Activities for Leptin in Bovine Trophoblast Cells

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ABSTRACT

Leptin is involved in various reproductive processes in humans and rodents, including placental development and function. The specific ways that leptin influences placental development and function in cattle are poorly understood. This work was completed to explore how leptin regulates hormone, cytokine and metalloprotease transcript abundance and cell proliferation in cultured bovine trophoblast cells. In the first set of studies, cells were cultured in the presence of graded recombinant bovine leptin concentrations (0, 10, 50, 250 ng/mL) for 6- or 24-h. Transcript profiles were examined from extracted RNA. Leptin supplementation did not affect abundance of the maternal recognition of pregnancy factor, interferon-tau (*IFNT*), but leptin increased ($P < 0.05$) abundance of chorionic somatomammotropin hormone 2 (*CSH2*; *i.e.* placental lactogen) at both 6- and 24-h at each concentration tested. At 24-h, the greatest *CSH2* abundance ($P < 0.05$) was detected in cells supplemented with 50 ng/mL leptin. Transcript abundance of the remodeling factor, metalloprotease 2 (*MMP2*), was greater ($P < 0.05$) in leptin-treated cells at 24-h but not at 6-h. The 24-h *MMP2* response was greatest ($P < 0.05$) at 250 ng/mL. Transcript abundance for *MMP9* was not altered by leptin treatment. In a separate set of studies, cell proliferation assays were completed. Leptin supplementation did not affect CT1 proliferation at any dose tested. In conclusion, leptin supplementation did not affect bovine trophoblast cell proliferation or *IFNT* expression, but leptin increases *CSH2* and *MMP2* transcript abundance. Both of these factors are involved with peri- and post-implantation placental development and function, and this implicates leptin as a potential mediator of early placental development and function in cattle.

Keywords

Placenta, trophoctoderm, leptin, gene expression, cattle

1. Introduction

Leptin is best known as an adipose-derived hormone that controls food intake and adiposity in mammals, but leptin also has noted effects on reproduction in various mammals, including cattle. The placenta is an apparent target of leptin action. There is an inverse relationship between circulating leptin concentrations and placental size in early pregnancy and at term in cattle [1]. Placenta-derived leptin may also influence pregnancy in cattle. Leptin and its receptor are produced by the bovine pre-implantation embryo, placenta and endometrium [2,3]. Placental leptin overexpression is associated with placentomegaly in nuclear transfer-derived bovine pregnancies [3]. These abnormal pregnancies contain excessive trophoblast cell proliferation and differentiation and produce greater circulating concentrations of placental lactogen (now termed chorionic somatomammotropin hormone 2 [CSH2]), a hormone produced by invasive binucleated trophoctoderm cells [3]. Errors in methylation status within the leptin promoter are implicated as one reason why leptin expression is altered in these nuclear-derived pregnancies [4].

This work tested the hypothesis that leptin acts in various capacities to impact early placental development and function in cattle. Studies were completed using a bovine trophoblast cell line (CT1) [5]. This cell line possesses a pre-implantation stage morphology and gene expression profile of bovine trophoblast cells and has been useful for describing how other endometrial- and embryo-derived factors impact pre- and peri-implantation events in bovine trophoctoderm [5,6]. In this work, CT1 cells were used to explore how leptin supplementation influences transcript profiles for several factors involved with maternal recognition of pregnancy factor, placental differentiation and hormone production, and placental attachment and implantation.

2. Materials and Methods

2.1 Bovine trophoblast cell culture

The bovine trophoblast cell line, CT1, was cultured as described previously [5]. In brief, cells were maintained on MatrigelTM Basement Membrane Matrix (BD Biosciences, Bedford, MA) with Dulbecco's Modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, ME, USA) containing 40 mM glucose, 10% [v/v] fetal bovine serum (FBS; Life Technologies), 100 μ M non-essential amino acids (Thermo Fisher Scientific), 55 μ M β -mercaptoethanol (Thermo Fisher Scientific), and antibiotic/antimycotic (100 U/mL penicillin G, 100 μ g/mL streptomycin sulfate, 250 ng/mL amphotericin B; Thermo Fisher Scientific). Cultures were maintained at 38.5°C with 5% CO₂ in air. For both the transcript profiling work and the proliferation assay, cells were serum-starved for 24-h prior to adding treatments.

