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SPECIFIC THYROXINE RECEPTORS IN MAMMARY CYTOSOL FROM LACTATING CATTLE

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SUMMARY: Specific thyroxine (T_4) binding was identified in bovine mammary cytosol preparations. Binding specificity of 3,5,3' triiodothyronine (T_3) with respect to T_4 was less than 1%. The halftime of T_4 binding and displacement at 4°C was 1 and 20 minutes, respectively. Scatchard analyses demonstrated the presence of two T_4 binding components with dissociation constants of 3.61×10^{-10} and 7.73×10^{-8}M. The high affinity binding component had a molecular weight of $\sim$ 100,000, a T_4 binding capacity of 5.05×10^{-12}moles/mg protein, and was destroyed by boiling or protease treatment. High-affinity, low capacity T_4 binding was not found in bovine serum and was unique to mammary tissue.

Milk secretion is a transient physiological process dependent upon many factors. The initiation and subsequent maintenance of lactation are influenced by an array of specific hormonal interactions (1), however precise details of these control mechanisms have not yet been elucidated. Thyroid hormones affect milk secretion in that injection or feeding of these hormones temporarily increases milk production in rats and cattle (2,3). Daily injections of L-thyroxine (T_4) for seven weeks increased milk secretion in cows by 27% when administered at dosages that were 25% above their normal thyroid secretion rates (4). Moreover, feeding thyroprotein (iodinated casein) to cows results in a 10% increase in overall milk production (5), and a 15-20% increase when administered during late lactation (6,7). How thyroid hormones cause increased milk production is unclear. They may serve to alter whole animal metabolism with greater milk secretion as a consequence. Alternatively, they may function independently or through synergistic interactions with other hormones, by altering specific mammary tissue function.

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It has been demonstrated that 3,5,3' triiodothyronine (T_3) synergizes with prolactin, when added to mouse mammary gland explants, to increase α-lactalbumin synthesis (3-5 fold), a major milk protein (8). This finding implicates receptor binding with consequent gene expression as the mode of action for thyroid hormones. Thyroid hormone binding has been reported in nuclei, mitochondria, cytosol and plasma membranes of various tissues (9-18). Conclusive evidence that the physiological effects of these hormones are exerted through receptor mediated processes has not been reported, however it would seem likely. The objective of this study was to determine if mammary tissue from lactating cows specifically bound thyroid hormones. Once this was established, preliminary biochemical characterization of the cytosolic T_4 binding component was carried out.

MATERIALS AND METHODS

Routine Procedures Mammary tissue (100 gm) was removed from normal nonpregnant Holstein cows, in mid to late lactation 15-30 min after slaughter and immediately transferred to ice cold .025 M Tris buffer pH 7.5 containing .25 M sucrose and .01 M $MgCl_2$. Subsequent operations were done at 4°C. Tissues were minced manually and homogenized with a Brinkman Polytron PCU-2 homogenizer Homogenates were filtered through cheesecloth (4 layers) and centrifuged, at 40,000 x g, for 30 min to remove large cellular fragments. Resulting supernatants were centrifuged, at 105,000 x g, for 90 min to eliminate microsomes. Cytosol was aliquoted and used immediately or frozen at -20°C, for future characterization. Boiled cytosol was prepared by pipetting 10 ml of fresh cytosol into polypropylene tubes and placing the capped tubes in a boiling water bath for 15 min. Boiled preparations were centrifuged, at 40,000 x g, for 30 min and the supernatants were used immediately.

$[^{125}I]T_4$ Binding in Mammary Cytosol Binding of T_4 to cytosol was tested by adding 20,000 cpm (∿12 fmol) $[^{125}I]T_4$ (Amersham IM.141) in .1 ml of .02 M Tris buffer, pH 7.5 containing .01 M $MgCl_2$ (assay buffer) to .1 ml of cytosol. The cytosol added to each assay tube contained approximately 40 µg of protein, determined by a modified Lowry method (19). The modification being that .9 ml of .1 N NaOH and 1% SDS was added to .1 ml of cytosol prior to boiling for 30 min. Specific binding was defined as binding that occurred in the absence of unlabelled T_4, minus that which occurred in the presence of excess (1 µg) unlabelled T_4 (NSB), expressed as a percentage of total radioactivity. The final incubation volume of each assay tube was made up to .8 ml with assay buffer. Except when binding kinetics were examined, routine assays were incubated at 4°C for at least 2 hr. Partitioning of bound and free T_4 was achieved by adding 1.0 ml of ice cold activated charcoal coated with dextran T-80 (DCC) in deionized water (.25% and .025% w/v, respectively) to each tube and incubated

at 4°C for 10 min. The tubes were centrifuged at 1000 x g and 4°C for 10 min and decanted. Radioactivity in both supernatants and pellets was measured in a gamma scintillation spectrometer.

