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Research article

Effect of fluoxetine on cyclic adenosine monophosphate response to follicle-stimulating hormone in HEK cells

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Abstract

Fluoxetine is an antidepressant that acts as a selective serotonin reuptake inhibitor. Fluoxetine has exhibited different mechanisms of action on many different cell lines. In this study, the effect of fluoxetine on cAMP production in human embryonic kidney (HEK) cells under the stimulation of bovine follicle-stimulating hormone were examined. HEK cells were incubated for 60 minutes with fluoxetine at different concentrations (0, 6.25, 12.5, 25 and 50 µM), and then cAMP accumulation, ATP level, and AMPK phosphorylation and cell viability were tested. Our results showed that fluoxetine at 25 and 50 µM attenuated cAMP levels in cells after bFSH stimulation. The depletion of cAMP at this concentration is accompanied by a decrease in ATP energy and an increased capacity to phosphorylate AMPK. As such, this study showed that fluoxetine induces a reduction in cAMP accumulation and that it is associated with the AMPK pathway in HEK cells.

Keywords: Adenyl cyclase, Adenosine triphosphate, AMPK, Cyclic AMP, Fluoxetine

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INTRODUCTION

Cyclic 3',5'-adenosine monophosphate (cAMP) is a second messenger and plays an important role in cell mechanisms. cAMP is formed from ATP via the enzyme adenyl cyclase upon activation of Gs protein-coupled receptors (GPCRs), and it subsequently mediates many biological reactions (Sutherland and Rall, 1958). cAMP regulates many intracellular mechanisms like those of protein kinase A, exchange protein activated by cAMP, or cyclic nucleotide-gated ion channels (Muñoz-Llancao et al., 2015). The increased production of cAMP in liver, fat, and muscle cells in response to β-adrenoceptor activation affects enzymes involved in the metabolisms of glycogen and fat (Boularan and Gales, 2015). The increase in cAMP levels inhibits NK cells (human natural killer) cytotoxic function (Bariagaber and Whalen, 2003) and impairs the inflammatory functions of monocytes (Wall et al., 2009). The increase of cAMP levels can also lead to the activation of cyclic nucleotide-gated ion channels in retinal photoreceptors and olfactory sensory neurons cells (Kaupp and Seifert, 2002). The cAMP/Exchange protein activated by cAMP (EPAC) pathway has also been shown to be involved in the maintenance of metabolic homeostasis in the body, such as by inducing leptin resistance, or increasing secretion and sensitivity of insulin (Almahariq et al., 2014). The inhibition of cAMP synthesis by fluoxetine was associated with the inhibition of hormone-induced steroidogenesis in rat testes Leydig cells and mouse Leydig tumor cells (mLTC-1) (Nguyen et al., 2019b).

Fluoxetine (FLX) can be used to relieve depression because it is a selective serotonin reuptake inhibitors antidepressant (Wong et al., 1995), but it also inhibits various ion channels (Pancrazio et al., 1998; Deák et al., 2000; Nahon et al., 2005; Dong et al., 2015; Thériault et al., 2015), as well as the activity of mitochondria (Curti et al., 1999; Hroudová and Fišar, 2012; Charles et al., 2017). FLX can improve memory and cognitive function (Huang et al., 2018), and it has been shown to inhibit β-amyloid production and prevent neuronal degeneration in a mouse model (Ma et al., 2017). Furthermore, Li et al. (2004) have shown that FLX can significantly enhance GSK3β phosphorylation and increase β-catenin levels (Pilar-Cuéllar et al., 2012).

Our most recent study showed that FLX inhibits adenyl cyclase activity, leading to reduced intracellular cAMP and steroidogenesis in mLTC-1 Leydig cells (Nguyen et al., 2019b). cAMP is a known mediator for steroidogenesis through StAR-dependent cholesterol entry in the mitochondria (Li et al., 2004). In this study, we further investigated the effect of FLX on adenyl cyclase activity and intracellular cAMP levels, in order to understand better the mechanism of FLX action.

MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Sigma-Aldrich (France) unless otherwise noted. pGlosensor-TM-22F cyclic AMP plasmid and CellTiter-Blue Cell viability assay (G8080) were purchased from Promega (Charbonnières-les-Bains, France), XtremeGENE HP DNA transfection reagent and protease inhibitor cocktail were purchased from Roche (Boulogne-Billancourt, France),

Tris/glycine buffer (10X) and Protein Standard (1610373) were obtained from Bio-Rad (Hercules, California, USA). Primary antibodies against total AMPKα and phospho-AMPKα (Thr172) were purchased from Cell Signalling technology, Inc (Danvers, MA, USA).