2.2 Transcript profiling

Procedures were completed as described previously [7]. CT1 cells were seeded onto 6-well plates (9.5 cm²/well) with a CellBIND® surface (Corning, Tewksburg, MA, USA) (~1 x 10⁵ cells/well). After 3 to 5-d of growth (appr. 50% confluency), cells were serum-starved for 1 d and then medium was replaced with fresh serum-free CT1 medium containing 0, 10, 50 or 250 ng/mL recombinant bovine leptin. After either 6- or 24-h, total cellular (tc) RNA was extracted using Trizol® and the PureLink® Micro-to-Midi Total RNA Purification System (Thermo Fisher Scientific). Purity and quantity of RNA was determined using a NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific). Only samples with an A₂₆₀/A₂₈₀ ratio > 1.8 were used.

For quantitative (q) reverse transcription (RT) PCR [7], total RNA (10 ng/reaction) was treated in RNase-free DNase (New England Biolabs, Ipswich, MA, USA) and reverse transcribed (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Austin, TX, USA). PCR was completed using SYBR Green Master Mix reagent (Applied Biosystems) and specific primer pairs (Table 1) using a RealPlex Master Cyclor (Eppendorf, Hamburg, Germany). All primers were validated for efficiency before use (range = 86-97%). Reactions were completed with 40 amplification cycles using 58 to 60 °C annealing/synthesis (depending on the primer set) for 1 min and 95 °C denaturation for 15-sec. Amplification of a single PCR product was verified for each PCR run by dissociation curve analysis. Non-reverse transcribed RNA samples were included as negative controls. Ribosomal protein S9 (*RPS9*) served as the internal reference control. *RPS9* mRNA abundance did not differ based on leptin treatment (data not shown). The threshold cycle (C_T) values were expressed relative to the abundance of the reference control ($2^{-C_T(\text{transcript of interest})}/2^{-C_T(RPS9)}$) [8].

2.3 CT1 cell proliferation assay

This assay was completed as described previously [6]. In brief, cells were seeded onto 24-well plates (1.9 cm²/well; (~1 x 10⁴ cells/well). After 2-d of growth and 1 d of serum-starvation, medium was replaced with fresh serum-free CT1 medium containing 0, 10, 50 or 250 ng/mL recombinant bovine leptin (DevaTal Inc., Hamilton, NJ, USA). After 23 h, 5-ethynyl-2'-deoxyuridine (EdU; 100 µM) was added and cells were incubated for 45-min. Cells were fixed (4% [w/v] paraformaldehyde), and the Click-ItTM EdU Cell Proliferation Assay containing Alexa Fluor® 488 detection (Thermo Fisher Scientific) was used to detect EdU-positive nuclei. Cells were counterstained with Hoechst 33342 (10 µg/ml) to visualize all nuclei. The EdU-positive

and total nuclei were counted in 5 representative fields (range: 30 to 150 cells/field) in each well using NIS-Elements Software (Nikon Instruments Inc., Melville, NY) after capture with an Eclipse Ti-E inverted microscope equipped with an X-Cite 120 epifluorescence illumination system and DS-L3 digital camera (Nikon Instruments Inc.). Each treatment was assessed in three wells in each replicate study. Mitotic index was calculated by averaging the percentage of EdU-positive nuclei for all of the readings for each treatment.

2.4 Statistical analysis

Data were analyzed by ANOVA using the general linear model (GLM) of the Statistical Analysis System (SAS institute, Cary, NC, USA) for the effects of leptin concentration on outcomes. The C_T ratio data were log-transformed before analysis. Individual means were compared by pair-wise comparisons (PDIF analysis, SAS). Data presented as arithmetic means and SEM.

3. Results

The first set of studies examined potential roles for leptin in regulating early placental hormone and cytokine production (Fig. 1). The first transcript examined was *IFNT*, the maternal recognition of pregnancy factor in ruminants [9]. Leptin did not influence *IFNT* mRNA abundance at either time point (Fig. 1A&B). Two time periods (6-h and 24-h) were chosen for this and other studies to maximize opportunities to detect leptin-dependent changes in gene expression.

