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Dexamethasone Attenuates the Estradiol-induced Increase of IGF-I mRNA in the Rat Uterus

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In recent years growth factors, e.g. insulin-like growth factor-I (IGF-I), transforming growth factor-α (TGF-α) and epidermal growth factor (EGF), have been considered as mediators of estradiol-stimulated growth in the uterus. In the liver, dexamethasone (Dex) has been shown to inhibit the IGF-I mRNA increase induced by growth hormone (GH). In the present study the influence of Dex on estradiol-stimulated induction of uterine IGF-I mRNA was examined. The concentration of IGF-I mRNA in the uterus and liver was monitored, as well as the levels of ER mRNA and estrogen receptor (ER). Since it has been previously shown that the maximal induction of uterine IGF-I mRNA after estradiol (E_2) stimulation occurs after 21–24 h, Dex was administered to ovariectomized (ovx) rats 3 h before an E_2 injection and 24 h before sacrifice. There was a significant decrease in IGF-I mRNA in the Dex + E_2 treated rats compared to the rats given E_2 only. In both groups an increase was seen compared to the level in the ovx control group. The uterine ER mRNA levels in E_2 and Dex + E_2 treated animals were significantly elevated compared to the ovx control. There were no significant changes in uterine ER content after hormone treatment compared to the level in ovx control rats. In the liver no effects on IGF-I mRNA were detected. Hepatic ER mRNA was significantly increased in the E_2 treated group, compared to both the ovx control group and the animals that received Dex + E_2. The hepatic ER level was also increased in the E_2 treated group compared to the ovx control and the group which received Dex + E_2. In conclusion, Dex does attenuate the estrogen-induced uterine IGF-I mRNA increase in ovx rats. In addition to this, Dex was found to inhibit the estrogen-induced increase in ER and ER mRNA in the liver of ovx rats.

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INTRODUCTION

Glucocorticoid hormones supress growth in general and they have also been shown to affect reproductive functions. The supression of reproductive functions by glucocorticoids is likely part of the mechanisms involved in delaying reproduction at times of stress [1]. Rabin *et al.* [2] suggested that this is an adaptive response in order to conserve energy during hardship. It has also been shown that glucocorticoids inhibit estradiol-mediated uterine growth and decrease uterine estradiol receptor concentration [2, 3]. It is known that glucocorticoids induce tissue resistance to insulin-like growth factor-I (IGF-I) either directly or via stimulating the secretion of an IGF-I inhibitor by the liver [4].

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Since it has been shown that estradiol (E_2) mediates at least part of its action via growth factors, e.g. IGF-I [5–8], the present study was initiated to examine whether the induction of IGF-I mRNA in the uterus after E_2 stimulation is affected by simultaneous dexamethasone (Dex) treatment. Luo and Murphy [9] have shown that an injection of Dex 3 h before growth hormone (GH) treatment of hypophysectomized (hx) rats inhibits the induction of IGF-I mRNA in the liver. They also found that IGF-I mRNA decreased in pituitary-intact rats after 6 days of daily Dex injections. The estrogen-induced IGF-I mRNA elevation is maximal 21–24 h after the initiation of treatment [8]. Thus, the present study was designed to examine the IGF-I mRNA levels in a 24 h experiment, in which rats were injected with Dex 3 h before the E_2 injection and 24 h before sacrifice. GH and GH + Dex were also administered to ovariectomized (ovx) rats to study if it would

influence the hepatic IGF-I mRNA in ovx rats in the same way as in hx rats.

MATERIALS AND METHODS

Animals and hormone administration

Adult, 55–60 days old, female Sprague–Dawley rats weighing approx. 250 g, were used. The animals were housed in a controlled environment at 20°C on an illumination schedule of 12 h of light–dark each day. Standard pellet food and water were provided *ad libitum*.

Ovariectomy was performed under light ether anaesthesia 7 days before hormone treatment.

Growth hormone (Genotropin®), was a gift from Kabi/Pharmacia, Stockholm, Sweden. Estradiol-17β and Dex, purchased from Sigma Co. (St Louis, MO), were dissolved in 99.5% ethanol at a high concentration and then diluted with 0.9% NaCl to the proper concentration. The final ethanol concentration in the injections was less than 5%. The animals were injected subcutaneously in the neck with 100 μl (Table 1).

