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Characterization of the hepatic insulin receptor undergoing internalization through clathrin-coated vesicles and endosomes

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Administration of insulin to rats caused a transient increase in the amount of hepatic insulin receptor present in clathrin-coated vesicles and endosomes. However, the total 'in vitro' insulin stimulated tyrosine kinase activity of the receptor present in endosomes did not vary when expressed per mg of protein and decreased when expressed per β-subunit content. A decrease in the endogenous phospho tyrosine content of the receptor β-subunit was observed in endosomes in response to insulin. This indicates that a fraction of the internalized receptor is dephosphorylated in endosomes, which renders it unable to become stimulated by insulin 'in vitro'.

Insulin receptor; Internalization; Clathrin-coated vesicle; Endosome; Protein phosphorylation; Rat liver

1. INTRODUCTION

Insulin administration to rats results in the accumulation of activated hepatic insulin receptors within endosomes [1,2]. Internalization starts with a clustering of the occupied insulin receptors in clathrin-coated domains of the plasma membrane, followed by invagination of the coated pits that leads to the formation of clathrin-coated vesicles that migrate to the endosomes. It is within this compartment where the sorting and degradation of the insulin receptor takes place [3]. The internalization is associated with a dephosphorylation of the β-subunit tyrosine residues and activation of receptor tyrosine kinase activity, a fact that has been characterized with the receptor present in endosomes [4]. Although clathrin-coated vesicles have been shown to participate in the pathway of receptor-mediated endocytosis of insulin [5]; there are no data available on the activity changes of the receptor present in these vesicles.

In the present work, we studied the impact of 'in vivo' insulin administration on the insulin receptor at different stages of internalization corresponding to total membranes, clathrin-coated vesicles and endosomes.

2. EXPERIMENTAL

2.1. *Materials*

Insulin was from Eli Lilly. The α-PY antibody was from ICN Biochemicals (PY 20) and the α-IR antibody was from Oncogene Science (Ab-3).

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2.2. *Animals*

Female Sprague–Dawley rats (5 weeks old) were kept under light anaesthesia with ether and insulin (2 μg/100 g body weight) was injected into the tail vein in 0.4 ml of phosphate-buffered saline. Animals were killed by decapitation at noted times after insulin injection (0–5 min).

2.3. *Subcellular fractions*

Total membranes were prepared as described previously [6]. The clathrin-coated vesicles were purified by differential centrifugation [5]. The vesicles were characterized by electron microscopy and by the protein pattern obtained by SDS-PAGE. The endosomes were prepared as described by Khan et al. [2].

2.4. *Purification of insulin receptor*

The proteins from total membranes, clathrin-coated vesicles or endosomes were extracted by solubilization with 2% Triton X-100 containing proteases and phosphatases inhibitors [4] and subjected to chromatography on wheat-germ lectin-Sepharose (WGA-Sepharose). The retained proteins were eluted by washing the column with 0.3 M *N*-acetyl-D-glucosamine as in [6].

2.5. *Tyrosine kinase activity (IRTK activity)*

IRTK activity was assayed using 1 mg/ml of poly (Glu/Tyr) (4:1) as a substrate and 65 μM [γ-^{32}P]ATP either in the absence or in the presence of 75 nM insulin.

2.6. *Immunological analysis*

The β-subunit content was assayed by Western blot using a polyclonal antibody (α-CT antibody) raised in rabbits against a peptide corresponding to the C-terminal region (residues 1341–1357) of rat insulin receptor [7] and it was a gift from Stalmans and colleagues (Leuven, Belgium). The endogenous phospho tyrosine was determined by immunoprecipitation of freshly prepared insulin receptor (100 μg of WGA-eluted protein) with a mouse monoclonal antibody generated against an internal epitope of human and rodent insulin receptor (α-IR) followed by a Western blot using a mouse monoclonal anti-phospho tyrosine antibody (α-PY). The scanning of the autoradiograms to determine the β-subunit and phospho tyrosine content of insulin receptor was done at immunoblot intensities that fell within the linear range of a standard curve.

