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Effects of Estrogen and Prolactin on Bovine BRCA1 Gene Expression

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Abstract : Primary cultures of bovine mammary secretory epithelial cells and spontaneously transformed mammary secretory epithelial cells were treated with estradiol 17-ß or prolactin separately and in combination with both hormones. Both estradiol and prolactin induced BRCA1 expression in the cells. Hormone induced expression of BRCA1 was highest in transformed cells. Moreover, the induction of BRCA1 gene expression was highest when both estrogen and prolactin were in the culture medium. Gel shift assays showed that STAT5a antibody bound to a 20-bp double stranded oligonucleotide, containing a potential STAT5 response element (5'-acagtttctaaggaacactg-3', 3'-tgtcaaagattccttgtgac-5'). This is the first report showing that bovine mammary cells, in culture, are responsive to estrogen and prolactin in inducing expression of bovine BRCA1 and that BRCA1 may contain a STAT5 response element directing expression of the bovine BRCA1 gene. Further work is needed to examine the presence of estrogen response element(s) in estradiol induced BRCA1 expression. One must determine if PRL induced BRCA1 induction occurs after transfection of STAT5 -/- cells with STAT5a and STAT5a mutant constructs. This will give the strongest evidence of the direct involvement of a STAT5–BRCA1 signaling pathway.

Key words: BRCA1, estrogen, prolactin, mammary cells

Introduction

Prolactin and estrogen influence mammary cell growth and differentiation (Tucker, 1974). Serum concentrations of these hormones increase during gestation and prolactin is believed to be involved in the initiation of stage two lactogenesis (Tucker, 1974). Both hormones and their receptors have been implicated in breast cancer and its metastases (Wennbo and Tornell, 2000). The BRCA1 (breast ovarian cancer susceptibility protein) gene encodes a 220 kDa secretory protein with tumor suppressor like properties (Chen *et al.*, 1996; Miki *et al.*, 1994; Nevalainen *et al.*, 1996). The protein contains a short sequence shared by granins, proteins in various types of secretory vesicles whose function(s) is not yet clear (Jensen *et al.*, 1996). Mutation in the BRCA1 gene is found in about two-thirds of heritable breast carcinomas and in an un-estimated number of ovarian cancers (Futreal *et al.*, 1994).

BRCA1 protein has been localized in both the nucleus and cytoplasm of cells (Jensen *et al.*, 1996). Its localization appears to be related to the type of cell and whether it is normal or of neoplastic origin. We have shown that BRCA1 is differentially expressed in bovine mammary tissue throughout its development and involution (Chung and Gorewit, 2001). We have also shown that the protein is present in milk from lactating cattle, suggesting that it can be a secreted protein during lactation (Chung and Gorewit, 2002).

It has been recently shown that estrogen and prolactin up-regulate (BRCA1) expression in human breast cancer cells (Favy *et al.*, 1999). Although it has been shown that BRCA1 is expressed in normal and malignant breast tissue, very little is known about the mechanism(s) whereby prolactin regulates its expression.

STAT family proteins (Signal Transducers and Activators of Transcription) are transcription factors that have been found in the interferon system (Schindler and Darnell, 1995). They interact with gamma interferon via the GAS element (Heim *et al.*, 1995; Schindler and Darnell, 1995). At present, eight different STATs have been molecularly cloned (Heim *et al.*, 1995). STAT proteins have common features, including SIC and SH3 domains that are important in protein-protein interactions.

STAT proteins bind to cytokine receptor via interactions between phosphorylated tyrosine on the receptor and the SH2 domain of the STAT (Heim *et al.*, 1995). STAT binding to receptors is activated by JAK (Janus kinase) (Schindler and Darnell, 1995). Activated STATs form homodimers or heterodimers and translocate into the nucleus, where they bind to their specific target sequences and control gene expression.

STAT5 was initially identified as mammary gland factor (MGF) regulated by prolactin (Raught *et al.*, 1994). In the mammary epithelium, although STATI, STAT3, STAT5A, STAT5B and STAT6 are present in a latent form, only STAT5A and STAT5B are activated. This selective activation of STAT5 by prolactin was also observed in COS-7 cells co-transfected with the long form of the mouse prolactin receptor (PRL-R) and expression vectors for STATI, STAT3, STAT5 and STAT6 (Mayr *et al.*, 1998).

