Dexamethasone Modulates ErbB Tyrosine Kinase Expression and Signaling Through Multiple And Redundant Mechanisms In Cultured Rat Hepatocytes

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Running Title: Dexamethasone Effects on ErbB Expression and Signaling

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Glucocorticoids paradoxically exert both stimulatory and inhibitory effects on the proliferation of cultured rat hepatocytes. We studied the effects of dexamethasone, a synthetic glucocorticoid, on the proliferation of cultured rat hepatocytes. The timing of growth factor addition modified the action of high dose dexamethasone (10^{-6}M) on DNA synthesis. When we added transforming growth factor-α (TGFα) at the time of plating, 10^{-6}M dexamethasone weakly stimulated DNA synthesis by 26% relative to cells cultured in dexamethasone-free media. When we delayed growth factor addition until 24-48 h after plating, 10^{-6}M dexamethasone inhibited DNA synthesis by 50%. Using immunologic methods, we analyzed the expression and signaling patterns of the ErbB kinases in dexamethasone-treated cells. High dose dexamethasone stabilized the expression of EGFr and ErbB3, and it suppressed the de novo expression in ErbB2 that occurs during the third and fourth day of culture in 10^{-8}M dexamethasone. High dose dexamethasone by 72 h suppressed basal and epidermal growth factor (EGF)-associated phosphorylation of ERK and AKT. The reduction in ERK 1/2 phosphorylation correlated with suppression of a culture-dependent increase in Son-of-sevenless 1 (Sos1) and ERK1/2 expression. High dose dexamethasone in hepatocytes stabilized or upregulated several inhibitory effectors of EGFr/ErbB2 and ERK, including receptor-associated late transducer (RALT) and MAPK Phosphatase-1 (MKP-1), respectively. Thus, 10^{-6}M dexamethasone exerts a time dependent and redundant inhibitory effect on EGFr-mediated proliferative signaling in hepatocytes, targeting not only the ErbB proteins but also their various positive and negative effectors.

Liver, EGF, TGFα, cell culture
Dexamethasone, a synthetic glucocorticoid, is frequently included in the medium of cultured hepatocytes to improve their survival and function. In most experimental models, dexamethasone inhibits hepatocyte growth \textit{in vivo}. For example, when injected into the rat, dexamethasone inhibits the striking increase in hepatocyte proliferation that occurs after 70% hepatectomy (28) or in transplanted livers following cold preservation and reperfusion (13). Likewise, dexamethasone has been reported to inhibit the proliferation of rat hepatocytes (35) (34) and liver derived cell lines (24). In primary hepatocyte culture, however, other investigators have reported that dexamethasone enhances epidermal growth factor (EGF)-stimulated DNA synthesis (30) (26). We hypothesized that these divergent observations result from differential effects of dexamethasone on one or more components of EGF receptor signaling.

Fetal hepatocytes express three of the four ErbB tyrosine kinase receptors (EGFr, ErbB2, and ErbB3), but by three weeks of age, ErbB2 is largely extinguished (10). When it binds ligands of the EGF/TGF\(\alpha\) family, EGFr can interact with other ErbB proteins to form activated homo (EGFr/EGFr)- or hetero (EGFr/ErbB2 or EGFr/ErbB3) dimers. In contrast, ErbB2 and ErbB3 cannot form active homodimers, and must dimerize with each other or with EGFr to form active tyrosine kinase signaling complexes. ErbB2 is structurally unable to bind an extracellular ligand, and ErbB3, which is the receptor for heregulin (Hrg) peptides, has an inactive tyrosine kinase domain. Relative changes in the cell surface expression of individual ErbB proteins can affect cellular signaling outcomes in response to EGF or Hrg peptides (10).

We recently showed that expression of these ErbB proteins changes as hepatocytes adapt to primary culture (10) (31). For example, adult hepatocytes, cultured for 24h in the presence of insulin and/or EGF, begin to express ErbB2, which is normally expressed in fetal and neonatal but not adult liver (10). EGF- or TGF\(\alpha\) - stimulated DNA synthesis in adult rat hepatocytes requires the presence of ErbB2 in a heterodimer with EGFr (31). The induction of ErbB2 is the likely explanation for the long lag phase (> 40 h from the time of plating) required for hepatocytes to initiate DNA synthesis in response to EGF.
Since the activated glucocorticoid receptor frequently acts as a transcription factor, altering gene expression, we hypothesized that dexamethasone blunts EGF or TGFα stimulated DNA synthesis by inhibiting the ErbB2 induction. Given the temporal changes of ErbB protein expression in vitro, we further speculated that the overall influence of dexamethasone on the hepatocellular response to EGF-type ligands depends on the time of growth factor addition. Our analysis of prior work suggested that the stimulatory effects of dexamethasone occurred when EGF and dexamethasone were added simultaneously at the beginning of the culture period (30). In contrast, the inhibitory action occurred when isolated hepatocytes were plated in a high concentration of dexamethasone for at least 24 h before EGF exposure (35).

