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Lithium chloride inhibits StAR and progesterone production through GSK-3β and ERK1/2 signaling pathways in human granulosa-lutein cells

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ABSTRACT

Lithium chloride (LiCl) is a widely-used medication to treat neurological disorders that has undesirable side effects on the female reproductive system. It has been show that LiCl can inhibit ovarian folliculogenesis, promote follicle atresia and suppress steroid hormone production in rodents. However, the effects of LiCl on human ovarian steroidogenesis remain completely unknown. In this study, both primary and immortalized human granulosa-lutein (hGL) cells were used to investigate the effects of LiCl on progesterone production and its related enzyme expression as well as the underlying mechanisms. Our results showed that LiCl significantly down-regulated the steroidogenic acute regulatory protein (StAR) expression and subsequent progesterone production in hGL cells. Additionally, LiCl induced the phosphorylation of GSK-3β and ERK1/2 but not AKT or CREB. Knockdown of endogenous GSK-3β or inhibition of ERK1/2 partially reversed LiCl-induced down-regulation of StAR. Furthermore, by using dual inhibition approaches, the results showed that both GSK-3β and ERK1/2 signaling mediated the regulatory effect of LiCl on StAR expression. Our findings deepen our understanding of the pathological effects and the underlying molecular mechanisms of how lithium might affect the female reproductive system.

1. Introduction

Successful implantation and subsequent conception require a competent embryo interacting with a receptive uterine environment. After ovulation, the follicular granulosa and theca cells develop into corpus luteum, which secretes progesterone and provides an appropriate uterine environment to support the conceptus and maintain a healthy pregnancy (Niswender et al., 2000). Progesterone synthesis starts from cholesterol, which is the common precursor of all steroid hormones. In brief, serum cholesterol enters into the cytoplasm, reaches mitochondria, and is transported to the inner mitochondria, a process requiring a transport protein called steroidogenic acute regulatory protein (StAR). This regulatory protein mediates the rate-limiting step of the progesterone synthesis (Lin et al., 1995). Next, cholesterol is catalyzed into pregnenolone by the cytochrome P450 side-chain cleavage enzyme ($P450_{SCC}$) in the mitochondria. Pregnenolone is finally transformed into progesterone by the reaction of 3β-hydroxysteroid dehydrogenase (3β-HSD), which mainly occurs in the cytoplasm (Miller and Auchus, 2011).

Lithium chloride (LiCl) is a lithium salt-based medicine that has been therapeutically used to treat bipolar disorders (Gurvich and Klein, 2002). This common psychopharmacological treatment has undesirable side effects on the female reproductive system (Strauss and Gross, 1984). Most patients who undergo lithium drug treatment may experience infertility and disrupted ovarian cycles (Strauss and Gross, 1984). In humans, lithium treatment causes a significant increase in serum luteinizing hormone (LH) levels without changing the serum testosterone level (Sheard et al., 1977). Lithium has been characterized as a teratogen because lithium

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exposure during pregnancy is associated with high risk of cardiac malformation in fetus (Hogan and Freeman, 2016; Patorno et al., 2017). In mature male albino rats, LiCl affects testicular steroidogenic and gametogenic functions (Ghosh et al., 1990). In rat ovaries, lithium induces tremendous follicular atresia (Mirakhori et al., 2013). Treatment of bovine cumulus oophoron complexes with LiCl during *in vitro* maturation (IVM) reduced the expansion of cumulus and decreased the progesterone secretion (Uzbekova et al., 2009). In mouse preimplantation embryos, LiCl inhibits embryo cleavage by disrupting the mitotic cell cycle (Acevedo et al., 2007). Mouse follicles treatment with LiCl leads to abnormal follicular development and decreases in estradiol and testosterone concentrations in the cultured medium (Li et al., 2014). However, it is still completely unknown whether LiCl treatment affects progesterone production in humans.

Glycogen synthase kinase-3β (GSK-3β) is a critical mediator of the canonical Wnt/β-catenin signaling (Clevers, 2006) that has been identified as a potential LiCl targets (Zhang et al., 2003). Lithium inhibits the activity of GSK-3β by increasing its phosphorylation, and this inhibitory effect can be directly competed by magnesium (Ryves and Harwood, 2001) or indirectly abolished by AKT-mediated reactions (Chalecka-Franaszek and Chuang, 1999). Moreover, GSK-3β inactivation directly regulates the CREB phosphorylation (Horike et al., 2008). Thus, LiCl may exhibit its cellular function via mimicking Wnt/β-catenin signaling activation or via activating the AKT/CREB signaling pathway. In fact, GSK-3β, AKT or CREB signaling is involved in the regulation of StAR/progesterone synthesis (Chen et al., 2007; Lee et al., 2015; Stapp et al., 2014). In addition, LiCl activates the MEK/ERK1/2 signaling pathway in acute promyelocytic leukemia (Zassadowski et al., 2015). Our previous study also demonstrated that the ERK1/2 signaling pathway is involved in TGF-β1-induced down-regulation of StAR/progesterone production (Fang et al., 2014). Collectively, all of these studies prompted us to propose a possible cellular mechanism by which LiCl regulates progesterone synthesis. In this study, we examined the effects of LiCl on StAR expression and the production of progesterone in human granulosa cells and potential underlying molecular mechanisms.

