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Ethanol Alters Production and Secretion of Estrogen-Regulated Growth Factors That Control Prolactin-Secreting Tumors in the Pituitary

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Abstract

Background—Chronic administration of ethanol increases plasma prolactin levels and enhances estradiol's mitogenic action on the lactotropes of the pituitary gland. The present study was conducted to determine whether ethanol's lactotropic cell-proliferating action, like estradiol's, is associated with alteration in the production of 3 peptides that regulate cell growth: transforming growth factor beta 1 (TGF- β 1), TGF- β 3 and basic fibroblast growth factor (bFGF).

Methods—Using ovariectomized Fischer-344 female rats, we determined ethanol's and estradiol's actions on lactotropic cell proliferation and growth-regulatory peptide production and release in the pituitary gland during tumorigenesis.

Results—Ethanol increased basal and estradiol-enhanced mitosis of lactotropes in the pituitary glands of ovariectomized rats. The level of growth-inhibitory TGF- β 1 was reduced in the pituitary following ethanol and/or estradiol treatment for 2 and 4 weeks. In contrast, ethanol and estradiol alone as well as together increased levels of growth-stimulatory TGF- β 3 and bFGF in the pituitary at 2 and 4 weeks. In primary cultures of pituitary cells, both ethanol and estradiol reduced TGF- β 1 release and increased TGF- β 3 and bFGF release at 24 hours. Ethanol's effect on growth factor levels in the pituitary or growth factor release from the pituitary cells was less than that of estradiol. When ethanol and estradiol were applied together, their individual effects on these growth factors were amplified.

Conclusions—These results confirm estradiol's modulation of pituitary growth factor production and release, and provide evidence that ethanol, like estradiol, alters the production and secretion of growth-regulatory peptides controlling lactotropic cell proliferation.

Keywords

Prolactinomas; Estradiol; Ethanol; Transforming Growth Factor Beta 1 and 3; Basic Fibroblast Growth Factor

Several reports provide evidence for the existence of high levels of prolactin in male and female chronic alcoholics. Two studies conducted in Europe and Japan using alcoholic women who reported daily alcohol intake of 84 to 170 g daily for a period between 2 and 16 years but had no clinical evidence of alcoholic liver cirrhosis showed persistent hyperprolactinemia (Seki et al., 1991; Välimäki et al., 1990). Studies conducted in the United States reported

hyperprolactinemia (22 to 87 ng/ml) in 6 of 12 alcohol-dependent women who had a history of drinking 75 to 247 g of alcohol per day for a minimum period of 7 years (Teoh et al., 1992). Alcohol-induced hyperprolactinemia is also reported in healthy, well-nourished women during residence on a clinical research ward for 35 days (Mendelson and Mello, 1988). Sixty percent of women in the heavy drinker category (blood alcohol level: 109 to 199 mg/dl) and 50% of moderate drinkers (blood alcohol levels: 48 to 87 mg/dl) showed elevated plasma prolactin levels, and many of these drinkers had elevated plasma prolactin several days after cessation of drinking. Alcoholic men also showed elevated levels of prolactin (Ida et al., 1992; Marchesi et al., 1992; Soyka et al., 1991). Male alcoholic patients frequently show evidence of feminization such as gynecomastia, spider angiomas, palmar erythema and changes in body hair patterns (Van Thiel, 1979). Alcohol-induced hyperprolactinemia and pituitary hyperplasia have been demonstrated in nonhuman primates (Kornet et al., 1991; Mello et al., 1988). In estrogen-sensitive Fischer-344 female rats, chronic ethanol administration increases plasma prolactin levels and pituitary weight in cyclic female rats and ovariectomized rats as well as potentiates estradiol's mitogenic effects in ovariectomized female rats (De et al., 1995; Sanchis et al., 1985). How ethanol increases lactotropic cell proliferation is not well understood.

In Fischer-344 rats, estradiol's mitogenic action is regulated by the interplay of various growth-inhibitory and growth-stimulatory peptides. It has been shown that lactotrope proliferation is inhibited by transforming growth factor beta 1 (TGF- β 1) (Hentges et al., 2000a; Sarkar et al., 1998) but stimulated by TGF- β 3 (De et al., 2002; Hentges et al., 2000a) and basic fibroblast growth factor (bFGF) (Chaturvedi and Sarkar, 2005; Hentges et al., 2000b). Estradiol, a potent mitogen for lactotropes, decreases TGF- β 1 levels but increases TGF- β 3 and bFGF levels by altering the gene transcription of these peptides in the pituitary (Chaturvedi and Sarkar, 2005; Hentges et al., 2000a,b; Pastorcic and Sarkar, 1997). The role of these growth-regulating peptides in ethanol's modulation of lactotropic cell proliferation was investigated in this study.

