Immunolocalization of Leptin Receptor and mRNA Expression of Leptin and Estrogen Receptors as well as Caspases in the Chorioallantoic Membrane (CAM) of the Chicken Embryo*

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The chicken chorioallantoic membrane (CAM) is an extra-embryonic structure formed by fusion of the chorion and allantois during early avian development. The CAM is responsible for carrying out three significant processes: (1) respiration, (2) storage of urea, ammonia and uric acid produced by the embryo, (3) resorption of Ca²⁺ from the shell through the chorionic epithelium to be transported into the vasculature of the embryo. CAM development starts at day 4 of incubation. Undifferentiated blood vessels grow very rapidly until day 8, originating a network of capillaries that migrate to reside in the area at the base of the chorion and mediate gas exchange with the external environment. Rapid capillary proliferation continues until day 11 and the vascular system attains its final arrangement three days before hatching (AUSPRUNK et al. 1974). Angiogenesis is the formation of new blood vessels from the existing vasculature, a complex multistep process involving the proliferation, migration, and remodeling of endothelial cells in response to growth factors and cytokines produced by endothelial and non-endothelial cells. One of these factors is leptin, a member of the polypeptide hormone family (SIERRA-HONIGMANN et al. 1998; PARK et al. 2001;
Leptin has been found in many vertebrates; however, the existence of leptin in birds has been controversial (SHARP et al. 2008). The interaction between leptin and leptin receptor (LEPR) is well established; it is conserved across diverse taxa (RØNNESTAD et al. 2010; HAMMOND et al. 2012; PROKOP et al. 2012, 2014), but surprisingly in the in-silico evaluation of 35 taxa of PROKOP (R 2014), and an alternative pathway involving inflammatory cytokines (ARAI et al. 1990) and induces pyroptosis (VANAJA et al. 2015). Similar to oncosis and unlike apoptosis, pyroptosis results in cellular lysis and release of cytosolic contents to the extracellular space (MIAO et al. 2011).

Over the past few decades efforts have been made to reduce, replace, and refine experiments for ethical use of experimental animals. The chicken CAM is used as an experimental model in tests of the biocompatibility/toxicology of organic and inorganic materials (SPANEL-BOROWSKI 1989; VALDES et al. 2002), assessments of the ability of bacterial strains to invade epithelial barriers (ADAM et al. 2002), analysis of metastatic (LOKMAN et al. 2012) and developmental growth potencies of grafted tumor and embryonic tissue samples (NAVARRO et al. 2003), and the angiogenic response to a defined stimulus (RIBATTI et al. 2001; RICHARDSON & SINGH 2003). However, the functional and physiological properties of the CAM are not fully understood. Experimental treatment of the CAM with human leptin stimulated neovascularization (BOULOMIE et al. 1998; RIBATTI et al. 2001), suggest the presence of leptin receptors in this tissue. In order to partially characterize the endocrine properties of the CAM, mechanism of CAM development and sensitivity to hormones, we immunolocalized leptin receptors (the leptin binding domain of the receptor) in this membrane and analyzed the mRNA expression of the long and short forms of the leptin receptor as well as estrogen receptors (α and β). Additionally, in order to assess the participation of programmed cell death in CAM growth and development, the mRNA expression of caspases (-1 and -3) was also analyzed.

Material and Methods

Fertilized eggs (n=50) of the Ross Broiler Breeder strain were set in an incubator (Masalles 65 DIGIT) and were incubated under standard conditions (1-18 days of incubation: t = 37.8°C, relative humidity (RH) = 55%). They were candled on day 5 of the incubation to eliminate unfertilized eggs and dead embryos and subsequently, 36 eggs were selected on embryonic days 12 (E12), 15 (E15) and 18 (E18) (six embryos of each group). They were decapitated and sexed after
analysis of morphology of gonads. Fragments of the chorioallantoic membrane of female and male embryos were isolated for histological analysis or kept frozen in liquid nitrogen until RNA isolation.