In this paper, we show that the inhibitory effect of dexamethasone on EGF-stimulated DNA synthesis in cultured rat hepatocytes does indeed depend on the time of growth factor addition. Furthermore, our work provides a molecular basis for the inhibitory effect by showing that the concentration of dexamethasone alters not only the expression and interaction of the ErbB proteins themselves, but also a number of positive and negative growth regulatory proteins in ErbB-regulated signaling pathways.
EXPERIMENTAL PROCEDURES

Peptides, Reagents, and Radiochemicals. Synthetic rat TGFα was from Peninsular Laboratories Inc. (Belmont, CA). Insulin (Novolin® R) was from Novo Nordisk Pharmaceuticals, Inc. (Princeton, NJ). 

\(^3\text{H}\)-Thymidine (6 Ci/mmol,) was from Perkin Elmer Life Science, Inc. (Boston, MA). Dexamethasone, pyruvate, bovine serum albumin (fatty acid-free), Percoll, and all buffer reagents were from Sigma (St. Louis, MO). Protein G-Sepharose and ECL reagents were from Pierce (Rockford, IL). Nitrocellulose membranes were from Osmonics (Minnetonka, MN).

Animals. Adult male Sprague-Dawley rats (250-300 grams) from Harlan (Indianapolis, IN) were raised under conditions of regulated lighting (lights on 0600-1800). They had ad libitum access to water and Purina rodent chow (Ralston-Purina, St. Louis, MO). The Animal Use Subcommittee of the Vanderbilt Animal Care Committee approved all protocols.

Culture Media and Supplies. Williams’ Medium E, supplemented with 20 mM pyruvate, and 50 µg/ml gentamicin, was the medium used for all culture studies. The media typically contained insulin (100 nM), needed to preserve EGF or TGFα responsiveness. The concentration of dexamethasone was adjusted for each experiment. Medium and calf serum were from Gibco, Invitrogen Corp. (Grand Island, NY). Type I collagenase was from Wako Pure Chemical Industries, Ltd. (Richmond, VA). Falcon six-well dishes were from Fisher Scientific.

Primary Cell Cultures. Hepatocytes were isolated from the livers of male rats between 10:00 and 11:30 AM to control for circadian variation using a two-stage, collagenase isolation protocol (9). To reduce nonhepatocyte contamination, cells were sedimented through Percoll (9). We plated cells (3.75×10^5/well) in type-1 collagen-coated 6 well 35-mm plates for 60 to 90 minutes before adding serum-
free medium, growth factors, or dexamethasone. In some experiments, growth factor was added at different times after the change from plating to growth medium.

**Immunoprecipitation and Western Blotting.** Hepatocytes were lysed in TGH buffer (20mM HEPES, 1% Triton X-100, 10% glycerol 50mM NaCl). This buffer included protease inhibitors (1mM PMSF, 1mM sodium orthovanadate, 10µg/ml aprotinin and 1µg/ml leupeptin) as well as phosphatase inhibitors (10mM sodium molybdate and 10mM β-glycerol phosphate). Lysates were microfuged at 20,800 x g for 30 min., and then immunoblotted or immunoprecipitated as previously described (4, 10). We used the following affinity-purified antibodies from Santa Cruz Biotechnology Inc. (Santa Cruz, CA): sc-03 for EGFR; sc-284 for ErbB2; sc-285 for ErbB3; sc-283 for ErbB4, sc-370 for MKP-1, sc-256 for Sos 1, sc258 for Sos 2 The anti-phosphotyrosine antibody used was PY99 (Santa Cruz, CA). The anti-phosphotyrosine [Y1112]-ErbB2 antibody was from Orbigen, Inc. (San Diego, CA). The anti-phosphotyrosine [Y845]-EGFr, [Y168]-EGFr,[Y1289]-ErbB3, phosphoMEK1/2, phospho-cRaf, the phospho and total AKT and Erk1,2 antibodies were from Cell Signaling Technology (Beverly, MA). The PI3 kinase rabbit antiserum (06-195) was from Upstate USA, Inc. (Charlottesville, VA). The anti-Ras mAb was from Transduction Laboratories (R02120).