MATERIALS AND METHODS

Animals

Female rats of the Fischer-344 strain (100 to 140 g of body weight) obtained from Simonsen Laboratories (Gilroy, CA) were housed in a controlled environment (temperature 22°C, lights on 05:00 to 19:00 hours), and provided with certified rodent chow meal (Purina Mills, Inc., St Louis, MO) or liquid diet (Bio-Serv Liquid Rat Diet L/D'82, Bio-Serv, Frenchtown, NJ) and water ad libitum. Rats underwent bilateral ovariectomy using sodium pentobarbitone anesthesia, followed by the surgical subcutaneous (sc) implantation of an estradiol-containing Silastic capsule or empty Silastic capsule. The estradiol capsule maintained plasma levels of estradiol 17- β between 120 and 150 pg/ml (De et al., 1995). The rats were either ad libitumfed rodent chow meal (ad lib-fed), pair-fed an isocaloric liquid diet (pair-fed) or fed an ethanol-containing liquid diet (alcohol-fed) for 14 or 28 days using the procedure as described by us previously (Chen et al., 2006). The concentration of ethanol in the diet was 8.7% v/v, which provided about 37% of the total dietary calories. The ethanol treatment regimen used has been shown to maintain blood alcohol levels within the range of 115 to 123 mg/dl between day 10 and 30 (De et al., 2002).

In Vivo Lactotropic Cell Proliferation

At the end of each experiment, animals were intraperitoneally (ip) injected with 50 mg/kg body weight of bromodeoxyuridine (BrdU) 4 hours prior to sacrifice. After sacrifice, anterior pituitaries were removed, weighed, fixed in 4% buffered formaldehyde, and used for immunocytochemical detection of BrdU-labeled lactotropes undergoing replicative DNA

synthesis (De et al., 1995). At least 5,000 labeled and unlabeled cells from each anterior pituitary were counted.

Primary Cultures of Anterior Pituitary Cells

Anterior pituitaries from ovariectomized rats implanted with estradiol-containing capsules for 7 to 10 days were dissociated enzymatically with Hank's balanced salt solution (HBSS) containing collagenase, DNase and bovine serum albumin (BSA), and grown on poly-L-lysine-coated coverslips (Hentges et al., 2000a). Cells were maintained in Delbecco's Modified Eagle's Medium (DMEM; 1:1; Sigma, containing 100 units/ml penicillin and 100 μ g/ml streptomycin) with high serum (10% fetal calf serum; FCS) for 1 day and then in medium containing 2.5% FCS and 10% horse serum for another 2 days. Cultures were then maintained in serum-free DMEM containing human transferrin (100 nM), insulin (5 μ M), putrescine (1 μ M), and sodium selenite (30 nM). The lactotropic cell population in these cell cultures, as determined by identifying prolactin-immunoreactive cells, was $59 \pm 2\%$ ($n = 8$).

Assays of TGF- β 1, TGF- β 3, bFGF, and Tissue Total Protein

Anterior pituitary glands from ethanol-fed and control-fed rats were excised, sonicated in 1 ml of 0.01 M PBS (pH 7.0), and centrifuged. The pituitary extracts as well as cell culture medium were acidextracted (final concentration: 4 mM HCl, 50 μ g/ml bacitracin, 1 KU/ml trasylol, 1% BSA), lyophilized, reconstituted in assay buffer, and used for measurement of TGF- β 1, TGF- β 3, and bFGF levels using ELISA kits (Research & Diagnostic System Inc., Minneapolis, MN). Tissue levels of protein were measured using bicinchoninic acid reagents (Pierce, Rockford, IL).

Statistics

The mean and SE of the data were determined. The 1-way analysis of variance test followed by Student–Newman–Keuls post hoc tests were used to determine the significance of differences between groups. A $p < 0.05$ was considered significant.

RESULTS

Ethanol and Estradiol Actions on Lactotropic Cell Proliferation and Growth Factor Production in the Pituitary

Previously, we have shown that ethanol administration increased pituitary lactotropic cell proliferation in both ovariectomized and estradiol-treated ovariectomized rats (De et al., 1995, 2002). These data were confirmed in the present study in which the BrdU-positive lactotropes in the pituitary gland were counted following ethanol feeding in ovariectomized and estradiol-treated ovariectomized rats. BrdU was utilized to identify and quantitate cells undergoing DNA synthesis in rat anterior pituitary glands. BrdU-stained cells were identified within the lactotropes by prolactin immunostaining. The percentage of BrdU- and prolactin-labeled cells (mitotic lactotropes) increased in a time-dependent manner following estradiol treatment (Fig. 1A). The percentage of mitotic lactotrope was also increased after ethanol treatment as compared with pair-fed or ad lib-fed control rats at 2 and 4 weeks in ovariectomized and estradiol-treated ovariectomized animals (Fig. 1A). The percentage of mitotic lactotropes was similar between pair-fed or ad lib-fed ovariectomized and estradioltreated ovariectomized animals.

