DEFECT IN LONG-TERM ACTIVATION OF PHOSPHATIDYLINOSITOL 3-KINASE BY INSULIN *IN VIVO*: STUDIES IN INSULIN-RESISTANT HHTG RATS

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Objective. To study the regulation of phosphatidylinositol (PI) 3-kinase by insulin *in vivo* in hereditary hypertriglyceridemic and insulin resistant rat (hHTg).

Methods. Total and insulin receptor substrate-1 (IRS-1) associated PI 3-kinase activities were measured in skeletal muscles and adipose tissue after an intense insulin induced glucose utilization as accomplished by 90 min euglycemic hyperinsulinemic clamp.

Results. In quadriceps femoris muscle, no stimulation of total or IRS-1 associated PI 3-kinase activities was found after hyperinsulinemia in both hHTg and control rats. In contrast, in soleus muscle of control rats total PI 3-kinase activity was stimulated by insulin ($P<0.001$), while any such effect was not found in hHTg rats. IRS-1 associated PI 3-kinase activity in soleus muscle was significantly decreased in hHTg rats when compared to control rats ($P<0.001$), but was not affected by insulin. In white adipose tissue (WAT), both the total ($P<0.05$) and IRS-1 associated PI 3-kinase activities ($P<0.001$) were increased after 90 min hyperinsulinemia in control animals but not in hHTg animals.

Conclusions. Long-term activation of PI 3-kinase activity by insulin *in vivo* involves IRS-1 in white adipose tissue, but not in skeletal muscle which implies tissue specificity. The impairment in the PI 3-kinase activation by insulin in hHTg rats may participate in insulin resistance of these animals.

Key words: Insulin – IRS-1 – PI 3-kinase – Insulin resistance – hHTg rat

Insulin induces a variety of physiological responses including the stimulation of glucose transport in muscle and adipose tissue, which is mainly due to the translocation of the GLUT4 glucose transporter from intracellular vesicles to the plasma membrane (Klip and Paquet 1990). Various effects of insulin are initiated by the binding of insulin to its cognate receptor and subsequent activation of the insulin receptor tyrosine kinase resulting in tyrosine phosphorylation of intracellular substrate proteins. The latter may operate as a messenger for various biological effects of insulin (Kahn 1994).

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The early postreceptor events leading to the activation of glucose transport are not yet fully characterized. The *in vitro* data, mainly obtained in various cell culture systems have accumulated substantial evidence suggesting that the stimulatory effect of insulin on glucose transport requires the activation of phosphatidylinositol 3-kinase (Kanai et al. 1993; Quon et al. 1994). This induces the association of 85 kDa regulatory subunit of phosphatidylinositol 3-kinase with the Tyr-phosphorylated form of insulin receptor substrate-1 (IRS-1) (Kahn 1994; Kanai et al. 1993; Quon et al. 1994; Folli et al. 1992).

Insulin induced activation of PI 3-kinase has been confirmed in animal experiments as well. Thus, the

insulin bolus administered into the portal circulation of anesthetized animals induced a rapid and short lasting (up to 10 min) stimulation of PI 3-kinase activity in intact rats (FOLLI et al. 1992; KELLY et al. 1991; FOLLI et al. 1993; SAAD et al. 1993). However, the long-term activation of the PI 3-kinase by hyperinsulinemia at euglycemia has not been studied so far. We have examined the activation status under the conditions of a hyperinsulinemic euglycemic clamp in control and insulin resistant rats. This approach enables the concomitant quantitation of glucose uptake in relation to the PI 3-kinase activation state.

The animal model of insulin resistance used in this study is the hereditary hypertriglyceridemic (hHTg) rat. These animals are characterized by insulin resistance in white adipose tissue and muscle (KLIMES et al. 1994) as supported by decreased glucose uptake in the white adipose tissue and in skeletal muscles (STOLBA et al. 1993) and 50 percent decrease of GLUT4 protein levels in skeletal muscle (SEBOKOVA et al. 1995). To evaluate the possible involvement of phosphatidylinositol (PI) 3-kinase in insulin resistance of these animals, the regulation of PI 3-kinase was studied in skeletal muscles and white adipose tissue from control and hHTg rats.

Materials and Methods

Animals. Adult male hereditary hypertriglyceridemic rats from the colony bred at this Institute (250-300 g) were housed in temperature (22± 2 °C) and light (12 h light:dark cycle; lights off at 18.00 h) controlled room and allowed access to standard laboratory chow and water ad libitum. Control normotriglyceridemic (NTg) Wistar rats (VELAZ, Prague, Czech Republic) were housed under the same conditions.

