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CORRELATION OF MEMBRANE PHOSPHORYLATION AND EPIDERMAL GROWTH FACTOR BINDING TO HEPATIC MEMBRANES ISOLATED FROM TRIIODOTHYRONINE-TREATED RATS

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The in vivo adminstration of L-triiodothyronine to normal adult rats produced a reduction in the number of binding sites in hepatic membranes for epidermal growth factor; hyperthyroidism had no effect on insulin binding. The decreased receptor number correlated with a decrease in epidermal growth factor-stimulated phosphorylation of isolated hepatic membrane proteins (180 and 165 kDa) with adenosine [γ-^{32}P]triphosphate.

Introduction

Recently it was shown that triiodothyronine (T_3) is actively transported into isolated hepatocytes [1]. Furthermore, it has been known for some time the thyroid hormones are involved in the maintenance, growth and differentiation of hepatic tissues [2]. Some of the hepatic changes associated with increased thyroid levels are increased rates of lipid turnover [2], and an increase in liver cholesterol esterase activity [3]. The administration of pharmacological doses of T_3 in vivo have been shown to enhance DNA, RNA and malic enzyme synthesis such that growth of hepatoma cells was promoted [4], to decrease the coupling of glucagon to adenyl cyclase [5] and to modulate the level of adrenergic receptors in hepatic, cardiac and adipose tissues [2,6].

Epidermal growth factor (EGF) has been shown to bind to specific receptors in many tissues and mammalian cell types. This binding is coupled to diverse biological responses which include enhanced amino acid uptake, increased thymidine incorporation, precocious development of eyelid opening, tooth eruption, and inhibition of palate fusion [7]. EGF binds to liver membranes and intact hepatocytes and as a consequence enhances the uptake of amino acids, the incorporation of thymidine and the phosphorylation of membrane proteins [8], such as the EGF receptor [9]. A variety of agents have been shown to cause modulation of EGF binding in many cell types. The number of EGF receptors was decreased in regenerating liver [10], in benzo(*a*)pyrene transformed Syrian hamster embryo fibroblasts [11], RNA tumor virus transformed cells [12], phorbol ester transformed cells [13] and in transformed cells which release tumor growth factors [14]. There are multiple mechanisms for this reduction in binding including competition for the receptor by tumor growth factors [14], induction of receptor internalization [13], a change in receptor affinity caused by tumor promoting compounds [13] or possibly a reduction in receptor synthesis at the translational step.

Abbreviations: EGF, epidermal growth factor; T_3, triiodothyronine; SDS, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Since both T_3 and EGF are involved in enhancement of growth and maintenance of hepatic function and since T_3 has a demonstrated role in regulation of receptor binding, the effect of the administration of pharmacological amounts of T_3 in vivo on the binding of EGF to normal liver membranes of intact hepatocytes has been examined. Changes in EGF binding have been correlated with changes in EGF-induced membrane phosphorylation. Stimulation of membrane phosphorylation has been suggested as part of the mechanism of action for EGF [9]. Therefore experiments were undertaken to test the hypothesis that there may be a direct correlation between receptor number and phosphorylation of membrane proteins.

Methods

Male Sprague-Dawley rats, purchased from Canadian Breeding Farms, LaPrairie, Quebec, were maintained on Lab-Blox Rat Chow (Allied Mill, Chicago), water ad libitum and a controlled light cycle (12 h light-12 h dark period initiated at 8:30 p.m.) for 2 weeks before and during the subsequent experimental treatment. Rats (200 g) were injected subcutaneously daily for three consecutive days with T_3 (Calbiochem, La Jolla, CA) in alkaline saline (50 μg per 100 g of total body weight). Effectiveness of T_3 treatment was monitored by the observation of a weight reduction over the three day treatment period [3]. The animals were killed and membranes or hepatocytes were prepared at 10:30 a.m. on the fourth treatment day.