Previously we have shown that estradiol-induced lactotropic cell proliferation is regulated by coordinated actions of growth-inhibitory TGF- β 1 and growth-stimulatory TGF- β 3 and bFGF (Sarkar, 2006). In agreement with this, as shown in Fig. 1B–D, estradiol time-dependently decreased TGF- β 1 levels but increased TGF- β 3 and bFGF levels in the anterior pituitary. Like

estradiol, ethanol alone reduced TGF- β 1 level and increased TGF- β 3 and bFGF levels in the anterior pituitary. Ethanol also potentiated estradiol's effects on TGF- β 1, TGF- β 3 and bFGF levels. Furthermore, estradiol-, ethanol-, and estradiol + ethanol-induced levels of TGF- β 1 were negatively associated with the percentage of mitotic lactotropes, while levels of TGF- β 3 and bFGF were positively associated (compare Fig. 1A–D).

Ethanol's and Estradiol's Actions on Growth Factor Secretion From the Anterior Pituitary Cells

To determine ethanol's and estradiol's actions on growth factor release from the anterior pituitary cells, we measured TGF- β 1, TGF- β 3 and bFGF release from pituitary cell cultures following ethanol and/or estradiol treatments. These studies revealed that treatment with estradiol or ethanol alone for a period of 24 hours reduced TGF- β 1 release but increased TGF- β 3 and bFGF release from pituitary cells in cultures (Fig. 2A–C). The effect of estradiol on the release of these growth factors was greater than that of ethanol. When both ethanol and estradiol were given, their individual effects were enhanced.

DISCUSSION

We have previously shown that estradiol treatment caused a time-dependent increase in the number of proliferating lactotropes and plasma levels of prolactin in Fisher-344 ovariectomized rats (Banerjee et al., 1994; Gottschall et al., 1986). Data presented in this study confirm estradiol's mitogenic action by demonstrating BrdU incorporation into a significant population of lactotropes in the anterior pituitary of ovariectomized rats. These data also show that repetitive administration of a moderate dose of ethanol for a period of 2 and 4 weeks increases the number of mitotic lactotropes and potentiates estradiol's mitogenic action on lactotropic cells in the pituitary. Additionally, these data provide evidence that ethanol, like estradiol, reduces pituitary levels of TGF- β 1 but increases levels of growth-stimulatory TGF- β 3 and bFGF. Furthermore, like estradiol, ethanol reduced TGF- β 1 release while increasing TGF- β 3 and bFGF release. Together, these data suggest that ethanol and estradiol similarly affect the production and secretion of lactotropic growth-regulatory peptides in the pituitary to promote prolactin-secreting tumors.

The mechanism by which ethanol increases lactotropic proliferation may involve cytokines locally produced in the anterior pituitary. Ethanol has been shown to modulate the production and activity of cytokines in various tissues that have an important role in cell proliferation and growth (Martinez et al., 1992). TGF- β 1, one of the cytokines, has been shown to have marked effects on cellular differentiation and growth in various cell types. It is a potent inhibitor of the epithelial cell types and stimulates the growth of some mesenchymal cell types (Arrick et al., 1990; Moses et al., 1985; Roberts et al., 1985; Sirbasku, 1978; Soto and Sonnenschein, 1987). We have previously shown that TGF- β 1 can inhibit cell proliferation both in vivo and in vitro (Burns and Sarkar, 1993; Hentges et al., 2000a,b; Sarkar et al., 1992). This growth factor is produced in the pituitary and inside the lactotropes. The production and secretion of TGF- β 1 are reduced during estradiol-induced tumorigenesis in the anterior pituitary. TGF- β 1 protein levels in estradiol-treated rats are further decreased by ethanol administration. In ethanol-treated rats, TGF- β 1 production and secretion are reduced during tumorigenesis in the anterior pituitary. Thus, like estradiol's action, ethanol's effect on pituitary growth may be partly mediated by alteration in TGF- β 1 production and action.

