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CHARACTERIZATION OF THE GUINEA PIG ADIPOCYTE THYROTROPIN RECEPTOR*

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SUMMARY ^{125}I-TSH binding to porcine thyroid and guinea pig fat resulted in curvilinear Scatchard plots with similar dissociation constants for the high and low affinity binding components. Antibodies from the sera of patients with Graves' disease inhibited binding to the high and low affinity binding components of both tissues. Covalent cross-linking of ^{125}I-TSH to membranes from each tissue resulted in the specific labeling of two protein bands. The guinea pig fat receptor subunits have M_r values of 52,000 and 38,000, whereas the porcine thyroid receptor subunits have values of 46,000 & 35,000. The labeling of the receptor subunits was inhibited by preincubation with Graves' autoantibodies. Despite possessing a different subunit composition, the receptors from these tissues exhibit similar affinity for TSH and share similar antigenic determinants for Graves' autoantibodies.

Specific binding sites for thyrotropin (TSH) are present on thyroid (1-3) as well as fat cell membranes (4,5). The physiological significance of TSH binding to fat cells is not clearly understood. However, Birnbaumer and Rodbell (6) have shown that TSH stimulates adenylate cyclase activity in fat.

The sera from some patients with Graves' disease contain anti-TSH receptor antibodies which compete with TSH for binding to thyroid plasma membranes (see ref. 7 for review). Such antibodies have also been shown to interact with membranes prepared from adipose tissue (4). Recently, Ingbar and coworkers have used guinea pig fat cell membranes to effect a partial purification of anti-TSH receptor antibodies from the sera of patients with Graves' disease (8). Baker and coworkers (9) have used guinea pig fat cell membranes in screening

* A preliminary report of these findings was presented at the 67th annual meeting of the Endocrine Society, Baltimore, Maryland, 1985.

Abbreviations: TSH - thyrotropin; PPM - partially purified membranes; PM - purified membranes; SM-solubilized membranes; IgG - immunoglobulin G; SDS - sodium dodecyl sulfate; PAGE - polyacrylamide gel electrophoresis; DSS - dissuccinimidyl suberate; M_r - average molecular mass.

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hybridomas for the production of monoclonal antibodies against the thyroid TSH receptor. The use of fat cell membranes rather than thyroid membranes, eliminates the detection of antibodies against extraneous thyroid antigens such as thyroglobulin and microsomes. The present studies were undertaken to characterize the physicochemical properties of the TSH receptor in guinea pig fat in comparison with that of thyroid gland.

MATERIALS AND METHODS

Purified bovine TSH (40 IU/mg), a gift from Dr. John G. Pierce (UCLA) was iodinated using the lactoperoxidase method (10). DEAE Affi-gel Blue was purchased from Bio-Rad (Richmond, CA.) and used to prepare protease-free IgG from sera (11).

Binding of ^{125}I-TSH to partially purified (PPM), purified (PM) or solubilized (SM) membranes was measured as previously described (10,12-14), and the binding data was analyzed by the method of Scatchard (15).

The method used for separating the high and low affinity binding components of the TSH receptor by discontinuous sucrose gradient ultracentrifugation is detailed in the legend of Fig. 2.

Radioiodinated TSH incubated for 1 h with porcine thyroid and guinea pig fat cell PM was cross-linked to the membranes using the bifunctional cross-linker dissuccinimidyl suberate (DSS) as previously described (16) and detailed in figure legend 4.

