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Food and Chemical Toxicology 43 (2005) 637–645
www.elsevier.com/locate/foodchemtox

Dynamic profiling of estrogen receptor and epidermal growth factor signaling in the uteri of genistein- and estrogen-treated rats

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Received 19 April 2004; accepted 12 December 2004

Abstract

The pharmacokinetics and time course actions of the soy isoflavone, genistein, and estradiol benzoate (EB) on sex steroid and growth factor signaling were compared in the rat uterus. Following one s.c. injection of 500 mg genistein/kg BW or 500 μg EB/kg BW, AUC for genistein was 20171.8 ng h/ml and was 15.7 ng h/ml for estradiol-17-β. Estrogen receptor-α (ER-α) decreased within 2 h of genistein or EB treatment, returning to basal levels within 24 and 48 h, respectively. In response to genistein and EB, progesterone receptors (PRs) A and B increased between 16 and 24 h, with a significant increase at 24 and 48 h. Epidermal growth factor receptor (EGFR) expression peaked 16 h after genistein or EB treatment, inversely correlating with extracellular regulating kinase (ERK) phosphorylation. These effects were inhibited by antiestrogen pretreatment, demonstrating a requirement for ER. At 16 h, uterine weight, epithelial cell height, and cell proliferation increased. While EGFR levels increased, phosphorylated EGFR was not altered. Reduced phosphorylation of downstream kinases corresponded with decreased stromal phosphorylated-ERK (P-ERK) immunolabeling, suggesting signal attenuation. Dynamic profiling of sex steroid receptors and EGF signaling molecules suggest a similar mechanism of action for genistein and EB in the uterus, albeit at approximately 1000-fold concentration.

Keywords: Genistein; Estrogen; EGF signaling; Receptors; Rat uterus

1. Introduction

The Asian diet, high in soy products, is associated with many health benefits, including reduced incidence of hormone-dependent cancers (Adlercreutz et al., 1991; Lee et al., 1991). These effects are thought to be due to the high amounts of isoflavones in soy, including genistein. Animal models have demonstrated that genistein has chemopreventive effects against mammary and prostate cancers (Lamartiniere et al., 2002). Due to structural similarity to estradiol, genistein has estrogenic properties, and elicits a dose–responsive uterotrophic effect in rats (Kanno et al., 2003). Since compounds capable of causing uterine proliferation are potentially toxic, characterization of their mode of action is needed.

Pharmacologic s.c. injections of genistein result in estrogenic responses (reviewed by Nilsson et al., 2001). However, given the high affinity of genistein for ER-β, compared with ER-α (Kuiper et al., 1997; Barkhem

Abbreviations: T1/2A and T1/2E, absorption and elimination half-lives; AUC, area under the drug concentration–time curve; CL, clearance; EGFR, epidermal growth factor receptor; EB, estradiol benzoate; ER, estrogen receptor; ERK, extracellular regulating kinase; C_{max}, maximal concentration; PRs, progesterone receptors; PCNA, proliferating cell nuclear antigen; T_{max}, time at maximal concentration; V_{ss}, volume of distribution.

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doi:10.1016/j.fct.2004.12.022

et al., 1998), it is possible for its mechanism to differ from that of estradiol. In the prepubertal rat uterus, genistein stimulated expression of stromal EGF and epithelial EGFR (Brown and Lamartiniere, 2000), which may mediate uterotrophic activity. Although genistein appears to act in a manner analogous to estradiol, specific inhibition of EGFR tyrosine kinase by genistein in vitro (Akiyama et al., 1987) represents the possibility for a unique action directly through the EGFR.

The EGF signaling pathway is involved in bidirectional crosstalk with ER-α (reviewed by Hall et al., 2001). The prepubertal rat uterus expresses ER (Wang et al., 1999) and EGFR (Mukku and Stancel, 1985a), providing a model to study this process. EGFR expression is stimulated by estrogen in the rat uterus (Mukku and Stancel, 1985b). In the mouse uterus, the mitogenic effects of estradiol were also achieved by treatment with EGF, and were blocked with antiestrogen, further indicating a requirement for ERs (Ignar-Trowbridge et al., 1993). Similarly, EGF treatment failed to stimulate uterine DNA and PR synthesis in mice lacking ER-α (ERKO) (Curtis et al., 1996), suggesting linkage between these pathways.