We normalized immunoprecipitations or immunoblots by using equal amounts of protein, as determined by either the Bio-Rad DC Protein Assay (Bio-Rad Laboratories; Hercules, CA) or the Micro BCA protein assay (Pierce Biotechnology, Inc., Rockford, IL). After each transfer, we confirmed equal protein loading and transfer by Ponceau S staining of immunoblots, scanning the image for future reference. Immunoreactive signal was detected using the ECL method (Pierce Biotechnology, Inc., Rockford, IL). We performed densitometry using an Epson scanner with Biosoft Quantiscan Software or the Image J program (1).

**DNA Synthesis Assays.** Cells were exposed to the Williams’ Medium E for varying periods of time before the addition of TGFα (typically at a concentration of 5-7.5 nM; TGFα was used in place of EGF because unlike EGF, which targets EGFr for lysosomal degradation, TGFα promotes recycling of
internalized EGFr to the hepatocyte surface (31). After stimulation, the cells were refed with medium containing specified growth factors and 1 µCi/ml of [³H-methyl] thymidine. In some experiments, the time of addition of TGFα after cell plating varied. After labeling for 24-48 h, cells were harvested, and the incorporation of tracer was determined as detailed previously (9). The results of assays in triplicate are expressed as the specific activity of the DNA (dpm/µg DNA ± S.D.).

Statistical Analysis. Statistical analysis was performed using an unpaired, two tailed Student’s t-test assuming equal variances between compared groups.
RESULTS

Prolonged exposure of hepatocytes to dexamethasone inhibits TGF\(\alpha\)-stimulated DNA synthesis, while simultaneous exposure stimulates DNA synthesis: To determine the effect of dexamethasone on TGF\(\alpha\)-stimulated DNA synthesis, we added different concentrations of dexamethasone from 0 to 10\(^{-6}\) M immediately after plating. We then added TGF\(\alpha\) (1 or 7.5 nM) either at the beginning of the culture period or 48 h later. Fig. 1A (left panel) shows that dexamethasone slightly increased 7.5 nM TGF\(\alpha\)-stimulated DNA synthesis in a dose-dependent manner when TGF\(\alpha\) was added immediately after cell plating. For example, in the 7.5 nM TGF\(\alpha\) treatment groups, dexamethasone at 10\(^{-7}\) M increased DNA synthesis by 26% compared with the dexamethasone-free group (P < .05). In contrast, when we delayed growth factor addition until 48 h after plating (right panel), dexamethasone strongly inhibited TGF\(\alpha\)-stimulated DNA synthesis. Dexamethasone (10\(^{-6}\) M) suppressed 1 nM TGF\(\alpha\) and 7.5 nM TGF\(\alpha\)-stimulated DNA synthesis by 78 and 62%, respectively, indicating that the higher concentration of TGF\(\alpha\) may partly override the action of dexamethasone. The data confirm previous reports that the delayed addition of EGF-like substances elicits a 3 fold greater peak DNA synthesis in cultured hepatocytes (25) (compare 1 nM TGF\(\alpha\) in left and right panels).

To define the kinetics of dexamethasone suppression of EGF-mediated DNA synthesis, we exposed cells to two different concentrations of dexamethasone and then added TGF\(\alpha\) at different times after plating, evaluating DNA synthesis between 48 and 96 h. Fig. 1B shows that dexamethasone did not inhibit TGF\(\alpha\)-stimulated DNA synthesis during the initial 18 h of culture, indicating that the inhibitory effect of dexamethasone likely required gene transcription. Maximal inhibition occurred when dexamethasone was present for 48 h before TGF\(\alpha\) addition.

Dexamethasone stabilizes the normal in vivo ErbB expression pattern during cell culture: When placed in culture, adult rat hepatocytes show an altered expression of ErbB proteins. For example, in vivo fetal/neonatal, but not adult, rat hepatocytes normally express ErbB2 (8). However, adult hepatocytes
cultured for 48 h re-express ErbB2, which then contributes to the EGFr signaling complex (31). To determine whether dexamethasone altered the ErbB expression profile, we cultured cells in the presence of $10^{-8}$ or $10^{-6}$ M dexamethasone for 72 h. We cultured the cells in both insulin (100 nM) and a subunitogenic dose of TGFα (0.1 nM) to enhance cell viability. Insulin was also added because it is required to preserve maximal TGFα responsiveness of hepatocytes after 24 h of culture (30). Lysates were immunoblotted for the four ErbB proteins at 24 and 72 h. Confirming our previous experience, no ErbB4 was detected. Fig. 2A shows that the low and high concentrations of dexamethasone had divergent effects on ErbB protein expression. Whereas $10^{-6}$ M dexamethasone stabilized the expression of EGFr and ErbB3 between 24 and 72 h, $10^{-8}$ M reduced it. The high and low concentrations of dexamethasone also differed in their effect on de novo ErbB2 expression. The overall expression level in the $10^{-8}$ M dexamethasone-containing medium consistently exceeded that in the $10^{-6}$ M dexamethasone medium. Fig. 2B shows the results of quantitative densitometry for ErbB3 and ErbB2 in cells harvested at either 24 or 72 h. Note that there is an inverse expression of ErbB3 (left panel) and ErbB2 (right panel) regardless of whether the medium contains dexamethasone at a low or high concentration.

