



The ontogenies of endometrial and myometrial leptin and adiponectin receptors in pregnant rats: Their putative impact on uterine contractility

Annamária Schaffer^a, Eszter Ducza^b, Nikolett Bódi^c, Mária Bagyánszki^c, Zita Szalai^c,
Mohsen Mirdamadi^a, Tamara Barna^a, Kálmán F. Szűcs^a, Róbert Gáspár^{a,*}

^a Department of Pharmacology and Pharmacotherapy, Albert-Szent-Györgyi Medical School, Faculty of Medicine, University of Szeged, Hungary

^b Department of Pharmacodynamics and Biopharmacy, Faculty of Pharmacy, University of Szeged, Hungary

^c Department of Physiology, Anatomy and Neuroscience, Institute of Biology, Faculty of Science and Informatics, University of Szeged, Hungary

ARTICLE INFO

Keywords:

Leptin
Adiponectin
Pregnant uterus
Rat
Contraction
Adipokine receptors

ABSTRACT

Aims: Limited data are available about the functions and expressions of leptin and adiponectin receptors (LEPR, AdipoRs) in the uterus. Our aim was to investigate the effects of leptin and adiponectin on the contractions of intact and denuded nonpregnant and pregnant uteri, as well as the changes in mRNA and protein expressions of LEPR and AdipoRs during the gestational period.

Main methods: Contractions of nonpregnant and 5-, 15-, 18-, 20- or 22-day pregnant uterine rings were measured in an isolated organ bath system. The tissue contractions were stimulated with KCl and modified by cumulative concentrations of leptin or adiponectin. The mRNAs, protein expressions and localizations of LEPR and AdipoRs were determined by RT-PCR, Western blot and immunohistochemistry, respectively.

Key findings: Both adipokines relaxed the nonpregnant intact uterus more effectively than the denuded myometrium. Leptin inhibited the contractions of endometrium-denuded uteri throughout pregnancy, while its action was weakened on intact uteri towards term. The changes in LEPR receptor densities were independent of the relaxing effect. Adiponectin inhibited contractions, but this effect ceased on pregnancy day 22, while a gradual decrease was detected towards term on denuded myometria. These modifications were in harmony with changes in the expressions of AdipoRs.

Significance: Both leptin and adiponectin play a role in the relaxation of the pregnant uterus, but their efficacy significantly decreases towards the end of gestation. Their endometrial receptors may have a fine-tuning role in uterine contractions, predicting the importance of these adipokines in uterine contractions under altered adipokine level conditions.

1. Introduction

Adipose tissue produces a group of proteins called adipokines. They are involved in multiple physiological functions, including reproduction.

Leptin is produced by white adipose tissue, but also expressed in the gastrointestinal tract, hypothalamus, placenta and mammary glands [1–4]. It has crucial roles in the regulation of appetite, metabolism, and neuroendocrine functions. Leptin is one of the most important signals to the central nervous system about the nutritional status, since its concentrations reflect the percentage of body fat [5]. Leptin binds to its receptor (LEPR) and activates primarily the JAK2/STAT cascade, but cross-talk with other pathways is also possible [6]. The lack of functional

leptin system plays a role in obesity and diabetes mellitus, but severely obese patients generally have hyperleptinemia. The fact that leptin fails to promote energy expenditure in these patients indicates leptin resistance, a phenomenon that is still not fully understood [7]. Low leptin concentration is a sign of energy deficiency resulting in interrupted physiological processes [8].

Leptin regulates the hypothalamic-pituitary-gonadal (HPG) axis [9]. Since LEPRs are not expressed in GnRH neurons, leptin indirectly stimulates GnRH secretion by acting on kisspeptin neurons [10,11]. The consequences of leptin system deficiency include delayed onset of puberty, infertility or impaired reproductive functions [12–14]. Maternal leptin concentrations are increased by body fat and gestational age, since the main sources of leptin are adipose tissue and placenta [15].

* Corresponding author.

E-mail address: gaspar.robort@med.u-szeged.hu (R. Gáspár).

<https://doi.org/10.1016/j.lfs.2022.120465>

Received 3 January 2022; Received in revised form 2 March 2022; Accepted 4 March 2022

Available online 7 March 2022

0024-3205/© 2022 Elsevier Inc. All rights reserved.

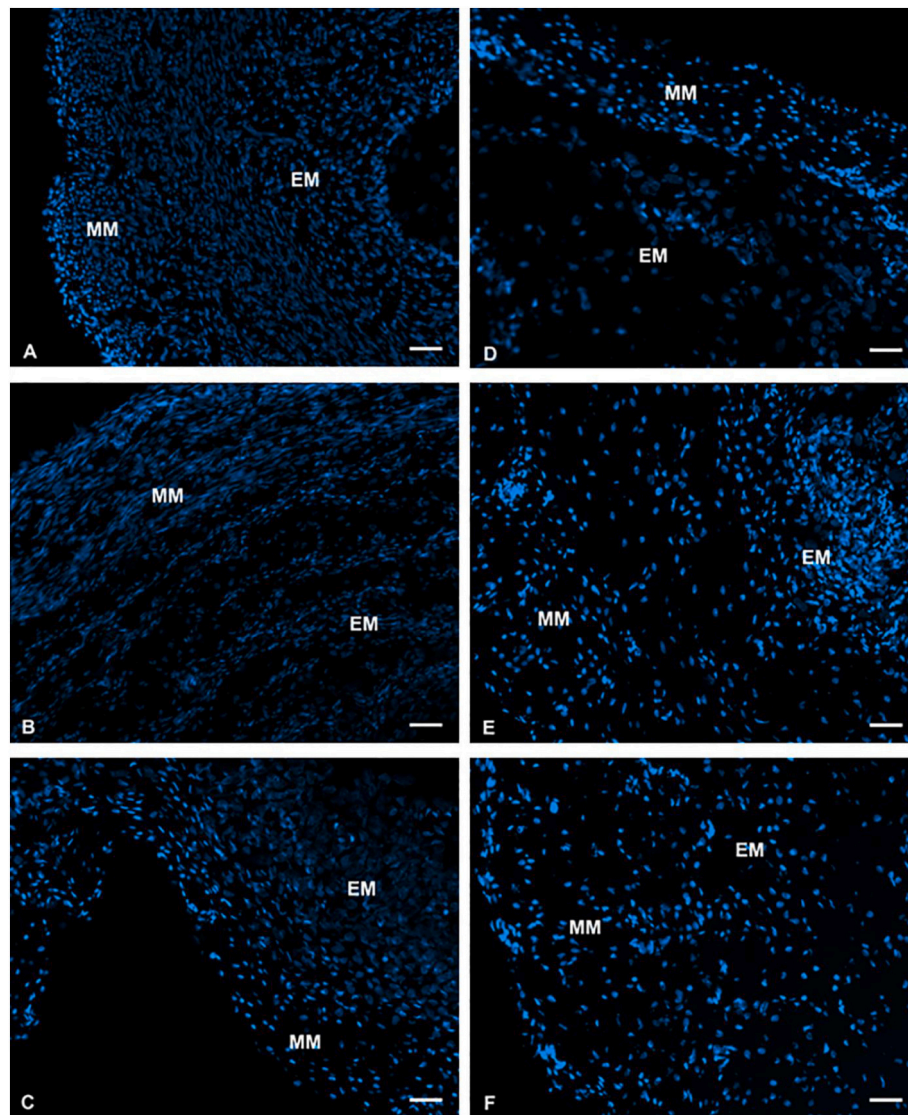


Fig. 1. Representative fluorescent micrographs of cryosections from nonpregnant and pregnant rat uterus showing nuclear stainings with DAPI (blue). A: nonpregnant, B: gestational day 5, C: gestational day 15, D: gestational day 18, E: gestational day 20, F: gestational day 22, MM: myometrium, EM: endometrium. Scale bar: 50 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Leptin affects gonadal organs modifying uterine activity. Leptin inhibits contractions of rat myometrium, normal and obese human pregnant myometrium, but the exact mechanism is still unidentified [16,17].

Unlike leptin, adiponectin serum concentrations correlate inversely with BMI, hence it is called a ‘beneficial’ adipocytokine. Obesity is linked with its reduced plasma levels, whereas weight loss induces an elevation in concentration [18–20]. Adiponectin is the most abundant adipokine in human circulation [21], females have 2–3 times higher concentrations than males [22]. Adiponectin has major regulatory roles in lipid metabolism and insulin sensitivity [23]. It acts through its main receptors: AdipoR1 and AdipoR2. It also binds to the T-cadherin, which has a role in vascularization [24,25]. Although the uterine presence of T-cadherin has not been confirmed yet, likely it may be expressed in the smooth muscle cells of the uterus. It can be hypothesized that T-cadherin regulates the uterine effects of adiponectin, partly by affecting vascularization [26]. AdipoR1 is expressed ubiquitously, while AdipoR2 can be detected in the liver [27]. Receptor stimulation activates AMPK cascade, p38 MAPK and PPAR α , mediated by an adaptor protein, called APPL1 [28].