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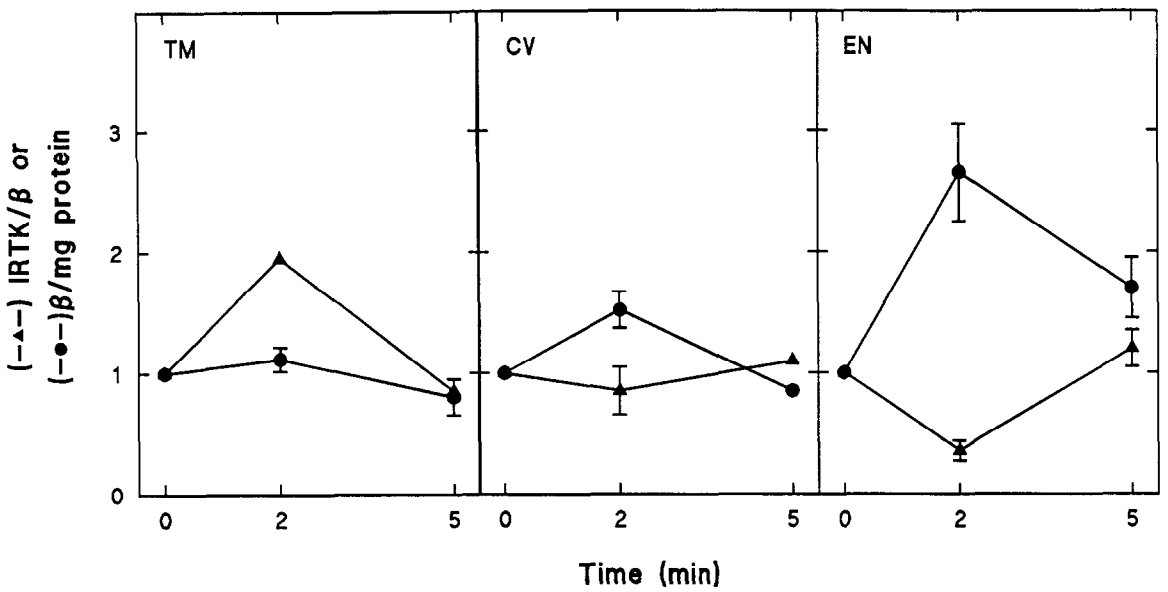

Fig. 1. Time course of change in insulin receptor content and exogenous tyrosine kinase activity following insulin injection. Insulin receptor from total membranes (TM), clathrin-coated vesicles (CV) or endosomes (EN) was prepared from control or insulin-injected rats at the indicated times. The insulin receptor were partially purified by WGA-Sepharose chromatography and their β-subunit content was measured by Western blot using an antibody raised against the C-terminal region of the β-subunit. The protein was determined by the Bradford method. Total IRTK-activity was assayed in the presence of 75 nM insulin using 1 mg/ml poly (Glu/Tyr) (4:1) and 65 μM [γ-^{32}P]ATP. The figure shows the ratio of β-subunit/mg protein (●) and of IRTK-activity/β-subunit (▲). The results are the means ± S.E.M. from two or three separate experiments, with each point determined in triplicate. All data are referred to control rats, to which a value of 1 was given.

3. RESULTS AND DISCUSSION

Administration of insulin to rats resulted in transient increases in the insulin receptor present in coated vesicles and endosomes as assessed by the presence of its β-subunit (Fig. 1). The magnitude and time course of the changes in β-subunit content in endosomes are in good agreement with those detected by Khan et al. [2] who studied the specific insulin binding to endosomes isolated from rat livers at different times after insulin injection. Increases of 3- to 4-fold in the internalization of insulin receptor have also been described to occur in Fao hepatoma cells in response to insulin [8]. The increase in clathrin-coated vesicles was lower than that observed in endosomes. This would be in agreement with a role of these vesicles as intermediates in the pathway of internalization of the hormone–receptor complexes that would give rise to endosomes [3,5]. Our results are similar to those obtained by Pilch et al. [5] that showed that the time course of insulin uptake into the coated vesicles from rat liver presented a maximum 3 min after insulin injection.