RESULTS AND DISCUSSION

Equilibrium binding of ^{125}I-TSH was studied using thyroid as well as guinea pig fat cell PPM. TSH binding in both tissues was characterized by curvilinear Scatchard plots (Fig. 1). Our laboratory has previously shown that the curvi-

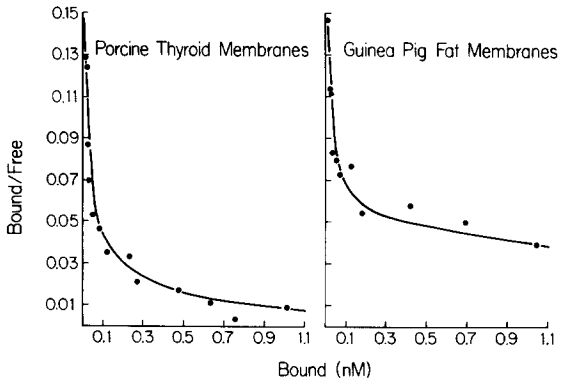

Figure 1

Scatchard plots of specific ^{125}I-TSH binding to porcine thyroid and guinea pig fat cell membranes. PPM (160 µg/ml) were incubated with ^{125}I-labeled TSH (100,000 CPM) and varying concentrations of unlabeled TSH (TS-10, Sigma) for 1 h at room temperature in a final volume of 250 µl of 50 mM Tris acetate buffer, pH 7.4, 0.25% bovine serum albumin. Nonspecific binding was determined in the presence of excess unlabeled TSH (0.5 IU/ml). ^{125}I-labeled TSH bound to PPM was separated from free TSH by filtration. Radioactivity on the filters was counted and specific binding was calculated by subtracting nonspecific and filter binding.

linear plot of TSH binding to thyroid membranes is indicative of two distinct binding components: one with high affinity and low capacity, the other with low affinity and high capacity (10,13). The apparent equilibrium dissociation constants as determined from the Scatchard plots (Fig. 1) were comparable for the two tissues.

We have also shown that the high and low affinity binding components of solubilized thyroid membranes can be separated using discontinuous sucrose density centrifugation (13). Solubilized fat cell membranes and thyroid membranes were labeled with radioiodinated TSH in the presence and absence of excess native TSH, and the labeled membranes were centrifuged on discontinuous sucrose density gradients. The gradients were fractionated, and specifically bound ^{125}I-TSH was determined as described in the legend of Fig. 2. Two peaks of bound TSH, representing the high (lighter peak) and low affinity (heavier peak) binding components, were present for both types of membranes (Fig. 2).

Antigenic similarity between the thyroid and fat cell receptors was examined by comparing the ability of antibodies from the sera of several patients with Graves' disease to inhibit ^{125}I-TSH binding to both membranes. Following

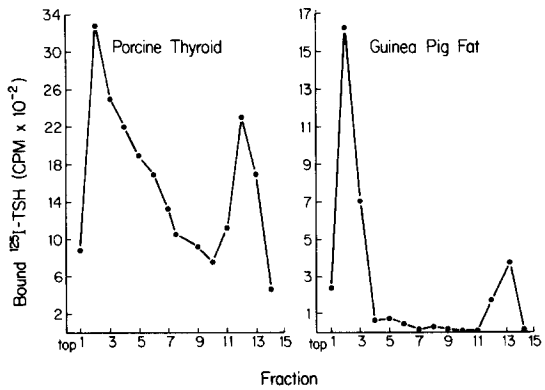

Figure 2
Sucrose gradient centrifugation of porcine thyroid and guinea pig fat cell SM. SM (100-200 µg membrane protein) in 50 mM Tris-acetate (pH 7.4) were labeled for 1 h at room temperature with ^{125}I-TSH (5-8x10^5cpm) in the presence and absence of excess native hormone. Samples of labeled SM (0.8 ml) were applied to a 5%, 10%, 20% and 50% (wt/vol) discontinuous sucrose density gradient and centrifuged for 2 h at 225,000 xg at 4°C. Fractions were collected from the bottom of the tubes, and bound hormone was separated from free by precipitation with polyethylene glycol (14) and filtration on cellulose acetate filters. The bound radioactivity was corrected for nonspecific and filter binding.

