Down regulation of epidermal growth factor receptors in liver proliferation induced by a mixture of triiodothyronine, amino acids, glucagon, and heparin (TAGH)

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Abstract

This study investigated the mechanisms by which TAGH solution (a mixture of triiodothyronine, amino acids, glucagon, and heparin) induces DNA synthesis in hepatocytes in the liver of intact rats, with particular reference to events at the epidermal growth factor (EGF) receptor. Both partial hepatectomy and infusion of TAGH stimulated DNA synthesis at 24 hours and both procedures resulted in a reduction of EGF receptors assessed in plasma membranes isolated from rat liver at this time. In cell cultures, while EGF strongly stimulated DNA synthesis and started EGF receptor down regulation, TAGH had only a minor effect (1.5× basal) on DNA synthesis and did not interact with or down regulate the EGF receptor. Membrane phosphorylation studies, however, showed that TAGH induced phosphorylation of tyrosine residues in the EGF receptor. The in vivo action of TAGH seems to entail recruitment of similar changes in the EGF receptor to those that occur after partial hepatectomy.

(Gut 1993; 34: 1601-1606)

The factors controlling hepatocyte proliferation are complex and incompletely understood.¹ Prominent events at the hepatocyte membrane after induction of DNA synthesis in rat livers by partial hepatectomy include down regulation and phosphorylation of the epidermal growth factor (EGF) receptor.^{2,3} These events can also be seen when EGF is used to start DNA synthesis in hepatocytes in vitro.⁴⁻⁷ Ligand binding to the EGF receptor therefore seems to be integrally associated with liver cell proliferation.

Short *et al*⁸ described the action of TAGH solution – a mixture of triiodothyronine, amino acids, glucagon and heparin – as an initiator of DNA synthesis, hepatocyte mitosis, and also causing an increase in liver size in intact rats. The time course of DNA synthesis after the start of the infusion was the same as that following 70% hepatectomy, enhancement of DNA formation occurring within 15 hours. The mechanism of action of TAGH has not been elucidated. As the constituents of TAGH solution are readily available as pharmaceutical preparations, this offers a potential treatment for severe liver diseases in which hepatic regeneration would be beneficial. We therefore investigated the mode of action of TAGH by comparing in vivo and in vitro phenomena induced in hepatocytes by EGF and TAGH. The findings show that in vivo TAGH

results in EGF receptor down regulation; in vitro hepatocyte cultures TAGH does not induce EGF receptor down regulation, although it was capable of inducing phosphorylation of tyrosine residues of the EGF receptor in liver plasma membrane preparations in vitro.

Materials and methods

ANIMALS

All experiments were performed on male August rats (250–300 g) (National Institute of Medical Research, Mill Hill, London, UK), and all procedures were carried out between 9 00 am and noon.

Seventy per cent hepatectomy was performed as described by Higgins and Anderson⁹ under ether anaesthetic. Sham operations consisted of laparotomy and gentle manipulation of the liver.

The TAGH solution (9 ml) was made up fresh at the time of the experiment and infused by the tail vein from 20 ml hyperdermic syringes mounted in horizontal syringe pumps set to deliver 3 ml/h. The TAGH solution consisted of 100 µg of triiodothyronine (200 µl), 1 mg of glucagon (1 ml), 100 units of heparin (100 µl) and 7.7 ml of an amino acid mixture (Synthamin 14*). Synthamin 14* is a commercial mixture of nine essential and six non-essential L-amino acids (8.5% wt/vol). The solution was adjusted to pH 7.2 with 0.01 M sodium hydroxide.

MATERIALS

[6-³H]-Thymidine (specific activity 25–30 Ci/mmol) and carrier free Na¹²⁵I were obtained from Amersham International PLC, Amersham, Bucks, UK. William's medium E, fetal bovine serum (FBS), penicillin/streptomycin, fungizone (amphotericin B), gentamycin, and Nunclon microwell plates were obtained from Gibco, Paisley, Renfrewshire, Scotland. Insulin (human monocomponent, actrapid) and glucagon were purchased from Novo, Copenhagen, Denmark. Triiodothyronine was from Sigma. Heparin was from Leo Labs Ltd, Princes Risborough, Bucks, UK. Collagenase (*Clostridium histolyticum*) was purchased from Boehringer Mannheim Ltd, Lewes, East Sussex, UK. The amino acid solution, Synthamin 14, was purchased from Travenol Laboratories Ltd, Compton, Berks, UK. Collaborative Research mouse EGF (tissue culture grade) was obtained from Universal Biologicals, London, UK. Other reagents and chemicals were from Sigma

