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Association Between LH receptor Regulation and Ovarian Hyperstimulation Syndrome in a Rodent Model

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Abstract

Ovarian hyperstimulation syndrome (OHSS) is a common complication of ovarian stimulation associated with the administration of human chorionic gonadotropin (hCG) during assisted reproduction. We have determined the expression of luteinizing hormone receptor (*Lhcgr*) mRNA, vascular endothelial growth factor (VEGF) and its transcription factor, HIF1α during the periovulatory period in a rodent model of OHSS and compared these results with normal ovulatory periods. These results showed that the downregulation of *Lhcgr* mRNA in response to conditions that mimic preovulatory LH surge was significantly impaired in the OHSS group compared to the complete downregulation seen in the control group. Most importantly, the downregulation of luteinizing hormone receptor mRNA expression following hCG administration was sustained in the control group up to 48h, whereas it remained at significantly higher levels in the OHSS group. This impairment of hCG-induced *Lhcgr* downregulation in the OHSS group was accompanied by significantly elevated levels of VEGF and its transcription factor, HIF1α. Furthermore, the downregulation of *Lhcgr* that occurs in response to a preovulatory LH surge in normal cycles was accompanied by low levels of VEGF. This study shows that while downregulation of *Lhcgr* as well as low VEGF levels are seen in response to a preovulatory LH surge in normal ovarian cycle, impaired *Lhcgr* downregulation and elevated VEGF levels were found in the OHSS group.

Keywords

Ovarian hyperstimulation syndrome; assisted reproduction; *in vitro* fertilization; human chorionic gonadotropin; luteinizing hormone receptor

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AUTHOR CONTRIBUTION STATEMENT

CZ and TG performed experiments, analyzed data and contributed to manuscript preparation. BM analyzed data and contributed to manuscript preparation. KMJM conceived and supervised the study, interpreted the data and contributed to manuscript preparation.

DISCLOSURE

The authors report no conflicts of interest in this work.

INTRODUCTION

Ovarian hyperstimulation syndrome (OHSS) is one of the most serious complications of assisted reproduction treatments, arising from excessive stimulation of the ovaries by exogenous gonadotropins administrated during *in vitro* fertilization procedures. Women with OHSS are mainly characterized by enlarged ovaries and increased vascular permeability (VP) with massive fluid shift and accumulation in the peritoneal cavity, which is almost always associated with the administration of human chorionic gonadotropin (hCG) (Kumar, et al. 2011). The reported incidence of OHSS among all IVF treatment cycles is as high as 23% for all forms, and up to 5% for severe OHSS (Aboulghar and Mansour 2003, Delvigne and Rozenberg 2002, Ishikawa, et al. 2003, Schenker and Weinstein 1978). Other clinical features of severe OHSS include hypovolemia, oliguria and renal failure, pleural effusion, hydrothorax, hemoconcentration, and thromboembolic phenomena, and even death as a result of electrolyte and fluid imbalance (Ajonuma, et al. 2005). Although this potentially fatal condition has been recognized for the past four decades, the exact mechanism remains obscure.

The vascular endothelial growth factor (VEGF) family consists of physiological regulators of angiogenesis and mediators of vascular endothelial cell permeability (Ferrara and Keyt 1997). VEGFA, a member of the VEGF family, is one of the most important regulators of ovarian angiogenesis, supporting normal follicular growth and corpus luteum function (Geva and Jaffe 2000, Harada, et al. 2010). Several studies in humans and rodents have suggested that increased vascular permeability mediated by the VEGF/VEGFR2 pathway is responsible for the massive fluid shift from the intravascular compartment into the peritoneal cavity (Abramov, et al. 1997, Brinsden, et al. 1995, Kitsou, et al. 2014, Senger, et al. 1983). Serum levels of VEGF have been proposed to be a useful predictor of the risk of OHSS (Artini, et al. 1998, Manau, et al. 2007, Quintana, et al. 2008). Accordingly, the rise in the serum VEGF concentration that occurs after human chorionic gonadotropin (hCG) administration is thought to be an important prognostic marker of OHSS in IVF procedures (Agrawal, et al. 1999). This is supported by studies showing dose-dependent increases in VEGF expression in response to hCG (Neulen, et al. 1995, Saylan, et al. 2010) suggesting that hCG plays a pivotal role in inducing OHSS. Pregnant Mare Serum Gonadotropin (PMSG) treatment alone has been shown to produce a slight increase in vascular permeability as well as in VEGF expression, but not sufficiently to cause OHSS (Gomez, et al. 2002). Previous studies from our laboratory have shown that there is a close association between LH receptor and VEGF expression in the ovary (Harada, et al. 2010). This is consistent with the findings that OHSS occurs in response to hCG treatment for ovulation induction (Guimera, et al. 2009). Thus, it appears that the transient downregulation of *Lhcgr* expression that occurs in response to the preovulatory LH surge in normal ovarian cycles might prevent premature VEGF secretion by the ovaries and reduces the incidence of OHSS. Therefore, using an animal model of OHSS, the present study examined whether the downregulation of Lhcgr is aberrantly disrupted to cause premature production of VEGF. Our results show that in the OHSS model, the aberrant *Lhcgr* upregulation occurs in parallel with increased VEGF secretion compared to the superovulation model where

