Molecular and Cellular Endocrinology 437 (2016) 11–21

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

β-arrestins regulate gonadotropin receptor-mediated cell proliferation and apoptosis by controlling different FSHR or LHCGR intracellular signaling in the hGL5 cell line

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ARTICLE INFO

Article history:
Received 14 January 2016
Received in revised form
8 July 2016
Accepted 3 August 2016
Available online 5 August 2016

Keywords:
hGL5
FSHR
LHCGR
Apoptosis
GPCR
Arrestin

ABSTRACT

Gonadotropin signaling classically involves proliferative, steroidogenic and apoptotic stimuli. In this study, we used the human granulosa cell line hGL5 to demonstrate how follicle-stimulating hormone (FSH) and luteinizing hormone (LH) differently control proliferative or apoptotic signals, revealing novel intrinsic properties of their receptors (FSHR, LHCGR). We found that, in this tumor-like cell line, the expression of endogenous FSHR and LHCGR is serum-dependent, but both receptors were unable to activate the canonical cAMP/PKA pathway upon gonadotropin stimulation, failing to produce cAMP, progesterone and G protein-coupled receptor (GPCR)-mediated apoptosis *in vitro*. Conversely, ligand treatment resulted in FSHR- and LHCGR-mediated ERK1/2 phosphorylation and cell proliferation due to receptor coupling to β-arrestins. The inactive cAMP/PKA pathway was unlocked by siRNA-mediated knock-down of β-arrestin 1 and 2, leading to progesterone synthesis and apoptosis. Surprisingly, FSH, but not LH treatment accelerated the cAMP/PKA-mediated apoptosis after β-arrestin silencing, an effect which could be reproduced by overexpressing the FSHR, but not the LHCGR. This work demonstrates that the expression of FSHR and LHCGR can be induced in hGL5 cells but that the FSHR-dependent cAMP/PKA pathway is constitutively silenced, possibly to protect cells from FSHR-cAMP-PKA-induced apoptosis. Also, we revealed previously unrecognized features intrinsic to the two structurally similar gonadotropin receptors, oppositely resulting in the regulation of life and death signals *in vitro*.

1. Introduction

The gonadotropins follicle-stimulating hormone (FSH), luteinizing hormone (LH) and choriogonadotropin (CG) are essential for the development and regulation of reproductive functions. These glycoprotein hormones act via their structurally related receptors (FSHR and LHCGR, respectively), expressed in the gonads, and regulate physiological functions by modulating both survival and death signals at the cellular level (Craig et al., 2007; Matsuda et al., 2012; Govindaraj et al., 2014). In addition, their role in the regulation of apoptotic and proliferative signals may be related with tumorigenesis and tissue invasion (Choi et al., 2007; Mertens-Walker et al., 2012; Tao et al., 2013). In this study, we demonstrate how tumor-like, gonadotropin-responsive cells deviate G protein-coupled receptor (GPCR)-linked pro-apoptotic towards proliferative signals via receptor coupling to β-arrestins.

1.1. FSHR and LHCGR structures and signaling pathways

FSHR and LHCGR belong to the superfamily of the GPCRs (Simoni et al., 1997; Ascoli et al., 2002). They have an extracellular ligand-specific portion, 7 transmembrane domains joined by intra- and extracellular loops, and an intracellular C-terminal tail which contributes to the activation of multiple intracellular signaling cascades (Costagliola et al., 2005). Due to structural differences characterizing the hinge region and the C-terminal tail of FSHR and LHCGR (Vassart et al., 2004; Bonomi et al., 2006), and receptor-specific downstream signaling pathways (Conti, 2002), different,

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http://dx.doi.org/10.1016/j.mce.2016.08.005
0303-7207/

Abbreviations

FSH	Follicle-stimulating hormone
LH	luteinizing hormone
CG	choriogonadotropin
FSHR	FSH receptor
LHCGR	LH and CG receptor
GPCR	G protein-coupled receptor
PKA	protein kinase A
ERK1/2	extracellular-regulated kinase
CREB	cAMP-responsive elements binding protein
KGN	human granulosa-like tumor
hCG	human CG
pCREB	phospho-CREB
pERK1/2	phospho-ERK1/2
siRNA	small interfering RNA
FBS	fetal bovine serum
FBS-cs	FBS stripped by activated charcoal-dextran
IBMX	3-Isobutil-1-methylxanthine
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PMA	phorbol 12-myristate 13-acetate
SD	standard deviation
DAPI	4,6-diamidino-2-phenylindole
MEK	mitogen-activated protein kinase kinase
Akt	protein kinase B
mTOR	mammalian target of rapamycin
EC50	50% effective dose

intrinsic receptor-mediated pro- and anti-apoptotic stimuli may occur.

