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Role of ErbB2 in the prostaglandin E_2-induced enhancement of the mitogenic response to epidermal growth factor in cultured hepatocytes

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ABSTRACT

Prostaglandin E_2 (PGE_2) enhances the mitogenic response to epidermal growth factor (EGF) in hepatocytes, but the underlying mechanisms are not clear. We previously observed that PGE_2 upregulates EGF-induced signalling in the MEK/ERK and PI3K/Akt pathways in hepatocytes. Other investigations have indicated that ErbB2 enhances the mitogenic effect of EGF in these cells. In the present study we found that treatment with PGE_2 increased ErbB2 and decreased ErbB3 expression at both the mRNA and protein level in cultured rat hepatocytes. Silencing of the ErbB2 expression with specific siRNA blocked the stimulation by PGE_2 and EGF of cyclin D1 expression and DNA synthesis. Both EGF and PGE_2 increased the expression of ERK and Akt, but while the effect of EGF was inhibited by ErbB2-directed siRNA, this did not affect the PGE_2-induced upregulation of ERK and Akt. These data suggest that PGE_2 can enhance the mitogenic effect of EGF both by increasing ErbB2 expression and by ErbB2-independent mechanisms.

1. Introduction

Adult hepatocytes are normally quiescent in vivo, but quickly enter the cell cycle and proliferate when needed [1,2]. This proliferative response is regulated by the concerted action of a number of growth factors, hormones, and locally acting molecules [3–5].

Studies in vivo and in vitro have shown that the epidermal growth factor (EGF) receptor (EGFR) [6] has an essential role in hepatocyte proliferation [4,7–9]. EGFR belongs to the ErbB family of receptor tyrosine kinases, comprising EGFR (ErbB1), ErbB2, ErbB3, and ErbB4 [10]. Fetal hepatocytes express EGFR, ErbB2, and ErbB3, but ErbB2 disappears by the time of weaning, and adult liver expresses EGFR and ErbB3, but not ErbB2 or ErbB4 [11]. However, ErbB2 re-emerges upon partial hepatectomy and in hepatocytes in primary culture, while the expression of ErbB3 decreases [12,13]. Insulin given early during culturing, which enhances the response to EGF [8,14], also increases the expression of ErbB2 in hepatocytes [12].

The mitogenic response of hepatocytes to EGF is also amplified by several agents that act through G-protein-coupled receptors (GPCRs), including several prostaglandins [4]. In regenerating liver the prostaglandin levels increase, and inhibition of prostaglandin synthesis suppresses the regenerative response [15,16]. In cultured hepatocytes, several prostaglandins act in comitogenic synergism with EGF [17–19]. In these cells, prostaglandin E_2 (PGE_2) exerts its growth-promoting effect early in the G1 phase of the cell cycle, primarily through the EP3 prostanoid receptor [20,21]. The downstream mechanisms that link PGE_2 to enhanced EGF receptor signalling and mitogenic response are not clear. PGE_2-enhanced cyclin D1 expression and DNA synthesis are associated with upregulation of the responsiveness of the PI3K/Akt and MEK/ERK signalling pathways, and several observations, including a latency time of several hours, suggest that this effect of PGE_2 is a result of transcriptional regulation [19,22]. While prostaglandins, like other GPCR agonists, may alter the expression of several proliferation-related genes in hepatocytes [22–25], the mechanisms underlying the comitogenic effect have not been identified. It has been reported that, under other conditions, not involving stimulation with PGE_2, expression of Akt and ERK can be upregulated in hepatocytes in an ErbB2-dependent manner [26].

We have investigated the effect of PGE_2 on the expression of ErbB family members, especially ErbB2, and examined the possible role of ErbB2 in the effect of PGE_2 on EGF-mediated proliferation in primary cultures of hepatocytes.

Abbreviations: PGE_2, prostaglandin E_2; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; PI3K, phosphoinositide 3-kinase; GPCR, G-protein-coupled receptor; RT-PCR, reverse transcription polymerase chain reaction.

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2. Materials and methods

2.1. Materials

Prostaglandin E_2 was obtained from Cayman Chemical Company (MI, USA). EGF was from Sigma Chemical Co. (MO, USA). Antibodies against EGF receptor (sc-03), ErbB2 (sc-284), ErbB3 (sc-285) and ErbB4 (sc-283) were from Santa Cruz Biotechnology (CA, USA). The sources of other materials have been described [19,21,22].

