FSH, oxytocin and IGF-I regulate the expression of sirtuin 1 in porcine ovarian granulosa cells

Alexander V. Sirotkin[a,b], Petra Dekanová[a], Abdel Halim Harrath[c]

[a] *Constantine The Philosopher University in Nitra, Nitra, Slovakia*

[b] *Research Institute of Animal Production, Lužianky, Slovakia*

[c] *College of Science, King Saud University, Saudi Arabia*

Corresponding author: Prof. Alexander V. Sirotkin, PhD., DrSc., Constantine The Philosopher University in Nitra, Tr. A. Hlinku 1, 949 74 Nitra, Slovakia. Tel: +421-903561120, e-mail: asirotkin@ukf.sk

ABSTRACT

The involvement of the mTOR system/enzyme sirtuin 1 (SIRT1) intracellular signaling system in the control of ovarian functions and its role in mediating hormonal action on the ovary has been proposed, but this hypothesis should be supported by a demonstrated influence of hormones on mTOR/SIRT1. Therefore, the aim of our in vitro experiments was to examine the effect of the known hormonal regulators of ovarian functions, such as follicle-stimulating hormone (FSH), oxytocin (OT) and insulin-like growth factor I (IGF-I), on mTOR/SIRT1. The accumulation of SIRT1 in porcine ovarian granulosa cells cultured with and without these hormones (at doses of 1, 10 or 100 $ng.ml^{-1}$) was evaluated using immunocytochemistry. It was observed that the addition of FSH (at 10 $ng.ml^{-1}$ but not at 1 or 100 ng/ml) and OT (at all tested doses) increased the expression of SIRT1 in ovarian cells. In addition, 100 $ng.ml^{-1}$,

but not at 1 or 10 ng.ml^{-1}, of IGF-I decreased SIRT1 accumulation. Our observations are the first demonstration that hormones can directly regulate the ovarian mTOR/SIRT1 system and that this system could mediate the action of hormonal regulators on the ovary.

Keywords: Pig; Ovarian granulosa cells; Sirtuin 1; FSH; Oxytocin; IGF-I.

1. Introduction

Studies of both extra- and intracellular regulators of reproduction as well as their interrelationships are important from both theoretical and practical viewpoints. The mTOR signaling system and its key enzyme, sirtuin 1 (SIRT1), have been extensively studied in recent years. SIRT1 is a member of the NAD+-dependent deacetylase family (Frye, 2000; Jin et al., 2009). It is widely expressed in numerous cell types, including ovarian cells. In non-ovarian cells, SIRT1 regulates metabolism, hormone secretion, cell cycle and cell differentiation and is protective against cellular oxidative stress, DNA damage, apoptosis, aging and inflammation (Bordone et al., 2006; Fu et al., 2006; Haigis and Guarente, 2006; Wolf, 2006; Rajendrasozhan et al., 2008; Rodgers et al., 2008). It is known that food restriction regulates both reproduction and SIRT1 activity and that food restriction can potentially control reproduction through SIRT1 (Wolf, 2006). SIRT1 is involved in control of various reproductive processes including basic ovarian functions (see Tatone et al., 2015 for review). SIRT1 directly stimulates spermatogenesis but not oogenesis (Coussens et al., 2008) and reduces ovarian cell viability and promotes ovarian progesterone release (Morita et al., 2012) in rodents. SIRT-1 overexpression was associated with promotion of mice ovarian follicullogenesis and fecundity (Long et al., 2019), whilst its knock-down has an opposite effect (Tatone et al., 2018). SIRT1 might control reproductive processes through the induction of GnRH expression, LH release (Kolthur-Seetharan, 2009) and the induction of LH receptors

(Morita et al., 2012). The transfection-induced overexpression of SIRT1 in cultured porcine ovarian granulosa cells reduced their proliferation, increased progesterone and insulin-like growth factor I (IGF-I) release and modified the response of granulosa cells to exogenous follicle-stimulating hormone (FSH; (Pavlová et al., 2013; Sirotkin et al., 2014; Sirotkin, 2016).

