

Effect of Leptin and Insulin on Chick Embryonic Muscle Cells and Hepatocytes

D. LAMOŠOVÁ¹, M. ZEMAN^{1,2}

¹*Institute of Animal Biochemistry and Genetics, Slovak Academy of Sciences, Ivanka pri Dunaji, and* ²*Department of Animal Physiology and Ethology, Comenius University, Bratislava, Slovak Republic*

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Summary

In the present study we used the primary cultures of chick embryonic muscle and liver cells as a model for potential mutual combination effects of leptin and insulin, respectively. The influence of both hormones on the proliferation and protein synthesis was dose-dependent and related to the age of embryos from which the cells were isolated. Leptin (10 and 100 ng/well) increased the proliferation (estimated by DNA content and incorporation of labeled thymidine into DNA) and protein synthesis (determined by incorporation of labeled leucine into proteins) of muscle cells. The effect of leptin and insulin in muscle cells was similar. In younger embryo (11-day-old) the lower dose of leptin was more effective than the higher one compared to the insulin effect. Mutual effects of leptin and insulin were neither additive nor synergistic and were equivalent to the effects of individual hormones. In hepatocytes the influence of leptin was dependent on the age at which the cells were isolated (11- and 19-day-old embryos). The presence of insulin neither potentiated nor inhibited the effect of leptin.

Key words

Chick embryo • Hepatocytes • Insulin • Leptin • Muscle cells

Introduction

Leptin, an *ob* gene product – produced in mammals mostly by white adipose tissue – plays an important role in body weight homeostasis (Zhang *et al.* 1994). In addition to its role as a feedback signal in the regulation of energy balance, leptin may have multiple physiological actions. It was shown that effects of leptin on food intake are mediated through leptin receptors in the hypothalamus (Tartaglia *et al.* 1995). Leptin receptors are found not only in the brain but also in many other tissues including lungs, kidneys, ovaries, liver and

skeletal muscles (Tartaglia *et al.* 1995, Tartaglia 1997, Cioffi *et al.* 1996). The pattern of leptin receptor distribution suggests that leptin might exert a wide spectrum of cellular effects in tissues other than the brain. The long signal-transducing isoform of leptin receptor is differently expressed than a short isoform in various organs. In chickens, the long isoform of leptin receptor was found in the ovary, brain, liver, intestine and the kidney (Ohkubo *et al.* 2000), while skeletal muscles were not analyzed in that study.

Both *in vivo* and *in vitro* studies using mammalian models showed conflicting results on the

effects of leptin and insulin. Leptin could mimic the insulin effects on glucose transport and glycogen synthesis in cultured rat myotubes (Berti *et al.* 1997). Extended exposure of cultured rat adipocytes to leptin inhibits insulin responsiveness (Muller *et al.* 1997). Leptin promotes *in vitro* fatty acid utilization in rat muscle tissue and this effect is opposite to that induced by insulin (Muoio *et al.* 1997). Leptin also modulates insulin sensitivity of the liver (Cohen *et al.* 1996, Wang *et al.* 1997). It has recently been shown that leptin released by rat adipocytes requires the presence of insulin for *ob* gene expression (Remesar *et al.* 1997). Insulin is necessary for leptin synthesis and secretion from adipocytes but not for leptin action in mammals. Hereby, functional leptin receptors are necessary not only for central leptin action but also for making the brain sensitive to insulin (Remesar *et al.* 1997).

Most information on leptin comes from studies performed on laboratory rodents and humans. In birds, the data related to the physiology of leptin are rather sparse. Leptin gene was cloned in chickens (Taouis *et al.* 1998). Surprisingly, leptin expression was found not only in adipose tissue but also in hepatocytes. The presence of leptin in avian hepatocytes underlies an important role of the liver in fat metabolism of chickens (Ashwell *et al.* 1999).

Muscle tissue plays a central role in the action of insulin determining energy metabolism. In the present study we therefore used primary cultures of chick embryo muscle and liver cells as a model for potential leptin and insulin effects and their mutual combination. Actions of both hormones were evaluated by the incorporation of labeled leucine into proteins and the incorporation of labeled thymidine into DNA.

Material and Methods

Animals

Fertilized eggs of broiler breeder hens were incubated in a forced draught incubator with automatic turning every hour at a temperature 37.5 ± 0.2 °C.