downregulation of *Lhcgr* expression is accompanied by lower levels of VEGF with no manifestations of OHSS.

MATERIALS AND METHODS

Reagents

Pregnant mare serum gonadotropin (PMSG) was purchased from Calbiochem (San Diego, CA, USA). Highly purified human chorionic gonadotropin (hCG, CR127) was purchased from Dr. A. F. Parlow through the National Hormone and Peptide Program (Torrance, CA, USA). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA). Real-time PCR primers for 18S rRNA, *Lhcgr*, and *Vegfa* (TaqMan Assay-on-Demand Gene Expression Product) and MultiScribe reverse transcriptase and TaqMan reverse transcription reagents were obtained from Applied Biosystems (Foster City, CA, USA). BCA reagents were obtained from Thermo Scientific (Rockford, IL, USA). Quantikine enzyme-linked immunosorbent assay (ELISA) for mouse VEGF was purchased from R&D Systems, Minneapolis, MN, USA. Antibodies for VEGF and HIF1α were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Animals

Immature (23-day-old) female Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA, USA). The rats were housed in a 12-hour light, 12-hour dark cycle and had free access to standard food and water. Animal handling and treatment were conducted in accordance with the accepted standards of humane animal care, as outlined in the Ethical Committee Guidelines of the University of Michigan and approved by the institutional animal use and care committee.

The OHSS and superovulation rat models

Rat models for OHSS were based on the previously published procedures (van de Lagemaat, et al. 2011). Briefly, a group of rats was injected subcutaneously with 20 IU of PMSG in 0.1 ml of saline for four consecutive days, followed by 30 IU of hCG on the fifth day. For the superovulation (normal ovulation control [NOC]) group, rats were injected subcutaneously with a single dose of 50IU of PMSG followed by 30 IU of hCG 48 h later. The body weights of the animals were recorded. The animals were euthanized by CO_2 asphyxiation at 0, 12, 24, and 48 hours after hCG treatment. The ovaries were collected, and the weight and diameter were measured before being frozen in liquid nitrogen for further use.

Vascular permeability test

Alterations in vascular permeability, leakage, and accumulation of albumin in the peritoneal cavity were measured by the Evans Blue dye method as previously described by Ujioka et al. (Ujioka, et al. 1997). Briefly, at the end of treatment periods, rats receiving different treatments were anesthetized with ketamine (100 mg/kg). A fixed volume (0.2 ml) of 5 mM Evans Blue (EB) dye (Sigma) diluted in sterile PBS was injected via the tail vein. Thirty minutes after injection of the dye, the peritoneal cavity was filled with 5 ml of 0.9% saline and massaged for 30 seconds. Subsequently, the fluid was carefully extracted with a vascular catheter to prevent tissue or vessel damage. To avoid any protein interference,

the peritoneal fluid was recovered in tubes containing 0.05 ml 0.1M NaOH. The ovaries were then removed and weighed (in pairs). After 10 minutes of centrifugation at 1000 g, the EB concentration of the peritoneal irrigated fluid was measured at 600 nm using a spectrophotometer (Biospec-1601, Shimadzu, Germany). The level of the extravagated dye in the peritoneal fluid was expressed as μg/100 g body weight.