HEK cell culture

HEK cells transfected with pGlosensor-TM-22F cyclic AMP plasmid and FSH receptor were obtained from INRAe (France), and were then cultured in MEM medium (Gibco, Invitrogen) supplemented with 10 % fetal bovine serum, 50 μg/ml gentamicin, 10 units of penicillin/ml, and 10 μg/ml streptomycin, and used from P8 to P35 passage. All cells were incubated at 37°C and 5 % CO_2.

Intracellular measurement of cAMP in HEK cells

HEK cells were grown in a 96-well plate (symbol: Greiner white/clear bottom), approximately 100.000 cells/well. After two days of culture, the medium was removed and replaced with 100 μL/well of medium without fetal bovine serum and containing the luciferase substrate luciferin and 1 mM isobutyl-methyl-xanthine (IBMX), a phosphodiesterase inhibitor. Then, plates were incubated for 60 min with FLX at different concentrations (0, 6.25, 12.5, 25 and 50 μM). Finally, bovine FSH (bFSH) from Aspen (Aspen BioPharma, Castle Rock, USA) was added at a volume of 10 μL/well, corresponding to a concentration of 2.5 ng/well. Each concentration was repeated 3 times. And then the kinetics of intracellular oxyluciferin luminescence were recorded using a Polarstar Optima photometer (BMG Labtech, Champigny-sur-Marne, France).

Measurement of ATP concentration

HEK cells were incubated with or without FLX at different concentration for 60 min and then ATP concentration in cells was measured by CellTiter-Glo 2.0 Assay (Promega, Madison, WI, USA). ATP standard (Promega) were prepared at concentrations of 1×10−10, 1×10−11 and 1×10−12 M. The assay buffer and substrate were equilibrated to room temperature for 120 min, and then mixed together. 50 μL of this solution were then mixed with 50 μL of luciferine/luciferase in a 96-well white plate, and then strongly shaken for 2 min before incubating for 30 min at room temperature. The luminescence was read using an Ascent Luminoskan Luminometer (Thermo) at integration time 1000 (ms). PBS solution was as a blank for each experiment.

Evaluation of the viability of HEK cells

HEK cells were grown in a 96-well plate at 100.000 cells/well. After two days, the medium was replaced with serum-free medium, 100 μL/well. The plate was then incubated with FLX of various concentrations for 60 min at 37°C before adding 20 μL of CellTiter-Blue Reagent (Promega, Madison, WI, USA) to each well and incubating for another 60 min. Then, the fluorescence changes were measured using a Spectra Gemini fluorescence spectrometer (Molecular Devices, Sunnyvale, CA) at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The fluorescence signal from the CellTiter-Blue Reagent is proportional to the number of viable cells.

Western-Blotting

Total proteins after being extracted from HEK cells in lysis buffer were centrifuged at 13000 g for 30 min at 4°C. Protein concentrations were quantified by a colorimetric assay (Bio-Rad). The proteins were then electrophoresed on a 10% SDS Polyacrylamide gel and transferred onto a nitrocellulose membrane. The membranes were incubated with primary antibodies overnight at 4°C, anti-AMPKα (1:1000), anti-phospho-Thr172-AMPKα (1:1000), diluted in 5% BSA in 0.1% TBS-Tween. Finally, these membranes were added anti-rabbit IgG (H+L) (CF 770 Conjugate) (1:2000) and incubated for 60 min at room temperature. Band intensity was analyzed using Odyssey Software, version 1.2 (LICOR Biosciences, Lincoln, USA). AMPKα was used as a loading control.

Area under curve (AUC) calculations and statistical analysis

GraphPad 5.0 was used for Area Under Curve (AUC) analysis of individual kinematics. The mean and SEM for each triplicate AUC were determined. The AUC ratio was used to compare the cAMP reaction kinetics. One-way ANOVA with Dunnett's test was performed using this package. Significance level was $P < 0.05$.

RESULTS

Effect of FLX on cAMP accumulation response to bFSH

After a 60 min incubation with FLX at different concentrations of 0, 6.25, 12.5, 25 and 50 µM, HEK cells showed a strictly dose-dependent reduction in intracellular cAMP accumulation kinetics to bFSH (Figure 1A). This reduction was also observed by calculating the area under the curve (AUC) (Figure 1B). Specifically, only 25 µM (or more) of FLX significantly reduced intracellular cAMP accumulation after 60 min of incubation.