**Dexamethasone modulates EGF-stimulated tyrosine phosphorylation of EGFr and ErbB2:** EGF binds EGFr monomers on the cell surface of hepatocytes, leading to the formation of EGFr homodimers or EGFr-ErbB2 or EGFr-ErbB3 heterodimers (10). The data of Fig. 2 show that $10^{-6}$ M dexamethasone attenuated the induction of ErbB2. To study the consequence of this on ErbB2 phosphorylation, we exposed cells at either 24 or 72 h of culture to EGF (50 nM) for 1, 3, or 6 min. We then immunoprecipitated ErbB2 and EGFr from soluble lysates. Tyrosine phosphorylation of immunoprecipitated protein was determined by immunoblot with a monoclonal antibody against phosphotyrosine (PY-99, IgG2b). We also evaluated the total ErbB2 and EGFr protein immunoprecipitated.

As shown in Fig. 3A (PY row), very little phosphorylated ErbB2 was detected at 24 h, consistent with the very low levels of ErbB2 expression at this time (ErbB2 row). In fact, immunoblot detection of
immunoprecipitated ErbB2 with PY-99 or the ErbB2 antibody itself at this time requires prolonged radiographic exposures (31). In contrast, there was significant tyrosine phosphorylation and expression of ErbB2 at 72 h for cells cultured in $10^{-8}$ M dexamethasone. Both phosphorylation and expression were decreased in cells exposed to the $10^{-6}$ M concentration. Similar results were obtained for an EGFr immunoprecipitate in a parallel lysate (data not shown).

To confirm these effects, we used antibodies that specifically recognize discrete tyrosine phosphorylation sites (pY) within the cytoplasmic domains of EGFr or ErbB2. We used antibodies that recognize pY845 and pY1068 for EGFr and pY1112 for Erb2. Parallel blots were performed to find out if the degree of tyrosine phosphorylation correlated to the total ErbB expression level. Fig 3 B shows the results. At 24 h, for both EGFr and ErbB2 the amount of acute tyrosine phosphorylation in response to exogenous EGF (10 nM) was slightly greater in the cells treated with $10^{-6}$ M compared with $10^{-8}$ M (data were reproducible in multiple experiments). In contrast, for both of these interactive ErbBs, the degree of tyrosine phosphorylation at 72 h in the cells cultured in $10^{-8}$ M dexamethasone greatly exceeded those cultured in $10^{-6}$ M. After normalizing for overall ErbB2 levels, the specific phosphotyrosine signal in ErbB2 immunoprecipitates from the $10^{-8}$ M dexamethasone exceeded that of ErbB2 immunoprecipitates from the cells treated in $10^{-6}$ by two-fold (data not shown). These data indicate that high dose dexamethasone exerts an inhibitory influence on EGF-stimulated hepatocyte DNA synthesis by preventing the expression and phosphorylation of ErbB2.

**Dexamethasone alters ERK1/2 signaling:** After showing that the level of ErbB phosphorylation varied in the dexamethasone-treated cells, we evaluated the expression level and phosphorylation state of components of the ERK1/2 pathway, which is one of the two signaling pathways required for EGF to exert its full mitogenic effect in hepatocytes (33). As shown in Fig. 4, dexamethasone had little or no effect on the expression level of Ras at 24 or 72 h of cell culture. Dexamethasone mildly suppressed the two Son-of-sevenless (Sos) guanine nucleotide exchange factors that interact with Grb2 upon growth factor binding. These two proteins showed an interesting reciprocal pattern of expression with time in
culture. Sos 2 predominated at 24 h whereas Sos 1 was strongly induced by 72 h of cell culture, when Sos 2 disappears.

Moreover, when we examined the phosphorylation state of Raf (ser 338), MEK1,2 (ser 217/221), and ERK1/2 (thr 202/tyr204) by use of phospho-specific antibodies, we observed that dexamethasone-free or low dexamethasone concentrations were associated at 72 h with higher baseline levels of the phosphorylated species. Moreover, the increase in the active ERK1/2 species partially correlated with an increase in total ERK1/2.

