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Follicular Fluid High Density Lipoprotein-associated Sphingosine 1-Phosphate Is a Novel Mediator of Ovarian Angiogenesis*

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Sören von Otte[‡1,2], Jürgen R. J. Paletta[§1], Steffi Becker[‡], Simone König[¶], Manfred Fobker[‖], Robert R. Greb[**], Ludwig Kiesel[**], Gerd Assmann[‖‡‡], Klaus Diedrich[‡], and Jerzy-Roch Nofer[‖‡‡]

From the [‡]Klinik für Frauenheilkunde und Geburtshilfe, Universitätsklinikum Schleswig-Holstein, Campus Lübeck, Ratzeburger Allee 160, 23538 Lubeck, Germany, the [§]Klinik und Poliklinik für Unfall-, Hand-, und Wiederherstellungschirurgie, [‖]Institut für Klinische Chemie und Laboratoriumsmedizin, and [**]Klinik und Poliklinik für Frauenheilkunde und Geburtshilfe, Westfälische Wilhelms-Universität Münster, 48149 Münster, Germany, the [¶]Integrierte Funktionelle Genomik, Interdisziplinäres Zentrum für Klinische Forschung der Medizinischen Fakultät der Universität Münster, 48149 Münster, Germany, and the [‡‡]Leibniz Institut für Arteriosklerosеforschung an der Universität Münster, 48149 Münster, Germany

Angiogenesis plays an important role in the development of the ovarian follicle and its subsequent transition into the corpus luteum. Accordingly, follicular fluid is a rich source of mitogenic and angiogenic factors such as basic fibroblast growth factor and vascular endothelial growth factor secreted by granulosa cells. In the present study, we show that follicular fluid deprived of basic fibroblast growth factor or vascular endothelial growth factor by means of thermal denaturation or antibody neutralization retains its capacity to stimulate endothelial proliferation and angiogenesis. Mass spectrometric analysis of chromatographic fractions stimulating endothelial growth obtained from follicular fluid revealed that the heat-stable mitogenic activity is identical with the subfraction α of high density lipoproteins purified from follicular fluid (FF-HDL). Further investigations demonstrated that sphingosine 1-phosphate (S1P), one of the lysophospholipids associated with HDL, accounts for the capacity of this lipoprotein to stimulate endothelial growth and the formation of new vessels. Activation of mitogen-activated protein kinase ($p42/44^{ERK1/2}$), protein kinase C, and protein kinase Akt represent signaling pathways utilized by FF-HDL and S1P to induce endothelial proliferation and angiogenesis. We conclude that FF-HDL represents a novel mitogenic and angiogenic factor present in follicular fluid and that S1P is one of the FF-HDL lipid components accounting for this activity.

Angiogenesis, the formation of blood vessels from endothelial cells, occurs during embryonic and adult life (1). One of the few organ systems in healthy adults in which angiogenic processes appear is the female reproductive system. In the cytogenic and hormone-secreting ovaries, angiogenesis occurs on a dynamic basis during the reproductive cycle (2). The transition of the follicle into the corpus luteum and subsequent luteolysis is accompanied by growth and subsequent regression of blood vessels (3). Massive proliferation and migration of endothelial cells preceding new vessel formation is especially visible during corpus luteum formation after the ovulating gonadotrophin surge (4). These dynamic processes are driven by growth factors and cytokines and are tempered by inhibitors of angiogenesis present in follicular fluid. Several pro- and antiangiogenic molecules involved in endothelial cell proliferation, the principal process underlying ovarian angiogenesis, have been identified in follicular fluid (5). These include in the first place basic fibroblast growth factor (bFGF)[3] and vascular endothelial growth factor (VEGF) (6–9), as well as angiopoietins 1 and 2 (10), SPARC (secreted protein acidic and rich in cysteine), and thrombospondin (11). The exact contribution of each of these factors to the formation of new vessels within ovarian tissues remains unclear.