2.2. Isolation and culture of hepatocytes

Rat hepatocytes were isolated by in vitro collagenase perfusion and low speed centrifugation and cultured in a defined serum-free medium that routinely was supplemented with insulin and dexamethasone as described [7,18,27,28], but insulin was omitted in some of the experiments, as indicated below.

2.3. Transfection with small interfering RNA (siRNA)

ErbB2 siRNA was obtained from Dharmacon Inc. (IL USA) as a set of 4 different siRNAs. The efficacy of the siRNAs to repress their target was validated with qRT-PCR and immunoblotting, and the two most efficient siRNAs for ErbB2 were used in this study: siRNA5: Sense; GGUAGACGCUGAAGAGUAUUU, antisense: 5′-P-AUACUCUUCA GCGU CUACCUU. siRNA7: sense; GACAGAAGAUCCG GAAGUAUU, antisense: 5′-P-UACUUCCGGAUCUUCUGUCUU. *Transfection:* Hepatocytes were cultured in standard medium for 3 h before transfection. Then the cells were transfected in the absence of antibiotics by adding 200 μl DMEM with 5 μl of siRNA (20 μM) and 2 μl Lipofectamine 2000™ (Invitrogen, CA) to a final siRNA concentration of 80 nM for 4 h before the medium was changed back to the standard medium with antibiotics.

2.4. Immunoblotting

Aliquots of 10–20 μg cell protein (prepared in Laemmli buffer) were separated in 8–10% polyacrylamide (30:1 acrylamide: *N′,N′*-bis-acrylamide) by gel electrophoresis (SDS–PAGE), before transfer to nitrocellulose membranes. Equal loading was verified by Ponceau S staining. Membranes were blocked with 5% low fat dry milk and 0.05% Tween-20 in T-TBS (10 mM Tris–HCl, pH 8.0, 150 mM NaCl). Incubation with primary antibodies was performed overnight at 4 °C, followed by incubation with secondary antibodies conjugated to horseradish peroxidase (HRP). The bands were visualized with LumiGLO Reserve Chemiluminescent Substrate Kit (KPL, Kirkegaard & Perry Laboratories, MD, USA) and digital images were captured with EpiChemi 2 Darkroom (UVP, Cambridge, UK). Densitometric analysis was done with the LabWorks 4.6 software (Cambridge, UK). Membranes were stripped in 0.5 M NaOH, blocked and reprobed with another antibody if needed.

2.5. Measurement of DNA synthesis

[^{3}H] Thymidine was added to the cultures (1 μCi/ml, 0.125 Ci/mmol) at 24 h after plating. DNA synthesis was assessed by measuring the amount of radioactivity cumulatively incorporated into DNA, as previously described [18]. Protein was determined using Coomassie Plus Protein Assay (Pierce, Rockford, IL, USA).

2.6. Quantitative realtime RT-PCR (qRT-PCR) mRNA-isolation

RNA was isolated with QIAGEN RNeasy kit (Cat. No. 74106) according to manufacturer's description. RNA was treated with DNAse (RNase-Free DNase Set, QIAGEN). *cDNA synthesis:* cDNA was synthesized from 2.5 or 5 μg RNA with Superscript® III reverse transcriptase (Invitrogen, CA) with oligo(dT) as primer according to manufacturer's recommendation. *Primers:* Primer sequences were designed according to guidelines given by Bustin [29]. Primers used in this study with NCBI reference sequences: ErbB1 (NM_031507.1); forward 5′-CGTA-CAGCGCCACCTGACGG-3′, reverse 5′-TCGGAACCTGTGGCAGACCAGA-3′. ErbB2 (NM_017003.2); forward 5′-GACATTGATGAGACAGAGTA CC-3′, reverse 5′-ACACAGTCACTCCATAGCTC-3′. ErbB3 (NM_01721 8.2); forward 5′-TAGTGGTGAAGAACAATGGTG-3′, reverse 5′-GATCGT CTTGGTCAATATCTGG-3′. ErbB4 (NM_021687.1); forward 5′-TAT-CCCACCTCCTATCTACAC-3′, reverse 5′-TACACAAACTGACTTCCCGA-3′. Rpl4 (NM_022510.1), forward 5′-AAGATGATGAACACAGACCT-3′, reverse 5′-AAGTTTGTGATTCCTAGCCT-3′. Rpl32 (NM_013226.2); forward 5′-AGGACCAAGAAGTTCATCAG-3′, reverse 5′-TTCTTGTTA CTCC CGTAACC-3′. GAPDH (NM_017008.3); forward 5′-CCAAGGTCATC-CATGACAACTT-3′, reverse 5′-AGGGGCCATCCACAGTCTT-3′. *Real-time quantitative PCR:* PCR assays were analyzed with an Applied Biosystems 7900HT Fast Real-Time PCR System using Platinum SYBR Green qPCR Supermix-UDG with ROX (Cat. No. 11744, Invitrogen, CA). Data were analyzed with the SDS software (ver. 2.2, Applied Biosystems), cycle of threshold (C_t) and variation in baseline were calculated from each amplification plot. Based on the C_t value and standard curves the relative input amount of mRNA was calculated. The data were normalized using several internal control genes as described [30]. GADPH, Rpl4 and Rpl32 were selected as internal control by the geNorm™ software (http://medgen.ugent.be/~jvdesomp/genorm/).