To explore the ability of dexamethasone to alter the responsiveness of hepatocytes to EGFr signaling, we cultured cells in the presence of increasing concentrations of TGFα instead of a single submitogenic dose (0.1nM) as above. Fig. 4C shows that after 72 h, cells cultured in the presence of 10^{-8} M dexamethasone had higher levels of ErbB2 (left panel) and ppERK1/2 (right panel) than cells cultured in the 10^{-6} M concentration. The overall levels of both ErbB2 and ppERK1/2 increased with increasing concentrations of TGFα.

**Dexamethasone inhibits EGF stimulation of ERK1/2 and PI-3 kinase:** Phospho-AKT is an indirect measure of the PI3 kinase pathway, a second pathway required for full mitogenic action of EGF (8, 33). In the prior experiment, we evaluated whether dexamethasone altered phospho-AKT and were unable to find a consistent effect (data not shown). Since the cells in the prior experiment had not been exposed to fresh TGFα for over 24 h (the TGFα was added at 0 time for the 24 h group and 48 h for the 72 h group), we analyzed the acute effect of ligand on AKT phosphorylation in cells cultured in different concentrations of dexamethasone. We examined the acute response of Erk1/2 to EGF in this experiment as well.

In Fig. 5, we stimulated cells with EGF and under conditions of low or high dexamethasone for 24 or 72 h. We then immunoblotted lysates for the total and phosphorylated forms of ERK1/2 or the singly phosphorylated pAKT (ser 473). As shown in Fig. 5, irrespective of the dexamethasone
concentration, cells cultured for 72 h showed a remarkable increase in the relative amount of total AKT and ERK1/2 compared with 24 h.

When cultured in $10^{-8}$ M or $10^{-6}$ M dexamethasone for 24 h, cells exposed to the highest concentration showed a greater activation of ERK1/2 and AKT that partly correlated with increased expression levels of these molecules at this time. In contrast, by 72 h, cells cultured in the high concentration showed decreased levels of phosphorylated AKT and ERK1/2. Again, the reduced phosphorylation partly correlated with a reduction in the total protein, particularly for ERK1/2.

**Dexamethasone induces molecules that inhibit ErbB signaling:** Dexamethasone inhibits proliferation of a number of other cell types using negative effectors, including RALT (2), MPK-1 (14), and the p85 regulatory subunit of PI3 kinase (17). We evaluated their expression in hepatocytes as a function of the concentration of dexamethasone (Fig. 6). We found that $10^{-6}$M dexamethasone stabilized the expression of RALT, which normally disappears by 48 h in the absence of dexamethasone. We observed that increasing concentrations of dexamethasone suppressed the expression of the p85 regulatory subunit of PI3 kinase at 24 h of culture. However, this effect was reversed by 72 h. Finally, we found that dexamethasone modulated MPK-1. In contrast to RALT and p85, hepatocytes express little or no MPK-1 unless they are cultured in dexamethasone, which increases MPK-1 in a dose-dependent manner.
DISCUSSION

Dexamethasone has complex effects on cell proliferation in different tissues and cell types (36). It may augment the effect of a specific growth factor on DNA synthesis in some cell types, but inhibit it in others. For example, it enhances the stimulatory effect of EGF in myoblasts (20) and human diploid fibroblasts, but inhibits the response in 3T3 cells. In a given cell type, the actions of dexamethasone can vary between growth factors. In 3T3 cells, dexamethasone inhibits the growth promoting action of EGF, but potentiates that of fibroblast growth factor (FGF) (18, 19). This suggests that the action of dexamethasone does not target a single cytoplasmic or nuclear mechanism distal to the interaction between growth factors and their cell surface receptors.

Many glucocorticoid effects are both time- and transcription-dependent. However, some actions that depend on glucocorticoid binding to its receptor do not require subsequent binding of the receptor to glucocorticoid response elements within DNA (6, 7, 22). For example, in the A549 human lung adenocarcinoma cell line, dexamethasone rapidly inhibits recruitment of signaling factors to EGF receptors, increasing the tyrosine phosphorylation of lipocortin in a transcription-independent manner. The phosphorylated lipocortin binds to GrbB2 docking sites on EGFr, thereby inhibiting the transmission of signals necessary for EGF-stimulated arachidonic acid release and cell growth (12).

