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APELIN DECREASED PLACENTAL HORMONE SECRETION BY HUMAN TROPHOBLAST BEWO CELLS *VIA* APELIN RECEPTOR, PROTEIN KINASE A AND EXTRACELLULAR SIGNAL-REGULATED KINASES 1/2 ACTIVATION

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Apelin was thought to be an adipocyte-specific hormone, but recent studies have indicated a link between apelin and placenta function *e.g.* cell proliferation. The aim of the study was investigating dose- and time-dependent effect of apelin on hormone secretion including steroids: progesterone (P4) and estradiol (E2) and proteins: chorionic gonadotropin (hCG), human placental lactogen (hPL), placental growth factor (PLGF), as well as protein expression of steroid enzymes (3βHSD, CYP19) and protein hormones (hCG, hPL and PLGF) in placental cells. Syncytiotrophoblast BeWo cells, as human trophoblast models, were treated for 24, 48, and 72 hours with the human recombinant apelin at doses 0.02, 0.2, 2.0, 20 and 200 ng/ml followed by culture medium. Concentrations of the above hormones were studied by ELISA kits. Furthermore, protein expression of steroid enzymes and protein hormones were measured using Western blot. Our results showed that apelin significantly decreased both steroid and protein hormones by inhibiting steroid enzymes or protein hormone expression. Moreover, we demonstrated that apelin at dose 2.0 ng/ml increased phosphorylation of protein kinase A (PKA) from 1 to 60 min of BeWo cell incubation. Inhibitory effect of apelin on P4, E2 and PLGF secretion were abolished when BeWo cells were cultured in the presence of ML221, an apelin receptor antagonist, PD98059, an extracellular signal-regulated kinases (ERK1/2) antagonist and KT5720, a PKA antagonist. In turn, secretion of hCG and hPL occurs only in the presence of ML221 and PD98059. In conclusion, our results indicate that apelin can be considered as a gestational hormone implied in the endocrine function of the human placenta, with an important role in controlling the production of steroid and protein hormones in placental BeWo cells.

Key words: *apelin, apelin receptor, progesterone, estradiol, chorionic gonadotropin, human placental lactogen, placental growth factor, protein kinase A, trophoblast, placenta*

INTRODUCTION

The major role of the placenta is to establish a crosstalk between maternal and fetal circulations. Additionally, during pregnancy, the placenta produces a large number of hormones like steroids: progesterone (P4), estradiol (E2) and proteins: chorionic gonadotropin (hCG), human placental lactogen (hPL), placental growth factor (PLGF), and cytokines that are secreted into the foetal or maternal circulation or both, making a unique endocrine function for this short-lived organ. Suitable concentration of steroid and protein hormones is very important for the establishment and maintenance of pregnancy. Several cytokines and growth factors, such as leptin, are known to influence trophoblast migration, proliferation, invasion and also endocrine function (1). For example, studies of Coya *et al*., (2) demonstrated that leptin led to a dose-dependent decrease in E2 release by human term placental cells in culture, without an effect on hCG and P4.

Apelin was isolated from bovine stomach extracts as an endogenous ligand of the previously identified apelin receptor - APJ (3). All known isoforms of apelin are derivatives of the 77 amino acid prepropeptide which, as a result of post-translational modifications, is transformed into endogenous: apelin-36, apelin-17, apelin-16, apelin-13 or exogenous apelin-12 (4). The individual forms are characterized by a difference in the length of the polypeptide chain. The most commonly isoform of apelin used in laboratory tests is apelin-36, present in the lungs, uterus and testes, while the most active isoform is apelin-13, located in the mammary gland and hypothalamus (5). Previous studies have confirmed the apelin action on numerous diseases associated with heart failure (6), obesity or type II diabetes (7). Moreover, the contribution of apelin was also demonstrated in the course of neoangiogenesis (8), apoptosis (9), cell proliferation (10) and action on gastric and pancreatic enzymes activity (11). The impaired apelin concentration associated with certain complications during pregnancy, such as preeclampsia (PE); where the concentration of apelin in the plasma during delivery was lower in women suffering from this disorder compared to the control group (12). In turn, intrauterine hypotrophy (IUGR) showed a decreased level of apelin in the plasma and placenta (13). It was observed that apelin exerts an inhibitory effect on the contractility of the human uterus (14). All of these results

suggested the physiological and pathophysiological significance of apelin in pregnancy and during maintenance of the fetoplacental unit physiology. Our previous study documented that apelin and the APJ at gene and protein levels were expressed in both syncytiotrophoblast (BeWo) and cytotrophoblast (JEG-3) cell lines, suggesting a direct activity of apelin to modulate placenta functions like proliferation, cell cycles and the activation of various signalling pathways like extracellular signal-regulated kinases (ERK1/2), phosphatidylinositol 3'-kinase/Akt (Akt), signal transducer and activator of transcription 3 (Stat3) and 5'-monophosphate-activated protein kinase (AMPKα) (15). However, to date, there is no data addressing the direct impact of apelin on the trophoblast endocrine functions.

Thus, the aim of the present study was investigating the role of apelin on the main placental hormones concentration like steroids: P4 and E2 or proteins: hCG, hPL and PLGF as well as protein expression of steroid enzymes 3βHSD, CYP19 and protein hormones hCG, hPL and PLGF. We used human choriocarcinoma BeWo cell line, as a valuable cell model for the study of the cellular, molecular and endocrine aspects of human trophoblasts (16). As for the molecular mechanism by which apelin regulates hormone secretion, we studied the role of APJ receptor as well ERK1/2 and protein kinase A (PKA). Both, ERK1/2 and PKA are signalling molecules involved in most types of cell growth, survival/apoptosis and also endocrinology (17-20) and in major molecular mechanisms of apelin in other cell types (21-24).

MATERIAL AND METHODS

Reagents

DMEM/F12 medium, phosphate buffered saline (PBS), and trypsin were purchased from ThermoFisher Scientific (Waltham, MA, USA). Fetal bovine serum (FBS, heat inactivated) was purchased from Biowest (France). Insulin, glycerol, Tween 20, PD098059, and human recombinant apelin-13 (cat. no. A6469) and Laemmli buffer (cat. no. 38733) were obtained from Sigma Aldrich (Merck KGaA, Darmstadt, Germany). Tris base, NaCl, sodium dodecyl sulfate (SDS), and bovine serum albumin (BSA) were purchased from Bioshop (Canada Inc., Burlington). ML221 and KT5720 were obtained from TOCRIS Bioscience (Bristol, UK). The WesternBrightTM Sirius kit (cat. no. K-12043-D20) was obtained from Advansta (Menlo Park, USA). 4 – 20% gels (cat. no 456-1093) and PVDF membranes (cat. no. 1704156) were obtained from Bio-Rad Laboratories (Hercules, CA, USA).

Trophoblast cell culture

Human choriocarcinoma BeWo cell line (cat. CCL-98) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). BeWo cells were routinely cultured in DMEM/F12 medium without phenol red, and supplemented with 0.01 mg/ml insulin and 10% heat-inactivated FBS. The culture medium from cell line was changed every 2 days. BeWo cells were grown in 75-cm^2 tissue culture flasks in a 37°C incubator with a humidified mixture of 5% CO_2 and 95% air. All experiments came from independent replicates carried out on cells from passage 221 – 224.