2.7. Statistical analysis

Quantitative data are expressed as the mean ± SEM, and analyzed using two-tailed Student's *t*-test where *P*-values are indicated.

3. Results

3.1. PGE_2 enhances ErbB2 and decreases ErbB3 expression in cultured hepatocytes

The expression of ErbB protein and mRNA in hepatocytes cultured in the presence of dexamethasone and insulin [28] is shown in Fig. 1A. EGFR/ErbB1 protein decreased the first 24 h after plating, consistent with earlier ligand binding studies [31], but thereafter the expression of both protein and mRNA was relatively stable up to 72 h. As reported previously [12], the ErbB2 protein was hardly detectable in the first 24 h in culture and mRNA levels were very low, but thereafter the expression of ErbB2 increased dramatically. ErbB3 protein was present from the plating, and the expression increased somewhat up to 24 h, before decreasing with longer culturing; the mRNA expression followed the same pattern. No expression of ErbB4 protein (Fig. 1A) or mRNA (data not shown) was detected in the hepatocytes at any time during the culturing, in accordance with previous results [11].

Treatment of the hepatocytes with PGE_2 at 3 h of culturing did not significantly affect the expression of EGFR, but increased ErbB2 expression, both at the protein and the mRNA level, and substantially decreased ErbB3 expression (Fig. 1B), thus enhancing the changes occurring with time during culturing (Fig. 1A). Since insulin has been found to influence ErbB2 and ErbB3 protein expression in hepatocytes [12,32], we also performed a series of experiments where insulin was omitted from the basal medium. Fig. 1C shows the effects of insulin, EGF, and PGE_2, separately, on the mRNA levels of ErbB1, ErbB2, and ErbB3. PGE_2 decreased the ErbB1 mRNA level by about 40%. The ErbB2 mRNA expression was increased by about 50% by insulin or EGF and by more than 150% by PGE_2. ErbB3 expression was slightly reduced in response to EGF and was decreased by 60–70% by insulin or PGE_2. We next investigated the effects of PGE_2 on ErbB mRNA levels in the absence and presence of insulin

