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INSULIN RECEPTOR KINASE ACTIVITY IN RAT ADIPOCYTES IS DECREASED DURING AGING

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The tyrosine kinase activity of the insulin receptor derived from rat adipocyte plasma membranes was examined during aging. In the absence of insulin, autophosphorylation and histone H2B phosphorylation activities, measured with equal numbers of insulin receptors, were comparable among 3- and 24-month-old rats. In contrast, insulin-stimulated kinase activity was significantly reduced in the old animals. We have also found that the insulin dependent phosphorylation of a putative endogenous substrate of 60 kDa was drastically reduced in old animals. These results suggest that the decrease in kinase activity in old rats could be related with the insulin resistance of aging.

Impaired glucose tolerance has long been known to accompany human and rat aging (1,2). The glucose intolerance of aging seems to be the consequence of peripheral insulin resistance in target tissues, caused by an alteration of the hormone signalling mechanism at a postbinding level (3). One of the earliest postbinding events in insulin action so far recognized is the autophosphorylation in tyrosine of its own receptor at the plasma membrane (4). Although the key role of this phosphorylation in insulin action has recently received strong support (5), and a decreased kinase activity was observed in several cases of insulin resistance (6,7), little attention has been directed to the study of this receptor function during the aging process.

In the present work we have studied the insulin-dependent receptor tyrosine kinase activity of adipocyte plasma membranes derived from 3- and 24-month-old rats. The choice of this two age groups was aimed at analyzing the effect of aging on adult rats, an effect that has been reported to be associated with only minor changes of "in vivo" insulin action (8). Our results demonstrate a significant reduction in insulin-dependent glucose metabolism and receptor autophosphorylation in 24-month-old rats. In addition, we have found a decreased insulin-dependent phosphorylation of a putative endogenous substate in old rats. The possible role of other age associated

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factors, like obesity, influencing the kinase activity is also discussed. A preliminary account of these results has been presented elsewhere (9).

MATERIALS AND METHODS

Materials: Porcine insulin (Velosulin) was purchased from Nordisk. Collagenase was from Boehringer Corp. Bovine serum albumin fraction V from Armour Pharmaceutical Co. Reagents for electrophoresis and osmium tetroxide were from Serva. D-(U-^{14}C) glucose (302 Ci/mmol), (γ-^{32}P) ATP (3000Ci/mmol) and human insulin (^{125}I-TyrA14) (1775 Ci/mmol) were obtained from Amersham. Insulin radioimmunoassay kit was obtained from Oris Co. Phenylmethyl sulfonyl fluoride (PMFS), histone H2B from calf thymus type II-AS, and all other reagents were obtained from Sigma.

Isolation of fat cells, cell size determination and plasma membrane preparation: Male adult (3-months) and 24-month-old Wistar rats fed "ad libitum" on a standard laboratory diet and water were used throughout this study. Adipocytes were prepared according to Rodbell (10) in Krebs-Ringer-Bicarbonate buffer, pH 7.4, containing 3% bovine serum albumin. After staining with osmium tetroxide (11), fat cell diameter was determined using a calibrated scanning cytophotometer 03 Zeiss, and the mean fat cell lipid content was determined from previously derived formulas (12). Adipocyte plasma membranes were purified as previously described by Massagué and Czech (13). These membranes were stored as a pellet at -70°C until they were used.

Preparation of detergent-solubilized plasma membranes and insulin binding assay: Adipocyte plasma membrane proteins were solubilized in 50 mM Tris-HCl buffer, pH 7.4, containing 1% Triton X-100 and 0.5 mM PMFS for 60 min at 4°C. Insoluble material was removed by centrifugation at 100,000 x g for 60 min. The resulting supernatant was used in receptor binding and phosphorylation experiments. 40 μg of solubilized insulin receptor were incubated at 4°C for 16 h with (^{125}I-TyrA14) insulin (20,000 cpm, 0.2 ng/ml), in a final volume of 0.5 ml of a medium containing 25 mM Tris-HCl, pH 7.4, 0.2% BSA, 0.1% Triton X-100 in the absence and presence of excess unlabeled porcine insulin (32μg/ml). Separation of the free and receptor bound insulin was achieved by precipitation with 10% polyethylene glycol/0.5 mg/ml γ-globulin and subsequent filtration in GF/C filters.