preincubation of membranes for 1 h with normal or Graves' disease IgG, ^{125}I-TSH was added to the assay tubes and the incubation continued for an additional hour. Receptor bound TSH was separated from free TSH and the inhibition of ^{125}I-TSH binding by each IgG preparation was expressed as a percent of control binding in the absence of IgG. The percent inhibition of ^{125}I-TSH binding by seven different Graves' IgG studied was similar for both types of membranes and varied between 17% and 81% (data not shown). Normal IgG had minimal effect on ^{125}I-TSH binding to both thyroid and guinea pig fat membranes. To determine if the inhibition of ^{125}I-TSH binding to these receptors by Graves' IgG was the result of the interaction of the antibodies with the high and/or low affinity binding components, solubilized membranes were preincubated with Graves' IgG, Normal IgG or buffer (control) for 1 h at room temperature. Radioiodinated TSH was then added and the incubation continued for another hour. The membranes were then subjected to discontinuous sucrose density gradient ultracentrifugation as detailed in legend of Fig. 3. Preincubation with normal IgG had minimal effects on the labeling of the high and low affinity binding components of both thyroid and fat membranes, whereas preincubation with Graves' IgG resulted in marked inhibition of TSH binding to the high and low affinity

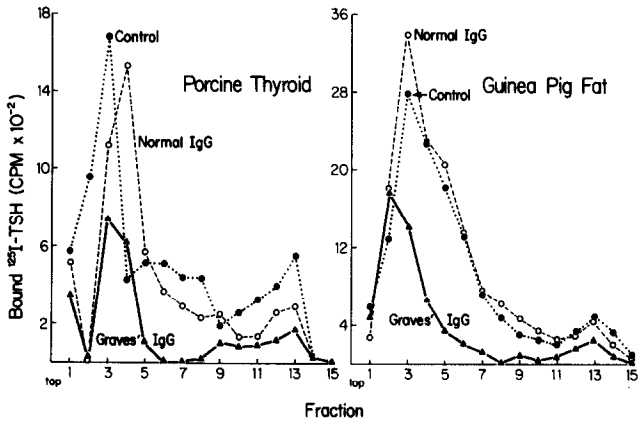

Figure 3
Effect of preincubation with normal and Graves' IgG on the labeling of the high and low affinity binding components separated by discontinuous sucrose gradient centrifugation. SM (100-200 µg) were preincubated for 1 h with 2.5 mg/ml of either normal IgG (0) or Graves' IgG (Δ) or buffer (0). The membranes were then incubated with ^{125}I-TSH for 1 h, and subsequently applied to discontinuous sucrose gradient and analyzed as described in the legend for figure 2.

binding components of both tissues. These data suggest that the high and low affinity binding components of both thyroid and fat cell receptors possess similar antigenic determinants for Graves' disease antibodies.

The subunit composition of TSH receptors of porcine thyroid and guinea pig fat cells was studied by covalently cross-linking ^{125}I-TSH to purified membranes, using DSS (Fig.4A, lanes 1-4). The effect of normal and Graves' IgG on the labeling of the receptor subunits was also examined (Fig. 4B, lanes 5-8). Under reducing conditions, SDS-PAGE of covalently labeled guinea pig membrane extracts resulted in the specific labeling of two protein bands (Fig. 4A, lane 1)

