Angiogenic Effect of Leptin in the Quail Chorioallantoic Membrane

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Received March 9, 2009
Accepted September 8, 2009

Abstract

Leptin, the product of *ob* gene, beside its key role in the control of body weight and food consumption, can be involved in the control of embryonic development. Leptin administration *in ovo* accelerated the embryonic and post-embryonic development in Japanese quail. Although the mechanisms of leptin effects on growth and development acceleration are not clear, stimulation of angiogenesis represents one of plausible explanations. Therefore, the aim of the present study was to investigate the pro-angiogenic effect of leptin *in vivo* in the quail chorioallantoic membrane (CAM). The recombinant murine leptin (10, 100, and 1000 ng) was applied either *ex ovo* on the CAM surface of *ex ovo* incubated embryos at embryonic day 7 (ED7) or *in ovo* into the egg albumen at ED5. Changes in blood vessels were quantified by the fractal analysis providing the fractal dimension (Df) estimate. Leptin administered *in ovo* was more efficient in stimulation of angiogenesis than the *ex ovo* treatment, since 10 ng dose elicited significantly higher ($P < 0.001$) stimulation of vessel development of the CAM under the air cell than it did after *ex ovo* cultivation. Our study confirmed that exogenously applied leptin was able to stimulate angiogenesis in CAM. Leptin-mediated stimulation of angiogenesis may improve nutrient utilization from the yolk and explain at least partially the accelerating effect of leptin on avian embryo growth and development.

Angiogenesis, leptin, chorioallantoic membrane, Japanese quail, fractal dimension

Angiogenesis is a process that refers to the growth of new blood vessels from a pre-existing vasculature. It is closely associated with endothelial cell migration and proliferation. Endothelial cells are particularly active during embryonic development and therefore the CAM assay has been considered as a very useful *in vivo* method for the quantification of angiogenesis and development of new drugs (Falkner et al. 2004).

The angiogenic effect has been demonstrated for numerous endogenous peptides including leptin (Ribatti et al. 2007). Leptin is an adipocytokine with pleiotropic effects. It is produced predominantly in adipocytes and its major role in mammals is the control of food intake and energy expenditure (Campfield et al. 1995). Since both processes are of key importance also for farm animals, possible roles of leptin in the neuroendocrine control of these species have been studied (Máčajová et al. 2004). The physiological role of leptin in poultry is not clear and the identity of the natural ligand for leptin receptors is controversial. Avian leptin cDNA has been cloned (Taouis et al. 1998) and its mRNA was detected in the adipocyte and liver (Taouis et al. 1998; Ashwell et al. 1999). However, a number of research groups raised the question whether the reported chicken leptin cDNA nucleotide sequence represents genuine chicken leptin (Ohkubo and Adachi 2008). On the other hand, the leptin receptor has been cloned and proved by several research groups (Horev et al. 2000; Ohkubo et al. 2000) in birds and various physiological responses were observed after exogenous leptin administration. Therefore, it is assumed that leptin-like activity exists also in birds (Ohkubo et al. 2008).

Previous studies showed that the administration of leptin *in ovo* accelerated the embryonic and post-embryonic development in Japanese quail (Máčajová et al. 2002; Lamošová et al. 2003). However, the mechanisms of how leptin can accelerate embryonic growth are not

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understood. Since leptin has been shown to stimulate angiogenesis under *in vitro* and *in vivo* conditions (Sierra-Honigmann et al. 1998; Talavera-Adame et al. 2008) we expect that activation of vessel development can contribute to better exchange of nutrients and gasses between the developing embryo and the yolk and gas environment. Therefore, the aim of our study was to determine the effect of leptin on the development of CAM in Japanese quail under *in vivo* conditions using two experimental approaches (*ex ovo* and *in ovo*).

Materials and Methods

Fertilized eggs of Japanese quail (*Coturnix japonica*) from our breeding colony (Laying Line 01 Ivanka pri Dunaji) were incubated in a forced draught incubator at 37 °C and 50-60% relative humidity. Three different doses of recombinant murine leptin (PeproTech, Great Britain), 10, 100, and 1000 ng, were administered under two treatment conditions: *ex ovo* (shell-less) and *in ovo*.

