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Low-Dose Dopamine Agonist Administration Blocks Vascular Endothelial Growth Factor (VEGF)-Mediated Vascular Hyperpermeability without Altering VEGF Receptor 2-Dependent Luteal Angiogenesis in a Rat Ovarian Hyperstimulation Model

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No specific treatment is available for ovarian hyperstimulation syndrome (OHSS), the most important complication in infertile women treated with gonadotropins. OHSS is caused by increased vascular permeability (VP) through ovarian hypersecretion of vascular endothelial growth factor (VEGF)-activating VEGF receptor 2 (VEGFR-2). We previously demonstrated in an OHSS rodent model that increased VP was prevented by inactivating VEGFR-2 with a receptor antagonist (SU5416). However, due to its toxicity (thromboembolism) and disruption of VEGFR-2-dependent angiogenic processes critical for pregnancy, this kind of compound cannot be used clinically to prevent OHSS. Dopamine receptor 2 (Dp-r2) agonists, used in the treatment of human hyperprolactinemia including pregnancy, inhibit VEGFR-2-dependent VP and angiogenesis when administered at high doses in animal cancer models. To test whether VEGFR-2-dependent VP and angiogenesis could be segregated in a dose-dependent fashion with the Dp-r2 agonist cabergoline, a well-established OHSS rat model supplemented with prolactin was used. A 100 μg/kg low-dose Dp-r2 agonist cabergoline reversed VEGFR-2-dependent VP without affecting luteal angiogenesis through partial inhibition of ovarian VEGFR-2 phosphorylation levels. No luteolytic effects (serum progesterone levels and luteal apoptosis unaffected) were observed. Cabergoline administration also did not affect VEGF/VEGFR-2 ovarian mRNA levels. Results in the animal model and the safe clinical profile of Dp-r2 agonists encouraged us to administer cabergoline to oocyte donors at high risk for developing the syndrome. Prophylactic administration of cabergoline (5–10 μg/kg·d) decreased the occurrence of OHSS from 65% (controls) to 25% (treatment). Therefore, a specific, safe treatment for OHSS is now available. (*Endocrinology* 147: 5400–5411, 2006)

IN REPRODUCTIVE-AGE women, a state of circulatory dysfunction is observed frequently during ovarian stimulation with gonadotropins used for infertility treatment (1). In symptomatic patients such a deregulation involves increased vascular permeability (VP) and accumulation of fluid in body cavities because of fluid shifts from the intravascular to the extravascular space. This massive extravasation causes hemoconcentration with reduced organ perfusion, especially the kidney, alterations in blood coagulation with the risk of thromboembolic events and leakage of fluid into the peritoneal cavity and lungs causing abdominal discomfort and respiratory distress syndrome respectively (2–4). The presence of these symptoms define the ovarian hyperstimulation syndrome (OHSS), which has caused several deaths (5, 6).

It is known that presence of LH-like activity like human chorionic gonadotropin (hCG) plus an ovary (corpus luteum and/or antral follicles) is an absolute requirement for OHSS onset because the syndrome resolves or does not develop when oophorectomy is performed (7) or the ovulation inducing medication hCG is not administered (8, 9) during controlled ovarian hyperstimulation with gonadotropins. Because hCG has no vasoactive activities by itself (10), its actions must be mediated through one or more angiogenic substances released by the ovaries in response to this gonadotropin.

The prime candidate, initially called vascular permeability factor (11), is vascular endothelial growth factor (VEGF), the most important mediator of hCG-dependent ovarian angiogenesis (12–14). In fact, it is known that VEGF levels increase after hCG administration at the mRNA level in granulosa cells (12–19) and secreted protein levels in serum, plasma, and peritoneal fluids (20–22). VEGF not only stimulates new blood vessel development (23, 24) including the ovary (19, 25) but also induces vas-

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Abbreviations: Cb2, Cabergoline; Dp-r2, dopamine receptor 2; EB, Evans Blue; hCG, human chorionic gonadotropin; OHSS, ovarian hyperstimulation syndrome; P4, progesterone; PECAM, platelet endothelial cell adhesion molecule-1; prl, prolactin; p-Tyr, phospho-tyrosine; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling; VP, vascular permeability; VEGF, vascular endothelial growth facto; VEGFR-2, VEGF receptor 2.

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cular hyperpermeability (26, 27) by interacting with its VEGF receptor 2 (VEGFR-2) (28, 29).

To functionally demonstrate the importance of the VEGF/VEGFR-2 pathway in OHSS, our group has previously shown, in a rodent OHSS model, that increased VP could be reversed by using SU5416 (10), a compound blocking the intracellular phosphorylation of VEGFR-2 (30). Due to its side effect (thromboembolism, vomiting) profile (31, 32) and the possibility that it might interfere with early pregnancy development by blocking implantation-related ovarian (33–37) and uterine (38, 39) angiogenesis, this drug cannot be used clinically to treat OHSS. Thus, there is a need to pharmacologically segregate the permeability component from the angiogenic portion of the VEGF/VEGFR-2 pathway to treat this disease.

In an attempt to address this issue, we decided to focus on the dopamine/dopamine receptor 2 (Dp-r2) pathway, whose activation is involved in the regulation of angiogenic events (40, 41) mediated by VEGF/VEGFR-2 signaling (42). Administration of high doses of Dp-r2 agonists simultaneously blocks tumor-related angiogenesis and VP in a mouse cancer model by interfering with VEGF/VEGFR-2 signaling (43). *In vitro* studies suggested that the molecular mechanism underlying this action involved the internalization of VEGFR-2 induced by the activation of the Dp-r2 (43). It is well known that doses of Dp-r2 agonists much lower than those used in the tumor model (44) are sufficient to activate the Dp-r2 pathway as demonstrated by the fact that they decrease prolactin (prl) secretion by the pituitary gland (45). Thus, low-dose Dp-r2 agonists are used for the treatment of hyperprolactinemia in humans (46–50). Interestingly, at these low doses, Dp-r2 agonists do not exert antiangiogenic activity because physiological states of high level VEGR2-dependent vascular activity like formation of corpora lutea (36) or pregnancy development (34) are not affected (46–50). It remains to be determined whether low doses of Dp-r2-activating drugs retain the ability to decrease VP without affecting angiogenesis and, if so, whether these effects are mediated by VEGFR-2.

To answer these questions, we used the well-established rodent model of OHSS for the following reasons: 1) vascular hyperpermeability can be consistently induced with gonadotropins (51), which stimulate the ovary to produce vasoactive substances, primarily VEGF (10, 52); 2) this hyperpermeable state does not seem to involve activation of other permeability-enhancing processes like inflammation (3); 3) hyperpermeability is mainly localized to the abdominal cavity, in which an increase in fluid, *i.e.* ascites, is consistently observed (3) and can easily and objectively be quantified (10, 51, 53); 4) Dp-r2 is present on vasculature lining the abdominal cavity (43); and 5) the activity state of Dp-r2 can be very easily monitored by measuring prl levels. The fact that VEGF/VEGFR-2-dependent ovarian angiogenesis (36) occurs simultaneously with pathologically increased VP (10), in response to gonadotropin stimulation, makes this OHSS model an ideal one to test whether a low-dose Dp-r2 agonist can segregate both components mediated by this pathway (36, 54).

