

Mechanisms of autophagy induction by sex steroids in bovine mammary epithelial cells

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Abstract

In dairy cattle, mammary gland involution serves to remodel the secretory tissue and occurs in a period of overlap between mammogenic stimulation caused by the next developing pregnancy and tissue regression induced by milk stasis. At this time, high concentrations of 17 β -oestradiol (E2) and progesterone (P4) support the regeneration of the mammary tissue, as well as enhance autophagy, a cellular process induced in response to stressful conditions for energy generation and homeostasis maintenance. This study aimed to elucidate the mechanisms of autophagy induction by E2 and P4 using an *in vitro* model of involution based on 20-fold reduction of FBS content (from 10% to 0.5%) in the culture medium of BME-UV1 bovine mammary epithelial cells (MECs). Real-time RT-PCR, Western blot and EMSA analyses demonstrated that addition of E2 and P4 caused a genomic effect in BME-UV1 cells, stimulating the expression of autophagy-related genes (ATGs): *BECN1*, *ATG5*, *LC3B* and their corresponding proteins. Furthermore, knockdown of oestrogen receptor (ER α) and experiments with the use of oestrogen and progesterone antagonists (4-hydroxytamoxifen and RU-486, respectively) demonstrated that the observed genomic effect is mediated by steroid receptors. Finally, both steroids were shown to form complexes with beclin1 and regulate Bcl-2 phosphorylation, indicating that an indirect, non-genomic effect of E2 and P4 may also contribute to autophagy induction in bovine MECs.

Key Words

- ▶ mammary epithelial cells (MECs)
- ▶ autophagy
- ▶ 17 β -oestradiol
- ▶ progesterone
- ▶ autophagy-related genes

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Introduction

In adult life of female mammals, the mammary gland undergoes cycles of developmental changes initiated by pregnancy. These changes can be divided into four consecutive, but partially overlapping stages: mammogenesis, lactogenesis, galactopoiesis and involution. Ovarian steroids 17 β -oestradiol (E2) and progesterone (P4) are significantly involved in coordination of the course of the lactation cycle. E2 and P4 mediate proliferative signals in mammary epithelial

cells (MECs), and their biological responses were proven to be dependent on the expression and activation of specific receptors: oestrogen receptors (ER α and ER β) and progesterone receptors (PRA and PRB) (Schams *et al.* 2003, Lamote *et al.* 2004). ER and PR induce genomic effects that regulate morphological growth and functional differentiation of the mammary gland during gestation, i.e. E2 activates ductal morphogenesis while P4 mediates lobulo-alveolar development (Lamote *et al.* 2004). At the

end of gestation an increase in the size of the mammary gland is observed due to a full expansion of the alveoli formed by MECs that undergo functional differentiation preparing for the extensive synthesis of milk constituents. Upon parturition, withdrawal of pregnancy hormones, mainly P4, stimulates milk secretion initiating the stage of lactogenesis, followed by galactopoiesis (maintenance of milk secretion) which is under control of pituitary hormones (prolactin and growth hormone).

In most mammals in the last stage of lactation cycle, involution, the mammary gland gradually regresses upon fulfilment of its functional purpose, returning to a state of development similar to the one prior pregnancy (Lamote *et al.* 2004). Regression of the mammary epithelium is tightly connected with increased rate of MECs' apoptotic cell death, decreased cell-cell and cell-extracellular matrix interactions, as well as degradation of extracellular matrix (ECM) by matrix metalloproteinases (Stefanon *et al.* 2002, Rabot *et al.* 2007). This scheme of events does not fully illustrate processes occurring in the mammary gland of ruminants, especially dairy cattle. Mammary involution in cattle is slower and less extensive due to the fact that dairy cows are typically pregnant when milk removal ceases (Capuco *et al.* 1997). Thus, in this species mammary involution promoted by milk stasis is at the same time opposed by mammogenic signals derived from the pregnancy hormones. In dairy cows the initial phase of the nonlactating period before parturition, termed dry period, is characterized by removal of senescent cells by apoptosis, followed by MECs proliferation during late dry period stimulated by sex steroids, preparing the mammary gland for the next lactation (Capuco & Akers 1999). Studies have shown that during dry period bovine MECs show signs of autophagy manifested by a high number of autophagic vacuoles observed and increased expression of autophagic protein beclin1 (Zarzyńska *et al.* 2007).

Autophagy, which developed in eukaryotic cells as an adaptive response to unfavourable conditions, is evolutionarily conserved, highly regulated self-degradative pathway activated for energy and macromolecules generation to maintain cellular homeostasis (Feng *et al.* 2014). Cytoplasm, organelles or other cellular components undergo sequestration in double-membrane vesicles called autophagosomes, which then fuse with lysosomes for final breakdown and recycling of sequestered substrates (He & Klionsky 2009). Autophagosome formation is driven by combined actions of a range of proteins designated as ATG (autophagy-related) proteins (Mizushima *et al.* 2011). Autophagy plays a crucial role in the turnover of long-lived proteins and damaged organelles, and therefore

is constitutively active in cells at a basal level. As a pro-survival process, autophagy is upregulated in response to the stressful conditions connected with nutrient limitation, oxidative stress or pathogen invasion (Yang & Klionsky 2010).

Studies have shown that autophagy is involved in mammary gland development and remodelling. *In vitro* studies on the course of alveologenesis, conducted with the use of 3D cultures on reconstituted basement membrane (rBM), have demonstrated that autophagy is induced in the cells localized in the centre of the developing alveoli, due to the lack of contact of those inner cells with ECM (Mills *et al.* 2004, Sobolewska *et al.* 2011). Furthermore, our research using BME-UV1 bovine mammary epithelial cells and a model of *in vitro* mammary involution showed that induction of autophagy may provide a survival mechanism for MECs during stress connected with temporal malnutrition (Sobolewska *et al.* 2009). The model of *in vitro* involution was based on significant reduction of foetal bovine serum (FBS) concentration in the culture medium, mimicking the naturally occurring decline in the access of bioactive compounds and nutrients in the mammary gland of dairy cows at the end of lactation and dry period, caused by concomitant pregnancy and related high energy demands of the developing foetus. In addition, we noted that autophagy induction was enhanced in bovine MECs in the presence of sex steroids: E2 and P4 (Sobolewska *et al.* 2009), but the exact mechanism of this regulation was not explored. Considering the fact that sex steroids exert their effects through genomic pathways regulating gene expression, in the present study we investigated the effect of E2 and P4 on the expression of chosen autophagic genes (*BECN1*, *ATG3*, *ATG5*, *LC3B*) and their protein products. Furthermore, we examined the potential steroid-induced regulation of other cell signalling pathways responsible for autophagy induction, to verify whether E2 and P4 may regulate autophagy in bovine MECs in the course of actions other than genomic.

Materials and methods

Media and reagents

BME-UV1 cell culture medium ingredients: Dulbecco modified Eagle's medium DMEM/F-12, RPMI-1640, NCTC135, α -lactose, lactalbumin hydrolysate, glutathione, bovine insulin, bovine holo-transferrin, hydrocortisone, L-ascorbic acid, 17 β -oestradiol (E2), progesterone (P4), LY294002, PD98059, 3-methyladenine (3MA), and

chloroquine (ChQ) were purchased from Sigma-Aldrich, whereas heat-inactivated foetal bovine serum (FBS), penicillin–streptomycin, fungizone and gentamycin were obtained from Life Technologies, Invitrogen. Antibodies against ER α (sc-543), PR (sc-539), β -actin (sc-47778), lamin A/C (sc-20681), p-Akt (Ser 473; sc-7985-R), total Akt (sc-1618), p-ERK (Tyr204; sc-7383), total ERK (sc-94), p-AMPK (Thr172; sc-33524), total AMPK (sc-25792), p-Bcl-2 (Ser70; sc-21864-R) and beclin-1 (BECN1; sc-11427) were purchased from Santa Cruz Biotechnology; antibodies against autophagic proteins ATG5 (NB110-53818), ATG3 (R-159-100) and LC3B (NB100-2220) were purchased from Novus Biologicals (Novus Biologicals, LLC, Littleton, CO, USA); secondary antibodies conjugated with IRDye680 or IRDye800, which were used for protein detection by Odyssey Infrared Imaging System, were purchased from LI-COR Biosciences (Lincoln, NE, USA). Plastic cell culture plates and flasks were purchased from Corning Incorporated. Sterile conical flasks, Lab-Tek Chamber Slides and disposable pipettes were supplied by Nunc Inc. (Naperville, IL, USA).

Cell culture

BME-UV1 bovine mammary epithelial cell line was purchased from the Cell Bank of The Lombardy and Emilia Romagna Experimental Zootechnic Institute, Italy. During routine culture, cells were grown in monolayer, in plastic culture flasks in growth medium comprising DMEM/F-12, RPMI-1640 and NCTC 135 in proportions of 5:3:2 by volume enriched with α -lactose (0.1%), glutathione (1.2 mM), bovine insulin (1.0 μ g/mL), bovine holo-transferrin (5.0 μ g/mL), hydrocortisone (1.0 μ g/mL), L-ascorbic acid (10 μ g/mL), 10% (v/v) heat-inactivated foetal bovine serum (FBS), penicillin–streptomycin (50 IU/mL), fungizone (2.5 μ g/mL), and gentamycin (50 μ g/mL). The cells were cultured in an atmosphere of 5% CO₂/95% humidified air at 37°C and routinely subcultured every second day. All experiments were performed with the use of *in vitro* model of bovine mammary gland involution. In this model, when cells cultured under routine conditions reached about 70% confluence, the medium was replaced with experimental medium containing reduced concentration of FBS from 10% to 0.5% (v/v). For the purpose of this study, cells grown in the medium containing 0.5% FBS were considered as a control group unless indicated differently. Additionally, in experimental conditions, medium was supplemented with E2 (1 nM), P4 (5 ng/mL) or a combination of both steroids (E2+P4). Concentrations of both steroids were

chosen based on literature data, showing that in dairy cows which are in advanced gestation at the time of dry period, circulating E2 gradually increases from 0.29 ng/mL (1.07 nM) at the beginning of drying off to over 1 ng/mL (3.68 nM) one day prepartum (Shah *et al.* 2006), whereas plasma concentration of P4 during the concurrent pregnancy fluctuates around 4–13 ng/mL (Stefanon *et al.* 2002, Gross *et al.* 2014).

GFP-LC3 construct and transfection

cDNA encoding bovine LC3B transcript was inserted into BamHI and EcoRI sites of pEGFP-C1, a GFP fusion protein expression vector (Clontech Laboratories), as previously described (Sobolewska *et al.* 2009). pEGFP-LC3 was transfected into the BME-UV1 cells using Lipofectamine 2000 (Thermo Fisher Scientific, Invitrogen) according to the protocol supplied by the producer. Twenty-four hours after transfection, the medium was replaced either with growth medium containing 10% FBS or with experimental medium (0.5% FBS) and in these conditions cells were cultured for the next 24 h. Next, the medium was replaced with experimental medium containing E2, P4 or both steroids together and the cells were cultured for consecutive 24 or 48 h. Simultaneously, control cells were maintained in growth medium. Then, cells were fixed in 3.7% paraformaldehyde for 20 min, washed twice with PBS, and finally mounted on microscope slides using SlowFade Gold reagent (Thermo Fisher Scientific, Invitrogen). Cells were visualized by confocal laser scanning microscope FV-500 system (Olympus Optical). The number of GFP-LC3II puncta with high intensity of green fluorescence was quantified using MicroImage analysis software (Olympus), together with the number of nuclei in a chosen image area. The results obtained were presented as the number of GFP-LC3II puncta per cell. At least 8 different images from each experimental condition were analysed quantitatively. For all conditions at least three independent experiments were performed.