Euglycemic hyperinsulinemic clamp. The rats were anesthetized by injection of xylasine hydrochloride (10 mg/kg) plus ketamine hydrochloride (75 mg/kg) and fitted with chronic tubings inserted into carotid artery and jugular vein and placed in metabolic cages equipped with special device to keep the tubings in vertical position. Three days later, after overnight fast (16-18 h) the conscious and freely moving animals were subjected to 90 min euglycemic hyperinsulinemic clamp (EHC, 6.4 $mU.kg^{-1}.min^{-1}$) according to KOOPMANS et al. (1991). After completion of the clamp the rats were sacrificed. The soleus and quadriceps muscles and white epididymal adipose tissue were immediately removed and frozen until assayed for PI 3-kinase activity.

Biochemical analyses. The plasma was obtained from blood sampled before, during and after the clamp and used for the estimation of insulin (RIA kit from Novo Nordisk, Copenhagen, Denmark) and glucose (Beckman analyzer, Fullerton, CA).

Polyclonal antisera against the 85 kDa subunit of PI 3-kinase and anti IRS-1 were generated by immunizing rabbits with particular recombinant proteins, as described in details by OUWENS et al. (1994).

For immunoprecipitation and measurement of the PI 3-kinase activity tissue homogenate (10 wt %) in lysis buffer (30 mM TRIS pH=7.5, 150 mM NaCl, 2 mM EDTA, 5 mM $MgCl_2$, 10 % glycerol, 0.5 mM sodium orthovanadate, 5 mM sodium fluoride, 0.1 mM PMSF, 1 µg/ml aprotinin and 1 µg/ml leupeptin) containing 0.5 % NP-40, was cleared by centrifugation for 10 min at 14,000 x g, at 4 °C, and incubated for 4 hrs at 4 °C with two different antibodies coupled to protein A Sepharose beads: either with the polyclonal anti P85 antiserum (5 µl) to examine the total PI 3-kinase activity or with the IRS-1 antiserum (5 µl) to measure the IRS-1 associated PI 3-kinase activity. Immunoprecipitates were washed twice with lysis buffer, containing 0.1 % NP 40 and twice with 30 M HEPES (pH 7.5). After that PI 3-kinase activity was determined by incorporation of ^{32}P labeled gamma ATP into phosphatidylinositol exactly as described by BURGERING et al. (1994). Incorporated ^{32}P was quantitated using a Phosphor Bio Imager System (Molecular Dynamics, USA). The intensity of the spots represents relative values for the PI 3-kinase activity. It is expressed as percentage where the PI 3-kinase activity in individual tissues of control (NTg) rats before the clamp commencement was considered as 100 %.

Statistical evaluation. Results are presented as mean± S.E. Statistical evaluation was carried out by the analysis of variance (ANOVA) followed by an appropriate *post hoc* test. Differences were considered statistically significant at $P<0.05$.

Results

Euglycemic hyperinsulinemic clamp. Plasma glucose levels before and during the clamp did not differ significantly between the hHTg and control rats (Tab. 1). Insulin values during the clamp were approximately 9-fold higher than basal fasting insulin levels (Tab. 1). The glucose infusion rate (GIR; an index of insulin sensitivity), which reflects the whole body insulin action on glucose uptake, was significantly decreased in hHTg rats in comparison to controls (Tab.1).

Effect of hyperinsulinemia on total and IRS-1 associated PI 3-kinase activity. Ninety minutes of euglycemic hyperinsulinemia stimulated the total PI 3-kinase activity in the soleus muscle and in the white adipose tissue of control rats. In contrast, hyperinsulinemia failed to activate the total PI 3-kinase activity in quadriceps femoris muscle of these animals. Similarly, no change of total PI 3-kinase activity was seen in response to euglycemic hyperinsulinemia in insulin resistant hHTg rats (Fig. 1a). There was also no difference in total PI 3-kinase activity between hHTg and control rats in all the tissues investigated.

IRS-1 associated PI 3-kinase activity was increased by euglycemic hyperinsulinemia only in the white adipose tissue of control rats (Fig. 1b). No effect of hyperinsulinemia on the activation PI 3-kinase bound to IRS-1 was observed in quadriceps femoris and soleus muscles of control animals. Hyperinsulinemia failed to activate the IRS-1 associated PI 3-kinase activity in any of the tissues obtained from insulin resistant hHTg animals. There was no difference in IRS-1 associated PI 3-kinase activity between control and hereditary hHTg rats under basal conditions, except the soleus muscle (i.e. after overnight fast) (Fig. 1b).