The second transcript examined was *CSH2*, which encodes placental lactogen, a well-characterized placental hormone produced primarily by differentiated, binucleate cells (BNCs) in

ruminants [10]. Increases ($P < 0.05$) in *CSH2* mRNA abundance were detected at 6 and 24 h with each leptin concentration tested (Fig. 1C&D). After 6-h of treatment, each leptin concentration increased ($P < 0.05$) *CSH2* mRNA abundance to the same extent (Fig. 1C), but a biphasic leptin dose-response was evident after 24-h of leptin exposure, where greater increases ($P < 0.05$) in *CSH2* mRNA abundance was detected in cells supplemented with 50 ng/mL leptin than when treated with either 10 or 250 ng/mL (Fig. 1D).

Another set of transcripts examined were matrix metalloprotease-2 and -9 (*MMP2/9*). Both enzymes play active roles in placental remodeling through gestation in cattle and other ruminants [11]. The relative abundance of *MMP2* mRNA was unaffected by 6-h leptin exposure, but a dose-dependent increase ($P < 0.05$) in *MMP2* abundance was detected after 24-h leptin supplementation (Fig. 2A&B). No changes in *MMP9* mRNA abundance were evident after 6-h or 24-h of leptin supplementation (Fig. 2C&D).

A final study examined whether leptin serves as a mitogen in bovine trophoblast cells. Supplementation with either 50- or 250-ng/ml leptin for 24 h did not affect the mitotic index of CT1 cells (Fig. 3).

4. Discussion

This project was undertaken to fill a gap in knowledge pertaining to leptin actions in the early developing bovine placenta. Much is known in cattle and other ruminants about leptin actions relating to metabolism, adiposity and milk/meat production, but little work exists for exploring actions of leptin within the bovine placenta. An expansive amount of information in rodents and humans indicates that leptin controls trophoblast cell proliferation, placental cell

161 survival and differentiation, placental hormone production, and implantation [12,13]. This work
162 explored if these events also are manipulated by leptin in bovine trophoblast cells.

163 The first action of leptin investigated was its ability to mediate trophoblast cell hormone
164 production. IFNT was examined because of its necessity for the establishment and maintenance
165 of pregnancy in ruminants [9]. Present findings indicate that leptin is not playing a direct role in
166 controlling *IFNT* expression. Previous work indicated that *IFNT* mRNA abundance mirrors
167 IFNT protein production [5]; therefore an effect for leptin on IFNT protein is unlikely. With that
168 said, indirect effects of leptin on maternal recognition of pregnancy are likely. Leptin and other
169 hormones (*e.g.* insulin-like growth factor 1, [IGF1]) altered by body condition and adiposity
170 impact uterine function, and this compromises conceptus development and IFNT production
171 [14].

172 The second hormone examined was CSH2. This prolactin-like hormone is produced by
173 the invasive and fusogenic binucleate cells (also known as trophoblast giant cells) from d 21 of
174 pregnancy onward [15]. CSH2 is involved with various maternal adaptations to pregnancy in
175 ruminants, including systemic effects such as increasing liver *IGF1* and *IGFBP3* mRNA
176 abundance [16] and local effects such as promoting uterine gland secretions [17]. An association
177 between leptin and CSH2 expression has been made previously. Both *leptin* and *CSH2* mRNA
178 abundance are increased in nuclear transfer-derived pregnancies [3]. It is not clear if leptin is the
179 causative factor regulating *CSH2* expression in these pregnancies, but the current work supports
180 the contention that leptin serves in this capacity.

181 The positive effect of leptin on *CSH2* expression also may encourage the continued use
182 of CT1 cells to study *CSH2* gene expression. Previous examinations of bovine or ovine *CSH2*
183 expression regulation required heterologous transfection studies using human and rodent

choriocarcinoma lines [18,19]. Marginal *CSH2* expression occurs in bovine trophoblast cell lines, including CT1 cells under normal growth conditions. This likely is due to the low incidence of binucleate cell formation in these cultures. Inducing binucleate cell development is challenging in these cultures [20]. It was interesting to observe increases in CT1 cell *CSH2* expression in the absence of binucleate cell formation, as observed by visual inspection of cell morphology. Thus, CT1 cells may serve as a useful homologous cell system to study *CSH* expression in ruminants.