Our previous investigations of angiogenic constituents of human follicular fluid indicated that a considerable portion of its mitogenic activity cannot be attributed to VEGF or bFGF (12). For instance, VEGF and bFGF added to endothelial cells at concentrations equivalent to those present in follicular fluid were revealed to be several times less potent in inducing endothelial proliferation than follicular fluid itself. Even the combined application of VEGF and bFGF to endothelial cells failed to induce mitogenic response comparable with that triggered by follicular fluid. These data clearly indicate that follicular fluid is a source of angiogenic activity distinct from traditional growth factors.

Follicular fluid HDL (FF-HDL) has been identified as a sole lipoprotein present in follicular fluid until the ovulation of the oocyte (13). Unlike serum HDL, FF-HDL particles are cholesterol-poor but contain significantly higher amounts of phospholipids. Apolipoproteins apoA-I and apoA-IV are major protein constituents of FF-HDL. The physiological role fulfilled by FF-HDL remains obscure. It has been proposed that FF-HDL is involved in sperm capacitation (14) or delivery of cholesterol to granulosa cells for progesterone production (15). Here we report that FF-HDL is a potent and endothelial cell-specific mitogen present in human follicular fluid. We further demonstrate that sphingosine 1-phosphate (S1P), the lysosphingolipid identified in FF-HDL, accounts for a significant portion of the mitogenic and angiogenic activity associated with this lipoprotein.

EXPERIMENTAL PROCEDURES

Reagents—S1P was purchased from Biomol (Hamburg, Germany). ApoA-I and apoA-IV were obtained from Sigma. The inhibitors PD98059, H7, and LY294002 were purchased from Calbiochem (Schwalbach, Germany).

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[1] These two authors contributed equally to this work.

[2] To whom correspondence should be addressed. Tel.: 49-451-500-2134; Fax: 49-451-500-2430; E-mail: svonotte@gmx.de.

[3] The abbreviations used are: bFGF, basic fibroblast growth factor; apo, apolipoprotein; FF, follicular fluid; FF-HDL, follicular fluid high density lipoprotein; S1P, sphingosine 1-phosphate; HUVEC, human umbilical vein endothelial cell(s); PKC, protein kinase C; VEGF, vascular endothelial growth factor; MALDI, matrix-assisted laser desorption ionization; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Human Follicular Fluid—Human follicular fluid (FF) was obtained from 10 women undergoing ovarian hyperstimulation with recombinant follicle-stimulating hormone (Gonal F®, Serono, Unterschleissheim, Germany) after pituitary desensitization with nafarelin acetate (Synarela®; Amersham Biosciences) for *in vitro* fertilization or intracytoplasmic sperm injection. Ovulation induction was performed by administration of 10,000 IU human gonadotrophin (hCG; Choragon®, Ferring, Kiel, Germany) when at least three follicles reached a diameter of at least 18 mm. After 36 h, follicular fluids were aspirated transvaginally under ultrasound guidance. For removal of cell debris, follicular fluids were immediately centrifuged after aspiration for 5 min at 1500 rpm. All samples were frozen at −80 °C for further analysis. For experiments, fluid samples of all aspirated follicles were pooled.

Isolation of Human Umbilical Vein Endothelial Cells (HUVEC)—HUVEC were isolated from human umbilical cord veins as described by Jaffe *et al.* (16) with minor modifications. Briefly, the venous lumen was washed with phosphate-buffered saline to remove coagulated blood, filled with 0.1% collagenase I (Worthington), and incubated for 15 min at 37 °C. The cell suspension was obtained by flushing the lumen with endothelial growth medium (Promocell, Heidelberg, Germany) and centrifuged at 173 × *g* for 10 min. The supernatant was removed, and the cell pellet was resuspended in endothelial cell medium. Cells were seeded at a density of 24,000 cells/cm^2 in cell culture dishes (BD Biosciences, Bedford, MA), cultured at 37 °C in a CO_2-enriched atmosphere and in medium containing 2% (w/v) fetal calf serum and gentamicin. Confluent monolayers were passaged twice at a ratio of 1:3 using 0.05% trypsin and 0.02% EDTA. Cells were either frozen at passage 2 or used for experiments in passage 3. Cell identity was confirmed by immunofluorescence microscopy using fluorescein isothiocyanate-labeled antibodies against CD31 (BD Biosciences).