FSH and other hormones as well as growth factors including oxytocin (OT) and IGF-I are the most well known regulators of ovarian functions. Their effect on ovarian cells could be mediated via various intracellular signaling molecules (Sirotkin, 2014). However, there is insufficient evidence for the role of mTOR/SIRT1 in mediating hormone action on ovarian cells. Such direct evidence could include (1) changes in the expression of SIRT1 under the influence of this hormone, (2) the ability of SIRT1 to affect ovarian functions controlled by this hormone and (3) the ability of SIRT1 to modify the action of this hormone on ovarian cells. Previously, we reported on the ability of SIRT1 to affect porcine ovarian cell functions and to modify FSH action on these cells (see above). Ovarian stimulation by gonadotropins has been associated with increased SIRT-1 level in serum, but not in ovarian follicular fluid (Bódis et al., 2018); however, the effect of hormonal regulators on ovarian SIRT1 has not yet been examined.

The aim of our in vitro experiments was to examine the effect of the addition of known hormonal regulators of ovarian functions, FSH, OT and IGF-I at various doses on SIRT1 accumulation in cultured porcine ovarian granulosa cells.

2. Materials and Methods

2.1. Preparation, culture and processing of ovarian cells

The major experimental protocols, including cell culture and their validation have been described in our previous publications (Meszarosova et al., 2008; Pavlova et al., 2011, 2013;

Sirotkin et al., 2014). Briefly, primary granulosa cells were aspirated from the ovaries of prepubertal gilts that were 100-120 days of age at a local abattoir; the cells were resuspended in Dulbecco's modified Eagle's medium (DMEM)/F-12 1:1 + 10% fetal calf serum and 1% antibiotic-antimycotic solution (all of which were purchased from Sigma, St. Louis, Mo, USA), dispensed to 16-well chamber slides (Nunc International, Naperville, TN, USA) in 200-µl aliquots and incubated at 37.5°C and 5% CO_2 humidified air until the formation of a 50-60% confluent monolayer (48-72 hours). After primary culture, the media from the plates were aspirated, and the cells were washed with fresh DMEM/F12. Then, the cells were cultured for two days with and without porcine FSH (Dr. A.P.F. Parlow, NHPP, Torrance, CA, USA), oxytocin (Sigma) or IGF-I (Sigma) added to the culture medium at concentrations of 0, 1, 10 or 100 $ng.ml^{-1}$. At the end of the culture, cell numbers and viability were determined by Trypan blue staining and cell counting with a hemocytometer. Cell viability was 70-80%. No statistically significant differences in these indices were observed between the control and experimental groups.

After removing the medium from the chamber slides, cells were washed in ice-cold PBS (pH 7.5), fixed in paraformaldehyde (4% in PBS, pH 7.2-7.4), dehydrated in a graded alcohol series (70, 80, 96; 10 min each) and stored at 4°C until the immunocytochemical analysis.

2.2. Immunocytochemical analysis

The immunocytochemical analysis method was previously validated for porcine granulosa cells (Meszarosova et al., 2008; Pavlova et al., 2011, 2013; Sirotkin et al., 2014). The presence of SIRT1 was detected using immunocytochemistry (Osborn and Isenberg, 1994). This method quantitative immunocytochemistry for SIRT-1 detection, quantification and changes in its accumulation in porcine granulosa cells has been previously validated by Western immunoblotting and other methods (Pavlova et al., 2013; Sirotkin et al., 2014;