The liver of the intact rat is exquisitely sensitive to the inhibitory actions of dexamethasone and other glucocorticoids, such as hydrocortisone, on cell proliferation. These hormones strongly inhibit DNA synthesis in the developing or regenerating liver and in all liver-derived cell lines (24). For example, hydrocortisone completely blocks DNA synthesis within a 24 h period when injected into weaning rats. It also partially inhibits DNA synthesis in the regenerating liver of young rats after partial hepatectomy, blunting the regenerative response by about 50%. Recently, it has been shown that dexamethasone inhibits the early regenerative response of the rat liver seen after cold preservation and transplantation (13). Furthermore, Nagy et al demonstrated that daily injections of dexamethasone (2 mg/kg) immediately before and after 70% hepatectomy in the rat inhibited not only hepatocyte proliferation but
also oval cell proliferation and differentiation. Interestingly, in this model, the liver restored its mass by the preferential hypertrophy of periportal hepatocytes. The action of dexamethasone was reversible. Forty h after dexamethasone withdrawal, the enlarged periportal cells entered the S-phase in a synchronized fashion (28).

Despite these growth inhibitory effects in the liver from the intact rat, dexamethasone or other glucocorticoids are usually included in the growth medium of cultured hepatocytes because they increase cell attachment and survival. They inhibit spontaneous apoptosis through the induction of Bcl-2 and Bcl-x (4), and they also inhibit the TNF and Fas death receptor-mediated apoptosis through the up-regulation of Flice (29). Some investigators have reported that dexamethasone increases EGF-stimulated DNA synthesis (30) (26), but others have reported that dexamethasone inhibits it (35) (34). These divergent effects are unrelated to differences in dose since they both varied in a dose-dependent manner. No clear concept to explain these discrepant findings has arisen until now.

In this paper, we show that the timing of the growth factor addition relative to the initial dexamethasone exposure is the critical determinant in defining whether dexamethasone has stimulatory or inhibitory effects on DNA synthesis (Fig. 1A). This conclusion is supported by the analysis of conflicting papers. Sand et al showed that in hepatocytes treated with insulin and EGF from the time of plating, dexamethasone increased the rate of DNA synthesis by 20-30% (30). In contrast, Vintermyr and Doskeland reported that when cells were exposed to EGF after 20 h of culture, DNA synthesis was inhibited by dexamethasone (34). The latter authors speculated that the use of collagen coated plates or the inclusion of pyruvate in their media may have been responsible for the discrepancies. We now show that neither collagen nor pyruvate can account for them since they are also components of our culture system. Instead, the inhibitory effect of dexamethasone is not immediate as described for some cell types (12) but requires cell exposure to dexamethasone for as long as 18 hours before growth factor addition (Fig. 1B).

Very little is known about the mechanisms used by dexamethasone to stimulate or inhibit DNA synthesis in the intact liver and in primary hepatocytes. Several possible explanations have been offered
for the inhibitory effect on hepatocytes, including the synthesis and release of a secreted growth inhibitory factor or an effect on the stability of gap junctions and cell contacts, which decrease during states of high cell proliferation, such as liver regeneration (34). Indeed, others reported that the inhibitory effect of dexamethasone was greater in areas of high cell density, suggesting that cell contact contributed to the effect (34).

We have recently shown that the ErbB expression profile in hepatocytes changes during adaptation to cell culture (31), coinciding with an increased responsiveness to EGF(25). The proliferative effects of EGF in cultured hepatocytes require the induction of ErbB2 shortly before DNA synthesis rises 48 h after plating. Since the timing of growth factor addition was critical to the inhibitory glucocorticoid effect on DNA synthesis (Fig. 1), we speculated that dexamethasone altered either the expression of the ErbB proteins or downstream signaling pathways. Indeed, Baker et al reported a precedent for a dexamethasone-induced growth altering change in the cell surface expression of growth factor receptors (5). In human diploid fibroblasts, dexamethasone enhanced EGF-stimulated DNA synthesis while increasing 125I-EGF binding to surface membranes by 1.5 fold at 8 h after addition and by 2-fold at 32 h.

We now report that dexamethasone has far-reaching effects on ErbB expression and activation (Figs. 2 and 3). Moreover, these changes in ErbB expression resulted in a decrease in EGF-stimulated tyrosine phosphorylation of EGFr and ErbB2 (Fig. 3). Dexamethasone also altered signaling downstream of the ErbB proteins, including the ERK and PI3 kinase pathways (Figs. 4 and 5). Both pathways are required for EGF to stimulate DNA synthesis in cultured hepatocytes based on studies using pharmacologic or genetic inhibitors (8) (33). Dexamethasone at a high dose suppressed both pathways in cells treated for 72 h of culture.