Phosphorylation assays: In all experiments equal numbers of receptors from 3-months- and 24-month-old rats, determined by binding assays, were used. Solubilized insulin receptors were preincubated for 10 min at room temperature in the absence or presence of 10^{-7} M insulin. The phosphorylation reaction was carried out in the presence of 12 mM $MgCl_2$, 4 mM $MnCl_2$, 25 mM Tris-HCl pH 7.4, 1 mM orthovanadate, 40 μM ATP/2μCi (γ-^{32}P) ATP. After 2 min incubation the reaction was terminated by the addition of electrophoresis sample buffer and heating to 95°C for 3 min, and samples were subjected to 7.5% SDS-PAGE under reducing conditions according to the method of Laemmli (14).

Exogenous substrate phosphorylations were conducted with solubilized insulin receptors preincubated in the phosphorylation buffer mentioned above in the presence or absence of 10^{-7} M insulin and 50 μM cold ATP for 10 min at room temperature. The phosphorylation reaction was initiated by the addition of the histone H2B (1mg/ml) final concentration and 3 μCi (γ-^{32}P) ATP, and continued for additional 10 min. The reaction was termined and histone H2B phosphorylation was analyzed by 10% SDS-PAGE as above. Radioactive proteins were identified by autoradiography and ^{32}P-incorporation was quantitated by densitometric analysis. Phosphoamino acid analysis of the receptor β subunit was determined by a described method (15). Treatment of gels with 1 N KOH was done as previously described (16).

Other methods: Plasma insulin levels were measured by the method of Desbuquois and Aubarch (17). Plasma glucose levels were determined by the glucose oxidase method (18). Production of (^{14}C)-CO_2 from D-(U-^{14}C) glucose was determined as described by Fain et al (19). Protein was determined by Bradford's method (20)

using BSA as standard. The reported values are the mean±SEM. Statistical comparisons were carried out using unpaired Student's t-test.

RESULTS

Table I summarizes the characteristics of the two groups of rats used in this study. Body and fat tissue weight increase markedly as rats age. This could be due partly to the enlargement of the fat cells, and also to the existence of fat cell hyperplasia, reported to occur in adult rats (21). Although there was some loss of adipose cells from old rats during incubation due to their increased susceptibility to collagenase digestion, we did not find any significant enlargement of the cells used in the experiments, since cell size (Table I) and lipid content (0.49±0.02 and 0.58±0.06 μg lipid/cell in 3- and 24-month-old rats respectively) remain constant throught the life span studied. These results suggest that fat cell proliferation which has been observed to occur even at old age (22) could be the cause of the fat weight increment in old animals. The insulin stimulation of glucose oxidation by fat cells was significantly reduced in old animals. Plasma insulin and glucose concentrations, reported to be elevated in isulin resistant genetically obese animals (23), were maintained or slightly reduced in old rats (Table I).

Since, the Bmax and K_D of insulin to its receptor in plasma membranes did not change with aging (data not shown), this study was focused on the kinase activity of the insulin receptor. The phosphorylation of plasma membranes derived from fat cells is shown in Fig. 1. It may be observed that among the various phosphorylated proteins, there is a 95 kDa band previously characterized as the β subunit of the insulin receptor (9), whose phosphorylation is stimulated by insulin (Fig. 1A). The insulin effect on the phosphorylation of the 95 kDa band is markedly reduced in 24-month-old animals. Since the autoradiograms correspond to gels loaded with equivalent amounts of insulin receptors (as determined by insulin binding activity), the reduction in ^{32}P incorporation into the 95 kDa band reflects a reduced kinase activity of the

Table 1. Metabolic characteristics of the two groups of rats

			Plasma			$^{14}CO_2$ production
Age (mo)	Rat Wt (g)	Fat Wt (g)	glucose (mM)	insulin (μU/ml)	Fat cell Ø (μm)	% insulin stimulation
3 (6-15)	381±5	5.9±0.5	7.96±0.5	37.4±3.2	94.5±2	149±4
24 (6-12)	678±23	28.9±2.2	7.44±0.5	30.7±3.6	101.5±4	118±6
	p<0.0005	p<0.0005	NS	NS	NS	p<0.005

Values are means±SEM; no. of rats given in parenthesis.

