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Specific involvement of $G_{\alpha i2}$ with epidermal growth factor receptor signaling in rat hepatocytes, and the inhibitory effect of chronic ethanol

Bao-Hong Zhang[1], Vikki Ho, Geoffrey C. Farrell*

Storr Liver Unit, Department of Medicine and Westmead Millennium Institute, University of Sydney at Westmead Hospital, Westmead, NSW 214 Australia

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Abstract

We have previously shown that chronic alcohol consumption inhibits liver regeneration by impairing epidermal growth factor receptor (EGFR)-operated phospholipase C-$_{\gamma 1}$ (PLC-$_{\gamma 1}$) activation and the resultant rise in intracellular $[Ca^{2+}]_i$. In hepatocytes, activation of PLC-$_{\gamma 1}$ by EGFR requires involvement of a pertussis toxin-sensitive inhibitory guanine nucleotide-binding regulatory protein ($G_{\alpha i}$) as an intermediate. In the present study, we first identified the $G_{\alpha i}$ protein isoform associated with the activated EGFR, and then examined whether the toxic effect of alcohol on EGFR signaling and liver cell proliferation was exerted on this association. In cultured hepatocytes from control rats, EGF rapidly induced association between EGFR and $G_{\alpha i2}$ but not other $G_{\alpha i}$ isoforms. In hepatocytes from rats fed alcohol for 16 weeks, EGF failed to stimulate this association of $G_{\alpha i2}$ with the EGFR. The impairment of EGFR-$G_{\alpha i2}$ complex formation caused by alcohol was associated with a decreased level of $G_{\alpha i2}$ in the plasma membrane fraction (~50% control). Pertussis toxin, an inhibitor of $G_{\alpha i}$ function, produced an analogous disruption of the association between $G_{\alpha i2}$ and the EGFR, as well as inhibiting EGF-induced DNA synthesis. It is concluded that, in hepatocytes, $G_{\alpha i2}$ is specific among $G_{\alpha i}$ isoforms in coupling activation of the EGFR to other signaling pathways that control cell proliferation. Impaired coupling of $G_{\alpha i2}$ of EGFR could contribute to the mechanism by which chronic alcohol exposure inhibits liver regeneration.

Keywords: EGFR signalling; $[Ca^{2+}]_i$; $G_{\alpha i2}$; Alcohol; Hepatocytes; DNA synthesis

1. Introduction

A rise in intracellular calcium concentration {$[Ca^{2+}]_i$} is one of the best characterized signal transduction pathways that is initiated by activation of the EGF receptor (EGFR) following attachment of EGF. Such, activation of EGFR generates inositol 1,4,5-trisphosphate {Ins(1,4,5)P_3} by activating phospholipase C-$_{\gamma 1}$ (PLC-$_{\gamma 1}$). Ins(1,4,5)P_3 elevates $[Ca^{2+}]_i$ by releasing stored Ca^{2+} from the endoplasmic reticulum [1]. This process, combined with receptor-stimulated Ca^{2+} influx, leads to an increase in $[Ca^{2+}]_i$ that is involved with the production of many physiological responses in the cell, including growth and proliferation [2,3].

In most cell types, activation of PLC-$_{\gamma 1}$ is triggered by tyrosine kinase activity intrinsic to the activated EGFR. This process involves a direct EGFR-PLC-$_{\gamma 1}$ interaction [1,4], but in hepatocytes it also requires involvement of a pertussis toxin sensitive inhibitory guanine nucleotide-binding regulatory ($G_{\alpha i}$) protein [5–8], as shown by the association of $G_{\alpha i}$ proteins with EGFR after EGF stimulation [7]. However, the isoform of $G_{\alpha i}$ involved in PLC-$_{\gamma 1}$ activation in hepatocytes has not been characterized.

Alcohol impairs liver regeneration, thereby worsening the prognosis of patients with alcoholic liver disease. Numerous studies have demonstrated experimentally that chronic ethanol consumption inhibits liver regeneration [9–11], but the precise molecular mechanism(s) are poorly understood. We and others have provided evidence that ethanol interferes with growth factor or cytokine-induced signal transduction pathways [12–18]. In particular, we recently postulated that ethanol impairs liver regeneration by abrogating EGFR-operated $[Ca^{2+}]_i$ signaling [18]. The tar-

* Corresponding author. Tel.: 612-98459006; fax: 612-98459100.
E-mail address: geoff_farrell@wmi.usyd.edu.au (G.C. Farrell).
[1]Present address: Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, MI 48109-0606, USA.