RNA extraction and quantitative real-time PCR analysis

Total RNA was extracted using TRIzol reagent and 1μg was reverse transcribed in a volume of 20 μl using 2.5 μM random hexamer, 500 μM dNTPs, 5.0 mM $MgCl_2$, 8 U RNase inhibitor, and 25 U multiscribe reverse transcriptase. The reactions were carried out in a PTC-100 (MJ Research, Inc.) Programmable Thermal Controller (25°C for 10 minutes, 48 °C for 30 minutes, and 95°C for 5 minutes). The resulting cDNAs were diluted with nuclease-free water (1:5). The real-time PCR quantification was performed using 5 μl of the diluted cDNAs in triplicate with predesigned primers and probes for rat *Vegfa*, designed to detect all three major transcript variants ($VEGF_{120, 164, 188}$), *Lhcgr*, and 18S rRNA. Reactions were carried out in a final volume of 25 μl using Applied Biosystems 7300 Real-Time PCR system (95°C for 15 seconds, 60°C for 1 minute) after initial incubation for 2 minutes at 50°C and 10 minutes at 95°C. The fold changes in *Lhcgr* and *Vegfa* mRNA expression were calculated by the ΔΔCt method using 18S rRNA as the internal control as previously described (Menon, et al. 2011).

Quantitation of VEGF by enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) was performed according to the manufacturer's instructions. After weighing, the ovaries were homogenized in 500 μl of 1× phosphate–buffered saline (PBS) and stored overnight at −20°C. After two freeze-thaw cycles to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 ×g. The supernatants were then given a 5-fold dilution for detection. The detection sensitivity range of the kit was 7.8–500 pg/ml. The optical density of each well for each sample was determined using the VERSA max microplate reader. Results were expressed as pictograms of VEGF per milligram protein (pg/mg protein).

Western blot analysis

Ovaries from the NOC and OHSS groups were homogenized using radio-immunoprecipitation assay (RIPA) buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 50 mM NaF, and 0.1% sodium dodecyl sulfate in phosphate buffered saline) containing protease inhibitors. The homogenates were centrifuged at 10,000 rpm for 15 minutes. Supernatants were collected and the total protein content was quantitated using the BCA assay reagent method. Sixty μg of total proteins were separated in SDS-PAGE (10%), transferred to nitrocellulose membranes, and probed with specific antibodies. Protein loading was normalized by stripping and re-probing the same blots with β-tubulin antibody as indicated in each experiment. Detection of signals was performed using the SuperSignal West Femto maximum sensitivity Western blotting detection kit (Thermo Scientific).

Statistical analysis

Two-way ANOVA and multiple comparisons (Sidak) were used to determine the significance of the differences between treatment groups (NOC and OHSS). All calculations were performed using GraphPad Prism 6 software. Data are expressed as mean ± SEM (n=6 per period and group). Probability (p) values of less than 0.05 were considered significant.

RESULTS

The OHSS rat model

In order to show the validity of the model, initial studies were conducted to determine the changes in body weight, ovary weight and diameter, and vascular permeability of both groups. Analysis of body weight revealed that the body weight gain (the difference between final weight and baseline weight before PMSG injection) at 48 hours was significantly higher in the OHSS group (41±0.5g vs. 31±0.8g; Fig. 1A), but not in the NOC group (37±0.4g vs. 35±0.5g). This indicated that the body weight gain changed significantly when the symptoms of OHSS were aggravated. In terms of ovary diameter and weight, there was no significant difference between baseline and 48 hours after hCG injection in the NOC group. However, the ovary weight and diameter at both time points in the OHSS group were significantly higher compared to the corresponding time points in the NOC group (ovary weight: 380±20mg vs. 140±14 mg at 24 hours; 380±12mg vs. 180±13mg at 48 hours; ovary diameter: 8±0.3mm vs. 6±04mm at 24 hours; 8±0.2mm vs. 6.5±02mm at 48 hours; Figs 1B& 1C, $p<0.01$ at 24 hours). As expected, there was a significant increase of EB dye concentration in the peritoneal fluid of the OHSS rats compared to the NOC rats at both time points measured (4.2±0.08μg/100g vs. 3.1μg (baseline) and 5.9μg/100g vs.3μg at 48 hours hCG, $p<0.05$), indicating an increased vascular permeability in the OHSS group (Fig. 1D). These results indicated that the treatment regimen was successful in inducing characteristics of OHSS in our experimental group.