It is well known that in the rat (55) but not humans (56), prl is important for the formation and function of the corpora lutea mediated partially through proangiogenic actions (57). Therefore, it is conceivable that administration of Dp-r2 agonists in our rat model could alter OHSS through not only its effects on VEGF/VEGFR-2 pathway but also the induction of luteolysis by low prl levels (58). To compensate for this undesirable action of Dp-r2 agonists observed in our pilot study, we modified the rat OHSS model by using animals with exogenous prl replacement in our subsequent studies.

Using the prl-supplemented OHSS model, our study demonstrates that the antipermeability and antiangiogenic effects derived from VEGFR-2 blockade can be dose-dependently segregated by the Dp-r2 agonist cabergoline (Cb2). Based on this observation, we conclude that low-dose Cb2 may provide a new, specific, and nontoxic approach to the treatment of diseases such as OHSS and others, in which increased VP mediated by the VEGF/VEGR2 pathway must be blocked without affecting angiogenesis.

Materials and Methods

Animals

Immature, 15-d-old, female Wistar rats were obtained from Harlam Iberica (Sant Feliu de Codina, Spain) and kept 1 wk in our laboratory so experiments could start with 22-d-old, 42- to 48-g animals. They were fed a standard diet and allowed free access to water with a 12-h light, 12-h dark schedule (lights on from 0700–1900 h). All animal studies were carried out using a protocol approved by the Animal Care and Use Committee of the Valencia University School of Medicine and in accordance with National Institutes of Health (NIH) guidelines for the humane use of laboratory animals.

Drugs and reagents

General chemicals of analytical grade were obtained from Sigma (St. Louis, MO) and Merck & Co., Inc. (Darmstadt, Germany). Pregnant mare's serum gonadotropin was purchased from Sigma, and hCG (Profasi) was obtained from Serono Laboratories (Madrid, Spain). Prl pellets were obtained from Innovative Research (Sarasota, FL) and Cb2 (Dostinex) from Pharmacia & Upjohn (North Peapack, NJ). The anesthetic ketamine (Ketolar) was purchased from Parke-Davis (Barcelona, Spain). Hormones progesterone (P4) and prl were measured by using a commercially available kit (Diagnostic Products) and a RIA kit supplied by NIH, respectively. The primary mouse antirat and immunoabsorbed biotinylated conjugated secondary antibodies to detect platelet endothelial cell adhesion molecule-1 (PECAM; a specific marker of endothelial cells) were obtained from PharMingen (San Diego, CA), whereas the diaminobenzidine substrate kit for peroxidase staining was from Vector Laboratories (Burlingame, CA). Apoptosis was detected by using the Apoptag kit from Chemicon (Temecula, CA). The TRIzol reagent was obtained from Life Technologies, Inc. (Paisley, Scotland, UK). The protease and phosphatase inhibitor cocktails were from Roche Diagnostics (Indianapolis, IN) and Sigma, respectively. The D_C protein assay reagent was from Bio-Rad Laboratories (Hercules, CA). Protein A/G beads as well as the primary rabbit IgG1 VEGFR-2 antimouse monoclonal antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The general and $_{1214}$VEGFR-2 phosphotyrosine antibodies were from Abcam (Irvine, UK), whereas horseradish peroxidase immunoglobulins to detect the above-mentioned primary antibodies were from Dako (Glostrup, Denmark).

The OHSS rat model

All the animals undergoing this study were subjected to the same well-described ovarian hyperstimulation protocol to induce the major manifestations of OHSS (51): to promote follicular development, immature female Wistar rats received pregnant mare's serum gonadotropin 10 IU sc injection during 4 consecutive days, starting on d 22 of life. On the fifth day (d 26 of life), animals were given hCG 30 IU to induce ovulation. The major symptoms of OHSS, like hyperluteinized ovaries,

increased VP, peaking 48 h after hCG (d 28 of life) administration, and ascitis (10, 51, 52) developed in animals treated with this ovarian stimulation protocol in the absence of pharmacological interventions.

Experimental design

As our test substances, we chose Cb2 because it has fewer side effects and a longer half-life in comparison with other commercially available Dp-r2 agonists like bromocriptine (59–61).

Pilot study: the effects of Cb2 in OHSS non-prl-supplemented rats. To find the minimal Cb2 dose able to decrease prl levels (activate the Dp-r2) and its effects on VP, a pilot, dose-response experiment was performed by administering OHSS rats with 0, 10, 50, 100, and 500 μg/kg body weight Cb2 doses (n = 8 per dosage) dissolved in 5% glucosaline on the day of hCG, when the syndrome just onsets (10, 52). The control group received glucosaline (n = 8).

Experiment 1: Cb2 for the prevention of increased VP in the OHSS prl-supplemented model. Cb2 was administered on the day of hCG injection to treatment group animals at the following dosages: 50 (minimal dose able to decrease prl levels in the pilot experiment) and 100 and 500 μg/kg·d. To prevent prl-dependent luteolysis, as observed in the pilot experiment, prl pellets (5 mg), producing continuous serum levels of approximately 50–75 ng/ml, were implanted sc in Cb2-treated animals on the day of hCG. In one control group, prl pellets were also placed to make sure that supplementation had no effect on the OHSS parameters evaluated. In the other control group, the pellets contained placebo.

Experiment 2: Cb2 for the treatment of increased VP in the OHSS prl-supplemented model. As demonstrated by the presence of ascitic fluid, OHSS is fully developed after the first 24 h after hCG administration (10, 52). To test whether Cb2 could reverse a hyperpermeability state once it had occurred, a single dose of compound (100 μg/kg·d) was given at such time point in prl-supplemented animals. Two control groups were given a single dose of glucosaline (n = 8 each group). In one control group, prl pellets were also placed to make sure that supplementation had no effect on the OHSS parameters evaluated. In the other control group, the pellets contained placebo.

Sample collection

Animals were anesthetized with ketamine, 5 mg/kg ip, 48 h after hCG administration to measure VP (51). Subsequently the animals were killed and blood obtained by cardiopuncture. After centrifugation, sera were stored at −20 C for subsequent analysis of P4 and prl. Both ovaries were removed. One ovary was dehydrated at 4 C by increasing gradients of 5, 10, and 15% sucrose in PBS followed by immersion in a mixture of sucrose 15% PBS and optimum cutting temperature medium (1:1) overnight. Samples were embedded in optimum cutting temperature compound and stored at −70 C until further use for evaluation of vascularization and apoptosis. The other ovary was cut in two similar-size pieces and stored at −80 C for later analysis of VEGF-VEGFR-2 mRNA expression and VEGFR-2 phosphorylation levels.

Vascular permeability

To measure VP, a previously described method was used (51). In brief, a fixed volume (0.2 ml) of 5 mM Evans Blue (EB) dye diluted in distilled water was injected via the femoral vein. Thirty minutes after dye injection, the peritoneal cavity was filled with 5 ml 0.9% saline (21 C; pH 6) and massaged for 30 sec. Subsequently the fluid was slowly extracted with a vascular catheter to prevent tissue or vessel damage. To avoid any protein interference, peritoneal fluid was recovered in tubes containing 0.05 ml 0.1 N NaOH. After centrifugation at 900 × *g* for 12 min, EB concentration was measured at 600 nm on a Shimadzu 1201 spectrophotometer (Barcelona, Spain). The level of the extravasated dye in the recovered fluid was expressed as microgram of EB per 100 g body weight.

Hormone measurements

Serum concentrations of prl (dilution 1:10) were measured in duplicate in 10-μl samples using the double-antibody RIA method and according to the method described previously (62). Concentrations were expressed in nanograms per milliliter. The intraassay and interassay coefficients of variations were 8 and 11.2%, respectively, and the sensitivity 10 ρg/tube.