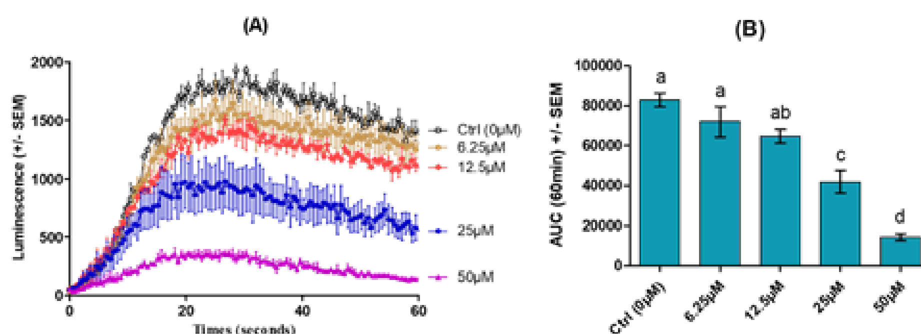

Figure 1 Effect of FLX on intracellular cAMP response to FSH in HEK cells. (A): Real-time recording of luminescence under bFSH stimulation in HEK cells in the presence of 0 µM (Ctrl) to 50 µM of FLX; (B): Dose-dependent response to FLX determined by the Area Under Curve (AUC) of individual kinetics in figure 1A. Data are means ± SEM of 3 independent experiments (n = 3). Results were analyzed by one-way ANOVA, followed by the Dunnett's test posttest. Different letters indicate significant differences between Ctrl and treatment at $P < 0.05$.

Effect of FLX on ATP concentration in HEK cells and HEK cell viability

In order to understand better the mechanism of action of FLX in HEK cells, we decided to investigate the effect of FLX on intracellular ATP levels and cell viability, because these are the main cause that directly affects intracellular cAMP levels. The results of Figure 2 show that FLX induced a decrease on intracellular ATP concentration after 60 min of incubation at 25 µM and 50 µM (decrease by 35.51% at 25 µM; by 64.49% at 50 µM compared to Ctrl (0 µM)) but cell viability was not affected when compared with the control (Figure 3).

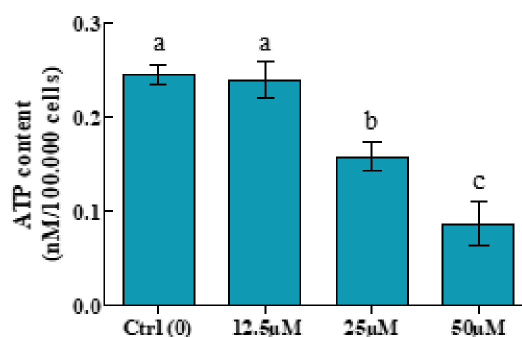

Figure 2 Effect of FLX on ATP content in HEK cells. Monitoring of ATP production in living cells using the Cell-Titer-Glo Assay. Cells were incubated at 37°C with FLX at 12.5, 25 and 50 µM, or without FLX (Ctrl, 0 µM). The experiment was performed 3 times; data are means ± SEM. Different letters indicate significant differences between Ctrl and treatment at $P < 0.05$.

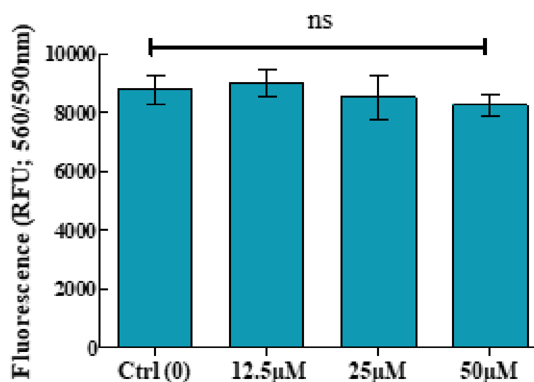

Figure 3 Effect of FLX on ATP content in HEK cells. Monitoring of ATP production in living cells using the Cell-Titer-Glo Assay. Cells were incubated at 37°C with FLX at 12.5, 25 and 50 µM, or without FLX (Ctrl, 0 µM). The experiment was performed 3 times; data are means ± SEM. ns: no significant difference.

Effect of FLX on AMPK phosphorylation in HEK cells

An increase in the intracellular AMP/ATP ratio leads to AMPK activation, so we monitored the level of AMPK phosphorylation in HEK cells in the presence of FLX. Figure 4 shows that FLX stimulates AMPK phosphorylation at 25 µM (increase by 40.11%) and 50 µM (increase 51.38%) compared to Ctrl (0 µM) after 60 min of incubation but not 12.5 µM.