Experimental procedure

Experiment 1

Time- and dose-dependent effect of human recombinant apelin on hormone secretion and protein expression of 3βHSD, CYP19, hPL, hCG, PLGF in trophoblast cells. BeWo cells were cultured in 96-well plate (4×10^3 cells/well) in DMEM/F12 with 10% FBS. After 24 hours, medium was replaced by DMEM/F12 with 1% FBS, and cells were treated with apelin at doses 0.02, 0.2, 2.0, 20 and 200 ng/ml. The concentrations of apelin were chosen based on our preliminary research and a previous study (15, 23). After 24, 48, 72 hours of treatment with apelin, culture medium was collected and stored at –20°C for the analysis of hormone secretion while cells were washed in PBS, then boiled in Laemmli buffer for 4 min and stored at 20°C to analyze the protein expression of enzymes 3βHSD, CYP19 or protein hormones hPL, hCG and PLGF.

Experiment 2

The effect of human recombinant apelin on PKA activation in BeWo cells. Our previous study documented that apelin increased the phosphorylation of ERK1/2 kinase in trophoblast cells (15) but we never studied the effect of apelin on PKA kinase activation in the placenta cells. So, BeWo cells (4×10^3 cells/per well of 96-well plate) were cultured in DMEM/F12 with 10% FBS. After 24 hours, medium was replaced by DMEM/F12 with 1% FBS, and cells were treated with apelin at dose 2.0 ng/ml (dose chosen based on experiment 1) for different incubation times of 1, 5, 15, 30, 45 and 60 minutes Study of Maldonado-Mercado *et al*. (25) documented that basal protein expression of PKA in isolated mitochondria from BeWo cells don't changes significantly during 0 – 20 minutes of incubation, so that in our study we performed effect of apelin on PKA phosphorylation during different time of incubation. Similar protocol we used in ovarian cells in the case of other metabolic hormone like ghrelin (26, 27), apelin (23), resistin (28) or vaspin (29). Then, cells were washed in PBS, boiled in Laemmli buffer for 4 min and stored at –20°C for measurment PKA protein expression.

Experiment 3

To test involvement of APJ receptor and signaling pathway of kinases in apelin action on trophoblast endocrine function, BeWo cells were pre-treated for 1 hour with selective inhibitors of APJ inhibitor (ML221 at 10 μM) or ERK 1/2 and PKA kinases (PD98059 at 10 μM and KT5720 at 50 ng/ml, respectively) after which apelin (2.0 ng/ml) was added. The concentrations of inhibitors were chosen based on our preliminary research and previous studies (15, 23). After 48 hours of cell incubation, culture medium was collected and stored at –20°C for the analysis of steroids (P4 and E2) and protein (hCG, hPL and PLGF) hormone secretion.

Western blot method

Western blotting and quantification were performed as a standard procedure described previously (15). In brief, proteins (80 μg) were separated by 4 – 12% Mini-Protean TGX System Precast Protein Gels and transferred to Trans-Blot Turbo Mini PVDF Transfer Packs. The membranes were blocked for 1 hour in 0.02M Tris-buffered saline containing 5% BSA or 5% milk and 0.1% Tween 20. Then, membranes were incubated overnight at 4°C with specific primary antibodies (*Table 1*), washed in TBST (Tris-buffered saline containing 0.1% Tween 20) and incubated for 1 hour at room temperature with a horseradish peroxidase-conjugated secondary antibody (*Table 1*). β-actin was used as a loading control. Signals were detected by chemiluminescence using WesternBrightTM Sirius, and visualized using a ChemiDoc-It Imaging System. All bands visualized by chemiluminescence were quantified using ImageJ analysis software (US National Institutes of Health, Bethesda, MD, USA).

ELISA kits

Steroids P4, E2 and proteins hormone hCG, hPL and PLGF levels in the culture medium were determined using commercially available ELISA kits (DRG Instruments GmbH, Germany) (*Table 2*) following manufacturer's instructions. Samples were run in triplicate within the same assay.

Statistical analysis

Statistical data are presented as means ± standard error of the mean (SEM) of four independent experiments performed in threefold. Distribution of normality was checked using a Shapiro-Wilk test. Statistical analysis was carried out using one-way ANOVA, followed by Tukey's test (GraphPad Software, La Jolla, CA, USA) to compare apelin effect to control cultures. Statistically significant differences $P < 0.05$ between treatment groups and controls are indicated with different letters, with $a < b < c < d < e < f$. Groups with the same letter are not significantly different from each other.

RESULTS

Dose- and time-dependent effect of human recombinant apelin on steroid hormones: P4 and E2 secretion by trophoblast BeWo cells

To determine whether apelin affects steroids secretion, BeWo cells were incubated for 24, 48 and 72 hours with 0.02, 0.2, 2.0, 20 and 200 ng/ml of human recombinant apelin. The secretion of P4 was significantly decreased by treatment with apelin at 20 ng/ml for 24 hours (8.658 compared with 15.778 ng/ml in control), all investigated doses of apelin for 48 hours (20.536, 20.624, 21.645, 21.185 and 21.215 ng/ml compared with 24.981 in control) and apelin at 0.02, 0.2, 2.0 and 20 ng/ml (16.524, 16.859, 14.171 and 21.425 ng/ml, respectively, compared with 24.517 in control) for 72 hours (*Fig. 1A*, $P < 0.05$).

Apelin at all investigated doses had no effect on E2 secretion after 24 hours of BeWo cell incubation. However, apelin at all concentrations 0.02, 0.2, 2.0, 20 and 200 ng/ml decreased E2 secretion by BeWo cells after 48 hours of incubation (736.131, 690.614, 627.007, 674.524 and 667.599 pg/ml, respectively, compared with 924.37 pg/ml in control; *Fig. 1B*; $P < 0.05$) and 72 hours of cells incubation (650.459, 522.469, 616.755, 577.805 and 539.819 pg/ml, respectively, compared with 751.023 pg/ml in control; *Fig. 1B*; $P < 0.05$). We observed time- and dose-dependent inhibitory effect of apelin on steroid hormone secretion by placenta BeWo cells.

Effect of human recombinant apelin on 3βHSD and CYP19 protein expression in trophoblast BeWo cells

Western blot analysis revealed that the treatment of BeWo cells with apelin 2.0 and 20 ng/ml decreased significantly 3βHSD protein expression after 24 hours of cell incubation and additionally apelin with 20 ng/ml after 48 hours of cell incubation (*Fig. 2*; $P < 0.05$). No effect was observed after 72 hours. Similarly, apelin at doses 2.0 and 20 ng/ml decreased significantly CYP19 protein expression after 24 and 72 hours of cell incubation, and only at 20 ng/ml after 48 hours of BeWo incubation (*Fig. 2*; $P < 0.05$).

Dose- and time-dependent effect of human recombinant apelin on protein hormones: hCG, hPL and PLGF secretion by trophoblast BeWo cells

Fig. 3A shows that apelin significantly decreased hCG secretion only at 20 ng/ml after 24 hours of incubation (5.483 compared to 6.600 mlU/ml in control). We observed that apelin significantly decreased hCG secretion also after 48 hours at 0.2, 2.0 and 20 ng/ml (7.579, 8.499 and 9.174 mlU/ml, respectively,

Table 1. Primary and secondary antibodies used for Western blotting.