Sirotkin, 2016). Primary mouse monoclonal antibodies against SIRT1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, catalogue number B-7, initial concentration 200 $\mu g.ml^{-1}$) were used at a dilution of 1:250. The visualization of the binding of primary antibodies was achieved with secondary polyclonal goat antibodies against mouse IgGs. The secondary antibodies labeled with the fluorescent marker fluorescein isothiocyanate (FITC) provided in a concentration 200 $\mu g.ml^{-1}$ by Santa Cruz Biotechnology, applied at a dilution of 1:1000 and mounted in a Vectashield with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA). The presence of SIRT1 was determined by fluorescent microscopy by using Leica Microsystems (Wetzlar, Germany). The cells expressing signal above background negative control levels were considered positive. The percentage of cells containing visible antigen was counted. Cells processed without the primary antibody were used as the negative control. Images of stained cells and negative control are shown in Fig.1.

2.3. *Statistics*

Each experimental group was represented by three chamber-slide wells. The data shown are the means of the values obtained in these three separate experiments performed on separate days with separate groups of granulosa cells, each obtained from 15-20 animals. At least 10 optical fields were analysed in each chamber. In each chamber (three per group), at least 1000 cells were scored. Each value represents the mean of nine replicates (at least 9000 cells in total). The percentage of cells containing antigen in different groups of cells was calculated. Significant differences between the experiments were evaluated with two-way ANOVA followed by chi-square test using SigmaPlot 11.0 software (Systat Software, GmbH, Erkhart, Germany). Differences from control at $P<0.05$ were considered significant.

3. Results and Discussion

Immunocytochemical analysis showed the presence of SIRT1 in porcine ovarian granulose cells after culture. SIRT1 was presented in the cells as cytoplasmic clusters (Fig.1). The percentage of cells containing SIRT1 varied between 3 and 75%, and this percentage was affected by hormonal treatments (Fig.2). The addition of FSH at a dose of 10 $ng.ml^{-1}$ but not at lower (1 ng/ml) or higher (100 $ng.ml^{-1}$) increased the expression of SIRT1 in the cells (Fig. 2A). OT increased the expression of SIRT1 in ovarian cells at all doses added (1, 10 or 100 $ng.ml^{-1}$) (Fig. 2B). IGF-I decreased SIRT1 accumulation when added at the highest (100 $ng.ml^{-1}$) but not at lower (1 or 10 $ng.ml^{-1}$) doses (Fig. 2Cc).

Our observations confirm our previous data (Pavlova et al., 2013; Sirotkin et al., 2014) on the presence of SIRT1 in cultured porcine ovarian granulosa cells. Furthermore, they are the first evidence that the expression of SIRT1 in ovarian cells might be controlled by FSH, OT and IGF-I. These hormones are well-known regulators of various ovarian functions, including cell proliferation, apoptosis, release of steroid and peptide hormones, ovarian folliculogenesis and follicle selection, oogenesis, ovulation and luteogenesis. The intracellular mechanisms and mediators of hormone action on these processes are not completely elucidated, but the involvement of receptors, protein kinases, transcription factors and small RNA has been documented (see Sirotkin, 2014 for review). It remains unknown whether the effects of hormonal regulators of ovarian functions could be mediated by SIRT1. We propose, that the hypothetical mediator of hormone action (1) changes under the influence of this hormone, (2) affects ovarian functions controlled by this hormone and (3) when changed, modifies the hormone effects. Our previous experiments with transfection of porcine ovarian granulose cells with a cDNA construct for SIRT1 (Pavlová et al., 2013; Sirotkin et al., 2014) and other studies of SIRT1 regulators effects (Sirotkin, 2016, Tatone et al., 2018) demonstrated the involvement of SIRT in both basic ovarian functions (proliferation, apoptosis, secretory activity and expression of transcription factors) and modifications of the

