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Inhibitory effect of fluvoxamine on β-casein expression via a serotonin-independent mechanism in human mammary epithelial cells

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ABSTRACT

Selective serotonin reuptake inhibitors (SSRIs) are widely used as a first-line therapy in postpartum depression. The objective of this study was to determine the mechanism underlying the inhibitory effects of the SSRI, fluvoxamine, on β-casein expression, an indicator of lactation, in MCF-12A human mammary epithelial cells. Expression levels of serotonin (5-hydroxytryptamine; 5-HT) transporter, an SSRI target protein, and tryptophan hydroxylase 1, a rate-limiting enzyme in 5-HT biosynthesis, were increased in MCF-12A cells by prolactin treatment. Treatment with 1 μM fluvoxamine for 72 h significantly decreased protein levels of β-casein and phosphorylated signal transducer and activator transcription 5 (pSTAT5). Extracellular 5-HT levels were significantly increased after exposure to 1 μM fluvoxamine, in comparison with those of untreated and vehicle-treated cells; however, extracellular 5-HT had little effect on the decrease in β-casein expression. Expression of glucose-related protein 78/binding immunoglobulin protein, a regulator of endoplasmic reticulum (ER) stress, was significantly increased after treatment with 1 μM fluvoxamine for 48 h. Exposure to tunicamycin, an inducer of ER stress, also decreased expression of β-casein and pSTAT5 in a manner similar to fluvoxamine. Our results indicate that fluvoxamine suppresses β-casein expression in MCF-12A cells via inhibition of STAT5 phosphorylation caused by induction of ER stress. Further studies are required to confirm the effect of fluvoxamine on the function of mammary epithelial cells.

1. Introduction

During pregnancy, numerous stimuli alter mammary gland function to facilitate milk production (Borellini and Oka, 1989). Mammary epithelial cell development and maturation are required for milk production and regulated by the dynamic interaction of numerous endocrine hormones, including estrogen, progesterone, and prolactin (PRL).

PRL, an anterior pituitary hormone, is essential for milk production during lactation and for mammary epithelial cell differentiation during pregnancy (Wilde et al., 1995). Signaling via the Janus kinase 2/signal transducer and activator transcription 5 (Jak2/STAT5) pathway is involved in milk production. PRL can induce STAT5 phosphorylation by Jak2 activation, resulting in expression of genes encoding milk proteins such as β-casein and whey acidic protein, which are important markers of mammary epithelial cell differentiation (Clevenger and Kline, 2001; Liu et al., 1996; Rui et al., 1994; Wagner et al., 2004).

5-Hydroxytryptamine (5-HT) is produced by the mammary epithelium and regulates lactation in many species, including mice, cows, and humans (Lauder, 2004). When the mammary gland fills with milk, 5-HT provides a negative feedback signal that suppresses further milk synthesis in the mammary epithelium (Lauder, 2004; Matsuda et al., 2004). Negative feedback is also important during weaning, when it initiates mammary gland involution, reduces milk production, and induces remodeling of the differentiated mammary epithelium to its pre-pregnancy state (Borellini and Oka, 1989). In mammary epithelial cell cultures, exogenous 5-HT increases tight junction permeability via activation of the 5-HT_7 receptor, which plays an important role in the

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initiation of mammary involution (Pai and Horseman, 2008; Stull et al., 2007).

Selective serotonin reuptake inhibitors (SSRIs) are widely used as first-line therapies in postpartum depression. A study of 431 women showed that those taking SSRIs were more likely to experience delayed activation of milk secretion (Marshall et al., 2010). Fluoxetine, an SSRI, increased tight junction permeability and decreased β-casein mRNA expression in bovine mammary epithelial cells (Hernandez et al., 2011). These reports imply that SSRIs inhibit the lactation function of mammary epithelial cells and may suppress human milk production. However, no studies have examined whether SSRIs inhibit expression of milk proteins such as β-casein in human mammary epithelial cells. Furthermore, the mechanism underlying SSRI-mediated suppression of milk protein expression has not been identified.

We previously showed that PRL induced β-casein expression in human mammary epithelial MCF-12 A cells via Jak2/STAT5 pathway activation (Chiba et al., 2014). In addition, PRL increased mRNA expression of tryptophan hydroxylase 1 (TPH1) (Chiba et al., 2015), which catalyzes the rate-limiting step in mammary 5-HT biosynthesis (Matsuda et al., 2004). We also showed that 5-HT decreased protein levels of β-casein and phosphorylated STAT5 (Chiba et al., 2014) via a 5-HT_7 receptor-mediated mechanism (Chiba et al., 2015). Therefore, endogenous 5-HT may be secreted from MCF-12A cells, subsequently affecting them in an autocrine and/or paracrine manner.

In the present study, we investigated the effect of fluvoxamine, an SSRI used in Japan, on β-casein expression in MCF-12A cells. Additionally, we explored the mechanisms underlying these effects.