Preparation of subcellular fractions

Detailed outlines of the procedures for preparation of subcellular tissue fractions are given in Eriksson [10, 11]. In short, the *vena cava* was cannulated and the portal vein severed. The liver was perfused *in situ* with 10 ml of body warm saline solution and then rapidly excised, weighed and trimmed. It was drained in cold saline for 5 min, dried briefly on Kleenex, a portion was removed for mRNA analysis and all were put in liquid nitrogen. It was stored at −70°C until used. After thawing it was cut into smaller pieces and homogenized in 3 vol of Tris–EDTA (10 mM Tris + 1.5 mM EDTA) buffer pH 7.4 in a Potter–Elvehjelm homogenizer with a Teflon pestle. The homogenate was centrifuged at 5000 *g* for 20 min in a Beckman JA-20 rotor. The lipid layer was removed and the supernatant recentrifuged at 184,000 *g* for 80 min in a Beckman 70Ti rotor. A small portion of the supernatant was removed for protein determination and the remainder transferred to a JS-13 tube. Solid ammonium sulphate was added under constant stirring to 35% saturation. The solution was stirred at 4°C for 60 min. The precipitate was collected following centrifugation at 12,000 *g* for 15 min in a Beckman JS-13 rotor. The precipitate was dissolved in 7 ml of TE buffer and used in binding experiments. Unless otherwise stated all procedures were performed at 0–4°C.

The uterus was removed, stripped of adhering fat and connective tissue, weighed, cut in two equal halves and immediately frozen in liquid nitrogen and stored at −70°C until analyzed. After thawing one half was analyzed with respect to receptor content. It was homogenized in TE-buffer in a glass–glass homogenizer, and the homogenate centrifuged at 1200 *g* for 20 min in a Beckman JA-20 rotor. The supernatant was recentrifuged at 192,000 *g* for 65 min in a Beckman SW55 rotor and the highspeed supernatant used for determination of cytosolic receptors, i.e. referring to an extractable pool of loosely bound nuclear ER that is found in the cytosol as a result of the cell fractionation procedures. The nuclear pellet obtained after the first centrifugation was used for determination of nuclear receptors.

Chemicals

[2,4,6,7-^{3}H]Estradiol-17β(3β,17β-dihydroxy-1,3,5(10)-estratriene) sp. act. 90.0 Ci/mmol was purchased from New England Nuclear (Boston, MA). Radioactivity was monitored in a LKB-Wallac 1215 Rackbeta liquid scintillation counter using Ready Safe (Beckman) as scintillator. Unlabelled diethylstilbestrol (DES) was purchased from Sigma Co. (St Louis, MO). Dextran T70 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden).

Analytical methods

Binding studies of cytoplasmic receptors were performed in the presence of a wide range of [^{3}H]estradiol concentrations (0.4–20.0 nM) ± 100-fold excess of cold DES. Tubes were vortexed and incubated at 4°C for 16 h and measurements of specific estrogen binding were performed by the dextran-coated charcoal method [12]. Determinations of transformed nuclear estrogen receptors were performed by the Nuclear Exchange Method (NEA) as described by Clark and Peck [13]. Data were plotted according to the method of Scatchard *et al.* [14] and protein determination performed according to Lowry *et al.* [15].

The results are shown as means ± SEM and the statistical calculations were done by ANOVA and

Table 1. Schedule over the hormonal treatment of the rats.

Experimental group	Time: 0 h	Time: 3 h	Time: 24 h
Ovariectomized control (OvxC)	Vehicle	Vehicle	Sacrifice
E_2	Vehicle	2.5 μg	Sacrifice
Dex	250 μg	Vehicle	Sacrifice
GH	Vehicle	100 μg	Sacrifice
Dex + E_2	250 μg	2.5 μg	Sacrifice
Dex + GH	250 μg	100 μg	Sacrifice

$n = 6$ rats in each group.

significances evaluated according to Scheffé [16], using WinSTAT computer program (Kalmia Co. Inc.).

Hybridization probes

The probe used for the IGF-I mRNA determinations was derived from a genomic clone of mouse IGF-I [17]. A 160 bp BanI–PvuI fragment from exon 3 (assuming homology to human IGF-I) was subcloned into the RNA expression vector pSP64.

The probe used for ER mRNA determinations was derived from pMOR101, a EcoRI fragment of 1.9 kb containing the whole open reading frame of the mouse estrogen receptor (ER) [18]. It was subcloned into the RNA expression vector pGEM-3. Restriction of this vector with Bgl II allows the synthesis of a probe corresponding to nucleotides 1470–2062 which encode the C-terminal half of the steroid binding domain E and all of domain F.