This study was designed to characterize and compare the actions of genistein and EB on EGF signaling proteins in the uterus, and to relate protein alterations to cell proliferation. Since analyses of biomarkers in cell signaling pathways at a single time point after s.c. injection does not reflect their dynamic nature, time course studies were used to assess the temporal relationship between uterine ER-α expression and EGF signaling.

2. Materials and methods

2.1. Animal treatments

Studies were carried out in accordance with the UAB Guidelines for Animal Use and Care. Female Sprague–Dawley rats (Charles River, Raleigh, NC) were housed under a 12 h light/dark cycle (10:00 h/22:00 h) and fed phytoestrogen-free AIN-76A diet (Harlan Teklad, Madison, WI) upon parturition. Prepubertal female offspring (23 days old) were used for all studies. For time course studies, rats were given one s.c. injection of either 500 mg genistein/kg BW (Hoffmann–LaRoche, Basel, Switzerland; 98.5% pure), 500 μg EB/kg BW (Sigma, St. Louis, MO), or the vehicle DMSO (Sigma) and sacrificed 2, 4, 8, 16, 24, or 48 h later. The genistein dose was chosen based on previous studies where three s.c. injections induced an uterotrophic response (Lamartiniere et al., 2002; Cotroneo et al., 2001; Brown and Lamartiniere, 2000) and protected against chemically-induced mammary cancer (Lamartiniere et al., 2002; Murrill et al., 1996). Control (zero time) animals were not injected. Dose response studies were done to determine the concentration of antiestrogen required to prevent EB-induced uterine weight increase. Rats were given 4.0 mg ICI 182,780/kg BW (Astrazeneca Pharmaceuticals, Cheshire, UK) 30 min prior to treatment with DMSO, genistein, EB, or ICI 182,780 alone, and were sacrificed 16 h later, based on the results of the time course.

2.2. Immunoblotting analyses

Immunoblotting methods are described in more detail elsewhere (Cotroneo et al., 2001; Brown et al., 1998). Briefly, uteri were homogenized in buffer containing 1% Triton-X-100 and protease/phosphatase inhibitors. Total protein content was determined using a commercially available kit (BCA Protein Assay Kit, Pierce, Rockford, IL). The following primary antibodies were used: anti-ER-α (NIDDKK); anti-EGFR (Calbiochem, La Jolla, CA); anti-PR (Neomarkers/Lab Vision, Fremont, CA); anti-Raf-1 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-P-Raf-1, anti-MEK 1/2, anti-P-MEKs 1/2, anti-ERKs 1/2, and anti-P-ERKs 1 and 2 (Cell Signaling, Beverly, MA). ERK antibodies recognized both isoforms (42 and 44 kDa), resulting in two distinct bands. MEK antibodies recognized both isoforms, but their close migration resulted in one band. Therefore, one band was used for data analyses for MEKs. After incubation with horseradish peroxidase-conjugated secondary antibody, proteins were detected with chemiluminescence (Pierce). Quantitation of band intensity on radiography film was done using scanning densitometry (Hewlitt Packard Scan Jet 4p, Boise, ID).

2.3. EGFR autophosphorylation

Immunoprecipitation of tyrosine-phosphorylated proteins from uterine extracts was done with anti-phosphotyrosine (Signal Transduction Laboratories, Carpinteria, CA) and protein A agarose beads (Calbiochem). The immunocomplexes were pelleted by centrifugation, washed with cold lysis buffer and resuspended for electrophoresis. To detect tyrosine-phosphorylated EGFR, membranes were incubated with anti-EGFR antibody. Proteins were detected and quantified as described above.

2.4. Serum analyses

Serum estradiol and genistein concentrations were determined in rats treated with genistein, EB or no s.c. injection (controls) and sacrificed 2, 4, 8, 16, 24, and 48 h later ($n = 4$–8 animals/time point). Radioimmunoassay for estradiol-17-β was conducted using a kit (Diagnostic Systems Laboratories, Webster, TX). Total (free and conjugated) genistein concentrations were