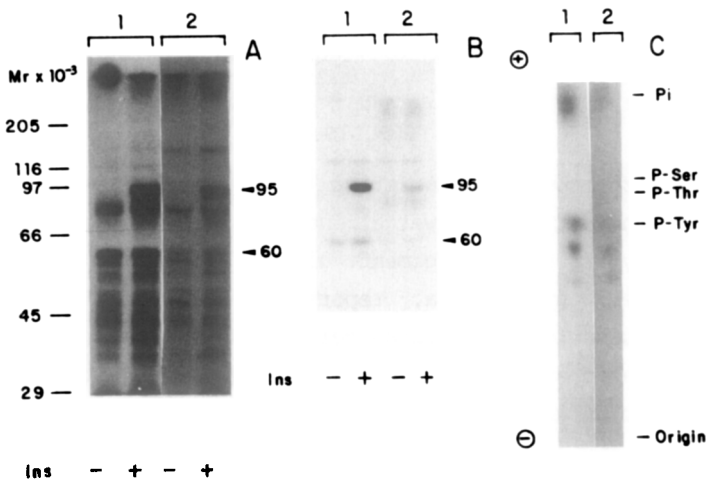

Figure 1: Effect of insulin on the phosphorylation solubilized plasma membrane proteins of fat cells from 3- (1) and 24-month-old rats (2). Autoradiogram shows the ^{32}P-incorporation in the absence or presence of 10^{-7}M insulin before (Fig. 1A) and after KOH treatment of the gels (Fig. 1B), and phosphoamino acid analysis of the β-subunit of insulin receptors (Fig. 1C). Results are representative of a single experiment, reproduced three times.

insulin receptors. The treatment of the gels with KOH to eliminate serine- and threonine- bound phosphate, shows that the 95 kDa band is the major phosphotyrosine-containing protein in adipocyte plasma membranes, and the differences in insulin-dependent ^{32}P incorporation between receptors from 3- and

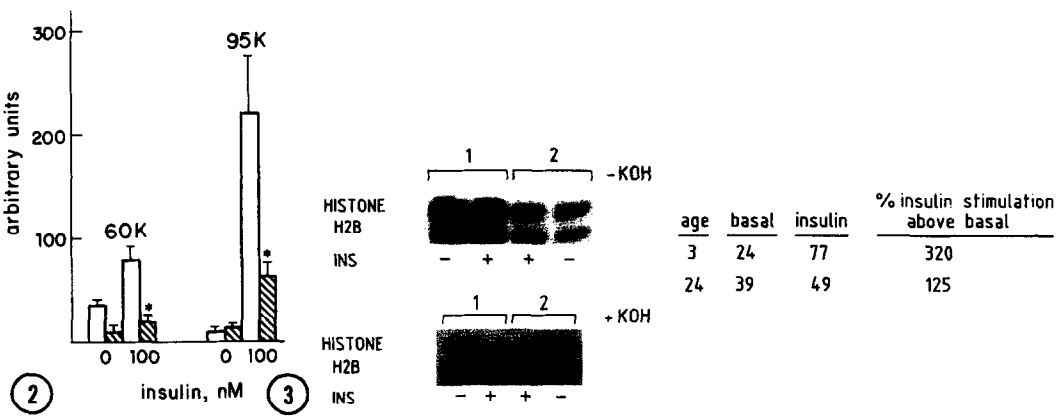

Figure 2: Effect of insulin on th ^{32}-P-incorporation into the 60 kDa and the 95 kDa bands after KOH treatment of the gels. Open and hatched bars correspond to 3- and 24-month-old rats respectively. The intensity of both proteins was measured by scanning densitometry and is expressed in arbitary units. the bars show mean values±SEM of three separate experiments, *p<0.05.

Figure 3: Effect of insulin on the H2B phosphorylation.
Left: Autoradiogram showing the ^{32}P-incorporation into histone H2B in the absence or presence of 10^{-7}M insulin before (upper) and after (lower) KOH treatment of the gels. Figure shows the results of a single representative experiment, reproduced three times.
Right: Quantitation by scanning densitometry of the histone H2B phosphorylation in the absence or presence of 10^{-7}M insulin after KOH treatment of the gels. The results are means of three separated experiment expressed in arbitrary units.