Prolactin receptor (PRLR) is a member of the cytokine/growth hormone/PRL receptor super family (Goffin *et al.*, 1999). The mechanism through which PRLR modulates STAT5 tyrosine phosphorylation, nuclear translocation, and DNA binding has been analyzed in HC11 cells, a mammary epithelial cell line, and 293-LA cells, a human kidney cell line stably over-expressing Jak2 kinase (Samir and Suhad, 1998). Wartmann *et al.*, 1996 have shown that in HC11 cells, STAT5 is specifically activated by PRL treatment, demonstrating that STAT5 is a physiological substrate downstream of the PRLR.

The pathways connecting extra cellular signals with the regulation of transcription factor activity are being investigated in detail. Considerable progress has been made in the description of the mechanism of action of steroid hormones and cytokines. Steroid hormones associate intra-cellularly with latent receptor molecules, cause the dissociation of masking proteins, the dimerization of receptors, and their binding to specific hormone response elements in the promoters of target genes (Beato *et al.*, 1996). It is well known that estrogen up regulates the expression of mammary gland prolactin receptors (Cassy *et al.*, 2000; Nevalainen *et al.*, 1996; Ormandy *et al.*, 1997).

Cytokines also activate latent transcription factors (STATs), but act through an enzymatic mechanism. For cytokines, tyrosine kinases associated with the trans-membrane cytokine receptors phosphorylate STAT molecules (Romagnolo *et al.*, 1998). The phosphorylated monomers dimerize and assume specific DNA binding ability. Both classes of transcription factors bind to different response elements and regulate different target genes and both signals, cytokines and steroid hormones, can affect growth differentiation and homeostasis of different cell types (Wartmann *et al.*, 1996).

Wyszomierski *et al.*, 2001 have recently shown that STAT5 functionally interacts with members of the steroid receptor family. They found that glucocorticoid receptor, mineralocorticoid receptor and progesterone receptor synergize with STAT5 in the induction of the transcription from the beta-casein gene promoter. The estrogen receptor decreased STAT5 mediated induction and the androgen receptor had no effect. Conversely, STAT5

negatively interfered with glucocorticoid receptor. The estrogen receptor induced transcription from an estrogen response element-containing promoter.
The objectives of the work described in this report were to determine if estrogen and prolactin could increase expression of BRCA1 in bovine mammary cells and explore the potential role of STAT5 in the process.

Materials and Methods

Cell Culture: Bovine primary mammary epithelial cells were obtained as described by (Zavizion *et al.*, 1992). Cells were cultured in medium containing a 1:1 (v/v) mixture of RPMI-1640 and Dulbecco's Modified Eagle Medium (Life Technologies, Inc) supplemented with 12% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 ng/ml) at 37 C in 5 % CO_2. Media were changed everyday the first week. After that, media were changed 2 to 3 times/week. Cells were passed before confluence using a 0.05 % trypsin solution (Life Technologies, Inc.) to maintain experimental cultures. A bovine mammary epithelial cell line (BME) was also cultured under the same culture conditions as primary epithelial cell (Zavizion *et al.*, 1992).

Effects of estrogen and prolactin on BRCA1 induction: In order to determine the effect of estrogen and prolactin on induction of BRCAl expression, primary cultures of bovine mammary epithelial cells and BME cell line cultures were treated with estradiol 17-ß or prolactin or a combination of estradiol 17-ß and prolactin and assayed for BRCA1 expression by RNAse protection assay (RPA). Cells (2×10^6) were plated in 100 mm dishes and left to grow to confluency. Before hormone treatment, cells were serum starved for 24h and media were changed without serum twice. Cells were then treated with E2 (0.5 ng/ml) or PRL (200 ng/ml) or a combination of E2 and PRL (.5ng/ml E2 and 200 ng/ml PRL). After 1 hour, medium was discarded and cells were lysed by Tri reagent LS (Molecular Research Center) to isolate total RNA using the manufacture's recommendation on assay procedures.