The third set of factors examined were the gelatinases, MMP2 and MMP9. These enzymes have been linked with key trophoblast invasion and placental remodeling events in mammals, including ruminants [21,22]. The interest in examining leptin as a controller of MMP2 and MMP9 was inspired by previous work observing increases in placental invasion and placentomegaly in placentae of nuclear transfer-derived pregnancies, which contain elevated leptin expression [3]. In CT1 cells, leptin stimulated *MMP2* mRNA abundance but not *MMP9* abundance. This is the first examination of leptin in bovine placental cells. Work in human cytotrophoblast cells also found that leptin preferentially increased *MMP2* mRNA abundance [12]. Unfortunately, the present study did not examine MMP activity. Leptin promotes both MMP2 and MMP9 enzyme activities in human placental cells [12,23]. Observing the same effects in bovine placental cells will be needed to firmly establish the link between leptin and placental remodeling in cattle.

It was interesting to note that leptin did not influence *MMP2* mRNA abundance after 6-h of treatment. Rather, 24-h was required to detect a leptin effect. The extended timing of this response suggests that leptin is not acting through a direct signaling cascade to stimulate *MMP2* transcription and/or limit *MMP2* mRNA degradation. Instead, an indirect action of leptin,

wherein leptin stimulates regulatory factors that must undergo transcription/translation before they can promote *MMP2* transcription likely occurred.

The final study examined whether leptin serves as a proliferative factor in bovine placental cells. Leptin's role as a proliferative agent in trophoblast cells is ambiguous in other species [24,25]. This work determined that leptin does not facilitate proliferation in bovine trophoblast cells. However, leptin may act by other means to promote trophoblast survival in cattle. Leptin is an anti-apoptotic factor in various cell types, including placental and embryonic cells [24]. Leptin also contains embryotrophic activities. It enhances bovine oocyte fertilization and subsequent embryo competency [26,27]. Therefore, leptin may well serve to control embryonic and early placental development through mechanisms other than directly influencing the mitogenic activity of cells.

In conclusion, the positive effect of leptin supplementation on *CSH2* and *MMP2* expression in bovine trophoblast cells and the lack of effects on *IFNT* expression and cell proliferation suggest that leptin may be acting as mediator of implantation and post-implantation hormone production in cattle. The work also suggests that leptin likely does not function as a mitogen in trophoblast cells. However, the positive effects of leptin on oocyte and embryo development in previous studies and present work describing positive effects of leptin on *CSH2* and *MMP2* expression implicate leptin as a mediator of embryonic development and implantation in cattle.