Antibody	Host species	Vendor/cat.no	Dilution
3βHSD	Mouse	abcam/ab75710	1:1000
CYP19	Rabbit	Thermo Fisher/ PA1-21398	1:200
hPL	Rabbit	abcam/ab15554	1:100
βhCG	Mouse	abcam/ab9582	1:500
PLGF	Rabbit	Proteintech/ 10642-1-AP	1:500
phospho-PKA	Rabbit	abcam/ab5815	1:700
total-PKA	Rabbit	abcam/ab187515	1:500
β-actin	Mouse	Sigma-Aldrich/ A5316	1:5000
Secondary	Goat anti-rabbit	Cell Signaling Technology/7074	1:1000
Secondary	Horse anti-mouse	Cell Signaling Technology/7076	1:1000

Table 2. ELISA kits using to study the concentration of hormones in the culture medium.

Hormone	Catalog no	Sensitivity of assay	Ranges of assay	Intra- experiment coefficients of variation	Inter- experiment coefficients of variation
P4	EIA-1516	0.045 ng/ml	0 – 40 ng/ml	6.99%	4.34%
E2	EIA-2693	9.714 pg/ml	0 – 2000 pg/ml	2.71%	6.72%
hCG	EIA-1911	< 1 mIU/ml	0 – 200 mIU/ml	4.00%	7.30%
hPL	EIA-1283	0.043 mg/l	0 – 20 mg/l	5.55%	7.14%
PLGF	EIA-4529	< 1.062 pg/ml	0 – 1000 pg/ml	1.70%	7.00%

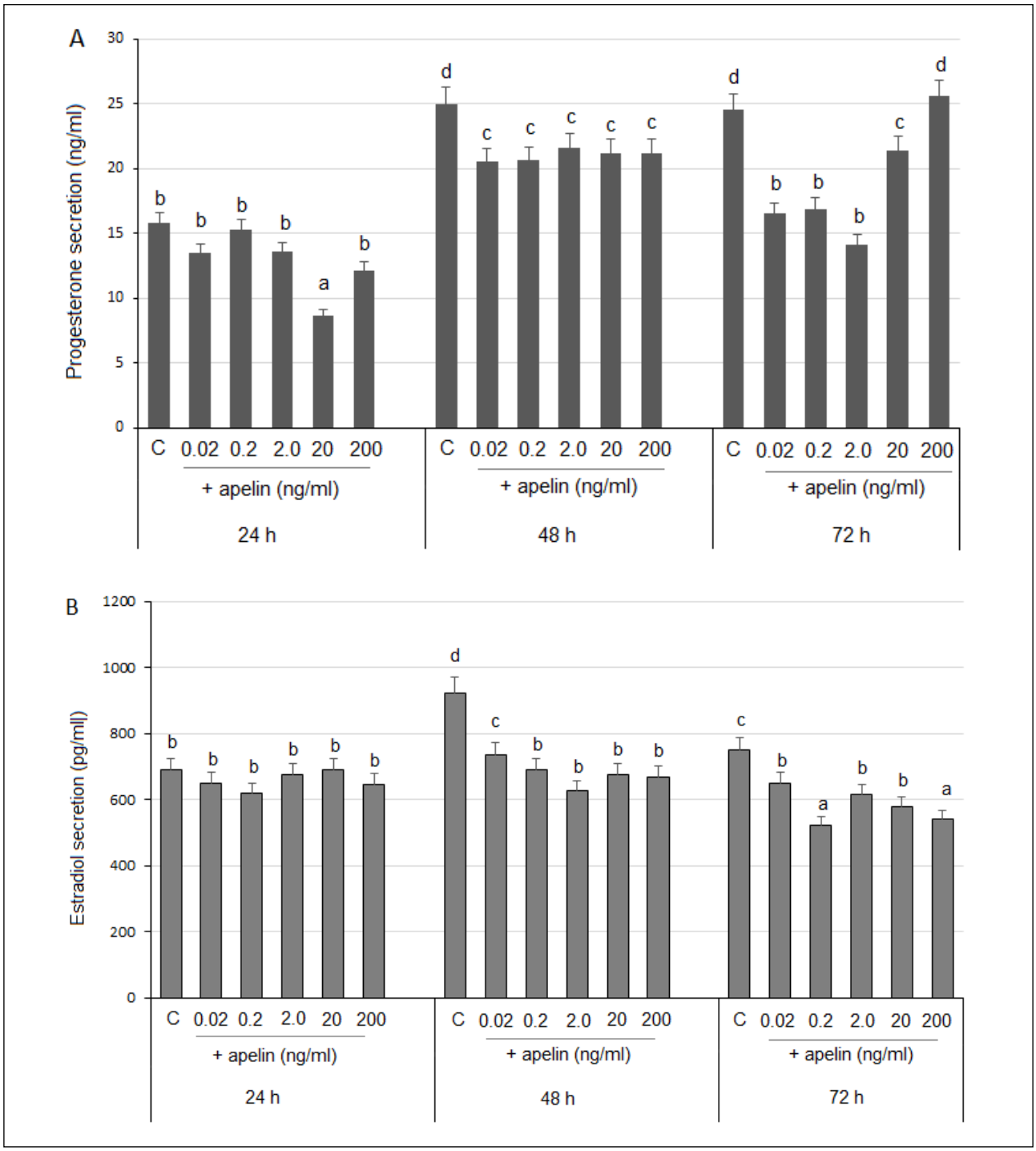

Fig. 1. Dose- and time-dependent effect of recombinant human apelin on progesterone and estradiol secretion by BeWo cells. 4×10^3 cells were cultured in DMEM/F12 with 10% FBS. After 24 hours, media were replaced by DMEM/F12 with 1% FBS, and cells were treated with apelin at doses 0.02, 0.2, 2.0, 20 and 200 ng/ml for 24, 48 and 72 hours. Steroid hormones: (A) progesterone and (B) estradiol were measured in a culture medium by ELISA kits as indicated in *Table 2*. Statistical analysis was carried out using one-way or two-way ANOVA, followed by Tukey's test (GraphPad Software, La Jolla, CA, USA). Statistically significant differences $P < 0.05$ between treatment groups and controls are indicated with different letters, with a < b < c < d. Groups with the same letter are not significantly different from each other.

compared to 10.187 mlU/ml in control) and 72 hours at all investigated doses 0.02, 0.2, 2.0, 20 and 200 ng/ml (6.620, 7.325, 5.831, 6.223 and 6.613 mlU/ml, respectively, compare to 8.553 mlU/ml in control) (*Fig. 3A*; $P < 0.05$).

We showed that after 24 hours all investigated doses of apelin reduced significantly hPL secretion by BeWo cells (0.773, 0.632, 0.705, 0.636, 0.730 mg/l compared to a control of 0.987 mg/l). However, in response to the 48 and 72 hours of incubation with apelin, we did not observe significant change in hPL secretion (*Fig. 3B*).