Proliferation Assay—Proliferation was assayed in 96-well plates. Cells were seeded at densities of 1×10^4 cells/well in 0.2 ml of endothelial cell medium in the absence or presence of follicular fluid (up to 80 μl/well). After incubation for 48 h at 37 °C, the proliferation was analyzed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) using a commercially available kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Heat Treatment of Follicular Fluid and Immunoneutralization—Heat inactivation was performed by incubation of follicular fluid for 30 min at 90 °C. After cooling, follicular fluid was centrifuged for 60 min at 2500 × *g* to remove denatured proteins, and the supernatant was retrieved for further processing. For immunoneutralization, anti-human VEGF antibody and anti-bFGF antibody (both from R&D Systems) were added to follicular fluid at an antibody/growth factor molar ratio of 1000:1 and incubated for 1 h at room temperature. The concentrations of bFGF and VEGF in native, heat-treated, and immunoneutralized follicular fluid were determined in duplicates by a quantitative sandwich-enzyme immunoassay technique using a Quantikine human VEGF immunoassay and a Quantikine human bFGF immunoassay (both from R&D Systems). The minimum detection limits of the assays were 5 and 3 pg/ml for VEGF and bFGF, respectively.

Purification of the Mitogenic Activity—All steps of the purification were carried out at 4–8 °C. Native follicular fluid (100 ml) was heat-treated and centrifuged as described above. The resulting supernatant (50 ml) was concentrated by ultrafiltration in a pressure chamber (Amicon, Beverly, MA), equipped with an YN30 membrane. The concentrate (5 ml) was applied to a DEAE-Sepharose column (2.6 × 22.0 cm; 116-ml bed volume (Amersham Biosciences) equilibrated with 10 mM Tris/HCl buffer (pH 7.0), with a flow rate of 0.5 bed volume/h. The column was washed with 2.5 bed volumes of buffer, and bound proteins were eluted with a linear NaCl gradient (0–1.0 mol/liter; 600 ml) at a flow rate of 1 ml/min. Fractions exhibiting high protein content were pooled, desalted, and subjected to further analysis.

Protein Separation and Identification—Proteins were separated under denaturing conditions in 11.5% (w/w) polyacrylamide gels by the method of Laemmli (17) and stained with Serva Blue R (Serva, Heidelberg, Germany). Bands were excised, and the proteins were tryptically digested in the gel following a slightly modified general procedure, which was previously published (18). Peptides generated by proteolysis were extracted, purified using C18 solid phase extraction (ZipZips; Millipore Corp., Bedford, MA), and subjected to MALDI time-of-flight mass spectrometry using TofSpec-2E (Waters/Micromass, Manchester, UK). Data base searches were performed using Mascot software (Matrix Science Ltd., London, UK) screening the NCBI and SwissProt data bases.

Lipoprotein Isolation, Characterization, and Modification—HDL (d = 1.125–1.210 g/ml) was isolated from follicular fluid by a discontinuous ultracentrifugation as described by Havel *et al.* (19) and dialyzed overnight against phosphate-buffered saline. The composition of lipoprotein in follicular fluid was investigated using one- or two-dimensional nondenaturing gel electrophoresis. A standard electrophoresis in agarose gel (1% w/v) was performed in the first dimension to separate major lipoprotein classes. The gels were stained with Sudan black and densitometered, or agarose strips were placed on a 2–15% polyacrylamide gradient gel with a 2% stacking gel. The electrophoresis was performed in a 25 mmol/liter Tris, 0.2 mol/liter glycine buffer (pH 8.2) for 3 h at 100 V. After electrophoresis, gels were electrotransferred to nitrocellulose membranes for immunoblotting with anti-apoA-I antibodies. Horseradish peroxidase-conjugated secondary antibodies were used for apolipoprotein visualization in a chemiluminescence-based procedure.