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References

- [1] Sullivan TM, Micke GC, Magalhaes RS, Phillips NJ, Perry VE. Dietary protein during gestation affects placental development in heifers. *Theriogenology* 2009;72:427-438.
- [2] Madeja ZE, Warzych E, Peippo J, Lechniak D, Switonski M. Gene expression and protein distribution of leptin and its receptor in bovine oocytes and preattachment embryos produced in vitro. *Animal* 2009;3:568-578.
- [3] Ravelich SR, Shelling AN, Ramachandran A, Reddy S, Keelan JA, Wells DN, Peterson AJ, Lee RS, Breier BH. Altered placental lactogen and leptin expression in placentomes from bovine nuclear transfer pregnancies. *Biol Reprod* 2004;71:1862-1869.
- [4] Kremenskoy M, Kremenska Y, Suzuki M, Imai K, Takahashi S, Hashizume K, Yagi S, Shiota K. Epigenetic characterization of the cpg islands of bovine leptin and pou5f1 genes in cloned bovine fetuses. *J Reprod Dev* 2006;52:277-285.
- [5] Michael DD, Alvarez IM, Ocon OM, Powell AM, Talbot NC, Johnson SE, Ealy AD. Fibroblast growth factor-2 is expressed by the bovine uterus and stimulates interferon-tau production in bovine trophectoderm. *Endocrinology* 2006;147:3571-3579.
- [6] Xie M, McCoski SR, Johnson SE, Rhoads ML, Ealy AD. Combinatorial effects of epidermal growth factor, fibroblast growth factor 2 and insulin-like growth factor 1 on trophoblast cell proliferation and embryogenesis in cattle. *Reprod Fertil Dev* 2015.
- [7] Yang QE, Johnson SE, Ealy AD. Protein kinase c delta mediates fibroblast growth factor-2-induced interferon-tau expression in bovine trophoblast. *Biology of Reproduction* 2011;84:933-943.
- [8] Yang QE, Fields SD, Zhang K, Ozawa M, Johnson SE, Ealy AD. Fibroblast growth factor 2 promotes primitive endoderm development in bovine blastocyst outgrowths. *Biol Reprod* 2011;85:946-953.
- [9] Ealy AD, Yang QE. Control of interferon-tau expression during early pregnancy in ruminants. *American Journal of Reproductive Immunology* 2009;61:95-106.
- [10] Warren WC, Keisler DH, Anthony RV. Synthesis and secretion of ovine placental lactogen and its biochemical properties. *Domest Anim Endocrinol* 1990;7:331-342.
- [11] Kizaki K, Ushizawa K, Takahashi T, Yamada O, Todoroki J, Sato T, Ito A, Hashizume K. Gelatinase (mmp-2 and -9) expression profiles during gestation in the bovine endometrium. *Reprod Biol Endocrinol* 2008;6:66.
- [12] Castellucci M, De Matteis R, Meisser A, Cancellato R, Monsurro V, Islami D, Sarzani R, Marzioni D, Cinti S, Bischof P. Leptin modulates extracellular matrix molecules and metalloproteinases: Possible implications for trophoblast invasion. *Mol Hum Reprod* 2000;6:951-958.
- [13] Schulz LC, Widmaier EP, Qiu J, Roberts RM. Effect of leptin on mouse trophoblast giant cells. *Biol Reprod* 2009;80:415-424.

- [14] Wiltbank MC, Baez GM, Garcia-Guerra A, Toledo MZ, Monteiro PL, Melo LF, Ochoa JC, Santos JE, Sartori R. Pivotal periods for pregnancy loss during the first trimester of gestation in lactating dairy cows. *Theriogenology* 2016;86:239-253.
- [15] Patel OV, Yamada O, Kizaki K, Todoroki J, Takahashi T, Imai K, Schuler LA, Hashizume K. Temporospatial expression of placental lactogen and prolactin-related protein-1 genes in the bovine placenta and uterus during pregnancy. *Mol Reprod Dev* 2004;69:146-152.
- [16] Currie MJ, Bassett NS, Breier BH, Klempt M, Min SH, Mackenzie DD, McCutcheon SN, Gluckman PD. Differential effects of maternal ovine placental lactogen and growth hormone (gh) administration on gh receptor, insulin-like growth factor (igf)-1 and igf binding protein-3 gene expression in the pregnant and fetal sheep. *Growth Regul* 1996;6:123-129.
- [17] Spencer TE, Gray A, Johnson GA, Taylor KM, Gertler A, Gootwine E, Ott TL, Bazer FW. Effects of recombinant ovine interferon tau, placental lactogen, and growth hormone on the ovine uterus. *BiolReprod* 1999;61:1409-1418.
- [18] Jeckel KM, Limesand SW, Anthony RV. Specificity protein-1 and -3 trans-activate the ovine placental lactogen gene promoter. *Mol Cell Endocrinol* 2009.
- [19] Liang R, Limesand SW, Anthony RV. Structure and transcriptional regulation of the ovine placental lactogen gene. *European journal of biochemistry / FEBS* 1999;265:883-895.
- [20] Koshi K, Suzuki Y, Nakaya Y, Imai K, Hosoe M, Takahashi T, Kizaki K, Miyazawa T, Hashizume K. Bovine trophoblastic cell differentiation and binucleation involves enhanced endogenous retrovirus element expression. *Reprod Biol Endocrinol* 2012;10:41.
- [21] Cohen M, Bischof P. Factors regulating trophoblast invasion. *Gynecol Obstet Invest* 2007;64:126-130.
- [22] Ulbrich SE, Meyer SU, Zitta K, Hiendleder S, Sinowatz F, Bauersachs S, Buttner M, Frohlich T, Arnold GJ, Reichenbach HD, Wolf E, Meyer HH. Bovine endometrial metalloproteinases mmp14 and mmp2 and the metalloproteinase inhibitor timp2 participate in maternal preparation of pregnancy. *Mol Cell Endocrinol* 2011;332:48-57.
- [23] Gonzalez RR, Devoto L, Campana A, Bischof P. Effects of leptin, interleukin-1alpha, interleukin-6, and transforming growth factor-beta on markers of trophoblast invasive phenotype: Integrins and metalloproteinases. *Endocrine* 2001;15:157-164.
- [24] Magarinos MP, Sanchez-Margalet V, Kotler M, Calvo JC, Varone CL. Leptin promotes cell proliferation and survival of trophoblastic cells. *BiolReprod* 2007;76:203-210.
- [25] Schulz LC, Widmaier EP. The effect of leptin on mouse trophoblast cell invasion. *Biol Reprod* 2004;71:1963-1967.
- [26] Arias-Alvarez M, Bermejo-Alvarez P, Gutierrez-Adan A, Rizos D, Lorenzo PL, Lonergan P. Effect of leptin supplementation during in vitro oocyte maturation and embryo culture on bovine embryo development and gene expression patterns. *Theriogenology* 2011;75:887-896.
- [27] Boelhauve M, Sinowatz F, Wolf E, Paula-Lopes FF. Maturation of bovine oocytes in the presence of leptin improves development and reduces apoptosis of in vitro-produced blastocysts. *Biol Reprod* 2005;73:737-744.