After 24 hours of incubation, apelin at a dose of 20 and 200 ng/ml significantly reduced PLGF secretion (273.23, 279.61

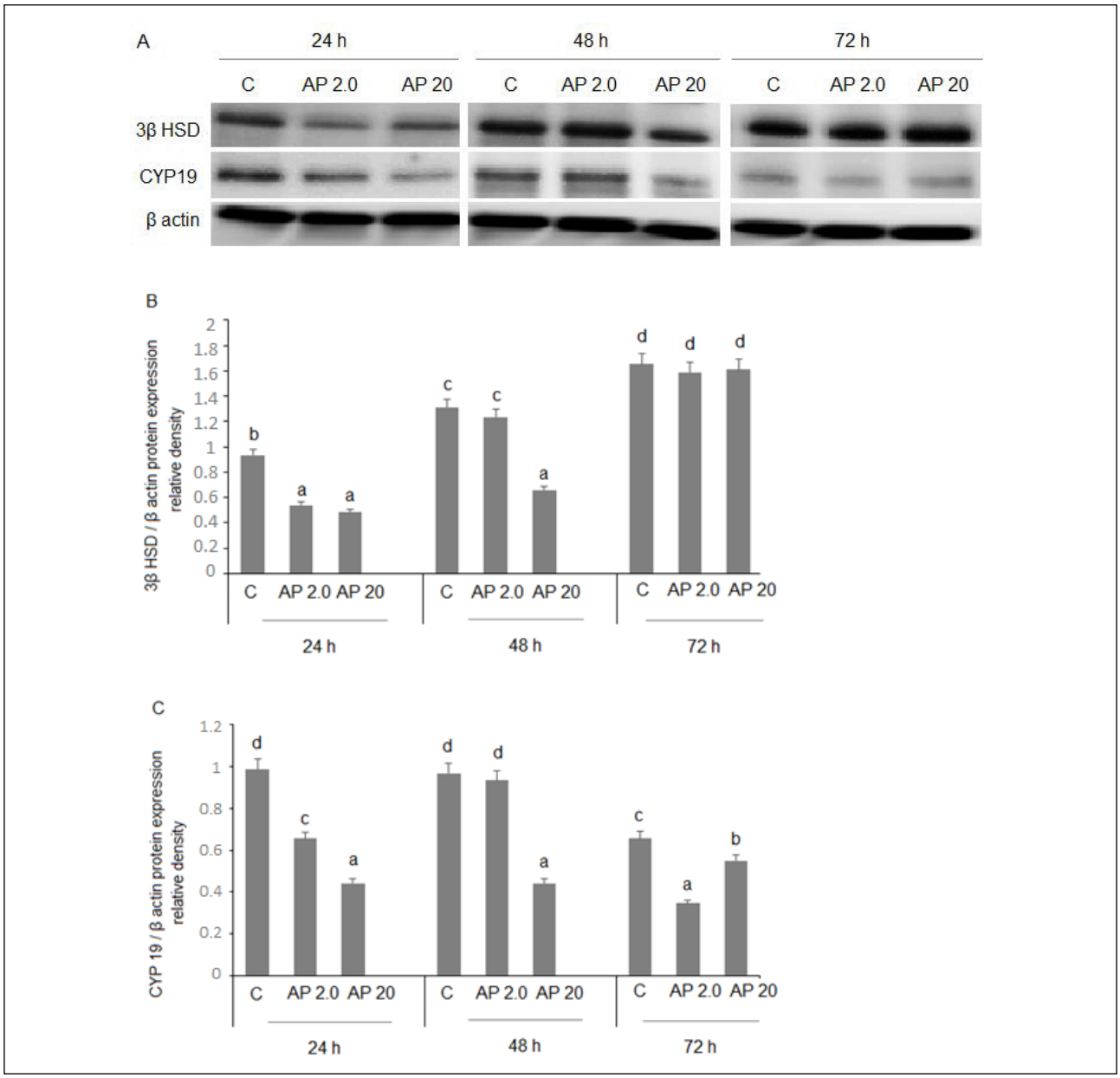

Fig. 2. The effect of recombinant human apelin on steroid enzymes 3βHSD and CYP19 protein expression (*A*) in BeWo cells. 4×10^3 cells were cultured in DMEM/F12 with 10% FBS. After 24 hours, media were replaced by DMEM/F12 with 1% FBS, and cells were treated with apelin at doses 2.0 and 20 ng/ml for 24, 48 and 72 hours. Steroid enzymes: (*B*) 3βHSD and (*C*) CYP19 were measurment in BeWo cells by Western blot as indicated in Material and Methods. Statistical analysis was carried out using one-way or two-way ANOVA, followed by Tukey's test (GraphPad Software, La Jolla, CA, USA). Statistically significant differences $P < 0.05$ between treatment groups and controls are indicated with different letters, with a < b < c < d. Groups with the same letter are not significantly different from each other.

pg/ml compared to the control 351.72 pg/ml). The same effect was observed for all tested doses of apelin after 48 hours of incubation (564.46, 577.68, 545.55, 485.37, 522.17 pg/ml relative to the control 710.65 pg/ml). Meanwhile, after 72 hours, a similar result was noted only in 20 ng/ml of apelin (310.31 pg/ml compared to the control sample 893.06 pg/ml) (*Fig. 3C*; $P < 0.05$).

Effect of human recombinant apelin on hCG, hPL and PLGF protein expression in trophoblast BeWo cells

The results of Western Blot analysis confirmed that apelin reduced the expression of all the protein hormones, which we studied. After 24 hours of incubation, apelin at a doses 2.0 and 20 ng/ml significantly reduced the expression of hPL and PLGF protein (*Fig. 4C* and *4D*; $P < 0.05$). The same result was noted after 48 hours for hCG hormone (*Fig. 4B*). In turn, after 72 hours, we observed that both doses of apelin 2.0 and 20 ng/ml reduced the expression of hCG and PLGF (*Fig. 4B* and *4D*; $P < 0.05$).

Effect of human recombinant apelin on PKA kinase phosphorylation in trophoblast BeWo cells

Our previous study clearly documented that apelin induced phosphorylation of several kinases including ERK1/2, Akt and