To prepare for *ex ovo* culture, the eggs at ED3 were wiped with 70% ethanol in a sterile laminar flow hood. The eggs were then opened and the embryos transferred into the six-well tissue culture plates (TPP, Switzerland), and returned to humidified incubator for the next 4 days (Parsons-Wingerter et al. 1998, 2000). At ED7, leptin was prepared to the desired concentrations in pre-warmed sterile phosphate buffered saline (PBS); 0.5 ml of solution containing either the leptin (10, 100, and 1000 ng) or the vehicle buffer was added gently in drops on the surface of each CAM. The embryos were then incubated at 37 °C for another 24 h, at which time they were fixed in pre-warmed fixative (4% paraformaldehyde/2% glutaraldehyde/ PBS) for 2 days at room temperature. Thereafter, the CAMs of fixed embryos were carefully dissected, mounted without folds onto glass slides and dried.

In the second approach we applied the same protocol as in previous studies (Máčajová et al. 2002; Lamošová et al. 2003). Leptin at doses of 10, 100, and 1000 ng in 50 µl of sterile PBS was injected into the egg albumen at ED5. The control group received 50 µl of PBS only. Before injection, the eggs were disinfected with 70% ethanol and after injection the holes in eggs were closed by wax. At ED10, the eggs were opened so that the air cells were not damaged and the embryos were removed. The parts of egg shells with air cells were rinsed with PBS and CAMs were fixed in pre-warmed fixative as above for 24 h at room temperature (22 ± 1 °C). Thereafter, the fixative was removed and CAMs were dried.

Images were obtained using digital camera (Canon EOS 40D with Canon EF 100 mm f/2.8 USM macro lens). The CAMs were illuminated using either transilluminator (Kaiser Slimlite 5000K Lightbox, for *ex ovo* CAMs) or ring flash (Canon MR-14EX Ring Lite, for *in ovo* CAMs) as a source of homogenous light. The subsequent image processing was performed with the ImageJ software (Abramoff et al. 2004). For quantification of the CAM arteries, the contrast and brightness of the obtained images were adjusted (Plate III, Fig.1). A square region (512 × 512 pixels) from the area with distal arterial branches was selected and used for further processing. The images were then binarized using manual tresholding and skeletonized to obtain final image where each vessel had the thickness of a single pixel (Plate IV, Fig. 2). The fractal coefficient (Df) was calculated following the procedures described by Parsons-Wingerter et al. (1998, 2000). Df, a statistical descriptor of space-vessel filling area and length (Kirchner et al. 1996), was determined using an ImageJ plugin, implementing the method of box counting. The CAM image was overlaid with a series of square boxes of decreasing size (denoted in pixel, p). The number of boxes (N_p) that contain at least one black pixel was counted. The negative value of the least squares regression slope of the plot of log N_p versus log p yielded D_f ($r^2 \geq 0.96$).

The results were statistically evaluated by one-way ANOVA followed by Fisher PLSD *post hoc* test.

Results

We found a significant dose-dependent effect of leptin on CAM angiogenesis in both *in vivo* experimental models. In the *ex ovo* model the dose of 10 ng of leptin administered onto the CAM surface did not influence the vessel density but the doses of 100 and 1000 ng significantly (both $P < 0.001$) stimulated angiogenesis (Fig. 3). Mortality of the embryos was maximum 35% and did not depend on the drug dose/control treatment.

In the *in ovo* approach, already 10 ng of leptin administered directly into the egg albumen significantly ($P < 0.001$) stimulated angiogenesis (Fig. 4). Embryo mortality upon drug administration was maximum 5% and did not depend on the drug dose. In both approaches the stimulating effect of leptin increased with the dose administered.

The comparison of both experimental conditions of CAM demonstrates that administration of the hormone into the egg albumen represented a more sensitive way of angiogenesis stimulation.