Abbreviations: EGFR, epidermal growth factor receptor; PLC-$_{\gamma 1}$, phospholipase C-$_{\gamma 1}$; $G_{\alpha i}$, inhibitory guanine nucleotide-binding regulatory protein; $[Ca^{2+}]_i$, intracellular calcium concentration; PMSF, phenylmethylsulfonylfluoride.

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gets for the toxic effect of ethanol on the $[Ca^{2+}]_i$ pathway appeared to be at or proximal to the level of PLC-$_{\gamma 1}$ activation [18,19], but the exact components affected were not clear.

The objectives of the present study were (i) to identify the isoform(s) of $G_{\alpha i}$ protein associated with EGFR in rat hepatocytes, and (ii) to examine whether the association of EGFR with $G_{\alpha i}$ protein is altered after chronic alcohol consumption. We also demonstrated the importance of the association between EGFR and $G_{\alpha i}$ protein for hepatocyte proliferation by mimicking the inhibitory effect of alcohol with pertussis toxin.

2. Materials and methods

2.1. Chronic alcohol consumption model

Male Wistar rats, initially weighing 180–220 g, were fed commercial rat pellets, with ethanol in the drinking water for 16 weeks as described previously [18]. Control rats were either pair-fed the same diet as ethanol-fed rats where ethanol was isocalorically substituted by dextrose in food, or were allowed *ad lib*. access to commercial rat pellets. This study was approved by the Animal Ethics Committee of the Western Sydney Area Health Service, and experiments conformed to the highest international standards of humane care of animals.

2.2. Isolation and culture of hepatocytes

After 16 weeks on the diets, hepatocytes were isolated from ether-anesthetized rats by non-recirculating collagenase perfusion through the portal vein, as described previously [20]. Freshly isolated hepatocytes were plated onto collagen I-coated culture dishes and then cultured at 37° in a humidified atmosphere of air containing 5% CO_2. Three hours after plating, the medium was changed to remove unattached hepatocytes.

2.3. Preparation of rat liver plasma membrane fraction

Preparation of rat liver plasma membrane fraction was performed as described previously [21], with minor modifications. In brief, livers were perfused with cold normal saline and removed from ether-anesthetized rats, minced and homogenized on ice in lysis buffer (20 mM Tris-HCl, 1 mM EDTA, 10% sucrose, 0.02% NaN_3, 5 μg/mL leupeptin, 0.3 mg/mL phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol, pH 7.4). The resulting homogenates were passed through a 200-μm nylon mesh and the eluent was centrifuged for 5 min at 500 *g* (4°) to remove nuclei and cell debris. Supernatant was collected, carefully layered onto a 44.5% sucrose pad and centrifuged for 40 min at 141,000 *g* (4°) using a SW28 rotor (Beckman L8-80). The interface was collected and washed three times with the wash buffer (20 mM Tris-HCl, 1 mM EDTA, 0.02% NaN_3, 5 μg/mL leupeptin, 0.3 mg/mL PMSF, 1 mM dithiothreitol, pH 7.4). Aliquots were taken for protein determination using a protein assay kit from Biorad (Hercules, CA). The final membrane pellet was diluted with wash buffer and stored at −70° until use.

2.4. Immunoprecipitation and immunoblotting

After 15–18 hr culture, hepatocytes were treated with EGF (200 ng/mL) for various times, as indicated in the figure legends. Cells were chilled on ice and washed twice with ice-cold PBS to terminate the reaction. Cells were lyzed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM NaF, 200 μM Na_3VO_4, and 1 mM PMSF) and harvested after scraping off the plates. Cell lysates were sonicated briefly, clarified by centrifugation and diluted to a protein concentration of 2 mg/mL using RIPA buffer.