To reduce S1P content in HDL isolated from follicular fluid, the treatment with alkalic phosphatase as described by Ruwisch *et al.* (20) was used. Briefly, 50 units of alkaline phosphatase were diluted in 0.45 ml of buffer containing 200 mmol/liter Tris-HCl (pH 4.5) and 75 mmol/liter $MgCl_2$ in glycine (2 mol/liter, pH 9.0) and added to 1.5 ml of HDL. After incubation for 30 min at 37 °C, pH was neutralized with HCl. Control samples were treated identically without the addition of phosphatase. The apoA-I-phospholipid complexes were prepared as described previously (21).

Western Blotting—HUVEC were lysed in 0.18 mol/liter Tris-HCl, 0.15 mol/liter NaCl, 10% (v/v) Nonidet P-40, 5% (v/v) sodium deoxycholate, 1% (v/v) SDS, 50 mmol/liter, 50 mmol/liter NaF, 1 mmol/liter EGTA, 1 mmol/liter orthovanadate, and the Complete® protease inhibitor mixture. Cell lysates (50 μg/lane) were subjected to SDS-gel polyacrylamide electrophoresis according to Laemmli (15). Thereafter, proteins were transferred to nitrocellulose membranes, which were blocked overnight in Tris-buffered saline containing 5% fat dry milk prior to incubations with antibodies. Loading controls were performed using an antibody against a ubiquitously expressed protein (α-actin).

Determination of Sphingosine 1-Phosphate—S1P levels were determined as described previously (20). Briefly, HDL was mixed with methanol/HCl (1:1; v/v), and lipids were extracted by the addition of 1 volume of chloroform/NaCl. After alkalization with NaOH, the alkaline aqueous phase was transferred into a siliconized glass tube, and the organic phase was re-extracted with methanol/NaCl/NaOH. The aqueous phases were combined, acidified, and extracted twice with chloroform. The organic phases were evaporated, and the dried lipids were dissolved in methanol/K_2HPO_4. The resolved lipids were derivatized with *o*-phthaldialdehyde. The derivatives were analyzed with a Merck-Hitachi LiChrom HPLC system (Merck-Hitachi, Darmstadt, Germany)