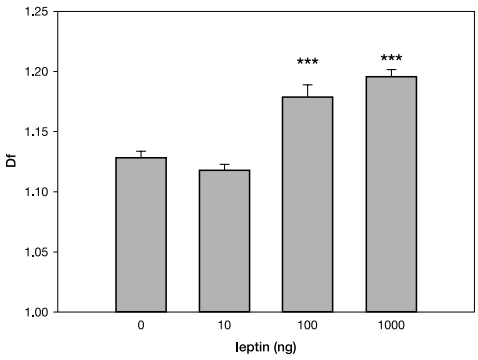

Fig. 3. Effect of leptin administered *ex ovo* at embryonic day 7 on angiogenesis in quail CAM expressed by fractal dimension (Df). Mean values ± SEM, n = 8-16, $^{***}P < 0.001$ vs. control group.

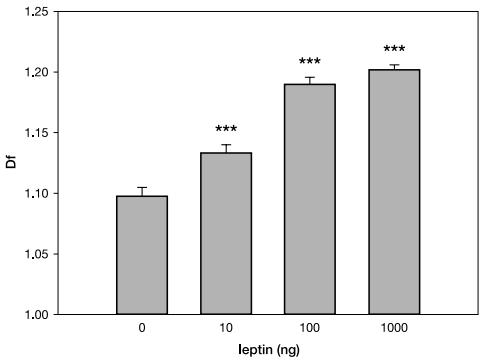

Fig.4. Effect of leptin administered *in ovo* at embryonic day 5 on angiogenesis in quail CAM expressed by fractal dimension (Df). Mean values ± SEM, n = 12-16, $^{***}P < 0.001$ vs. control group.

Discussion

Recent studies have addressed the possibility that leptin may either induce angiogenesis or influence efficiency of angiogenic factors (Sierra-Honigmann et al. 1998; Cao et al. 2001). Our study of quail CAM under two experimental conditions clearly demonstrated that leptin enhanced the formation of new blood vessels. The results are in accordance with the data obtained using chicken CAM (Bouloumié et al. 1998; Ribatti et al. 2001). Bouloumié et al. (1998) applied leptin at ED9 at higher doses (0.2, 1, and 3 µg of leptin) onto methylcellulose discs. The doses of 1 and 3 µg resulted in a marked stimulation of neovascularization. Thus, the stimulation of angiogenesis was observed after administration of higher concentrations of leptin than in our experiment. Ribatti et al. (2001) confirmed the stimulatory effect of leptin on angiogenesis after its administration onto chick CAM at ED8 on gelatine sponges at doses of 250, 500 a 1000 ng, but not 100 ng. The stimulating effect of leptin was specific, since the exposure to anti-leptin antibodies significantly inhibited the angiogenic response. The angiogenic response of leptin was similar to that obtained with fibroblast growth factor 2 (FGF-2). However, the administration of leptin to CAM together with anti FGF-2 antibodies reduced the angiogenic effect of leptin by approximately 40%, indicating that the activation of endogenous FGF-2 mediated at least in part leptin action (Ribatti et al. 2001). Recent data obtained with quail CAM demonstrate that leptin acts also in cooperation with the vascular endothelial growth factor (VEGF) inducing more significant changes than VEGF in vessel length and tortuousity (Talavera-Adame et al. 2008). An important finding supporting the idea of leptin effects on angiogenesis was the identification of leptin receptors in endothelial cells and mouse adipose tissue while its expression was not proved in adipose tissue and endothelial cells in leptin receptor-deficient (*db/db*) mice (Bornstein et al. 2000).

The murine leptin administration *in ovo* accelerated growth and development in quail embryos (Máčajová et al. 2002; Lamošová et al. 2003). Injection of 100 and 1000 ng leptin into egg albumen at ED5 of incubation stimulated the embryonic and postnatal development. The treated quail embryos hatched 18–22 h earlier than controls. Therefore, we used the same doses and experimental conditions also in this *in ovo* study. The mechanisms of avian embryonic growth and development acceleration by leptin are not clear. An increased vascular fenestration was documented in new corneal blood vessels after leptin treatment (Cao et al. 2001). Therefore, we speculate that the increased leptin content can stimulate development of CAM and subsequent transport and exchange of

small molecules between the embryo and yolk. Since the existence or structure of the natural ligand for the leptin receptors in birds is still a matter of discussion, we suggest CAM as a promising model for solving this question.