Cell lysates were precleared by incubating for 1 hr at 4° with 50 μL of a 50% slurry of protein A-Sepharose, after which the beads were pelleted. Precleared samples were incubated with the appropriate primary antibodies for 45 min followed by incubation by rotation with 50 μl of 50% protein A-Sepharose for 1 hr at 4°. Immunoprecipitates were washed three times with ice-cold RIPA buffer and once with PBS. Immunoprecipitates or plasma membrane samples were denatured in Laemmli sample buffer (250 mM Tris, pH 6.8; 5% SDS; 25% glycerol; 0.002% bromophenol blue; and 50 mM dithiothreitol) and resolved by 12% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to nitrocellulose membranes. Blots were blocked with 5% milk powder in TBST buffer (50 mM Tris, pH 7.4; 200 mM NaCl; and 0.1% Tween-20). The blocked nitrocellulose membranes were probed with primary antibodies followed by horseradish peroxidase (HRPO)-linked anti-rabbit or anti-mouse secondary antibodies. Blots were developed by the chemiluminescence (ECL) method and semi-quantified by scanning laser densitometry.

2.5. ADP-ribosylation of $G_{\alpha i}$ by pertussis toxin

Pertussis toxin catalyzed ADP-ribosylation of $G_{\alpha i}$ was performed as described by Crouch *et al* [22], with minor modification. Pertussis toxin was preactivated in HEPES buffer (mM: HEPES, 20 [pH 7.4], NaCl, 138; KCl, 2.9; NaH_2PO_4, 3.3; $MgCl_2$, 1.0) containing 40 mM dithiothreitol and 2 mM ATP for 30 min at 37°. EGFR-$G_{\alpha i}$ co-immunoprecipitates were incubated with a medium containing 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 mM thymidine, 5 μM NAD^+, 50 μM $NADP^+$, 0.5 μCi/mL [^{32}P]NAD, 20 mM dithiothreitol, 1 mM ATP and 20 μg/mL preactivated pertussis toxin for 90 min at 30°. The reaction was terminated by addition of 2× SDS-PAGE sample buffer followed

by boiling for 5 min. Proteins were separated using 12% SDS-PAGE. ^{32}P-Labeled $G_{\alpha i}$ proteins were visualized by autoradiography.

2.6. RNA isolation and Northern blot hybridization

Total hepatic RNA was extracted from ~200 mg liver and its concentration was determined spectrophotometrically at 260 nm. RNA (20 μg) was electropheresed in 1% agarose gel containing formaldehyde, transferred to hybond N^+ membrane and hybridized to a [γ-^{32}P] ATP end-labeled oligodeoxynucleotide $G_{\alpha i2}$ probe (5′-CGACAGGTCTTC-CGGAAGCATGCCTTG-3′), which is complementary to bases encoding amino acids 108–116. Assessment of the RNA loading accuracy and transfer efficiency were estimated by hybridizing membranes with ^{32}P-labeled oligonucleotide complementary to 18S rRNA (5′-CGGCATGTAT-TAGCTCTAGAATTACCACGA-3′).

2.7. DNA synthesis

DNA synthesis was determined by a [^{3}H]thymidine incorporation assay, essentially as described previously [20]. Hepatocytes were pretreated with pertussis toxin (100 ng/mL) for 3 hr followed by addition of EGF (10 ng/mL) to the cell culture. [^{3}H]thymidine (2 μCi/mL, sp act 40 Ci/mmol) was added to the medium 48 hr after addition of EGF, and hepatocytes were harvested 24 hr later for DNA extraction. The specific activity of the extracted DNA was determined with an LKB RackBeta (Model 1215) liquid scintillation spectrometer with dpm package (LKB, Turka, Finland); an external standard and the channels ratio method was used to correct for quenching. Values were expressed as disintegrations per minute per microgram of DNA (dpm/μg DNA).

2.8. Expression of data and statistical analysis

Results are expressed as mean ± SD. Analysis of variance (ANOVA) and Fisher's least significant method were used for comparisons among more than two means, and the Student's *t* test was used for comparison between two means. A *P*-value less than 0.05 was considered to be significant.