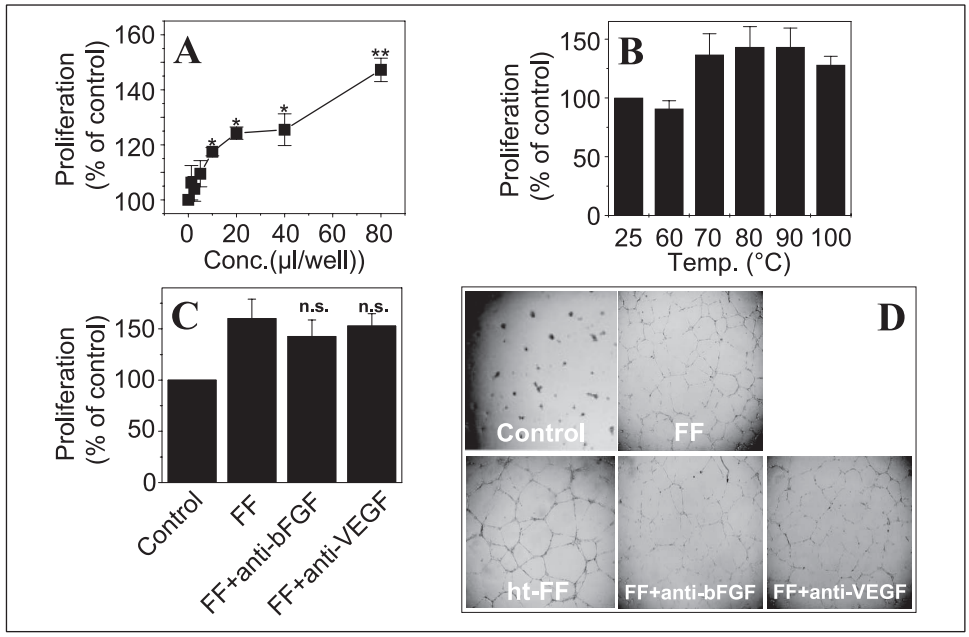

FIGURE 1. **Effect of follicular fluid on endothelial cell growth.** *A*, HUVEC were seeded in a 96-well plate at a density of 1×10^4 cells/well and exposed for 48 h to follicular fluid at the indicated concentrations. The proliferation rate was determined with an MTT assay as described under "Experimental Procedures." Data represent the mean ± S.D. from four separate determinations. *, $p < 0.01$; **, $p < 0.001$ (follicular fluid *versus* control). *B*, follicular fluid was heat-treated at the indicated temperature and added to HUVEC for determination of proliferation using an MTT assay. Data represent mean ± S.D. from three separate determinations. *C*, follicular fluid was preincubated for 1 h at 37 °C with monoclonal antibodies against bFGF and VEGF. The molar ratio of each antibody to the respective growth factor was 1000:1. HUVEC were exposed to antibody-treated follicular fluid for 48 h, and the proliferation rate was determined with an MTT assay. Isotype-matched irrelevant antibody was used for control. Data represent mean ± S.D. from three separate determinations. *n.s.*, not significant (anti-bFGF or anti-VEGF *versus* irrelevant antibody). *D*, HUVEC were seeded on Matrigel in a 96-well plate at a density of 1×10^5 cells/well for 18 h in the absence (control) or presence of FF, heat-treated follicular fluid (*ht-FF*), or follicular fluid preincubated with antibodies against bFGF (*FF + anti-FGF*) or VEGF (*FF + anti-VEGF*). Sprouting tubuli were observed under a light microscope at × 40 magnification. Shown are *images* representative of one experiment of three.

using an RP 18 Kromasil column (Chromatographie Service GmbH, Langerwehe, Germany). Separation was done with a gradient of methanol/K_2HPO_4 (0.07 mol/liter). The recovery of S1P was calculated using dihydro-S1P as a standard (20, 22).

Angiogenesis Assay on Matrigel—Growth factor-reduced Matrigel matrix was purchased from BD Biosciences. Gels were allowed to polymerize in a 96-well plate for 30 min at 37 °C. HUVEC were seeded at 1×10^4/well and grown in endothelial cell medium supplemented with 1% fetal calf serum and without growth supplement for 18 h in a humidified 37 °C, 5% CO_2 incubator. Tube formation was observed using a light microscope (Olympus BX51, Hamburg, Germany), and pictures were captured with a computer system and subjected to image processing using Image-Pro 4.5 image analysis software (CyberView Corp., Suwanee, GA). The extent of tube formation on matrix gels was expressed as total length of sprouts per captured area. Each control or test compound was assayed in duplicate, and the assays were performed three times.

Statistical Analysis—Data are presented as mean ± S.D. unless indicated otherwise. Results were analyzed using Student's *t* test, and statistical significance for all comparisons was assigned at $p < 0.05$.

RESULTS

Mitogenic and Angiogenic Activities of Human Follicular Fluid—First, we examined the ability of native human follicular fluid to stimulate endothelial cell proliferation as determined by MTT assay. As shown in Fig. 1*A*, follicular fluid induced HUVEC proliferation at different concentrations tested (10–80 µl of follicular fluid/well, protein concentration, ~47 g/liter). Basal endothelial proliferation was used as control and compared with proliferation after the addition of follicular fluid. Depending on the amount of follicular fluid added, the increases in proliferation varied from 25 to 60%.