The CAM serves as the respiratory organ for exchange of gases between pores of the eggshell and the dense network of capillaries and plays a role in electrolyte transport and mobilization of calcium transport from the shell (Reizis et al. 2005). The CAM assay is a useful and convenient model that has been used for understanding the role of different compounds in angiogenesis control and development of a promising anti-cancer drug (Richardson and Singh 2003). There are several modifications of CAM assay performed under *ex ovo* and *in ovo* conditions that have their advantages and disadvantages. For example the *ex ovo* approach allows better quantification of blood vessels over a wider area of the CAM than *in ovo* approach, which is limited by a small window in the eggshell. However, physiological conditions are better mimicked by the *in ovo* approach. In addition to the standard *ex ovo* approach we used in our study also *in ovo* administration of leptin that interferes less with the physiological status of embryo and offers better quantification of vessels. Quantification of angiogenic effects *in ovo* was done in the planar part of CAM under the egg's air cell. This CAM part seems interesting in connection with earlier findings related to regional variations of shell pore density and gas conductance (Rokitka and Rahn 1987) as well as blood vessel morphometry (Reizis et al. 2005). The capacity for CAM gas exchange can be assessed by the ratio between the eggshell pore density and CAM blood vessel density. Rokitka and Rahn (1987) found regional differences in shell pore density and shell gas conductance in six different avian species. All species displayed a decrease in regional shell conductance and pore density from the blunt end to the pointed end. Reizis et al. (2005), studying blood vessel morphometry in the chick CAM, determined higher blood vessel numerical density, area fraction of blood vessels and total length of blood vessels in the CAM under the air cell in relation to the rest of the CAM at ED10–ED20.

In conclusion, our results confirmed the angiogenic action of leptin in the quail CAM. We expect that the leptin-mediated stimulation of angiogenesis may contribute to the acceleration of quail embryo growth and development and this model can be useful for identification of avian leptin and its mechanism of action in birds. New modification of CAM assay in unfenestrated eggs can be advantageous for studying physiological effects of biological compounds on vessel development.

Účinok leptínu na angiogenézu v chorioalantoickej membráne prepelice japonskej

Leptín, produkt *ob* génu, môže okrem funkcie endokrinného hormónu zodpovedného hlavne za reguláciu telesnej hmotnosti a príjmu potravy, pôsobiť aj parakrinne, ako rastový faktor ovplyvňujúci vývin. Cieľom našej štúdie bolo sledovať účinok leptínu *in vivo* na angiogenézu v prepeličej chorioalantoickej membráne (CAM), a tak poukázať na jeho rastový efekt potvrdený v predchádzajúcich štúdiách. Rekombinantný myšací leptín (10, 100 a 1000 ng) bol aplikovaný buď *ex ovo* na povrch CAM izolovaných embryí v 7. dni embryonálneho vývinu alebo *in ovo* v 5. dni do bielka. Zmeny v cievnej štruktúre CAM boli kvantifikované pomocou fraktálnej analýzy, určením fraktálnej dimenzie (Df). Leptín podaný *in ovo* bol aktívnejší v stimulovaní angiogenézy meranej v CAM pod vzduchovou komôrkou, kde už 10 ng dávka vyvolala štatisticky významne vyššiu stimuláciu ($P < 0.001$) v porovnaní s *ex ovo* aplikáciou. Získané výsledky potvrdzujú angiogénny efekt exogénneho leptínu v chorioalantoickej membráne prepelice japonskej. Leptínom sprostredkovaná stimulácia angiogenézy môže zlepšiť využitie živín zo žĺtka a prispievať k akcelerácii rastu a vývinu vtáčích embryí.

Acknowledgements

This study was supported by the Grant Agency for Science of the Slovak Republic VEGA 1/4343/07. We are grateful to Helena Rojčíková for her technical assistance.