Our previous work (summarized in the review by Sarkar, 2006) indicates that estradiol exposure leads to increased lactotropic cell proliferation via orchestrated events involving TGF- β isoforms and their receptors as well as factors secondary to TGF- β action. These data demonstrate that estradiol decreases TGF- β 1, which leads to loss of growth-inhibitory control by this peptide in the lactotropes. The steroid also increases TGF- β 3 production and secretion

from lactotropes. The secreted TGF- β 3 is transported to the neighboring folliculostellate (FS) cells, where it acts to induce the release of bFGF. The FS cell-derived bFGF stimulates lactotropic cell proliferation, as lactotropes are not affected by TGF- β 1 growth inhibition and are activated by estradiol to express FGF receptors. In this study, we confirmed estradiol stimulatory effect on both TGF- β 3 and bFGF production and secretion. Additionally, we showed that ethanol increased the production and secretion of these 2 peptides in the anterior pituitary. Thus, it appears that ethanol alters the levels of lactotrope cell-growth regulating factors in the anterior pituitary. Additionally, ethanol mirrors the action of estradiol on the pituitary levels of the growth-regulatory peptides. Whether these peptide growth factors mediate ethanol's action on lactotropic cell proliferation needs further experimentation. Also, it is not apparent how ethanol alters the production of these growth factors.

Our laboratory has recently identified a signaling cascade for lactotrope growth control involving the TGF- β 1 and dopamine (Sarkar, 2006). Dopamine plays a key role in maintaining the normal function of lactotropes in the pituitary gland. Abnormalities in dopamine receptors and dopamine transporter function lead to hyperplasia of lactotropes (Bosse et al., 1997; Kelly et al., 1997; Laccarino et al., 2002; Saiardi et al., 1997). It is well known that estradiol inhibits dopamine release from the hypothalamus and downregulates dopamine D2 receptor activity in lactotropes (reviewed in Ben-Jonathan and Hnasko, 2001). The D2 receptor is a 7-transmembrane segment protein with a long third intracellular loop and a short intracellular C-terminus. The sixth exon of the D2 receptor gene is often excluded in the mature transcript, resulting in a short (29 amino acids shorter) isoform (D2S). Estradiol strongly favors the expression of the long isoform (D2L) mRNA over the short isoform D2S (Guivarc'h et al., 1998). It is interesting to note that ethanol, which increases lactotropic cell proliferation, also favors production of the D2L over the D2S isoform (Oomizu et al., 2003). Hence, we propose that during sustained exposure, ethanol or estrogen cancels the inhibitory effect of dopamine and thereby downregulates TGF- β 1's inhibitory effect on cell proliferation. This may cause an alteration in the balance between positive and negative regulators of cell growth, resulting in abnormal lactotropic cell proliferation.

Although data implicating ethanol as a risk factor in human pituitary tumor development are not available, several reports indicated chronic abnormalities in prolactin secretion in both alcoholic men and women (Mello et al., 1993). Our animal data, showing increased lactotropic cell proliferation, decreased lactotropic production and secretion of growth inhibitory polypeptide but increased pituitary production of lactotropic cell growth regulatory factors, suggest that ethanol consumption may promote or aggravate the pathology of existing pituitary tumors.

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Fig. 1.

Ethanol increases basal and estradiol-altered lactotropic cell proliferation and growth factor production in the pituitary. The changes in lactotropic growth and growth-regulatory polypeptides in the anterior pituitary were determined in ovariectomized rats sc implanted with an empty capsule (No E, 2 and 4 weeks combined) or estradiol-treated capsule (E) and fed rat chow ad libitum (Ad lib-fed) or given ethanol-containing liquid diet (Alcohol-fed) or pair-fed isocaloric diet (pair-fed) for 2 and 4 weeks. (A) The changes in the percentage of mitotic lactotopes as determined by the percentage of prolactin-stained lactotopes that are double stained with BrdU. B-D. Showing the changes in pituitary levels of TGF- β 1 (B), TGF- β 3 (C), and bFGF (D) in ovariectomized and estradiol-treated ovariectomized rats fed with control diet or ethanol diet for 2 and 4 weeks. Data are means $\pm$ SE obtained from 6 rats. ^a $p < 0.05$ compared with the similarly treated ovariectomy groups. ^b $p < 0.05$ compared with pair-fed group in similar steroid treatment.

Fig. 2.

Ethanol increases basal and estradiol-altered secretion of growth factors from the pituitary cells in primary cultures. Effect of ethanol, estradiol and ethanol + estradiol on TGF- β 1 (**A**), TGF- β 3 (**B**), and bFGF (**C**) release from mixed pituitary cells (5×10^4) in primary cultures. TGF- β 1, TGF- β 3, and bFGF levels in media samples were measured by ELISA. Treatment time was 24 hours. Data are means $\pm$ SE of 6 cultures. ^a $p < 0.001$, significantly different from the rest of the group.