3. Results

3.1. Specific interaction of $G_{\alpha i2}$ with EGFR in hepatocytes from ethanol-fed and pair-fed rats

$G_{\alpha i}$ has been implicated in mediating EGFR signalling in hepatocytes (5–8). To examine the isoforms of $G_{\alpha i}$ involved, hepatocytes were treated with EGF (200 ng/mL) for various times and the levels of four individual pertussis toxin-sensitive G_α isoforms ($G_{\alpha 0}$, $G_{\alpha i1}$, $G_{\alpha i2}$, and $G_{\alpha i3}$) were determined in immunoprecipitates of EGFR. EGFR immunoprecipitates were separated by SDS-PAGE and blotted with individual antibodies to these isoforms. Autographs were quantified by scanning laser densitometry and the results are expressed as fold increase over unstimulated cells from EF rats in individual experiments. $G_{\alpha 0}$ and $G_{\alpha i3}$ were not detected in EGFR immunoprecipitates from unstimulated or EGF-stimulated hepatocytes (data not shown), whereas $G_{\alpha i1}$ (Fig. 1A) and $G_{\alpha i2}$ (Fig. 1B) were regularly co-immunoprecipitated with EGFR. The amounts of $G_{\alpha i1}$ were not altered by EGF stimulation (Fig. 1A). In contrast, addition of EGF stimulated the association of $G_{\alpha i2}$ with EGFR in a time-dependent manner (Fig. 1B), peaking at 1 min. These changes in $G_{\alpha i2}$ in EGFR immunoprecipitates were not attributable to protein loading of EGFR, since Western blotting indicated equal loading of each sample (see Fig. 2C and 2D). These results indicate that $G_{\alpha i2}$ is the only isoform involved in EGF-induced signaling through the EGFR.

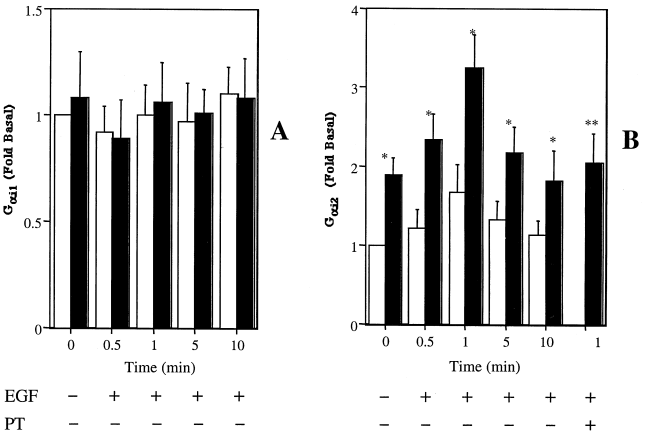

Fig. 1. Specific interaction of $G_{\alpha i2}$ with EGFR in hepatocytes from ethanol-fed and pair-fed rats. Equal amounts of cell lysates from ethanol-fed (EF) and pair-fed (PF) rats were immunoprecipitated with anti-EGFR antibody. Proteins were separated by SDS-PAGE, transferred to nitrocellulose and blotted with specific antibodies for $G_{\alpha i1}$ or $G_{\alpha i2}$. Autographs were quantified by scanning laser densitometry, and the results for $G_{\alpha i1}$ (A) and $G_{\alpha i2}$ (B) were presented as fold increase over unstimulated cells from EF rats. EF: □. PF: ■. The data shown represent the mean ± SD from at least three separate experiments. PT: pertussis toxin. Hepatocytes were pretreated with 100 ng/mL pertussis toxin for 3 hr followed by EGF stimulation for 1 min.

To determine whether chronic ethanol consumption alters the interactions between EGFR and individual $G_{\alpha i}$ isoforms, the levels of $G_{\alpha i1}$ and $G_{\alpha i2}$ in EGFR immunoprecipitates were determined in hepatocytes prepared after different treatments. As shown in Fig. 1A, there were no differences at any time point in the amount of $G_{\alpha i1}$ associated with EGFR between hepatocytes from ethanol-fed and pair-fed controls (compare open and shaded bars in Fig. 1A). However, hepatocytes from ethanol-fed rats exhibited lower basal levels of $G_{\alpha i2}$ in EGFR immunoprecipitates (Fig. 1B-compare the open with the shaded bar at time 0, that is without EGF stimulation). Further, while addition of EGF was followed by an increase in $G_{\alpha i2}$ associated with

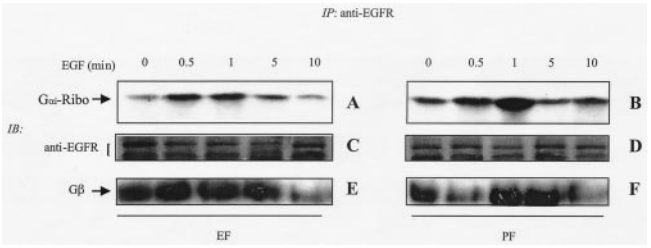

Fig. 2. ADP-ribosylation of $G_{\alpha i}$ in EGFR-$G_{\alpha i}$ co-immunoprecipitates from ethanol-fed and pair-fed rat hepatocytes. EGFR was immunoprecipitated from ethanol-fed (EF) and pair-fed (PF) rat hepatocytes at different time points after EGF stimulation. A, B: ADP-ribosylation of $G_{\alpha i}$ in EGFR immunoprecipitates catalyzed by pertussis toxin in EF and PF rats, respectively. C, D: Western blotting of EGFR in EGFR immunoprecipitates from EF and PF rats, respectively. E, F: levels of G_{β} in EGFR-$G_{\alpha i2}$ complex in EF and PF rats.