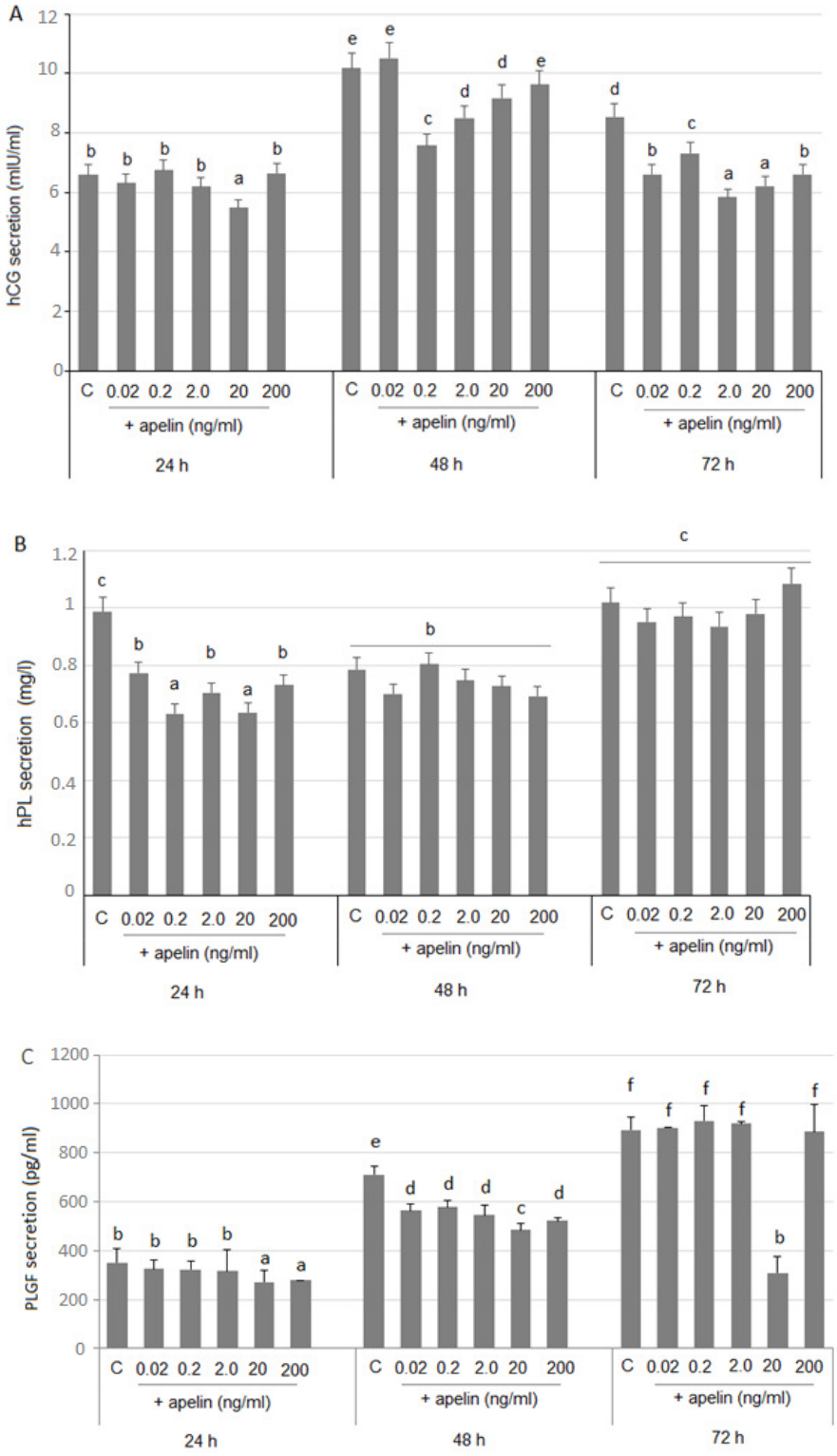

Fig. 3. Dose- and time-dependent effect of recombinant human apelin on protein hormone secretion by BeWo cells. 4×10^3 cells were cultured in DMEM/F12 with 10% FBS. After 24 hours, media were replaced by DMEM/F12 with 1% FBS, and cells were treated with apelin at doses 0.02, 0.2, 2.0, 20 and 200 ng/ml for 24, 48 and 72 hours. Protein hormones: (*A*) chorionic gonadotropin (hCG), (*B*) human placental lactogen (hPL) and (*C*) placental growth factor (PLGF) were measured in culture medium by ELISA kits as indicated in *Table 2*. Statistical analysis was carried out using one-way or two-way ANOVA, followed by Tukey's test (GraphPad Software, La Jolla, CA, USA). Statistically significant differences $P < 0.05$ between treatment groups and controls are indicated with different letters, with a < b < c < d < e < f. Groups with the same letter are not significantly different from each other.

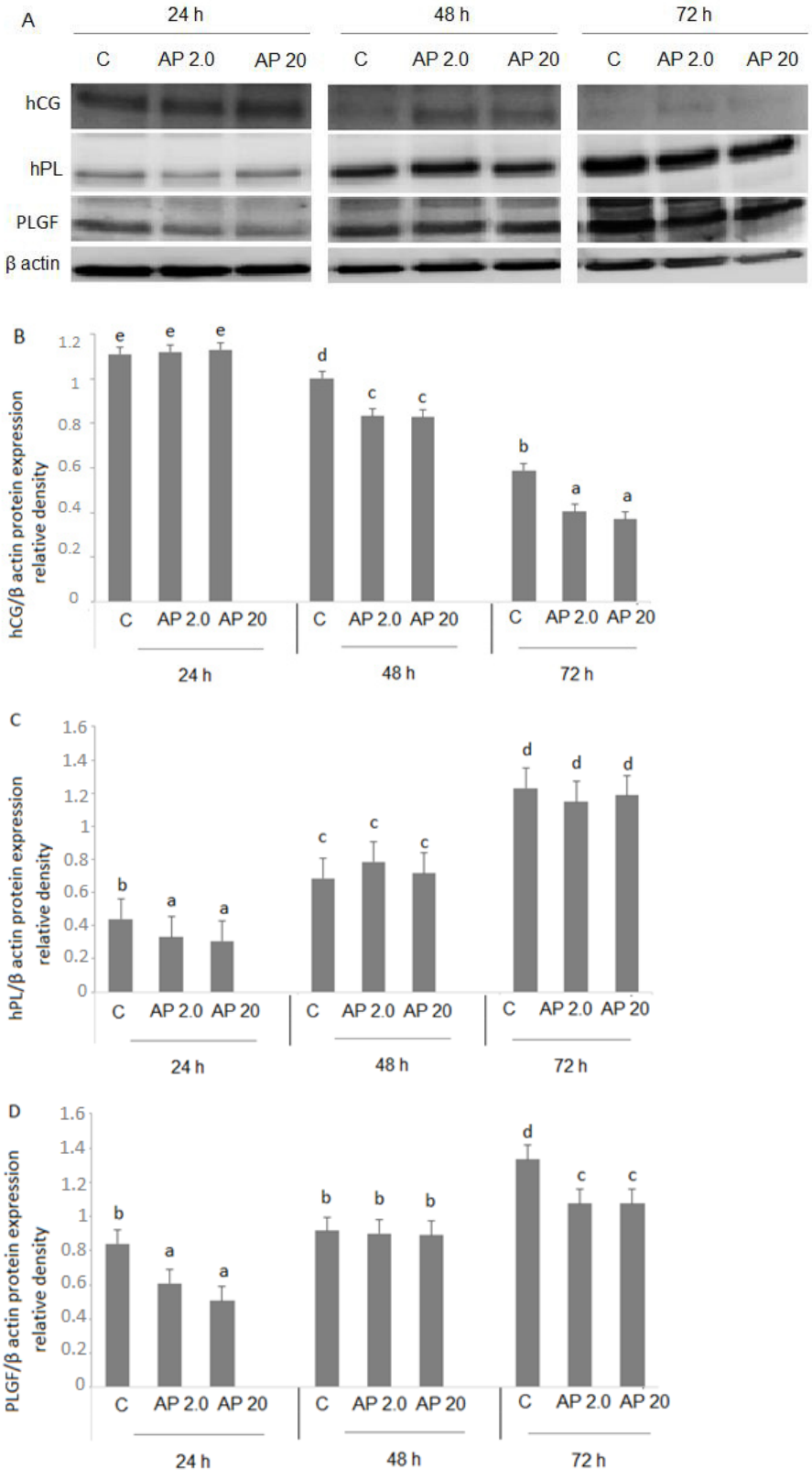

Fig. 4. Effect of recombinant human apelin on protein hormone expression (*A*) in BeWo cells. 4 × 10^3 cells were cultured in DMEM/F12 with 10% FBS. After 24 hours, media were replaced by DMEM/F12 with 1% FBS, and cells were treated with apelin at doses 2.0 and 20 ng/ml for 24, 48 and 72 hours. Protein hormones: (*B*) chorionic gonadotropin (hCG), (*C*) human placental lactogen (hPL) and (*D*) placental growth factor (PLGF) expression were measured in BeWo cells by Western blot as indicated in Material and Methods. Statistical analysis was carried out using one-way or two-way ANOVA, followed by Tukey's test (GraphPad Software, La Jolla, CA, USA). Statistically significant differences $P < 0.05$ between treatment groups and controls are indicated with different letters, with a < b < c < d < e. Groups with the same letter are not significantly different from each other.