Cell culture and incubation

Cells were isolated by trypsinization of 11- and 19-day-old chick embryo thigh and breast muscles or liver tissue (Königsberg 1979). The isolated cells were seeded (2.0 - 3.0×10^6 cells/well in 12 well-plate) in Dulbecco's modified Eagle's medium (DMEM) with

4.5 g/l glucose, sodium pyruvate (2 mmol/l), L-glutamine (2 mmol/l) supplemented with 10 % fetal calf serum and antibiotics (penicillin (10^5 U/l), streptomycin (25 mg/l), kanamycin (100 mg/l) and grown at 37 °C in a humidified incubator in air supplemented with 5 % CO₂ for 24 h. After 24 h of preincubation, the medium was replaced with a fresh medium containing a definite dose of leptin (10, 100 or 1000 ng/ml of medium) or insulin (2.57 or 25.7 mU/ml of medium) or their mutual combination (100 ng of leptin plus 2.57 mU of insulin/ml of medium). One ml of the medium was used for one well. The cells were evaluated after 2 h (incorporation of [³H]leucine into proteins) or 24 h (incorporation of [³H]thymidine into DNA). In our experiments, the recombinant murine leptin (Pepro Tech, EC Ltd, London, England) and insulin from the bovine pancreas (Sigma, St. Louis, Mo, USA) were used. Leptin and insulin were dissolved in DMEM up to the appropriate experimental concentration.

Incorporation of [³H]leucine

Two hours before the end of the treatment period, L-[4,5-³H]leucine (1620 GBq/mmol, Amersham) was added to cell culture to reach a final concentration of 6 µCi/ml (240 kBq/ml) of the medium. At the end of incubation, the medium was removed and the cells were processed by the method described by Robinson *et al.* (1976). The protein content was quantified with bovine serum albumin as a standard (Lowry *et al.* 1951).

Incorporation of [³H]thymidine

For determination of thymidine incorporation into DNA, [methyl-³H]thymidine (1500 GBq/mmol, Amersham) was added to the cell culture 24 h before the end of treatment. Its final concentration was 6 µCi /ml (240 kBq/ml) of the medium. At the end of incubation, the medium was removed and cells were processed by the method of Robinson *et al.* (1976). For RNA and DNA assays, yeast RNA and calf thymus DNA were used as standards.

Radioactivity measurements and statistical analysis

A Beckman LS-6000 SE (USA) was used for the measurement of radioactivity. Experimental values are given as means \pm S.E.M. Control and treatment groups were compared by Student's t-test. The influence of different concentrations of leptin on protein synthesis in muscle cells and hepatocytes was evaluated by ANOVA followed by Tukey's test.

Table 1. Effects of different doses of leptin on protein synthesis in muscle cells and hepatocytes isolated from 11-day-old chick embryos.

	Control group	10 ng /well	100 ng /well	1000 ng /well
Muscle cells	10.40 ± 1.04	12.32 ± 1.11	13.82 ± 0.99*	12.95 ± 0.88
Hepatocytes	64.32 ± 7.59	67.58 ± 9.70	71.56 ± 9.39	38.58 ± 3.76**

Data on the incorporation of [³H]leucine into cell proteins (cpm/μg protein) are expressed as mean from eight wells ± S.E.M. ** *P*<0.01, * *P*<0.05 vs. control.

Results

In preliminary experiments, the response of chick embryonic muscle cells and hepatocytes isolated from 11-day-old embryos was tested to different doses of leptin. Both muscle cells and hepatocytes were found to be sensitive to recombinant murine leptin. The most

effective dose, estimated on the basis of incorporation of labeled leucine into cell proteins, was 100 ng/dish (*P*=0.033, ANOVA) for chick muscle cells (Table 1). The highest tested dose of leptin (1000 ng/dish) had no significant effect in muscle cells and it inhibited protein synthesis in hepatocytes (Table 1).