The insulin stimulation of the receptor content is accompanied with an increase in the total tyrosine kinase specific activity of the receptor present in total rat liver membranes (as expressed relative to the β-subunit content). In contrast, this parameter did not essentially vary for the receptor present in clathrin-coated vesicles and showed a decrease for the receptor present in endosomes at 2 min after insulin administration; a fact that was reversed at 5 min post-injection. However, when the tyrosine kinase activity was expressed per mg of protein (Fig. 2A) no significant changes in tyrosine kinase activity was detected in clathrin-coated vesicles or endosomes from injected rats respect to controls. In contrast, a clear increase in tyrosine activity was still detected in total membranes. These data suggested that a large proportion of the receptor present in endosomes after insulin administration was in a form that could not be activated by insulin 'in vitro'.

It is widely accepted that tyrosine phosphorylation enhances the insulin receptor tyrosine kinase activity towards exogenous substrates [9]. In order to study if the changes in the tyrosine kinase activity were due to alterations in the phosphorylation state of the receptor, the content of phospho tyrosine in its β-subunit was determined. As shown in Fig. 2B, the receptor present in total membranes after 2 min of insulin-injection to rats showed a phospho tyrosine content per β-subunit about 7.5-fold higher than that of control rats. In contrast, the phospho tyrosine content in the receptor from clathrin-coated vesicles did not significantly vary whereas that of the receptor present in endosomes decreased about 2.5-fold with respect to controls. Dephosphorylation of internalized insulin receptor has been detected during recycling in Fao cells [8]. More recently, dephosphorylation of the hepatic receptor present in endosomes has been detected in rats after

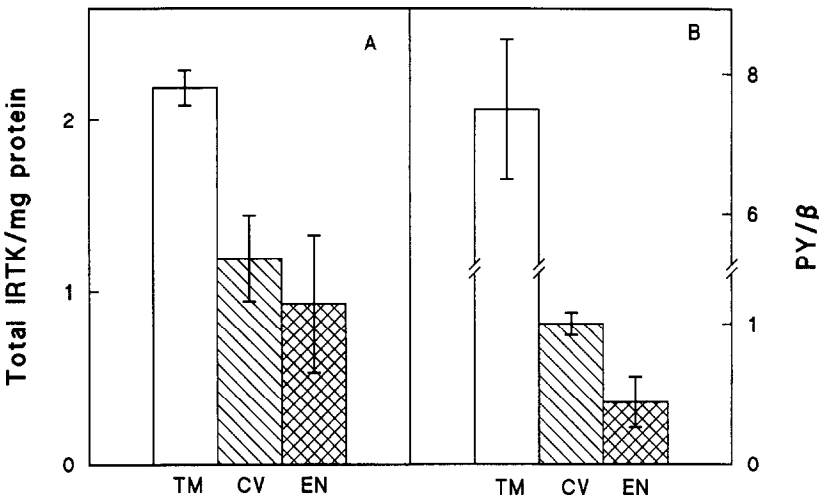

Fig. 2. Relative changes in β-subunit phospho tyrosine content and exogenous tyrosine kinase activity of the insulin receptor 2 min after insulin injection. Insulin receptor from total membranes (TM), clathrin-coated vesicles (CV) or endosomes (EN) was prepared from control or insulin injected rats at 2 min. In all cases, the data shown correspond to the ratios between the values detected in insulin injected rats and control rats. (A) The tyrosine kinase activity was measured in the presence of 75 nm insulin as described in the legend of Fig. 1. (B) The endogenous phospho tyrosine (PY) content was determined by immunoprecipitation of the insulin receptor by using the α-IR antibody, followed by Western-blot using the α-PY antibody, as described in the Experimental section. Each bar is the means ± S.D. of three separate experiments.

administration of insulin, but this fact has been correlated with activation rather than inactivation of the tyrosine kinase of the receptor [4]. In contrast, deactivation of internalized insulin receptors probably by dephosphorylation has been observed in rat adipocytes [10]. Our present data on the concomitant decrease in tyrosine kinase activity and in phospho tyrosine content in endosomal insulin receptors from injected rats supports that the dephosphorylation is associated with the inactivation of the receptor.

Summing up, the data show that the hepatic insulin receptor internalized in response to insulin is dephosphorylated as it proceeds through the endocytotic pathway with a concomitant loss of its activity and ability to become stimulated by insulin 'in vitro'.

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