Specificity of T_4 binding was determined by competitive binding experiments between [^{125}I]T_4 and several structurally similar compounds. These were DL-thyronine (T), L-thyroxine (T_4), 3,5,3'-triiodothyronine (T_3) 3,5,5'-triiodothyronine (reverse T_3), 3,3',5,5'-tetraiodothyroacetic acid (T_4A), 3,3',5-triiodothyroacetic acid (T_3A), 3,3',5-triidothyropropionic acid (T_3P), and 2,3,5,6-tetraiodobenzoic acid (T_4B). These compounds were obtained from Aldrich, Calbiochem-Behring or Tridom/Fluka. Each compound was dissolved in .1 M NaOH at a concentration of 1.0 mg/ml and dilutions of each were made with assay buffer in the range of 1 to 10^5 nM.

Effects of Enzymes and Chemical Agents on T_4 Binding in Cytosol Cytosol was preincubated with DNAse, RNAse, Lipase, Protease, Hyaluronidase or Neuraminidase to determine susceptability of T_4 binding to enzymatic degradation. Subsequent binding assay procedures were identical to those described above. The effects of 10 mM dithiothreitol, .3 mM sodium iodide and .2 mM 1, 8 anilinonapthalene sulphonic acid (ANS) on specific T_4 binding were also determined (available from Malinckrodt or Sigma Chemical Company).

Cytosol Binding and Displacement Kinetics of T_4 The time-course of T_4 binding in mammary cytosol was investigated using assay conditions described above. Binding was stopped by adding DCC at predetermined time intervals. The rate of T_4 displacement from cytosolic binding sites was determined by a similar method, except 125 pmol of unlabelled T_4 was added to specific binding tubes after a 2 hr incubation. Percent specific T_4 binding was calculated and the time-course of binding and displacement was plotted.

Dissociation constants (K_d) and binding capacities (B_{max}) for T_4 were determined by the method of Scatchard (20). Cytosolic preparations were incubated with [^{125}I]T_4 and increasing quantities of unlabelled T_4 added to respective tubes. The negative reciprocal slope of each linear component of the plot was calculated as the K_d. The high affinity B_{max} for T_4 was determined by linear extrapolation of the corrected low K_d component to the abscissa. Boiled cytosol and bovine serum were analyzed similarly.

Gel Filtration of T_4 Binding Components Mammary cytosol samples (1.0 ml) were fractionated on columns (1.5 cm I.D., 45 cm long) packed with Sephadex G-200 using .1 M Tris buffer pH 7.5, containing .05 M EDTA, and .005% sodium azide as the eluant. Protein content of each fraction (2.6 ml) was estimated by absorbance at 280 nm and assayed for specific T_4 binding properties as previously described. Columns were calibrated with blue dextran 2000 (void volume), ovalbumin (M.W. 45,000) and Na[^{125}I] (bed volume). Each cytosol

boiled or not, was concentrated 5-fold by centrifugal ultrafiltration through Amicon Membrane Cones (2100 CF 50) prior to gel filtration. This insured sufficient yield of fractionated cytosol for evaluation. Ten-fold dilutions of bovine serum were fractionated and treated in the same manner. Elution profiles and specific [^{125}I]T_4 binding (corrected for absorbance) of fractionated cytosol were plotted.

RESULTS

[^{125}I]T_4 Binding in Mammary Cytosol Specificity of T_4 binding in unfractionated cytosol preparations, is represented in Fig. 1. The amount of T_4, rT_3 and T_3 required to displace 50% of bound [^{125}I]T_4 was 7, 90, and 1,000 pmol, respectively. Other effective competitors for binding were T_4A, T_3A, T_3P in the range of 200 to 1000 pmol. DL-thyronine and T_4B (10,000 pmol) did not compete with [^{125}I]T_4 for binding. The slope of the T_3 displacement curve was quite different from the rest in Fig. 1. This may have resulted from competition between the two primary thyroid hormones for different affinity binding sites.