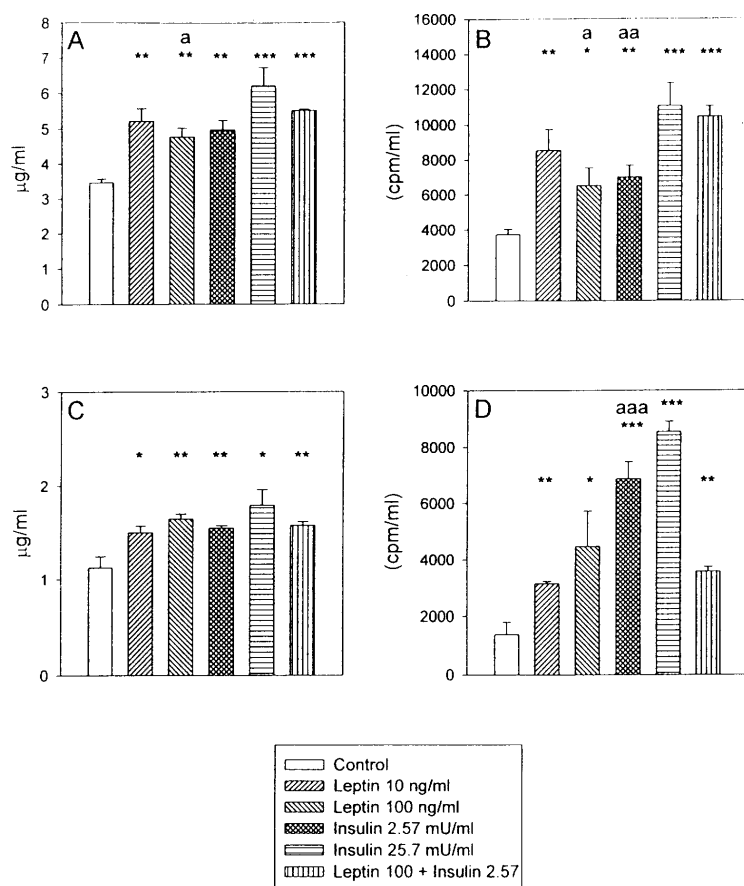


Fig. 1. Proliferation of chick muscle cells isolated from 11-day-old embryos (A, B) and 19-day-old embryos (C, D) after different doses of leptin and insulin application. DNA content (A, C) (μg/ml of hydrolysate) and 24 h incorporation of [³H]thymidine into DNA (B, D) (cpm/ml of hydrolysate). Data are means from six wells ± S.E.M. * *P*<0.05, ** *P*<0.01, *** *P*<0.001 vs. control; a *P*<0.05, aa *P*<0.01, aaa *P*<0.001 vs (leptin 100 ng + insulin 2.57 mU/ml).

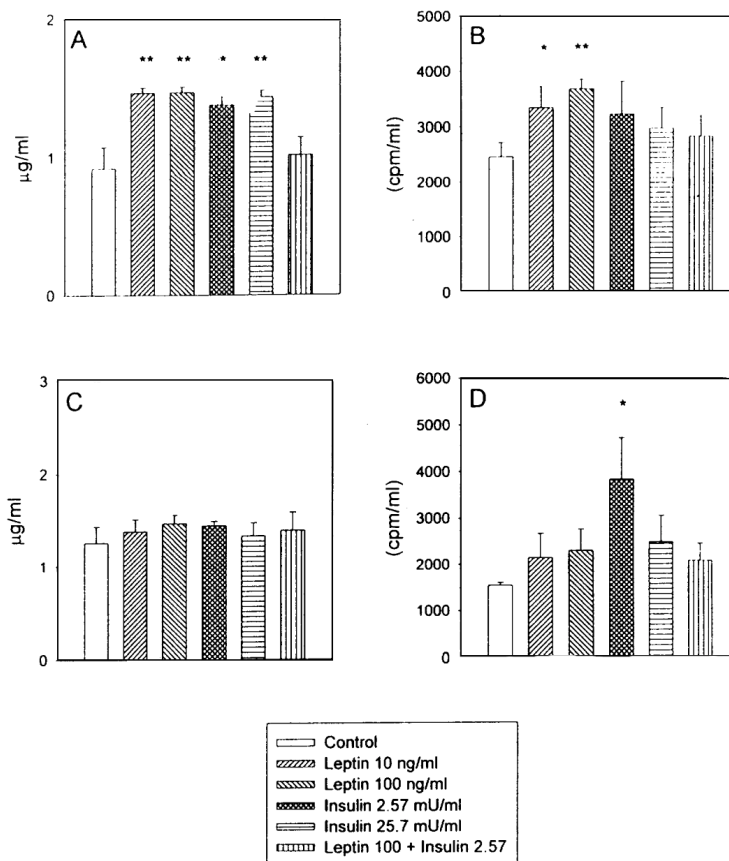
On the basis of these results, doses 10 and 100 ng of leptin per dish alone or in combination with insulin were used in subsequent experiments. Both concentrations of leptin enhanced the proliferation of