The adiponectin system is present in the endometrium [29], placenta [30] and uteri [31]. The deletion of the receptors does not cause

impaired fertility, implying its low significance in reproduction [32], but it may have influence partially through the regulation of metabolic processes [33]. Concentration is elevated in early-pregnancy but correlates negatively with maternal BMI. Hypoadiponectinaemia and insulin resistance in obese women may cause abnormalities during pregnancy. One study revealed that adiponectin reduces uterine contractility in mouse, suggesting a link between nutritional status and pregnancy outcome [34].

2. Aims of the study

The ontogenies of leptin and adiponectin receptors and the modifications of leptin and adiponectin effects on uterine contractions have not been clarified during pregnancy. Our aim was to identify the modification of these receptor expressions during gestation, and to measure the changes of the actions of leptin and adiponectin on normal and endometrium-denuded uterine contractions to obtain information about the significance of endometrial and myometrial receptors.

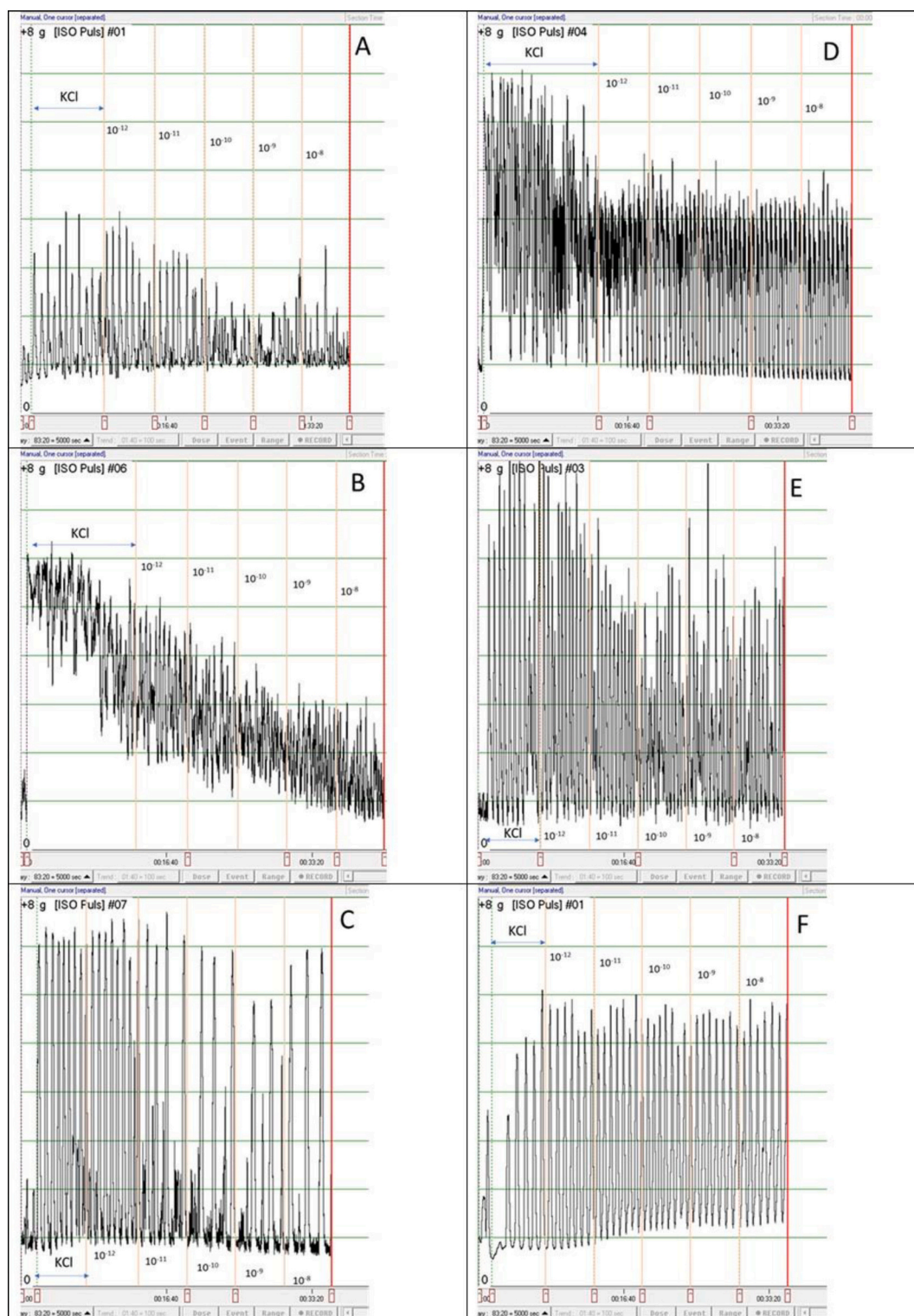


Fig. 2. Representative raw traces of KCl-stimulated uterine contractions and the effects of cumulative doses of leptin on nonpregnant (A) and pregnant (B-F) uteri.

3. Materials and methods

3.1. Housing and handling

The animals were treated in accordance with the European Communities Council Directive (2010/63/EU) and the Hungarian Act for the Protection of Animals in Research (Article 32 of Act XXVIII). All experiments involving animal subjects were carried out with the approval

of the National Scientific Ethical Committee on Animal Experimentation (registration number: IV./3071/2016.). The animals were kept in rooms with controlled temperature (22 ± 3 °C), humidity (30%–70%), and light (12-hour light/dark cycle), with tap water and standard rodent pellet (Animalab Hungary Ltd., Vác, Hungary) available ad libitum.

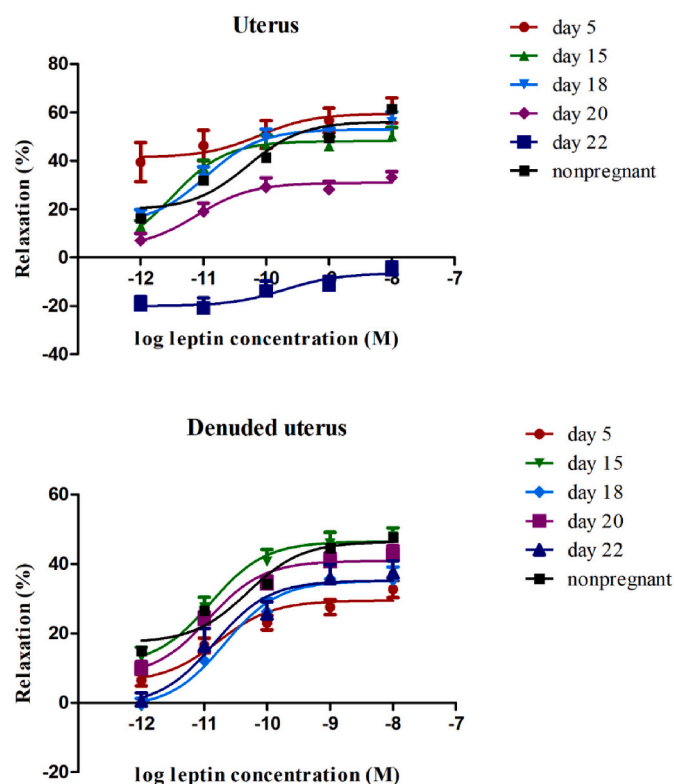


Fig. 3. Effects of leptin on KCl-induced contractions of nonpregnant and pregnant rat uteri or endometrium-denuded uteri. Cumulative concentrations of leptin in the range of 10^{-12} to 10^{-8} M reduced nonpregnant uterine contractions, while the removal of the endometrium weakened its action. Leptin also reduced the contractions of the pregnant intact uteri in the early phase of gestation. In the same concentration range, leptin was able to maintain its relaxing effect in the denuded uterus through the whole gestational period, however, the lack of endometrium weakened (day 5) or even enhanced (days 20 and 22) its efficacy. The change in contraction was calculated via the area under the curve and expressed in % \pm S.E.M., $n = 6$ for each group.

3.2. Mating and selection of nonpregnant females

Mature female (180–220 g) and male (220–260 g) Sprague-Dawley rats (Animalab Hungary Ltd., Vác, Hungary) were mated in a special mating cage. Female and male rats were separated with a metal door, which could be opened with a time-controlled electronic switch before dawn (5.00 a.m.). Within 4 h after possible mating, vaginal smears taken from the female rats were checked under microscope at 1200 \times magnification. In the case of spermatozoa in the sample or visible sperm plug in the vagina, the pregnancy was confirmed, and the day of copulation was designated as first day of pregnancy.

Nonpregnant fertile female rats in oestrous phase were also used for the experiments. On the day of the experiment, the oestrous cycle was detected by vaginal impedance measurement with Oestrus Cycle Monitor (IM-01, MSB-MET Ltd., Balatonfüred, Hungary). Rats with vaginal impedance values between 4.5 and 7.5 k Ω were chosen for the mating process or nonpregnant experiments, respectively.

3.3. Isolated organ bath studies

3.3.1. Uterus preparation

The animals were sacrificed by carbon dioxide inhalation in the morning of the experimental day. After abdominal incision, uterine tissues were removed from nonpregnant rats in the oestrous phase or from pregnant rats on gestational days 5, 15, 18, 20 or 22. Tissues were then prepared for the in vitro contractility measurements. Briefly, two

Table 1

EC50 values and the mean maximal relaxing effect of leptin (10^{-12} – 10^{-8} M) on rat uteri and denuded myometria.

