

Primary Cultured Human Breast Epithelial Cells Up-regulate Protein Disulfide Isomerase in Response to Zeranol

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Abstract. *Background:* Experimental data at the molecular, cellular and organismal levels have implicated dietary components in cancer. Exposure to numerous growth factors, hormones and environmental agents, that can regulate signaling events, is involved in carcinogenesis. Research targets on gene-nutrient interactions may give useful information for the development of a novel diet-based intervention for the at-risk population. *Materials and Methods:* To examine the proteomic effect of a low level dietary compound on potential breast cancer, primary human breast epithelial cells were isolated and cultured in media with or without zeranol, an anabolic, non-estrogenic growth promoter with estrogenic activity, used in beef cattle and naturally found in some fungus in grain. The cells then underwent proteomic analysis. *Results:* 2-D electrophoresis showed that protein disulfide isomerase (PDI) was up-regulated 5-fold in the breast epithelial cells exposed to zeranol. PDI has been shown to be up-regulated in a variety of cancerous tissues, although this is the first reported up-regulation of PDI in breast tissue. *Conclusion:* PDI may be a useful marker of dietary exposure to zeranol.

If the incidence of breast malignancies in women continues to follow current trends, one in ten women born in 2001 will develop a breast malignancy (1, 2). Basic and clinical research has contributed to a progressive understanding of the factors involved in the development of breast cancer. Genetic factors predominant in the manifestation of breast cancer include gene amplification, deletion, point mutation, loss of heterozygosity, chromosomal rearrangement and aneuploidy.

Age and the duration of exposure to endogenous and exogenous estrogens may be one of the best-defined risk factors linked to human breast cancer. Breast cancer is uncommon among women younger than 30 years of age, but the incidence of disease increases dramatically with the advance of age. Age may be the driving force for the accumulation of mutational load, telomere dysfunction and increased epigenetic cancer suppressor gene silencing, as well as oncogene activation. Reproductive functions that change estrogen status influence the risk of breast cancer. For example, early age at menarche and late age at menopause are associated with increased risk of breast cancer. The risk of developing malignant breast disease in young adult women who have completed full term pregnancies is much reduced in comparison to women who have first conceived later in their lives (3).

Following menopause, the major source of endogenous estrogen is the conversion of androgen associated with high levels of aromatase activity in adipose tissue. Evidence indicates that obese postmenopausal women produce higher levels of endogenous estrogen and are, therefore, at greater risk for developing breast cancer (4, 5). Postmenopausal hormone use increases breast cancer risk depending on the duration of use and whether estrogen alone or estrogen in combination with progestin is administered (6). The administration of antiestrogens, such as tamoxifen, is beneficial in the treatment of breast cancer and may reduce breast cancer incidence in high-risk women (7). Exposure to growth stimulators, such as estrogens and endocrine disruptors increases the likelihood of histological changes in human breast epithelial cells, which may lead to the promotion of cell proliferation. The interaction between the breast epithelial cells and stromal cells within the breast microenvironment may profoundly affect various steps of breast cancer progression. Experimental cancer models have demonstrated that the extracellular microenvironment influences the efficiency of tumor formation, the rate of

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proliferation, the extent of invasiveness and the ability of the cancer cells to metastasize. In cancers, the influence of the microenvironment is mediated, in part, by paracrine signaling between epithelial cancer cells and the surrounding stromal cells (8, 9).

Zeranol is a nonsteroidal, resorcylic acid lactone compound produced by fungi of the genus *Fusarium*. *Fusarium* spp., and, thus, the product zeranol, are natural and virtually unavoidable contaminants of crops used for animal feed and for human consumption, including corn, wheat, barley, oats and sorghum (10). Zeranol is classified as a mycotoxin due to its estrogenic activity and consequent effects on the fertility of susceptible animals (11). Based on carcinogenicity bioassay studies conducted by the National Toxicology Program (1982), zeranol was classified as a chemical for which there is positive evidence of carcinogenicity (12). The International Agency for Research in Cancer (1983) also categorized Zeranol with compounds for which there is evidence of carcinogenicity.

Zeranol has been shown to have estrogenic activity. This evidence includes: (i) zeranol-induced increases in uterine weight and synthesis of uterine induced protein (13); (ii) zeranol stimulation of mammary gland growth in beef heifer (14) and mouse with, in the latter case, a potency similar to that of estradiol (15); (iii) specific binding of zeranol to uterine cytosolic and nuclear estrogen receptors (13); (iv) zeranol-stimulated proliferation of cultured normal human breast cells (16) and the estrogen-sensitive human breast cancer cell (HBCC) line, MCF-7 (17, 18); (v) zeranol-induced stimulation of estrogen-inducible gene expression (pS2) in normal human breast cells (16) and the HBCC line, MCF-7 (19); (vi) zeranol-induced reduction of estrogen-suppressible breast cancer suppressor gene, protein tyrosine phosphatase g (PTP γ) expression in cultured normal and cancerous human breast cells and HBCC lines (16, 17, 20, 21); and (vii) zeranol induction of hepatotoxicity and subsequent hepatic neoplasia in the Armenian hamster, an animal that is especially susceptible to exogenous estrogen-induced liver damage, and prevention of this carcinogenic process by the antiestrogen, tamoxifen (22). Recently, we reported the involvement of the human breast epithelial and stromal compartments in PTP γ expression mediated by estrogenically active agents, including zeranol (21). Furthermore, new evidence from our laboratories showed that the down-regulation of PTP γ expression by estrogenically active agents, including zeranol, is mediated by ER α , not ER β as was previously believed (20). Moreover, zeranol induced human breast epithelial cell neoplastic transformation in normal breast cells (16).