muscle cells (estimated on the basis of DNA content, Figs 1A and 1C, and [³H]thymidine incorporation into DNA, Figs 1B and 1D). This effect was significant both in cells isolated from 11- and 19-day-old chick embryos. Leptin

and insulin exerted similar effects on muscle cells. The differences in effectiveness of both hormones were dose-dependent and related to the age of embryos from which the muscle cells had been isolated. In the younger embryos, a lower dose of leptin (10 ng) was more effective than the higher one (100 ng) compared to lower dose (2.57 mU/ml) of insulin (DNA content: 105 % and 95.9 %, respectively; incorporation of labeled thymidine: 122.3 % and 93.3 %, respectively). In older embryos, the

higher dose of leptin (100 ng) was more effective than the lower one (10 ng) compared to insulin (2.57 mU/ml) (DNA content: 106.3 % and 97.0 %, respectively; incorporation of labeled thymidine: 65 % and 45 %, respectively). The mutual effect of leptin and insulin was neither additive nor synergistic and was equivalent to the effect of each hormone given separately. In younger embryos, a combined action of leptin and insulin was more pronounced than in older ones.

Fig. 2. Effect of leptin and insulin on DNA content (A, C) ($\mu\text{g/ml}$ of hydrolysate) and incorporation of [^3H]thymidine into DNA (B, D) (cpm/ml of hydrolysate) in chick hepatocytes isolated from 11-day-old embryos (A, B) and 19-day-old embryos (C, D). Data are means from six wells \pm S.E.M., * $P < 0.05$, ** $P < 0.01$ vs. control.



The effects of leptin and insulin in hepatocytes were related to the age of embryos, at which the cells had been isolated. In younger embryos, leptin and insulin in both concentrations significantly increased the proliferation of hepatocytes compared with the control group (Fig. 2). The effects in hepatocytes isolated from 11-day-old embryos to both doses of leptin (10 and 100 ng/dish) were similar and higher than the effects of insulin (Figs 2A and 2B) (DNA content 106.5 % and 105.8 % respectively; incorporation of labeled thymidine: 114.3 % and 104.0 %, respectively compared to 2.57 mU/ml insulin). The mutual effect of leptin and insulin was lower than the effects of both substances applied alone (69.4 % of the leptin and 73.9 % of the insulin

effect on DNA content; 76.4 % of the leptin and 87.3 % of the insulin effect on incorporation of [^3H]thymidine). In hepatocytes isolated from 19-day-old embryos, the proliferation of cells was not significantly influenced either by leptin alone or by its combination with insulin (Fig. 2C). Incorporation of labeled thymidine for 24 h was significantly increased by the lower dose of insulin (Fig. 2D).

Discussion

It was found that the amino acid sequence of chicken leptin is 97 % identical to that of the mouse (Taouis *et al.* 1998). Because of this close similarity in

the primary structure of the mammalian and avian leptin we used commercially available mouse leptin in our experiments.

The fat deposits in mammals correlate with the leptin produced by adipose tissue and leptin expression is exclusively localized in the adipose tissue. Fat deposition in birds is regulated by interactions between environmental and genetic factors mediated by several hormonal pathways (Buyse *et al.* 1999), but the involvement of leptin in this process has not yet been studied. However, the presence of leptin was also proved in chickens. The expression of the chicken leptin gene was localized not only in adipose tissue but also in the liver (Taouis *et al.* 1998). Further studies proved leptin gene expression in adipose tissue and the liver and localized leptin also in the embryonic liver and yolk sac (Ashwell *et al.* 1999). These observations have confirmed the important role of the avian liver in processes of lipogenesis. Moreover, a positive correlation was found in broiler breeder hens between liver weight and leptin concentrations, suggesting that the liver may be a major source of leptin in chickens (Bruggeman *et al.* 1999). Therefore, we have chosen hepatocytes and muscle cells for studying the *in vitro* effects of leptin on embryonic growth and development.