	EC50 (M \pm S.E.M.)		Emax (% \pm S.E.M)		Comparison of intact and denuded uteri	
	Uterus	Denuded uterus	Uterus	Denuded uterus	EC50	Emax
Nonpregnant	$4.9 \times 10^{-10} \pm 1.7 \times 10^{-10}$	$1.1 \times 10^{-10} \pm 2.1 \times 10^{-11}$	61.2 ± 2.9	51.4 ± 1.9	#	#
Day 5	$7.7 \times 10^{-10} \pm 5.3 \times 10^{-11}$	$3.3 \times 10^{-11} \pm 6.9 \times 10^{-12}$	63.0 ± 5.1	30.9 ± 2.0	ns	###
Day 15	$2.9 \times 10^{-11} \pm 1.6 \times 10^{-12}$	$2.5 \times 10^{-11} \pm 6.5 \times 10^{-12}$	57.1 ± 3.3	$46.0 \pm 2.9^{***}$	ns	ns
Day 18	$6.0 \times 10^{-11} \pm 1.8 \times 10^{-12}$	$2.4 \times 10^{-11} \pm 5.0 \times 10^{-12}$	56.9 ± 4.5	39.7 ± 2.6	ns	###
Day 20	$3.0 \times 10^{-10} \pm 1.9 \times 10^{-11}$	$2.1 \times 10^{-11} \pm 3.9 \times 10^{-12}$	$32.2 \pm 2.2^{***}$	40.3 ± 2.3	ns	#
Day 22	$1.1 \times 10^{-9} \pm 5.5 \times 10^{-10}$	$1.0 \times 10^{-10} \pm 4.7 \times 10^{-11}$	$-4.5 \pm 3.4^{***}$	37.7 ± 3.5	ns	###

*: compared to the previous gestational day; #: compared to the intact uteri; ns: not significant.

$p < 0.05$.

$p < 0.001$.

*** $p < 0.001$.

rings were sliced from the middle region of each horn (4 rings from 1 rat), including implantation sites in the case of pregnancy, and then immediately placed in a heated chamber containing de Jongh solution (137 mM NaCl, 3 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 12 mM NaHCO₃, 4 mM Na₂HPO₄, and 6 mM glucose [pH 7.40]). Carbogen (95% oxygen and 5% carbon dioxide) was continuously bubbled through the buffer. Each muscle ring was thoroughly cleaned of fat and connective tissue, and in pregnant tissues all foetuses and placentas were removed as well. The cleaned muscle strips were individually mounted on tissue holders and were immediately placed in the isolated organ bath chambers. Each chamber contained 10 ml of buffer, its temperature was maintained at around 37 °C, and carbogen was bubbled through it. After mounting, the initial resting tension was set to about 1.5 g, and the strips were allowed to equilibrate for 60 min with a buffer change every 15 min. Rhythmic contractions of uterine rings were achieved by adding 25 mM KCl to the chambers. When the response became stable (after about 5–7 min), contractions were recorded for 5 min. All the above-mentioned experiments were also carried out after the removal of the endometrium (denuded uteri). This means that first the cleaned uterine strip was carefully turned inside out, and then the endometrial layer was gently scraped off with the blunt side of a blade.

3.3.2. Leptin and adiponectin studies

Leptin (PeproTech EC, Ltd., London, United Kingdom) or adiponectin (Sigma-Aldrich Kft., Budapest, Hungary) were added to each chamber of the organ bath at 5-minute intervals in a cumulative manner. Contractile responses were observed in the concentration range of 10^{-12} – 10^{-8} M. Stock solutions of both drugs were prepared with distilled water and were stored at -20 °C. The working dilutions were made right before the start of the experiment. The tension of the myometrial rings was measured with a strain gauge transducer (SG-02; MDE

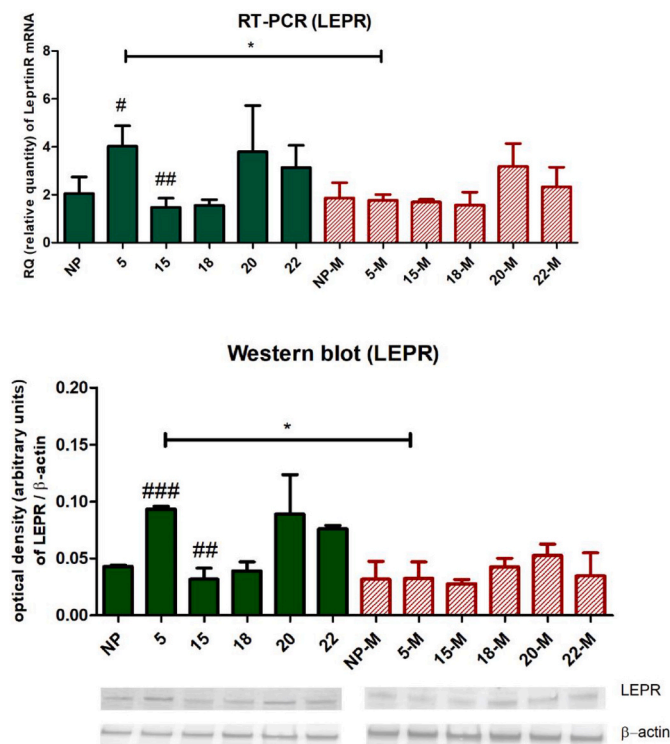


Fig. 4. RT-PCR and Western blot analysis of the expression of leptin receptor (LEPR, 156 kDa) and β -actin (42 kDa). The full columns show the expressions in the intact uterus, and the striped columns represent the myometrial expressions. NP: nonpregnant intact uterus, NP-M: nonpregnant denuded myometrium, #: compared to the previous gestational day; *: compared to the intact uteri; * $p < 0.05$, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$.

GmbH., Walldorf, Germany). For recording and analysis, we used the SPEL Advanced ISOSYS Data Acquisition System (MDE GmbH, Walldorf, Germany). The areas under curves of 5-minute periods were evaluated, and the effects of leptin or adiponectin were expressed as a percentage of the KCl-induced contractions. Statistical analysis was done with ANOVA Tukey's test using Prism 5.01 software (GraphPad Software, San Diego, CA, RRID:SCR_002798).

3.4. Receptor expression measurements

3.4.1. Real-time quantitative reverse transcription-PCR (RT-PCR)

3.4.1.1. Tissue isolation. Pregnant uterus samples were placed in RNA-later Solution (Sigma-Aldrich Kft., Budapest, Hungary) and then stored at -75°C until the extraction of total RNA.

Total RNA preparation from tissue. Total cellular RNA was isolated by extraction with guanidinium thiocyanate-acid-phenol-chloroform according to the protocol of Chomczynski and Sacchi [35]. After precipitation with isopropanol, the RNA was washed with 75% ethanol and resuspended in diethyl pyrocarbonate-treated water. RNA purity was measured at an optical density of 260/280 nm with BioSpec Nano (Shimadzu, Japan); all samples exhibited an absorbance ratio in the range of 1.6–2.0.

3.4.1.2. RT-PCR. Reverse transcription and amplification of the PCR products were performed by using the TaqMan RNA-to-CT-Step One Kit (Thermo Fisher Scientific, Budapest, Hungary) and an ABI StepOne Real-Time cyclers. Reverse-transcriptase PCR amplifications were performed as follows: at 48°C for 15 min and at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. The samples of qPCR experiments contained “no-template” control. The generation of specific

PCR products was confirmed by melting curve analysis. The following primers were used: assay ID Rn01483784_m1 for AdipoR1, Rn01463173_m1 for AdipoR2, Rn01433205_m1 for LEPR and Rn00667869_m1 for β -actin as endogenous control (Thermo Fisher Scientific, Budapest, Hungary). All samples were run in triplicate. The fluorescence intensities of the probes were plotted against PCR cycle number. The amplification cycle displaying the first significant increase in the fluorescence signal was defined as the threshold cycle (CT).

3.4.2. Western blot analysis

The uterine tissues from pregnant animals (tissue between two implantation sites) were homogenized using a Micro-Dismembrator (Sartorius AG, Germany) and centrifuged at $5000 \times g$ for 15 min at 4°C in RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Budapest, Hungary) with protease and phosphatase inhibitor cocktail. Total protein amounts from the supernatant were determined with spectrophotometry (BioSpec-nano, Shimadzu, Japan).

25 μg of protein per well was used for electrophoresis on 4–12% NuPAGE Bis-Tris Gel in XCell SureLock Mini-Cell Units (Thermo Fisher Scientific, Budapest, Hungary). Proteins were transferred from gels to nitrocellulose membranes by using the iBlot Gel Transfer System (Thermo Fisher Scientific, Budapest, Hungary). Antibody binding was detected with the WesternBreeze Chromogenic Western blot immunodetection kit (Thermo Fisher Scientific, Budapest, Hungary). The blots were incubated overnight, at 4°C , on a shaker with AdipoR1 (1:300, Bioss Antibodies, Cat# bs-0610R, RRID:AB_10857625), AdipoR2 (1:300, Bioss Antibodies, Cat# bs-0611R, RRID:AB_10857621), leptin receptor (1:300, Bioss Antibodies, Cat# bs-0109R, RRID:AB_10852741) and β -actin (1:300, Bioss Antibody, Cat# bs-0061R, RRID:AB_10855480) polyclonal primary antibody in the blocking buffer. The incubation of the secondary antibody solution was carried out based on the protocol of the WesternBreeze® Chromogenic Immunodetection Kit. Images were taken with the EDAS290 imaging system (Kodak Ltd., Hungary), the optical densities of immunoreactive bands were determined with Kodak 1D Images analysis software. Optical densities were expressed as arbitrary units after the subtraction of the local area background.