Thus, ER mRNA and IGF-I mRNA were detected in rat tissue using heterologous mouse probes. Rat IGF-I and mouse IGF-I show a high degree of homology. The feasibility to use the mouse IGF-I probe to specifically detect rat IGF-I mRNA has previously been demonstrated by Möller *et al.* [19].

The homology between mouse ER (pMOR) and rat ER is 97% and, on Northern blot analysis, the mouse derived probe hybridized to a rat liver mRNA of the approximate size of 6.5 kb [20]. This size is in good agreement with already published data on the ER mRNA [18]. The RNase protected hybrids were large enough to be precipitated by trichloroacetic acid (TCA).

A 1300 cDNA of rat glyceraldehyde-3-phosphate dehydrogenase (GAP DH), subcloned into the RNA expression vector pIBI-30, was used to synthesize RNA probes [21].

β-actin mRNA hybridizations were performed with a 51 bp probe, corresponding to amino acids 331–347 in exon 6 of the rat cytoplasmatic β-actin gene.

Hybridization analysis of mRNA

A solution hybridization assay of specific mRNA was used and performed as follows. Total nucleic acids (TNA) were prepared by digestion of homogenized tissue with proteinase K in a SDS-containing buffer, followed by subsequent extraction with phenol–chloroform as described by Durnam and Palmiter [22]. The concentration of DNA in the samples was measured fluorometrically at a wavelength of 458 nm. For measurements of specific mRNA, probes were synthesized *in vitro* and radiolabeled with ^{35}S-UTP (Amersham, Bucks, U.K.) The *in vitro* synthesis of radioactive cRNA was performed essentially as described by Melton *et al.* [23] using reagents supplied from Promega Biotech (Madison, Wisconsin). ^{35}S-UTP labeled cRNA was hybridized (20,000 cpm/incubation) at 70°C to TNA samples as described elsewhere [17]. Incubations were performed in duplicates in microcentrifuge tubes (Treff AG, Switzerland) in a total volume of 40 μl 0.1 × SET buffer containing 0.6 M NaCl, 0.75 mM dithiothreitol (DTT), and 25% formamide (v/v). After overnight incubation under two drops of paraffin oil, each sample was treated for 45 min at 37°C with 1 ml RNase buffer containing 0.3 M NaCl, 10 mM TRIS (pH 7.5), 2 mM EDTA, 40 μg RNase A, 0.3 μg RNase T1 (Boehringer-Mannheim, Germany) and 100 μg calf thymus DNA, to digest non-hybridized RNA. Labeled hybrids protected from RNase digestion were precipitated by addition of 100 μl 6 M trichloroacetic acid (TCA) and collected on filters (Whatman GF/C).

The inter-assay variation in the solution hybridization assay was found to be 8.2% and in the same set of experiments the intra-assay coefficient was 4.0% [20].

RESULTS

It is hard, if not impossible, to find an internal mRNA standard that is not affected by hormone stimuli. β-actin mRNA and GAP DH mRNA are often used as internal controls. Hsu and Frankel [24] have shown that β-actin mRNA is stimulated by E_2 in the immature rat uterus, and de Leeuw *et al.* [25] and Verneaux *et al.* [26] have reported that both β-actin mRNA and glyceraldehyde phosphate dehydrogenase (GAP DH) mRNA are subject to considerable variation in mammalian tissues. In this study GAP DH mRNA was measured in all samples. In the uterine samples β-actin mRNA was also analyzed. Since at least three different mRNA species have been measured in each sample it was possible to distinguish between specific induction and a variation in the whole mRNA population.

The uterine wet weights were determined after sacrifice. The weight increased in the E_2 treated group (mean ± SD; 112 ± 28 mg) compared to ovx controls (80 ± 9 mg). The weight in the Dex + E_2 treated group (95 ± 21) did not differ from either of these two groups.