Real-time PCR

Cells were pelleted by centrifugation, disrupted in 600 μ L of RLT Buffer from the Qiagen RNeasy Mini Kit (Qiagen; cat. no: 74104), and stored at –80°C until further use. Total RNA was extracted from cells with the RNeasy Mini Kit according to the protocol supplied by the producer. RNA concentration and purity were determined spectrophotometrically, and quality was confirmed using microcapillary electrophoresis (Bioanalyzer 2100, Agilent

Technologies). During reverse transcription, 2 µg of isolated total RNA was converted to cDNA with High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Applied Biosystems), and the reaction was carried out in a Mastercycler pro (Eppendorf, Hamburg, Germany). Real-time PCR was performed in triplicate using SYBR Select Master Mix (Applied Biosystems, Thermo Fisher Scientific). Each 10 µL reaction contained a final concentration of 0.5 µM each of forward and reverse primers, 1× master mix, and 1 µL cDNA (100 ng). Reaction was performed in Mx3005P QPCR machine (Stratagene). Cycling condition started with two initial steps at 50°C for 2 min and 95°C for 2 min, which were followed by 40 cycles: 95°C for 15 s; 58°C for 15 s; 72°C for 1 min each, respectively. Standard curves were run for each transcript to ensure exponential amplification, and 'no RT' controls were run to exclude nonspecific amplification. *GAPDH* was used as a reference gene. All primers were purchased in Oligo.pl (Laboratory of DNA Sequencing and Oligonucleotide Synthesis, Institute of Biochemistry and Biophysics (IBB), Polish Academy of Science, Warsaw, Poland). The sequences are listed in Table 1. Comparative CT method (Livak & Schmittgen 2001) was used to calculate the fold change in gene expression normalized to reference *GAPDH* gene. The expression of each *ATG* gene under basic experimental conditions (0.5% FBS medium) was appointed as 1 at each time point, and the levels of analysed mRNA were normalized according to the relative *GAPDH* mRNA expression in each sample. A change in expression was considered significant when at least 1.5-fold increase was noted above the control value. At least three independent experiments in three replicates were performed.

Western blot analysis

Cells were pelleted by centrifugation at 1500 *g* and stored at -80°C until protein isolation. Whole cell protein

extracts were isolated by lysing the collected cell pellets with RIPA buffer according to the previously published protocol (Sobolewska *et al.* 2009). For the purpose of experiment analysing the expression of oestrogen receptor in the nucleus, separate nuclear and cytoplasmic fractions were isolated using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific) according to the protocols provided by the producer. Total or nuclear extracts were subjected to SDS-PAGE, electrotransfer and immunostaining of the PVDF membranes according to the previously published protocol (Zielniok *et al.* 2014). Odyssey Infrared Imaging System (LI-COR Biosciences) was used to analyse the protein expression. Scan resolution of the instrument was set at 169 µm and the intensity was set at 5. Quantification of the integrated optical density (IOD) was performed with the analysis software provided with the Odyssey scanner (LI-COR Biosciences). Immunoblot analysis was performed on samples from three independent experiments. For the purpose of publication the colour immunoblot images were converted into black and white images by the Odyssey software.

Co-immunoprecipitation

Co-immunoprecipitation was performed using Pierce Co-Immunoprecipitation Kit (Thermo Fisher Scientific) according to the instructions provided by the producer. This co-immunoprecipitation kit enables isolation of native protein complexes from a lysate by directly immobilizing purified antibodies onto an agarose support as opposed to the traditional methods that use protein A or G, resulting in co-elution of the antibody heavy and light chains that may co-migrate with relevant bands, masking important results. After immunoprecipitation, the samples were further analysed by Western blot according to the above-mentioned protocol. Samples analysed were from three independent experiments.

Table 1 Primer sequences of examined *ATGs* and parameters of real-time RT-PCR assay.

Target gene	Nucleotide sequence	Real-time RT-PCR
<i>ATG5</i>	FRD: 5'-TTT GAA TAT GAA GGC ACA CC-3' REV: 5'-TGT AAA CCC ATC CAG AGT TG-3'	SYBR Select Master Mix (catalogue no. 4472908; Applied Biosystems) I. 50°C for 2 min II. 95°C for 2 min III. 40 cycles of: • 95°C for 15 s • 58°C for 15 s • 72°C for 1 min
<i>ATG3</i>	FRD: 5'-GGT TGT TCG GCT ATG ATG AG-3' REV: 5'-GGG AGA TGA GGG TGA TTT TC-3'	
<i>BECN1</i>	FRD: 5'-AGT TGA GAA AGG CGA GAC AC-3' REV: 5'-GAT GGA ATA GGA ACC ACC AC-3'	
<i>MAP1 LC3B</i>	FRD: 5'-TTA TCC GAG AGC AGC ATC C-3' REV: 5'-AGG CTT GAT TAG CAT TGA GC-3'	
<i>GAPDH</i>	FRD: 5'-CTT CAA CAG CGA CAC TCA-3' REV: 5'-CCA GGG ACC TTA CTC CTT-3'	

Primers were designed using Primer3 software, on the basis of the bovine sequences from NCBI database.

Table 2 siRNA sequences used for ER α gene silencing (accession no. NM_001001443.1).

	siRNA sequence (5'–3')	Start	Target sequence (5'–3')
siER α _1 FRD	GCUAUGAAGUGGGCAUGAUdTdT	889	GCTATGAAGTGGGCATGAT
siER α _1 REV	AUCAUGCCCACUUCUAGCdtT	889	ATCATGCCCACTTCATAGC
siER α _2 FRD	CAUAAUCUAUUCUGAGUAUdTdT	1130	CATAATCTATTCTGAGTAT
siER α _2 REV	AUACUCAGAAUAGAUUAUGdTdT	1130	ATACTCAGAATAGATTATG
siER α _3 FRD	CAUCUUGCUUAAUUCUGGAdTdT	1508	CATCTTGCTTAATTCTGGA
siER α _3 REV	UCCAGAAUUAAGCAAGAUGdTdT	1508	TCCAGAATTAAGCAAGATG

ER α gene silencing

The siRNA knockdown of *ER α* gene (accession no. NM_001001443.1) was performed using siRNA duplexes designed and synthesized by Sigma-Aldrich. To obtain a sufficient level of knockdown three different siRNA duplexes (Table 2) were simultaneously transfected into BME-UV1 cells. Prior transfection cells ($\sim 1 \times 10^6$) were plated onto 100mm culture plates and allowed to grow until 40% confluence. Twelve hours before transfection growth medium was replaced with antibiotic-free medium. On the day of transfection the medium was replaced with Opti-MEM (Gibco, Thermo Fisher Scientific). siRNA mix was transfected into BME-UV1 cells at a concentration of 100 pmol/plate, using Lipofectamine RNAiMAX (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's protocol. Two types of controls were used: (1) cells incubated in the same volume of transfection reagent without siER α , (2) cells transfected with BLOCK-iT Fluorescent control (fluorescein-labelled dsRNA oligomer) used at concentration of 100 pmol/plate (Invitrogen, Thermo Fisher Scientific). Twenty-four hours post-transfection the medium was replaced with growth medium and cells were cultured for the next 24h (until they reached about 70% confluence). On the next day, the medium was replaced with experimental medium (0.5% FBS) with addition of E2 (1nM) or E2+P4 (P4, 5 ng/mL). Controls were not supplemented with steroid hormones. Following 24-h exposure, whole cell extracts were harvested for Western blot analysis. Experiment was performed in triplicate.

Electrophoretic mobility shift assay (EMSA)

Cells were pelleted by centrifugation at 1500 *g* and nuclear proteins were extracted using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific). EMSA was performed on DNA oligonucleotides 5' end-labelled with IRDye 700 phosphoramidite and nuclear protein extracts using Odyssey Infrared EMSA Kit (LI-COR Biosciences) according to the protocol provided by the

producer. Oligonucleotide probes were designed on the basis of *in silico* analysis performed using MatInspector software (MatInspector Release professional ver. 7.7.3.1, Genomatix) and represented fragments of bovine *LC3B* promoter with ERE site and *ATG5* promoter with ERE site. The DNA probes were synthesized by Oligo.pl (Laboratory of DNA Sequencing and Oligonucleotide Synthesis, IBB Polish Academy of Science, Warsaw, Poland). Sequences of probes used in EMSA are listed in Table 3. In addition, control non-labelled DNA probes used as competitors were purchased from Oligo.pl. In each EMSA reaction, 6 μ g proteins were pre-incubated with a constant amount (50 nM) of labelled DNA probe for 30 min at room temperature in darkness, in a buffer provided by the producer (LI-COR Biosciences). To reduce nonspecific binding, poly(dI-dC) (1 μ g/mL) was added to the reaction, and 25 mM DTT/2.5% Tween20 was added to stabilize the dye and enhance the signal. In competition experiments, confirming the binding specificity, appropriate unlabelled DNA probes were added to the reaction at 200-fold molar excess. After incubation, protein-bound and free DNAs were separated by electrophoresis in 5% TBE native acrylamide gel in 0.5 \times TBE running buffer in darkness. Finally, gels were scanned using Odyssey scanner (LI-COR Biosciences). For the purpose of publication, colour EMSA gels were converted into black and white images by Odyssey software. All analyses were performed on samples from three independent experiments.

Table 3 DNA probes used in EMSA.

	DNA sequence 5'-end-labelled with IRDye700
ERE on <i>LC3B</i> promoter region – FRD	5'-GGTGCAGTTCCTGTAGAGT-3'
ERE on <i>LC3B</i> promoter region – REV	5'-ACTCTACAGGAAGTGCACC-3'
ERE on <i>ATG5</i> promoter region – FRD	5'-TGGACCCGTTGCGGTGGAAG-3'
ERE on <i>ATG5</i> promoter region – REV	5'-CTTCCACCGCAACGGGTCCA-3'

Measurement of apoptosis by flow cytometry

Apoptosis incidence was detected in BME-UV1 cells cultured for 24 or 48 h in experimental medium (0.5% FBS) supplemented or not supplemented with E2, P4 or both steroids simultaneously. In addition the effect of autophagy inhibitors 3-methyladenine (5 mM) and chloroquine (10 μ M) was tested. In this part of experiment, inhibitors were administered to the medium 1 hour before addition of steroid hormones and cells were cultured for the next 24 or 48 h. In parallel, cells cultured in standard growth medium (10% FBS) were used as additional control. Apoptosis was assayed using Dead Cell Apoptosis Kit with Alexa Fluor 488 Annexin V and propidium iodide (PI) (Molecular Probes, Thermo Fisher Scientific) according to the protocol provided by the producer. Apoptotic cells showing positive staining for Annexin V-Alexa Fluor 488 and negative for PI (early apoptosis) and those that were double positive (late apoptosis) were counted using BD FACSAria II (BD Biosciences, USA). The data are presented as percentage of apoptotic cells (early+late apoptosis) within the total number of analysed cells. At least 2×10^4 events were recorded per sample. The data were collected for three independent experiments.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 software (GraphPad Software). One-way analysis of variance (ANOVA) with Tukey's multiple-comparison post-test were used to determine the significance of effects between the treatments (control and different experimental conditions: E2, P4, E2+P4) at a specific time point, or between different time points of particular treatment (e.g. in the case of IOD data from Western blot analysis of kinases activity). In the case of data from Annexin V/PI apoptosis assay, the significance of effect was calculated in comparison to standard culture conditions (10% FBS medium), as well as in comparison to basic experimental conditions (0.5% FBS medium). *P* value of ≤ 0.05 was considered statistically significant, and $P \leq 0.01$, or $P \leq 0.001$ as highly significant.