Hepatocytes were isolated using the perfusion method of Seglen [15]. Following a 10–30 min digestion with 0.33 mg/ml collagenase (Worthington type II; Worthington Biochemical Corp., Freehold, NJ) in Krebs-Henseleit bicarbonate buffer, the liver was minced and incubated for 10 min with 60 ml of oxygenated Krebs-Henseleit bicarbonate buffer which contained 1% bovine serum albumin (Sigma, St. Louis, MO). The dispersed cells were filtered through a 100 mesh nylon screen and washed three times with the above Krebs-Henseleit buffer and resuspended in the binding assay buffer (defined below). Viability, determined by 0.4% Trypan blue exclusion was greater than 90%. Liver microsomal membranes were prepared from fresh liver by differential centrifugation as described by Hock and Hollenberg [16]. EGF was isolated from frozen mouse submaxillary glands [17] and insulin was a gift from Eli Lilly (Indianapolis, IN). Insulin and EGF were iodinated using the Chloramine T method [18] with ^{125}I purchased from Amersham Corp. (Oakville, Ontario). Radioactivity was measured by crystalline scintillation counting (85% efficiency).

The binding of ^{125}I-labeled peptides was measured by filtration on Millipore EGWP filters (Millipore Corp., Bedford, MA) as previously described [19]. Either hepatic membranes (0.5 mg/ml) or hepatocytes ($0.5 \cdot 10^6$ cell/ml) were suspended in a pH 7.4 buffer containing Earles salts, 20 mM Hepes and 0.1% bovine serum albumin. The ^{125}I-labeled peptides were incubated for 18 h at 4°C with 200 μl of the suspended sample with and without the addition of 100-fold excess unlabeled insulin or EGF. The difference in radioactivity bound in the presence and absence of excess unlabeled peptide was defined as specific peptide binding.

Membrane phosphorylation was studied by incubating isolated hepatic membranes (suspended to 100 μg of protein/100 μl in 20 mM Hepes, 1 mM $MnCl_2$ and 0.1% bovine serum albumin at pH 7.4), at 37°C for 10 min with and without $8.3 \cdot 10^{-8}$ M EGF followed by the addition of 30 μCi of [γ-^{32}P]ATP (New England Nuclear, Boston, MA). The phosphorylation reaction (0°C for 1 min) was terminated by first adding 100 μl of a solution (warmed to 100°C) containing 12.5 mM Tris-HCl, 4% SDS, 1 mM EDTA, 20% sucrose, 0.1% Bromophenol blue, and 4% 2-mercaptoethanol (pH 6.8) followed immediately by incubation for 5 min in a boiling water bath. Aliquots (100 μl) of the denatured membrane proteins were electrophoresed on SDS/7.5% polyacrylamide gels with a 3% stacking gel [20]. The gels were stained with Coomassie blue, destained, dried on Whatman paper and autoradiographed on Kodak X OMAT RP film using 2 Dupont Cornex intensifying screens (DuPont DeNemours & Co., Wilmington, DE) for 14–48 h. Molecular weights were determined using human erythrocyte ghost markers [21]. Radioactive phosphate incorporation into the protein bands separated by electrophoresis were

quantitated by cutting out the bands, digesting the polyacrylamide with 30% hydrogen peroxide, and then liquid scintillation counting in Aquasol II (New England Nuclear, Boston, MA) using a Packard Tri-Carb 400C counter.

Results

The specific binding of ^{125}I-labeled EGF and ^{125}I-labeled insulin to freshly isolated hepatocytes was determined following administration of T_3 to rats for 3 days. As shown in Table I, the amount of specific EGF bound to cells from T_3-treated rats was decreased to 30% of the control. However, binding of insulin to freshly isolated hepatocytes from T_3-treated rats was not altered. Preparations of liver membranes from T_3-treated rats also displayed a suppression of ^{125}I-labeled EGF binding (Table I) which was similar to the decrease observed in isolated hepatocytes. The quantity of insulin bound by rat membranes from T_3-treated animals was unchanged from the controls.

In order to determine if the decrease in EGF binding was due to a decrease in receptor affinity for EGF or a decrease in receptor number, the specific binding of EGF was determined with varying concentrations of EGF (Fig. 1A); Scatchard analysis of these data is shown in the insert to Fig. 1B. The EGF receptors in these hepatic membrane preparations are saturable, and T_3 suppressed the quantity of EGF bound by decreasing the number of receptor sites available for binding from 164 fmol/mg of protein to 106 fmol/mg protein without a change in the affinity of EGF for the liver receptor. Similar analysis of the insulin binding data (data not shown) demonstrated no change in either the receptor number or the affinity of the receptor for insulin.