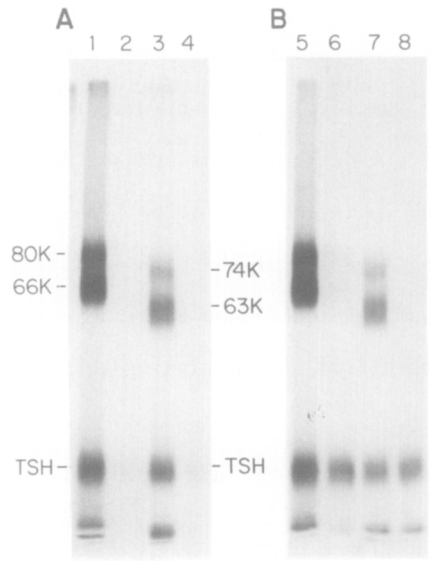

Figure 4

A, cross-linking of ^{125}I-TSH to guinea pig fat cell and porcine thyroid membranes. B, the effect of normal and Graves' disease IgG on the affinity labeling of the receptor subunits. Guinea pig fat cell (lanes 1,2,5 and 6) and thyroid membranes (lanes 3,4,7 and 8) were incubated with buffer (lanes 1-4), 2.5 mg/ml normal IgG lanes (5 and 7) or 2.5 mg/ml Graves' IgG (lanes 6 and 8) for 1 h at room temperature. The membranes (0.2 mg) were then incubated for 1 h with ^{125}I-TSH (1 x 10^6CPM). Nonspecific binding was determined in the presence of excess native TSH (lanes 2 and 4). The membranes were washed with 1.0 ml cold 10 mM sodium phoshate buffer, pH 7.4 and resuspended in 0.2 ml of the same buffer. DSS was dissolved in dimethyl sulfoxide and was added at a 1:50 ratio to a final concentration of 0.3 mM. The cross-linking was allowed to proceed for 15 min at room temperature and was terminated by the addition of excess 25 mM tris-acetate pH 7.4. The covalently-labeled membranes were pelleted by centrifugation and resuspended in 25 mM Tris-acetate pH 7.4 containing 1 M $MgCl_2$ and allowed to incubate for 1 h at room temperature to dissociate noncovalently bound ^{125}I-TSH. The membranes were then boiled for 5 min in 2% SDS in the presence of 50 mM DTT and analyzed by SDS-PAGE according to the method of Laemmli (17). Labeled proteins were visualized by exposure of the gel to Kodak X-Omat XAR-5 X-ray films for 4-7 days at -90°C. Dupont Cronex Lightning Plus intensifying screens were used to enhance the autoradiography.

with M_r values of 80,000 and 66,000 (before correction for M_r of TSH, 28,000). Similar analysis of porcine thyroid membranes resulted in the specific labeling of two protein bands (Fig. 4A, lane 3) with M_r values of 74,000 and 63,000.

The labeling of these bands was specific, for when the membranes were incubated in the presence of excess native hormone their labeling was completely inhibited (Fig. 4A, lanes 2 and 4). The band near the bottom of the gel represents free cross-linked TSH a contaminant of the membrane preparations (16). Under non-reducing conditions, SDS-PAGE of the affinity-labeled receptor complexes of both membranes resulted in a slight increase in the mobility of the labeled bands (corresponding to a decrease in M_r of 2,000-5,000) without the disappearance of either labeled species (data not shown). These observations indicate that neither of the two bands was composed of disulfide linked receptor complexes. The decrease in mobility of these bands in the presence of DTT may possibly be due to the reduction of intramolecular disulfide bonds.

When membranes of guinea pig fat cells (Fig. 4B, lane 6) and porcine thyroid (Fig. 4B, lane 8) were preincubated with Graves' IgG, prior to cross-linking of ^{125}I-TSH, the labeling of the two bands of both tissues, shown in Fig. 4A, lanes 1 and 3, was completely inhibited. Preincubation of the membranes with normal IgG, however, had very little effect on the labeling of these two bands (Fig. 4B, lanes 5 and 7). These data are consistent with the conclusion that the two labeled bands observed on gels are components of the TSH receptors in thyroid as well as fat tissue (18,19).

Taken together the results suggest that guinea pig fat cell and porcine thyroid TSH receptors are noncovalently linked oligomers. Under reducing conditions, the guinea pig fat receptor subunits have M_r values of 52,000 and 38,000 (after correction for the contribution of TSH, M_r = 28,000), whereas porcine thyroid receptor subunits have M_r of 46,000 and 35,000. Despite this difference in subunit composition, these receptors exhibit similar affinities for TSH and share similar antigenic determinants for Graves' disease antibodies.

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