Upon hormone binding, FSHR and LHCGR mediate a complex signaling network by coupling to the Gαs subunit, which leads to the adenylyl cyclase, cAMP and protein kinase A (PKA) activation. Phosphorylation of the extracellular-regulated kinase (ERK1/2) and cAMP-responsive elements binding protein (CREB) are downstream signaling events required for progesterone production (Seger et al., 2001; Amsterdam et al., 2002; Conti, 2002; Gloaguen et al., 2011).

Beside its steroidogenic potential, some studies found that sustained intracellular cAMP increase results in cell rounding (Schiffer et al., 1996; Maizels et al., 1998), a cytoskeletal modification that determines changes in the cell shape preceding apoptosis *in vitro* (Amsterdam et al., 1999; Zwain and Amato, 2001). On the other hand, the canonical cAMP/PKA pathway may be strongly linked to cell death via a cross-talk involving steroidogenic and pro-apoptotic signals, especially in cultured granulosa cells (Aharoni et al., 1995; Amsterdam et al., 1996), although opposite effects have also been reported, depending on the cell type and its metabolic state (Conti, 2002). ERK1/2 is triggered mainly via G proteins or β-arrestins (Ahn et al., 2004, 2009) and acts as modulator, mainly inhibitor, of steroidogenic and apoptotic signals (Amsterdam et al., 2002; Perry et al., 2002; Miyoshi et al., 2007) via receptor kinases (Pitcher et al., 1999). β-arrestins are involved in turning off GPCRs activation and trafficking (Laporte et al., 2002; Reiter and Lefkowitz, 2006), and the signaling cascades involved in life or death decisions (Kook et al., 2014). They counterbalance the effects of the cAMP/PKA pathway activation by uncoupling the receptor to the Gαs protein (Heitzler et al., 2012) and enhancing cell growth (Strungs and Luttrell, 2014). Therefore, the GPCR-mediated cell signaling may be the net result of a balance between life and death signals activated through distinct transduction mechanisms.

1.2. Crosstalk between pathways controlling life and death

Previous studies suggested the existence of a gonadotropin receptor-mediated mechanism regulating the crosstalk between signals controlling steroidogenesis and programmed cell death in primary as well as immortalized granulosa cells (Amsterdam et al., 2003). Indeed, it is well known that steroidogenic signals are inhibited, in favor of activation of proliferative signals, in tumor-derived or immortalized steroidogenic cells, e.g. the human granulosa-like tumor (KGN) cell line (Taniguchi et al., 2004). KGN cell proliferation is enhanced by ERK1/2 phosphorylation and inhibition of PKA, resulting in a blockade of steroid production, and revealing a mutually inhibitory cross-talk between PKA and ERK pathways (Taniguchi et al., 2004). This supports the view that, in granulosa cell lines, cell fate may be the result of a balance between life and death factors activated by GPCRs (Amsterdam et al., 2003; Sasson et al., 2004). In this regard, the ability of GPCRs to activate multiple signaling pathways, counterbalancing relatively high levels of intracellular cAMP by mediating life-promoting signaling such as ERK1/2, is crucial to shift the balance towards cell survival and growth instead of steroidogenesis and apoptosis (Breckwoldt et al., 1996; Gebauer et al., 1999; Seger et al., 2001; Maillet et al., 2002; Amsterdam et al., 2003). FSHR and LHCGR may elicit distinct downstream events through alternative second messengers, such as calcium ions and phospholipids, resulting in specific gonadotropin signaling and downstream events (Conti, 2002; Lee et al., 2002).

1.3. The human granulosa cell line hGL5

The development of ovarian granulosa cell lines is important for understanding the molecular mechanisms underlying reproduction and the response to glycoprotein hormones (Havelock et al., 2004). In this regard, the simplistic and clean nature of an *in vitro* system, which lacks confounding variables such as paracrine factors, is an optimal tool to unmask the receptor functions at the molecular level. However, no consistent steroidogenic, FSHR- and LHCGR-expressing cell lines exist, due to decreased long-term cell viability, loss of FSHR and LHCGR expression upon transformation (Amsterdam and Selvaraj, 1997), or lack of some important features such as the steroidogenic activity (Choi et al., 2004; Desai et al., 2015).

Immortalized human granulosa cells were previously described (Rainey et al., 1994). Interestingly, hGL5 cells maintain key steroidogenic pathways and are highly proliferating, offering an optimal tumor-like system for studies focused on gonadotropin-dependent proliferation, cell survival and apoptosis. However, the activation of adenylyl cyclase could only be achieved by Forskolin treatment, whereas neither FSH nor human CG (hCG) treatment results in the activation of the canonical cAMP/PKA pathway, suggesting that the FSHR and LHCGR expression is downregulated (Patel et al., 2009). It was demonstrated that the steroidogenic potential of cAMP is increased by oxytocin in hGL5 cells. Since the expression of the oxytocin receptor is serum-dependent (Copland et al., 2002), a metabolic control of GPCRs' expression and steroidogenic pathways' activation was suggested.