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Accepted for publication
10 March 1993

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IN VIVO ³H-THYMIDINE INCORPORATION

Rats received an intraperitoneal injection of ³H-thymidine (0.5 µCi/g body weight) one hour before death. Twenty four hours after partial hepatectomy or the start of the TAGH infusion, rats were anaesthetised and the liver perfused in place with ice cold saline to remove excess blood. The liver was rapidly removed and placed on ice. A small section of liver was frozen for measurement of thymidine incorporation. The remaining tissue was used to prepare liver cell plasma membranes. ³H-thymidine incorporation into liver DNA was measured biochemically by a modification of the method of Munro and Fleck,¹⁰ as described in Vesey *et al.*¹¹

ISOLATION AND CULTURE OF HEPATOCYTES

Adult rat hepatocytes were isolated using a modification of the Berry and Friend procedure,¹² as described in Vesey *et al.* 1992.¹¹ Cells were resuspended at 3×10⁵ or 2×10⁵ viable cells/ml in William's medium E, without L-glutamine, supplemented with penicillin (200 IU/ml); streptomycin (200 IU/ml); gentamycin (80 µg/ml); fungizone (1.25 µg/ml); 5% (vol/vol) heat inactivated fetal bovine serum and dexamethazone (10⁻⁸ M) (plating medium) and seeded on rat tail collagen (type-1) coated Nunclon 24-well cluster trays or 96-well microtitre tissue culture plates, at densities of 7.5×10⁴/cm² and at 6.25×10⁴/cm² respectively.

PLASMA MEMBRANE PREPARATION

Rat liver plasma membranes prepared by the method of Aronson and Touster¹³ were resuspended in 5 mM HEPES buffer pH 7.5 at approximately 5 mg/ml and stored in liquid nitrogen until used. Protein concentrations were determined by a modification of the method of Lowry¹⁴ using BSA as a standard.

IODINATION OF EGF

EGF was iodinated by a modification of the chloramine-T method to give a specific activity of between 20 to 60 µCi/µg as previously described.¹⁵

EGF BINDING STUDIES

On hepatocytes – binding studies on whole cells were carried out in 24 well cluster trays.¹¹ For analysis of receptor numbers and affinity, cells were incubated with increasing amounts of ¹²⁵I-EGF (30–4000 fmoles) for 2.5 hours, and non-specific binding determined at each point by inclusion of a 1–2 µg/ml of unlabelled EGF in replicate cultures. This was subtracted from total counts bound to obtain specific binding. Points represent the means of triplicate measurements.

On plasma membranes – increasing amounts of ¹²⁵I-EGF (50–3000 fmoles) were incubated with between 60 and 100 µg of membrane protein for

60 minutes.¹¹ Measurements were made in triplicate. Binding data were analysed and curves fitted using the GraphPAD Inplot computer program (San Diego, CA, USA).

IN VITRO ³H-THYMIDINE INCORPORATION

DNA synthesis was assessed in cultured hepatocytes by measurement of ³H-thymidine incorporation into cell DNA after stimulation with insulin (10⁻⁷ M) and EGF (20 ng/ml) or plating medium containing the dilutions of TAGH for 24 hours (see Tables).¹¹ The amount, in quadruplicate, of ³H-thymidine incorporated was assessed biochemically as described by Selden and Hodgson.¹⁶

EGF RECEPTOR PHOSPHORYLATION STUDIES

Phosphorylation studies were carried out essentially as Rubin *et al.*³ The reaction was performed at 0°C in a total volume of 50 µl, containing 50 µg of membrane protein, 50 mM Pipes at pH 7.0, 30 mM MgCl₂, 10 mM 2-mercaptoethanol, and 0.32 mM ethylene glycol BIS-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid. Factors, EGF (5 µg/ml in H₂O), or TAGH solution (10 µl), or water (10 µl) as control were added to the tubes. After 15 minutes preincubation at 0°C the reaction was started by addition of 15 µCi (15 µl) of γ ³²P ATP to a final concentration of 2 mM. It was stopped after one minute with 25 µl of 9% SDS sample buffer (0.0015% bromophenol blue, 10 ml of 2% SDS, 25 mM TRIS pH 6.8, and 20% sucrose). Tubes were sealed and heated for three minutes at 100°C. Aliquots (40 µl) were subjected to electrophoresis on 5% SDS PAGE slab gels.