Results

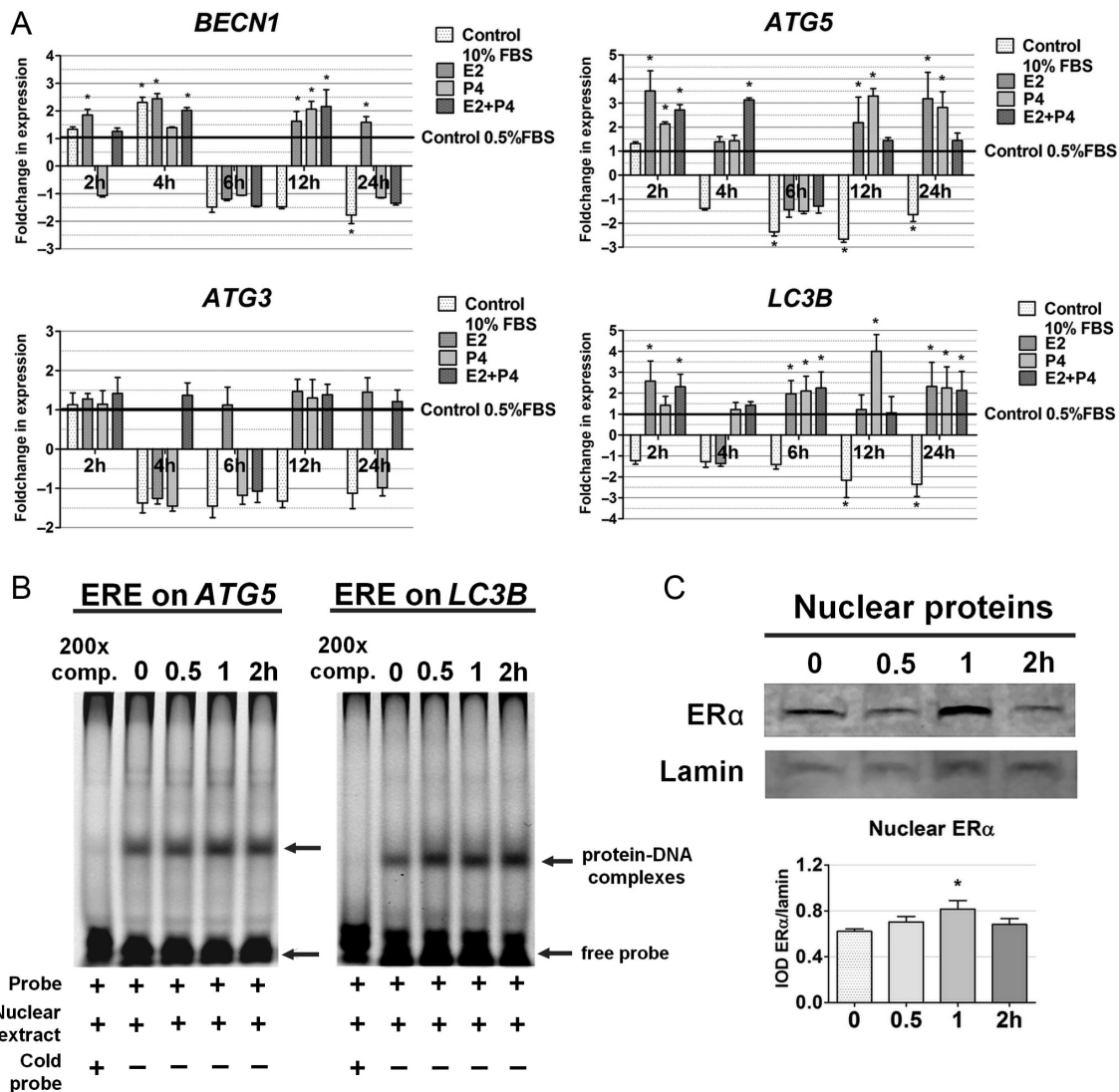
17 β -Oestradiol and progesterone regulate the expression of autophagy-related genes (ATGs)

Our previous studies have demonstrated that sex steroids (E2 or P4) increased the level of autophagosomal marker LC3 in its cleaved and lipidated form LC3II in BME-UV1

cells, indicating that these hormones may be involved in induction of autophagy in bovine MECs (Sobolewska *et al.* 2009, 2011). To test whether E2 and P4 regulate autophagy by the classical genomic pathway we investigated the expression of four autophagy-related genes: *BECN1*, *ATG5*, *ATG3* and *LC3B* in BME-UV1 cells cultured for 2, 4, 6, 12 or 24 h in 0.5% FBS medium with or without addition of E2 (1 nM), P4 (5 ng/mL) or E2+P4.

Among the investigated genes only *ATG3* did not show any significant difference in expression. The expression of *BECN1*, *ATG5* and *LC3B* were significantly increased in the presence of sex steroids when compared with basic experimental conditions (0.5% FBS medium), although the patterns of changes were not uniform (Fig. 1A). It is worth noting the expression of these genes was also significantly higher when compared with the standard growth medium conditions (10% FBS). 17 β -Oestradiol stimulated the expression of *BECN1* above the level induced by 0.5% FBS medium at 2, 4, 12 and 24 h of incubation; *ATG5* expression was increased at 2, 12 and 24 h, whereas *LC3B* expression was significantly elevated in the presence of E2 at 2, 6 and 24 h of culture. Progesterone stimulated the expression of *BECN1*, *ATG5* and *LC3B* genes after 12-h culture. After 24 h significantly increased levels of mRNA were noted in the case of *ATG5* and *LC3B*, and both of these genes also showed stimulation of expression by P4 at earlier time points (2 h and 6 h, respectively) (Fig. 1A). Simultaneous addition of E2 and P4 to 0.5% FBS medium also resulted in significant increase in expression of *BECN1*, *ATG5* and *LC3B*. *ATG5* showed elevated mRNA level at the earliest time points: 2 and 4 h; *BECN1* at 4 and 12 h, whereas *LC3B* at 2, 6 and 24 h of culture (Fig. 1A). Interestingly, all analysed genes showed a certain pattern of fluctuation in gene expression within the 24 h of experiment. The lowest level of expression of *LC3B* was observed at 4 h, *BECN1* and *ATG5* showed the lowest expression at 6 h, whereas in the case of *ATG3* it was noted between 4 and 6 h. The variability of the results suggests that the expression of *ATG* genes in this cellular model is not only regulated by extrinsic factors, but may also undergo regulation by intrinsic factors which depend on circadian rhythms. Recent studies suggest that circadian rhythm does not exclusively refer to behavioural changes and biological processes, but is also reflected by temporal changes in gene expression described as transcriptional rhythms, which are thought to be organ specific (Dooner *et al.* 2008, Zhang *et al.* 2014).

To further determine whether the observed gene-regulatory effect of steroids resulted from direct binding

**Figure 1**

Regulation of expression of chosen autophagy-related genes (ATGs) by 17 β -oestradiol (E2) and progesterone (P4) in BME-UV1 bovine mammary epithelial cells. (A) Results of real time RT-PCR analysis showing changes in expression of *BECN1*, *ATG5*, *ATG3* and *LC3B* genes in BME-UV1 cells cultured for 2, 4, 6, 12 or 24 h in 0.5% FBS medium with or without addition of E2 (1 nM), P4 (5 ng/mL) or both steroids (E2+P4); the levels of analysed mRNA were normalized according to the relative *GAPDH* mRNA expression of each sample; the expression of each ATG in cells cultured in basic experimental medium (0.5% FBS) was appointed as 1; a change in expression was considered significant (*) when at least 1.5-fold increase was obtained above the border value (control 0.5% FBS medium). (B) EMSA performed using IRDye700-labelled DNA oligonucleotide probes containing ERE site of *ATG5* and *LC3B* gene promoters and nuclear extracts from BME-UV1 cells cultured in 0.5% FBS medium for 24 h with E2 (1 nM) added for subsequent 0.5, 1 or 2 h; first lane on each EMSA image represents addition of 200-fold molar excess of unlabelled probe. (C) Western blot analysis showing changes in expression of ER α in nuclear extracts isolated from BME-UV1 cells non-treated (time 0) or treated with E2 for 0.5, 1 or 2 h, expression of lamin A/C was used as a loading control; graph below the images show the analysis of integrated optical density (IOD) of each band normalized to IOD of lamin A/C; all images are representative for three separate experiments.

of steroid receptors (ER and PR) to the promoter regions of investigated ATGs we performed EMSA analysis. Promoter regions of bovine ATGs: *BECN1* (*Bos taurus* chromosome 19, accession no: NC_007317), *ATG3* (*Bos taurus* chromosome 1, accession no: NC_007299), *ATG5* (*Bos taurus* chromosome 9, accession no: NC_007307), *ATG9A* (*Bos taurus* chromosome 2, accession no:

NC_007300), *ATG12* (*Bos taurus* chromosome 10, accession no: NC_007308) and *MAP1LC3B* (*Bos taurus* chromosome 18, accession no: NC_007316) were analysed by Genomatix EIDorado 4.7.0. Computer analysis of potential binding sites for steroid receptors on the promoters of bovine ATGs was performed with the use of MatInspector software (MatInspector Release

professional ver. 7.7.3.1, Genomatix). This *in silico* analysis revealed oestrogen response element (ERE) on the promoter regions of bovine *ATG5*, and *LC3B* genes. No progesterone response elements (PRE) were found on the promoters of any of the genes analysed. On the basis of these results, DNA oligonucleotide probes were designed, containing predicted ERE sequences on the promoters of bovine *ATG5* and *LC3B* genes. Nuclear extracts were isolated from BME-UV1 cells cultured for 0.5, 1 or 2 h in 0.5% FBS medium with addition of E2 (1 nM), or without supplementation (time 0).

EMSA analysis revealed formation of a characteristic protein–DNA complex on both used probes (Fig. 1B). In the case of probe designed on the basis of ERE site on *ATG5* promoter, the protein–DNA complex was the most pronounced after 1- and 2-h incubation with E2 when compared to time 0 (culture in non-supplemented 0.5% FBS medium). EMSA analysis using the probe containing ERE site of the *LC3B* promoter revealed augmented formation of the protein–DNA complex sooner, already after 0.5-h incubation with E2 when compared with time 0. Two-hundred fold molar excess of unlabelled probe demonstrated the specificity of the DNA-binding complexes (Fig. 1B). In addition, Western blot analysis was performed to determine the expression of ER in the nuclear extracts. Increased level of ER was detected in the nuclear extracts isolated from BME-UV1 cells incubated for 1 h in 0.5% medium supplemented with E2 (Fig. 1C).

