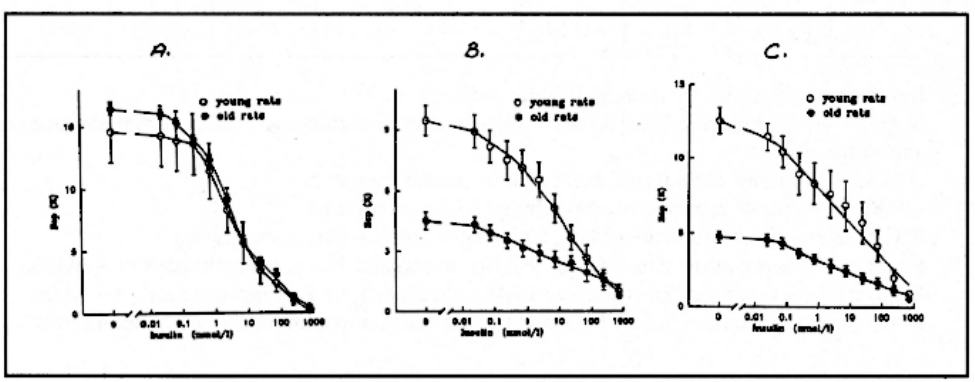

Fig. 2. Displacement of (^{125}I) — iodoinsulin by native insulin from liver plasma membranes (A), skeletal plasma membranes (B) and heart plasma membranes (C). The membranes (0.25 mg of protein/ml) were incubated with 80 pg/ml of (^{125}I) — iodoinsulin in the absence or presence of unlabelled insulin over a range of insulin concentration from 0—700 nmol/l. Data are expressed as the percentage of insulin bound per mg protein.

DISCUSSION

A number of studies indicate that not only liver and skeletal muscle but also heart muscle is an important target for insulin, which acts via specific receptors.

Cloning of the human insulin receptor (HIR) cDNA has revealed two forms of the insulin receptors, differing by the absence (HIR-12) or presence (HIR + 12) of 12 amino acids near the COOH-terminus of the alfa-subunit. As a consequence, distribution of the receptor isoforms differs among tissues; for instance, HIR + 12 is predominant in the liver, skeletal muscles express HIR-12, whereas the heart posesses both insulin receptor isoforms (7). In view of this facts it is not suprising, that the response of this three tissues to hyperglycemia and hyperinsulinemia observed in our study has not been identical.

As shown by Codina *et al.*, level of basal circulating glucose did not change in aging rats whereas insulin concentration increased and glucagon decreased linearly with animal weight (3).

Net insulinogenesis as well total insulin secretion increases linearly as a function of aging (12). Of equal importance is the fact that pancreases from the older rats exhibited the same degree of secretory responsiveness to changing glucose concentrations as did pancreas from the younger rats. The results of Reaven also suggest that aging leads to marked changes in both insulin secretion and insulin action. The decline in glucose — stimulated insulin secretion per unit endocrine pancreas appears to be an inevitable consequence of the aging process (13). Studies of the effect of aging on glycemia and blood lipid concentration reveal some gender differences. There was a significant age — dependent decline in glucose level in male animals whereas in females there was an increase in plasma glucose with aging (14). Our study performed on male animals has revealed that aging is associated not only with hyperinsulinemia but also with hyperglycemia that, in turn, confirms the development of insulin resistance. It has been shown that twelve-month-old rats are characterized by peripheral insulin resistance and decreased glucose tolerance. Although a decreased level of the variant with exon 11 correlates with insulin resistance of whole body glucose uptake, the changes in the expression of the insulin receptor variants seem to be secondary events and thus not the cause of the insulin resistance in old rats (15). As shown by Diaz and Blazquez changes in ^{125}I-insulin uptake depend on the tissues tested and on the age of animals (16). According to their data specific insulin uptake in the liver was much greater in 10 day-old animals than in adult rats. Comparing three different muscle tissues they discovered that heart insulin uptake was much higher in younger animals than in adult rats, while in the diaphragm it was significantly smaller in both groups. In skeletal muscle the hormone uptake was much smaller than in the other two muscle tissues and was even absent in the fetuses. These results indicate that insulin binding sites appear at different times and fluctuate in a different manner according to the tissues tested during rat development. Aging is associated with insulin resistance but the exact molecular mechanism is unknown. It has been established that there is no changes in the insulin receptor concentration in the liver and muscle

during rats aging as determined by immunoblotting using antibody to the COOH-terminus of the receptor (17).