PHOSPHOAMINO ACID ANALYSIS

The band corresponding to the EGF receptor was excised and the protein eluted from the gel strip in 0.05 M ammonium bicarbonate containing SDS (0.1%) and β-mercaptoethanol (5%) at

TABLE I DNA synthesis in the liver of rats after TAGH infusion

Treatment	³ H-Thymidine incorporation (dpm×10 ⁻⁴ /g liver)*	No
Saline	7.9 (4.8–11.2)	4
Hepatectomy	469 (283–711)	8
TAGH	63.4 (39–118)	8

*Mean (range).

³H-thymidine incorporation in rat liver 24 hours after infusion of TAGH compared with the response to hepatectomy. Thymidine was injected 1 hour before death at 24 hours. Data represent the means (range).

TABLE II EGF binding characteristics of plasma membranes isolated from whole liver 24 hours after 70% partial hepatectomy or TAGH infusion

Treatment	High affinity site	
	B _{max}	K _D
Sham	1138 (132)	1799 (385)
Hepatectomy	627 (278)	1690 (278)
Saline	1463 (114)	1520 (231)
TAGH	1165 (90)	1462 (260)

B_{max} values are given in fmoles/mg membrane protein (SEM) and those of the dissociation constant, K_D, in pM (SEM).

Figure 1: Effect of TAGH induced liver proliferation on liver plasma membrane EGF receptors. Plasma membranes were isolated by the method of Aronson and Touster¹³ from rats 24 hours after the start of TAGH (●) or saline infusion (○). Saturation binding curve (A) and Scatchard analysis (B).

37°C. After hydrolysis in hydrochloric acid (5.7 M) at 110°C it was lyophilised and the sample dissolved in a mixture of the phosphoamino acids, phosphoserine, phosphothreonine, and phosphotyrosine. The phosphoamino acids were separated by high voltage thin layer chromatography on cellulose plates in a mixture of pyridine, acetic acid, and water in the ratio 5:50:945 for 1000 volt hours. Standards were visualised with ninhydrin and the radioactive phosphoamino acids by autoradiography.

Results

IN VIVO STUDIES

Liver cell proliferation was stimulated in vivo by both 70% partial hepatectomy and by an intravenous infusion of 9 ml of TAGH solution over three hours. The mean increase in incorporation of ³H-thymidine into liver compared with controls was 60-fold 24 hours after partial hepatectomy and eightfold after TAGH infusion (Table I).

Figure 2: Effect of time in culture on hepatocyte cell surface EGF receptors. (A) Specific binding of increasing concentrations of ¹²⁵I-EGF by primary hepatocytes in culture at 5 (○), 22 (●), and 30 h (▲) after plating. (B) Scatchard analysis of binding data.

TABLE III Down regulation of EGF receptors; effect of time and EGF

Treatment	¹²⁵ I-EGF bound (fmol/10 ⁵ cells) at various times after start of treatment (h)				
	1	5	10	22	42
Control	24.1 (1.6)	24.0 (0.4)	16.3 (0.7)	8.9 (0.2)	5.2 (0.1)
EGF (5 ng/ml)	3.9 (0.2)	4.6 (0.4)	4.2 (0.2)	3.5 (0.1)	3.7 (0.1)

Cells were plated on collagen coated 24 well plates and allowed to adhere for 2 hours. At this time fresh medium with or without EGF (5 ng/ml) was added to the cells. At various times binding assays were performed on the cells. ¹²⁵I-EGF at 3 nM was used. All cells received an acid wash before the binding assay. Numbers represent the mean (SD) of quadruplicate measurements from a typical experiment. Qualitatively similar results were obtained from five similar experiments.

The well described fall in EGF receptor numbers after partial hepatectomy was confirmed. Scatchard analysis (Table II) of binding data (not shown) show a 45% reduction 24 hours after a 70% hepatectomy. An infusion of TAGH reduced ¹²⁵I-EGF binding to liver plasma membranes, isolated 24 hours after the start of infusion, by 20% compared with saline (Fig 1). Table II shows the EGF binding parameters of these membranes.