effect of FSH on transcription factor NF-κB. Curcumin-induced activation of SIRT1 gene was associated with reduction in plasma FSH level and activation of murine reproductive processes (Azami et al., 2019), as well as with changes in rabbit ovarian hormones release and fecundity (Sirotkin et al., 2018). Curcumin changed also response of rabbit ovarian cells to LH (Sirotkin et al., 2018). Furthermore, resveratrol-induced stimulation of SIRT1 accumulation was associated with changes in character of IGF-I action on apoptosis and testosterone release by porcine granulosa cells (Sirotkin et al., 2019a). Food restriction affecting mTOR/SIRT1 was able to modify also ghrelin action on chicken (Sirotkin et al., 2017b) and rabbit (Sirotkin et al., 2017a) hormones and reproduction in vivo and on response of rabbit ovarian cells to IGF-I (Sirotkin et al., 2017a). The present observations related to the action of FSH, OT and IGF-I on the expression of SIRT in porcine ovarian cells represent the first demonstration of direct regulation of ovarian SIRT-1 by hormones and indication that SIRT1 may be involved in mediating the effect of these hormones on the ovary. Moreover, the differences in character of hormones action on SIRT-1 indicate, that FSH, OT and IGF-I can affect SIRT1 via different signaling pathways documented previously (Sirotkin, 2014). Alternatively, these hormones can affect not only SIRT-1, but also other signaling molecules (Sirotkin, 2014) whose in turn could affect SIRT-1 directly or via feedback mechanisms. On the contrary, mTOR/SIRT1 system can modify FSH release (Tatone et al., 2015; Azami et al., 2019), as well as FSH (Morita et al., 2012; Sirotkin et al., 2019b), LH (Sirotkin et al., 2018) and IGF-I (Sirotkin et al., 2019a) action. Taken together, these observations demonstrate the involvement of mTOR/SIRT1 system in endocrine control of ovarian functions.

4. Conclusions

The hypothesis concerning the role of SIRT1 as a mediator of hormone action on ovarian cells requires further confirmation. Moreover, the involvement of SIRT1 in the

control of particular ovarian functions and the ability of SIRT1 to modify the action of various hormones on these functions should be supported by more data. Nevertheless, our present preliminary study is the first to provide evidence that hormones can direct regulate ovarian SIRT-1, and that some effect of hormonal regulators on the ovary may be mediated by the mTOR/SIRT1 intracellular signaling system.

Conflict of interest

The authors declare that they have no conflict of interests, no any financial interest or benefit that has arisen from the direct applications of their research

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Figure legends

Figure 1. Immunocytochemistry images showing cultured porcine granulosa cells containing sirtuin 1 (Leica Microsystems, Wetzlar, Germany). A. – specific staining by using primary antiserum agains sirtuin 1 and secondary antiserum labelled with fluorescein isothiocyanate (FITC; green fluorescence). B. – negative control (staining with secondary antibody with FITC without primary antibody). Scale bars: 1 cm = 20 μm.

Figure 2. Effect of FSH (A), OT (B) and IGF-I (C) on the percentage of porcine granulosa cells expressing sirtuin SIRT1.

Monolayers of granulosa cells from prepubertal gilts were cultured with or without FSH, OT or IGF-I (0, 1, 10, 100 $ng.ml^{-1}$ medium). After two days of culture, the cells were analyzed by immunocytochemistry. Data are the mean $\pm$ S.D. * - Effect of hormone addition: significant ($P < 0.05$) differences between the cells cultured with (1, 10 or 100 ng/ml) and without (0 ng/ml) hormone.

Fig.1.

