THYROTROPIN INTERACTIONS WITH HUMAN FAT CELL MEMBRANE PREPARATIONS AND THE FINDING OF A SOLUBLE THYROTROPIN BINDING COMPONENT

Brian R. Mullin,[1] George Lee,[1] Fred D. Ledley,[1] Roger J. Winand,[2] and Leonard D. Kohn[1]

[1] Section on Biochemistry of Cell Regulation, Laboratory of Biochemical Pharmacology, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014, U.S.A.

[2] Département de Clinique et de Sémiologie Médicales, Institut de Médecine, Université de Liège, B4000 Liège, Belgium

Received December 22, 1975

SUMMARY: Both human and guinea pig adipocyte membranes are shown to have specific thyrotropin receptor sites, and the thyrotropin binding to human retro-orbital tissue adipocyte membranes is shown to be enhanced by gamma globulin from patients with exophthalmos. In addition, supernatant solutions from crude human and guinea pig membrane preparations are shown to contain a thermolabile, non-dialyzable inhibitor of thyrotropin binding which acts by forming a thyrotropin-inhibitor adduct rather than by directly interacting with the membrane receptors.

In previous reports we showed that purified thyrotropin (TSH)† could cause exophthalmos in a mammalian model, the guinea pig, and that this activity resided in a fragment of the TSH molecule lacking thyroid stimulating ability (1-4). We further showed that TSH or its exophthalmogenic fragment (EPF)† could specifically bind to plasma membranes obtained from guinea pig retro-orbital tissue (the Harderian gland) and that gamma globulin preparations from patients with Graves' disease and malignant exophthalmos significantly increased the extent of this binding. We therefore suggested that exophthalmos might be the result of two interacting factors, an abnormal serum gamma globulin and either TSH or an exophthalmogenic derivative of the TSH molecule (3-9). The human retro-orbital space is composed chiefly of adipose tissue and has no equivalent of the guinea pig Harderian gland (10). An extrapolation of this two-factor theory to human exophthalmos thus requires that TSH interact with human fat cell membranes obtained

† Abbreviations are: TSH, thyrotropin or thyroid-stimulating hormone; EPF, the exophthalmos-producing fragment of the thyrotropin molecule produced by partial pepsin digestion and consisting of an intact or nearly intact β subunit of the thyrotropin molecule and the amino-terminal half of the α subunit of the thyrotropin molecule (3).

from the retro-orbital space. The present report demonstrates such an interaction, *i.e.*, adipocyte membranes from human retro-orbital tissue exhibit both specific ^{125}I-TSH binding and TSH stimulation of adenylate cyclase activity. The present report further demonstrates that both human and guinea pig cell extracts contain a soluble component capable of specific TSH binding.

MATERIALS AND METHODS

TSH and ^{125}I-TSH were purified bovine preparations prepared as previously described (1, 2, 8). Human and guinea pig adipocytes were obtained by the method of Rodbell and Krishna (11); adipocyte membranes were prepared from the isolated adipocytes by a modification of their method (11). Adipocytes were washed twice with a medium containing 0.25 M sucrose, 1 mM ATP, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.4. Cells were suspended in an equal volume of this medium and aspirated 9 times through a Swinny filter (Becton-Dickinson) at room temperature. Following centrifugation at 16,000 rpm for 15 minutes at 2° in a Sorval RC-2 centrifuge, the fat cake was removed and the pellet taken up in a small amount of the same sucrose medium. The pellet was washed 3 times, then homogenized by 10 strokes in a Teflon pestle-glass homogenizer (Thomas). Membranes were stored in liquid nitrogen until use. Human fat tissues were either surgical or necroscopy specimens, the latter obtained within 3 hours postmortem from patients with cardiac ailments. All tissues were normal in gross and microscopic detail.

TSH binding was assayed as previously described (5-8); adenylate cyclase assays were performed using the conditions of Rodbell and Krishna (11) as modified by Wolff and Jones (12). Cyclic AMP was measured using a protein binding assay (13).

RESULTS

Table I shows that ^{125}I-TSH, 1.4×10^{-9} M, binds to human retro-orbital tissue fat cell membranes, as well as to retro-orbital tissue membranes obtained from the Harderian gland of the guinea pig. Cold TSH, 1.8×10^{-6} M, completely suppresses this binding, whereas equivalent concentrations of insulin, albumin, glucagon, prolactin, follicle-stimulating hormone, and human chorionic gonadotropin have no effect on binding, *i.e.*, the binding appears to be specific. The interaction of ^{125}I-TSH with fat cell membranes is not unique to retro-orbital tissue since fat cell membranes from perirenal and subcutaneous tissue adipocytes also exhibit specific TSH binding (Table I).