IN VITRO STUDIES – HEPATOCYTE CULTURES

Primary cultures of adult hepatocytes showed EGF binding, but with a progressive reduction with time in culture (Fig 2). Although there is a slight reduction in the number of cells per well over the culture period this could not account for the reduction in EGF binding seen. During the period from five to 30 hours in culture the number of receptors per cell fell from 2.3 × 10⁵ to

Figure 3: Autoradiograph of phosphorylated liver plasma membrane proteins from sham operated and partially hepatectomised rats. Membrane phosphorylation was carried out as described in the methods after preincubation with EGF, water, or insulin. Protein (35 µg/well) was subjected to SDS PAGE on a 5% slab gel (Laemmli discontinuous buffer, pH 8.3) and the gel autoradiographed for 1.5 hours at -70°C. Lanes 1-4 control membranes, lanes 5-8 membranes from animals 24 hours after hepatic resection. Lanes 1 and 5; 5 µg/ml EGF, lanes 2 and 6; 20 ng/ml EGF, lanes 3 and 7; insulin 10 µM, lanes 4 and 8; 10 µl water.

Figure 4: Effect of EGF and TAGH on phosphorylation of the EGF receptor in liver plasma membranes from TAGH treated and control rats. TAGH (10%), EGF (5 µg/ml). 30 µg protein loaded per well. Gel was exposed to photographic film for one hour at -70°C .

Figure 5: Autoradiograph of ^{32}P -labelled phosphoamino acids in normal rat liver plasma membrane EGF receptors preincubated with EGF, TAGH, or water. Phospho-serine, -tyrosine, and -threonine were separated along side as standards and visualised with ninhydrin.

TABLE IV DNA synthesis in hepatocytes in primary culture in response to TAGH

Treatment	^3H -Thymidine incorporation (dpm $\times 10^{-3}/2 \times 10^4$ cells)*
No addition	30 (6)
EGF + Insulin	108.6 (18)
TAGH 1/10	27.5 (2.0)
TAGH 1/100	30.9 (1.7)
TAGH 1/200	35.0 (2.6)
TAGH 1/500	41.6 (4.1)
TAGH 1/1000	47.7 (3.5)
TAGH 1/5000	50.9 (2.5)
TAGH 1/10 000	50.1 (7.9)
TAGH 1/10 000 + EGF	98.4 (17)
EGF + Insulin + TAGH 1/10 000	135.8 (32)

Values are the mean and SD of three measurements of a representative experiment. *n=3.

TABLE V Effect of TAGH on cultured hepatocyte cell surface ^{125}I -EGF binding

Treatment	Time preincubated with TAGH (h)			
	2	5	15	23
Control	21 (0.8)	18 (1)	16 (0.9)	10 (0.3)
TAGH 1/5000	18 (1.1)	18 (0.7)	15 (0.7)	11 (0.2)

Mean (SD) from six measurements, n=2. TAGH solution was made up in 9 ml and diluted in medium to give the dilutions shown.

a typical increase in DNA synthesis of eight to 12-fold over basal in the presence of 10 ng/ml EGF.

Addition of TAGH to hepatocytes in culture gave a low level of stimulation of DNA synthesis which did not exceed $1.5 \times$ basal at any concentrations tested (Table IV). TAGH did not compete with binding of labelled EGF, or start down regulation of the EGF receptor on the isolated cells (Table V).

MEMBRANE PHOSPHORYLATION STUDIES

The ability of EGF to induce autophosphorylation of the EGF receptor in plasma membranes isolated from normal rat liver and liver membranes of animals 24 hours after partial hepatectomy and TAGH infusion was compared. Incubation of normal membranes with EGF (5 µg/ml) in the presence of γ ^{32}P labelled ATP, induced a striking enhancement of phosphorylation of the 170 kDa band corresponding to the EGF receptor. Less noticeable phosphorylation of the receptor was seen in response to EGF in liver plasma membranes isolated 24 hours after partial hepatectomy and in TAGH infusion (Figs 3 and 4).

Normal plasma membranes were exposed to TAGH to investigate its effects on membrane phosphorylation. TAGH (1/10 dilution) induced a similar degree of phosphorylation of the 170 kDa band as was induced by EGF (Fig 4).

There was no evidence of enhanced phosphorylation of proteins at other molecular weights by TAGH. Phosphoamino acid analysis performed on acid hydrolysates of the phosphorylated 170 kDa band, showed that both EGF and TAGH specifically induced phosphorylation of tyrosine residues (Fig 5).