Progesterone levels were measured using an enzyme immunoassay kit. Samples (50 μl) were diluted 1:2 using reagents provided by the kit and assayed in duplicate. Concentrations were expressed in nanograms per milliliter. The intraassay and interassay coefficients of variations were 6.4 and 8.6%, respectively.

Tissue processing for immunohistochemistry and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL)

Several series of eight (5 micras gross) sections were obtained from the middle of each ovary. The series with the largest diameter sections was considered as representative of the whole ovary and was therefore processed. The first (+1) and last section (+8) in each series were stained with hematoxylin as a tissue quality sample control. The sections +3, +5, and +7 were used to detect PECAM with immunohistochemical techniques. The sections +2, +4, and +6 were selected for TUNEL techniques.

PECAM and TUNEL techniques

The immunohistochemical localization of the corpus luteum vasculature was evaluated by using a primary antibody against PECAM diluted 1:200; according to the manufacturer's instructions followed by counterstaining with hematoxylin.

The TUNEL assay was selected to study DNA fragmentation. Apoptotic cells detected by Apoptag kit were revealed by the presence of brown nuclei we counterstained with methyl green.

Quantitative image analysis

For quantitative analysis, two slides of each representative ovarian series were photographed in three random high-power (×40) fields with an Eclipse E800 camera (Nikon, Melville, NY) linked to an image analysis system (version 4.01; ImageProPlus, Silver Spring, MD). By using this method, a total of 48 photographs (two sections × three fields × eight ovaries) were analyzed for vascular density and apoptosis quantification purposes in each group.

For analysis of the blood vessel density, the photographed area of interest was outlined, highlighted, and measured in square millimeter. The density of PECAM was calculated by forming the ratio of the area of specific staining divided by the total area of the structure of interest (usually corpora lutea) multiplied by 100.

Because of the diffuse (brown) staining, the ImageProPlus program failed to appropriately segment the number of dark-stained nuclei in the quantification of apoptosis. Thus, this parameter was evaluated with a semiquantitative method by randomly positioning over the photographs a grid (established constant size of 40,000 square pixels) automatically generated by the Image J program (developed by NIH, free domain at http://rsb.info.nih.gov/ij). Only cells with a dark brown (apoptotic) or clear green (viable) color were counted in all diagonal squares. Cells positioned over left or upper margins in each individual square were excluded form the counting. Apoptosis was calculated by forming the ratio of the average number of apoptotic cells by the total cell number counted and multiplied by 100.

Quantitative fluorescence RT-PCR

VEGF and VEGFR-2 ovarian mRNA expression was quantified using the ABI PRISM 7700 thermocycler (PerkinElmer Corp., Foster City, CA), specific primers, and universal PCR conditions as previously described by our group (52). A similar approach was used to amplify Dp-r2 with a pair of primers, ^{711}GACACCACTCAAGGGCAACTGT^{732} forward and ^{816}AGCATCCATTCTCCGCCTG^{798} reverse (National Center for Biotechnology Information accession no. NM_012547) designed to respectively encompass the Dp-r2 exons 2–3 and 3–4, giving rise to an expected 106-bp PCR product. To reassure that no genomic DNA was being amplified, aliquots from the original mRNA lysates not subjected to reverse transcription were used as controls (−RT, see Fig. 1). At the end

of the PCR, the products were subjected to melting analysis and sequenced to verify that the presence of each single band corresponded to the expected ones.

Protein isolation from ovarian samples

Total protein was extracted from each tissue ovarian sample (half an ovary approximately) individually according to standard methods by using modified radioimmunoprecipitation assay buffer containing 0.2 mg/ml sodium orthovanadate, a protease, and a phosphatase inhibitor cocktail at the recommended concentrations. Protein concentration was measured using a D_C protein assay reagent. Individual lysates corresponding to animals in each group (n = 8) contributed equally (125 μg) to make a pool of 1000 μg protein.

VEGFR-2 immunoprecipitation

Each pooled lysate was incubated with 15 μl of anti-VEGFR-2 antibody in 1 ml of modified radioimmunoprecipitation assay buffer overnight at 4 C, and 30 μl of protein A/G beads were then added and incubated 4 h in a cold room with gentle rocking. The beads were pelleted by gentle centrifugation, the supernatant discarded, and the beads washed three times with PBS, resuspended in 30 μl of electrophoresis loading buffer, and heated at 99 C for 3 min before SDS-PAGE following standard methods.

Western blot

The blot was first probed for 90 min at room temperature with mouse antiphospho-tyrosine (p-Tyr) 1:1000 and a goat antimouse secondary antibody conjugated to horseradish peroxidase at a dilution of 1:2000. After stripping, reblotting was performed with phY1214 mouse antirat antibodies at a dilution 1:500, respectively. A dilution 1:1000 was used to detect VEGFR-2 (Flk-1), with the secondary antibody used at a concentration 1:5000.

Densitometric analysis

Blot bands obtained using anti-VEGFR-2, anti-p-Tyr and antiphosphotyrosine 1214 VEGFR-2 (phY1214) antibodies were photographed. Only the intensity of the upper band specifically recognized by VEGFR-2 antibody, corresponding to the mature form of the protein (~220 kDa) (63) in each line was analyzed using the Image Scion software (Scion Corp., Houston, TX) for densitometric purposes. Thus, only same-size bands recognized by p-Tyr and ph1214 antibodies were also included in the densitometric analysis. The whole levels of VEGFR-2 phosphorylation in each group were expressed as the ratio p-Tyr to total VEGFR-2 whereas specific phosphorylation levels for 1214VEGFR-2 site were expressed as phY1214 VEGFR-2 to total VEGFR-2 resulting from the densitometric analysis of the corresponding bands.

Statistical analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS, Inc., Chicago, IL). Data were expressed as the mean ± SEM. In the VP and mRNA expression experiments, after performing a Kruskal-Wallis, a nonparametric Mann-Whitney test was used to compare OHSS control values with those found in each specific treatment group. An ANOVA test (*post hoc* Scheffé) was used for detecting differences in prl and P4 measurements among groups. This test

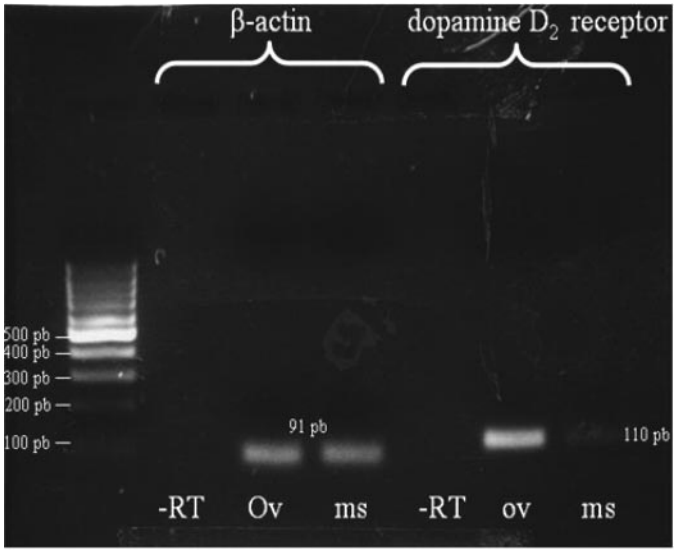

FIG. 1. RT-PCR Dp-r2 mRNA expression. Dp-r2 expression detected in ovarian (Ov) and mesenteric tissue (ms) from OHSS rats. Melting analysis after quantitative fluorescent RT-PCR provided detection of single-amplification products in both tissues. Subsequent electrophoresis in a 4% agarose gel reveals band sizes in the range of the expected length for Dp-r2 (106 bp) and β-actin (91 bp). Dp-r2 was present in the ovary (Ov) and at a lower concentration in the mesentery (ms) (very faint band). No products/bands were amplified/obtained when the reverse transcription process was omitted (−RT), indicating that specific primers did recognize mRNA and not genomic DNA.