Many metabolic processes in vertebrates are controlled by insulin. In comparison with mammals, birds are resistant to insulin and it is commonly thought that glucagon plays a more important role than insulin in the control of bird fat metabolism (Simon 1995). Insulin is essential for the induction of lipogenic enzymes in the liver and is able to decrease the glucose content only marginally (Simon 1988, 1995). In our experiments, the effects of leptin and insulin on proliferation and protein synthesis in embryonic muscle and liver cells were investigated. We used bovine insulin because it was found that bovine insulin can serve as an alternative to chick insulin (Laurin and Cartwright 1993). Since chicken hepatocytes are unresponsive to insulin given in physiological concentrations (Harvey *et al.* 1977, Rosebrough and Steele 1987), we administered higher doses of insulin.

The concentrations of leptin and insulin used in our experiments effectively stimulated the proliferation and protein synthesis of hepatocytes isolated from 11-day-old chick embryos. Cells isolated from older embryos did not respond to leptin application. We demonstrated that hepatocytes isolated from chick embryos are sensitive to leptin and insulin and the effects of both substances are age-dependent. Our results

obtained with insulin correspond to the data found in birds in *in vitro* and *in vivo* experiments during the postnatal period (Laurin and Cartwright 1993, Ashwell *et al.* 1999, Bruggeman *et al.* 1999). The role of leptin in the development of chick embryos is not known, but leptin may have paracrine and endocrine effects on embryogenesis in mammals (Hoggard *et al.* 1997). Its presence during early stages of embryogenesis suggests that it may also have similar effects in birds. It was suggested that leptin controls nutrient transfer to the developing embryo (Ashwell *et al.* 1999). Our data demonstrating physiological effects of leptin on embryonic muscle cells and hepatocytes provide further information supporting the functional role of leptin in avian embryonic development. Thus leptin may serve as a trophic factor for the chick embryo. Action of this hormone can be connected with utilization of lipids from the yolk. Its stimulatory effect was more pronounced in younger embryos than the older ones in which the yolk sac was almost resorbed.

Muscle tissue plays a central role in insulin action and in the pathogenesis of insulin resistance in mammals. The effects of acute exposure to insulin on protein synthesis were examined in primary, differentiated cultures of embryonic chick heart and skeletal muscle cells (Airhard *et al.* 1982). Doses of insulin in the physiological range produced significant stimulation of protein synthesis in both cell types and the effects were dose- and time-dependent. Our data confirmed these findings and, moreover, showed stimulation of protein synthesis in chick cultured muscle cells by leptin. The stimulatory effect of leptin on protein synthesis and proliferation of cultured muscle cells in our experiments was comparable with results of other authors evaluating the effects of insulin on chick embryo fibroblasts (DePhilip *et al.* 1979).

The mechanism of the stimulatory action of leptin on protein synthesis in embryonic chick muscle cells is completely unknown. In mammals, leptin was found to have a dual concentration-dependent effect on insulin function in C₂C₁₂ myotubes. In low concentrations, leptin shows insulin-like effects but in high concentrations it impairs insulin action (Berti *et al.* 1997). An impairment of the first steps of insulin signaling chain, i.e. autophosphorylation of the insulin receptor or tyrosine phosphorylation of the insulin receptor substrate by leptin, was also demonstrated in rat fibroblasts, NIH3T3 cells and HepG2 cells (Kroder *et al.* 1996). In our experiments, we found a stimulatory effect of leptin on the proliferation and protein synthesis in

muscle and liver cells, especially in younger embryos. However, the concomitant action of leptin and insulin did not show additive or synergistic effects. We therefore suppose in analogy with the situation in mammals, that leptin can also interact with the insulin signaling chain in birds. More pronounced effect found in younger embryos may be due to the more important role of leptin in earlier developmental stages.

Our results have demonstrated direct effects of mouse leptin on primary culture of muscle cells and

hepatocytes isolated from broiler chick embryos. The significant stimulation of protein synthesis and proliferation suggests that leptin may be a trophic factor for chick embryos acting in an autocrine or paracrine manner at early stages of avian embryogenesis.

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Reprint requests

Ing. Dalma Lamošová, CSc., Institute of Animal Biochemistry and Genetics, Slovak Academy of Sciences, Moyzesova 61, 900 28 Ivanka pri Dunaji. Slovak Republic. Fax: +421-7-45943 932, E-mail: ubzlamo@nic.savba.sk