T a b l e 1

Plasma glucose and insulin levels before (" - Insulin") and after an euglycemic hyperinsulinemic clamp (" + Insulin"), and glucose infusion rate (GIR) in hereditary hypertriglyceridemic (hHTg) and control (NTg) Wistar rats

Strain	NTg		hHTg	
	- Insulin	+ Insulin	- Insulin	+ Insulin
Glucose [mmol.l^{-1}]	6.1±0.2^{a}	5.9±0.2^{a}	6.8±0.6^{a}	5.9±0.2^{a}
Insulin [μU.ml^{-1}]	35.8±12.5^{a}	296.9±15.4^{b}	40.7±4.8^{a}	319.2±34.5^{b}
GIR [mg.kg^{-1}.min^{-1}]	27.2±0.2^{a}		23.0±0.3^{b}	

Data are expressed as mean ± SEM, n=8. Values without a common superscript $^{(a,b)}$ within a row are significantly different $p<0.05$)

Discussion

Based on studies in cultured cells, insulin has been shown to activate the phosphatidylinositol 3-kinase (Kanai et al. 1993; Quon et al. 1994). Besides, the inhibition of PI 3-kinase by wortmannin and Ly 294002 abolished the stimulation of hexose uptake by insulin in 3T3-L1 adipocytes (Cheatham et al. 1994). Insulin has been also shown to stimulate the activity of this enzyme under *in vivo* conditions after a bolus injection to anesthetized rats (Folli et al. 1992). However, it is not known to what extent the activation status of this enzyme is related to the rate of glucose disposal *in vivo*. It is possible that changes in peripheral insulin action leading to a state of insulin resistance are reflected in some situations by the activity of PI 3-kinase.

In the present study we have been able to demonstrate that PI 3-kinase in the soleus muscle and in white adipose tissue undergoes insulin stimulation after 90 min of hyperinsulinemia at euglycemia. Surprisingly, no stimulation of PI 3-kinase activity was found in the quadriceps femoris muscle. Soleus muscle differs from quadriceps femoris muscle in fibre type composition, and the latter was shown to be related to insulin sensitivity (James et al. 1985). In fact, the insulin stimulated glucose transport is higher in red, more oxidative fibers (soleus muscle) than in the white, more glycolytic fibers (quadriceps femoris muscle) (Handberg et al. 1993).

Furthermore, after insulin stimulation a significant fraction of PI 3-kinase activity was associated with IRS-1 as determined by immunoprecipitation with antibodies directed against IRS-1. This phenomenon was observed only in white adipose tissue, but not in skeletal muscle of control rats. These results may suggest that the long-term activation of PI 3-kinase by insulin *in vivo* may involve the IRS-1 in white adipose tissue, while in skeletal muscle related proteins such as IRS-2 might be involved.