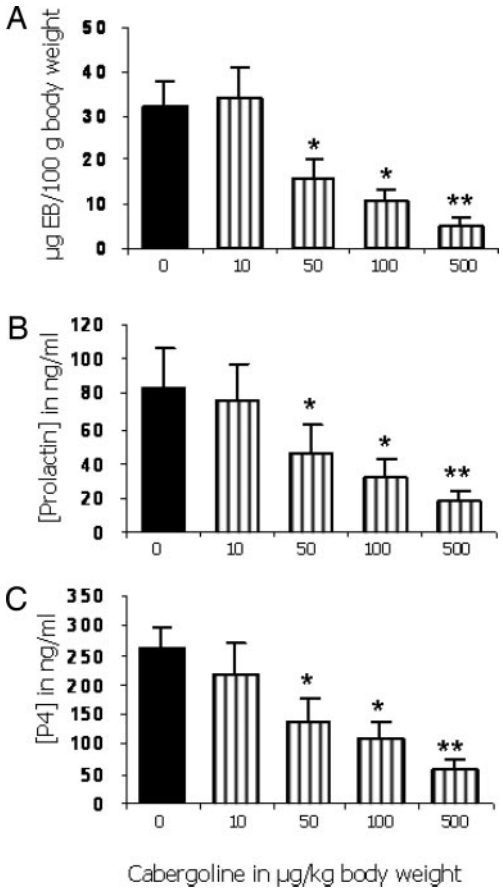

FIG. 2. The effects of Cb2 administration to nonsupplemented OHSS rats. A dose-response experiment was performed to find the minimal Cb2 dose activating the Dp-r2 in OHSS animals. Cb2 was given coinciding with the day of hCG administration. The following dosages were administered: 0 (control), 10, 50, 100, and 500 μg/kg·d (n = 8 animals per Cb2 dose). Vascular permeability (A) (as micrograms of extravasated EB dye per 100 g animal weight), serum prl (B) (nanograms per milliliter), and progesterone (C) (nanograms per milliliter) levels were measured 48 h after hCG injection. Please note that in the absence of exogenous prl replacement, a significant decrease in all parameters evaluated is observed starting at a 50 μg/kg·d Cb2 dose. *Bars*, mean + SEM in each group. *, $P < 0.05$; **, $P < 0.01$, comparison of OHSS control group (0) with different treatment groups.

was also used to find differences in vascularization and apoptosis. Significance was defined as $P < 0.05$.

Results

The presence of the Dp-r2 in the rat mesentery and corpus luteum

Before testing our hypothesis that low-dose Dp-r2 agonist blocks VEGF/VEGFR-2-mediated increase in peritoneal VP without affecting angiogenesis in the corpus luteum, we first demonstrated that Dp-r2 is present in the vasculature of corpora lutea and mesentery. The mesentery served as a positive control because Dp-r2 had been previously demonstrated to be expressed in rodent mesenteric endothelial cells (43). Primers used to amplify Dp-r2 showed a 106-bp PCR product in accordance with expected size (see Fig. 1). The sequentiation and posterior blast analysis showed a 100% homology with Dp-r2 rat mRNA (National Center for Biotechnology Information accession no. NM_012547), indicating that Dp-r2 was present in both tissues. The higher expression of Dp-r2 in the ovaries, compared with the mesentery, is very likely due to the higher abundance of vasculature in this tissue.

Pilot experiment: delimiting Cb2 doses able to potentially exert antipermeability effects in OHSS rats. The lowest Cb2 dose able to decrease VP in the OHSS model was 50 μg/kg·d, but such a dose also significantly decreased prl secretion, which in turn induced luteolysis as shown by a decline in serum P4 levels. Higher doses of Cb2 showed a similar pattern. A Cb2 dose of 10 μg/kg·d, which had no effect on prl secretion, affected neither VP nor P4 secretion when compared with controls (Fig. 2) Therefore, we were unable to find a Cb2 dose that significantly decreased VP without affecting negatively prl secretion and luteal function. From these experiments we learned that prl supplementation was necessary to adequately study the effects of Cb2 on VEGF/VEGFR-2-mediated events in our OHSS model. The prl levels measured in the OHSS control animals served as a guide for the amount of prl needed for proper replacement in Cb2-treated OHSS animals.

Experiment 1: 100 μg/kg·d low-dose Cb2 prevents the occurrence of vascular hyperpermeability without affecting angiogenesis. In control groups (with and without prl replacement), the major symptom of OHSS (10, 51, 52), increased VP with ascites, developed in all animals treated with the OHSS stimulation protocol and reached a peak 48 h after hCG administration. No significant differences between controls were observed. In the experimental 100 μg/kg·d group, Cb2 significantly inhibited VP evaluated 48 h after hCG administration (Fig. 3A) but interestingly did not affect luteal angiogenesis (Figs. 3B and 4C) at that point or when evaluated 96 h after hCG administration (data not shown), indicating the absence of inhibitory delayed effects on angiogenesis. A lower dose of Cb2, as in the 50 μg/kg·d group, also did not affect luteal angiogenesis and simultaneously seemed to block VP, but such a decrease was not statistically significant ($P = 0.089$). On the other hand, a high 500 μg/kg·d dose provided a 2-