The ^{125}I-TSH binding has been demonstrated using intact human adipocytes from retro-orbital tissue (14), as well as using plasma membrane preparations derived from these adipocytes (Table I). In both cases ^{125}I-TSH binding is

TABLE I. *Binding of ^{125}I-TSH to fat cell membranes in the presence and absence of gamma globulin from patients with exopthalmos.*

Source of fat cell membranes	Species	^{125}I-TSH bound/mg membrane protein [a]	
		- Exophthalmogenic gamma globulin	+ Exophthalmogenic gamma globulin [b]
		cpm x 10^{-2}	
Retro-orbital	Human	29,350	72,000
	Guinea pig	34,200	89,540
Subcutaneous	Human	49,600	not done
	Guinea pig	22,600	not done
Perirenal	Human	38,260	not done
	Guinea pig	31,900	not done

[a] Binding assays contained 125,000 cpm (1.4×10^{-9} M) ^{125}I-TSH. In each case binding was performed as a function of membrane protein concentration; values presented are derived from the linear portions of the curves obtained. Control incubations contained either no membranes or contained unlabeled TSH at a concentration of 1 mg/ml. The values presented are the specific binding, *i.e.*, the ^{125}I-TSH which could be displaced by unlabeled TSH.

[b] The "abnormal" gamma globulin was present at a concentration of 200 µg/ml. Control assays which contained 200 µg/ml pooled normal gamma globulin yielded values 10% less than incubations in the absence of any gamma globulin.

enchanced by gamma globulin from patients with Graves' disease and exophthalmos [Table I and (14)]. ^{125}I-TSH binding to human retro-orbital tissue adipocyte membranes is not enhanced by gamma globulin from the sera of normal controls or from the sera of patients with Graves' disease who do not have exophthalmos.

The TSH binding to the human fat cell membranes is associated with stimulation of adenylate cyclase activity (Table II).

In the course of these studies, it was noted that crude membrane suspensions from both guinea pig retro-orbital tissue and human adipocytes contained an inhibitor of TSH binding. The existence of the inhibitor was suggested by the increased ability of washed membrane preparations to bind TSH (Fig. 1A); its existence was confirmed by the demonstration that the supernatant phase of crude membrane preparations contained a factor which inhibited TSH binding either to the

TABLE II. *TSH stimulation of adenylate cyclase activity in human adipocyte membranes*

Addition	Membrane Source		
	Retro-orbital	Subcutaneous	Perirenal
	picomoles cAMP formed/15 min/mg membrane protein [a]		
TSH [b]	410	665	133
Fluoride [b]	540	731	133

[a] Net values above basal level. The control of membranes in the absence of TSH or fluoride was effectively 0, *i.e.*, the basal adenylate cyclase activity of membrane preparations resulted in insufficient cAMP formation to result in displacement in the cAMP binding protein assay.

[b] TSH concentration was 76 μm; fluoride concentration was 10 mM.

washed membrane preparations (Fig. 1B) or to sucrose gradient purified membrane preparations (not shown).

The present data suggest that this inhibitor is a soluble TSH binding factor located within the cell. That it is intracellular in location is suggested by the finding that the inhibitor is present in the supernatant phase of membrane homogenates obtained from isolated cell preparations which had been washed free of extracellular fluid components (Fig. 1B). Specific interaction of the inhibitor with TSH was shown by the forward displacement of ^{125}I-TSH on Sephadex G-100 columns after the ^{125}I-TSH and inhibitor were preincubated together (Fig. 2). Formation of the ^{125}I-TSH--inhibitor adduct was not prevented by preincubation with albumin, insulin, glucagon, or FSH at concentrations at least 1,000-fold greater than ^{125}I-TSH, but was prevented by preincubation with a 1,000-fold excess of unlabeled TSH.

The ^{125}I-TSH--inhibitor adduct obtained from the Sephadex G-100 columns (fractions 55-60, Fig. 2) did not bind to washed membranes when tested under standard assay conditions or under conditions previously shown to measure binding of solubilized TSH receptor preparations (15).