EGF immunoprecipitates from both experimental groups, the amount detected in hepatocytes from ethanol-fed rats was considerably less than in controls (Fig. 1B, compare open with shaded bars at each time point). There was no significant difference in the amount of $G_{\alpha i1}$ or $G_{\alpha i2}$ associated with EGFR between pair-fed and *ad lib*.-fed rat hepatocytes (data not shown).

If $G_{\alpha i2}$ is the isoform specifically associated with the EGFR, treatment with pertussis toxin, an inhibitor of $G_{\alpha i}$ function, should perturb this involvement. To address this, hepatocytes were pretreated with 100 ng/mL pertussis toxin for 3 hr followed by EGF stimulation for 1 min. As shown in Fig. 1B, treatment with pertussis toxin (PT) appreciably reduced the amount of $G_{\alpha i2}$ associated with EGFR following addition of EGF ($P < 0.01$, compared with the value of PF cells at 1 min after EGF stimulation).

3.2. ADP-ribosylation of $G_{\alpha i}$ in EGFR-$G_{\alpha i}$ complex from ethanol-fed and pair-fed rat hepatocytes

$G_{\alpha i}$ is a substrate for ADP-ribosylation, and ADP-ribosylation of $G_{\alpha i}$ can be catalyzed by pertussis toxin. To investigate whether the $G_{\alpha i2}$ associated with EGFR maintains its substrate specificity for pertussis toxin mediated ADP-ribosylation, and to determine the effect of chronic alcohol consumption on this, EGFR-$G_{\alpha i}$ co-immunoprecipitates were incubated with [^{32}P]NAD and activated pertussis toxin. The products were separated on SDS-PAGE, and ADP-ribosylation of $G_{\alpha i}$ was then determined by autoradiography. As shown in Fig. 2, the changes in ADP-ribosylation of $G_{\alpha i}$ following addition of EGF to hepatocytes were identical to those of $G_{\alpha i2}$ protein accumulation in EGFR immunoprecipitates. Similarly, the increase in ADP-ribosylation of EGFR-$G_{\alpha i}$ complexes in hepatocytes from ethanol-fed rats was greatly reduced compared to controls (Fig. 2A vs 2B). Western blotting results demonstrated the equal EGFR protein loading of each sample used for G_{α} ribosylation assays (Fig. 2C and D). These results indicate that the $G_{\alpha i2}$ associated with EGFR retains its substrate specificity for pertussis toxin ADP-ribosylation.

To examine whether the increased ribosylation of $G_{\alpha i2}$ at 1 min after EGF stimulation in control hepatocytes was due to the changes in amount of $G_{\beta\gamma}$ associated with $G_{\alpha i2}$, we examined the levels of $G_{\beta\gamma}$ in the EGFR-G complex by probing the EGFR immunoprecipitates with anti-G_{β} antibody. As shown in Fig. 2F, in control hepatocytes, the levels of G_{β} in the $G_{\alpha i}$-EGFR complex decreased at 30 sec after EGF stimulation. The amount of G_{β} recovered to the pre-stimulation level after 1 min and reduced again at 10 min. A similar pattern was obtained for G_{β} in ethanol-fed hepatocytes except that there was only a single decrease in G_{β} at 10 min after EGF stimulation (Fig. 2E). These results demonstrate that the increased ADP-ribosylation of $G_{\alpha i2}$ was not because of the changes in the amount of $G_{\beta\gamma}$ associated with $G_{\alpha i2}$.