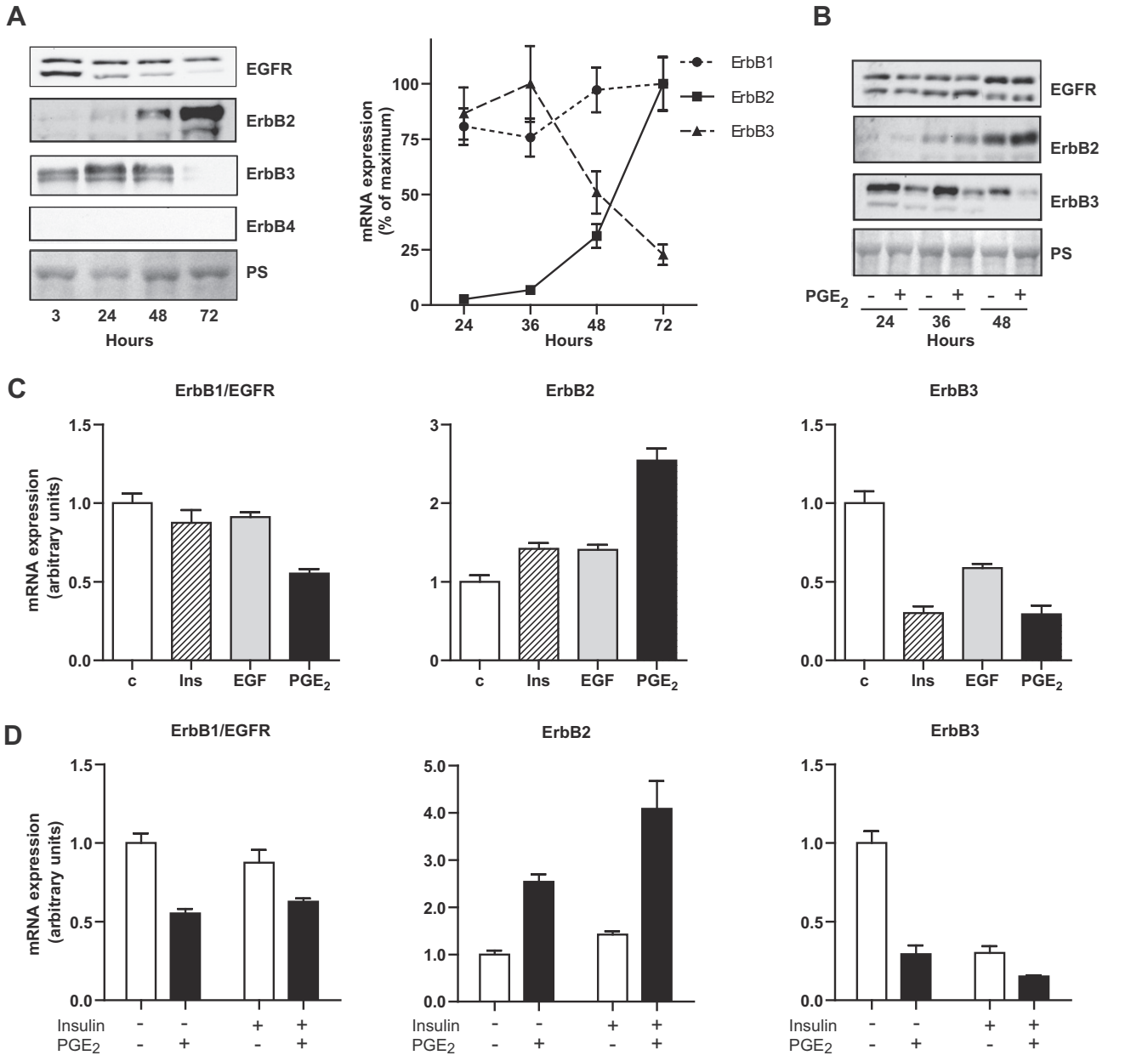

Fig. 1. ErbB expression in cultured hepatocytes and the effect of PGE_2, insulin, and EGF. (A) Immunoblots of ErbB proteins and qRT-PCR of ErbB mRNA at the indicated time points after plating. ErbB mRNA expression presented is relative to the maximum expression. (B) The effect of PGE_2 (100 μM added at 3 h after plating) on the expression of EGFR, ErbB2, and ErbB3 proteins. (C) The effects of insulin (100 nM at 3 h), EGF (10 nM at 24 h), and PGE_2 (100 μM at 3 h) on ErbB1/EGFR, ErbB2 and ErbB3 mRNA expression at 36 h after plating. (D) The effects of insulin (100 nM) and/or PGE_2 (100 μM), both added at 3 h, on the mRNA expression of ErbB1/EGFR, ErbB2, and ErbB3 mRNA at 36 h after plating. Quantitative data represent the mean ± SEM of three independent experiments. Immunoblots are from a representative experiment of at least three (Abbreviation: PS = Ponceau S staining).

(Fig. 1D). ErbB1 expression was lowered moderately by PGE_2. The enhanced ErbB2 expression in response to PGE_2, measured at the mRNA level, was evident also in the presence of insulin. Finally, PGE_2 decreased ErbB3 mRNA expression, both in the absence and presence of insulin.

3.2. ErbB2-directed siRNA inhibits the mitogenic response induced by PGE_2 and EGF

To assess the role of ErbB2 expression in mitogenic responses, we transiently transfected the cells with siRNA against ErbB2. Experiments to evaluate the specificity showed that, as compared to a non-silencing RNA control, the siRNA directed at ErbB2 strongly reduced ErbB2 mRNA and protein expression without reducing ErbB1 expression (Fig. 2A) and blocked the PGE_2- and EGF-induced upregulation of the ErbB2 protein (Fig. 2B). It was noted that the ErbB3 mRNA and protein expression was modestly increased when ErbB2 expression was blocked, in accordance with a previous report [26].