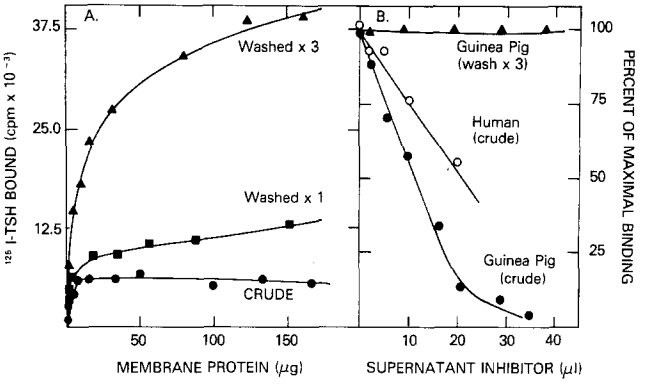

FIG. 1. *(A) Binding of ^{125}I-TSH to crude membrane preparations from guinea pig retro-orbital tissue and to the same membrane preparation after being washed with buffer 1 to 3 times.* Crude guinea pig retro-orbital tissue membrane preparations were obtained using procedures for tissue disruption and homogenization previously described (5, 6). The washing procedure involved pelleting the membranes by centrifugation at 10,000 x g and suspending them in an equal volume of buffer. Assay conditions were standard and included 60,000 cpm ^{125}I-TSH (~ 0.7 x 10^{-9} M). *(B) Inhibition of binding by the supernatant solution obtained from crude membrane suspensions prepared from guinea pig retro-orbital tissue (●) or human adipocytes (○).* The membranes used in this experiment were the guinea pig retro-orbital tissue membranes which had been washed 3 times; assay conditions were the same as in Fig. 1A. Data obtained using human retro-orbital or subcutaneous tissue adipocyte membranes which had been washed 3 times were analogous. The last supernatant wash solution obtained from either the guinea pig membranes washed 3 times or the human adipocyte membranes washed 3 times, had no effect on binding (▲). The supernatant solutions were centrifuged at 100,000 x g for 2 hours to remove any possible particulate components capable of binding TSH; none of the supernatant solutions could bind TSH when tested in standard binding assays or in soluble TSH receptor assays we have previously described (15).

In contrast to these experiments involving preincubation of inhibitor and TSH, preincubation of inhibitor with membranes did not result in inhibition of TSH binding. The inhibitor thus appears to have no direct interaction with the TSH receptor.

The inhibitor is destroyed by heating at 90°. It is found in the clear supernatant phase rather than in the lipid cap of the supernatant phase obtained after centrifuging the adipocyte extracts. It is not removed by centrifugation at 100,000 x g for 2 hours.

DISCUSSION

These experiments demonstrate that membranes from human retro-orbital tissue fat cells can bind TSH at specific receptor sites and that gamma globulin from

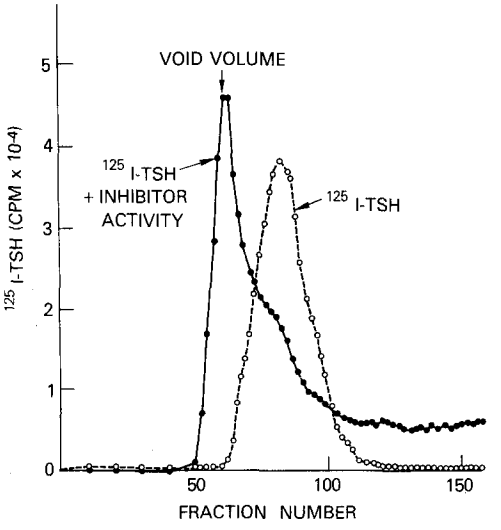

FIG. 2. *Sephadex G-100 chromatography of ^{125}I-TSH (0) and ^{125}I-TSH incubated with the supernatant inhibitor obtained from crude preparations of guinea pig retro-orbital tissue (●).* ^{125}I-TSH (1 x 10^6 cpm) was incubated with the supernatant inhibitor (200 µl) under assay conditions and chromatographed on a 50 x 1 cm Sephadex G-100 column (●——●). An identical aliquot of ^{125}I-TSH was incubated under assay conditions in the absence of the supernatant inhibitor and was analogously chromatographed (0-----0) on the same column. When albumin (5 mg/200 µl), insulin (0.2 mg/200 µl), glucagon (0.2 mg/200 µl), or FSH (1 mg/200 µl) were preincubated with ^{125}I-TSH under assay conditions, the ^{125}I-TSH eluted in the same position as free ^{125}I-TSH (0-----0); similarly, when these same agents were preincubated with ^{125}I-TSH and the inhibitor, the forward displacement of the ^{125}I-TSH by the inhibitor (●——●) was unaffected. In contrast, displacement of the ^{125}I-TSH by inhibitor was not seen if the preincubation with ^{125}I-TSH was analogously performed in the presence of unlabeled TSH (0.2 mg/200 µl). Elution in all cases was with 0.01 M Tris-chloride, pH 7.0, containing 0.1% albumin. Elution with 0.025 M Tris-acetate, pH 6.0, with a 0.1% or 1% albumin did not alter these results. Studies using the supernatant inhibitor from crude membrane preparation of human adipocytes yielded analogous data.