Immunohistochemical localization of leptin receptor in the chicken chorioallantoic membrane

Fragments of the CAM were fixed in 4% formaldehyde in phosphate-buffered saline (pH 7.4) and embedded in paraffin by routine procedure. The 6 µm thick sections of the CAM were stained by: (1) routine haematoxylin-eosin technique or (2) immunohistochemical technique with specific antibody for chicken leptin receptor, for which sections were dewaxed in xylene and hydrated through a series of graded ethanol and microwaved in 10mM citric acid (pH 6.0) for antigen retrieval. Next, they were incubated in 1% H2O2 to inhibit endogenous peroxidase activity. Afterwards, sections were incubated with normal goat serum and then with rabbit antiserum raised against recombinant leptin-binding domain (anti-LBD; aminoacids 419-624) of chicken leptin receptor purified by affinity chromatography. The sections were then incubated with the secondary antibody (biotinylated goat anti-rabbit, 1:350 in PBS; Vector Laboratories) and with the avidin-biotin-peroxidase complex StreptABComplex/HPR (DakoCytomation, Denmark). The colour reaction was developed by incubation with a DAB-H2O2 mixture. The negative control was incubated without primary antibody or the primary antibody was preincubated with recombinant chicken LBD before incubation with tissue sections. The resulting immunocytochemical staining was the same as in the negative control, confirming the specificity of the primary antibody against leptin receptor.

RNA isolation

The total RNA of each tissue was isolated by using TRI-Reagent (Molecular Research Center, Cincinnati, USA), according to the manufacturer’s protocol (modified method described by CHOMCZYSKII and SACCHI (1987)). The tissues were homogenized by using UltraTurrax T25 (IKA-Labortechnik, Staufen, Germany) and subsequently phase separation was done with bromo-chloropropane. RNA was precipitated from the aqueous phase by mixing with isopropanol, washing with 75 % ethanol and dissolving in pure RNase free water (Promega, Madison, USA). The concentration of RNA was measured spectrophotometrically using a Biophotometer (Eppendorf, Hamburg, Germany). Each sample was tested for RNA degradation by separation on agarose gels and spectrophotometrical analysis of absorbance at 260/280 nm ratio. The 260/280 nm ratio of RNA was 1.65-1.85. To exclude genomic DNA contamination, RNA samples were treated with DNase (RQ1 RNase-Free DNase, Promega).

Reverse transcription and PCR reactions for leptin receptors, estrogen receptors and caspases

Reverse transcription (RT) reactions were performed in 20 µl volume. The reaction mixture contained 5 µg of total RNA, 200 U M-MuLV reverse transcriptase (Fermentas, Vilnius, Lithuania), 0.5 µg oligo(dT)₁₈ as a primer, 1 mM of each dNTP, 20 U ribonuclease inhibitor (Fermentas) and 4 µl of 5 x reaction buffer containing 250 mM Tris-HCl, 250 mM KCl, 20 mM MgCl₂, and 50 mM DTT (Fermentas). The resulting first strand cDNA (1 µl) was used for PCR reactions. The expression of mRNA encoding leptin receptor (long and short isoforms), estrogen receptors (α and β) and caspases (-1 and -3) in the CAM was determined. 18S rRNA was used as a reference gene. The primer sequences, their localization in the genome, and product size are shown in Table 1. The 25 ml PCR reaction mixture contained 1 µl of cDNA, 0.2 mM of each dNTP, 0.2 µM of each primer, 1.5 mM MgCl₂ and 0.625 U of TaqDNA polymerase (Fermentas). 18S rRNA gene expression was acquired in a separate sample. All PCR reactions were performed in a Thermocycler Gradient (Eppendorf). The cycle profiles were programmed as follows: initial template denaturation for 5 min at 95°C, denaturation at 95°C for 30 s, annealing at gene specific temperature (Table 1) for 30 s, followed by extension at 72°C for 30 s. Table 1 lists the number of cycles and PCR profiles; the final extension step for amplification was increased to 7 min at 72°C. Amplification products were separated by electrophoresis on 1.5 % agarose gels containing ethidium bromide. The PCR products were semi-quantitatively estimated from the density of the gel band using Scion Image for Windows Software (Scion Corporation, Maryland, USA). The relative density of each gene product was compared with 18S rRNA products, and expressed as the mean of 6 samples.

