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Trichostatin A reduces GnRH mRNA expression with a concomitant increase in retinaldehyde dehydrogenase in GnRH-producing neurons☆

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ABSTRACT

Trichostatin A (TSA) is a selective inhibitor of mammalian histone deacetylase and is widely used to modify the ability of DNA transcription factors to bind DNA within chromatin by interfering with histone deacetylation. In the GnRH-producing neuronal cell line GT1-7, TSA significantly reduced expression of GnRH mRNA. Kisspeptin, a known regulator of GnRH release, failed to increase GnRH mRNA expression and did not modify TSA-induced reduction of GnRH expression. TSA, but not kisspeptin, increased histone acetylation in whole-cell lysates and significantly stimulated the expression of retinaldehyde dehydrogenase (RALDH), a retinoic acid (RA)-synthesizing enzyme that is known to be involved in cell differentiation. In addition, treatment of the GT1-7 cells with RA dose-dependently inhibited the expression of GnRH mRNA. Whereas, TSA-induced reduction of GnRH mRNA was not modulated by treatment with the pan-RA receptor inverse agonist BMS493 or the RA metabolism inhibitor liarozole.

Our current results suggest that the RALDH and RA might not be directly involved in the reduction of GnRH expression induced by TSA, however these substances could be a novel regulator of GnRH.

1. Introduction

Gonadotropin-releasing hormone (GnRH), which is released from the hypothalamus, plays a central role in regulating reproductive function by stimulating the pituitary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Knobil, 1980), and is defined as a crucial component of the hypothalamic–pituitary–gonadal axis. Most recently, the physiological control of the reproductive axis was elucidated after the identification of an essential role for kisspeptin and its receptor, G-protein-coupled receptor 54 (GPR54) (de Roux et al., 2003; Seminara et al., 2003) in reproductive functions. It is now generally agreed that kisspeptin-secreting neurons activate GnRH neurons through GPR54 and are implicated in normal reproductive cycles in females (Skorupskaite et al., 2014).

Previous studies demonstrated that the expression of GnRH in the hypothalamus changes according to age and season (Parry et al., 1997; Ubuka and Bentley, 2009). Sex steroids such as estradiol (Sun et al., 2001) and testosterone (King et al., 1989) also change the pattern of GnRH synthesis. More recent studies revealed the role of kisspeptin in the feedback control of GnRH (Gottsch et al., 2006) and in increasing GnRH mRNA levels in GnRH-secreting neuronal cells (Novaira et al., 2009). Furthermore, several transcription factors that interact with GnRH promoter regions were shown to regulate GnRH expression (Kelley et al., 2000; Wolfe et al., 2002; Rave-Harel et al., 2005). In addition, epigenetic mechanisms have been shown to regulate GnRH expression, providing a divergent level of control that could be involved in the development and maturation of GnRH neurons (Kurian et al., 2010; Gan et al., 2012).

Trichostatin A (TSA) is an antifungal antibiotic that was first isolated from metabolites of *Streptomyces hygroscopicus* (Tsuji et al., 1976). It is now widely used in experimental reagents as a selective inhibitor of histone deacetylase, and is also known as a potential anti-cancer drug (Drummond et al., 2005).

In this study, we found that TSA decreased GnRH mRNA expression in the GnRH-producing cell line GT1-7, with a

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concomitant increase in the retinoic acid (RA)-synthesizing enzyme retinaldehyde dehydrogenase (RALDH). In addition, we found that RA, which is converted from retinol (vitamin A) by RALDH, has the ability to reduce GnRH mRNA expression in GT1-7 cells. Our current findings suggest the possibility that TSA and/or RA could be a novel regulator of GnRH. The effects of kisspeptin on this phenomenon were examined as well.

2. Materials and methods

2.1. Materials

The following chemicals and reagents were obtained from the indicated sources: fetal bovine serum (FBS), trypsin (Invitrogen, Carlsbad, CA); GnRH, Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, TSA (Abgent, San Diego, CA); kisspeptin (AnaSpec, Fremont, CA); all-trans RA (Sigma–Aldrich, Saint Louis, MO); BMS 493 and liarozole hydrochloride (Tocris Bioscience, Bristol, UK); anti-acetyl histone monoclonal antibodies, RALDH2 antibodies and suitable horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, Dallas, TX).

2.2. Cell culture and stimulation

GT1-7 cells, a mouse GnRH-producing neuronal cell line (Mellon et al., 1990) kindly provided by Dr. P. Mellon of the University of California (San Diego, CA), were plated in 35-mm tissue culture dishes and incubated in high-glucose DMEM containing 10% heat-inactivated FBS and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 24 h, the culture medium was changed to high-glucose DMEM containing 1% heat-inactivated FBS and 1% penicillin-streptomycin and incubated without (control) or with test reagents (TSA or RA) for the indicated times. TSA and RA were prepared as stock solutions in DMSO and working concentrations were prepared by diluting in DMEM. The total volume of DMSO in each stimulation condition was equalized by adding DMSO without test reagents. Kisspeptin was dissolved in distilled water as a stock solution. When BMS 493 and liarozole were used, cells were pretreated with these reagents for 60 min prior to stimulation.