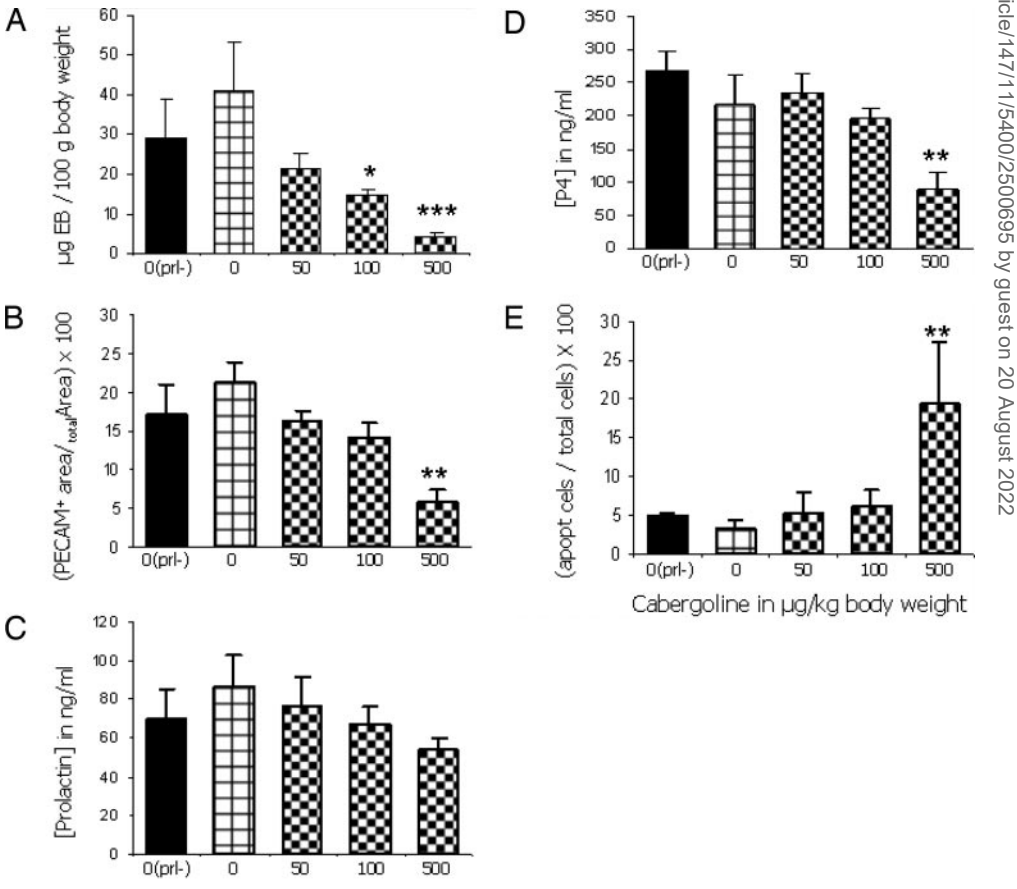

FIG. 3. Cb2 to prevent the occurrence of increased VP in prl supplemented OHSS rats. Vascular permeability (A) (as micrograms of extravasated EB dye per 100 g animal weight), luteal vascular density (B) (as the percentage of PECAM, a specific endothelial cell marker, stained area per total area), serum prl level (C) (nanograms per milliliter), serum P4 levels (D) (nanograms per milliliter), and percentage of luteal apoptosis (E) (as the percentage of apoptotic cells per total cells) 48 h after hCG injection in control OHSS rats supplemented with prl 5 mg and treated with Cb2 at 0 (control), 50, 100, or 500 μg/kg·d doses. An additional Cb2 untreated, non-prl-supplemented OHSS control [0(prl−)] group was also included in the analysis. Experimental groups received a single dose of Cb2 on the day of hCG administration, whereas both controls received glucosaline (n = 8 animals/group). Note 100 μg/kg is the only Cb2 dose able to significantly decrease VP without altering luteal angiogenesis. *Bars*, mean + SEM in each group. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.005$, comparison of 0 (prl−) control group against the prl-supplemented control (0) and experimental (50, 100, 500) groups.

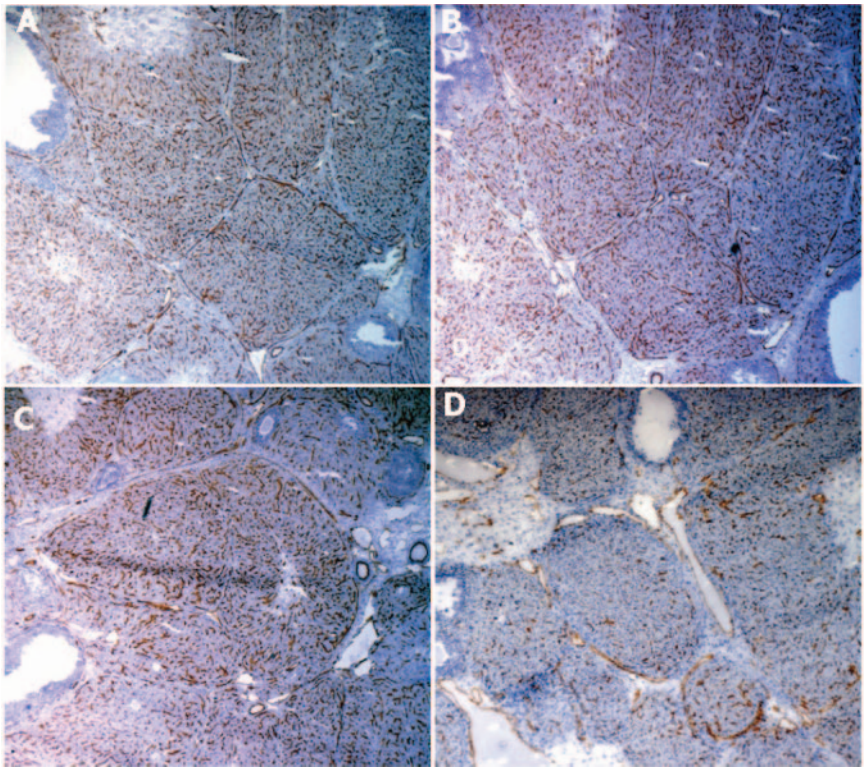

FIG. 4. High-dose Cb2 blocks luteal angiogenesis in prl-supplemented OHSS rats. Representative images of vascular staining (*brown*) with an antibody against PECAM (endothelial cell marker) 48 h after hCG administration in the corpus luteum of control OHSS rats not supplemented (A) and supplemented with 5 mg prl (B) and in experimental prl-supplemented OHSS rats treated with a dose of 100 (C) and 500 (D) μg/kg·d Cb2. Glucosaline or Cb2 was administered coinciding with hCG injection (n = 8 animals/group). Note no differences among the controls (A and B) and the 100 μg/kg·d Cb2 dose experimental group (C) compared. Also note the decrease (disruption) in vascular density in the 500 μg/kg·d Cb2 group (D), compared with any of the other groups.

to 3-fold greater inhibition of VP than the 100 μg/kg·d Cb2 when compared with untreated controls (Fig. 3A), but such a high dose disrupted luteal vessel proliferation (Figs. 3B and 4D), indicating that angiogenesis had been decreased in this organ.

Differences in P4 levels (Fig. 3D) and luteal apoptosis (Fig. 3E), two clear indicators of functional and structural luteolysis, were observed only in 500 μg/kg·d group when compared with untreated controls and the experimental groups. No significant differences were found when prl levels in all the experimental groups were compared with Cb2-untreated controls (Fig. 3C); therefore, it was very likely that the luteolytic effects observed in the 500 μg/kg·d dose (Fig. 3D) were not due to prl deprivation but because of Cb2 actions on the VEGFR-2 angiogenic component (Figs. 3B and 4D). Because the segregation of the VP and angiogenic effects was consistently accomplished only when using 100 μg/kg Cb2, we decided to using only such a dose of the Dp-r2 agonist for subsequent functional experiments.

Experiment 2: low-dose 100 μg/kg·d Cb2 reverses increased vascular hyperpermeability in OHSS rats without affecting angiogenesis. In the above-described experiments, Cb2 prevented the occurrence of hyperpermeability states in our OHSS prl-supplemented model. In the next set of experiments, we tested whether Cb2 could reverse a hyperpermeability state once it occurred. A dose of 100 μg/kg·d Cb2 given 24 h after hCG administration decreased VP when compared with the non-prl and prl-supplemented OHSS controls (Fig. 5A). These results demonstrated that Cb2 not only prevents the occurrence of a VEGF/VEGFR-2-dependent hyperpermeability state but also reverses such a state once it has occurred. Similar to results obtained in experiment 1, luteal vessel density was unaffected by a 100 μg/kg·d Cb2 dose when compared with controls (Fig. 5B), suggesting that dependent VP had been specifically blocked without affecting the angiogenic component of VEGFR-2. No differences in P4 levels (Fig. 5D) and luteal apoptosis (Fig. 5E), two clear indicators of functional and structural luteolysis, were observed between the two control and experimental groups. These observations indicate that inhibition of VP was not due to the alteration of luteal function mediated by prl deprivation or the disruption of VEGFR-2-dependent angiogenesis.