2. Materials and methods

2.1. Cell culture

MCF-12A cells were cultured as previously described (Chiba et al., 2014). Briefly, the cells were seeded at a density of 1×10^5 cells/cm^2 on dishes coated with Matrigel® (Corning, NY, USA) and cultured in a growth medium consisting of Dulbecco's modified Eagle's medium and F12 (1:1; Invitrogen, Grand Island, NY, USA) supplemented with 10 μg/ml human insulin (Sigma-Aldrich, St. Louis, MO, USA), 0.5 μg/ml hydrocortisone (Sigma-Aldrich), 20 ng/ml human recombinant epithelial growth factor (hEGF; BD Biosciences, San Jose, CA, USA), 5% horse serum (Invitrogen), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich). Unless otherwise stated, the growth medium was changed to a differentiation medium, modified by the addition of PRL and removal of hEGF, 24 h after cell seeding, and the cells were cultured in differentiation medium for 6 days. The cells that were not treated with PRL were cultured in growth medium for 7 days. For the cells treated with fluvoxamine (Sigma-Aldrich), 5-HT (Wako Pure Chemical Industries, Osaka, Japan), or other reagents, no horse serum was added to the differentiation medium and all reagents were added to this medium on day 7 of the culture period.

2.2. Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was isolated from the cultured cells using an RNeasy Mini Kit and DNase Set (Qiagen N.V., Venlo, Netherlands). cDNA was prepared using a High-Capacity RNA to cDNA™ kit (Applied Biosystems®, Life Technologies, Waltham, MA, USA). mRNA levels were evaluated using a 7500 Real-time PCR System (Applied Biosystems) with TaqMan Universal Master Mix II (Applied Biosystems) under the following conditions: 2 min at 50 °C and 10 min at 95 °C, followed by 60 cycles of 95 °C for 15 s and 60 °C for 1 min. The pre-designed primer and probe sets for human β-casein and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts were obtained from Applied Biosystems (β-casein, Hs_00914395_m1; GAPDH, Hs_02758991_g1). Quantitative values were obtained from the threshold cycle (Ct) number. The mRNA level of each target gene was normalized to the GAPDH mRNA level of each sample. All samples were analyzed in triplicate and the data were expressed as the average relative transcript level.

2.3. Western blotting

Protein was extracted from cultured cells using M-PER® mammalian protein extraction reagent (Pierce®, Thermo Fisher Scientific, Waltham, MA, USA). Protein concentrations were determined using a BCA™ Protein Assay kit (Pierce®, Thermo Fisher Scientific). The protein samples were subjected to electrophoresis on a 7.5% or 12.5% e-PAGEL® (ATTO, Tokyo, Japan) and transferred onto polyvinylidene difluoride membranes (GE Healthcare, Tokyo, Japan). The membranes were probed overnight at 4 °C with primary antibodies specific for β-casein (1:200; Novus Biologicals, Littleton, CO, USA), STAT5 (1:1000; Abcam, Tokyo, Japan), phosphorylated STAT5 (pSTAT5, 1:400; Abcam), TPH1 (1:250; Abcam), glucose-related protein 78/binding immunoglobulin protein (GRP78/BiP, 1:400; Abcam), 5-HT transporter (SERT, 1:500, Abcam), or GAPDH (1:2000; Calbiochem®, EMD Millipore, Billerica, MA, USA). Protein bands were visualized using horseradish peroxidase-conjugated secondary antibodies (1:2000; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and observed using an Image Reader LAS-3000 System (Fuji Photo Film, Tokyo, Japan). The immunoblot films were scanned and blot density was analyzed using ImageJ version 1.47 (National Institutes of Health, Bethesda, MD, USA). The density of the target protein band was divided by that of the GAPDH band for each sample, and these data were presented as relative protein levels.

2.4. Extracellular 5-HT assay

The 5-HT in the medium was concentrated using a Supel™-Select SCX SPE solid extraction column (Supelco, Bellefonte, PA, USA). Briefly, 6 ml of the medium was applied to the column, which was conditioned by methanol containing 0.1% formic acid and water before use. The column was then washed with water and the desired fraction was eluted with methanol and 10% ammonium hydroxide solution. The eluate was evaporated in a vacuum with a centrifugal evaporator. The residue was dissolved in water and 20 μl of reconstituted solution was analyzed by high-performance liquid chromatography (HPLC).

The apparatus used for HPLC was a Shimadzu Prominence system (Kyoto, Japan) equipped with an LC-20AD pump, SIL-20A autosampler, RF-10AXL fluorescence detector (excitation: 280 nm; emission: 340 nm), CBM-20A controller, and CTO-20A column oven. A Shim-pack VP-ODS C18 column (5 μm, 150 × 4.6 mm internal diameter, Shimadzu) was used for reverse-phase HPLC. A mixture (75:25) of sodium acetate buffer (pH 6.8) containing 1 mM 1-octanesulfonic acid sodium and methanol was used as an isocratic mobile phase. The flow rate of the mobile phase was 1.5 ml/min. Procaine hydrochloride was used as the internal standard. The data were expressed as the amount of 5-HT in the medium per 10^6 cells.

2.5. Statistical analyzes

All values are reported as mean ± standard error of the mean (S.E.M.). Student's *t*-test was used for comparisons of 2 groups. One-way or two-way analysis of variance (ANOVA) followed by