Uterine IGF-I mRNA increased 6-fold in the E_2 treated group, and 4-fold in the Dex + E_2 treated group. There was a significant difference between the IGF-I mRNA levels of these two groups. The levels of uterine IGF-I mRNA in the groups treated with Dex, GH or Dex + GH were all unaffected (Fig. 1). Uterine ER mRNA was significantly increased in E_2 treated and Dex + E_2 treated animals. There was no significant difference between these two groups. The ER mRNA levels in the groups treated with Dex, GH or Dex + GH were unchanged compared to the ovx control group (Fig. 1). The GAP DH mRNA increased 6-fold in the E_2 treated group and 10-fold in the Dex + E_2 treated group. No significant changes were seen in the other treatment groups (Fig. 1). The amount of β-actin mRNA was doubled in the E_2 and

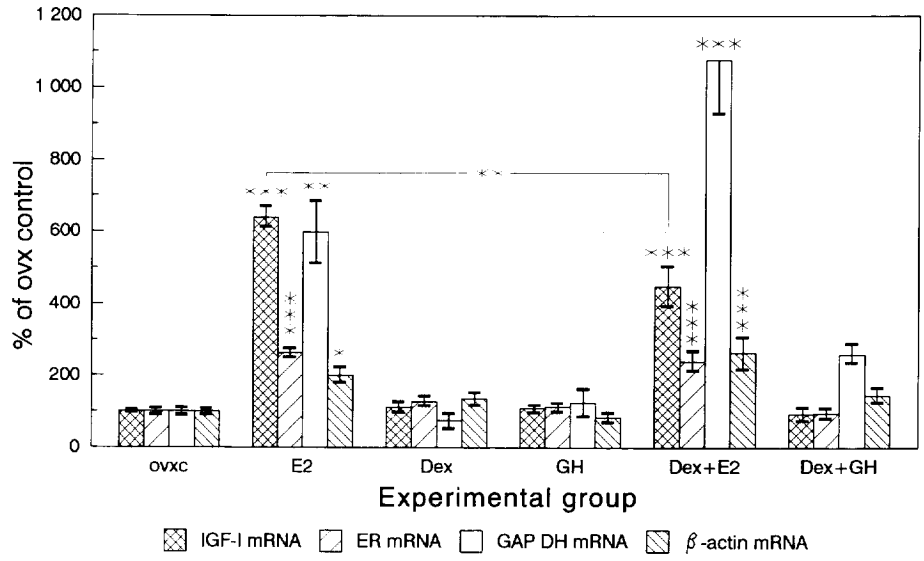

Fig. 1. The results on uterine IGF-I mRNA, ER mRNA, GAP DH mRNA and β-actin mRNA from ovx control (ovxc) rats treated with E_2, Dex, GH, Dex + E_2 and Dex + GH measured by solution hybridization. Dexamethasone or vehicle were given at time 0. Estradiol, GH or vehicle were given after 3 h. The rats were sacrificed after 24 h. There were six rats in each group. In the mRNA determinations the ovx animals were set to 100%. Values are expressed as mean ± SEM. Significances are evaluated according to Scheffé. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Dex + E_2 treated groups, whereas the Dex, GH and Dex + GH treated groups were unaffected (Fig. 1).

Uterine ER was not significantly changed in the hormone treated groups compared to the ovx controls (data not shown).

Hepatic IGF-I mRNA levels displayed no significant changes (Fig. 2). The ER mRNA in the liver increased in the E_2 treated animals, and this increase was also significant compared to the group treated with Dex + E_2. The hepatic ER mRNA level in the groups treated with Dex, GH, Dex + E_2 or Dex + GH was unaffected as compared to the ovx controls (Fig. 2). The GAP DH mRNA level increased in the Dex (2-fold), Dex + E_2 (1.8-fold) and Dex + GH (1.6-fold) treated groups compared to the ovx control (Fig. 2). The hepatic ER was increased in the E_2 treated group compared to the ovx control and also compared to the Dex + E_2 treated group. The ER concentration in the