Ribonuclease Protection Assay (RPA): Total BRCA1 transcript levels were measured using a ribonuclease protection assay and a Multi-NPA hybridization procedure (Ambion, Inc.). The template to generate a ribo-probe was prepared by PCR from bovine BRCA1 cDNA. BRCA1 primer pair, forward (+85/+104): 5'tctgctctgggtaagttca 3' and backward (+640/+620): 5'atttaggtgacactatagaatgatgttgtatccgctgctt 3' was used for preparation of template. The reverse primer contained the SP6 RNA polymerase-binding site to make antisense ribo-probe.
A 500 nt biotin labeled antisense ribo-probe was synthesized by MAXI Script (Ambion) according to the manufacturer's recommendations. The synthesized probe was gel purified and hybridized with 15 :g of total RNA to protect a fragment of the expected length. After nuclease digestion, the protected fragment was electrophoresed and transferred to nylon membrane by electro-transfer. Protected fragments were detected by Ambion's Bright star biodetection kit. BRCA1 mRNA was quantified by Photo Image IS 1000 with arbitrary units corrected for the expression of the control, actin mRNA (BRCA1/Actin).

Gel shift assays: Bovine mammary epithelial cell line (BME) was cultured until confluency, serum starved for 24h and media were changed without serum twice. The cells were then treated with estrogen (0.2, 0.8 ng/ml) or prolactin (100, 200 ng/ml) or a combination of estrogen (0.2 ng/ml) and prolactin (100 ng/ml) for 1 hour. BME Nuclear extracts were prepared according to the method of Lemkin *et al.*, 2000. A 20-bp double stranded oligonucleotide, containing potential STAT5 response element, (5'-acagtttctaaggaacactg-3', 3'-tgtcaaagattccttgtgac-5') was synthesized based on the human BRCA1 promoter sequence (NID: gl 147602). Oligonucleotide was purified by polyacrylamide gel electrophoresis, and equimolar quantities of complementary strands were annealed in buffer (10 mM Tris, pH 8.0, lmM EDTA, 10 mM NaCL) by heating at IOO °C for 5 min and cooling to room temperature. Annealed oligonucleotide pairs were 3' end labeled with biotin-dATP by terminal transferase (Life Technologies, Inc.). Ten nanograms of nuclear extracts were pre-incubated for 10 min in a buffer containing 20 mM HEPES, pH 7.9,50 mM NaCl, I mM $MgCl_2$, I mM dithiothretol, I mM EDTA, I ng of poly (dA-dT)-poly (dA-dT), and 10 % glycerol. Probes (15 ftnol) were then added and the incubation continued at room temperature for 15 min.
In experiments to identify the protein component of the protein-DNA complexes (super shift assay), antibodies raised against STAT5a, were purchased from Santa Cruz Biotechnology (CA). Antibodies were incubated with the nuclear extracts for 15 min at room temperature before addition of the probe. Protein-DNA complexes were separated on a 5% nondenaturing polyacrylamide gel (38:1, Acrylamide-bisacrylamide; 2 % glycerol; 22 mM Tris-borate, 0.5 mM EDTA, pH 8.3) at 15 mA (l h at 4 °C). Gels were transferred onto nylon membrane and cross linked and DNA-protein antibody complexes were detected by Ambion's CDP-star biotin detection kit.