3.3. Expression of $G_{\alpha i}$ isoforms in liver plasma membrane fractions from ethanol-fed and pair-fed rats

It remained possible that the decreased association of $G_{\alpha i}$ with EGFR in chronically ethanol-fed rat hepatocytes was attributable to a nonspecific, generalized decrease in G_{α} protein expression by alcohol. To test this, the expression of four different pertussis toxin-sensitive G_{α} proteins was determined in plasma membrane fractions from four rats in each group. As shown in Fig. 3, there were no differences in expressions of $G_{\alpha i1}$, $G_{\alpha i3}$ and $G_{\alpha 0}$ between ethanol-fed and pair-fed rats. However, levels of $G_{\alpha i2}$ in plasma membrane fractions from ethanol-fed rats were reduced approximately 50% compared with pair-fed rats (Fig. 3B). There were no significant differences in any isoforms of G_{α} proteins between pair-fed and *ad lib*.-fed rats (data not shown).

3.4. $G_{\alpha i2}$ mRNA expression in livers from ethanol-fed and pair-fed rats

Next we examined whether $G_{\alpha i2}$ protein was down-regulated at the pretranslational level, $G_{\alpha i2}$ mRNA was extracted from three individual rats in ethanol-fed, pair-fed or *ad lib*.-fed group, and $G_{\alpha i2}$ mRNA levels were determined using Northern blotting. As shown in Fig. 4, hepatic levels of $G_{\alpha i2}$ mRNA were not altered by long-term ethanol consumption compared with pair-fed or *ad lib*.-fed rats.

3.5. Effects of pertussis toxin on EGF-induced hepatocyte proliferation

The overall results demonstrate that chronic ethanol consumption inhibits EGFR-$G_{\alpha i2}$ interactions in rat hepatocytes. To elucidate whether this effect on $G_{\alpha i2}$ could also contribute to the impairment of hepatocyte proliferation produced by alcohol, control hepatocytes were pretreated with pertussis toxin for 3 h at a concentration of 100 ng/ml, the concentration shown earlier to impair the association of $G_{\alpha i2}$ with EGFR (Fig. 1B). As shown in Fig. 5, such pre-

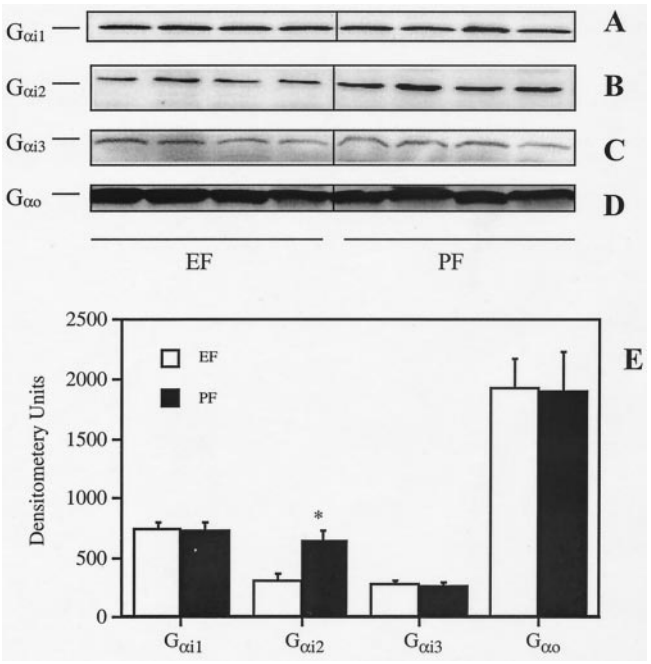

Fig. 3. Levels of $G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$ and $G_{\alpha 0}$ protein in liver plasma membrane fractions from ethanol-fed and pair-fed rats. Liver plasma membranes were extracted from 4 ethanol-fed (EF) and 4 pair-fed (PF) rats as described in "Methods". Proteins from equal amounts of plasma membrane fractions were separated by SDS-PAGE and blotted by antibodies to $G_{\alpha i1}$ (A), $G_{\alpha i2}$ (B), $G_{\alpha i3}$ (C) and $G_{\alpha 0}$ (D) isoforms. Autographs were quantified by scanning laser densitometery and the results were expressed as densitometry units (E). Results are mean ± SD from four different membrane preparations. $*P < 0.01$, as compared with the corresponding EF group.