3.5. Fluorescent immunohistochemistry

The dissected tissue samples of uterus were processed for fluorescent microscopy. For triple-labelling fluorescent immunohistochemistry, 5 μm -thick cryosections were prepared from different days of gestation. After washing in TBS with 0.025% Triton X-100 and blocking in TBS containing 1% bovine serum albumin (Sigma-Aldrich, Budapest, Hungary) and 10% normal goat serum (Sigma-Aldrich, Budapest, Hungary) (2 h, room temperature), the samples were incubated overnight with anti-AdipoR1 (rabbit; Novus Biologicals, Cat# NBP2-67631, RRID:AB_2893422 final dilution 1:100), anti-AdipoR2 (mouse; Santa Cruz Biotechnology, Cat# sc-514045, temporary ID is RRID:AB_2895612 final dilution 1:100) and anti-Leptin R (chicken; Novus Biologicals, Cat# NBP2-21062, temporary ID is RRID:AB_2895613 final dilution 1:200) primary antibodies at 4°C . After washing in TBS, samples were incubated with anti-rabbit Alexa Fluor 488 (Thermo Fisher Scientific Cat# A32731, RRID:AB_2633280; final dilution 1:200), anti-mouse CyTM3 (Jackson ImmunoResearch Laboratories Cat# 115-165-146, RRID:AB_2338690; final dilution 1:200) and anti-chicken Alexa Fluor 647 (Jackson ImmunoResearch Laboratories Cat# 103-605-155, RRID:AB_2337392; final dilution 1:200) secondary antibodies for 1 h at room temperature. Negative controls were performed by omitting the primary antibodies when no immunoreactivity was observed. Sections were mounted in Fluoroshield™ with DAPI mounting medium (Sigma-Aldrich, Budapest, Hungary) (Fig. 1), observed and photographed with a Zeiss Imager Z.2 fluorescent microscope equipped with an Axiocam 506 mono camera. Simultaneous immunostainings of all investigated receptors were done, but the results of triple-labelling are not shown.

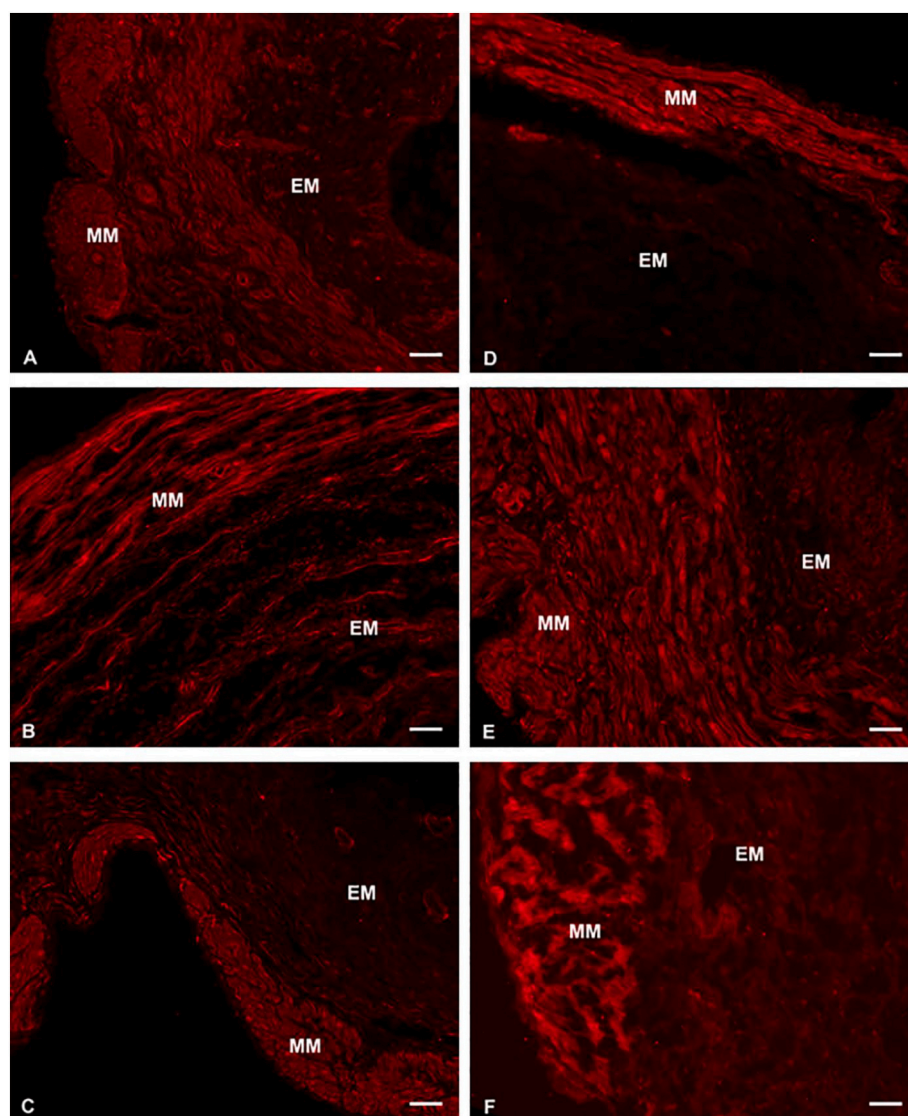


Fig. 5. Representative fluorescent micrographs of cryosections from nonpregnant and pregnant rat uterus after LEPR (red) immunohistochemistry. A: nonpregnant, B: gestational day 5, C: gestational day 15, D: gestational day 18, E: gestational day 20, F: gestational day 22, MM: myometrium, EM: endometrium. Scale bar: 50 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Instead, leptin and adiponectin receptors are shown individually for better visibility and interpretability.

4. Results

4.1. Leptin

4.1.1. *In vitro* contractility

Leptin modified the KCl-induced contraction responses both in nonpregnant and pregnant uteri (Fig. 2).

Leptin reduced nonpregnant uterine contractions in a concentration-dependent manner. This relaxant effect was significantly decreased in denuded uteri.

In pregnant uteri, leptin also showed an inhibitory effect on the contractions. Leptin had a strong relaxing effect in the intact uterus in the early phase of pregnancy, but its action dramatically decreased towards the end of gestation. Particularly, the relaxant effect of leptin ceased on the last day of pregnancy.

In contrast, the contractions of the denuded pregnant uteri were reduced in the presence of leptin on each investigated gestational day. A gradual decrease was observed in the relaxing effect from day 5 to day

15, but from there the mean maximal relaxation did not change significantly until the end of pregnancy.

Comparing the maximum of the relaxing effect (E_{max} values) in intact and denuded uteri, the mean leptin-induced relaxations of denuded uteri were significantly reduced on days 5 and 15, and significantly increased on days 20 and 22 (Fig. 3, Table 1).

4.1.2. Receptor expression (LEPR)

Leptin receptor is expressed both in nonpregnant and pregnant rat uteri. On pregnancy day 5 the highest expression was detected during the gestational period. On days 15 and 18 the expression was reduced, but higher expressions were detected again in the late phase of gestation (days 20 and 22) although the increase was not significant. The removal of the endometrial layer significantly reduced the amount of receptor mRNA and protein on day 5. However, the denuded uteri did not contain significantly fewer leptin receptors either on other gestational days or in nonpregnant samples (Fig. 4A and B).