Impaired downregulation of *Lhcgr* mRNA in OHSS rats following hCG treatment

To determine if there is a relationship between *Lhcgr* expression and OHSS induction, real-time PCR analysis was performed using ovaries from both the OHSS and NOC models. As expected, there was a significant downregulation of *Lhcgr* mRNA at 12 hours after hCG injection in the NOC group (80% compared to the saline-treated controls; $p<0.05$). The OHSS group also exhibited downregulation of *Lhcgr* mRNA, but it was significantly lower than that of the NOC group (50% vs. 80% downregulation at 12 hours). Furthermore, in the NOC group, the recovery of *Lhcgr* from downregulation was very gradual (50% recovery at 24 hours and 65% recovery at 48 hours). Conversely, in the OHSS group, the recovery of *Lhcgr* from downregulation was more rapid (94% recovery at 24 hours) and the *Lhcgr* mRNA levels were significantly upregulated (2.8±0.6-fold compared to the NOC group (Fig. 2). Collectively, these results show that the ligand-induced downregulation of *Lhcgr* appeared impaired in the OHSS group. Consequently, there were significantly elevated levels of *Lhcgr* expression post-hCG injection in the rats from the OHSS group.

Impaired ligand-induced downregulation of *Lhcgr* was accompanied by an increase in the expression of VEGF (mRNA and Protein) in OHSS rats

In order to investigate the relationship between the early recovery of *Lhcgr* from hCG-induced downregulation, expression of VEGF, and the onset of OHSS, we compared the expression levels of *Vegfa* mRNA and protein in the ovaries from the OHSS model with those from the NOC group by real-time PCR, Western blot analysis, and ELISA, respectively. The results of the real-time PCR analysis showed that, as expected, there was a significant downregulation of *Vegfa* mRNA at 12 hours (40% downregulation vs. control) in the NOC group. However, in the OHSS group, there was no sign of downregulation of *Vegfa* mRNA at 12 hours of hCG treatment. Instead, it showed a significant increase in a time-dependent manner compared to the corresponding time points in the NOC group (1.25-fold vs. 0.6-fold at 12 hours, 1.5-fold vs. 0.82-fold at 24 hours, and 1.9-fold vs. 0.9-fold at 48 hours, $p<0.01$; Fig. 3A). These results were further supported by the Western blot analyses of the ovary lysates, which showed a significant increase of VEGF protein expression in the OHSS group at all the time points examined, as compared to the corresponding time points in the NOC group (Fig. 3B). These results were further confirmed by ELISA, with quantitation of VEGF protein showing a significant time-dependent increase in the accumulation of VEGF in the ovaries of OHSS rats when compared to the NOC group (OHSS group vs. NOC group: 54±0.3 pg/mg protein vs. 46±0.5pg at 12 hours; 89±0.4 pg/mg vs. 49±0.3 pg at 24 hours, and 93±0.4pg/mg vs. 50±0.2pg/mg at 48 hours; Fig. 3C). There were increased basal levels of VEGF in OHSS animals at 0h prior to hCG administration. This increase is most likely due to stimulation by the LH activity associated with PMSG. Using highly purified preparations of PMSG, it has been previously demonstrated that PMSG has LH-like activity associated with the molecule (Moore and Ward 1980). It is possible that this causes an increase in the basal level expression of VEGF at 0 time hCG treatment.