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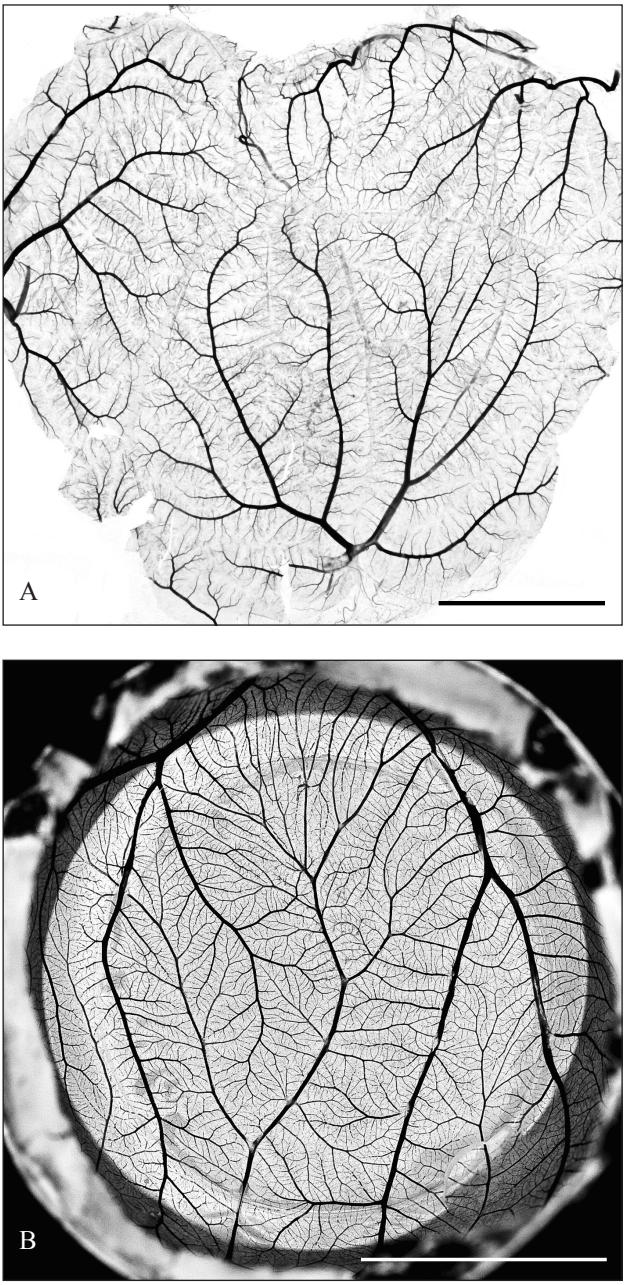

Fig. 1. Images of CAM after greyscale conversion. A – *ex ovo* approach, membrane mounted on glass slide; B – *in ovo* approach, membrane under air cell. Bar = 5 mm.

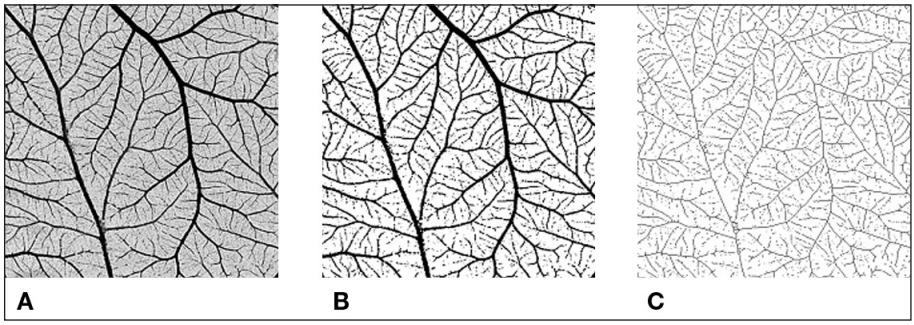

Fig. 2. Example of square 512 × 512 pixel selection (3.5 × 3.5 mm) used for analysis. A – adjusted grayscale image; B – binarized image; C – skeletonized image