Other groups have shown that dexamethasone suppresses these pathways in other cell types through negative effectors that either directly bind to the ErbB proteins or indirectly inhibit ErbB action by inhibiting ERK or PI3 kinase. Dexamethasone can induce unique adaptor-like molecules that bind to discrete regions of the ErbB cytoplasmic domains and inhibit signaling. Such proteins include RALT, (also known as gene 33 and MiG-6) and Dok-1 (21). RALT suppresses the tyrosine kinase activity of
both ErbB-2 and EGFR (2, 15, 16, 38). Dexamethasone also inhibits signaling components downstream from the ligand-ErbB interaction. Inhibition of ERK primarily occurs through the induction of the dual specificity phosphatase MKP-1, which dephosphorylates phosphoserine and phosphothreonine residues within ERK, attenuating the transmission of growth factor signals (14, 23). Dexamethasone also exerts some of its anti-proliferative and pro-apoptotic effects in certain cell types by upregulating the p85 regulatory subunit and thereby inhibiting PI3K (11, 17, 32). In hepatocytes, we found that dexamethasone influenced the expression of p85, RALT, and MKP-1 in a manner that generally correlated with its inhibitory effect on DNA synthesis (Fig. 6).

Hepatocytes in primary culture are a popular model to study and define the regulation of hepatocyte growth, function, and signaling. However, primary hepatocytes are a dynamic system that adapt to the conditions of culture. In doing so, they undergo striking changes in the expression patterns of cell surface receptors, such as the ErbB proteins (Figs. 2, 3) downstream growth promoter elements, such as ERK and AKT (Figs. 4, 5), and growth inhibitors, such as RALT and MKP-1 (Fig. 6). These changes, which are frequently unappreciated and undocumented, may lead to conflicting results, particularly when cells cannot be used for a period of time to induce or knockdown gene expression experimentally. Our present work shows how consideration of this factor reconciles contradictory reports that dexamethasone stimulates or inhibits EGF-stimulated hepatocyte proliferation. In this paper, we have shown that the ability of dexamethasone to stimulate or inhibit proliferation is highly dependent on the time that EGF ligands are introduced into culture. Dexamethasone augments EGF-stimulated DNA synthesis when dexamethasone and EGF are added together shortly after cell isolation and plating. In contrast, prolonged exposure (> 24 h) to high concentrations of dexamethasone before ligand activation of EGFr inhibits hepatocyte DNA synthesis. Dexamethasone in this instance causes extensive changes in signaling elements, curbing numerous and redundant culture-dependent changes in the ErbB proteins and their effectors that collectively optimize the hepatocellular proliferative response to EGF.

The ability of dexamethasone to regulate in a coordinate fashion the many positive and negative signaling elements involved in ErbB signaling may be a consequence of its effects on differentiation. For
example, dexamethasone alone is sufficient to transform pancreatic cells into hepatocyte-like cells that can support replication of hepatitis B virus (37). Dexamethasone also has dramatic effects on hepatocyte differentiation in organoid cultures, suppressing growth and inducing hepatocyte maturation (27). Indeed, dexamethasone activates an enhancer element 6 kb upstream of the HNF4α-1 promoter that positively regulates the expression of this transcription factor, which is weakly expressed in the embryonic liver and strongly expressed in the adult liver (3). The relative contributions of individual ErbB signaling elements in the actions of dexamethasone requires further study. Indeed, some of the effects of dexamethasone on DNA synthesis may proceed through other complex signaling pathways not monitored in the current work. Future work involving direct modulation of various signaling proteins may allow us to define their relative importance in mediating the effects of dexamethasone on DNA synthesis.

GRANTS

We acknowledge the generous support of student research stipend from the Vanderbilt Diabetes Center to RB. This work was supported by DK 53804 (to WER).
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Figure Legends

**Fig. 1:** Prolonged dexamethasone exposure inhibits cellular responsiveness to EGFr stimulation. A. The effect of dexamethasone on DNA synthesis depends on the time of growth factor addition. TGFα was added at either 0 h (left panel) or 48 h (right panel). [3H] TdR incorporation was measured for 48 h beginning at either 48 h (left panel) or 72 h (right panel). Dark bars = 7.5 nM TGFα and hatched bars = 1.0 nM TGFα. B. Time course of dexamethasone inhibition. The addition of TGFα (7.5 nM) was delayed for up to 48 h in hepatocyte cultures in either 10⁻⁶ or 10⁻⁸ M dexamethasone. DNA synthesis was assessed between 48 and 96 h of culture. P<0.05 at 30, 36, and 48 h.