Statistical analysis

Two-way analysis of variance (ANOVA), with two main effects: sex and stage of embryo development, followed by Duncan’s multiple range test was applied to analyze the data. Values are expressed as the mean ± SEM from six determinations and considered significantly different at P<0.05.
Results

Immunohistochemical analysis of leptin receptor in the CAM tissue

Immunohistochemical analysis of the CAM showed that leptin receptor is localized both in the female and male embryos at embryonic days 12 (E12), 15 (E15) and 18 (E18). The intensity of the immunopositive reaction for leptin binding domain of leptin receptor did not significantly differ between the chorionic epithelium and allantoic layer with extended blood vessels. There were no differences in leptin receptor immunoreactivity among the analyzed stages of development or between sexes. Preincubation of 1° antibody against leptin binding domain with chicken LBD or incubation of tissue slides with TBST instead of 1° antibody against leptin receptor abolished staining (Fig. 1).

Fig. 1 Haematoxylin and eosin staining of chorioallantoic membrane (CAM) of chicken embryos and immunolocalization of leptin receptors in the female and male embryonic CAM on day 12, 15 and 18 of embryogenesis (E12, E15 and E18). Negative control tissue was incubated without 1° antibody or 1° antibody was preincubated with recombinant protein – chicken LBD (leptin binding domain of chicken leptin receptor). ABV – allantoic blood vessels, CE – chorionic epithelium, AE – allantoic epithelium, ML – mesodermal layer, ▶ immunoreactive cells (leptin receptor). Scale bar = 20 μm.
Table 1

Positions of oligonucleotide primers in mRNA sequence, GeneBank Accession Numbers, sequences of amplified gene primers, annealing temperature of PCR reaction, number of cycles and product size

<table>
<thead>
<tr>
<th>Gene</th>
<th>Position</th>
<th>Gene Bank Accession Number</th>
<th>Primers sequences: (5’-3’)</th>
<th>Annealing temperature (°C)</th>
<th>Number of cycles in PCR</th>
<th>Product size (bp)</th>
</tr>
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<tr>
<td>LepR (Long isoform)</td>
<td>2641-2660</td>
<td>AF169827</td>
<td>F: CCAAGAGTTGGGATCCGCTGAGT R: GCCACCCTGATCTGGAGTTA</td>
<td>60</td>
<td>30</td>
<td>504</td>
</tr>
<tr>
<td>LepR (Short isoform)</td>
<td>1-18</td>
<td>197-216</td>
<td>F: GAATGAAGAAACTGCTCTGG R: TACAACATCATGCTGATT</td>
<td>56</td>
<td>30</td>
<td>216</td>
</tr>
<tr>
<td>ERα</td>
<td>1522-1542</td>
<td>X03805.1</td>
<td>F: GTGCCCTTAAGTCCATCATCTGCT R: CGTCCAGCATCTCCAGTAAAG</td>
<td>58</td>
<td>30</td>
<td>300</td>
</tr>
<tr>
<td>ERβ</td>
<td>1374-1394</td>
<td>AB036415</td>
<td>F: TGATATGCTCTGGCCATGAC R: CTCTCTGCTCAGCAGTGGTC</td>
<td>55</td>
<td>30</td>
<td>304</td>
</tr>
<tr>
<td>Caspase-1</td>
<td>1183-1203</td>
<td>AF031351.1</td>
<td>F: GATACGTAATCCATCGACCC R: CCATCTCAGCATTGAGCTC</td>
<td>55</td>
<td>27</td>
<td>313</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>696-715</td>
<td>934-956</td>
<td>F: TTAGATTTCTGGTATTGAAGC R: GAAATCCTGTCGAGTGGAGCAG</td>
<td>62</td>
<td>30</td>
<td>261</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>160-179</td>
<td>308-326</td>
<td>F: CGCGTGCATTATCAGCACC R: ACCCGTGGTCACCAGTGA</td>
<td>60</td>
<td>30</td>
<td>167</td>
</tr>
</tbody>
</table>

Fig. 2. Semiquantitative analysis of mRNA expression of long (A) and short (B) isoforms of leptin receptor in the chicken CAM (means ± SEM, n=6). F – female group, M – male group. E12, E15, E18 – days 12, 15, and 18 of chicken embryogenesis.