Results and Discussion

Effects of erstrogen and prolactin on BRCA1 induction: The bovine mammary epithelial cell line (BME) was more responsive to E2 and PRL, within an hour of incubation, than primary epithelial cells (Fig.1) for BRCA1 gene induction. Estradiol 17-ß and PRL induced significant increases in BRCA1 expression in BME cells. The combination of these two hormones showed an additive effect, which was two times the increase compared to BRCA1 gene induction seen in the control (Fig.1).
Marquis *et al.*, 1995; Gudas *et al.*, 1995 reported up-regulation of BRCA1 by ovarian hormones, 17B-estradiol and progesterone (P) in ovariectomized mice and in breast cancer cells. More recently, Favy *et al.*, 1999 reported prolactin (PRL)-dependent up-regulation of BRCA1 expression in human breast cancer cells. However, molecular mechanisms explaining how E2, and PRL up regulate BRCA1 are unknown.
In previous studies, breast cancer cells were incubated with hormones 18 or 24 h before analysis of BRCA1 mRNA expression. However, our cells were incubated for only one hour with these hormones. The BME cells showed high sensitivity within an hour to E2 and PRL for BRCA1 gene induction. The E2 and PRL combination induced additive BRCA1 mRNA expression. In BME cells, BRCA1 responded to mammotropic hormones like an early response gene. The primary epithelial cells did not show the same degree of response.
This result posed two questions. Why was the response different in these two different cells and do these hormones directly effect BRCA1 gene induction? To answer the first question, let us consider primary characteristics of these two cells. BME cells are spontaneously mutated cells. We believe this cell line is much more proliferative than primary epithelial cells in response to E2 and PRL. This characteristic may have resulted in the difference of BRCA1 induction seen, even though its response was within an hour. Therefore, we speculate that protective feedback mechanisms of cells induce the growth regulator, BRCA1.

Gel shift assays : We selected BME cells for the gel shift assay because the BME cells were more responsive to E2 and PRL individually, than primary epithelial cells. We used oligo nucleotides of potential STAT5 response elements for the assay. A potential GAS/STAT5 binding element was incubated with nuclear extract and resulted in bands on membrane (Fig. 2). Hormone treated cells showed more intense bands than untreated control cells. Moreover, the band intensity was sensitive to PRL concentration (Fig. 2). Super-shift assays showed reactions of STAT5a antibody with BRCA1. Shifted bands were seen compared to controls without STAT5a antibody (Fig. 2).
The best-known functions of PRL in the mammary gland are its ability to induce lobuloalveolar growth (Wennbo and Tornell, 2000) and to stimulate postpartum lactogenesis (Wennbo and Tornell,

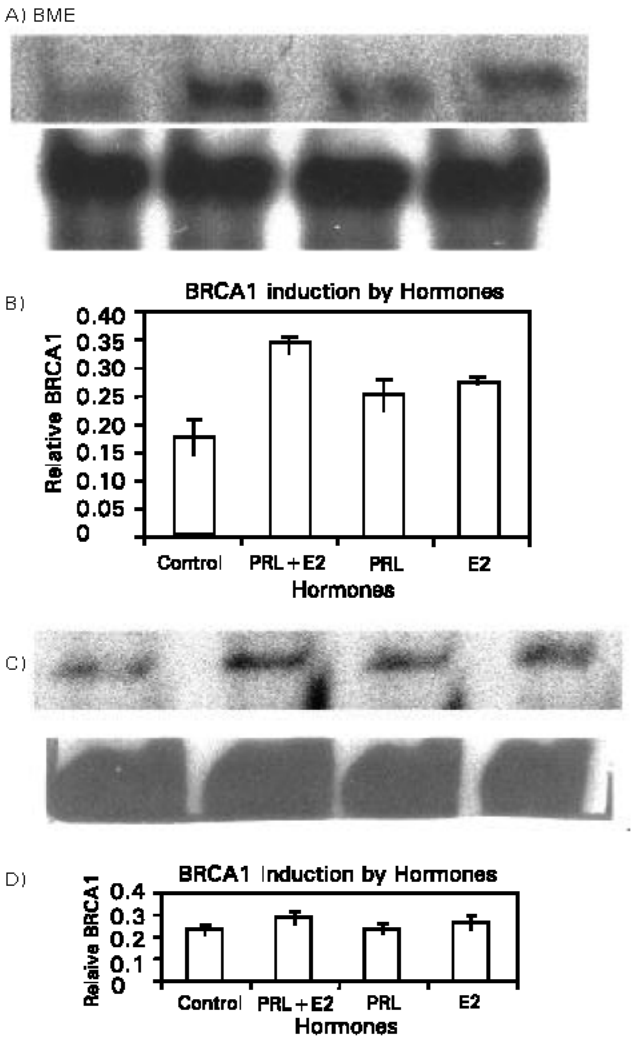

Fig. 1: BRCA1 gene expression in cultured bovine mammary epithelial cells. An RNAse protection assay was performed for bovine BRCA1 gene induction within 1 h of incubation with E2 (0.5 ng/ml) or PRL (200 ng/ml) or a combination of E2 and PRL (.5ng/ml E2 and 200 ng/ml PRL) in BME cells (A) and primary cultured bovine mammary epithelial cells (C) using biotin anti-sense probes of BRCA1 and ß-actin. (B) and (D) quantification of BRCA1 mRNA expression is shown in (A) and (C), respectively. Relative BRCA1 mRNA expression to ß-actin mRNA expression is also shown. Experiments were done in triplicate and means and standard errors of the means are shown as bars on graphs. The radiographs are examples of the replicates.