A. B.

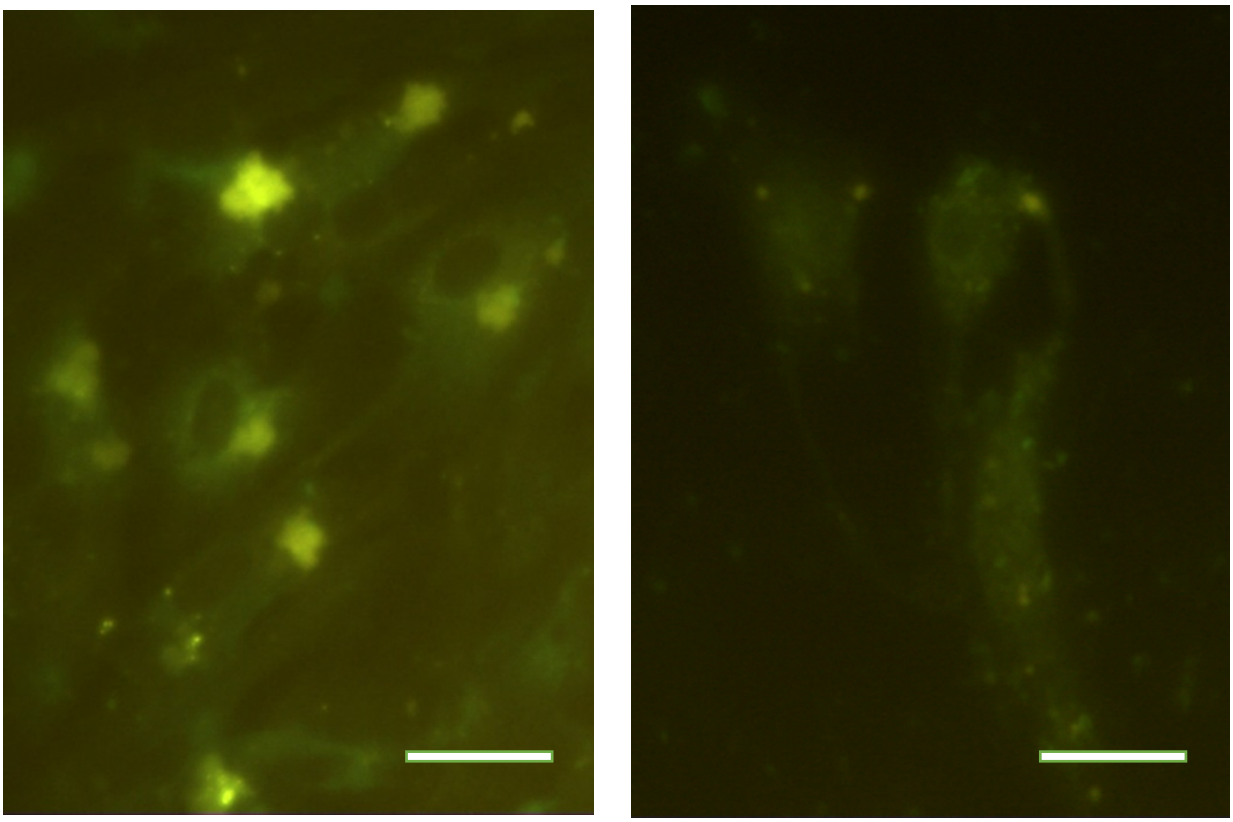
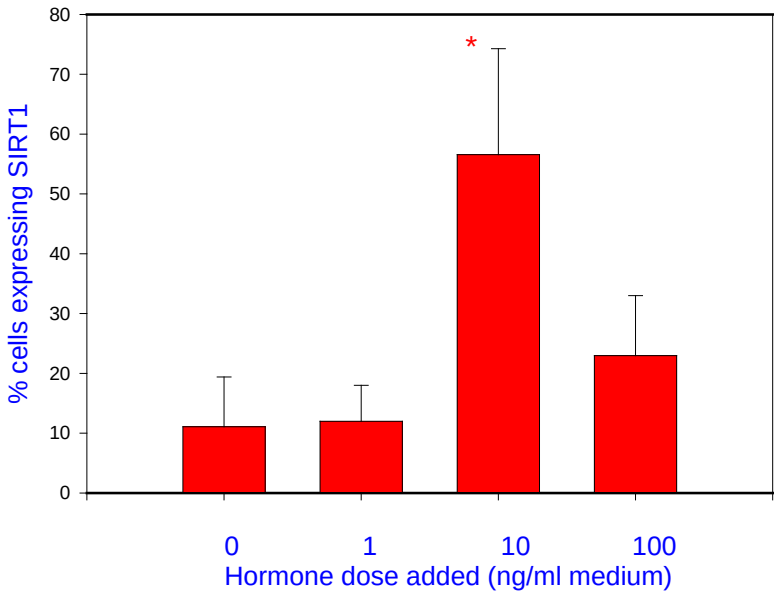
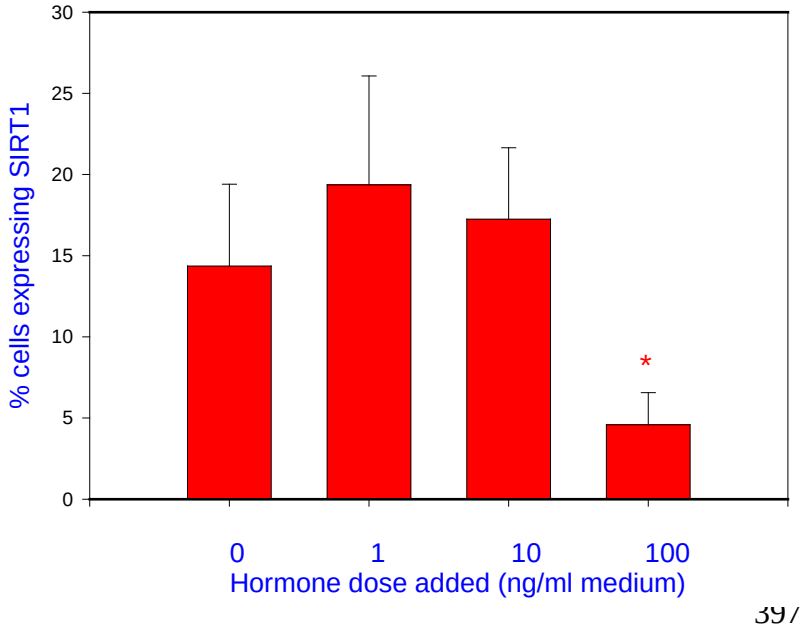