The ErbB2-directed siRNA inhibited EGF- and PGE_2-induced mitogenic responses in the hepatocytes. Fig. 2C shows that in cells treated with siRNA against ErbB2, the increased expression of cyclin D1 occurring upon stimulation with EGF or PGE_2, or the combination of the two, was almost abolished. As shown in Fig. 2D, blocking of the ErbB2 expression also suppressed the EGF-induced DNA synthesis and the comitogenic effect of PGE_2. These findings

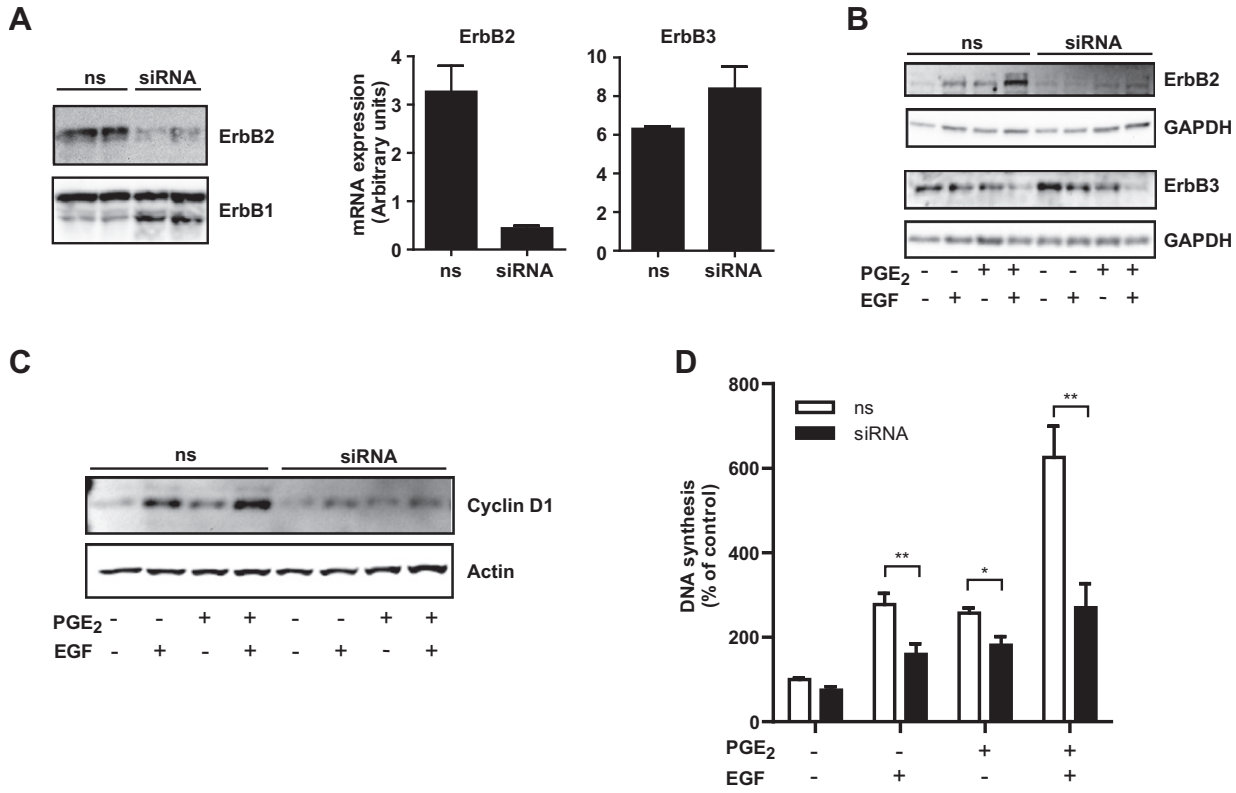

Fig. 2. Effect of ErbB2-directed small interfering RNA (siRNA) on the expression of ErbB2, ErbB3, cyclin D1, and the mitogenic response. (A) Hepatocytes transfected with non-silencing (ns) or ErbB2-directed siRNA (siRNA) were cultured for 72 h. Immunoblots showing ErbB2 and EGFR/ErbB1 protein expression and qRT-PCR of ErbB2 and ErbB3 mRNA (Data shown from one experiment of three). (B) Effect of ErbB2 siRNA on PGE_2- and EGF-stimulated ErbB2 and ErbB3 expression at 48 h after plating. PGE_2 (100 μM) and EGF (10 nM) were added at 7 h and 24 h, respectively. Immunoblots with ErbB2, ErbB3 and GAPDH antibodies. (C) Effect of ErbB2 siRNA on cyclin D1 expression at 48 h after plating. Hepatocytes were treated as in 2B. (D) DNA synthesis induced by EGF and PGE_2. The experiments were performed as described in 2B, with the exception that [^{3}H]-thymidine was added at 24 h after plating and the cells were harvested at 52 h after plating. [^{3}H]-thymidine incorporation was measured as described. Mean ± SEM of seven independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, immunoblots are from a representative experiment of at least three.

are consistent with an involvement of ErbB2 in the EGF-stimulated mitogenic signal, and suggest that PGE_2 enhances the effect of EGF through upregulation of ErbB2.

3.3. Differential effects of ErbB2 silencing on PGE_2- and EGF-induced upregulation of ERK and Akt expression

We have reported that pretreatment with PGE_2 enhances the responsiveness of the ERK and PI3K/Akt pathways to EGF in hepatocytes [19]. Other data have indicated that EGF also increases the expression of ERK and Akt by a mechanism involving ErbB2 [26]. We now investigated whether ErbB2 mediates a PGE_2-induced upregulation of ERK and/or Akt protein expression. Fig. 3 shows that both EGF and PGE_2 upregulated ERK and, to a lesser extent, Akt. The effects of EGF were blocked by ErbB2 siRNA. In contrast, ErbB2 silencing had no effect on the upregulation of ERK and Akt induced by PGE_2, alone or in combination with EGF. These data suggest that although PGE_2 upregulates ErbB2, which may contribute to its comitogenic effect, it also acts through other, ErbB2-independent, mechanisms to upregulate signalling in the ERK and Akt pathways.