4.2. Immunohistochemistry

The intensity of LEPR staining was strong both in nonpregnant and

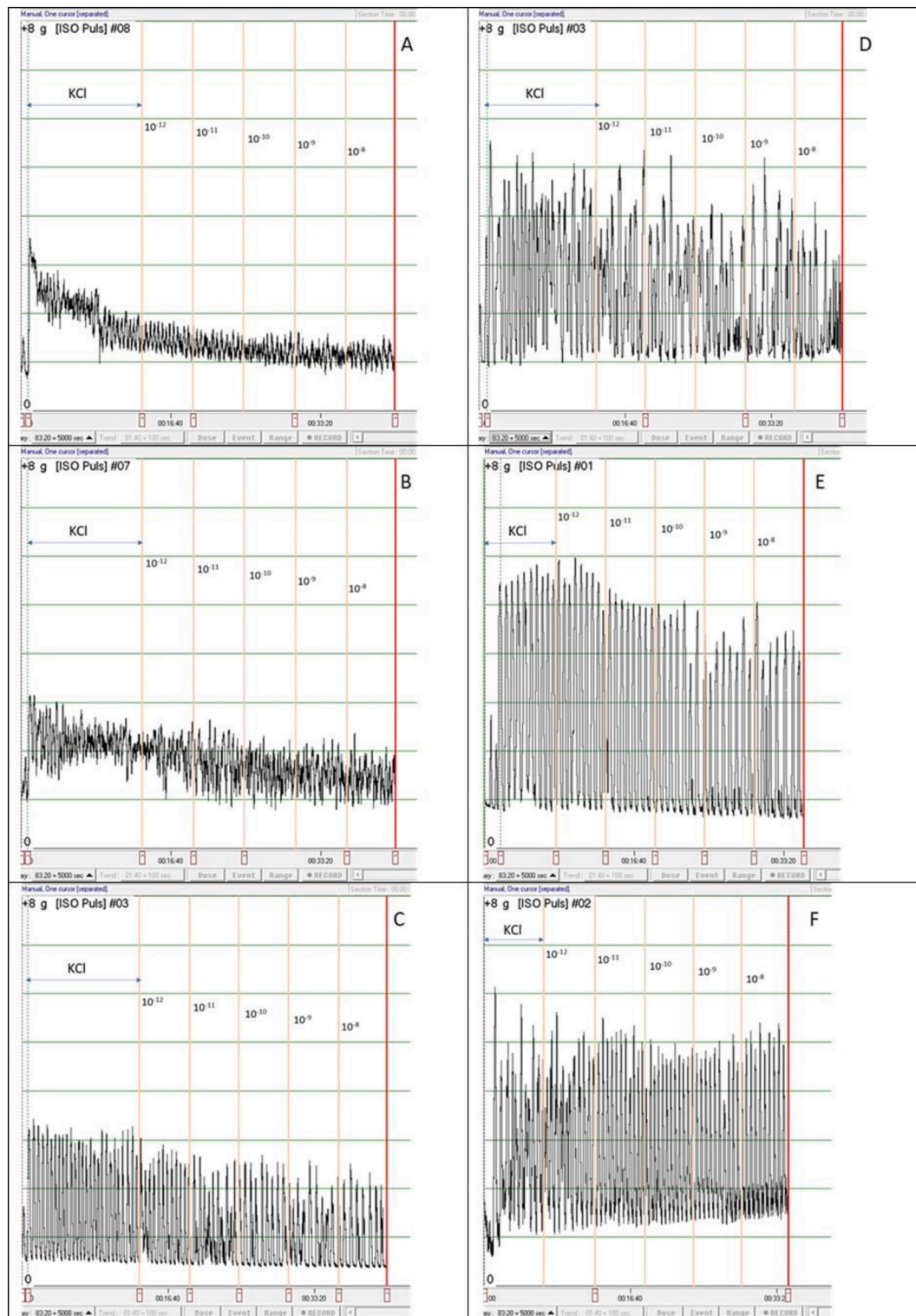


Fig. 6. Representative raw traces of KCl-stimulated uterine contractions and the effects of cumulative doses of adiponectin on nonpregnant (A) and pregnant (B-F) uteri.

pregnant samples. In the nonpregnant uteri, LEPRs were distributed mainly in the circular and longitudinal layers of the myometrium. In the pregnant samples, staining revealed that the presence of LEPR was mostly restricted to the myometrial layer, except on day 5, when higher intensities were detected in the endometrial layer (Fig. 5).

4.3. Adiponectin

4.3.1. *In vitro* contractility

Adiponectin modified the KCl-induced contraction responses both in nonpregnant and pregnant uteri (Fig. 6).

In nonpregnant rat uterus, adiponectin caused dose-dependent uterine relaxation. A significant reduction was detected in the relaxing

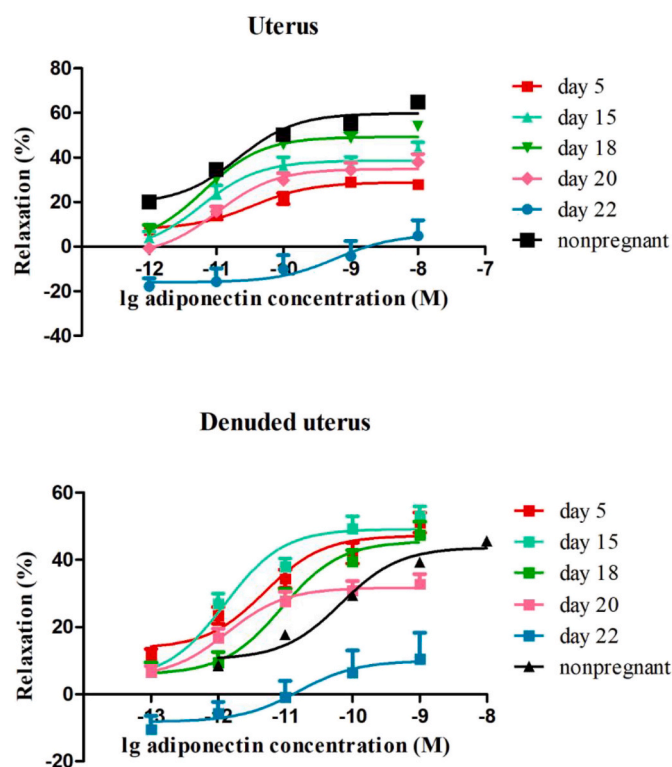


Fig. 7. Effects of adiponectin on KCl-induced contractions of nonpregnant and pregnant rat uterus or endometrium-denuded uterus on different days of gestation. Cumulative concentrations of adiponectin in the range of 10^{-12} to 10^{-8} M reduced uterine contractions in each case. Adiponectin caused dose-dependent relaxation in the early, mid, and late phases of pregnancy. The change in contraction was calculated via the area under the curve and expressed in $\% \pm$ S.E.M., $n = 6$ for each group.

effect when the endometrial layer was removed.

Adiponectin reduced the contractility of pregnant uteri. The relaxant effect of adiponectin on pregnant uteri showed an increase from pregnancy day 5 to day 18. A decrease in the inhibitory effect was seen on the 20-day pregnant uterus, and its relaxant action practically ceased on the last day of gestation.

In denuded uteri, a potent relaxing effect was observed in the early phase of pregnancy (days 5, 15 and 18), but a significant decrease was seen in the mean maximal inhibition on day 20. On the last day of pregnancy, the effect of adiponectin ceased in the case of denuded uteri, too. Accordingly, the mean maximal relaxations of denuded uteri were significantly higher on days 5, 15 and 22 as compared with the whole uteri. The gradual decreases in relaxing effect both in whole and denuded uteri from gestational days 18 to 22 were spectacular (Fig. 7, Table 2).

4.3.2. Receptor expression (AdipoR1 and AdipoR2)

The expression of AdipoR1 was the highest in nonpregnant and 5-day-pregnant uteri, in both cases the removal of the endometrium reduced mRNA and protein expressions, although the decrease in protein expression was not significant. From gestational day 15, the expressions of the receptors were moderated, and endometrial denuding did not significantly modify mRNA and protein levels from day 18 (Fig. 8).

AdipoR2 was identified both in nonpregnant and pregnant uteri. Receptor mRNA and protein expressions peaked on gestational day 15, but after that a significant decrease was detected till the end of pregnancy. The endometrial removal dramatically reduced receptor mRNA and protein expressions on gestational days 15 and 20. However, AdipoR2 was basically minimized in late pregnant uteri (days 18–22),

Table 2

EC50 values and the mean maximal relaxing effect of adiponectin (10^{-12} – 10^{-8} M) on rat uteri and denuded myometria.

	EC50 (M \pm S.E.M.)		Emax (% \pm S.E.M)		Comparison of intact and denuded uterus	
	Uterus	Denuded uterus	Uterus	Denuded uterus	EC50	Emax
Nonpregnant	$4.0 \times 10^{-11} \pm 8.6 \times 10^{-12}$	$2.3 \times 10^{-10} \pm 9.9 \times 10^{-11}$	62.7 ± 2.6	46.4 ± 2.8	ns	###
Day 5	$5.2 \times 10^{-11} \pm 1.6 \times 10^{-12}$	$4.7 \times 10^{-12} \pm 7.8 \times 10^{-13}$	30.2 ± 2.5	48.8 ± 2.8	##	###
Day 15	$2.1 \times 10^{-10} \pm 1.1 \times 10^{-11}$	$2.3 \times 10^{-12} \pm 6.0 \times 10^{-13}$	37.6 ± 3.0	49.7 ± 2.9	#	##
Day 18	$2.3 \times 10^{-11} \pm 7.9 \times 10^{-12}$	$1.6 \times 10^{-11} \pm 5.7 \times 10^{-12}$	$50.8 \pm 2.7^{***}$	45.9 ± 3.7	ns	ns
Day 20	$1.1 \times 10^{-11} \pm 1.5 \times 10^{-12}$	$2.6 \times 10^{-12} \pm 5.9 \times 10^{-13}$	$36.2 \pm 2.8^{**}$	$32.9 \pm 2.5^{**}$	###	ns
Day 22	$8.4 \times 10^{-10} \pm 6.8 \times 10^{-11}$	$2.8 \times 10^{-11} \pm 7.3 \times 10^{-12}$	$6.5 \pm 6.8^{***}$	$17.0 \pm 6.4^{**}$	ns	#

*: compared to the previous gestational day; #: compared to the intact uteri; ns: not significant.