After successful treatment of OHSS, we decided to perform preliminary descriptive experiments to investigate how Cb2 had affected VEGFR-2 at the molecular level to allow inhibited hyperpermeability without affecting luteal angiogenesis. Control and Cb2-treated animal ovarian samples collected in this experiment were used for such purposes.

Cb2 does not affect VEGF/VEGFR-2 expression in OHSS animals

Ovarian samples obtained from experiment 2 were used to test whether Cb2 had an inhibitory effect on VEGF and/or VEGFR-2 mRNA expression in the ovary. We found mRNA expression of both genes was similar in the experimental and control OHSS groups (Fig. 6). These data indicated that the mechanism of inhibition of VP by low dose Cb2 was not through the inhibition of VEGF or VEGFR-2 production in the ovary.

Low-dose Cb2 blocks VP through inhibition of VEGFR-2 phosphorylation sites

Cb2 at 100 μg/kg·d did not affect vessel proliferation, indicating that the angiogenic activity component of VEGF/

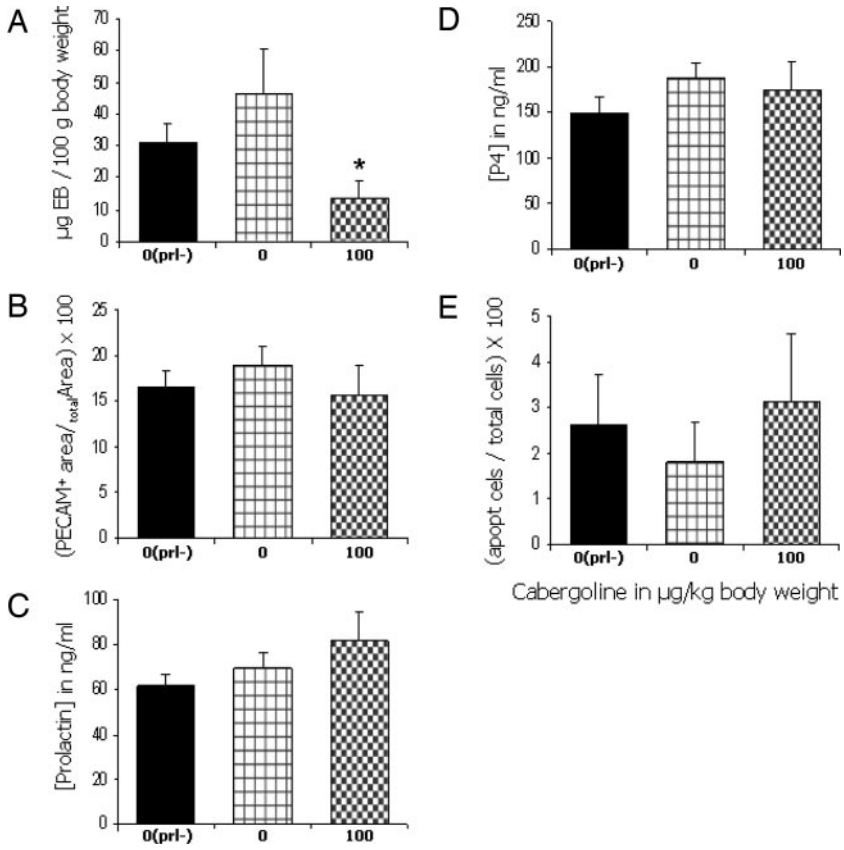

FIG. 5. The effects of low-dose (100 μg/kg·d) Cb2 in the treatment of increased VP in prl-supplemented OHSS rats. Vascular permeability (A) (as micrograms of extravasated EB dye per 100 g animal weight), luteal vascular density (B) (as the percentage of PECAM, a specific endothelial cell marker, stained area per total area), serum prl level (C) (nanograms per milliliter), serum P4 levels (D) (nanograms per milliliter), and percentage of luteal apoptosis (E) (as the percentage of apoptotic cells per total cells) 48 h after hCG injection in control OHSS rats supplemented with placebo [0(prl−)] or prl 5 mg (0) and in experimental OHSS rats supplemented with prl and treated with a 100 μg/kg·d Cb2 dose (100). Cb2 or glucosaline was administered 24 h after hCG (n = 8 animals/group). Note that a 100 μg/kg·d Cb2 dose administration was able to significantly reverse a state of increased VP in prl-supplemented OHSS rats. Also note changes in VP in the experimental group were not mediated by antiangiogenic or luteolytic effects (unaffected serum prl and P4 levels, unaffected luteal vessel density, and apoptosis). *Bars*, mean + SEM in each group. *, $P < 0.05$, compared with the non-prl-supplemented, 0(prl−) control group against the prl supplemented control (0), and experimental Cb2-treated (100) OHSS groups.

VEGFR-2 pathway remained intact. Thus, it was likely that the VEGFR-2 had not been completely inactivated/blocked by Cb2 but still remained active and able to transduce effective signaling. To test for direct evidence that supported this fact at the molecular level, we checked for the phosphorylation state of VEGFR-2 tyrosine 1214. The activation of this site has been described as critical for the transduction of ligand-dependent VEGFR-2 signaling (64, 65). We did not observe any significant differences in this parameter when ovarian samples from Cb2-treated prl-supplemented OHSS rats were compared with untreated controls (Fig. 7B).

Because Cb2 did not block the angiogenic component of VEGFR-2, we wondered whether Cb2 had really decreased VP by affecting VEGFR-2 itself or perhaps any other component of the downstream cascade. The phosphorylation of the tyrosine sites in the transmembrane and C-terminal regions of the receptor are known to onset subsequent VEGFR-2 downstream signaling (66). Therefore, analysis of the activation state of these amino acids could provide a clear answer to this question.

Thus, Western blot, using a general phosphotyrosine antibody, was used to check for global analysis of the activation of all the VEGFR-2 phosphorylated sites. Subsequent densitometric analyses revealed that Cb2 administration decreased the general phosphorylation of VEGFR-2 by 42%, compared with controls (Fig. 7A). These data suggested to us that the effects of Cb2 on the reduced phosphorylation of a single or several tyrosine sites, other than the one critical for the activation of VEGFR-2, could probably be involved in the segregation of the VP and angiogenic components.

Discussion

Using a prl-supplemented OHSS rat model, we herein show that, at a low dose, Cb2 inhibits vascular hyperpermeability without affecting angiogenesis. Cb2 action seems to be mediated through activation Dp-r2 because endogenous prl secretion, a marker of Dp-r2 activity, was consistently reduced by the administration of Cb2. Activation of Dp-r2 is associated with changes in the VEGF/VEGFR-2 pathway as indicated by altered phosphorylation state of this receptor. Our findings indicate that the permeability component of VEGF/VEGFR-2 pathway can be segregated from the angiogenic component by Dp-r2 agonists in a dose-dependent manner. These findings suggest that for the first time, a specific nontoxic treatment of OHSS has become available without affecting reproductive angiogenesis.