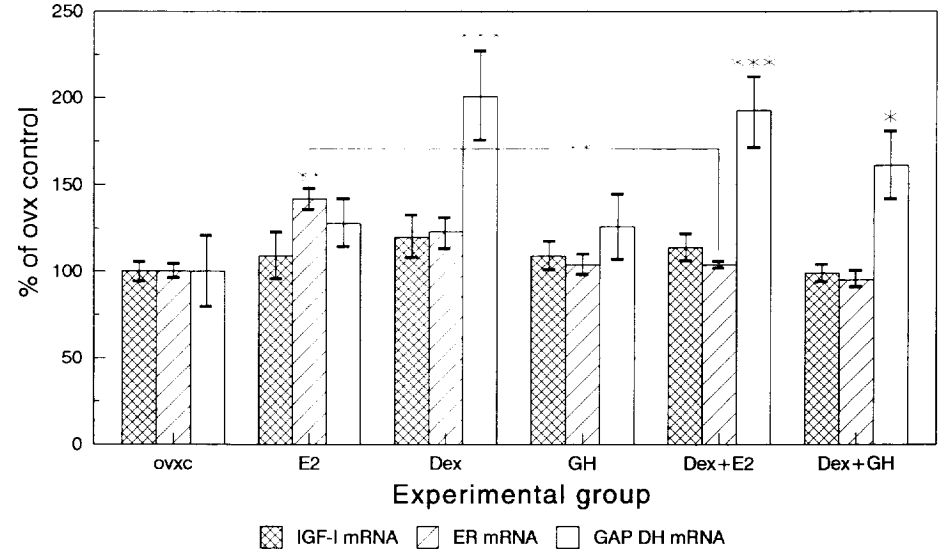

Fig. 2. The results on hepatic IGF-I mRNA, ER mRNA and GAP DH mRNA from ovx control (ovxc) rats treated with E_2, Dex, GH, Dex + E_2 and Dex + GH measured by solution hybridization. Dexamethasone or vehicle were given at time 0. Estradiol, GH or vehicle were given after 3 h. The rats were sacrificed after 24 h. There were six rats in each group. In the mRNA determinations the ovx animals were set to 100%. Values are expressed as mean ± SEM. Significances are evaluated according to Scheffé. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

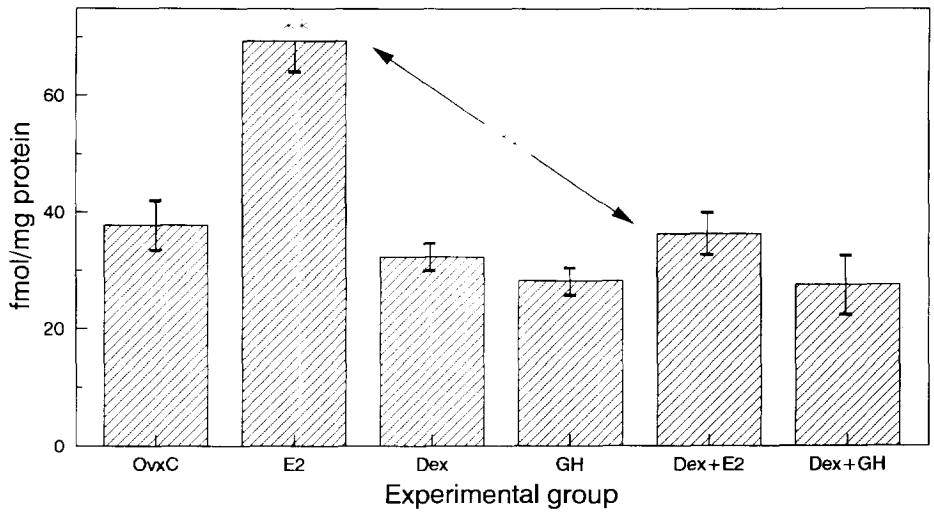

Fig. 3. The results on ER in the liver from ovx control (ovxc) rats treated with E_2, Dex, GH, Dex + E_2 and Dex + GH. Dexamethasone or vehicle were given at time 0. Estradiol, GH or vehicle were given after 3 h. The rats were sacrificed after 24 h. There were six rats in each group. Values are expressed as mean ± SEM. Significances are evaluated according to Scheffé. $^{**}P < 0.01$.

groups treated with Dex, GH, Dex + E_2 or Dex + GH was not affected by the treatment (Fig. 3).

DISCUSSION

Estrogens stimulate uterine growth during two physiological phases designated early and late responses. The early response includes events such as increased uterine blood flow, histamine release, cAMP production and increased concentration of osmotically active electrolytes that subsequently induce water imbibition [13, 27–29]. Furthermore, in recent years the activation of proto-oncogenes, which encode nuclear regulatory proteins, are considered important in the proliferative response [30–32], as well as the enhanced expression of growth factors and their receptors [5, 33–38] during this phase. The late phase is characterized by nuclear synthetic events and increased metabolic activity, leading to increased protein- and RNA-synthesis and subsequently to increased uterine dry weight and DNA content [39–41].