$p < 0.05$.

$p < 0.01$.

$p < 0.001$.

** $p < 0.01$.

*** $p < 0.001$.

although the expression of AdipoR2 was significantly reduced in the denuded myometrium (Fig. 9).

4.4. Immunohistochemistry

In consistence with the results of RT-PCR and Western blot analysis, the uterine smooth muscle and the endometrial layers of nonpregnant and early pregnant samples exhibited a strong immunostaining for AdipoR1. Towards the end of gestation, lower immunoreactivities were observed, and receptors were distributed mostly in the muscular layer (Fig. 10).

The lowest intensities of AdipoR2 stainings were seen in nonpregnant, 5-day and 18-day pregnant samples. However, uterine smooth muscle cells showed the highest immunoreactivities on gestational day 15. Also, the endometrial presence of AdipoR2 was stronger in the mid and late phases of pregnancy (days 15 and 20) (Fig. 11).

5. Discussion

Besides their main roles as regulators of metabolic functions and glucose homeostasis, certain adipokines have been proved to affect smooth muscle functions in different organs [36]. Relevantly, it was shown that leptin inhibits uterine contractions both in humans and rats [16,17]. However, there is no data about how the effect of leptin changes during the period of pregnancy. As part of this study, we investigated the impact of leptin on uterine contractions on different gestational days.

The results from Western blot analysis and immunohistochemical studies show that leptin receptors are mainly expressed in the

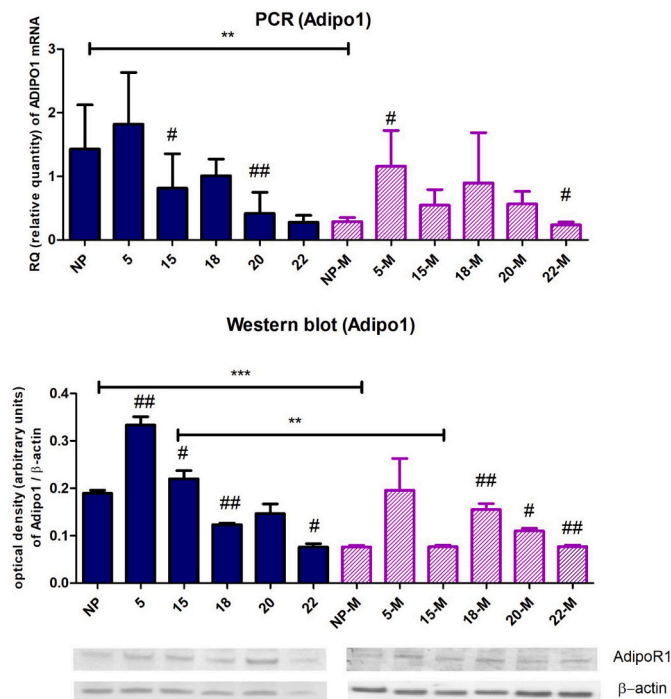


Fig. 8. RT-PCR and Western blot analysis of the expression of adiponectin1 receptor (Adipo1, ~52 kDa) and β -actin (42 kDa). The full columns show the expressions in the intact uterus, and the striped columns represent the myometrial expressions. NP: nonpregnant intact uterus, NP-M: nonpregnant denuded myometrium, #: compared to the previous gestational day; *: compared to the intact uteri; ** $p < 0.01$, *** $p < 0.001$, # $p < 0.05$, ## $p < 0.01$.

myometrial layer of nonpregnant uteri. Although the reduction in LEPR expression induced by endometrium removal was not significant, we detected a decreased relaxant response to leptin in nonpregnant myometrium. This finding suggests that even a very small amount of endometrial LEPR may modify contractility in nonpregnant uteri.

In early gestation (day 5), the uterine presence of LEPR is significantly increased with a considerably higher expression in the endometrium. However, in the mid-term (days 15, 18), its expression decreases, and most of the receptors seem to be distributed in the myometrium. The amount of both endometrial and myometrial leptin receptors tends to rise in the last phase of gestation (days 20, 22). The leptin-induced relaxant effect in the intact uterus gradually decreases towards the end of pregnancy, but when the endometrial layer is removed, leptin can maintain its uterorelaxant effect throughout gestation. We found that the lack of endometrium reduces the relaxing effect of leptin on gestational day 18, but from day 20, the absence of endometrial receptors does not allow a further decrease in the relaxing effect and maintains the action of leptin on a constant level. Although the immunohistochemical studies were not quantitative, the tendency of alterations in endometrial and myometrial LEPRs is spectacular and confirms the RT-PCR and Western blot results. On the last days of gestation (days 20 and 22), the expression of endometrial receptors is negligible, therefore myometrial LEPRs are responsible for the responses, but they may lose their significance in relaxation towards labor. This phenomenon would not be unique, since we made a similar assumption in our previous studies with kisspeptin [37]. We hypothesize that in nonpregnant cases and in the early and mid-terms, both endometrial and myometrial leptin receptors are responsible for relaxation. However, it is obvious that the change in the relaxing effect of leptin is not parallel with the modification of LEPR expression during the gestational period, which suggests that the alteration of signal mechanisms or the sensitivity of LEPRs might be responsible for the weaker or even ceasing inhibitory effect at term. Since the major signal transduction pathway for LEPRs is the JAK/STAT

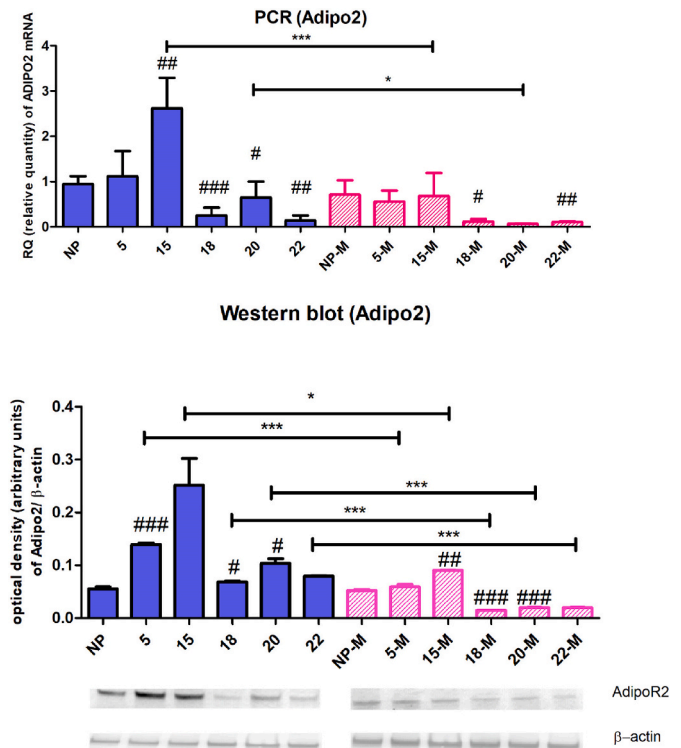


Fig. 9. RT-PCR and Western blot analysis of the expression of adiponectin2 receptor (Adipo2, ~52 kDa) and β -actin (42 kDa). The full columns show the expressions in the intact uterus, and the striped columns represent the myometrial expressions. NP: nonpregnant intact uterus, NP-M: nonpregnant denuded myometrium, #: compared to the previous gestational day; *: compared to the intact uteri; * $p < 0.05$, *** $p < 0.001$, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$.

cascade, it is possible that the modified phosphorylation/dephosphorylation of different proteins is responsible for this alteration [38]. Additionally, leptin can alter numerous signal pathways, such as inducing vasoconstriction in vascular tissues by increasing intracellular Ca^{2+} levels [39], or provoking endothelium-dependent relaxation by inducing nNOS expression [40]. Any change in these mechanisms might also modify the final response in uterine smooth muscle.

Current knowledge about the role of adiponectin in uterine contractions is very limited, therefore the experimental protocol followed during our experiments was the same as that for leptin. Earlier, the presence of AdipoR1 and AdipoR2 receptors was confirmed in pregnant uterus [41], but now we have proved that adiponectin has an inhibitory effect on myometrial contractions. The interpretation of the effect of adiponectin is more complex because its action is mediated via two receptors. The stimulation of these receptors activates mainly the AMPK and PPAR α pathways [42].