However insulin stimulation of receptor autophosphorylation was reduced in both the liver and muscle of rats at 20 month. Interestingly, IRS-1 protein levels decreased at an early stage (5 months) by 58% and remained at low levels only in skeletal muscle, not in liver. The phosphotyrosine — associated PI 3-kinase activity after insulin stimulation was dramatically reduced in liver and muscle of 20 month old rats. These data suggest that changes in the early step of insulin signal transduction may have an important role in the insulin resistance observed in old animals (17). As the first step in insulin action is its specific binding to alfa-subunits we have decided to study the insulin binding kinetics in plasma membranes of the liver, heart and skeletal muscle in order to establish whether their ability to bind the hormone is altered with aging. Binding defects may be manifested by decreased number of receptors or decreased affinity of the receptor for insulin. The number of insulin receptors can be determined on the basis of the maximum binding capacity as we did. The affinity for hormone can be measured as the dissociation constant (K_d) of insulin receptor complex because the inverse relationship exists between the value of K_d and affinity itself, or as the association constant (K_a) of the insulin-receptor complex which is proportionate to the affinity for insulin (18).

In our opinion, the product of $K_a \times R$ (affinity constant multiplied by binding capacity) may serve as a very convenient index which described the functional features of insulin receptors taking into account both number of insulin receptors and their affinity (19, 20). Taking all the parameters of insulin binding tested in our study we can conclude that in the liver, overall ability for insulin binding does not change with aging, whereas in the skeletal muscle and specifically in the heart is significantly altered in the old animals.

In vitro results of Autuori *et al.*, have shown that in hepatocytes isolated from prenatal, postnatal and adult rat there is a constant increase in the number of insulin binding sites per cell, whereas the affinity of plasma membrane receptors for hormonal ligand remains unaltered from prenatal to adult hepatocytes (21).

Similar results were obtained by Baldini *et al.*, showing that insulin binding to intact hepatocytes does not seem to be age — dependent (22). However, plasma membrane mediated intracellular responses are definitely impaired leading to a decreased age related insulin responsiveness in rat hepatocytes.

By contrast, Linnik and Gatsko have shown that an age — related decrease in ^{125}I — insulin binding to rat liver plasma membranes results from the loss of binding sites in old rats (24—26 month) whereas the affinity of insulin receptors to the hormone does not change with age (23). In addition, the mechanism of down regulation of insulin receptors in the rat liver does not work in aging (24). It has been shown that older animals (24—26 month) exhibit a less obvious

response of insulin receptor to 2 — week insulin administration. These results are in good agreement with data of Barzilai *et al.*, who has proved marked hepatic insulin resistance ensued with aging (25). Probably the differences between our results and those of Barzilai can be accunted for by differences in age of rat studied.

The study of potential age-related changes in cardiac and skeletal muscle insulin receptors, carried out recetly (26) has revealed that cardiac and skeletal muscle demonstrate different responses of age — associated alterations. Cardiac insulin receptor and the p85 alpha subunit of PI3 — kinase both decline by about 40% whereas the skeletal muscle content of these two parameters tested is unaffected by aging. Consistent with these findings are data of Reed *et al.*, showing that skeletal muscle insulin receptor binding, autophosphorylation and tyrosine kinase activity do not differ between young and mature rats (27).

However our results indicate that if we take into account both maximum binding capacity and affinity for insulin, entire ability to bind insulin in skeletal muscle of old (12 month) rats is lower as compared to the newborns but mainly in the high affinity receptor class. This phenomenon probably results from the lack of spare high affinity insulin receptors in skeletal muscle as shown by Camp *et al.* (28).

Our results are, to same extent, in good agreement with results of Johnston *et al.* (29) who have shown that specific insulin binding and the number of high-affinity insulin receptor are both increased in newborn canine heart and skeletal muscle as compared with adults.

According to our present results, the most dramatic changes in insulin binding occur in the heart during aging where both high and low affinity insulin receptors are affected.

It has been well documented that isolated cardiac myocytes posses specific receptors for insulin which consist of high- and low-affinity binding sites. According to Eckel and Reinauer low — affinity sites can mediate a biological response comparable to that of high — affinity sites (30).

The biological significance of the class of low affinity receptor sites has been questioned by Cuatrecasas (31) and Marinetti (32) who have reported that only the high affinity sites are associated with the physiological action of the hormone. The data of Eckel and Reinauer suggest a functional role of low-affinity receptors in cardiac muscle. For instance, the low-affinity insulin receptors in cardiac myocytes may be involved in the process of internalization in this tissue (33).

It has been recently shown that insulin stimulates the L-type calcium current in isolated rat ventricular myocytes in a dose — dependent and reversible manner. The L-type calcium current (I Ca L) in cardiac myocytes may serve as a possible target of insulin action in the regulation of cardiac

function and this effect is mediated by insulin receptors and cAMP-dependent protein kinase (34, 35). In view of this, our results showing that insulin binding in the cardiac muscle is greatly affected by aging may have some implications for heart function including heart rate and force of cardiac contraction.

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