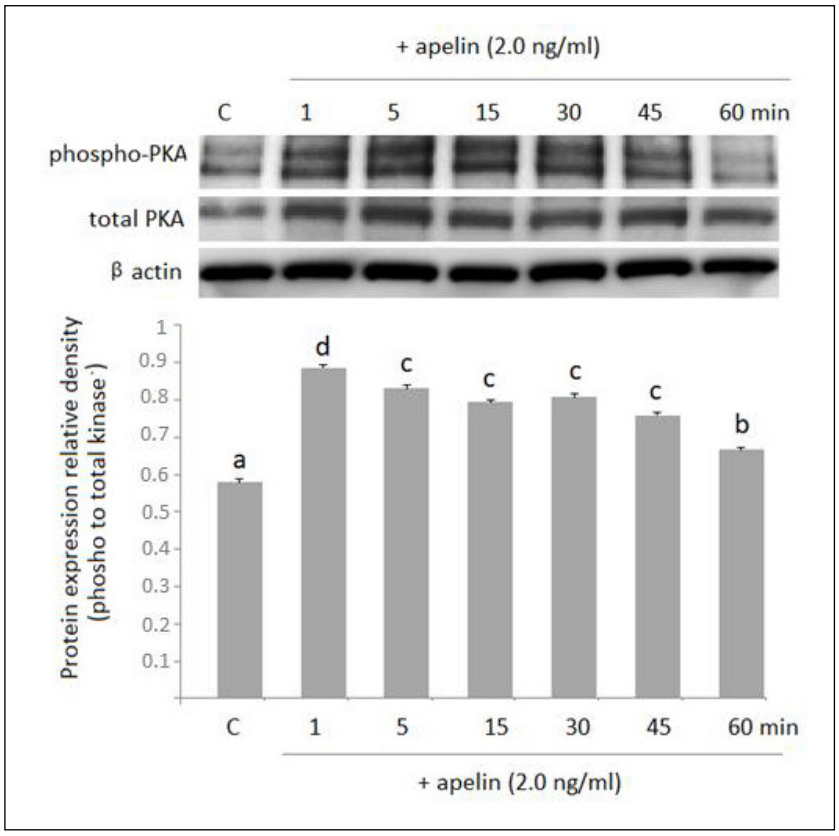

Fig. 5. Effect of recombinant human apelin on phosphorylation of kinase A (PKA). 4 × 10^3 cells were cultured in DMEM/F12 with 10% FBS. After 24 hours, media were replaced by DMEM/F12 with 1% FBS, and cells were treated with apelin at dose 2.0 ng/ml for variable incubation durations of 1, 5, 15, 30, 45 and 60 min. Then, BeWo cells were harvested as indicated in Material and Methods to measure PKA protein expression by Western blot. Statistical analysis was carried out using one-way ANOVA, followed by Tukey's test (GraphPad Software, La Jolla, CA, USA). Statistically significant differences $P < 0.05$ between treatment groups and controls are indicated with different letters, with $a < b < c < d$. Groups with the same letter are not significantly different from each other.

Stat3 (15). In the present study we focused on PKA activation because phosphorylation of this kinases is involved in hormone secretion (20). We observed that apelin (2.0 ng/ml) increased significantly the phosphorylation of kinase PKA from 1 to 60 min of BeWo cell incubation (*Fig. 5*; $P < 0.05$).

Role of receptor APJ and kinases ERK1/2 and PKA in apelin action on hormones secretion by trophoblast BeWo cells

BeWo cells were cultured with potent antagonists of APJ (ML221), PKA (KT5720) or ERK1/2 (PD98059). The APJ and both kinases antagonists reversed the inhibitory activity of apelin (2.0 ng/ml) on both steroids P4 and E2 secretion as well as PLGF in comparison to the control group values, suggesting that APJ and both PKA and ERK1/2 are involved in the action of apelin on above placental secretion (*Fig. 6*; $P < 0.05$). Moreover, we observed that levels of protein hormones hCG and hPL, were returned to the control when we used ML221 and PD98059, suggesting that APJ and ERK1/2 participate in the apelin action on protein hormones secretion.

DISCUSSION

The results of this study show, the *in vitro* effect of the human recombinant apelin on the endocrine function of placental syncytiotrophoblast BeWo cells. Our results clearly documented that apelin significantly decreased both steroid and protein hormones by the inhibition of enzymes 3βHSD and CYP19 or protein hormones expression. As a molecular mechanism of apelin action on placental BeWo cell endocrinology, we propose the activation of receptor APJ and phosphorylation of kinase ERK1/2 and PKA. Steroids (P4 and E2) and PLGF secretion were abolished when BeWo cells were cultured in the presence of APJ and both kinases antagonist, while hCG and hPL secretion occurred only in the presence of APJ and ERK1/2 antagonist, suggesting that the APJ receptor and both ERK1/2 and PKA kinases participate in the apelin action on placenta endocrinology (*Fig. 7*). In our study we used BeWo cell line because this type of placenta cells commonly used to study endocrine function, while JEG-3 are widely used to study the molecular mechanisms underlying the proliferation and invasive potential of cytotrophoblast (30). Data of Burleigh *et al*. (31) documented that microarray analysis indicated that approximately 2700 genes are differentially expressed between BeWo and JEG-3 cells, suggesting that they are suited only for specific experimental paradigms. These differences in gene expression patterns suggest that JEG3 and BeWo cell lines will vary in their capacity to respond to an identical experimental treatment. Our previously study compared apelin action on BeWo and JEG-3 cells proliferation as well cell cycle and we observed no difference between placenta cells in apelin response (15).

The results of the presented study indicate that apelin changes the hormonal profile of placental cells. Placental hormones have many important functions that determine the correct course of pregnancy. All sorts of secretion abnormalities can lead to numerous pregnancy pathologies. P4 and E2 are the two most important steroid hormones produced by the human placenta. In this study, we documented that the human recombinant apelin during 24 – 72 hours of BeWo cells culture significanly decreased P4 secretion by inhibition 3βHSD protein expression. P4 until about 10 weeks of pregnancy is produced by the corpus luteum (32), then between 10 and 12 weeks the full secretory function of this hormone takes over the placenta (33). During pregnancy, P4 is involved *inter alia* in: silencing the activity of the uterus, preventing miscarriages, promoting insulin resistance and preparing mammary glands for lactation (34). Results of our study have documented that the human