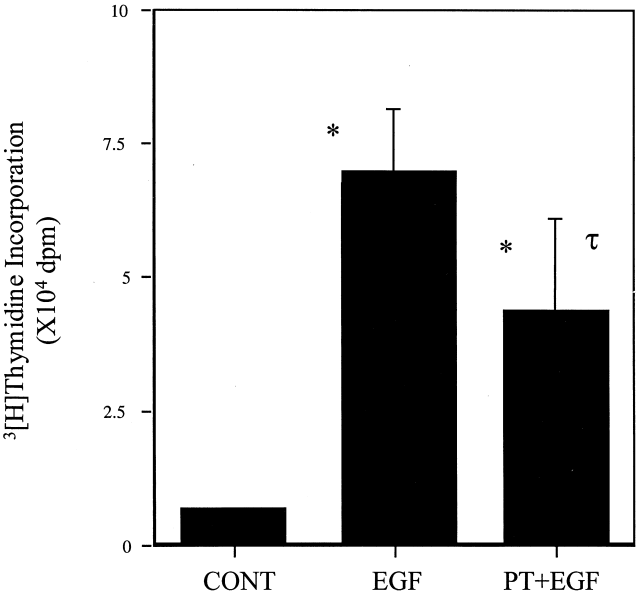

Fig. 5. Effects of pertussis toxin on EGF-induced hepatocyte proliferation. Hepatocytes from *ad lib*.-fed control rats were treated with 100 ng/mL pertussis toxin (PT) for 3 hr followed by stimulation with of EGF (10 ng/mL). 48 hr after stimulation, [*methyl*-^{3}H]thymidine (2 μCi/mL) was added to the cell culture. Incubation was continued for a further 24 hr, after which cells were processed for determination of [^{3}H]thymidine incorporation into hepatic DNA. Data shown are mean ± SD, with group sizes of 6 to 14. CONT, no additions to cells; EGF, addition of EGF; PT + EGF, pertussis toxin (100 ng/mL for 3 hr) added before EGF. $*P < 0.001$, compared with control. $\tau P < 0.005$, compared with EGF.

treatment with pertussis toxin significantly ($P < 0.005$) impaired EGF-stimulated hepatocyte proliferation.

4. Discussion

In many cell types, including fibroblasts and EGFR overexpressing A431 cells [23,24], PLC-$_{\gamma 1}$ is activated directly by the intrinsic tyrosine kinase of the EGFR upon EGF stimulation. In contrast, in several highly differentiated cell types, including hepatocytes [5–8], inner medullary collecting tubule cells [25,26] and pancreatic acinar cells [27], EGF-induced PLC-$_{\gamma 1}$ activation appears to be more complicated. For instance, in these cells a pertusis toxin-sensitive $G_{\alpha i}$ protein is required for EGFR-induced PLC-$_{\gamma 1}$ activation. In hepatocytes, $G_{\alpha i}$ proteins are known to be involved in EGFR signaling as coupling intermediates for PLC-$_{\gamma 1}$ activation by attaching to EGFR [7]. However, the exact isoform(s) of $G_{\alpha i}$ proteins that are involved has not been characterized to date. Results in the present study clearly demonstrate that $G_{\alpha i2}$ is the only form specifically involved in EGFR signalling in hepatocytes.

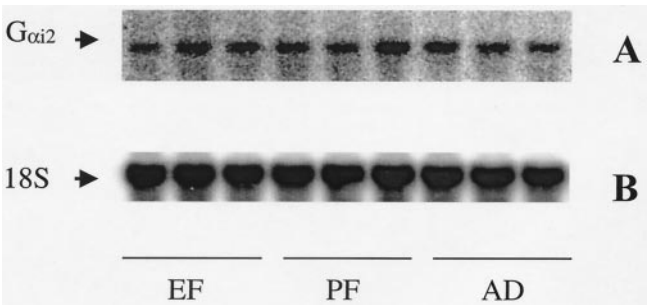

Fig. 4. $G_{\alpha i2}$ mRNA levels in livers from ethanol-fed and pair-fed rats. Total RNA was prepared from livers of three individual rats in ethanol-fed (EF), pair-fed (PF) or *ad lib*.-fed (AD) groups. 20 μg of total RNA was separated on a 1% formaldehyde agarose gel and probed with radiolabeled oligodeoxynucleotide $G_{\alpha i2}$ probe (A). Membranes were stripped and reprobed with 18 S rRNA to assess equal loading of sample (B). There were no differences between the experimental groups.