2000) . These properties are mediated through the activation of genes involved in many growth control and differentiation. STAT5 knockout mice show poor mammary gland development (Lemkin *et al.*, 2000). Further, Yang *et al.*, 2000 recently showed that STAT5 is differentially expressed throughout various stages of lactation in cows, thus suggesting that the STAT system is involved in the regulation of mammary gland growth and differentiation. The expression of STAT5 was identical to the expression patterns that were found for bovine mammary gland BRCA1 (Chung and Gorewit, 2001).

In our experiments, the potential GAS (-177/-160) element showed a shifted band with STAT5a antibody. Therefore, there is a

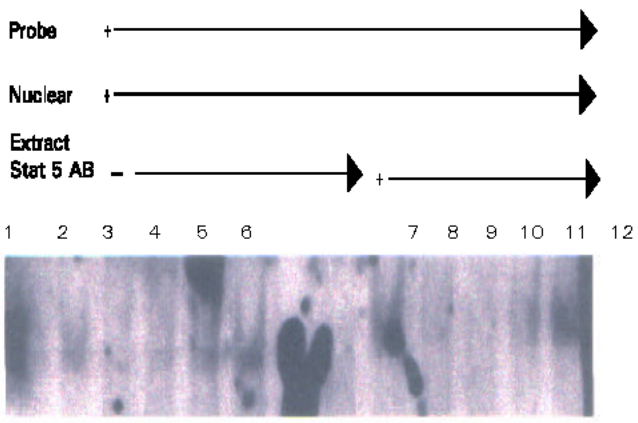

Fig. 2: Identification of a GAS(-290/-309) oligonucleotide in the BRCA1 promoter. The potential GAS biotin labeled oligonucleotide was incubated with nuclear extract. Nuclear extracts from control cells (lane 1), nuclear extracts from cells treated with 100 ng/ml PRL (lane 2) and 10 ng/ml PRL (lane 3). Lane 4 represents extracts treated with 0.2ng/ml E2. Lanes 5 and 6 represent extracts treated with a combination of 100 ng/ml PRL + 0.2 ng/ml of E2. Lanes 7-12 represent extracts incubated with STAT5a antibody alone. Experiments were done in triplicate and the radiograph is an example of the replicates.

possibility that the PRL-STAT5 pathway may be a direct BRCA1 regulator. To confirm a PRL-STAT5-BRCA1 pathway, it is necessary to perform further studies. One must determine if PRL induced BRCA1 induction occurs after transfection of STAT5 -/- cells with STAT5 and STAT5 mutant constructs. This will give the strongest evidence of the direct involvement of a STAT5 -BRCA1- signaling pathway. Further work is needed to determine if the estrogen receptor response element is involved in the estrogen induced up regulation of BRCA1 in bovine mammary cells.

Estrogen and prolactin enhance expression of the bovine BRCA1 gene in cultured bovine mammary secretory epithelial cells. The combination of estrogen and prolactin enhance BRAC1 above that seen for either hormone given alone. Antibody to STAT5a binds to an element that binds to the BRCA1 gene suggesting that a STAT 5 signaling system may be involved in BRCA1 gene expression in bovine mammary epithelial cells.

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Abreviation key: BME = bovine mammary epithelial cells, BRCA1 = breast ovarian cancer susceptibility protein one, E2 = estradiol 17-ß , PRL = prolactin, STAT = signal transducer and activator of transcription, PRLR = prolactin receptor, RPA = RNase protection assay, RNA = ribonucleic acid, PCR = polymerase chain reaction, GAS = interferon gamma activated site, JAK = Janus kinase.