4. Discussion

Previous studies have shown that pretreatment of hepatocytes with PGE_2 or certain other prostaglandins enhances the mitogenic response to subsequently administered EGF [17–19,33]. The mechanisms behind this effect are not fully understood. The expression of the ErbB family proteins in liver undergoes great changes during development and is regulated by external factors such as insulin and other hormones [11,12], and in this study we found that PGE_2 induced an upregulation of ErbB2 expression at the protein and mRNA level and a concomitant downregulation of ErbB3 in cultured rat hepatocytes. Furthermore, silencing of the ErbB2 expression with specific siRNA blocked the stimulation by PGE_2 and EGF of cyclin D1 expression and DNA synthesis. Together, these data suggest that PGE_2-induced upregulation of ErbB2 may be one of the mechanisms involved in the synergistic effect of PGE_2 on EGF-stimulated mitogenesis.

The increased ErbB2 and decreased ErbB3 expression in response to PGE_2 was qualitatively similar to, but more pronounced than, the reported regulation by insulin of ErbB2 and ErbB3 expression in hepatocytes [12,32], and these effects of PGE_2 on ErbB2 and ErbB3 were observed both in the absence and presence of insulin. The downregulation of ErbB3 could be either an independent effect of PGE_2 stimulation or a secondary event mediated by the rise in ErbB2. Support for the latter mechanism might be found in the observation in our experiments that silencing of ErbB2 by siRNA led to increased expression of ErbB3 both at the mRNA and protein level (Fig. 3B and C). On the other hand, protein levels of ErbB3 decreased markedly before ErbB2 levels increased detectably (Fig. 1C).

The relationship between cyclooxygenase/prostanoids and ErbB2 has been studied in other cells. In breast cancer cells PGE_2 can induce increased expression of ErbB2 [34]. Upregulation of COX-2 in combination with ErbB2 positivity (and ER-negativity) in breast cancer is associated with poor prognosis [35]. On the other hand, in colorectal cancer cells, activation of ErbB2 led to expression