17 β -Oestradiol and progesterone regulate the expression of autophagic proteins

To test whether the changes in *ATGs* expression induced by both steroids also resulted in increased levels of corresponding autophagic proteins, we performed Western blot analysis using whole cells extracts isolated from BME-UV1 cells incubated for 6, 12, 24 and 48 h in experimental medium with or without addition of E2, P4 or E2+P4. Our results demonstrated that *ATG3* was the only protein that did not show any significant change in the presence of steroid hormones added to the experimental medium (0.5% FBS) (Fig. 2C), which corresponded with the results of real-time RT-PCR. On the other hand, a statistically significant increase in beclin1 protein expression was noted at 6- and 12-h incubation with P4 and E2+P4 and after 48-h incubation with E2 in comparison to control conditions (Fig. 2A). Significantly higher levels of *ATG5* were observed after treatment of bovine MECs with P4, as well as E2+P4 after 6 and 12 h when compared with control, and at the later time points a significant decrease of *ATG5* expression was noted in these conditions (Fig. 2B). E2 administered alone did not exert any significant effect on *ATG5* protein level throughout the time of experiment, indicating that progesterone may play a dominant role in the enhancement of *ATG5* expression in bovine MECs (Fig. 2B). Formation of LC3-II was noted earlier when cells were treated with sex steroids administered separately or together in comparison to

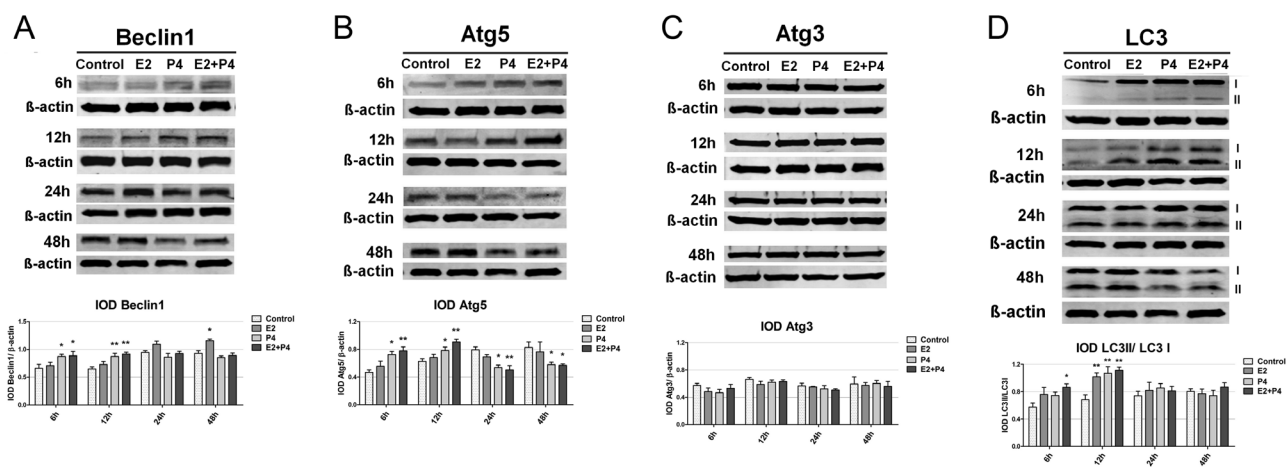


Figure 2

Results of Western blot analysis showing changes in expression of (A) beclin1, (B) *ATG5*, (C) *ATG3*, (D) LC3I and LC3II proteins in BME-UV1 cells cultured for 6, 12, 24 or 48 h in 0.5% FBS medium without (control) or with addition of E2 (1 nM), P4 (5 ng/mL) or both steroids (E2+P4); expression of β -actin was used as a loading control. Graphs below the images show the analysis of integrated optical density (IOD) of each band normalized to IOD of β -actin, with an exception of LC3 in which case the IOD values represent the ratio LC3II/LC3I. Results of densitometry are presented as means \pm s.e.m. from at least three separate experiments; * statistically significant difference ($P < 0.05$), ** statistically significant difference ($P < 0.01$), *** statistically significant difference ($P < 0.001$) in comparison with control conditions.

control conditions (Fig. 2D). When E2 and P4 were added simultaneously to the experimental medium a clear band of LC3II protein was noted already after 6h, and this result was significant in comparison to control. After 12-h treatment of BME-UV1 cells with E2, P4 or E2+P4 a significant increase in the level of LC3II was observed when compared with control. The tendency of augmented LC3II protein expression was maintained at 24-h treatment; however, according to densitometric analysis this change could not be regarded as statistically significant. At the latest time point, the difference in LC3II expression was insignificant between control and experimental conditions (Fig. 2D).

We also confirmed the induction of autophagy in conditions of FBS deficiency and medium supplementation with sex steroids by performing an experiment on

BME-UV1 cells transfected with a GFP-LC3 construct (Clontech Laboratories). In standard conditions (10% FBS), the cells showed mostly a diffused pattern of weak GFP-LC3 fluorescence indicating mainly the cytoplasmic localization of this fusion protein (Fig. 3A). When BME-UV1 cells were cultured in the 0.5% FBS medium for 24 or 48 h, a more punctuated pattern of fluorescence was observed pointing at localization of GFP-LC3II within the autophagosomal membranes (Fig. 3A). In the presence of sex steroids administered separately or together the number of GFP-LC3II puncta further increased. Quantitative analysis of the number of GFP-LC3II puncta per cell revealed a significant increase in formation of the autophagosomes in cells cultured in 0.5% FBS medium, especially after 48-h incubation period in comparison to the standard growth conditions (10% FBS medium)

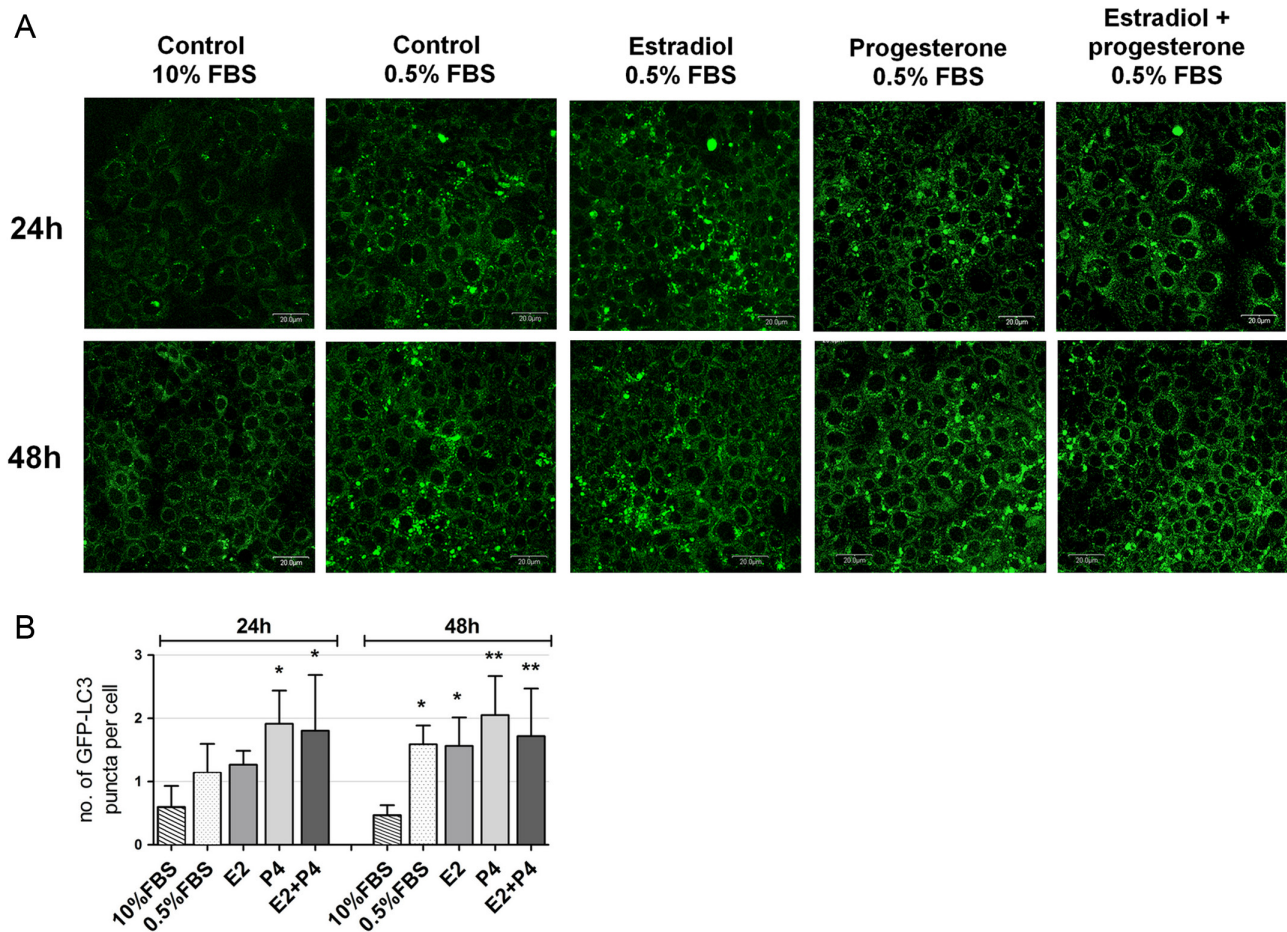


Figure 3

(A) Confocal images of BME-UV1 cells transfected with GFP-LC3 construct cultured in standard growth medium (10% FBS) or in experimental medium (0.5% FBS) with or without addition of E2 (1 nM), P4 (5 ng/mL) or both (E2+P4). Cells were cultured for 24 or 48 h in the experimental conditions. Punctuated pattern of GFP-LC3 fluorescence indicates autophagosomes localization in cells; (B) bar graph representing the number of GFP-LC3 puncta per cell in control and experimental conditions. Results are presented as means \pm s.e.m. from at least three independent experiments; * statistically significant difference ($P < 0.05$), ** statistically significant difference ($P < 0.01$) in comparison with 10% FBS medium.

(Fig. 3B). The effect was augmented in the presence of P4 and E2+P4; however, due to high values of s.d. the effect could not be regarded as significant when compared with 0.5% FBS medium conditions (Fig. 3B).

Effect of steroid receptors inhibition on autophagy induction

In order to further confirm the role of both sex steroids in autophagy induction we performed experiments with the use of steroid receptor antagonists: 4-OHT (4-hydroxytamoxifen, ER antagonist) and RU-486 (also known as Mifepristone, PR modulator). BME-UV1 cells cultured in 0.5% FBS medium were pre-treated with 4-OHT (1 μ M) or RU-486 (10 nM) for 1 h and then incubated for consecutive 24 h in the medium containing E2 (1 nM) or P4 (5 ng/mL), respectively. Cells were used for RNA and protein extraction in order to determine the expression of beclin1, ATG5 and LC3 on both mRNA and protein levels

using real-time RT-PCR and Western blot, respectively (Fig. 4A).

Pre-treatment of cells with 4-OHT resulted in only slight decrease in the level of ATG5, beclin1 and LC3II/LC3I ratio in comparison with cells treated with E2 but without ER antagonist; however, the total level of LC3 was shown to be significantly decreased in the presence of 4-OHT. Addition of RU-486 to the culture medium 1 h prior to P4 treatment caused a significant decrease in expression of ATG5 and total LC3 in comparison to cells treated only with P4 (Fig. 4A). The results obtained indicate that 4-OHT and RU-486 primarily affected the expression of LC3II protein, not causing a significant change in autophagy induction. In parallel we performed gene expression analysis and showed that pre-treatment of cells with ER or PR antagonist resulted in significant reduction of *ATG5* and *LC3B* mRNA levels, whereas *BECN1* expression was significantly decreased only in the presence of 4-OHT (Fig. 4A).