The expression of AdipoR1 receptor is significantly decreased after endometrium removal in nonpregnant samples. In contrast, AdipoR2 receptors are mainly distributed in the myometrium of nonpregnant uteri. Adiponectin relaxed both the intact and the denuded nonpregnant uteri, but its effect was decreased in the absence of endometrium. These findings suggest that AdipoR1 receptors have a crucial impact on nonpregnant uterine contractions, and endometrial AdipoR1 receptors enhance the adiponectin-mediated relaxation in nonpregnant uteri. We also found that adiponectin can reduce the contractions of both the intact and the denuded uteri in early and mid-term pregnancies, but on the last day of gestation, adiponectin could not retain its uterorelaxant effect in either case. In positive correlation with these results, the expressions of both investigated AdipoRs are reduced towards the end of gestation, and these modifications are clearly revealed by

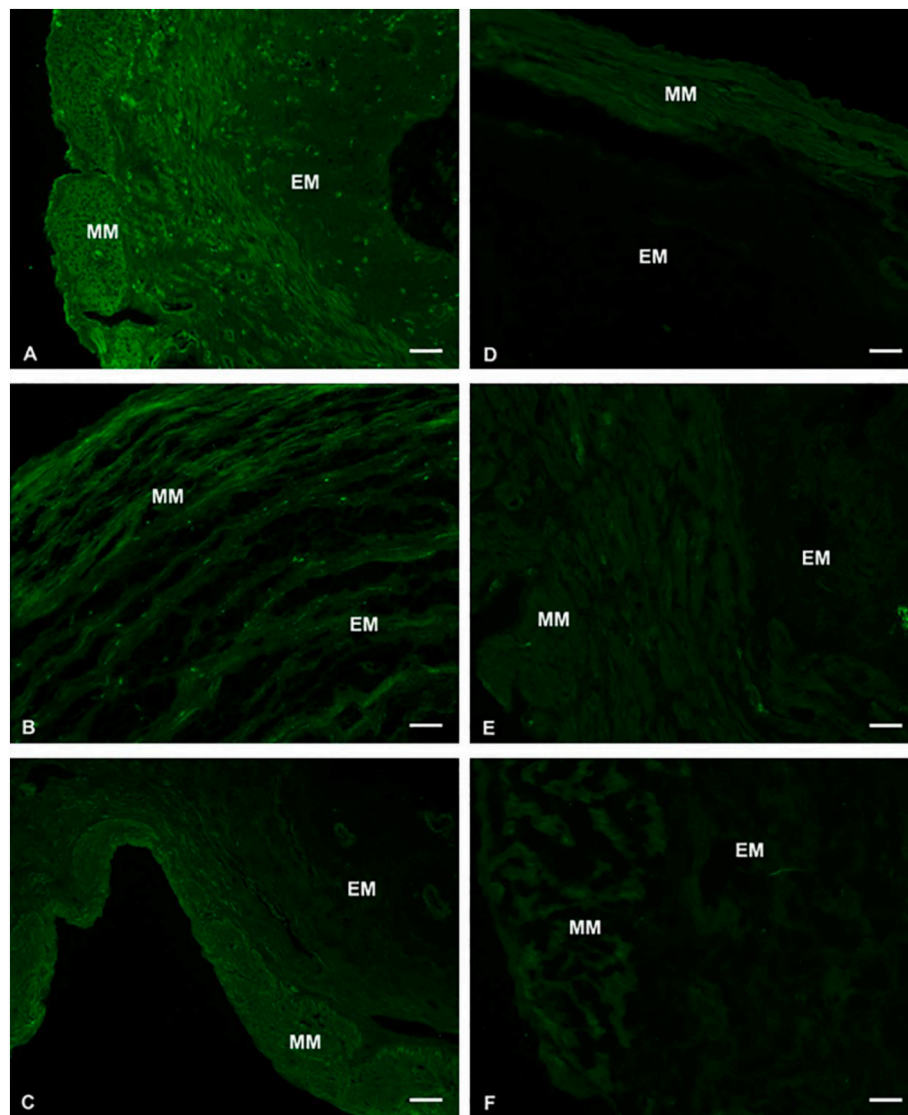


Fig. 10. Representative fluorescent micrographs of cryosections from nonpregnant and pregnant rat uterus after AdipoR1 immunohistochemistry. A: nonpregnant, B: gestational day 5, C: gestational day 15, D: gestational day 18, E: gestational day 20, F: gestational day 22, MM: myometrium, EM: endometrium. Scale bar: 50 μ m.

immunohistochemistry both in the myometrial and the endometrial layers. While endometrial AdipoRs play a role in the relaxation of nonpregnant uteri, the opposite can be seen during most of the gestational period. On gestational days 5 and 15, the relaxing effects of adiponectin are increased in the absence of the endometrium, presumably because of the lack of endometrial receptors. We hypothesize that in early pregnancy, endometrial AdipoRs are likely to mediate smooth muscle contraction. Interestingly, on days 18, 20 and 22 of gestation, no significant differences were measured in the mean maximal relaxation values in the denuded myometrium, suggesting that endometrial AdipoRs have no significant impact on uterine response. On the last gestational day, however, the removal of the endometrium again enhances relaxation. However, this increase in the E_{\max} value is less than that in the case of leptin, implying the major role of endometrial LEPR in uterine contractions in term. The modification of the relaxing effect of adiponectin was in parallel with the alteration of AdipoRs, suggesting that the sensitivities and the signal mechanisms of AdipoR1 and AdipoR2 are not significantly changed during the gestational period.

Few limitations of our study must be revealed. Investigations of the signaling pathways of LEPR and AdipoRs were not carried out in these series of experiment. The present study also does not cover the identification of possible modifications in signaling mechanism regarding

LEPRs and AdipoRs, and the differentiation between the roles of endometrial and myometrial AdipoR1 and AdipoR2 receptors in uterine contractility.

6. Conclusion

Both leptin and adiponectin play a role in the relaxation of the pregnant uterus, but their efficacy significantly decreases towards the end of gestation. We suppose that both adipokines have a role in the maintenance of uterine quiescence in early and mid-pregnancy, but their involvement is gradually reduced towards labor. Besides other factors, endometrial LEPR and AdipoRs have regulatory roles in the actions of these adipokines and possibly in the fine-tuning of uterine contractions both in nonpregnant and pregnant conditions. To the best of our knowledge, this is the first evidence for the ontogeny of LEPR, AdipoR1 and AdipoR2 receptor expression and distribution, and for the modification of these adipokines in uterine contractions throughout gestation. Our results help to better understand the uterine effects exerted by these adipokines during pregnancy and might predict the importance of these adipokines in uterine contractions under altered adipokine level conditions, such as obesity.

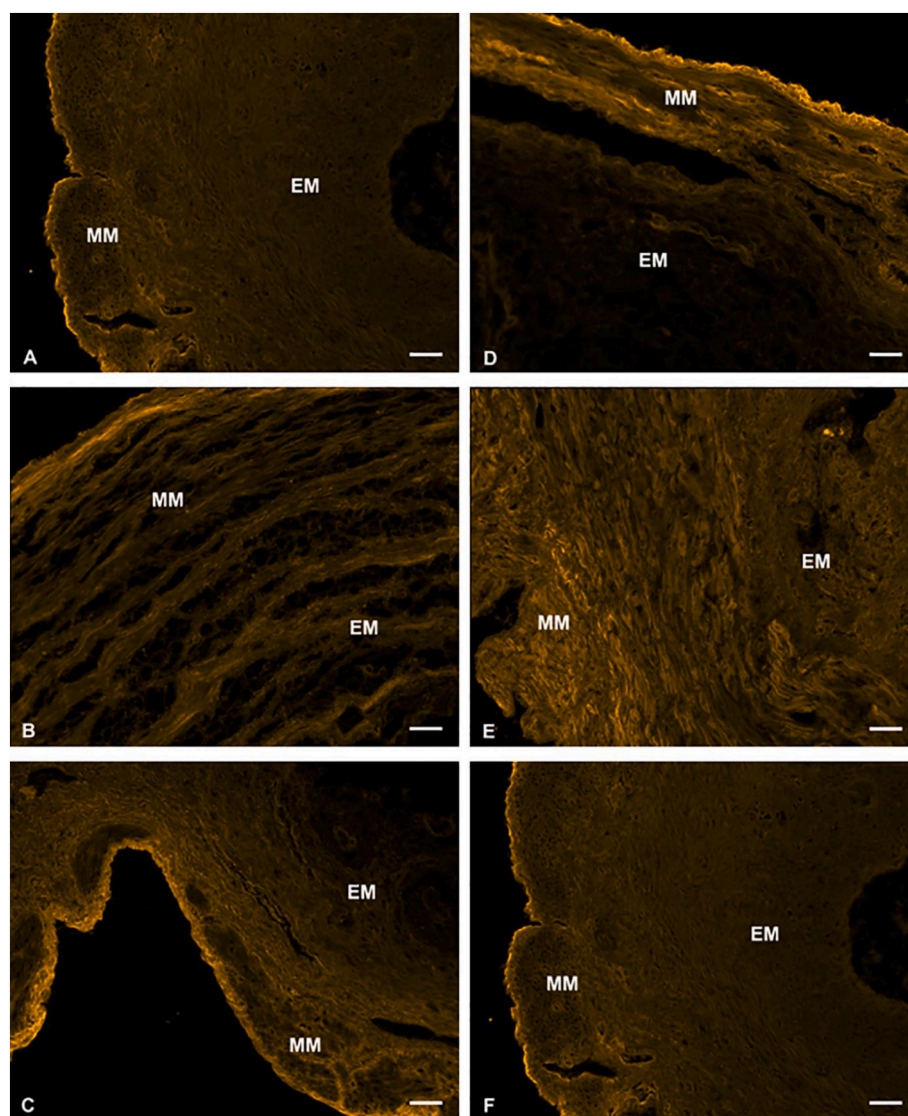


Fig. 11. Representative fluorescent micrographs of cryosections from nonpregnant and pregnant rat uterus after AdipoR2 immunohistochemistry. A: nonpregnant, B: gestational day 5, C: gestational day 15, D: gestational day 18, E: gestational day 20, F: gestational day 22, MM: myometrium, EM: endometrium. Scale bar: 50 μ m.