Zeranol, a nonsteroidal anabolic growth promoter, has been used in the U.S. beef industry to improve the weight gains, carcass quality and feed efficiency for almost the past three decades. Concern exists regarding the potential human breast cancer health risks of zeranol residues in the edible

tissues of the food-producing animals implanted with zeranol. This is largely based on its demonstrated estrogenic activity.

The overall goal of our study was to employ an *in vitro* human breast cell culture model to investigate the effect of the dietary, tumorigenic component zeranol on the proteomic status of breast tissue. Our hypothesis was that expressed, unique proteins will be associated with an increase in hyperplastic growth exhibited by human breast cells after incubation with zeranol.

Materials and Methods

Human breast tissues. Normal human breast tissues were obtained through the Tissue Procurement Program of The Ohio State University Hospital in Columbus, OH, USA. The tissue samples were placed in a mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12) (1:1) without phenol red (Sigma Chemical Co., St. Louis, MO, USA) and stored at 4°C before transfer to the laboratory.

Tissue dissociation. Tissues were sterilized in 70% ethanol for 30 sec and then washed three times with fresh DMEM/F12. Tissue samples were minced and then dissociated overnight at 37°C with 0.1% collagenase (GibcoBRL, Bethesda, MD, USA) in phenol red-free DMEM/F12 medium (1 g tissue/ml) supplemented with 5% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA, USA) and an antibiotic-antimycotic (100 unit/ml penicillin G sodium, 100 µg/ml streptomycin sulfate and 0.25 µg/ml amphotericin B) (GibcoBRL).

Cell culture. The digested mixture was centrifuged at 200 xg for 5 min at 25°C. The cell pellet was re-suspended and allowed to settle by gravity. The supernatant, containing mostly stromal cells, was then discarded. The sedimented cells, mostly epithelial cells, were washed three times with phenol red-free DMEM/F12 medium and allowed again to settle by gravity. The sedimented epithelial cells were re-suspended in phenol red-free low calcium DMEM/F12 (0.04 mM CaCl₂) supplemented with Chelex-100 (Bio-Rad Laboratories, Richmond, CA, USA) – treated FBS (10%).

Cell treatments. All experiments were performed on primary cultured normal human breast epithelial cells not propagated beyond the third passage. Breast epithelial cells were allowed to grow to 80% confluence, before being passaged using 0.5% trypsin – 5.3 mM EDTA (GibcoBRL) with a 1/4 splitting ratio.

Breast epithelial cells were placed in T-75 flask (2x10⁶ viable cells/flask) and cultured in low calcium DMEM/F12 (0.04 mM CaCl₂) supplemented with Chelex-100 – treated FBS (10%) to allow cells to attach onto the flask. The media was changed to phenol red-free low calcium DMEM/F12 (0.04 mM CaCl₂) supplemented with Dextran-Coated Charcoal (DCC) (Dextran T-70; Pharmacia; activated charcoal; Sigma Chemical Co.) – stripped Chelex-100-treated FBS (10%) for 24 h. Cells were then treated with 30 nM zeranol or vehicle control (DMSO) in the same media for 24 h.

Control and zeranol-treated human primary cultured normal human breast epithelial cells were solubilized in IEF buffer (9.5M urea, 0.8% NP-40, 10% β-ME, 2% ampholines; Fisher Scientific, Fair Lawn, NJ, USA) and the proteins separated by 2-D PAGE according to Görg *et al.* (23). Gels were washed in water, fixed with 20% trichloroacetic acid (Fisher Scientific) and stained with 0.1% colloidal