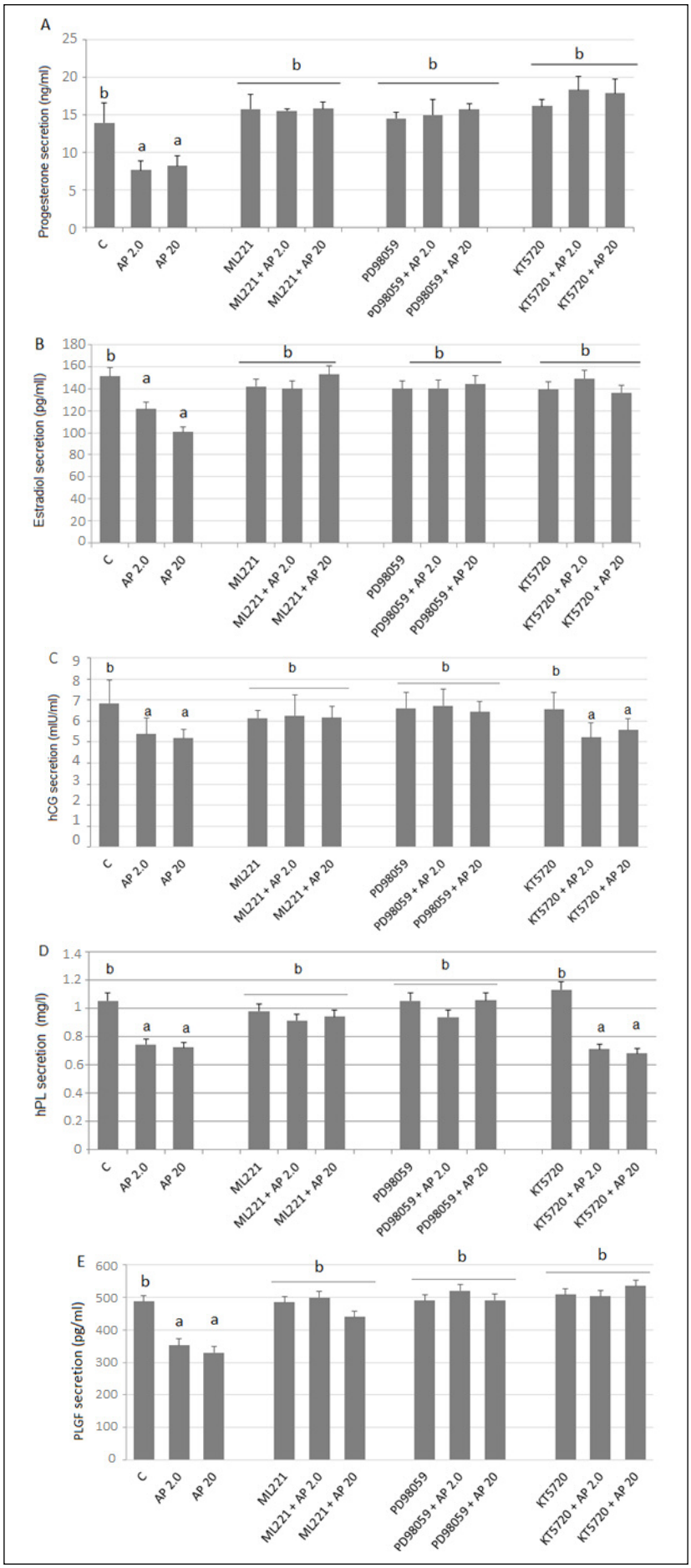

Fig. 6. Molecular mechanism of inhibitory action of apelin on hormone secretion. (*A*) progesterone, (*B*) estradiol, (*C*) chorionic gonadotropin (hCG), (*D*) human placental lactogen (hPL) and (*E*) placental growth factor (PLGF). BeWo cells (4×10^3 cells) were cultured in DMEM/F12 with 10% FBS. After 24 hours, media were replaced by DMEM/F12 with 1% FBS, and cells were pre-treated for 1 hour with selective inhibitors of APJ receptor (ML221) or ERK 1/2 or PKA kinases (PD98059 or KT5720, respectively), after which apelin (2.0 ng/ml) was added for 48 hours and culture medium was collected for hormone secretion measurement. Statistical analysis was carried out using one-way or two-way ANOVA, followed by Tukey's test (GraphPad Software, La Jolla, CA, USA). Statistically significant differences $P < 0.05$ between treatment groups and controls are indicated with different letters, with a < b. Groups with the same letter are not significantly different from each other.

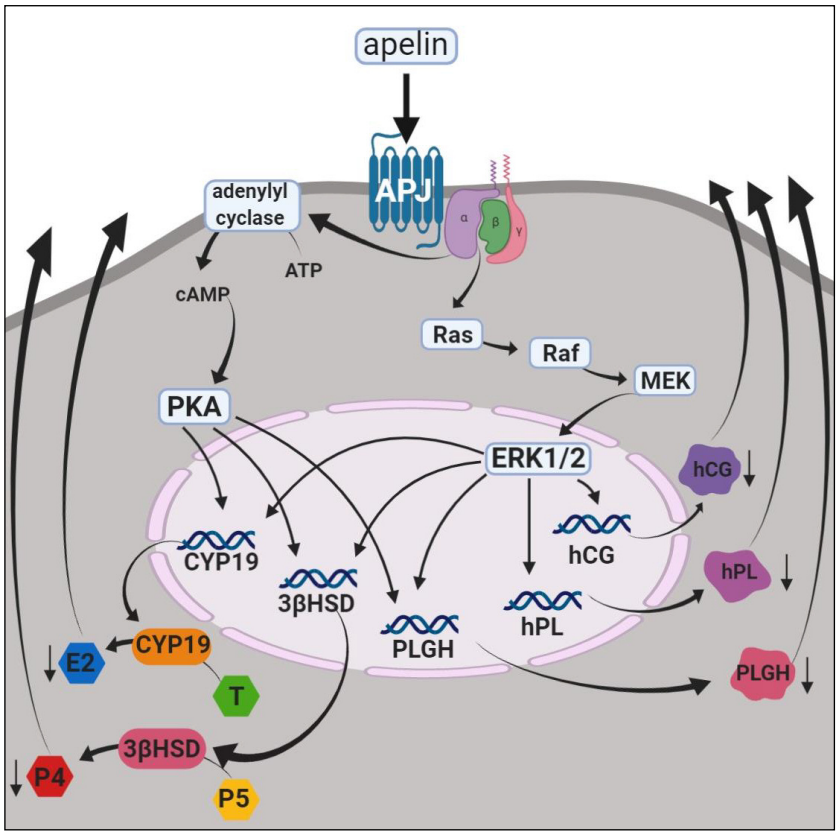

Fig. 7. Schemat summarizing inhibitory effect of apelin on BeWo cell hormone secretion: progesterone (P4), estradiol (E2), chorionic gonadotropin (hCG), human placental lactogen (hPL) and placental growth factor (PLGF). Apelin receptor (APJ) and both kinases: protein kinase A (PKA) and an extracellular signal-regulated kinases (ERK1/2) are involved in P4, E2 and PLGF secretion, while in the secretion of hCG and hPL activation of APJ and ERK1/2.

recombinant apelin can cause disorders of the aforementioned processes by decreasing P4 secretion and inhibiting 3βHSD protein expression in syncytiotrophoblast cells. The results of our study further suggest that the effect of apelin on placental cells may be one of the reasons for premature termination of pregnancy and the occurrence of PE. Literature data have shown that pregnant women with low serum P4 levels (< 12 ng/ml) have a higher risk of miscarriages (35) and also in PE. Last paper of Gurlek *et al*. (36) showed that apelin-13 and apelin-36 are moderately successful markers to differentiate subjects with PE. However, a review paper by Georgiadou *et al*. (37) showed opposing results of apelin levels in placenta tissue and blood of PE patients compared to the control subject. In any case, our findings are in agreement with published studies indicating that leptin, like apelin, decreased P4 secretion by cultured human first-trimester cytotrophoblast cells (38). Bone morphogenetic protein-15 (BMP-15), an oocyte-derived growth factor, has been shown also to play stimulatory role on P4 synthsis by oocyte cumulus complexes (39).