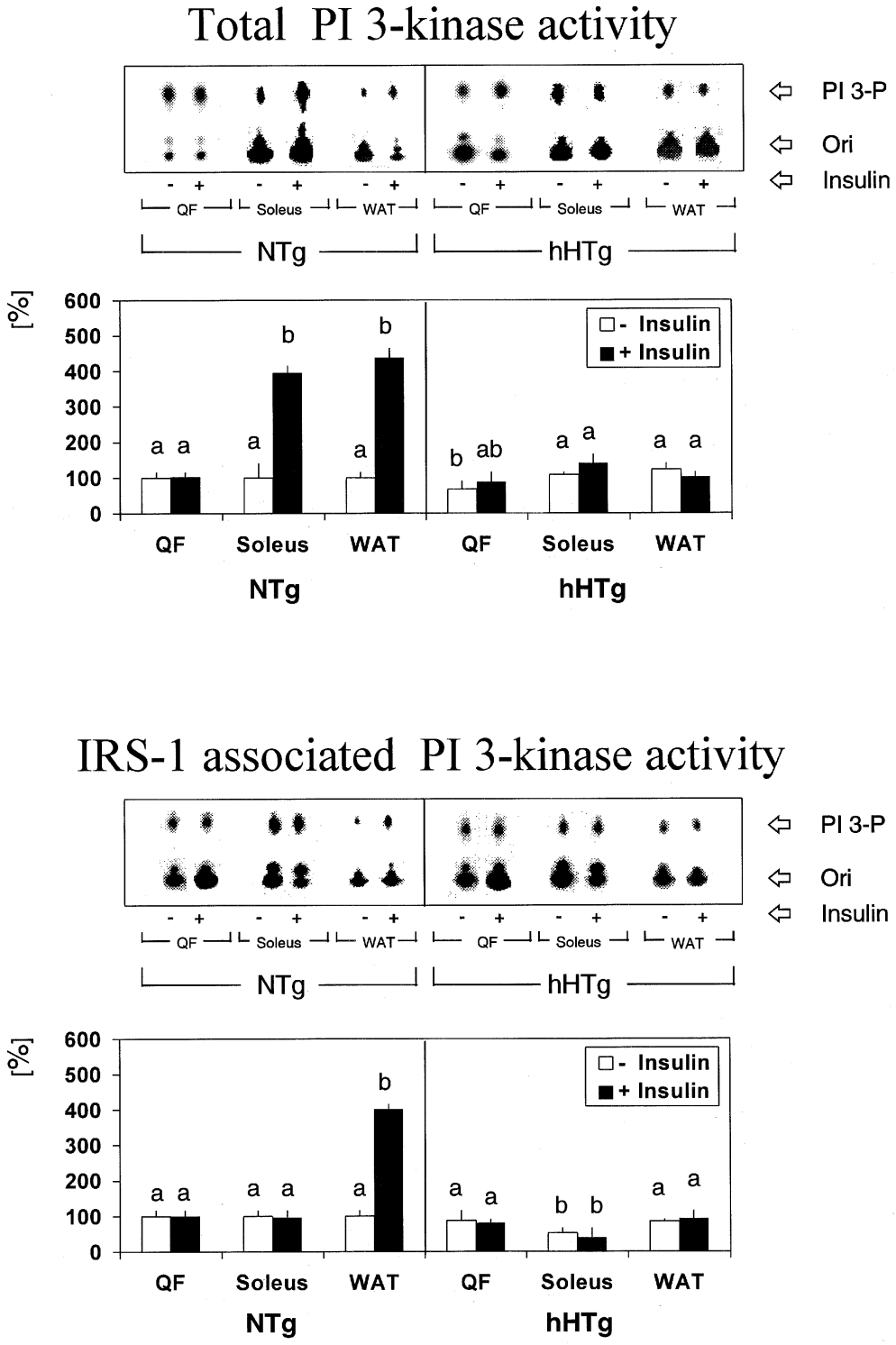

Fig. 1 Total (upper panel) and IRS-1 associated activity of PI 3-kinase (lower panel) in the soleus, quadriceps femoris muscle and white adipose tissue of control and hHTg rats after 90 min of hyperinsulinemia at euglycemia (symbolized as " + Insulin"). Data are presented in percentage as mean ± SEM, n=4, where the PI 3-kinase activity for individual tissue of control rats before the clamp commencement was considered as 100 %. Values without a common superscript [(a,b)] are significantly different (P<0.05). NTg = control normotriglyceridemic rats; hHTg = hereditary hypertriglyceridemic rats; QF = quadriceps femoris muscle; WAT = white adipose tissue.

Folli et al. (1992) demonstrated a rapid stimulation (within minutes) of PI 3-kinase activity and its association with IRS-1 in hindlimb muscle of anesthetized rats after the bolus injection of insulin into the portal circulation. Moreover, in isolated rat adipocytes insulin at physiological concentrations has been shown to stimulate the PI 3-kinase for few minutes only (Kelly et al. 1991). In view of the observations that IRS-1 and IRS-2 phosphorylations are transient, the situation may arise that under clamp conditions, IRS-1 and IRS-2 may in various tissues activate the PI 3-kinase differentially.

The defect in the ability of insulin to stimulate glucose utilization by skeletal muscle and adipose tissue (Klimes et al. 1994) was demonstrated in the hereditary hypertriglyceridemic and insulin resistant rat. Our data on decreased glucose infusion rate reconfirmed our previous observations on a defect in insulin action in these animals (Stolba et al. 1993) which has been thought to result from postbinding steps in the insulin action pathway.

Major objective of this study was to explore the effect of insulin in hHTg rats on the activation of PI 3-kinase through the IRS-1, the main pathway in insulin signaling. We have presented evidence that the activation of PI 3-kinase by insulin is impaired in skeletal muscle and adipose tissue of hHTg rats in comparison to control animals. Our data are in harmony with several recent studies showing a defect in the stimulation of PI 3-kinase in muscle of various animal models of insulin resistance (Folli et al. 1993; Saad et al. 1993). Our results, however, expand the recent knowledge by reporting on a failure of insulin to activate the PI 3-kinase/IRS-1 system under *in vivo* conditions.

Thus, it seems likely that the stimulation of PI 3-kinase may play a significant role in mediating some of the metabolic effects of insulin *in vivo.* The derangements in this insulin signalling pathway may help to explain some of the post receptor signalling defects related to the insulin resistance of hereditary hypertriglyceridemic rat.

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