2.3. RNA preparation, reverse transcription, and real-time quantitative RT-PCR

Total RNA from untreated and treated GT1-7 cells was extracted using Trizol-S (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. To obtain cDNA, 2.0 μg of total RNA was reverse transcribed using an oligo(dT) primer (Promega, Madison, WI) and a First Strand cDNA Synthesis Kit (Invitrogen) with reverse transcription (RT) buffer. This reaction was supplemented with 0.01 M dithiothreitol (DTT), 1 mM dNTPs, and 200 units of RNase inhibitor/human placenta ribonuclease inhibitor (Ribonuclease Inhibitor, Code No. 2310, Takara Bio, Tokyo, Japan), adjusted to a final volume of 25 μl, and incubated at 37 °C for 60 min. Quantification of GnRH, RALDH1 and RALDH2 mRNA expression was obtained through real-time quantitative PCR (ABI Prism 7700 Sequence Detection System; Life Technologies) utilizing Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA). The PCR primers were designed based on the published sequences of GnRH (forward: 5′-ACTGTGTGTTTGGAAGGCTGC-3′ and reverse: 5′-TTCCAGAGCT CCTCGCAGATC-3′), RALDH1 and RALDH2 (Fujiwara et al., 2007), while the internal reference GAPDH primer was purchased from Sigma–Aldrich. Real-time PCR amplification and product detection were performed using the ABI PRISM 7700 Sequence Detection System as recommended by the manufacturer (User Bulletin No. 2). The simultaneous measurement of mRNA (GnRH, RALDH1, RALDH2) and GAPDH permitted normalization of the amount of cDNA added per sample. Each assay included a duplicate standard curve sample, a no-template control, and triplicate cDNA samples from the treated GT1-7 cells. For each set of primers, a no-template control and a no-reverse transcriptase control were included. The thermal cycling conditions were: 2 min denaturation at 92 °C followed by 40 cycles of 92 °C for 30 s, 54 °C for 30 s, 75 °C for 30 s, and extension at 75 °C for 5 min. Reaction conditions were programmed on a Power Macintosh 7100 (Apple Computer, Santa Clara, CA) linked directly to the ABI PRISM 7700. The crossing threshold was determined using PRISM 7700 software. Post-amplification dissociation curves were obtained to verify the presence of a single amplification product in the absence of DNA contamination.

2.4. Western blotting

GT1-7 cells were rinsed with PBS and then lysed on ice with RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) containing 0.1 mg/ml phenylmethylsulfonyl fluoride, 30 mg/ml aprotinin, and 1 mM sodium orthovanadate, scraped for 20 s, and centrifuged at 14,000 × *g* for 10 min at 4 °C. Protein concentration was measured in the cell lysates using the Bradford method of protein quantitation. Ten micrograms of denatured protein per well were separated in a 10% SDS-PAGE gel according to standard protocols. Protein was transferred onto polyvinylidene difluoride membranes (Hybond-P PVDF, Amersham Biosciences, Little Chalfont, UK), which were subsequently blocked for 2 h at room temperature in Blotto (5% milk in TBS). To determine the levels of histone acetylation, membranes were incubated with an anti-acetyl histone monoclonal antibody (1:200 dilution; Santa Cruz Biotechnology), followed by incubation with an HRP-conjugated secondary antibody. To determine the levels of RALDH2, a 1:100 dilution of an anti-RALDH2 antibody (Santa Cruz Biotechnology) was used. Following chemiluminescence detection (Amersham Biosciences), membranes were exposed to X-ray film (Fujifilm, Tokyo, Japan).

2.5. Statistical analysis

All experiments were repeated independently at least three times. Each experiment in each experimental group was performed using triplicate samples (luciferase assays) or duplicate samples (western blot). Data are expressed as means ± standard error of the mean (SEM). Statistical analysis was performed using a one-way ANOVA followed by Duncan's multiple range test using Microsoft Excel 2000. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of TSA on GnRH mRNA expression in GT1-7 cells

GT1-7 cells were incubated with increasing concentrations of TSA for 24 h, and GnRH mRNA levels were then measured by quantitative real-time RT-PCR. TSA significantly inhibited basal levels of GnRH mRNA in GT1-7 cells. The expression of GnRH mRNA was reduced 0.24 ± 0.08-fold and 0.33 ± 0.12-fold by 1 μM and 5 μM TSA, respectively, compared to the untreated cells (Fig. 1A). As expected, histone acetylation in whole-cell lysates from GT1-7 cells was significantly increased by TSA treatment (Fig. 1B).