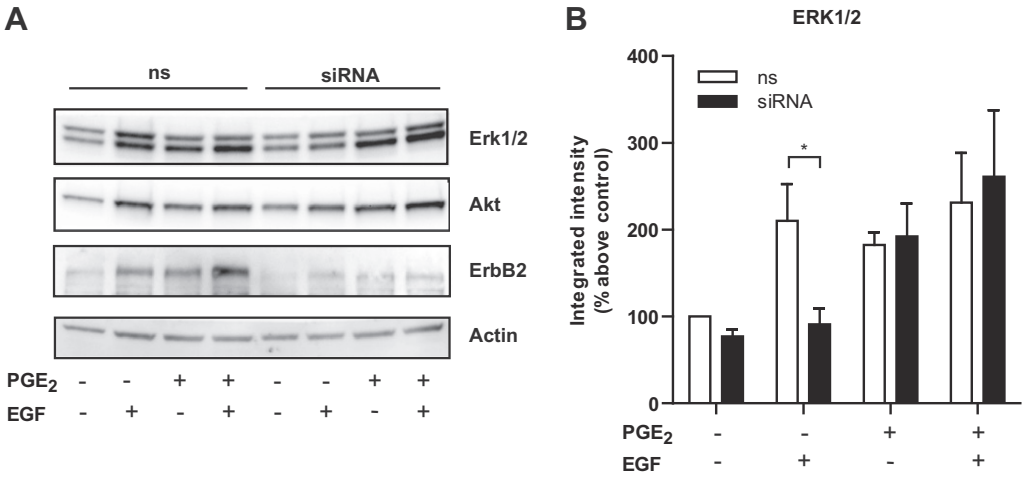

Fig. 3. Effect of ErbB2-directed siRNA on the expression of ERK and Akt. Hepatocytes were transfected and stimulated with PGE_2 (100 μM at 7 h), and/or EGF (10 nM at 24 h) and harvested at 48 h. (A) Immunoblots of ERK1/2, Akt, ErbB2, and actin. One representative of three independent experiments. (B) Densitometric analysis of ERK1/2 immunoblots. The data are the integrated intensity of bands quantified, normalized to the integrated intensity of the actin expression and presented as percent of untreated. Data from three independent experiments. Mean ± SEM. $^*P < 0.05$.

of COX-2 and secretion of PGE_2 [36]. Together, these results might suggest a positive feed-forward interaction between the COX-2/PGE_2 and ErbB2 signalling pathways. We have found no published data on PGE_2 and ErbB3 receptor expression.

The enhancing effect of PGE_2 on the mitogenic response to EGF in hepatocytes is exerted early in the G1 phase of the cell cycle, several hours before the EGF stimulation, suggesting that gene regulation and protein expression is involved [18,19]. Prostaglandins and other GPCR agonists alter the expression of several cell cycle-active genes in hepatocytes [22–25], but it is not clear which genes are involved in the comitogenic effects of PGE_2. A role of increased ErbB2 expression is suggested by several observations. We found here that PGE_2, like insulin [12], increases the expression of ErbB2 and decreases ErbB3. This mimics the pattern of ErbB expression in fetal and regenerating liver and may represent changes associated with more mitogenically sensitive hepatocytes. Furthermore, siRNA silencing of ErbB2 suppressed the stimulation by PGE_2 and EGF of cyclin D1 expression and DNA synthesis. In addition to supporting the evidence that a high ErbB2 level enhances the mitogenic response to EGF [12], these data suggest that upregulation of the ErbB2 expression has a role in the comitogenic effect of PGE_2 in hepatocytes.

The mechanisms by which ErbB2 may participate in the comitogenic effect of PGE_2 are not known. It is likely, however, that a higher density of ErbB2 receptors will increase the formation of EGFR-ErbB2 heterodimers and thereby lead to altered and amplified intracellular signalling and biological responses [37–39]. Previous work has demonstrated the importance of persistent and coordinated activation of ERK and Akt for an optimal DNA synthesis in hepatocytes [40] and have shown that PGE_2 induces upregulation of EGF-responsive signalling in the Akt and ERK pathways [19]. In the present study we found that upon prolonged culturing, PGE_2, like EGF, induced an increase in the expression of ERK and Akt proteins. However, while inhibition of ErbB2 expression blocked the EGF-stimulated increase of ERK and to a lesser extent Akt, the PGE_2-induced increase of ERK and Akt was not affected by ErbB2 siRNA.

Taken together, this study shows that PGE_2 increases ErbB2 and decreases ErbB3 expression. Furthermore, the data suggest that the PGE_2-induced upregulation of ErbB2 increases the mitogenic response to EGF. However, PGE_2 apparently also acts through other mechanisms to enhance the EGF effect, since it upregulates the ERK and Akt expression independently of ErbB2.

Acknowledgments

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