Effects of Enzymes and Chemical Agents on T_4 Binding in Cytosol Protease digestion of cytosol, prior to incubation with [^{125}I]T_4 reduced specific binding (Fig. 2); Neuraminidase, Hyaluronidase, Lipase, DNAse, and RNAse had no effect. This suggested that the T_4 binding component(s), of unfractionated mammary cytosol, was a protein. Specific binding was also inhibited by ANS, a well known blocking agent of T_4 binding to serum proteins. Sodium iodide and dithiothreitol had no effect on T_4 binding.

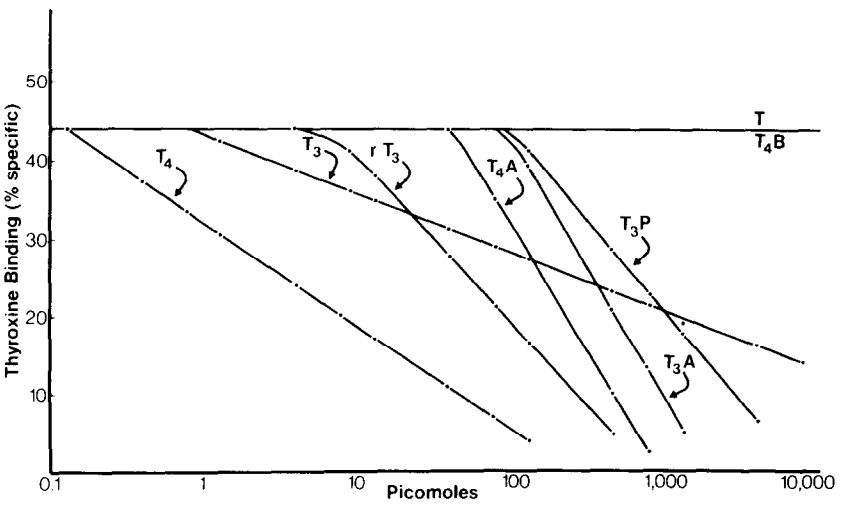

Figure 1
Effect of thyronine and similar iodinated compounds on displacement of [^{125}I]T_4 from mammary cytosol binding sites. The abscissa indicates the quantity of each compound added to respective tubes. Means are represented from experiments done on cytosol from 3 cows.

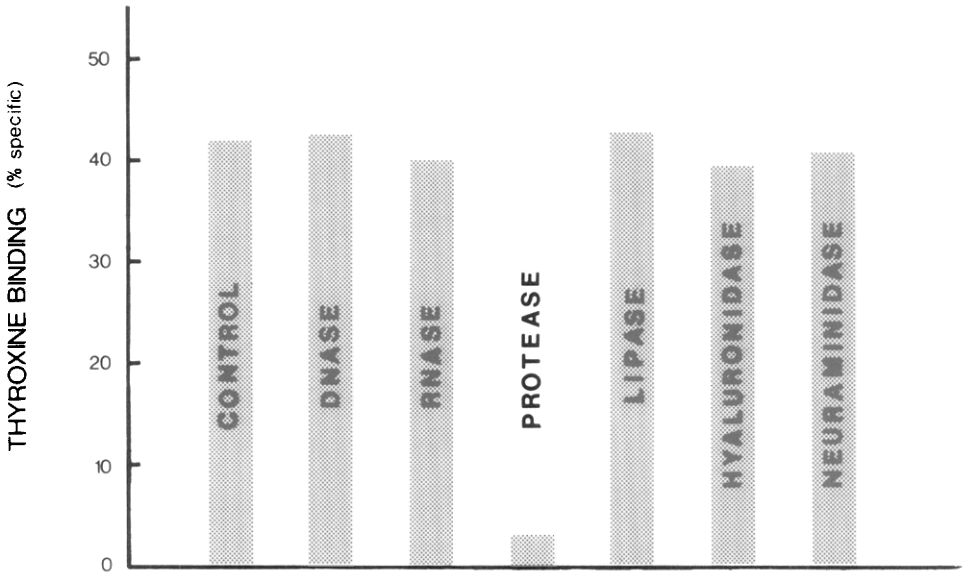

Figure 2
Histograph of the effects of enzymatic digestion on specific cytosolic [^{125}I]T_4 binding. Enzymes (50 µg in .6 ml assay buffer) were incubated with cytosol (40 µg protein) for 2 hr at room temperature prior to [^{125}I]T_4 binding assays. Means are represented from experiments done on cytosol from 3 cows.