patients with Graves' disease and exophthalmos enhances this binding. The findings are analogous to those obtained with guinea pig retro-orbital tissue membrane preparations despite the morphologic differences in the two tissues. The data thus allow our extrapolation of the "two-factor" mechanism for experimental exophthalmos to the human condition, although they are not a direct proof of its validity.

The existence of TSH receptors on membranes of adipocytes isolated from other tissues is compatible with our previous *in vivo* study indicating a TSH effect on fat cells. In that report, TSH was shown to induce the same glycos-

aminoglycan changes in guinea pig perirenal tissue as in guinea pig retro-orbital tissue (4). Whether or not a *direct* TSH effect on adipocytes reflects itself in the lipid changes known to occur in hypo- and hyperthyroidism is not clear but warrants further investigation. We have previously noted that pretibial myxedema might be an extension of the "two-factor" mechanism involving its own abnormal gamma globulin (4); hence the effect of the gamma globulin from patients with Graves' disease on binding to human adipocyte membranes other than those of retro-orbital origin is under active investigation.

The existence of a soluble TSH binding factor is at least of experimental importance and may have significant physiological relevance. From the experimental point of view, binding of TSH to poorly washed membrane preparations might yield confusing data, since specific TSH-receptor binding would be hidden by the TSH interaction with a soluble factor which is not only not measured by the binding assay but competes with the receptor for the ^{125}I-TSH added to the incubations. From a physiological point of view, this binding component might play a significant role in transmitting the TSH message to the cell machinery; for example, it might play a role in the TSH effect on thyroglobulin biosynthesis. Such a factor would be analogous to those involved in the intracellular binding of estrogen and steroid hormones and more recently of thyroid hormone (16). The nature of this inhibitor and its relationship to both cell function and the TSH plasma membrane receptor is under active investigation.

REFERENCES

1. Winand, R. J., and Kohn, L. D. (1970) *J. Biol. Chem.* 245, 967-975.
2. Kohn, L. D., and Winand, R. J. (1971) *J. Biol. Chem.* 246, 6570-6575.
3. Kohn, L. D., and Winand, R. J. (1975) *J. Biol. Chem.* 250, 6503-6508.
4. Winand, R. J., and Kohn, L. D. (1973) *Endocrinology* 93, 670-680.
5. Winand, R. J., and Kohn, L. D. (1972) *Proc. Nat. Acad. Sci. U.S.A.* 69, 1711-1715.
6. Bolonkin, D., Tate, R. L., Luber, J. H., Kohn, L. D., and Winand, R. J. (1975) *J. Biol. Chem.* 250, 6516-6521.
7. Amir, S. M., Carraway, J. F., Jr., Kohn, L. D., and Winand, R. J. (1973) *J. Biol. Chem.* 248, 4092-4100.
8. Tate, R. L., Schwartz, M., Holmes, J. M., Kohn, L. D., and Winand, R. J. (1975) *J. Biol. Chem.* 250, 6509-6515.

9. Kohn, L. D., and Winand, R. J. (1974) *Israel J. Med. Sci.* 10, 1348-1364.
10. Weglius, O. (1971) in *The Thyroid* (S. S. Werner and S. H. Ingbar, eds.), New York, Harper & Row, pp. 520-528.
11. Rodbell, M., and Krishna, G. (1974) *Methods Enzymol.* 31, 103-115.
12. Wolff, J., and Jones, A. B. (1971) *J. Biol. Chem.* 246, 3939-3947.
13. Gilman, A. C. (1970) *Proc. Nat. Acad. Sci. U.S.A.* 67, 305-310.
14. Etienne, J., Kohn, L. D., and Winand, R. J. Proc. 7th Internatl. Thyroid Conference, Boston, June 9-13, 1975. *Excerpta Medica*, Amsterdam, The Netherlands, *in press*.
15. Tate, R. L., Holmes, J. M., Kohn, L. D., and Winand, R. J. (1975) *J. Biol. Chem.* 250, 6527-6533.
16. Jensen, E. V., and DeSombre, E. R. (1972) *Annu. Rev. Biochem.* 41, 203-230.