Until recently the pathophysiology of OHSS was poorly understood. We (10, 20, 52) and others (67–72) have shown that development of OHSS is probably mediated by increased ovarian production and secretion of ovarian vasoactive substances, with VEGF being the main candidate among them (12–14). VEGF signaling although VEGFR-2 not only stimulates new blood vessel development (24, 28, 29) including in the ovary (35, 36, 54) but is also an important regulator of VP (11, 12, 26–29). Validation of the importance of the VERGF/VEGFR-2 pathway in OHSS comes from the findings in our previous study (10) in which development of this condition could be prevented by the administration of SU5416, a substance that blocks VEGFR-2 phosphorylation. This study demonstrated for the first time that gonadotropin-

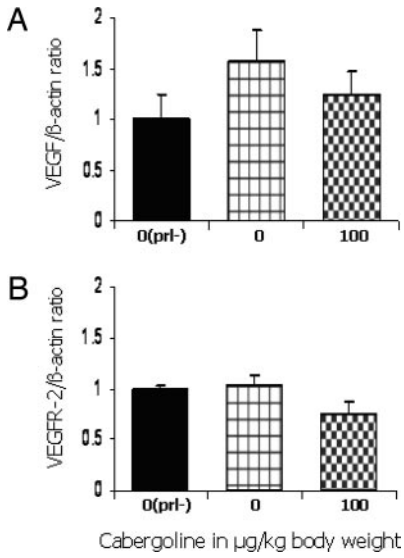

FIG. 6. The effects of low-dose (100 μg/kg·d) Cb2 treatment on VEGF and VEGFR-2 mRNA levels in prl-supplemented OHSS rats. QF-PCR measurements of VEGF (A) (as arbitrary units of intensity resulting from the ratio of normalized VEGF to β-actin expression) and VEGFR-2 (B) (as arbitrary units of intensity resulting from the ratio of normalized VEGFR-2 to β-actin expression) mRNA levels 48 h after hCG administration in the ovaries of control OHSS rats supplemented with placebo [0(prl−)] or prl 5 mg (0) and in experimental prl-supplemented OHSS rats treated with a 100 μg/kg·d Cb2 dose (100), Cb2, or glucosaline were administered 24 h after hCG injection (n = 8 animals/group, half an ovary per animal). No differences were found after comparison of group 0(prl−) against the other two groups.

induced changes of VP can be prevented by interference with the VEGF/VEGFR-2 signaling pathway. Unfortunately, SU5416 cannot be used clinically due to its side effect profile (31, 32). Also, our study could have been criticized because we did not explore the possibility that prevention of OHSS by SU5416 might have occurred through direct blockage of ovarian angiogenesis, which could have inactivated structures like corpora lutea (34, 36), the main source of vasoactive substances causing OHSS. These observations motivated us to look for inhibitors of VP with a more benign side effect profile, not inducing luteolysis, which interferes with pregnancy development and less antiangiogenic effects.

Based on previous animal studies (43) and observations in the humans (46–50, 73), we theorized that the specific actions of Cb2 or other similar Dp-r2 agonists, like bromocriptine (74), on VP and angiogenesis might be dose dependent. For example, administration of high doses interferes with three actions, which include angiogenesis, VP, and prl secretion (43). On the other hand, administration of low doses, as used in humans who suffer from hyperprolactinemia, decreases prl levels without affecting physiologic angiogenesis as it occurs in reproductive organs (46–50). The effect of low-dose Dp-r2 agonist on VP had not been studied before. The testing of our hypothesis that administration of low-dose Cb2 through activation of its receptor specifically inhibited VEGF/VEGFR-2-dependent VP was reliant on selecting the appropriate OHSS model. The model required that VEGF/VEGFR-2-dependent changes in hyperpermeability and angiogenesis could be activated simultaneously, whereas at the same time, information about the activation state of the Dp-r2 had to be available. In addition, Dp-r2 had to be present in the blood vessels of interest, in which we expected that changes in VP and angiogenesis would occur. The rat OHSS model fulfilled these criteria. Because lowering of prl by Dp-r2 activation made corpora lutea, the source of substances regulating VP in OHSS (7, 10), dysfunctional, we performed our experiments in prl-replaced animals.

Dosages of Cb2 decreasing VP in OHSS prl-supplemented animals (experiments 1 and 2) decreased prl levels in the pilot experiment, suggesting that Dp-r2 activation was required to decrease VEGFR-2-dependent VP. Evidence supporting interdependence between Dp-r2 and VEGFR-2 comes from studies in Dp-r2 knockout models. In these animals VEGFR-2 phosphorylation is increased in the absence of the Dp-r2 inhibitor stimuli and cannot be reversed by the administration of Dp-r2 agonists (75).

To study, at the molecular level, how low-dose Cb2 inhibited increased VP without affecting angiogenesis, we tested whether this Dp-r2 agonist had affected VEGFR-2 activation in the ovaries of prl-replaced OHSS animals. We observed a 42% decrease in VEGFR-2 phosphorylation levels in Cb2-treated animals when compared with controls. Studies analyzing biological actions other than VP induced by VEGFR-2 have suggested that each specific tyrosine site could be involved in the regulation of one of the different biological actions mediated by this receptor (76). In agreement with those studies, we suggest that Cb2 might have acted by inducing the targeted inactivation of one or several VEGFR-2 sites involved in inducing VP and not affecting tyrosine sites activating the angiogenic component. Unfortunately, the specific VEGFR-2 tyrosine amino acid/s regulating VP (or angiogenesis) have not yet been defined. Thus, further analysis to demonstrate this hypothesis would require antibodies/inhibitors and/or rat knockout models targeting for each of the eight VEGFR-2 phosphotyrosine sites described (77), but both elements are not yet available.

Support of the assumption that Cb2 did not affect the angiogenic component of VEGFR-2 comes therefore from our findings that luteal blood vessel proliferation, a process in which VEGFR-2 activation is essential (36, 54), was similar between controls and treatment.

It is of note that a similar percentage of decreased total phosphorylation of VEGFR-2 had been reported by a previous work studying the effects of high doses of Dp-r2 agonist in cultured endothelial cells (43). These authors suggested that high doses of Dp-r2 agonists reduced the density of VEGFR-2 on the membrane of endothelial cell through a process of induced internalization. Their findings suggested that the receptor became unreachable for VEGF, and this led to a general inhibition of the VEGF/VEGFR-2 pathway, which resulted in not only decreased VP but also angiogenesis. In our experiments it was very likely that VEGFR-2 had not been internalized by low-dose Cb2; otherwise, not only VP but also angiogenesis would have been blocked. We obtained direct evidence supporting this assumption at the molecular level by studying the phosphorylation state of VEGFR-2 tyrosine amino acid at position 1214, a site critical for the overall transduction of the VEGFR-2 signaling (64), which remained unaffected by Cb2. Therefore, these data suggest that the molecular mechanism of Dp-r2 agonist ac-