Cytosol Binding and Displacement Kinetics of T_4 The time-course of [^{125}I]T_4 binding in unfractionated cytosol was very rapid. The halftime of maximum binding was less than 1 min (Fig. 3). Binding stability was tested for 48 hr, during which time [^{125}I]T_4 binding decreased by only 5% at 4°C (Fig. 3 insert). The halftime of displacement was approximately 20 min and displacement rate appeared exponential (Fig. 4).

A representative Scatchard plot of T_4 binding by mammary cytosol preparations is shown in Fig. 5. Unfractionated cytosol exhibited two components with affinity for T_4. A relatively low affinity component had a K_d of 7.73 $\pm$ 1.28 x 10^{-8} moles T_4/liter and was practically unsaturable. The K_d of the higher affinity component was 3.61 $\pm$ .24 x 10^{-10} moles T_4/liter with a B_{max} of 5.05 $\pm$ .49 x 10^{-12} moles T_4/mg protein. Bovine serum exhibited a single, relatively low affinity, component with a virtually unsaturable capacity for T_4 (not shown)

Boiled cytosol did not possess high affinity binding components for T_4. Scatchard analyses of boiled cytosol demonstrated a single T_4 binding component with a K_d (7.58 $\pm$ .15 x 10^{-8} M) similar to the low affinity component of untreated cytosol (Fig. 5). Selective denaturation of cytosol proteins, by boiling, probably accounted for higher bound/free ratios.

Gel Filtration of T_4 Binding Components Chromatography of mammary cytosol yielded two T_4 binding components. These components were represented by two

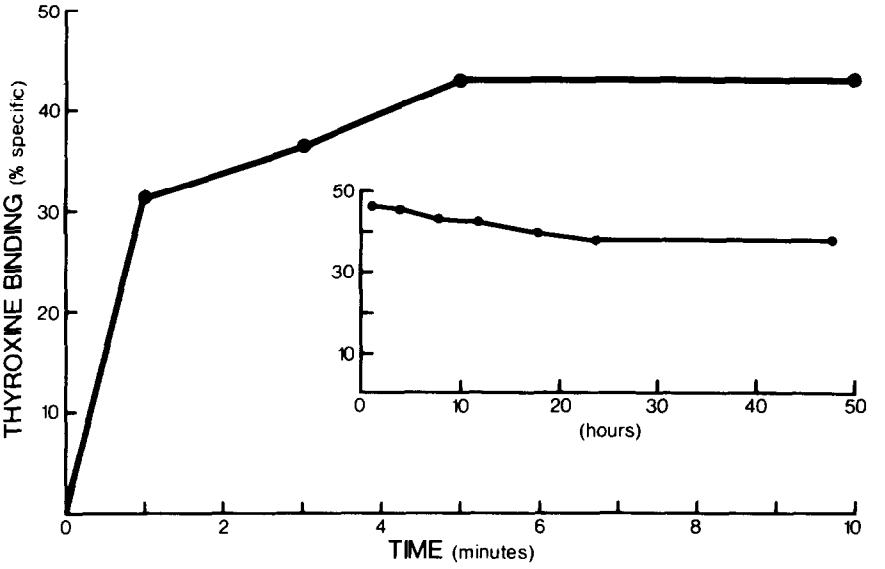

Figure 3
Time-course of specific [^{125}I]T_4 binding in cytosol. Means are represented from experiments done on cytosol from 2 cows.

predominant eluant peaks with specific T_4 binding properties (Fig. 6a). Molecular weights for these T_4 binding proteins were approximately 100,000 and 60,000. Chromatography of bovine serum yielded one predominant specific T_4 binding component with a molecular weight of 50,000 (Fig. 6b). Chromato-