Author contributions

A. Schaffer performed experiments, acquired, analyzed, interpreted the data and wrote the manuscript, E. Ducza, N. Bódi performed the research, acquired, analyzed and interpreted the data; Z. Szalai, M. Mirdamadi, T. Barna performed experiments; M. Bagyánszki, K. F. Szűcs analyzed and interpreted the data; R. Gáspár designed and supervised the research and wrote the manuscript. All authors were involved in drafting and revising the manuscript.

Funding source

Project No. TKP2021-EGA-32 has been implemented with the support provided by the Ministry of Innovation and Technology of Hungary from the National Research, Development, and Innovation Fund, financed under the TKP2021-EGA funding scheme.

Declaration of competing interest

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

Acknowledgment

We thank Cedars-Sinai Medical Center's International Research and Innovation in Medicine Program, and the Association for Regional Cooperation in the Fields of Health, Science and Technology (RECOOP HST Association) for their support.

References

- [1] S.M. Smith-Kirwin, et al., Leptin expression in human mammary epithelial cells and breast milk, *J. Clin. Endocrinol. Metab.* 83 (1998) 1810.
- [2] A. Sahu, Minireview: a hypothalamic role in energy balance with special emphasis on leptin, *Endocrinology* 145 (2004) 2613–2620.
- [3] H. Masuzaki, et al., Nonadipose tissue production of leptin: leptin as a novel placenta-derived hormone in humans, *Nat. Med.* 39 (3) (1997) 1029–1033.
- [4] S. Guilmeau, R. Ducroc, A. Bado, Leptin and the gastrointestinal tract, *Handb. Biol. Act. Pept.* (2006) 1071–1076, <https://doi.org/10.1016/B978-012369442-3/50150-1>.
- [5] Y. Zhang, S. Chua, Leptin function and regulation, in: *Comprehensive Physiology* 8, John Wiley & Sons, Inc., 2017, pp. 351–369.

- [6] D.L. Morris, L. Rui, in: *Recent Advances in Understanding Leptin Signaling and Leptin Resistance* 297, 2009, pp. 1247–1259, <https://doi.org/10.1152/ajpendo.00274.2009>.
- [7] M.G. Myers, R.L. Leibel, R.J. Seeley, M.W. Schwartz, Obesity and leptin resistance: distinguishing cause from effect, *Trends Endocrinol. Metab.* 21 (2010) 643–651.
- [8] P. Tataranni, et al., Adiposity, plasma leptin concentration and reproductive function in active and sedentary females, *Int. J. Obes.* 219 (21) (1997) 818–821.
- [9] K. Ogura, et al., Effects of leptin on secretion of LH and FSH from primary cultured female rat pituitary cells, *Eur. J. Endocrinol.* 144 (2001) 653–658.
- [10] K. Skorupskaitė, J.T. George, R.A. Anderson, The kisspeptin-GnRH pathway in human reproductive health and disease, *Hum. Reprod. Update* 20 (2014) 485–500.
- [11] Y.A. Pankov, Kisspeptin and leptin in the regulation of fertility, *Mol. Biol.* 49 (2015) 631–637.
- [12] D. Israel, S. Chua, Leptin receptor modulation of adiposity and fertility, *Trends Endocrinol. Metab.* 21 (2010) 10–16.
- [13] A.M. Clempson, et al., Evidence that leptin genotype is associated with fertility, growth, and milk production in Holstein cows, *J. Dairy Sci.* 94 (2011) 3618–3628.
- [14] E. Dos Santos, et al., Adiponectin and leptin systems in human endometrium during window of implantation, *Fertil. Steril.* 97 (2012) 771–778.e1.
- [15] S. Moschos, J.L. Chan, C.S. Mantzoros, Leptin and reproduction: a review, *Fertil. Steril.* 77 (2002) 433–444.
- [16] A.T. Moynihan, M.P. Hahir, S.V. Glavey, T.J. Smith, J.J. Morrison, Inhibitory effect of leptin on human uterine contractility in vitro, *Am. J. Obstet. Gynecol.* 195 (2006) 504–509.
- [17] S. Mumtaz, S. Alsaif, S. Wray, K. Noble, Inhibitory effect of visfatin and leptin on human and rat myometrial contractility, *Life Sci.* 125 (2015) 57–62.
- [18] N. Stefan, M. Stumvoll, Adiponectin - its role in metabolism and beyond, *Horm. Metab. Res.* 34 (2002) 469–474.
- [19] Y. Arita, et al., Paradoxical decrease of an adipose-specific protein, adiponectin, Obesity. *Biochem. Biophys. Res. Commun.* 257 (1999) 79–83.
- [20] W.-S. Yang, et al., Weight reduction increases plasma levels of an adipose-derived anti-inflammatory protein, Adiponectin. *J. Clin. Endocrinol. Metab.* 86 (2001) 3815–3819.
- [21] U.B. Pajvani, et al., Structure-function studies of the adipocyte-secreted hormone Acrp30/Adiponectin: implications for metabolic regulation and bioactivity, *J. Biol. Chem.* 278 (2003) 9073–9085. *
- [22] T.P. Combs, et al., Sexual differentiation, pregnancy, calorie restriction, and aging affect the adipocyte-specific secretory protein adiponectin, *Diabetes* 52 (2003) 268–276.
- [23] A.H. Berg, T.P. Combs, P.E. Scherer, Acrp30/adiponectin: an adipokine regulating glucose and lipid metabolism, *Trends Endocrinol. Metab.* 13 (2002) 84–89.
- [24] C. Hug, et al., T-cadherin is a receptor for hexameric and high-molecular-weight forms of Acrp30/adiponectin, *Proc. Natl. Acad. Sci.* 101 (2004) 10308–10313.
- [25] A. Sowka, P. Dobrzyn, Role of perivascular adipose tissue-derived adiponectin in vascular homeostasis, *Cells* 10 (2021) 1485.
- [26] T. Takeuchi, Y. Adachi, Y. Ohtsuki, M. Furihata, Adiponectin receptors, with special focus on the role of the third receptor, T-cadherin, in vascular disease, *Med. Mol. Morphol.* 403 (40) (2007) 115–120.
- [27] T. Yamauchi, et al., Cloning of adiponectin receptors that mediate antidiabetic metabolic effects, *Nat* 4236941 (423) (2003) 762–769.
- [28] S.S. Deepa, L.Q. Dong, in: *APPL1: Role in Adiponectin Signaling and Beyond* 296, 2009, pp. 22–36, <https://doi.org/10.1152/ajpendo.90731.2008>.
- [29] Y. Takemura, et al., Expression of adiponectin receptors and its possible implication in the human endometrium, *Endocrinology* 147 (2006) 3203–3210.
- [30] J.E. Caminos, et al., Expression and regulation of adiponectin and receptor in human and rat placenta, *J. Clin. Endocrinol. Metab.* 90 (2005) 4276–4286.
- [31] S.T. Kim, et al., Adiponectin and adiponectin receptors in the mouse preimplantation embryo and uterus, *Hum. Reprod.* 26 (2011) 82–95.
- [32] T. Yamauchi, et al., Targeted disruption of AdipoR1 and AdipoR2 causes abrogation of adiponectin binding and metabolic actions, *Nat. Med.* 133 (13) (2007) 332–339.
- [33] K. Brochu-Gaudreau, et al., Adiponectin action from head to toe, *Endocr.* 371 (37) (2009) 11–32.
- [34] V. Vyas, et al., Adiponectin links maternal metabolism to uterine contractility, *FASEB J.* 33 (2019) 14588–14601.
- [35] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, *Anal. Biochem.* 162 (1987) 156–159.
- [36] S. Alsaif, S. Mumtaz, S. Wray, A short review of adipokines, smooth muscle and uterine contractility, *Life Sci.* 125 (2015) 2–8.
- [37] A. Schaffer, et al., The ontogeny of kisspeptin receptor in the uterine contractions in rats: its possible role in the quiescence of non-pregnant and pregnant uteri, *Eur. J. Pharmacol.* 896 (2021), 173924.
- [38] H.K. Park, R.S. Ahima, Leptin signaling, *F1000Prime Rep.* 6 (2014).
- [39] S. Gomart, et al., Leptin-induced endothelium-independent vasoconstriction in thoracic aorta and pulmonary artery of spontaneously hypertensive rats: role of calcium channels and stores, *PLoS One* 12 (2017), e0169205.
- [40] S. Benkhoff, et al., Leptin potentiates endothelium-dependent relaxation by inducing endothelial expression of neuronal NO synthase, *Arterioscler. Thromb. Vasc. Biol.* 32 (2012) 1605–1612.
- [41] N. Smolinska, et al., Expression of adiponectin and adiponectin receptors 1 and 2 in the porcine uterus, conceptus, and trophoblast during early pregnancy, *Theriogenology* 82 (2014) 951–965.
- [42] T. Kadowaki, T. Yamauchi, Adiponectin receptor signaling: a new layer to the current model, *Cell Metab.* 13 (2011) 123–124.