Impaired ligand-induced downregulation of *Lhcgr* was accompanied by increased expression of HIF1α in OHSS rats

Several studies have shown that hypoxia is a potent stimulator of *Vegfa* expression and that the transcription factor hypoxia-inducible factor-1α (HIF1α) regulates *Vegfa* gene expression (Ahluwalia and Tarnawski 2012, Critchley, et al. 2006, Kurihara, et al. 2014, Zhang, et al. 2017). Furthermore, it has been shown that LH/hCG upregulates HIF1α in luteinized human granulosa cells (van den Driesche, et al. 2008). In light of these facts, we investigated whether the expression of HIF1α changes in the OHSS model compared to the NOC group following hCG treatment. Results of the Western blot analysis showed that there was a significant increase in HIF1α protein expression in OHSS rat ovaries at all the time points tested compared to the corresponding time points in the NOC group (OHSS group vs. NOC group: 3.8-fold vs. 1.8-fold at 12 hours, 4.8-fold vs. 2-fold at 24 hours, and 5.5-fold vs. 2-fold at 48 hours; $p<0.05$; Fig. 4). These results suggest that unlike the NOC group, the increased responsiveness of the ovary to hCG in the OHSS group might lead to an increase in VEGFA production that possibly contributes to the onset of OHSS.

DISCUSSION

Using an animal model of ovarian hyperstimulation syndrome (OHSS), the present study examined the acute changes in ovarian response to hCG administration in rats treated with multiple doses of PMSG to induce OHSS (OHSS group). These results were compared with those of animals treated with a single dose of PMSG to induce superovulation, followed by treatment with hCG (NOC group). While the OHSS animals manifested all known characteristics of OHSS such as massive cystic enlargement of the ovaries associated with increased capillary permeability, the triad of ascites, pleural effusion, and hemoconcentration (data not shown), the NOC animals were devoid of these characteristics and produced a normal response to hCG, such as the ability to undergo downregulation of *Lhcgr* mRNA expression similar to that seen in response to the preovulatory LH surge during the normal ovarian cycle. Since it is now recognized that increased angiogenesis is primarily responsible for OHSS, we focused our attention on the pattern of VEGF production in the OHSS group to explain the dysregulation of angiogenesis associated with OHSS.

VEGF is now recognized as a major regulator of angiogenesis. Since VEGF is known to play an important role in all phases of ovarian angiogenesis including follicle development as well as formation and maintenance of corpus luteum (Robinson, et al. 2009), it is likely that dysregulation of VEGF production might be a contributing factor in the onset of OHSS. We hypothesized that dysregulation of LHCGR following hCG administration might cause alterations in VEGF production and that this might, in turn, dysregulate angiogenesis. This is based on previous studies showing that OHSS occurs as a consequence of hCG administration (Elchalal and Schenker 1997, Kasum 2010, Rizk, et al. 1997). Furthermore, in our previous studies, we have demonstrated that during the ovarian cycle, the levels of expression of *Lhcgr* mRNA and *Vegfa* mRNA in the ovarian tissue show similar patterns consistent with the notion that VEGF production in response to hCG requires functional LHCGR (Harada, et al. 2010). *Lhcgr* expression during the ovarian cycle is a dynamic process since its expression shows continuous changes in response to a constantly changing milieu of gonadotropins and other paracrine factors. In the rat, during follicle development, *Lhcgr* expression rises gradually in response to increasing FSH levels, reaching maximum levels at the preovulatory stage (Zeleznik 2004). In response to the LH surge, *Lhcgr* expression shows a sudden decrease that lasts for about 72 hours before the levels begin to rise, reaching maximum levels by the mid portion of the luteal phase (Hoffman, et al. 1991, Peegel, et al. 1994). The levels then decrease with the regression of the corpus luteum. We have shown that the expression of *Vegfa* mRNA parallels *Lhcgr* mRNA levels during the ovarian cycle (Harada, et al. 2010). Detailed studies of the mechanism of *Lhcgr* downregulation following treatment with ovulatory doses of hCG revealed that the decline of *Lhcgr* mRNA is due to its increased decay mediated by an RNA binding protein rather than decreased transcription (Nair, et al. 2002, Nair and Menon 2004, 2005, Nair, et al. 2008). While the physiological role of LH receptor downregulation is not fully understood, it likely plays a role in regulating VEGF secretion since the downregulation of *Lhcgr* decreases VEGF levels.