Our results showed that the human recombinant apelin has an inhibitory effect also on E2 secretion after 48 and 72 hours of cell incubation, as well as CYP19 protein expression during all durations of BeWo culture (24 – 72 hours). One hypothesis to explain the difference between steroid hormone levels and protein expression of enzymes is that apelin could modulate the activity of steroid enzymes. However, this needs to be further investigated. One of the main functions of E2 is to increase the blood flow between the uterus and the placenta, which has effects on embryo implantation, preparing the mammary glands for breastfeeding and can cause contraction in isolated myometrial cells (34). Decreasing the level of E2 during pregnancy can have a significant effect on pregnancy pathology like IUGR, which is manifested by a very low fetal weight in relation to the stage of pregnancy, and also associated with the inhibition of E2 secretion and an increase in CYP19 expression. An increased level of CYP19 protein expression may be a feedback signal aimed at compensating for E2 secretion pathway disorders in IUGR (40). In IUGR placentas, apelin staining was strongly decreased in both syncytiotrophoblast and stroma of the placental villi compared to control (13). A reduced level of E2 was also observed in PE (41). Interestingly, leptin like apelin in our results, also reduced E2 secretion by cultured human placental cells (2). The role of apelin on steroid hormone secretion has been demonstrated in different steroidogenic gonadal tissues including ovarian follicles (23), corpus luteum (22) or testis (42). Interesting, *in vitro* observation documented that the secretion of steroid hormones and the endometrial steroidogenesis are modulated by opioid receptors activation (43), however in placental steroidogenesis it was still not examined.

We showed that human recombinant apelin decreased secretion and protein expression of hCG, hPL and PLGF in trophoblast BeWo cells. The presence of hCG in the mother's serum can be detected as early as 8 days after fertilization, and from this time until the 10th week of pregnancy, the level increases, later the secretion of hCG decreases (44). Additionally, hPL presence in the placenta is detectable already in the second week of pregnancy, then, after 3 – 6 weeks of gestation, it is released mainly into the mother's bloodstream, constantly increasing its level until the delivery of the placenta (42). In the mother's serum, PLGF can be detected between 15 – 20 weeks of pregnancy, and the maximum level is observed in the third trimester (16). Several observations suggest the role of protein hormones like hCG, hPL or PLGF in feto-placental units including stimulations of the proliferation of placental microvascular endothelial cells (45), increases secretion of P4 by the corpus luteum (46), suppresses the maternal immune system (33) and has a relaxation effect on the uterine tissue (47). The

placental protein hormone also participates in the lipolysis of human adipose tissue, and the resulting free fatty acids are an indirect source of energy for the fetus (48). The inhibitory effect of apelin on the placental protein hormone level are in agreement with previous data showing that leptin decreased hCG secretion by primary cells of human syncytiotrophoblast (2). Other studies have shown that adiponectin reduced hCG and hPL secretion, while visfatin increased significantly PLGF level by syncytiotrophoblast cells (2, 49). On the other hand, it was observed that hPL has an inhibitory effect on leptin secretion by primary cells of human syncytiotrophoblast (2), while E2 increased leptin levels in BeWo cells (50). However, there are no studies documenting apelin regulation in the placenta cells. Further, levels of placental protein hormones decreased in the IUGR and PE (51, 52), so the results of our study suggest that apelin, *via* inhibiting the placenta endocrine function, can be involved in pregnancy pathologies. Moreover, apelin could be implicated in the pathophysiology of PE (through the regulation of blood pressure and angiogenesis), in the regulation of fetal growth (through its effects on plasma volume expansion) and in the pathophysiology of gestational diabetes mellitus (through the regulation of glucose metabolism) (53). However, the pathophysiology of preeclampsia, preterm delivery, and gestational diabetes are all different and cannot be explained by single molecule - apelin. Thus, the complex study on effect of several hormones/factors on placenta pathology should be further validated.

The mechanism involved for inhibitory action of apelin on placental steroid secretion is the activation of APJ receptors and both kinases ERK1/2 and PKA. This is because after the stimulation of BeWo cells with the pharmacological blockers of APJ and kinases PKA and ERK 1/2, we observed an abolition of the apelin inhibitory effect. Involvement of the APJ receptor in steroidogenic action of apelin was observed in human (54) and pig (22, 23) ovarian cells as well as the mitogenic effect of apelin in trophoblast BeWo cells (15). In our study, for the first time, we also observed that both kinases PKA and ERK 1/2 are involved in the steroidogenic action of apelin. It is well known fact that both PKA and ERK1/2 are also involving in proliferation, survival, apoptosis or differentiation. Our previously study clearly documented that apelin induced phosphorylation of kinases ERK1/2, Akt, Stat3 and AMPKα and we noted mitogenic action of apelin in BeWo and JEG-3 cells by activation of ERK1/2, Stat3 and AMPKα signaling (15). Data of Gomez-Concha *et al*. (55) demonstrated the significant contribution of PKA kinase in P4 sythesis by placental cells. Other studies indicated that the conversion of androstenedione to E2 *via* calcitriol is mediated by the PKA signaling pathway, whereas the effect of calcitriol on the 3βHSD expression, which is involved in the P4 formation, seemed to be regulated by another mechanism independent of cAMP (56). Latest data of Liong *et al*. (57) showed that in trophoblast cells, ERK1/2 signalling is required for the secretory function in the placenta including IL-6, IL-8, MCP-1, and GRO-α secretion. Moreover, we observed that PLGH secretion was abolished when BeWo cells were cultured in the presence of APJ and both kinases antagonists, while hCG and hPL secretion only in the presence of APJ and ERK1/2 antagonist. MAPK and ERK 1/2 are involved in forskolin-induced hCG release by BeWo cells (58). Additionally, activation of the MAPK pathway plays a significant role in the molecular hormone of hPL secretion by stimulation of apolipoprotein A-I (59).

Successful pregnancy depends on a well-developed and functional placenta regulated by steroid and peptide hormones, growth factors and cytokines, acting in an autocrine/paracrine fashion. Using trophoblast BeWo cells cultures, we showed the effect of apelin on some placental hormone secretions, which suggest that apelin could be one important participant of such regulation of the human fetoplacental unit. The limitation of our studies is using placental cell lines. However, more experiments on other models of cultured primary human placental cells or explants as well *in vivo* animals are necessary to confirm the obtained results.

Abbreviations: 3βHSD, 3β-hydroxysteroid dehydrogenase; Akt, phosphatidylinositol 3'-kinase/Akt pathway; AMPKα, 5'-monophosphate-activated protein kinase; APJ, apelin receptor; cAMP, cyclic adenosine monophosphate; CYP19, aromatase cytochrome P450; E2, estradiol; ERK1/2, extracellular signal-regulated kinases; GRO-α, growth-regulated oncogene-alpha; hCG, chorionic gonadotropin; hPL, human placental lactogen; IL-6, interleukin-6; IL-8, interleukin-8; IUGR, intrauterine hypotrophy; KT5720, PKA antagonist; MCP-1, monocyte chemoattractant protein-1; ML221, APJ antagonist; P4, progesterone; PD98059, ERK1/2 antagonist; PE, preeclampsia; PKA, protein kinase A; PLGF, placental growth factor; PVDF, polyvinylidene fluoride; Stat3, signal transducer and activator of transcription 3.

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