Incubation of membranes from control (Fig. 2; channel B) and T_3-treated rats (channel D) with $8.3 \cdot 10^{-8}$ M EGF at 37°C followed by a 1 min pulse of [γ-^{32}P]ATP at 0°C resulted in a prompt phosphorylation of two bands with molecular weights of 180000 (180 K) and 165000 (165 K). The amount of ^{32}P incorporated into each band

TABLE I

SPECIFIC BINDING OF ^{125}I-LABELED EGF AND ^{125}I-LABELED INSULIN TO HEPATOCYTES AND HEPATIC MEMBRANES FROM NORMAL AND T_3 INJECTED RATS

Freshly isolated hepatocytes and isolated membranes suspended in Earles-Hepes buffer with 0.1% albumin (pH 7.4) were incubated at 0°C with ^{125}I-labeled EGF (3.0 nM; $8.0 \cdot 10^5$ cpm/pmol) or ^{125}I-labeled insulin (1.2 nM; $1.5 \cdot 10^6$ cpm/pmol) for 18 h with and without 100-fold excess unlabeled peptide. The samples were filtered and washed at 0°C on EGWP filters followed by counting of the filter. Results are mean ± S.E.

Preparation	*n*	Specifically bound ligand (cpm/mg protein)	
		^{125}I-EGF	^{125}I-Insulin
A. Hepatocytes			
Control	3	45500 ± 6200	3540 ± 400
T_3-treated	3	14100 ± 1600	3600 ± 550
B. Hepatic membranes			
Control	4	1550 ± 400	697 ± 165
T_3-treated	4	417 ± 100	576 ± 65

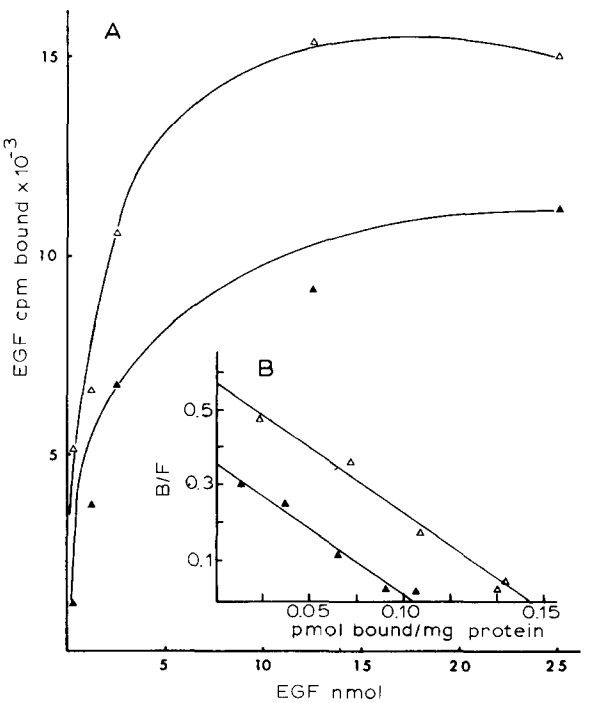

Fig. 1. Typical binding of ^{125}I-labeled EGF to rat liver microsomal membranes as described in Table I. Liver membranes were incubated with various concentrations of ^{125}I-labeled EGF. Specific binding to control membranes (△) and T_3-treated membranes (▲). The inset (B) is a Scatchard plot of the binding data from Fig. 1A; control (△), T_3-treated membranes (▲). *B/F* is bound/free.

was compared with the reduction in regulation of EGF binding to that particular membrane preparation. In each experiment, the reduction in EGF binding due to T_3-treatment correlated well with the reduction in EGF enhanced phosphorylation which was maximized at 1 min. Similar results were obtained with a ^{32}P pulse of 15 s (Table II).