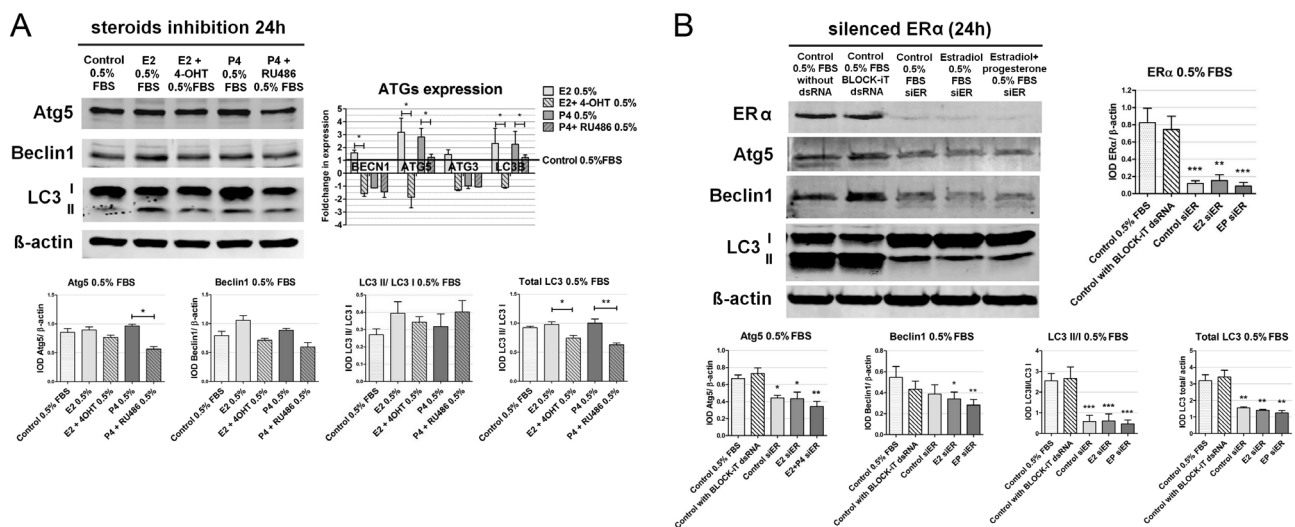


Figure 4

Effect of steroid receptors' inhibition on the expression of autophagic genes and proteins in BME-UV1 bovine mammary epithelial cells. (A) Western blot and quantitative RT-PCR analyses showing changes in expression of mRNA and proteins: ATG5, beclin1, LC3I and LC3II in BME-UV1 cells cultured in the presence of E2, P4 added alone or with their antagonists: 4-hydroxytamoxifen (4-OHT) and RU-486, respectively. Cells were cultured for 24 h in 0.5% FBS medium (control) supplemented with E2 (1 nM) or P4 (5 ng/mL), or treated with 4-OHT (1 μ M) or RU-486 (10 nM) for 1 h prior to addition of E2 or P4, respectively. Graph besides the Western blot images presents real-time RT-PCR results of the levels of analysed mRNA, which were normalized according to the relative *GAPDH* mRNA expression of each sample. The expression of each ATG in cells cultured in basic experimental medium (0.5% FBS) was appointed as 1. A change in expression was considered significant (*) when at least 1.5-fold difference was obtained between samples treated with steroid hormone (E2 or P4) and samples incubated simultaneously with steroid and its receptor antagonist. (B) Western blot analysis of total protein extracts isolated from BME-UV1 cells transfected with siER α , non-transfected cells and cells transfected with non-specific BLOCK-iT Fluorescent dsRNA oligomer that were used as controls. Graph besides the Western blot images show the IOD analysis of each ER α band normalized to IOD of β -actin. In all Western blot analyses, expression of β -actin was used as a loading control. Graphs below the images (A) and (B) show the IOD analysis of each band representing the expression of autophagic proteins normalized to IOD of β -actin. In addition, the IOD values for LC3 protein are also presented as the ratio LC3II/LC3I to show the changes in autophagy induction. Results of densitometry are presented as means \pm s.e.m. from at least three separate experiments; * statistically significant difference ($P < 0.05$), ** statistically significant difference ($P < 0.01$), *** statistically significant difference ($P < 0.001$) in comparison to relevant control conditions.

We also used siRNA technique to knockdown steroid receptors genes in order to further evaluate whether decreased levels of steroid receptors transcripts will result in lower expression of autophagic proteins, despite the addition of sex steroids to the experimental medium. Despite our efforts, we were not able to obtain a sufficient knockdown of progesterone receptor (PR); however, we were successful in the case of *ERα* transcript. Following transfection with siER and post-transfection recovery period BME-UV1 cells were cultured for 24 h in 0.5% FBS medium with or without the addition of E2 or E2+P4. Western blot analysis revealed a significant reduction of *ERα* protein expression in cells transfected with siERα in comparison to both control conditions (cells treated with Lipofectamine alone or transfected with BLOCK-iT dsRNA) (Fig. 4B). In parallel, we observed a significant decrease in the level of ATG5, beclin1 and LC3 proteins in siERα-treated cells incubated in 0.5% FBS medium with addition of E2 or E2+P4 in comparison to both control conditions. Interestingly, the expression of ATG5 and LC3 proteins was significantly reduced also in siERα-transfected cells cultured in 0.5% medium without steroids. This may indicate that *ERα* may be involved in autophagy regulation also in a ligand-independent manner (Fig. 4B).

Steroid-induced signalling pathways regulating autophagy

Aside from the classical genomic pathway, steroid hormones can induce cell signalling phosphorylation cascades, i.e. activation of adenylyl cyclase, mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase (PI3K) or

AMP-activated protein kinase (AMPK) pathways in various cells (Cheski *et al.* 2007, D'Eon *et al.* 2008). Some of these pathways have been shown to be involved in activation of autophagy. For example, signalling pathway mediated by AMPK is induced during periods of intracellular metabolic stress, leading to inhibition of mTOR activity, which subsequently stimulates autophagy induction (He & Klionsky 2009). Furthermore, activation of autophagy in response to nutrients limitation is tightly connected with hyperphosphorylation of Bcl-2, the binding partner of beclin1. This leads to dissociation of beclin1 from the complex formed with Bcl-2, and allows for autophagy induction (He & Klionsky 2009).

Thus, we aimed to determine whether E2 and P4 may modulate the phosphorylation status of AMPK and Bcl-2 in BME-UV1 cells cultured in conditions mimicking the state of mammary involution. In this experiment, confluent BME-UV1 cells were cultured in 0.5% FBS medium for 24 h prior to the addition of E2, P4 or E2+P4 and subsequently the culture was conducted for a short period of time: 15, 30, 60 or 120 min in order to observe the rapid 'non-nuclear' actions of steroids. Control conditions comprised cells cultured in 0.5% medium prior to the addition of sex steroids (time 0). Western blot analysis revealed a slight increase in the level of phosphorylated AMPK in cells treated with sex steroids added alone or simultaneously when compared with control at 15 min of experiment; however, these results were not statistically significant (Fig. 5A). In the course of experiment, the level of pAMPK gradually decreased in all tested conditions when compared with time 0. On the other hand, the level of phosphorylated Bcl-2 was significantly increased in

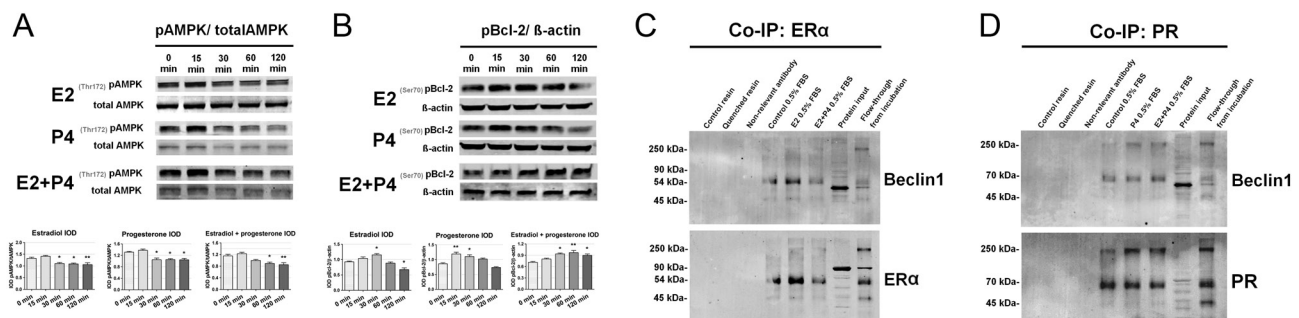


Figure 5

Western blot analysis of the levels of (A) activated/phosphorylated and total AMPK and (B) phosphorylated (Ser70) Bcl-2 in BME-UV1 cells cultured in 0.5% FBS medium for 24 h (time 0) or additionally supplemented with oestradiol (1 nM), progesterone (5 ng/mL), or both steroids for 15, 30, 60 or 120 min; expression of β-actin was used as a loading control. Graphs below the images show the IOD analysis of each band expressed as the ratio of phosphorylated/total AMPK kinase, or phosphorylated Bcl-2/β-actin. Results are presented as mean ± s.e.m. from at least three separate experiments; * statistically significant difference ($P < 0.05$) and ** statistically significant difference ($P < 0.01$) in comparison with control conditions (time 0). (C and D) Co-immunoprecipitation analysis showing complexes of beclin1 with ERα (C) and PR (D); nuclear extracts from BME-UV1 cells cultured for 12 h in 0.5% FBS medium with or without addition of E2, or P4 or both (E2+P4) were immunoprecipitated using anti-ERα or anti-PR antibodies, after electrophoresis membrane was blotted with anti-ERα, anti-PR or anti-beclin1 antibodies. In addition, Western blot images present lanes with loaded control samples.

comparison to control (time 0) when BME-UV1 cells were cultured in the presence of sex steroids (Fig. 5B). Increased level of pBcl-2 was noted already between 15 and 30 min of incubation with P4, after 30-min treatment with E2, whereas simultaneous addition of E2 and P4 resulted in sustained elevation of pBcl-2 levels between 30 and 120 min of incubation.

In addition, in recent years there have been a few studies demonstrating the possible interactions between beclin1 and oestrogen receptor, suggesting that complexes formed by these proteins may contribute to autophagy regulation, or may modify oestrogenic functions (John *et al.* 2008, Van Dorst *et al.* 2011). Knowledge about this phenomenon is still scarce and the studies were conducted only on cancer models. Therefore, we decided to use the co-immunoprecipitation (co-IP) method to determine whether beclin1 forms complexes with ER α and PR in bovine MECs. BME-UV1 cells were cultured for 12 h in 0.5% FBS medium with or without the addition of E2, P4 or E2+P4. The incubation period was chosen on the basis of our observation demonstrating that 12 h are sufficient to induce the expression of autophagic genes and their proteins (Figs. 1 and 2). Co-IP was done using Co-Immunoprecipitation (Co-IP) Kit (Thermo Scientific), which enables isolation of native protein complexes from a lysate by covalently coupling antibodies onto an amine-reactive resin. To make sure that specific protein-protein interactions were identified we used relevant controls according to producer's recommendations. The following controls were used: control resin, not activated immunoprecipitation resin (negative control); quenched antibody coupling resin, instead of the antibody against ER or PR quenching buffer, was added to the coupling resin; non-relevant antibody control, we coupled antibody against beta-actin to the antibody coupling resin. In addition, when the co-IP results were analysed by SDS-PAGE we examined the expression of ER α or PR and beclin1 in the protein input (cell lysate prior IP) as well as in the collected flow-through formed after first wash with appropriate wash buffer (Fig. 5C and D).