The results of our present study show that in the OHSS model, the duration and amplitude of *Lhcgr* expression following treatment with an ovulatory dose of hCG is significantly lower than that seen in the NOC group. VEGF production was also elevated in ovaries from the OHSS model compared to the NOC group. Most interestingly, the levels of the transcription factor HIF1α, which is widely considered to be one of the transcription factors involved in *VEGF* expression was significantly elevated in the ovaries of OHSS animals following hCG administration compared to that seen in the NOC animals. The levels of both mRNA and protein levels of HIF1α was significantly elevated in the ovaries from the OHSS model. Recent studies have shown that the transcription factor HIF1α regulates VEGF-induced angiogenesis during corpus luteum development (van den Driesche, et al. 2008, Wu, et al. 2015), and decreases the oxygen concentration in the follicular fluid of growing follicles (Basini, et al. 2004). Our results show that there is a time-dependent upregulation of hypoxia-inducible factor in response to hCG treatment in OHSS-induced rat ovaries. Such an increase was not observed in the NOC group. These results suggest that high expression of LHCGR in the OHSS group creates a hypoxic environment thereby activating the expression of HIF1α and leads to the upregulation of VEGF. It should be pointed out that our results on the relationship between *Lhcgr* mRNA downregulation and *Vegfa* expression is only correlative at the present time, but it provides a foundation for embarking on future studies to unravel the regulatory role of LHCGR downregulation on VEGF production and its potential role in the onset of OHSS. Based on our present results, we speculate that the downregulation of LHCGR in response to a preovulatory LH surge probably prevents the onset of OHSS during the normal ovarian cycle. Conversely, dysfunction of *Lhcgr* downregulation might act as a contributing factor in the onset of OHSS.

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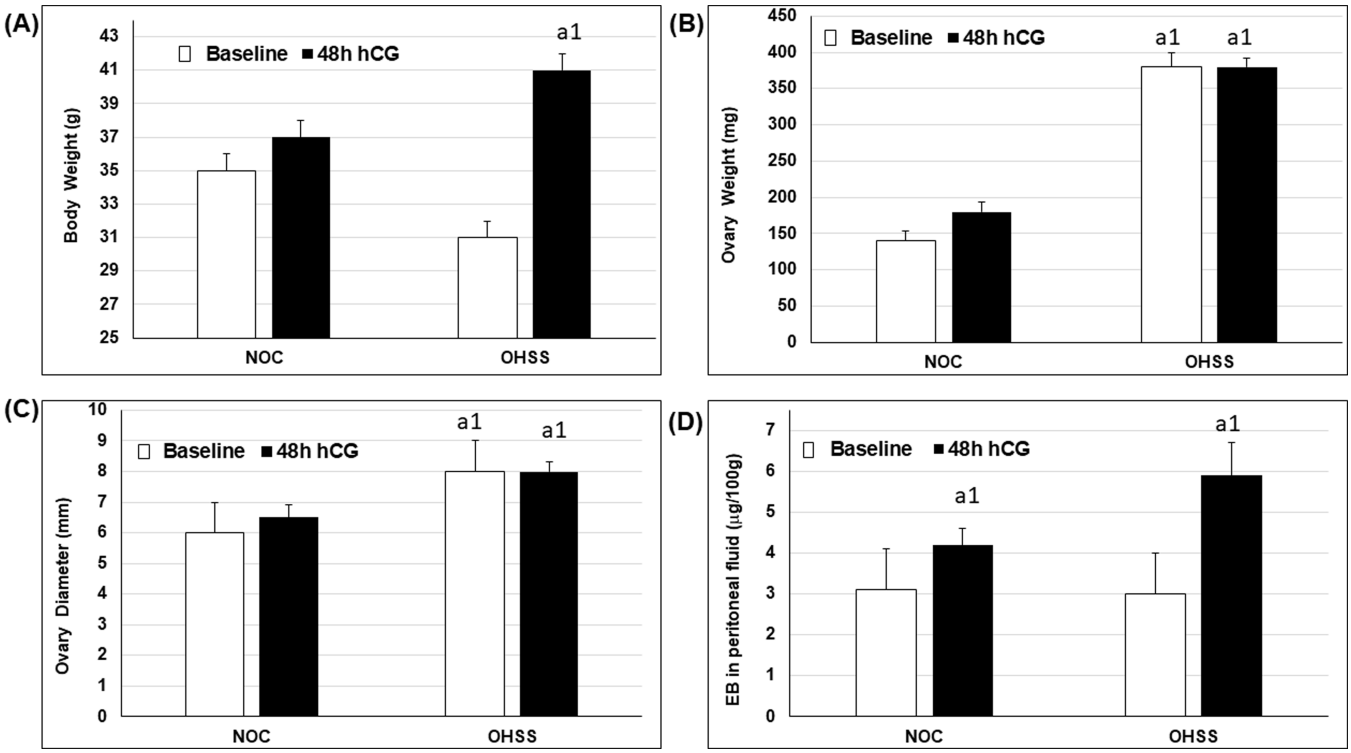