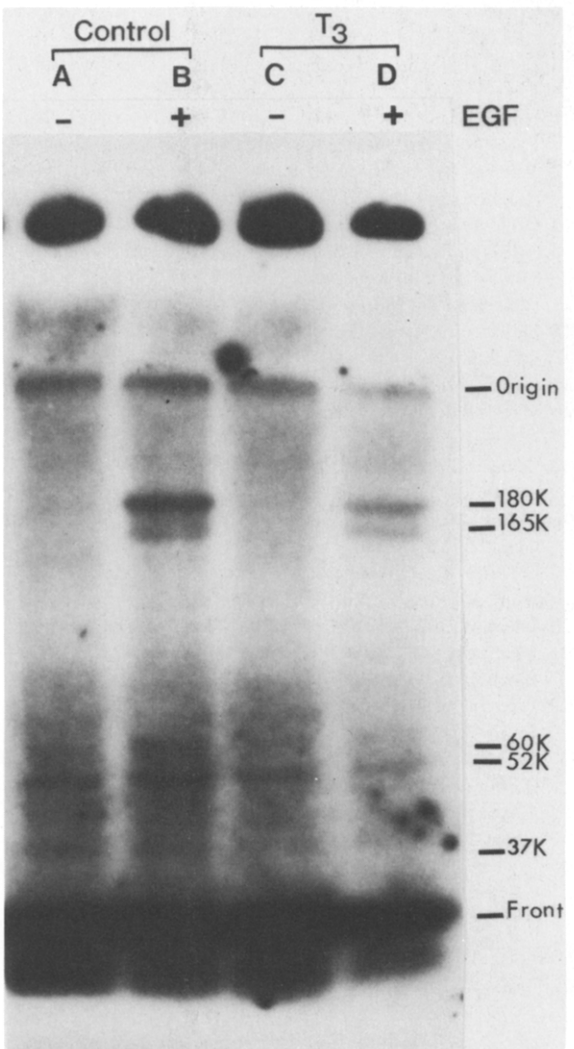

Fig. 2. Autoradiographic analysis of [γ-^{32}P]ATP pulse labeled membranes from conrol liver microsomal membranes (channel A and B) and liver microsomal membranes from rats treated in vivo with T_3 (channels C and D) following separation of the membrane proteins by polyacrylamide electrophoresis. Membranes were pulse labeled as described in the methods following incubation with (channels B and D) and without $8.3 \cdot 10^{-8}$ M egf at 37°C for 10 min.

TABLE II

COMPARISON OF EGF BINDING AND THE PHOSPHORYLATION OF SPECIFIC MEMBRANE PROTEINS

Binding was determined as in Table I. Incorporation is presented in terms of mg of protein applied to each channel.

	EGF bound (cpm/mg protein)	^{32}P incorporated (cpm/mg protein	
		15 s	60 s
Experiment A			
Control	40000	2800	4400
T_3-treated	21200	1350	2700
% reduction	53	48	61
Experiment B			
Control	38000	3400	5600
T_3-treated	15200	1300	2540
% reduction	40	38	45

Using rat microsomal membranes from control and T_3-treated rat, it was possible to demonstrate that isolated control membranes (Fig. 2; channel A) have a phosphorylated protein band with a molecular weight corresponding to 52000 (52 K) following a 1 min pulse labeling with [γ-^{32}P]ATP. Incubation of EGF with control membranes did not result in any change in the level of phosphorylation of the 52 kDa protein band (channel B).

Discussion

These studies demonstrate that in vivo pharmacological doses of T_3 caused a marked decrease in the binding of EGF to rat hepatocytes and isolated rat liver membranes. Furthermore, this reduction was correlated with a decrease in EGF stimulated phosphorylation of membrane proteins. The 180 kDa and 165 kDa phosphorylated proteins have molecular weights which are similar to those reported for the EGF receptor and one of its proteolytically processed products. EGF stimulated the phosphorylation of tyrosine residues in major membrane proteins in regenerating rat liver tissue [8] and with the human carcinoma cell line A431 [23]. Cohen et al. [24] have shown that the EGF receptor copurified with the EGF stimulated protein kinase and the phosphorylated protein. The data from this report suggest that the

receptor, EGF stimulated protein kinase and the tyrosine substrate membrane complex can be regulated by the addition of T_3 and not another non-phosphorylatable receptor which might exist. The failure of T_3 to reduced the insulin binding in isolated microsomal membrane (Table I) would indicate that the loss of EGF receptor is not the result of a generalized alteration in membrane proteins induced by T_3. It is, therefore, possible that T_3 downregulates the EGF receptor mechanism by a specific modulation of the synthesis and/or degradation of the EGF receptor or by synthesis of a specific cellular protein which masks the EGF receptor as in transformed cells [14].

These data also suggest that the downregulation of EGF receptor demonstrated in regenerating rat hepatic tissue may be secondary to T_3 modulation since an intact thyroid was required for maximum rats of liver regeneration [4] and T_3 is required for optimum growth of transplantable Morris hepatomas 7777 [25]. These results are consistent with other studies which indicate that T_3 may have an overall role in control of hormonal response with modulation at the receptor level and of hormonal concentration [2–6].

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