Results obtained showed that both ER α and PR were able to co-immunoprecipitate with beclin1. A weak ER α -beclin1 complex was formed even in the absence of steroids in the medium. The amount of beclin1 precipitating with ER α further increased when E2 was added, whereas simultaneous addition of E2 and P4 caused a decrease in formation of this complex (Fig. 5C). It is possible that at that time progesterone induced formation of PR-beclin1 complexes, thus both steroid receptors were competing

for the same ligand. Similar results were observed in the case of co-IP of beclin1 and PR (Fig. 5D). PR-beclin1 interaction was noted even without the presence of P4 in the medium, as a band of PR and beclin1 was observed when beclin1 was co-immunoprecipitated with PR in control conditions. Addition of P4 alone or together with E2 caused a further slight increase in the interactions between these proteins (Fig. 5D).

Other steroid-induced signalling pathways connected with autophagy regulation and involved in MECs survival

As mentioned previously, 17 β -oestradiol and progesterone have been shown to induce PI3K/Akt and MAPK/Erk signalling pathways, whose activation is also linked to autophagy regulation. Therefore, we also investigated the level of Akt and ERK1/2 kinases in their total and phosphorylated forms after treatment of BME-UV1 cells with E2, P4 or E2+P4 (Fig. 6). The culture conditions of bovine MECs, as well as Western blot analysis, were performed according to the experimental layout described in the previous section.

A significant increase in the level of active Akt kinase (phosphorylated at Ser473) was noted after 15 min of incubation with both steroids added separately or together, when compared with control conditions (time 0–0.5% FBS) (Fig. 6A). On the other hand, a significant increase in ERK1/2 activation (phosphorylation at Thr202/Tyr204) was observed only when cells were incubated with P4 for 15 min (Fig. 6C). In the case of E2 administered alone or in combination with P4, the observed increase in pERK1/2 level was not significant. It is worth noting that the level of pERK was quite high even in control conditions, i.e. in cells cultured in non-supplemented 0.5% FBS medium, which could be related to the unfavourable conditions caused by FBS deprivation, because the cells were maintained in the 0.5% FBS medium 24 h prior to the addition of sex steroids.

In addition, we examined whether the transient activation of PI3K/Akt and MAPK/Erk signalling pathways by sex steroids significantly influences autophagy induction in BME-UV1 cells. Thus, we designed an experiment in which cells were pre-treated with PI3K inhibitor LY294002 (20 μ M) or MEK1/2 inhibitor PD98059 (50 μ M) for 1 h prior to the addition of E2, P4 or both steroids to the medium. Since in our model autophagy induction, monitored by conversion of LC3, could be clearly observed starting from 12-h incubation

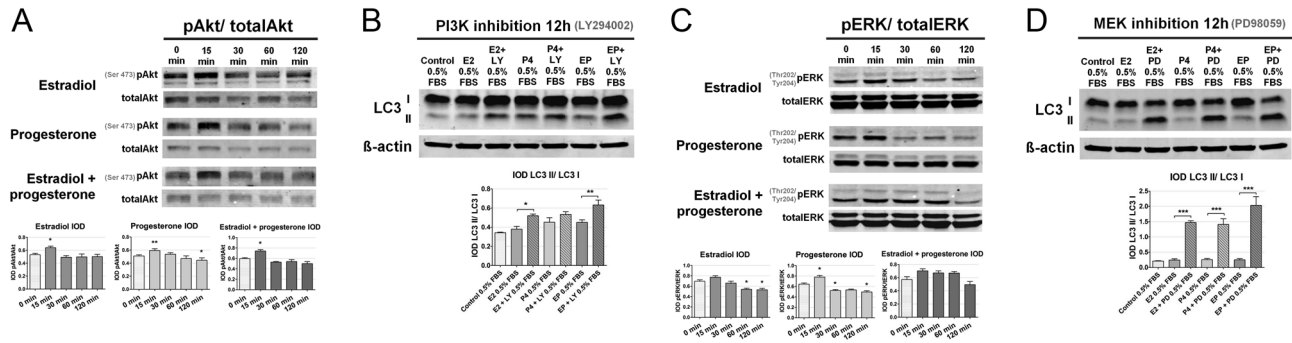


Figure 6

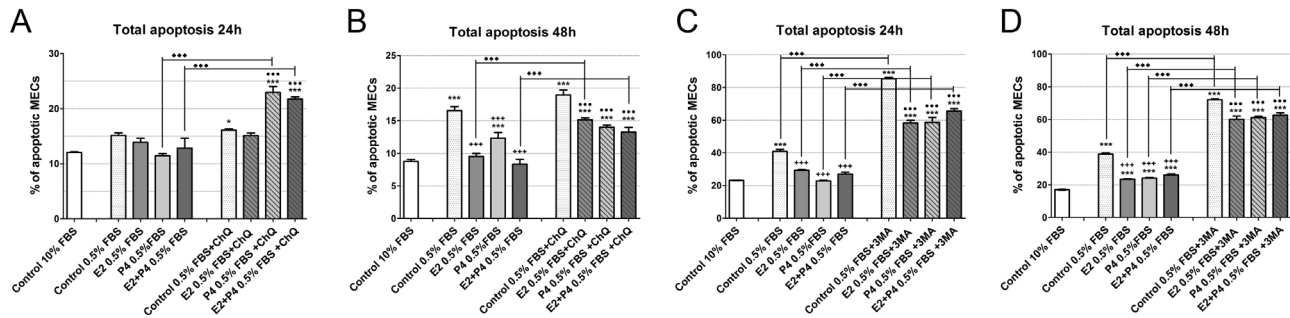
Western blot analysis of the levels (A) of activated/phosphorylated and total Akt kinase, (C) of activated/phosphorylated and total ERK1/2 kinase in BME-UV1 cells cultured in 0.5% FBS medium for 24 h (time 0) or additionally supplemented with oestradiol (E2, 1 nM), progesterone (P4, 5 ng/mL), or both steroids for 15, 30, 60 or 120 min; (B) and (D) Western blot analysis of LC3I and LCII protein expression in cells cultured in 0.5% FBS medium for 12 h in the presence of sex steroids: E2, P4 or both (EP) and additionally supplemented with PI3K inhibitor LY294002 (20 μ M) – B or MEK inhibitor PD98059 (50 μ M) – D for 1 h prior to administration of steroid hormones to the medium; β -actin was used as a loading control in all Western blot analyses; graphs below images A and C show the IOD analysis of each band expressed as the ratio of phosphorylated/total kinases; graphs below images B and D show IOD values for LC3 protein presented as the ratio LC3II/LC3I. Results are presented as mean \pm S.E.M. from three separate experiments, * statistically significant difference ($P < 0.05$) and ** statistically significant difference ($P < 0.01$) in comparison with control conditions (time 0), or between samples treated and not treated with kinase inhibitors.

with experimental factors, MECs were cultured in these conditions for 12 h and the levels of LC3I and LC3II were determined by Western blot (Fig. 6B and D). The results obtained demonstrated that co-treatment of MECs with PI3K inhibitor or MEK1/2 inhibitor and sex steroids administered separately or together (E2+P4) enhanced formation of the lipidated LC3II form, thus increasing autophagy (Fig. 6B and D, respectively). The effectiveness of blocking PI3K and MEK1/2 activity by LY294002 and PD98059, respectively, was investigated in short-term experiment, in which BME-UV1 cells were first cultured in 0.5% FBS medium for 24 h, and then treated for 1 h with kinases inhibitors prior to the addition of E2, P4 or E2+P4 for 15 min. Western blot analysis confirmed significant reduction in the levels of pAkt (Ser 473) and pERK1/2 (Thr202/Tyr204) in samples treated with 20 μ M LY294002 and 50 μ M PD98059, respectively (data not shown).

Rapid phosphorylation of Akt in the presence of E2 and P4 may be connected with the survival effect of these steroid hormones, other than autophagy induction. In fact, phosphorylation of Akt is connected with mTORC1 activation, and the resulting inhibition of autophagy (He & Kliensky 2009). In order to verify the survival effect of E2 and P4 in bovine MECs cultured in conditions mimicking the state of *in vitro* mammary involution, we determined the number of apoptotic cells after 24- and 48-h culture in 0.5% FBS medium with or without addition of sex steroids using the Annexin V/PI assay with flow cytometric analysis. Furthermore, a similar experiment was performed in the presence of two types of autophagy

inhibitors: 3-methyladenine (3MA, 5 mM), which inhibits class III PI3K therefore blocking autophagosomes formation, and chloroquine (ChQ, 10 μ M), which causes alkalization of lysosomal content, preventing fusion of autophagosomes with lysosomes and blocking autophagic progress. ChQ or 3MA was added to 0.5% FBS medium 1 h prior to administration of sex steroids, followed by 24- or 48-h culture. In this experiment growth conditions (10% FBS medium) as well as basic experimental medium (0.5% FBS) were used as control. Results are presented as the percentage of apoptotic cells (Annexin V+/PI- and Annexin V+/PI+) in control and experimental conditions in relation to the total number of counted cells in a sample (Fig. 7).

In general, the number of apoptotic cells differed between the experiments in which ChQ and 3MA were used as autophagy inhibitors. These discrepancies resulted from differences in solvents used during preparation of the inhibitors. Chloroquine is water soluble, thus no additional vehicle was added to the culture medium when the effect of ChQ and sex steroids was investigated. On the other hand, 3MA was initially dissolved in dimethylformamide (DMF), and next dissolved in the culture medium to the final concentration of 5 mM. Knowing that DMF shows cytotoxic properties, and that its concentration in the medium with 3MA reached 1% (v/v), the same amount of pure vehicle was also added to all culture conditions when the effect of 3MA was investigated. Therefore, the results obtained were analysed in two sets separately: (1) the protective effect

**Figure 7**

Evaluation of apoptosis by Annexin V/PI double staining in BME-UV1 cells. Cells were cultured for 24 and 48 h in standard growth medium with 10% FBS or experimental medium (0.5% FBS) with/without addition of E2 (1 nM), P4 (5 ng/mL) or E2+P4. In addition, cells were pre-treated with autophagy inhibitors: (A, B) chloroquine (ChQ, 10 μ M) or (C, D) 3-methyladenine (3MA, 5 mM) for 1 h prior to addition of sex steroids followed by 24- or 48-h culture. Bar graphs show the percentage of apoptotic cells (sum of Annexin V+/PI- and Annexin V+/PI+ cells) in control and experimental conditions based on flow cytometric analysis. The data were collected for three independent experiments. All values are expressed as mean \pm s.d. The statistical significance was evaluated using one-way ANOVA with the Tukey's *post hoc* test; * significantly different from 10% FBS ($*P < 0.05$), *** $P < 0.001$; + significantly different from 0.5% FBS (*** $P < 0.001$); ● significantly different from 0.5% FBS+inhibitor (●●● $P < 0.001$); ◆ significantly different experimental conditions with and without addition of inhibitors (◆◆◆ $P < 0.001$).

of sex steroids against apoptosis was investigated in cells grown in 0.5% FBS medium with or without ChQ and sex steroids (Fig. 7A and B); (2) the protective effect of sex steroids against apoptosis was investigated in cells grown in 0.5% FBS medium containing DMF, with or without 3MA and steroid hormones (Fig. 7C and D).