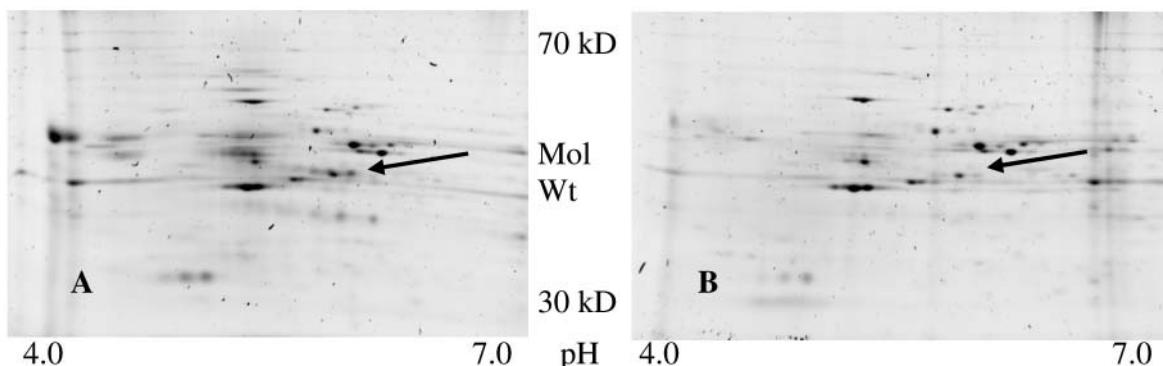


Figure 1. Two-dimensional gels of total cell extracts of primary cultured human breast epithelial cells cultured with (A) and without (B) zeranol (30 nM) in the media. 2-D image analysis identified a spot (identified by the arrow) that was increased 5-fold in the cells exposed to zeranol compared to the control cells. This spot from gel was excised and sent for mass spectrometry analysis.

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1 MRLRRRALFP GVALLAAAR LAAASDVLEL TDDNFESRIS DTGSAGLMLV EFFAPWCGHC
61 KRLAPEYEAA ATRLKGIVPL AKVDCTANTN TCNKYGVSGY PTLKIFRDGE EAGAYDGRT
121 ADGIVSHLKK QAGPASVPLR TEEEFKKFIS DKDASIVGFF DDSFSEAHSE FLKAASNLRD
181 NYRFAHTNVE SLVNEYDDNG EGIILFRPSH LTNKFEDKTV AYTEQKMTSG KIKKFIQENI
241 FGICPHMTED NKDLIQGKDL LIAYYDVDE KNAKGSNYWR NRVMMVAKKF LDAGHKLNFA
301 VASRKTFSSHE LSDFGLESTA GEIPVVAIRT AKGEKFVMQE EFSRDGKALE RFLQDYFDGN
361 LKRYLKSEPI PESNDGPVKV VVAENFDEIV NNENKDVLIE FYAPWCGHCK NLEPKYKELG
421 EKLSKDPNIV IAKMDATAND VPSPYEVRGF PTIYFSPANL KLNPKKYEGG RELSDFISYL
481 QREATNPPVI QEEKPKKKKK AQEDL

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Figure 2. The sequence of human protein disulfide isomerase. The underlined sequences were determined from tryptic fragments using MALDI-TOF and nano-LC/MS/MS analyses.

Coomassie blue in 20% trichloroacetic acid overnight. The images were acquired with an Alpha Innotek (San Leandro, CA, USA) CCD camera and imported into Progenesis PG200 (v2006) (Nonlinear Dynamics Limited, Newcastle-upon-Tyne, UK). A differentially expressed spot was excised and sequenced through in-gel digestion and analyses using MALDI-TOF and nano-LC/MS/MS as previously elsewhere (24).

Results

The 2-D gels of zeranol-exposed breast epithelial and normal breast epithelial cells are shown in Figure 1. The spot selected had a 5-fold increase in normalized volume in the zeranol-exposed cells compared to the control cells. The primary protein sequence as determined by nano-LC/MS/MS for the spot is shown in Figure 2. A match with these sequences was found with human protein disulfide isomerase (PDI).

Discussion

That protein disulfide isomerase is up-regulated in response to cancer-causing agents, and is consistent with studies in other cell types. Protein disulfide isomerase expression was demonstrated in human multiple myeloma (25), SH-SY5Y neuroblastoma, A549 lung adenocarcinoma, LoVo colon adenocarcinoma, and Sup-B15 acute lymphoblastic leukemia

(B cell) cell lines and ovarian tumor cells (26). In addition its presence in 2-D PAGE has been proposed to be employed as a marker of B-cell chronic lymphocytic leukemia (27). The role of this protein in the mechanisms of zeranol induction of cell proliferation is unknown. However, the fact that it is inducible in primary cultured normal human breast epithelial cells *in vitro* makes it an excellent biomarker for investigating its mechanistic role in the process of breast tumorigenesis, as well as a potential molecular bio-marker of dietary zeranol exposure.

While zeranol containing implants are still used in the US beef cattle industry, the European Union has banned the use of it and other growth promoters because of their potential to cause cancer. Although the *in vitro* exposure of primary cultured normal human breast epithelial cells to zeranol at 30 nM for 24 h did result in an increase in PDI present in the cells, no assumptions that zeranol or its metabolites contained in beef may be any health risk to human consumers can be drawn.

Conclusion

Protein disulfide isomerase may be useful as a molecular biomarker for examining the involvement of zeranol from beef in human breast tumorigenesis.

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