24-month-old rats are maintained after alkaline treatment (Fig. 1B). The phosphoamino acid analysis of the 95 kDa phosphoprotein extracted from the gels (before KOH treatment) is shown in Fig. 1C and confirms that tyrosines are the only amino acid residues that become phosphorylated after incubation with insulin. Fig. 1C also shows that the phosphotyrosine content of the 95 kDa band is markedly lower in 24- than in 3-month-old rats.

We would like to point out that, in addition to stimulating the phosphorylation of the insulin receptor, the hormone also enhances the alkali resistant ^{32}P incorporation into a protein of Mr 60,000 (Fig. 1B). This protein could be related with the 60 kDa band recently reported by Momomura et al, (24) in rat adipocytes. Interestingly, insulin dependent phosphorylation of the 60 kDa band was also drastically reduced in old animals (Fig. 1B). Quantitation of ^{32}P incorporation into both the 95 kDa and 60 kDa bands demonstrates a significant decrease in insulinstimulated phosphorylation of both proteins in old rats (Fig. 2). The decreased kinase activity of the insulin receptor in 24- as compared to 3-month-old animals was also tested using exogenous substrates. Figure 3 shows that, under maximally stimulating insulin concentrations the phosphorylation of histone H2B by receptor preparations from old animals was also drastically reduced.

DISCUSSION

The development of insulin resistance during growth in rats seems to take place in two periods. The greatest decline in insulin action is achieved during adolescence and is associated with marked increases in body weight, and adipose cell size, whereas during the adult life insulin action is unaffected or little diminished (8). A major question is studies on aging is to choose the optimal ages to be compared. Nariyama et al. (8) found little differences in insulin action on glucose disposal in rats (Sprague-Dawley strain) as they grow from 4- to 12-months of age. Craig et al. (25) compared 12- and 24-month-old rats (Long-Evans strain) which were similar in body weight, fat content and fat cell volume, and did not find any difference in insulin action on glucose uptake. In the Wistar rats used throughout this study none of the parameters characteristic of obesity was found, except an increase in body and fat weight. As shown in table I, plasma glucose and insulin concentrations did not increase as rats grow from 3- to 24-months of age. Moreover, the adipose cell size as well as its lipid content appears to be similar in both groups of rats suggesting an increment in fat cell number rather than a modification of the adipocyte itself. However, the insulin effect on $^{14}CO_2$ production from (U-^{14}C-glucose) was significantly reduced in old rats. This impairement of insulin action has also been observed in old compared to adult rats if they were under food restriction and mild exercise (unpublished observations).

The data presented herein provide evidence that the insulin receptor tyrosine kinase activity in adipocyte plasma membranes is significantly

reduced in response to insulin in old (24-months) as compared to adult (3-months) rats. Since it has been suggested that cellular signalling by insulin may be transmitted via tyrosine phosphorylation of several endogenous substrates by the intrinsic tyrosine kinase activity of the receptor (26), the decreased alkali resistant ^{32}P incorporation observed for the 60 kDa protein in 24-month-old rats could be related with the insulin resistance in aged rats. Moreover, the diminished autophosphorylation rate of the receptor β subunit is associated with a decreased kinase activity of the receptor towards exogenous substrates. While the molecular basis of the kinase activity defect in old rats is yet unknown, it is possible that modification by other protein kinases may be involved. The insulin receptor β subunit, undergoes phosphorylation "in vivo" and "in vitro" (27,28) by cAMP-dependent kinase leading to an attenuation of insulin stimulation of the receptor kinase activity. Since old rats have been shown to be hyperglucagonemic (29), it can be speculated that higher intracellular activity of PK-A could lead to the inhibition of the insulin receptor kinase activity.

In conclusion, the results in the present study show that during rat aging, insulin action on glucose metabolism in adipocytes decreases, confirming previously reported results in human aging (30), and this is paralleled by a significant reduction of insulin stimulation of receptor autophosphorylation, a feature common to several cases of insulin resistance. It is concievable that this same process may occur in other peripheral tissues, and therefore, give rise to the overall insulin resistance of old animals. The alteration in kinase activity appears to be the consequence of the aging process itself and not of the enlargement of fat cells and increased plasma glucose and insulin concentrations commonly associated with obesity. However, a contribution of the increment in body weight and fat content cannot be ruled out. It should be pointed out, however, that while experimentally induced obesity in mice causes a decrease in the muscle insulin receptor autophosphorylation (31), the spontaneous obesity per se, at least in humans, has not influence on the receptor kinase activity (32).

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