Fig. 2.

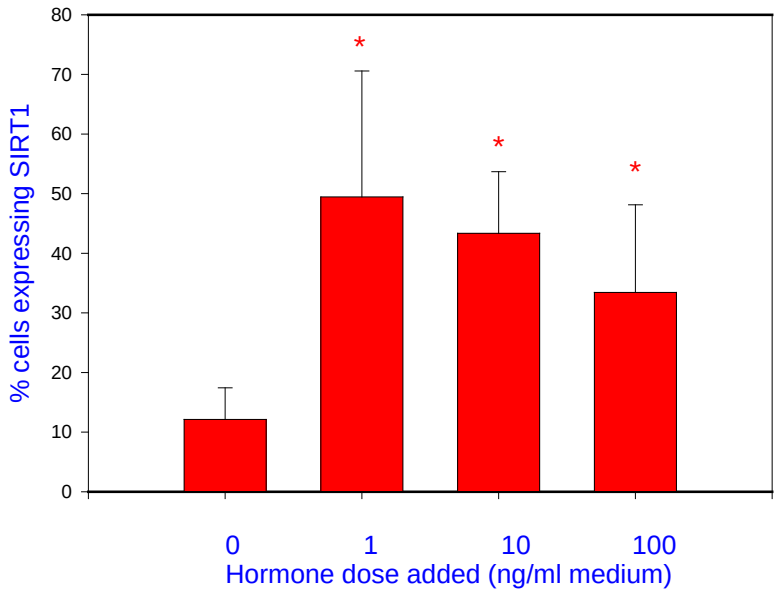
A. FSH



B. OT



C. IGF-I


80
70
60
50
40
30
20
10
0
% cells expressing SIRT1
*
*
*
0
1
10
100
Hormone dose added (ng/ml medium)