2. Materials and methods

2.1. Simian virus 40 large T antigen-immortalized human granulosa cell (SVOG) culture

In the present study, we used an immortalized human granulosa-lutein cell line (SVOG) that was previously produced from human granulosa-lutein cells (from *in vitro* fertilization patients) by transfecting with the simian virus 40 large T antigen (Lie et al., 1996). This cell line has been previously used to study human ovarian biology (Chang et al, 2016a, 2016c, 2016d, 2016e). SVOG cells were seeded in 6-well plates ($4–8 \times 10^5$ cells per well) and cultured in DMEM/F-12 medium (Sigma-Aldrich Corp., Oakville, ON, USA) supplemented with 5% charcoal/dextran-treated fetal bovine serum (FBS) (HyClone, Logan, UT, USA), 100 μg/ml streptomycin sulfate (Invitrogen, Life Technologies, Inc./BRL, Grand Island, NY, USA), 100 U/mL penicillin (Invitrogen, Life Technologies) and 1X GlutaMAX (Invitrogen, Life Technologies). The cultures were maintained at a humidified atmosphere containing 95% air and 5% CO_2 at 37 °C.

2.2. Preparation of primary human granulosa-lutein (hGL) cells

The hGL cells were obtained with informed patient consent after the approval from the University of British Columbia Research Ethics Board. Follicular aspirates were obtained from women patients undergoing *in vitro* fertilization and the controlled ovarian stimulation protocol was performed as previously described (Chang et al., 2014, 2016b). The follicular aspirates were centrifuged at 500 g for 15 min at room temperature, and then the supernatant was removed. The pellets were re-suspended with 4 ml of DMEM/F-12 medium and then were slowly transferred onto the top of 8 ml of Ficoll-Paque Plus (GE Healthcare Life Sciences, Piscataway, NJ, USA) in a 15 ml-Falcon tube. The tube was centrifuged at 600 g for 20 min at room temperature. The granulosa cell layer was collected and washed with 5 ml of DMEM/F-12 medium. The number of viable cells was counted, and the cells were seeded in DMEM/F-12 medium supplemented with 10% charcoal/dextran-treated FBS and cultured in a humidified 5% CO_2 air atmosphere at 37 °C. The culture medium was changed every other day in all experiments.

2.3. Antibodies and reagents

Polyclonal rabbit anti-*StAR* (sc-25806) and monoclonal mouse anti-GAPDH (sc-23948) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal mouse anti-β-catenin (610153) antibody was obtained from BD Biosciences (Mississauga, ON, Canada). Monoclonal rabbit anti-non-phospho (Active)-β-Catenin^{Ser45} (19807), monoclonal rabbit anti-phospho-GSK-3β^{Ser9} (5558), monoclonal rabbit anti-GSK-3β (12456), monoclonal mouse anti-phospho-p44/42 MAPK (Erk1/2)$^{Thr202/Tyr204}$ (9106), polyclonal rabbit anti-p44/42 MAPK (Erk1/2) (9102), monoclonal rabbit anti-phospho-CREB^{Ser133} (9198), monoclonal rabbit anti-CREB (9197), polyclonal rabbit anti-phospho-AKT^{Ser473} (9271) and polyclonal rabbit anti-AKT (9272) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse and goat anti-rabbit IgG were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Lithium chloride was obtained from Fisher Scientific Co. (Pittsburgh, PA, USA).

2.4. Reverse transcription quantitative real-time PCR (RT-qPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. A total of 2 μg RNA was reversely transcribed into first-strand cDNA with dNTP, random primers and moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). The Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used for RT-qPCR. The total amount of the reaction system is 20 μl, including 10 μl of 2 × SYBR Green qPCR MaterMix (Applied Biosystems), 7.5 μl of primer mixture (25 μM) and 20 ng of cDNA. The primers used in our experiments were as follows: *StAR*, 5′- AAACTTACGTGGCTACTCAGCATC-3’ (sense) and 5′- GACCTGGTTGATGCTCTTG-3’ (antisense); P450 side chain cleavage enzyme (P450scc) (*CYP11A1*), 5′-CAGGAGGGGTGGACACGAC-3’ (sense) and 5′-AGGTTGCGTGCCATCTCATAC-3’ (antisense); 3β-hydroxysteroid dehydrogenase (*3β-HSD*), 5′-GCCTTCCAGACCAGAATTGAGAGA-3’ (sense) and 5′-TCCTTCAAGTACAGTCAGCTTGGT-3’ (antisense); β-catenin (*CTNNB1*), 5′-CTGC TGTTTTGTTCCGAATGTC-3’ (sense) and 5′-CCATTGGCTCTGTTCTGAAGAGA-3’ (antisense); glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 5′- GAGTCAACGGATTTTGGTCGT-3’ (sense) and 5′-GACAAGCTTCCCGTTCTCAG-3’ (antisense). All of the experiments were repeated at least three times and each sample was assayed in triplicate. Relative quantification of mRNA levels was performed using a comparative cycle threshold (Ct) method with the 2-ΔΔCt formula using GAPDH as the reference gene.

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