Fig. 1. Classical symptoms of OHSS were observed in OHSS-induced rats

OHSS and superovulation were induced in 23-day-old immature rats using the procedure detailed in the Methods section. Ovaries were harvested after different time intervals of hCG treatment and comparisons were made of (A) body weight, (B) ovary weight, (C) ovary diameter, and (D) vascular permeability of both NOC and OHSS-induced rats. The graphs represent ± SEM of the three independent experiments. [a1] $p<0.05$ vs. corresponding time points in the NOC group.

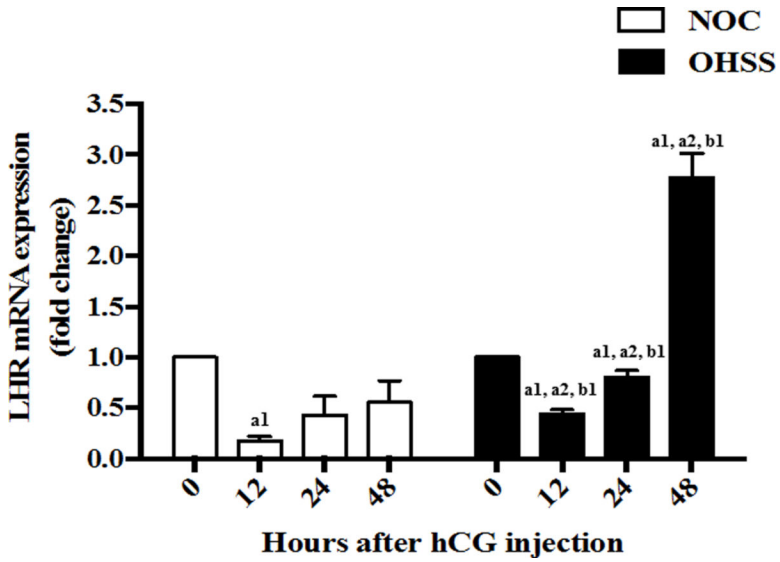

Fig. 2. Comparison of ligand-induced downregulation of *Lhcgr* mRNA in NOC and OHSS rat ovaries

Total RNA was extracted from the OHSS and NOC rat ovaries and real-time PCR was performed using specific primers and probes for LHCGR and 18S rRNA. The graphs represent the changes in LHCGR mRNA levels normalized with 18S rRNA. Error bars represent the mean SEM., $^{a1}p<0.05$ vs. 0 hours within each group, ^{a2} $p<0.01$ vs. 24 hours and 48 hours within each group, $^{b1}p<0.01$ vs. the same time points in the NOC group, respectively. Downregulation of *Lhcgr* was impaired in the OHSS rat ovaries compared to the control (NOC) ovaries.

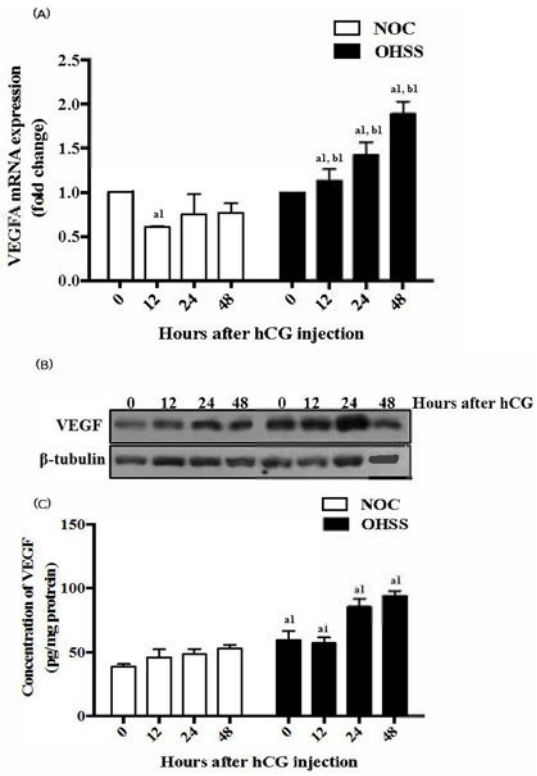

Fig. 3. RNA and protein expressions of VEGF in control (NOC) and OHSS rat ovaries