312 **Table 1.** Primer sequences used to complete quantitative RT-PCR.

Gene Name	Primer Sequence (5' to 3')
<i>CSH2</i>	Forward: CGGATTCCCCTTCAAAGCCT Reverse: CCTCAACAGGGCTTCGTCAA
<i>IFNT</i>	Forward: GCCCGAATGAACAGACTCTC Reverse: CCATCTCCTGAGGAAGACCA
<i>MMP2</i>	Forward: GGCATCTCTCAGATCCGTGG Reverse: TGTGGGTCTTCGTACACAGC
<i>MMP9</i>	Forward: AGGGTAAGGTGCTGCTGTTC Reverse: AGGAGGTCTGAAGGTCACGTA
<i>RPS9</i>	Forward: GAGCTGGGTTTGTGCGCAA Reverse: GGTCGAGGCGGGACTTCT

313 * All primers were chosen based on Primer Design Software from NCBI.

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Figure Legends:

Figure 1. Bovine leptin supplementation does not affect *IFNT* expression but increases *CSH2* mRNA abundance in bovine trophoblast cells. CT1 cells were exposed to 0, 10, 50 or 250 ng/mL recombinant bovine leptin for either 6 h (Panels A & C; n=3 replicate studies) or 24 h (Panels B & D; n = 4 replicate studies), and then RNA was extracted and analyzed to quantify the relative abundance of *IFNT* (Panels A & B) and *CSH2* (Panels C & D) mRNA. Different superscripts within each panel represent differences ($P < 0.05$).

Figure 2. Bovine leptin supplementation increases *MMP2* mRNA abundance but does not affect *MMP9* abundance in bovine trophoblast cells. CT1 cells were exposed to 0, 10, 50 or 250 ng/mL recombinant bovine leptin for either 6 h (Panels A & C; n=5 replicate studies) or 24 h (Panels B & D; n = 5 replicate studies), and then RNA was extracted and analyzed to quantify the relative abundance of *MMP2* (Panels A & B) and *MMP9* (Panels C & D) mRNA. Different superscripts within each panel represent differences ($P < 0.05$).

Figure 3. Bovine leptin supplementation does not affect bovine trophoblast cell proliferation. CT1 cells were serum-starved overnight, and then were challenged with 0, 10, 50 or 250 ng/mL recombinant bovine leptin. After 23 h, EdU reagent was added for 45 min, and then cells were processed and examined by epifluorescence microscopy for the percentage of EdU positive cells (Mitotic Index) (n = 3 replicate studies). Different superscripts within each panel represent differences ($P < 0.05$).

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A**6 hr** **B****24 hr** **C****6 hr** **D****24 hr**

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