When cells were grown in basic experimental medium containing 0.5% FBS, a significant increase in the number of apoptotic cells was noted after 48-h incubation when compared with 10% FBS medium (Fig. 7B). Addition of chloroquine caused a significant increase in apoptotic cell number already after 24 h and a further increase was noted at 48-h culture (Fig. 7B). Addition of E2, P4 or both steroids together significantly lowered the percentage of apoptotic cells after 48 h when compared with 0.5% FBS medium (Fig. 7B). After 48 h, the protective effect of sex steroids was also observed when MECs were pre-treated with ChQ; however, the number of apoptotic cells was significantly higher in ChQ+E2 and ChQ+E2+P4 samples in comparison to adequate conditions without ChQ (Fig. 7B). On the other hand, during the first 24-h treatment, inhibition of autophagy with chloroquine abolished the protective effect of sex steroids in MECs, because incubation of cells in medium containing ChQ and P4, or ChQ with E2+P4 resulted in significantly increased percentage of apoptotic cells when compared with other experimental conditions (10% FBS, 0.5% FBS, as well as adequate condition without ChQ). This observation supports the hypothesis that during unfavourable conditions of temporal malnutrition, progesterone exerts prosurvival effect in bovine MECs largely by its ability to induce autophagy (Fig. 7A).

Analysing the results of experiment in which 3MA was used as autophagy inhibitor, we noted significantly pronounced differences at both investigated time points (24 and 48 h) between samples treated or not treated with 3MA (Fig. 7C and D). In general, addition of vehicle (DMF) to the culture medium in a concentration of 1% (v/v) caused a twofold increase in the number of apoptotic cells even when MECs were grown in growth medium (10% FBS) (Fig. 7C, D vs 7A, B). This experiment also proved that lowering the content of FBS to 0.5% (v/v) augmented the number of apoptotic cells in culture, and the results were significant after 24 and 48 h when compared to 10% FBS conditions (Fig. 7C and D). Addition of E2, P4 or E2+P4 decreased the percentage of apoptotic cells to the values similar to 10% FBS conditions (Fig. 7C and D). When bovine MECs were grown in the presence of 3MA, a highly significant increase in the number of apoptotic cells was noted at 24- and 48-h culture in comparison to basic experimental medium (0.5% FBS) as well as to 10% FBS medium. Addition of sex steroids significantly diminished the effect of 3MA in comparison to 0.5% FBS+3MA condition (Fig. 7C and D). Nevertheless, the registered percentage of apoptotic cells remained significantly higher in comparison to adequate conditions not supplemented with 3MA (the values were over 2 times increased). The results obtained partially support our previous observations pointing at the role of 17 β -oestradiol and progesterone in the induction of autophagy. Blocking the first steps of autophagic pathway with 3MA substantially diminished to a high extent the protective effect of sex steroids in bovine MECs. In addition, this weaker protection of bovine MECs could

have resulted from the fact that 3MA may also inhibit class I PI3 kinase, which is known to stimulate the prosurvival pathway mediated by Akt kinase.

Discussion

Autophagy is a powerful promoter of cellular homeostasis maintenance, providing the balance between biosynthesis and degradation of cellular components. Beyond its fundamental role in cell growth and development, the primary role of autophagy is to mediate protective function under a variety of extracellular and intracellular stress conditions (Chen & Klionsky 2011). Induction of autophagy in MECs during involution of bovine mammary gland was shown for the first time by Zarzynska *et al.* (2007). The following studies on mouse and bovine mammary glands confirmed autophagy induction in the early stages of involution, demonstrating increased expression of autophagic marker LC3II in the mammary epithelium of mice after forced pup-weaning, and cows during the early dry period (Teplova *et al.* 2013, Wohlgenuth *et al.* 2016). It seems, however, that the role of autophagy in the mammary glands of rodents and ruminants may be different. In majority of species, in which lactation cycles are clearly separated, most secretory alveolar MECs undergo apoptosis during involution. Teplova and coworkers (Teplova *et al.* 2013) showed that at that time some of the epithelial cells remaining within the alveoli begin reabsorbing the residual milk, and undergo changes in their phenotype from secretory to phagocytic, enabling them to actively eliminate the apoptotic cells from the lumens of the alveoli by efferocytosis (Teplova *et al.* 2013). The authors suggested that in murine mammary gland autophagic proteins may have a function of efferocytosis mediators (Teplova *et al.* 2013). On the other hand, in ruminants, especially dairy cows, consecutive lactation cycles overlap, since these animals are typically pregnant when the involution is induced by termination of milking. Thus, during the dry period, the combined effects of various factors, including enhanced competition of intensively developing foetus and mother organism for nutritional and bioactive compounds, decreased level of lactogenic hormones, increased expression of auto/paracrine apoptogenic peptides (IGFBPs, TGF- β 1) and high concentrations of sex steroids may lead to autophagy induction in bovine mammary epithelium (Motyl *et al.* 2007). High level of progesterone as well as increasing concentration of 17 β -oestradiol observed during the dry period induces regeneration of the mammary secretory

epithelium prior to the next lactation, stimulating MECs proliferation. Our previous and present studies suggest that these steroid hormones may also exert protective effect on bovine MECs exposed to stress connected with nutrient limitation by enhancement of autophagy induction (Sobolewska *et al.* 2009). We used a very simplified model of *in vitro* mammary involution of BME-UV1 cells, based on twenty-fold reduction of FBS concentration in the culture medium. This model intends to mimic the stress connected with reduced levels of nutritional and bioactive compounds in the intramammary environment, which is caused by increasing needs of the developing foetus that take priority over the cow's needs for body tissue maintenance. Our previous study has shown that FBS deficiency causes a progressive increase in the number of BME-UV1 cells with elevated content of caspase-3 and LC3II, reaching a peak after 48-h culture, which proves that in this *in vitro* model both apoptosis and autophagy are induced in bovine MECs (Sobolewska *et al.* 2009). These findings were also confirmed in the present study, as we detected significantly increased number of apoptotic cells after 48-h incubation of BME-UV1 cells in FBS-deficient conditions. In parallel, we detected significantly increased expression of autophagy-related genes: *BECN1*, *ATG5* and *LC3B* between 2- and 24-h culture in 0.5% FBS medium and increased level of LC3II protein between 6- and 48-h culture. These results indicate that in serum-deficient conditions autophagy is induced prior to apoptosis activation.

As mentioned above, our previous study presented the first evidence of autophagy activation by 17 β -oestradiol and progesterone in bovine MECs (Sobolewska *et al.* 2009). We further explored this phenomenon and investigated the changes in expression of autophagic genes: *BECN1*, *ATG5*, *ATG3* and *LC3B* in BME-UV1 cells cultured in 0.5% FBS medium supplemented with E2, P4 or both steroids simultaneously. We have chosen these genes based on the fact that their protein products play an important role in different stages of autophagosome formation: beclin1 is involved in nucleation and assembly of the initial phagophore membrane, being a part of a large complex of the class III phosphatidylinositol 3-kinase (PtdInsK); ATG5 belongs to the first conjugation system (ATG5-ATG12-ATG16) controlling the early steps of phagophore membrane elongation; ATG3 has the activity of the E2 enzyme of the ubiquitination system and is involved in the conjugation of cleaved LC3 with phosphatidylethanolamine (PE); LC3-I protein undergoes C-terminal cleavage and conjugation with PE,

which allows for its permanent binding to both sides of elongating phagophore membrane, and controlling the size of the autophagosome (He & Klionsky 2009). The results obtained demonstrated that E2 and P4 were able to augment the expression of *BECN1*, *ATG5* and *LC3B*, above the levels detected after serum deprivation, which was also reflected by elevated expression of encoded proteins in cells cultured in 0.5% medium supplemented with P4 or E2+P4. In addition, we observed increased number of GFP-LC3 puncta in bovine MECs cultured in 0.5% FBS supplemented with sex steroids. However, these results were not statistically significant most probably due to observed high variation in the number of GFP-LC3 per cell within the analysed samples, which caused high s.d. Among the investigated genes, only *ATG3* expression did not change significantly under the influence of FBS deficiency and steroid treatment. Since *ATG3* is an E2-like enzyme required for lipid conjugation of LC3 it is possible that the expression of *ATG3* on mRNA and protein level is stable in cells, whereas the enzymatic activity is regulated, which was not investigated in the present study. Stimulation of other *ATGs* expression by both steroids was further confirmed by blocking the effect of hormones with oestrogen or progesterone receptors antagonists, 4-OHT and RU-486, respectively. Addition of RU-486 to the 0.5% FBS medium 1 h prior to supplementation with P4 caused a significant decrease in the mRNA and protein level of *ATG5* and *LC3* after 24-h treatment. In the case of 4-OHT the expression of *BECN1*, *ATG5* and *LC3B* genes was significantly reduced after 24-h culture in E2+4-OHT medium, but changes in protein expression were significant only in reference to total LC3. The experiment with steroid receptor inhibitors were also tested at earlier time points of cell culture, however the inhibitory effect of 4-OHT as well as RU-486 was not observed at shorter exposure. In addition, when we used the technique of siRNA-mediated ER α gene knockdown, resulting in a significant decrease in the level of ER α receptor, we also observed a significant reduction of beclin1, *ATG5* and *LC3* protein expression. The data obtained indicate that steroid-induced regulation of autophagy is mediated by their receptors ER α and PR. So far the mechanisms of autophagy regulation by sex steroids have been poorly explored and the available data most often concern pathological models of mammary cancer cells. Recent studies on cells and tissues of steroid hormone-responsive organs, such as uterus, the ovary, bone and mammary glands, describe results that are contradictory to each other. For example, E2 and P4 caused a reduction in autophagy in uterine epithelial cells (Choi *et al.* 2014), and

it was demonstrated that murine uterus shows the highest levels of autophagic activation manifested by increased expression of LC3II and *ATG5* proteins under hormone deprivation conditions after ovariectomy (Choi *et al.* 2014, Park *et al.* 2016). In contrast, a recent study by Yang and coworkers (Yang *et al.* 2013a,b) demonstrated that oestradiol enhanced autophagy in osteoblasts in serum-deprived conditions, causing increased expression of beclin1, LC3 and ULK1 after 48-h starvation, which is in agreement with our present findings in bovine MECs. It seems that regulation of autophagy by sex steroids exhibits a tissue type or cell type specific patterns, most probably because of the differences in hormone responsiveness and physiological or pathophysiological context.