OHSS and superovulation (NOC) were induced in 23-day-old immature rats by the procedure outlined in the Methods section. (A) Total RNA isolated and real-time PCR analyses were performed using specific primers and probes for *VEGF* and *18S* rRNA. The graphs represent the changes in *VEGF* mRNA levels normalized with *18S* rRNA and are shown as fold change vs. control. Error bars represent the mean SE, $^{a1}p<0.05$ vs. 0 hours within each group, $^{a2}p<0.01$ vs. 24 hours and 48 hours within each group, $^{b1}p<0.01$ vs. the same time points in the NOC group, respectively. (B) Western blot analyses were performed using the ovarian RIPA lysates. Sixty μg of total proteins from the RIPA lysates of NOC and OHSS rat ovaries were separated on 10% SDS-PAGE. The proteins were transferred to nitrocellulose membrane and probed with VEGF antibody. The membranes were stripped and re-probed with βtubulin antibodies. The blots shown here are representative of the three independent experiments. (C) The levels of VEGF in the ovaries of NOC and OHSS rats were quantitated by ELISA. Error bars represent ± SE. $^{a1}p<0.05$ vs. the corresponding time points in the NOC group. VEGF protein and mRNA were increased in OHSS ovaries in comparison to NOC ovaries.

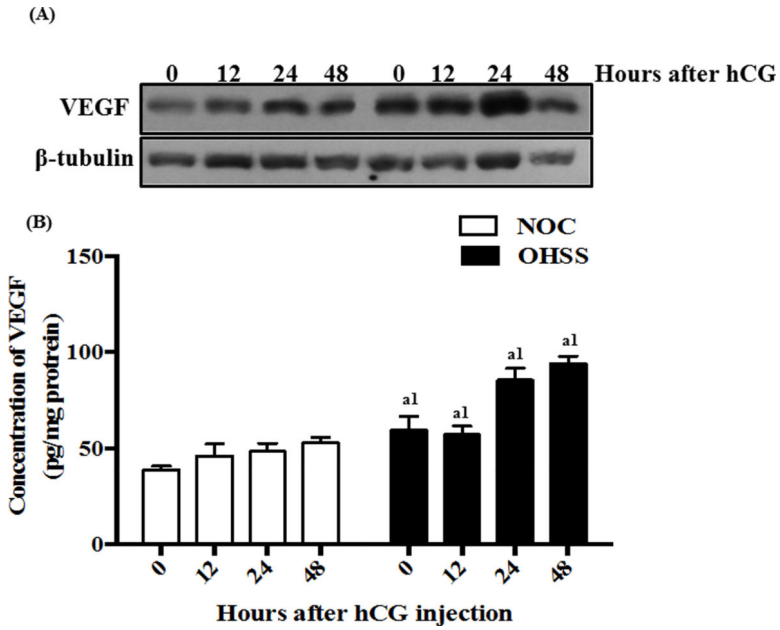

Fig. 4. HIF1α expression during ligand-induced downregulation of *Lhcgr* expression in control (NOC) and OHSS ovaries

Western blot analyses were performed using the ovarian RIPA lysates. Sixty μg of total proteins from the RIPA lysates of NOC and OHSS rat ovaries were separated on 10% SDS-PAGE. The proteins were transferred to nitrocellulose membrane and probed with HIF1α antibody. The membranes were stripped and re-probed with β-tubulin antibodies. The bar graphs represent the densitometry scanning of HIF1α blots with β-tubulin blots and expressed as fold change vs. control. The blots shown here are representative of the three independent experiments. The error bars represent ± SEM. $^{a1}p<0.05$ vs. the corresponding time points in the NOC group. HIF1α expression was induced in the OHSS group compared to the NOC group.