**Fig. 2:** Dexamethasone alters the expression of individual ErbB tyrosine kinase proteins. A. Cells were cultured for 24, 48, and 72 h in the presence of either 10⁻⁸ or 10⁻⁶ M dexamethasone in the presence of a mitogenic dose of TGFα (0.1 nM). Cell lysates were immunoblotted for the presence of EGFr, ErbB3, and ErbB2. B. Cells were cultured in the presence of either 10⁻⁸ or 10⁻⁶ M dexamethasone for 24 or 72 h. Lysates were prepared and immunoblotted for ErbB2 or ErbB3 (n=3 wells). Scanning densitometry was done to show the inverse expression patterns of ErbB2 and ErbB3 at 72 h under conditions of 10⁻⁸ or 10⁻⁶ M dexamethasone. P<0.05 for ErbB2 or ErbB3 groups in 10⁻⁸ or 10⁻⁶ M dexamethasone at 72h.

**Fig. 3:** Dexamethasone inhibits EGF stimulated tyrosine phosphorylation of ErbB2. A. Cells were cultured in the presence of either 10⁻⁸ or 10⁻⁶ M dexamethasone for 24 or 72 h. The cells were then exposed to EGF (100 nM) for 0, 1.5, 3 or 6 min. ErbB2 was immunoprecipitated from Triton X-100 solubilized cell lysates and then probed with a monoclonal antibody against phosphotyrosine (PY99; A, upper panel). Equal loading was shown by stripping the blot and probing it with an ErbB2 polyclonal antibody (A; lower panel). B. Cells were cultured in the presence of either 10⁻⁸ or 10⁻⁶ M dexamethasone for 24 or 72 h. The cells were then treated with EGF (10 nM) for 0, 1, or 3 min. and then analyzed for phosphorylated EGFr (B; top panels) or ErbB2 (B; lower panels) by immunoblot using phospho-EGFr or ErbB2 antibodies.

**Fig. 4:** Dexamethasone alters ERK1/2 signaling. A. Cells were treated with different concentrations of dexamethasone for 24 or 72 h and evaluated for the total protein expression of Grb2, Sos1, Sos2, and RAS. Note that Sos1 increases with culture time, but the higher concentrations of dexamethasone suppress this increase. B. Cells were treated as described in A and then evaluated by immunoblot for the phosphorylated forms of Raf, Mek1/2, ERK1/2, or total ERK1/2. Note that the total amount of ERK1 increases with culture time, although dexamethasone suppresses this increase. There is increased baseline phosphorylation of Raf, Mek 1/2, and ERK1/2 in the cells in the dexamethasone-free or 10⁻⁸ M dexamethasone for 72 h. C. Cells were cultured (n=3 wells) in the presence of increasing concentrations of TGFα (0, 0.1, 1, or 5 nM) in 10⁻⁸ or 10⁻⁶ M dexamethasone. Immunoblots were analyzed by scanning densitometry for ErbB2 (left panel) and ERK1/2 (right panel) at 72 h. Note that 10⁻⁸ M dexamethasone diminishes ErbB2 and ERK1/2 expression.

**Fig. 5:** Dexamethasone regulates acute EGF-dependent phosphorylation of AKT and Erk1/2. We treated cells with either 10⁻⁸ or 10⁻⁶ M dexamethasone for 24 or 72 h and then exposed them to EGF (50 nM) for 0, 1, 5, 3, or 6 min. This figure shows a representative experiment. Note that 10⁻⁸ M dexamethasone increases the total amount of AKT and ERK at 24 h. Both AKT and ERK increase at 72 h, but the increase in the total amount of ERK1, but not ERK2 or AKT, is suppressed by the highest concentration of dexamethasone. All of the phosphorylated forms of ERK1/2 and AKT are diminished in cells culture in the highest concentrations of dexamethasone at 72 h, which indicates that other mechanisms besides protein density account for the inhibitory effect of dexamethasone in this model.
**Fig. 6: Dexamethasone modulates inhibitors of MAPK and PI3K-signaling pathways:** We treated cells for 24, 48 or 72 h with different concentrations of dexamethasone and evaluated the total protein expression RALT (left panel), MKP-1 (middle panel), and the 85 kDa regulatory subunit of PI3K (right panel). Note higher concentrations of dexamethasone stabilize the expression of RALT while inducing that of MKP-1. Dexamethasone is required for p85 expression at 72 h, but suppresses its expression at 24 h in a dose-dependent manner.
A.

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Culture Time (h) 24 48 72 24 48 72

B.

![Graph showing ErbB3 and ErbB2 expression levels over time for different concentrations of Dex (M).](image)
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