The present study demonstrated that 17 β -oestradiol exerts its stimulatory effect on *ATGs* through a classic genomic pathway mediated by activated ER α and its transcription activity on the promoters of chosen *ATGs*. Computer analysis showed potential oestrogen-responsive elements (ERE) on the promoters of *ATG5* and *LC3B* genes, and EMSA revealed increased formation of specific protein–DNA complex with DNA probes containing ERE sites of *ATG5* and *LC3* promoters, which was increased upon treatment with E2. In parallel, Western blot analysis showed elevated expression of ER α in nuclear extracts from BME-UV1 cells treated with E2. In the case of progesterone indirect pathways have to be involved in autophagy induction, since sequence analysis did not confirm the presence of any canonical progesterone-responsive element (PRE) on the promoter regions of *BECN1*, *ATG5* or *LC3B* genes. Nevertheless, a significant increase of *BECN1*, *ATG5* and *LC3B* expression was noted between 2- and 24-h incubation of bovine MECs in 0.5% FBS medium supplemented with P4, suggesting that the transcriptional effect of P4 on *ATGs* expression is mediated through the cooperation of PR with other DNA-binding proteins. Our preliminary studies revealed that an indirect genomic effect of progesterone may result from interactions of PR with transcription factor AP1, since multiple AP1-binding motifs were detected on the promoters of investigated *ATGs*, and co-immunoprecipitation assay proved that PR is able to associate with c-Jun subunit of AP1 (data not shown). Further experiments using EMSA and chromatin immunoprecipitation assays are needed to confirm this hypothesis and gain further insight into the mechanism involved in *ATGs* transactivation induced by P4.

Increasing evidence suggest that there are several distinct pathways by which steroid hormones and their receptors regulate cellular processes. As mentioned previously, ligand-bound steroid receptors can bind directly

to hormone-responsive elements (HRE) on the promoters of target genes or can interact with other transcription factor complexes like Fos/Jun (AP-1-responsive elements) and influence transcription of genes whose promoters do not harbour HREs (Heldring *et al.* 2007). In addition, sex steroids exert rapid effects by activation of other cellular signalling pathways mediated by various kinases, and this function of steroids may be either mediated by classical steroid receptors, or by a distinct membrane-associated type of receptors (Levin 2001, Salazar *et al.* 2016). Recently, several studies on different cell types have demonstrated that E2 and P4 are involved in activation of signalling pathways regulating autophagy. E2 was shown to induce autophagy via activation of ERK1/2 kinase in osteoblasts, human neuronal cell line SH-SY5Y and papillary thyroid cancer cells (Yang *et al.* 2013a,b, Fan *et al.* 2015, Li *et al.* 2015), whereas P4 induced autophagy by indirect downregulation of PI3K/Akt pathway mediated by increased expression of PTEN (De Amicis *et al.* 2014). We investigated whether these sex steroids induce changes in the activity of three kinases: AMPK, Akt and ERK1/2, which mediate signal transduction cascades of three distinct pathways regulating autophagy. The results obtained did not confirm significant stimulation of AMPK activity by E2 and P4 administered separately or together to BME-UV1 cells, whereas phosphorylation of ERK1/2 was transiently upregulated only in the presence of P4, although a prolonged but not significant increase of pERK1/2 levels was also noted when P4 was added with E2. It is worth noting, however, that in our experiment AMPK and ERK1/2 also showed high activity prior to addition of steroids to the medium. This effect could have resulted from exposition of MECs to stressful conditions related to 24-h incubation in FBS-deficient medium prior to addition of sex steroids. Signalling pathways mediated by these kinases are upregulated during nutrient deprivation and reduced energy levels, inducing autophagy as survival process (He & Klionsky 2009). Among investigated kinases only Akt showed transient but significant increase in activation 15 min after addition of E2, P4 or E2+P4 to the 0.5% FBS medium. After this time the levels of phosphorylated Akt gradually decreased. This rapid, steroid-mediated activation of Akt indicates the non-genomic effect of both hormones, which is rather connected with other survival mechanisms induced by E2 and P4 in bovine MECs, than with autophagy regulation. PI3K/Akt signalling is one of the most critical pathways that regulate cell survival and provide a signal to withstand apoptotic stimuli (Song *et al.* 2005). Rathmell and coworkers (Rathmell *et al.* 2003) have demonstrated that Akt can maintain long-

term cell survival even in the absence of growth factors by preventing pro-apoptotic Bax activation. Activation of PI3K/Akt pathway, subsequently leading to mTOR1 complex activation, is also known to inhibit autophagy in various cells and tissues (Kadowaki *et al.* 2006, Ballou & Lin 2008). However, in our study long-term (24, 48 h) culture of BME-UV1 cells in 0.5% FBS medium containing E2, P4 or both steroids clearly enhanced autophagy induction in bovine MECs. Furthermore, previous study showed that during the 48-h exposure of BME-UV1 cells to E2 and P4 the level of phosphorylated mTOR was also significantly diminished (Sobolewska *et al.* 2009). Thus, activation of Akt signalling by E2 and P4 under nutrient deprivation may constitute another protective mechanism which supports mammary cells survival, while inhibiting apoptotic signalling. Both protective mechanisms may be induced in parallel. In fact, we observed that inhibition of PI3K/Akt pathway by LY294002 (20 μ M), as well as inhibition of MEK/ERK pathway by PD98059 (50 μ M) both resulted in augmented level of LC3II indicating that when these pathways are blocked in bovine MECs autophagy is being enhanced. Interestingly, both inhibitors have been shown to block autophagy in various experimental models (Liu *et al.* 2012, Yang *et al.* 2013a,b). In our study administration of LY294002 prior to addition of E2 or E2+P4 most probably caused inhibition of class I PI3K signalling pathway, blocking activation of mTORC1, the main negative regulator of autophagy. Similar observations were described in the study by Xing and coworkers (Xing *et al.* 2008). Surprisingly, inhibition of MEK pathway, followed by co-treatment with E2, P4 or E2+P4 also significantly augmented LC3I conversion to LC3II when compared with samples treated only with steroid hormones. Although ERK signalling pathway has been connected with increased formation of autophagic vacuoles, some recent studies demonstrated that inhibition of this pathway by PD98059 may result in increased level of LC3II (Wang *et al.* 2016). Inactivation of ERK1/2 and increased autophagy induction may be caused by stress connected with blocking cell cycle progression, as ERK1/2 is connected with assembly of the cyclin E/cdk2 complex (Wang *et al.* 2016). In the present study, 12-h incubation of bovine MECs in the presence of PD98059 could have exerted a similar effect, but further investigation is needed to verify this hypothesis.

To further test the hypothesis of different survival mechanisms induced by sex steroids in bovine MECs undergoing temporal malnutrition, which in our research model was caused by 20-fold reduction of FBS content, we used flow cytometric analysis to determine the number of

apoptotic cells in these conditions. BME-UV1 cells were cultured in 0.5% FBS medium with or without addition of sex steroid. In parallel, the same experiment was conducted in the presence of autophagy inhibitors: 3MA or chloroquine. The results obtained demonstrated that blocking autophagy significantly reduced cell survival, by increasing the number of apoptotic cells when MECs were cultured in stressful conditions (0.5% FBS), especially after 48-h culture. This underlines the important role of autophagy as a protective mechanism in bovine MECs. However, in the presence of E2 and P4 the proapoptotic effect of 3MA and chloroquine was partially reversed, suggesting that both sex steroids have the ability to induce other prosurvival signalling pathways. Activation of Akt signalling by E2 and P4 under nutrient deprivation may constitute one of the protective mechanism which supports mammary cells survival, while inhibiting apoptotic signalling.

In addition to the kinases, we also determined the level of phosphorylated Bcl-2, since Bcl-2 phosphorylation is necessary to release beclin1 from Bcl-2/beclin1 complex, and this step is required for autophagy induction (Pattingre *et al.* 2005, Wei *et al.* 2008). Interestingly, we observed a rapid increase in the level of phosphorylated Bcl-2 in bovine MECs treated with P4 (after 15 min), E2 (after 30 min) or both steroids simultaneously (between 30 and 120 min). These findings suggest that 17 β -oestradiol and progesterone not only regulate autophagy via their genomic actions, but may also accelerate autophagy induction by non-genomic pathways. Further studies are necessary to evaluate whether this effect is mediated by membrane-associated steroid receptors, or other independent mechanisms. However, these observations may also explain the accelerated formation of autophagosome-associated LC3II form in BME-UV1 cells treated with E2, P4 or E2+P4, which was already detected after 2-h culture in 0.5% FBS medium supplemented with steroids, whereas in FBS-deficient medium LC3II formation was induced at later time points (after 6 h).

Finally, in the present study we demonstrated that beclin1 is able to form complexes with both ER α and PR, which may constitute another link of steroid signalling pathways with autophagy machinery. Beclin1 is a well-established regulator of autophagic pathway, which is able to interact with a number of binding partners due to its characteristic structure. The protein consists of three structural domains: N-terminal BH3 domain, through which beclin1 interacts with Bcl-2 family members, a coiled-coiled domain (CCD) which allows beclin1 to bind with i.e. UVRAG, and the C-terminal evolutionary

conserved domain (ECD) required for the regulation of autophagy by beclin1, through its interaction with PI3KC3/Vps34 (Sahni *et al.* 2014). Co-immunoprecipitation of beclin1 with ER α was previously described by John and coworkers (John *et al.* 2008) in MCF-7 breast cancer cell line. The authors reported that administration of E2 to the culture medium caused a movement of beclin1 and ER α towards each other and the formation of beclin1/ER α complexes was shown to increase in the presence of ER α ligands: twofold after addition of E2 and 4-hydroxytamoxifen, and sixfold after raloxifene. Next, Van Dorst and coworkers (Van Dorst *et al.* 2011) confirmed beclin1 as a potential binding partner for ER using the T7 Select TM Human Breast cDNA phage library to screen for cellular targets of E2. In the case of PR, to our knowledge this is the first report showing the formation of PR/beclin1 complexes. The role of these complexes in autophagy regulation in bovine MECs is unknown, and requires further studies. In the context of breast cancer cells, it is hypothesized that binding of beclin1 to steroid receptor results in sequestration of ER α away from the promoter site of target genes inhibiting the oestrogenic cell signalling and growth response, thus contributing to the development of anti-oestrogen resistance (John *et al.* 2008). In light of the findings obtained in the present study, it seems that the role of steroid receptor/beclin1 complexes may differ in normal MECs, and may actually contribute to enhanced autophagy induction. Further investigation will be needed to determine the function of such complexes in the cascade of cellular events which take place in epithelial cells during development and remodelling of the mammary gland.

Conclusions

The present study demonstrated that 17 β -oestradiol and progesterone enhance autophagy induction in bovine mammary epithelial cells by stimulating the expression of autophagy-related genes (*BECN1*, *ATG5*, *LC3B*), as well as the levels of corresponding autophagic proteins. Our results indicate that E2 may be involved in regulation of *ATG5* and *LC3B* expression via ligand-activated oestrogen receptors that bind to promoters of these genes directly, whereas progesterone exerts its actions by indirect stimulation of *ATGs* expression. Furthermore both steroids may stimulate autophagy induction by regulating the phosphorylation status of Bcl-2, thus enabling beclin1 dissociation from the complex with Bcl-2 in bovine MECs, although this indirect, non-genomic effect may be more relevant in the case of progesterone. In addition,

it is possible that direct interactions of ER α and PR with beclin1 may contribute to induction of autophagy in bovine MECs. These results indicate that during involution of bovine mammary gland the role of E2 and P4 in supporting regeneration of the secretory tissue and preventing the extensive death of mammary epithelial cells are at least partially connected with the ability of both sex steroids to stimulate autophagy induction in MECs.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Authors' contribution statement

K Zielniok performed the experiments, analysed the data and co-wrote the manuscript. A Sobolewska prepared and isolated the pEGFP-LC3 plasmid. M Gajewska designed the study, was involved in analysis and interpretation of the results, and co-wrote the manuscript.

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