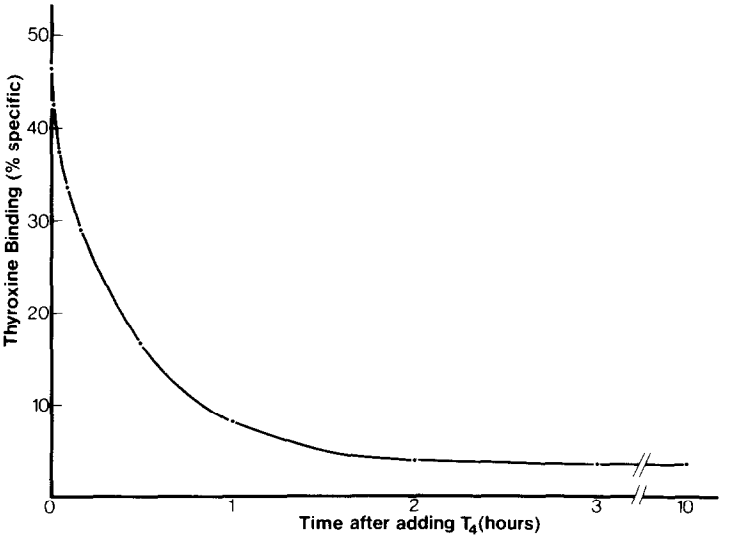

Figure 4
Time-course of specific [^{125}I]T_4 displacement in cytosol. Means are represented from experiments done on cytosol from 2 cows.

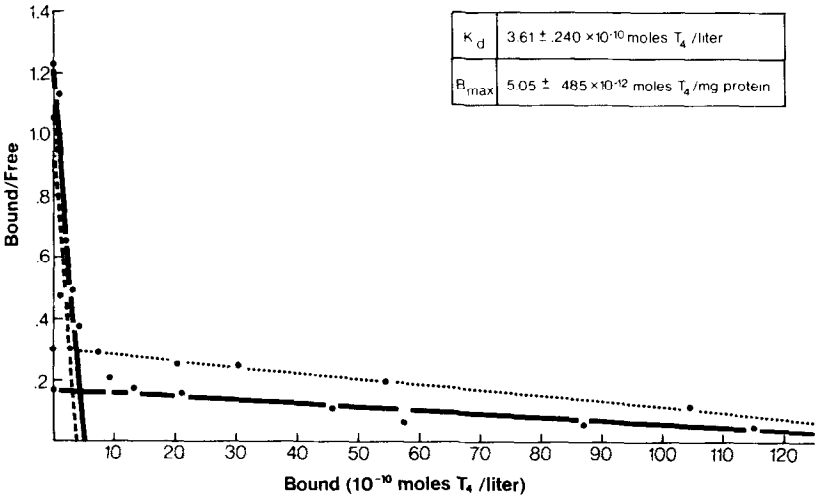

Figure 5
Scatchard analysis of specific [^{125}I]T_4 binding. Non-specific binding has been subtracted from bound/free calculations. The solid line represents uncorrected cytosol analysis. The dashed line represents high affinity binding corrected for low affinity contribution. The dotted line indicates characteristic analysis of boiled cytosol. K_d and B_{max} values are the average of preparations from 3 cows.

graphy of boiled cytosol (not shown), also yielded one 60,000 molecular weight specific T_4 binding component.

DISCUSSION

Data presented from this study provides evidence for the presence of a T_4 receptor in bovine mammary cytosol. The T_4 binding properties reported here, such as rapid, specific, high-affinity, low-capacity binding by a macromolecular protein corresponds to the operational definition of a biological receptor, except that this receptor-like T_4 binding has not, as yet, been associated with a specific physiological process. Cytosolic binding of T_4 has previously not been considered to be biologically important, however, the mammary gland has never been the target tissue under investigation. The mammary gland differs from other tissues and its function may very well be regulated by different mechanisms than other thyroid hormone targets. At the onset of lactation the metabolism of the gland changes drastically during transition from inactive to active milk secretion. In view of the pronounced effect of T_4 administration on milk secretion, in the cow, it is likely that this hormone is somehow involved in functional transition to, and maintenance of, lactation.