Fig. 3. Semiquantitative analysis of mRNA expression of estrogen receptor α (A) and β (B) in the chicken CAM (means ± SEM, n=6). F – female group, M – male group. E12, E15, E18 – day 12, 15, and 18 of chicken embryogenesis.
mRNA expression of leptin receptors, estrogen receptors and caspases in the CAM

The analysis of mRNA expression revealed that both long and short isoforms of leptin receptor mRNA are expressed in the chicken CAM on the 12th, 15th, and 18th day of embryogenesis. The mean relative expression of the long form of leptin receptor was not significantly different among analyzed stages of embryogenesis or between sexes. The values were $0.437 \pm 0.041$ in female and $0.401 \pm 0.066$ in male CAM. The expression of mRNA encoding the short form of leptin receptor was significantly lower than expression of the long form of receptor ($0.120 \pm 0.020$ and $0.420 \pm 0.061$, respectively). There were no differences in the short isoform of leptin receptor mRNA expression between female and male CAM ($0.138 \pm 0.018$ and $0.101 \pm 0.018$, respectively) (Fig. 2).

The mRNA expression of estrogen receptors $\alpha$ and $\beta$ was detected in the chicken CAM in each analyzed stage of embryogenesis. The expression of ER$\alpha$ ($0.613 \pm 0.064$) was significantly higher than the expression of ER$\beta$ ($0.179 \pm 0.021$). Neither mRNA expression of ER$\alpha$ nor ER$\beta$ was significantly different in male and female CAM (Fig. 3).

As shown in Fig. 4, mRNA expression of caspase-1 and caspase-3 was detected in all examined experimental groups. The relative expression of caspase-1 was significantly lower than caspase-3 ($0.597 \pm 0.041$ and $1.193 \pm 0.031$, respectively). The mRNA expression of caspase-1 as well as caspase-3 did not differ between female and male CAM (Fig. 4).

Discussion

In the present study, for the first time the leptin binding domain of leptin receptors was immunolocalized in the male and female CAM. There were no differences in the intensity of reactions between sexes and among analyzed stages of embryogenesis, confirmed by mRNA expression analysis. It was also shown that both long and short isoforms of leptin receptor mRNA are expressed in the CAM membrane. It cannot be excluded that the presence of receptors for leptin in the CAM may be related to the process of angiogenesis. This assumption is supported by an investigation in which quail embryos treated with leptin had a significant increase in vascular complexity and density (TALAVERA-ADAME et al. 2008). On the other hand, SU et al. (2012) observed the opposite effect, i.e. significant antiangiogenic activity of leptin, but, interestingly, only in females. This suggests that the mechanism of leptin action on angiogenesis is complicated and requires further study. Nevertheless, detection of leptin receptor mRNAs and protein in the CAM tissue suggest that the influence of leptin on CAM development may depend on the ability of leptin receptors localized in the CAM tissue to interact with the circulating leptin hormone.

Beyond affecting angiogenesis, leptin may also be involved in the regulation of calcium absorption from the egg shell, the major source of calcium for the development of high-calcium consuming organs, e.g. the skeleton, muscles and brain (TUAN et al. 1991; BLOM & LILJA 2004). The domestic chicken begins to mobilize calcium between days 7 and 8 of incubation, corresponding to the initiation of mineralization of skeletal tissue. However, until day 10, the egg yolk is the main source of calcium (TUAN et al. 1991; KARLSON & LILJA 2008), between days 10 and 12, calcium is also mobilized from the shell. At the end of embryonic development, more than 80% of the calcium is derived from the shell (SIMKISS 1975; TUAN 1987). It cannot be excluded that leptin also participates in the process of calcium resorption from the egg shell and incorporation into the embryonic body. The mRNA expression of leptin receptors did not change among analyzed stages of development, whereas the rate of incorporation of calcium into the chorioallantois is not stable during embryogenesis. It is greater for 15- than 12-day-old embryos (CROOKS & SIMKISS 1975). The key step for ion transport through the membrane is the 14th
day of incubation (MORIARTY & HOGBEN 1970) and leptin may be one of many regulatory proteins involved in the process of mineralization in chicken embryos.

This assumption can be supported by a previous study (GRZEGORZEWSKA et al. 2008) that detected the expression of leptin receptor mRNA and protein in the oviduct of the laying hen. Taking into consideration this observation, it cannot be excluded that leptin is involved in a variety of calcium transport processes in birds. It is well known that leptin may act as a regulator of the bone formation process (PARHAMI et al. 2001; GALUS & WLODARSKI 2004; HANDSCHIN et al. 2007; DIMITRI et al. 2015), corroborated by the expression of leptin receptor in chicken bones (MAURO et al. 2010).