Discussion

A substantial body of evidence implicates events

at the EGF receptor as the start of hepatocyte proliferation after partial hepatectomy. EGF can start liver DNA synthesis when infused into intact rats¹⁷ and when added to primary hepatocyte in cultures¹⁶ – these events are associated with EGF receptor phosphorylation and down regulation^{2-5,7}; EGF given to partially hepatectomised rats is targeted in enhanced amounts to the nucleus – the site of maximum hepatic uptake corresponds to those areas of the liver where regeneration is most active¹⁸; partial hepatectomy results in substantial down regulation of the EGF receptor.² Recent studies have suggested that in vivo, at the time of partial hepatectomy, the ligand responsible for down regulation of the EGF is in fact TGF α , which is synthesised in the liver immediately after partial hepatectomy, and is a powerful hepatic mitogen via the EGF receptor.¹⁹⁻²¹

In this study we have induced hepatocyte DNA synthesis in the intact liver with an infusion of TAGH, and shown similar changes in the EGF receptor to those occurring after partial resection. Both partial hepatectomy and TAGH infusion lead to EGF receptor down regulation shown by reduced binding of EGF to liver plasma membranes isolated 24 hours later. In studies on such membranes isolated after partial hepatectomy or TAGH infusion, EGF induced less receptor phosphorylation than in normal membranes: reduced EGF induced autophosphorylation of the EGF receptor in membranes from the livers of hepatectomised rats has previously been attributed to the reduced receptor density on such membranes.³ These findings suggest TAGH infusion leads to a similar process of EGF receptor phosphorylation and internalisation as occurs after partial hepatectomy.

One hypothesis for the similarity of the events started by TAGH and liver resection at the EGF receptor is that TAGH (or a component thereof) directly interacts with the EGF receptor at the hepatocyte membrane. In cell culture conditions, however, in which exogenous EGF can readily be shown to down regulate its receptor and start hepatocyte DNA synthesis, TAGH showed no ability to down regulate the receptor, nor did it competitively inhibit EGF binding. Thus the modest increase in DNA synthesis started in hepatocyte cultures by TAGH (1:5000) is of similar magnitude to that reported for triiodothyronine alone¹⁷ and must act by a pathway other than that of direct interaction with the EGF binding site of the EGF receptor.

One shortcoming of the experiments reported here is that the effect of TAGH in inducing phosphorylation of membranes in vitro was only detected at high doses of TAGH. The necessity for using very high doses of reagents, however, to detect in vitro phosphorylation of membranes, is well recognised. EGF itself is used, by others^{3,7} as well as ourselves, at doses 100 to 1000 times that used for inducing DNA synthesis in cell cultures, because the phosphorylation is not readily detectable at a lower dose. The high dose of TAGH, when used in the cell culture system, did not induce DNA synthesis, but in contrast there was a substantial decrease (by greater than 90%) in amino acid incorporation into protein (data not shown), showing that at those concen-

trations there was local toxicity to the whole cell. It seems, however, very unlikely that 'toxicity' to isolated membranes could induce the specific tyrosine phosphorylation of the EGF receptor that we have shown.

If TAGH can cause EGF receptor phosphorylation not only in isolated liver cell membranes, but in vivo, this may constitute the mechanism by which it stimulates DNA synthesis. TAGH might be acting by enhancing the effect of the physiological levels of ligands of the EGF receptor in the hepatocyte environment by phosphorylation of the EGF receptor. Our cell culture experiments do not support this suggestion as they did not show the synergy between EGF and TAGH that would be predicted, but because of the inhibitory effect of TAGH alone at the concentrations used, they cannot refute this possibility.

As a direct interaction of a component of TAGH with the EGF binding site on the receptor is not apparent, the mechanism by which TAGH leads to EGF receptor phosphorylation is unclear. It is possible that the tyrosine phosphorylation caused by TAGH is not autophosphorylation of the receptor – that is, by activation of the receptor's own tyrosine kinase, but is induced by other tyrosine kinases. Whether the tyrosine phosphorylation pattern of the receptor in response to TAGH is the same as that caused by EGF or TGF α was not investigated. Different sites or patterns of phosphorylation might be expected to result in different secondary events within the hepatocyte. Further consideration of this would also require elucidation of whether TAGH induces similar phosphorylation of the receptor in vivo as occurs on membranes in vitro.

It is possible that the striking proliferative events started by TAGH in vivo reflect secondary actions of TAGH. TAGH might stimulate formation or release of EGF from duodenum, submaxillary glands, or platelets, or stimulate TGF α synthesis or release in the intact liver. This second possibility is under investigation.

This work was supported by grants from The Medical Research Council and the Cancer Research Campaign.

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