Cytosolic binding proteins for thyroid hormones have been found in other tissues, although none possesses identical properties. The only common characteristic appears to be that all reported binders are proteins. The

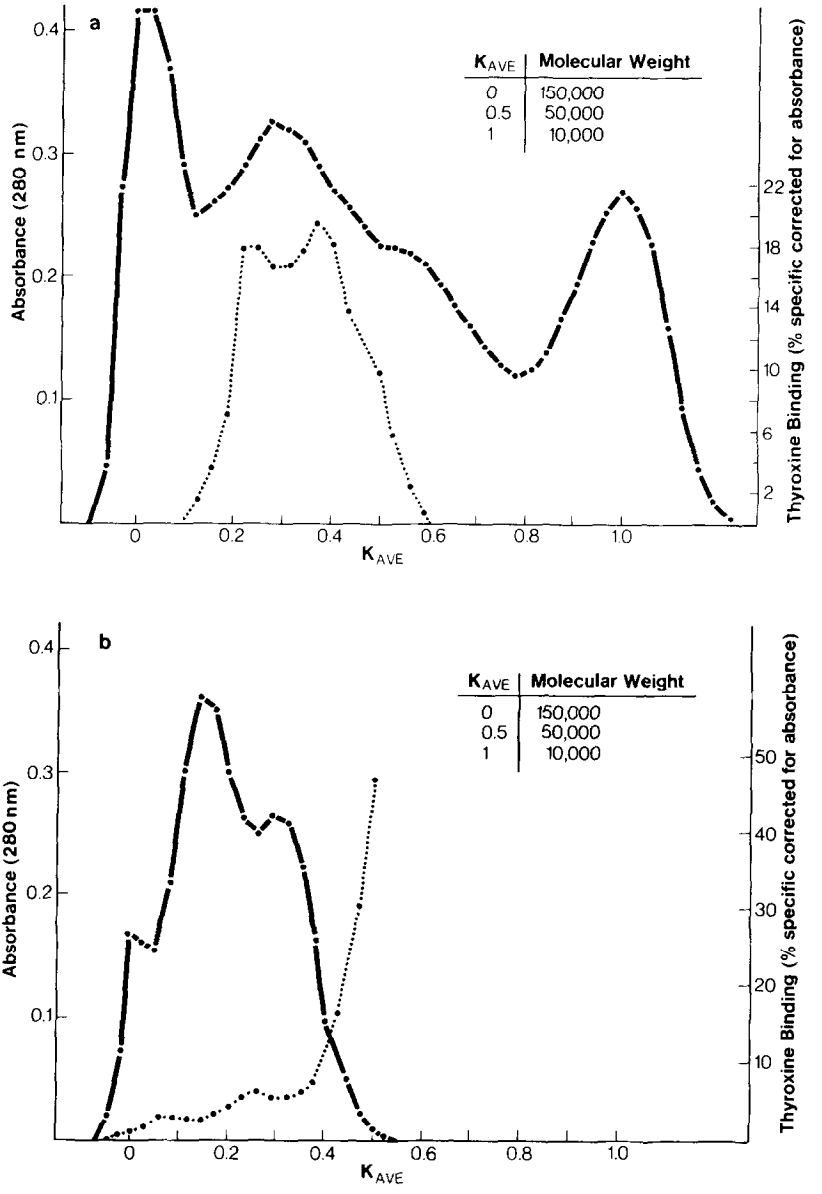

Figure 6
a) Sephadex G-200 gel filtration of mammary cytosol.
b) Sephadex G-200 gel filtration of bovine serum.

Column fractions were monitored for U.V. absorbance at 280 nm (·————·) and specific [^{125}I]T_4 binding expressed per unit absorbance (o·····o)

presence of a high-affinity, low-capacity T_3 binder in rat liver and kidney cytosol has been reported (9). In dog liver and kidney cytosols a protein has been described (21) with similar affinities to that in the rat, however binding

was preferential for T_4. Cytosolic binding proteins for thyroid hormones have been found in other tissues, such as dog brain and pig anterior pituitary (22, 17). One can only conclude that the properties of thyroid hormone binding in cytosol differ greatly depending upon the tissue studied. The action of T_4 on bovine mammary tissue may be exerted through translocation of the cytosol recep tor-hormone complex to nuclear acceptors or other cellular organelles. This is the first reported demonstration of specific T_4 binding in normal mammary tissue of any species. Further investigations regarding the significance of this observation are ongoing.

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