Dexamethasone inhibits GH mediated induction of hepatic and tibial IGF-I mRNA and serum IGF-I in hypophysectomized rats [9]. Adamo *et al.* [42] reported that Dex decreased steady state levels of IGF-I mRNA in cultured rat neuronal and glial cells. Their *in vitro* data together with the *in vivo* data reported by Luo and Murphy [9] suggest that the inhibition of IGF-I gene expression may be a mechanism whereby glucocorticoids retard growth.

Campell [3] administered 1 mg of Dex 20–25 min before 1 μg of E_2 to immature rats weighing 45–55 g and observed significant reductions in uterine growth after 3 and 24 h of E_2 treatment. The reduction was more pronounced after 3 h (26%) than after 24 (9.0%). In the present study the decrease in uterine growth after Dex treatment of E_2 stimulated ovx rats was 15%, but the decrease was not significant. Campell [3] used 20 μg Dex and 20 ng E_2/g immature rat whereas in this study the corresponding doses were 1 μg Dex and 10 ng E_2/g ovx rat. Thus, the effect of Dex could be a dose-dependent phenomenon.

Rabin *et al.* [2] showed a reduction in uterine growth after Dex treatment to estrogen-stimulated ovx rats. They performed a 5 day study and used a dose of Dex corresponding to 4 μg/g ovx rat/day. In the same study Dex was also shown to decrease E_2-induced uterine cytosolic and nuclear ER levels after 5 days of treatment. In the present study there was no significant difference in the induction of uterine ER mRNA between the Dex + E_2 treated rats and the E_2-stimulated group. Both groups showed an increase in uterine ER mRNA as compared to the ovx control group, but no difference compared to the ovx controls with regards to the ER proteins. If the induction of ER mRNA is compared to the internal standards, it seems likely that the 2-fold increase in ER mRNA reflects the general mRNA stimulation due to E_2 treatment, especially since no induction in ER can be observed.

Rabin *et al.* [2] proposed as one explanation to the inhibitory effect of Dex, that glucocorticoids may inhibit estrogen-dependent growth factor secretion. The present study supports this hypothesis. The E_2-induced IGF-I mRNA increase was indeed attenuated by a preceding Dex injection. The attenuation is only partial, but the difference between the E_2 treated and the Dex + E_2 treated animals is significant ($P < 0.01$). Although the expression of IGF-I mRNA in the Dex + E_2 treated animals was decreased compared to the E_2 treated group, their level of IGF-I mRNA was increased compared to the ovx control group. This

could explain why no significant decrease in uterine wet weight was seen after the Dex treatment of E_2 stimulated rats. Another explanation, which cannot be excluded, is that IGF-I is not the only growth factor responsible for E_2-stimulated growth and that the other factor/s is/are not affected by Dex. Both the internal standards are induced after E_2 treatment, GAP DH mRNA in the same range as IGF-I mRNA, but as β-actin mRNA and ER mRNA are induced to 1/3 of these they are considered to reflect the general induction. The decrease in IGF-I mRNA after Dex + E_2 treatment compared to E_2 treatment is not seen for GAP DH mRNA. On the contrary, GAP DH mRNA is increased even more in the Dex + E_2 group compared to the E_2 treated group. Therefore the induction of IGF-I mRNA after E_2 treatment is considered specific just as the attenuation seen in IGF-I mRNA after an injection with Dex prior to the E_2 treatment.

The effect of GH on hepatic IGF-I mRNA that is seen in hx rats [43], was not seen in ovx rats in this study.

In hypophysectomized animals GH treatment increases ER and ER mRNA concentrations, although not to the levels seen in intact animals [20, 44]. In ovx rats, E_2 increases the levels of hepatic ER and ER mRNA [8], whereas GH has no effect when injected, as in this study. The stimulating effects of E_2 on hepatic ER and ER mRNA are thought to be indirect via increased trough levels of serum GH [45]. Glucocorticoid treatment of intact animals has been shown to decrease GH secretion in general [46]. Thus, the finding that Dex inhibits E_2-induced increase of ER and ER mRNA in the liver of ovx rats, could be explained by the Dex-mediated decrease of GH antagonizing the estrogen-induced increase in serum GH levels. Dexamethasone treatment to ovx rats does not decrease hepatic ER and ER mRNA, indicating that Dex is selectively antagonizing the elevated GH levels due to E_2 stimulation.

An additional finding in this study is that GAP DH mRNA is regulated by Dex in the liver. All three treatment groups where Dex is given exhibit significant increases in GAP DH mRNA.

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