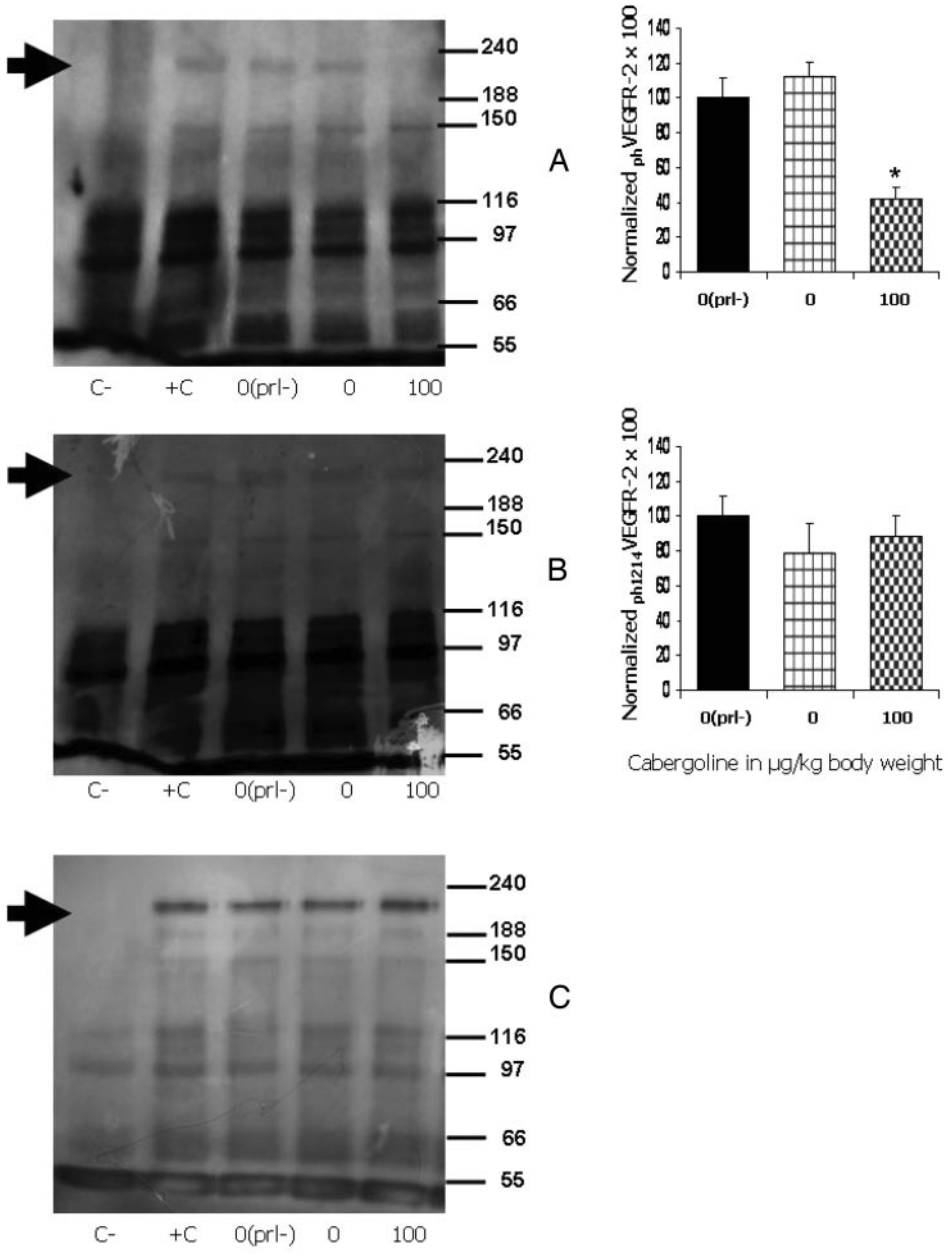

FIG. 7. The effects of low-dose (100 μg/kg·d) Cb2 treatment on VEGFR-2 phosphorylation levels in prl-supplemented OHSS rats. Effects of Cb2 on VEGFR-2 phosphorylation levels 48 h after hCG injection using pooled VEGFR-2 immunoprecipitated ovarian protein extracts (n = 8 samples/pool, each sample obtained from half an ovary coming from a single animal) from control OHSS rats supplemented with placebo [0(prl−)], or 5 mg prl (0) and from experimental 5 mg prl-supplemented OHSS rats treated with a 100 μg/kg·d Cb2 dose (100). Controls and experimental group respectively received glucosaline or Cb2 24 h after hCG administration (n = 8 animals/group). A positive control (+C), lung rat tissue, and a negative control (C−) consisting of ovarian samples from OHSS placebo rats incubated with a VEGFR-2 antibody competitor peptide were also included. Upper, medium, and lower Western blots are representative pictures obtained in one of the five replicated experiments and show bands obtained using a general phosphotyrosine (A), a specific 1214VEGFR-2 phosphotyrosine (B), and a VEGFR-2 (C) antirat antibody. Note 116 kDa and lower bands (band size in kilodaltons is indicated on the *right margin* of each Western picture) are unspecific because they also appear in the C line. *Black arrows* indicate the expected band size corresponding to the mature, membrane bound, functionally active form of VEGFR-2 protein (~220 kDa). Specific staining for the approximately 150- and approximately 190-kDa band sizes correspond to immature VEGFR-2 protein forms (see references in *Materials and Methods* for details). Please note the absence/decreased phosphotyrosine signaling (total phosphorylated VEGFR-2) for the approximately 220-kDa band in the (100) line corresponding to the Cb2-treated group. Also note no apparent staining differences are observed for total and ph1214 VEGFR-2 signaling between controls and treatment group. Graphs beside each Western represents the densitometry of expected size bands (~220 kDa) recognized by p-Tyr (A) and ph1214VEGFR-2 (B) antibodies in the 0(prl−), 0, and 100 groups divided by the corresponding total VEGFR-2 band size densities (C), normalized to the 0 (prl−) control group and multiplied by 100. Mean ± SEM represents five replicate experiments. Results have been expressed as arbitrary units of density corresponding to the ratio of total phosphorylated VEGFR-2 to total VEGFR-2 (A) and phosphorylated 1214 VEGFR-2 to total VEGFR-2 (B) obtained through the process just mentioned above. Note Cb2 did not affect ph1214VEGFR-2 phosphorylation levels (B). More than a 50% decrease in total VEGFR-2 phosphorylation level was observed in the Cb2-treated group, compared with controls (A). *, $P < 0.05$, compared with the non-prl-supplemented, 0(prl−) control group against the prl supplemented control (0), and experimental Cb2-treated (100) OHSS groups.

tion on VEGFR-2 must be different when administered at high and low doses or under *in vivo* and *in vitro* conditions. Further studies on dopamine-mediated vascular mechanisms may help to gain insight into which other components of the VEGFR-2 pathways are specifically involved in the regulation of VP and angiogenesis.

Based on results obtained in our animal model and the known safe profile of Dp-r2 agonists, even in pregnant women (47, 50), we tested whether Cb2 could also prevent the occurrence of OHSS in humans. We tested the effects of Cb2 in egg donor patients at high risk for developing OHSS (>25 preovulatory follicles, estradiol > 3000 ρg/ml in serum) using a dosage that is sufficient to block prl secretion. It is of note that in humans the dose used (5–10 μg/kg·d) is 5–10 times lower than in rodents (50 μg/kg·d) and that a decrease in prl secretion does not interfere with ovarian function (56). Results have shown that prophylactic administration of Cb2 prevents the onset of OHSS symptoms like ascitis (>500 ml), increase in hematocrit (>42%), and oliguria in more than 75% in the treatment group (n = 63), whereas placebo administration is effective in only 35% of control group patients (n = 57). Partial results derived from this study have been very recently published in abstract form (78), whereas the manuscript detailing procedures and including whole results is now under preparation.

In summary we have shown that the low-dose Cb2 blocks VP without affecting angiogenesis. Its mode of action seems to be through partial blockage of VEGFR-2-specific phosphorylation sites. Cb2 blocks not only hyperpermeability in a rat OHSS model but also a similar condition in humans, which can occur during infertility treatment. Cb2 is a well-established, widely used, safe, nontoxic medication (47, 48, 50, 56, 61, 79–81). Our study suggests a new clinical application for Cb2 in the treatment of OHSS. Further studies are needed to establish whether Cb2 has a role in the treatment of clinical conditions such as stroke (82), myocardial infarction (83), respiratory distress syndrome (84), acute Kawasaki disease (85), or atopic dermatitis (86) in which the permeability component of the VEGF/VEGFR-2 pathway is suspected to be abnormal and angiogenesis must be preserved.

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