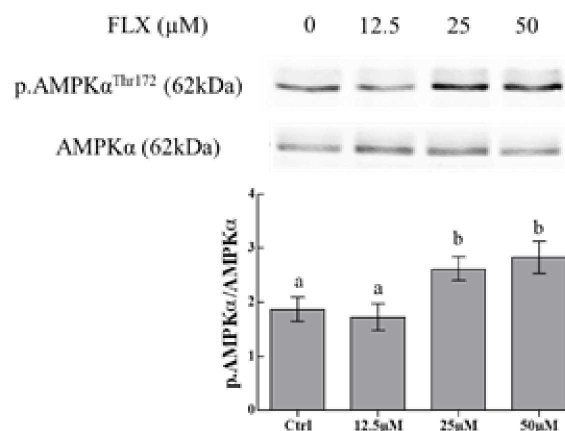

Figure 4 Effect of FLX on AMPK phosphorylation in HEK cells. The band of phospho-AMPKα (Thr172) was detected at 62 kDa (top bands). Total AMPKα (62 kDa, bottom bands) were used as loading controls and p.AMPKα (Thr172)/total AMPKα ratio is shown at the bottom. Data are means ± SEM of 4 independent experiments (n = 4). Different letters indicate significant differences between Ctrl (0 µM) and treatment at P<0.05.

DISCUSSION

We have previously shown that FLX exerts an inhibition on LH-stimulated cAMP accumulation in mouse Leydig tumoral mLTC-1 cells (Nguyen et al., 2019b), and human ovarian tumor COV434 cells (Nguyen et al., 2021). This study demonstrates that FLX also inhibits cAMP accumulation under bFSH stimulation in HEK cells. These results are very similar to those observed when assessing the effect of FLX on the LH-dependent stimulation of cAMP accumulation in mLTC-1 cells (Nguyen et al., 2019b). They had shown that the effect of FLX on cAMP accumulation response to hLH in mLTC-1 cells operates via two mechanisms: AMPK-dependent for high concentrations of FLX (25, 50 and 100 µM), and AMPK-independent for low concentrations of FLX (12.5 µM) (Nguyen et al., 2019b). In this study, we showed that the effect of FLX on cAMP response to FSH in HEK is dependent on the AMPK signaling pathway.

As previously mentioned, ATP is the substrate to synthesize cAMP through adenyl cyclase. Therefore, our data suggest that the decrease in cAMP synthesis for bFSH in HEK cells may be related to decreased ATP content, but not to decreased cell viability. Many previous studies have shown that FLX has an indirect effect on electron transport activity and (F1F0) -ATPase, thereby inhibiting oxidative phosphorylation in mitochondria, and leading to reduced ATP (Curti et al., 1999). FLX will also inhibit mitochondrial activity when present at high concentrations in cells (Hroudová and Fišar, 2012).

Furthermore, AMPK is a key regulator of cellular energy homeostasis, which is involved in the regulation of fatty acids, cholesterol synthesis (Carling et al., 1987), and many other anabolic pathways (Viollet et al., 2010; Kim et al., 2016). AMPK activation is triggered by various factors such as: the phosphorylation of threonine 172 at its α-subunit catalyzed by protein kinases such as Liver kinase B1 (LKB1), Ca2+/calmodulin-dependent protein kinase kinase β (CaMKK β) (Kim et al., 2016), the effects of pharmacological agents such as metformin, A769662, or Compound C, or increased AMP:ATP and ADP:ATP ratios (Nguyen, 2019a).

CONCLUSIONS

In this study, we show that FLX induces a decline in ATP levels, leading to increased AMPK phosphorylation and decreased cAMP accumulation through stimulation of bFSH in HEK cells. Therefore, our hypothesis is that AMPK activation causes a decrease in cAMP synthesis through adenyl cyclase under bFSH stimulation. However, we do not rule out the possibility that FLX directly affects adenyl cyclase and reduces cAMP production independently of AMPK.

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AUTHOR CONTRIBUTIONS

Conceptualization and study design: T.M.D.N; Methodology: T.M.D.N. and N.T.T.; Formal analysis: T.M.D.N.; Investigation: T.M.D.N.; Data curation: T.M.D.N.; Writing-original draft preparation: T.M.D.N.; Manuscript finalization: T.M.D.N. and N.T.T.

CONFLICT OF INTEREST

We have no conflict of interest.

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