The mRNA expression of estrogen receptors α and β has been detected in the CAM tissue of both male and female chicken embryos at E12, E15 and E18. So far, in the available literature, the simultaneous expression of both isoforms of estrogen receptors in the chicken CAM has not been reported, however ALBERGOTTI et al. (2009) demonstrated mRNA expression of ERα in the chicken CAM, whereas in the chorioallantoic membrane of American alligator (Alligator mississippiensis) mRNA expression of ERα (CRUZE et al. 2012) and in the Turtle (Pseudemys nelsoni) mRNA expression of ERα as well as ERβ were detected (CRUZE et al. 2013). The expression of ERα mRNA decreased 2-fold between embryonic stages (19 and 25), whereas ERβ expression increased 12.3-fold between embryonic stages 19 and 25 (CRUZE et al. 2013). In the present study, such differences in mRNA expression of estrogen receptor mRNA among analyzed stages of embryogenesis were not observed, however parallel to the reptilian CAM, in the chicken CAM mRNA expression of estrogen receptor α was significantly higher than expression of ERβ. A similar relation was previously observed in the laying hen ovary (HRABIA et al. 2008). This may be the result of the different physiological roles of the two isoforms of estrogen receptors. ERα activity may be associated with proliferation, whereas ERβ with stimulation of apoptosis and differentiation of cells (ZHAO et al. 2008; PATERNI et al., 2014). Moreover, the CAM of the chicken shares the capability of chorioallantoic placenta to synthesize and respond to the signaling of progesterone (P4), indicating that steroidogenic and steroid responsive extraembryonic membranes are not exclusive characteristics of viviparous amniotes (ALBERGOTTI et al. 2009). In addition, it was also demonstrated by CRUZE et al. 2013 that the CAM of the American alligator exhibits mRNA expression of steroidogenic enzymes critical in the synthesis of progestins, androgens and estrogens. They also detected mRNA expression of androgen receptor and glucocorticoid receptor suggesting that oviparous extraembryonic membranes have the potential to respond to steroid hormone signaling and to synthesize steroid hormones, which are mainly produced in gonads and adrenal glands (BRUGGEMAN et al. 2002; JOHNSON et al. 2002).

The last aim of this study was to assess the expression of mRNA encoding caspase-1 and -3 in the chicken CAM. The mRNA expression of caspase-1 and -3 was detected in chicken CAM at all three analyzed stages of embryogenesis. Expression of caspase-1 was significantly lower than that of caspase-3. Apoptotic cells have been previously detected during the early phase of chicken embryogenesis at sites of active growth, morphogenesis and active cell migration (HIRATA & HALL 2000). Studies on cell death in chicken embryos have been frequently focused on individual organ systems (JEFFS et al. 1992; HURLE et al. 1995; MILLER & BRIGLIN 1996; SANDERS 1997). Apoptotic cells have been previously localized three hours after the onset of incubation (HIRATA & HALL 2000), as well as in the gastrulating embryo (SANDERS & WRIDE 1997). Our results demonstrate that mRNA expression of caspase-1 and caspase-3 does not change among the analyzed stages of embryogenesis. This may suggest the participation of apoptosis and/or pyroptosis in the process of CAM development in the chicken embryo.

In summary, leptin receptor was immunolocalized, and leptin and estrogen receptors and caspase-1 and -3 mRNA was detected in the chicken CAM at all examined stages of embryogenesis. The results show the potential of this membrane to respond to different stimuli (leptin, estrogens) during early chicken embryogenesis. Additionally, the presence of caspase-1 and -3 mRNA expression in CAM tissue at E12, E15 and E18 indicates that chicken CAM may be used as a model in analysis of the mechanism of cell death after in ovo manipulations.

These results suggest that the CAM is a target tissue for leptin and estrogens and additionally, that the development of this membrane is associated with apoptosis and/or pyroptosis. Our results should be taken into consideration in studies with the CAM as an experimental model, in tests of endocrine disrupting potential of chemicals, and analysis of therapeutic methods and metastatic potential of cancers.

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References