The mechanism by which $G_{\alpha i2}$ protein interacts with EGFR to form EGFR-$G_{\alpha i2}$ complexes, and how $G_{\alpha i2}$ is involved in activation of PLC-$_{\gamma 1}$, are poorly understood. Liang and Garrison [24] proposed that EGFR might interact with $G_{\alpha i2}$ protein through a short intracellular sequence in the receptor, a hypothesis which is supported by the observation of Okamoto *et al* that a small region in the intracellular domain of insulin-like growth factor-II is capable of directly activating $G_{\alpha i2}$ protein [28]. An interesting finding in the present study was that there were detectable levels of $G_{\alpha i1}$ and $G_{\alpha i2}$ associated with the unactivated EGFR (Fig. 1), indicating that $G_{\alpha i1}$ and $G_{\alpha i2}$ are structurally connected to EGFR in resting hepatocytes.

The increased association of $G_{\alpha i2}$ with EGFR after EGF stimulation suggests that activation of the EGFR promotes its association with $G_{\alpha i2}$, which may then act as a permissive factor for PLC-$_{\gamma 1}$ activation. This proposal is supported by previous indirect evidence that pretreatment of hepatocytes with pertussis toxin abolishes activation of PLC-$_{\gamma 1}$ [5–8]. It is also consistent with the observation in the present study that decreased expression or inactivation of $G_{\alpha i2}$ was associated with disruption of EGFR-$G_{\alpha i2}$ complex formation in hepatocytes from ethanol-fed rats. Further reconstitution studies using purified EGFR and $G_{\alpha i2}$ are required for a clearer understanding of the physical mechanisms by which EGFR-$G_{\alpha i2}$ is formed after EGF stimulation of hepatocytes.

Previous studies have shown that effects of chronic alcohol on $G_{\alpha i}$ expression vary between cell types, and between *in vivo* and *in vitro* studies in the liver. For instance, in the N1E-115 related neuroblastoma cell line, chronic alcohol exposure increased the level of $G_{\alpha i}$ protein, whereas in the N18TG2 parental cell line, alcohol produced no change in $G_{\alpha i}$ [29]. In primary rat hepatocyte cultures, chronic alcohol diminished $G_{\alpha i}$ protein expression [30], but $G_{\alpha i}$ was not altered in liver membrane fractions after chronic alcohol feeding [21,31]. An interesting observation of the present study is that ethanol selectively down-regulated $G_{\alpha i2}$ at the post-transcriptional level without affecting other pertussin toxin-sensitive G_{α} proteins. The latter is generally in agreement with previous studies that chronic alcohol consumption does not alter the general $G_{\alpha i}$ protein after chronic ethanol feeding [31]. However, our results in the present study that $G_{\alpha i2}$ is specifically decreased conflict with those observed by Diehl *et al*., who noted that $G_{\alpha i2}$ was not altered after chronic ethanol feeding [21]. The reasons for this discrepancy are not clear, but one explanation may be the duration of ethanol feeding, which was longer in the present study.

The reduced expression of $G_{\alpha i2}$ and the resultant decrease in association of EGFR and $G_{\alpha i2}$ could be relevant to the molecular mechanisms by which chronic alcohol consumption impairs hepatocyte proliferation. Several lines of evidence support this notion. First, long-term alcohol feeding in rats reduces hepatic $G_{\alpha i2}$ expression (Fig. 3), interrupts EGFR-$G_{\alpha i2}$ complex formation (Fig. 1) and impairs liver regeneration [18]. Second, pertusis toxin, which dissociates $G_{\alpha i2}$ from EGFR, also inhibits hepatocyte proliferation (Fig. 5). Third, reduced expression of $G_{\alpha i2}$ protein impairs normal growth and development of the liver [32]. Lastly, a potential role for $G_{\alpha i2}$ in cell growth has also been recognized in other cell types [33]. For example, transfection of activated mutant forms of $G_{\alpha i2}$ induced transformation in NIH 3T3 cells [34,35], whereas transfection of a dominant-negative mutant inhibited cell growth in murine melanoma K-1735 cells [36] and the murine macrophage cell line BAC 1.2F5 [37].

In summary, the present results provide the first evidence that $G_{\alpha i2}$ is the isoform specifically associated with the activated EGFR in hepatocytes. Chronic ethanol consumption diminished the association between EGFR and $G_{\alpha i2}$, a phenomenon that appeared to be proportional to a specific decrease in $G_{\alpha i2}$ protein levels in ethanol-fed rats. The essential role of $G_{\alpha i2}$ in hepatocyte proliferation was given credence by studies in which disruption of EGFR-$G_{\alpha i2}$ association by pertussis toxin resulted in inhibition of hepatocyte proliferation.

Acknowledgments

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