In order to characterize the temperature stability of mitogenic mediators, follicular fluid was subjected to incubation at different temperatures (from 25 to 100 °C) for 30 min. Denatured proteins were removed by centrifugation, and the supernatant was used for determination of growth factor concentration by immunoassay or mitogenic activity by proliferation assay. No detectable amounts of bFGF or VEGF were found in the follicular fluid after heat treatment (not shown). Interestingly, the addition of heat-treated follicular fluid to endothelial cells resulted in a marked increase in endothelial proliferation rate, and the heat stability could be demonstrated for up to 100 °C (Fig. 1*B*). The mitogenic effect of follicular fluid increased with rising temperature during pretreatment of follicular fluid and reached a maximum at 90 °C. This might reflect a temperature-dependent removal of antiangiogenic proteins by heat denaturation. To further characterize the contribution of bFGF and VEGF to mitogenic activity of follicular fluid, the growth rate of endothelial cells exposed to follicular fluid, in which bFGF and VEGF were neutralized with specific monoclonal antibodies, was determined. To achieve this, follicular fluid was incubated for 1 hour at room temperature with antibodies against bFGF and VEGF at an antibody/growth factor molar ratio of 1000:1. Concentrations of bFGF and VEGF in pooled native follicular fluid were 160 pg/ml and 3.6 ng/ml, respectively, and were reduced below the detection limit after pretreatment with antibodies. However, the mitogenic activity of follicular fluid under these experimental conditions was reduced only by 12 ± 5% ($n = 3$, not significant) and 29 ± 4% ($n = 3$, not significant), respectively, as compared with untreated follicular fluid (Fig. 1*C*), indicating that some unidentified residual angiogenic activity was present in follicular fluid after immunoneutralization.

Although endothelial proliferation is critical for the formation of new vessels, it may be argued that proliferative effects alone are not sufficient for supporting effective angiogenesis. Therefore, we next sought to

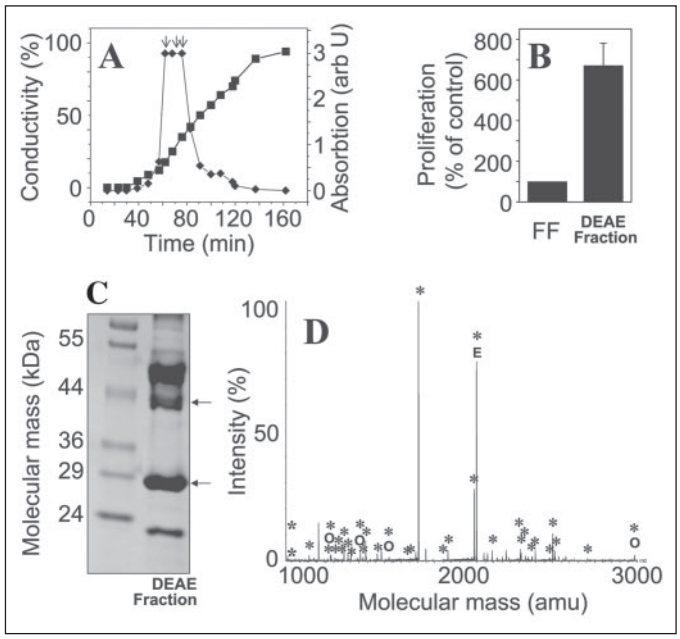

FIGURE 2. **Demonstration of HDL in mitogenically active fractions of follicular fluid.** *A*, follicular fluid was loaded onto a DEAE-Sepharose column, and ion exchange chromatography was performed as described under "Experimental Procedures." Fractions with retention times between 65 and 80 min (*arrows*) were collected, pooled, and used for further experiments. *B*, HUVEC were exposed to the pooled chromatographic fraction for 48 h, and the proliferation rate was determined with an MTT assay. Data represent mean ± S.D. from three separate determinations. *C* and *D*, proteins were separated using 12.5% SDS-PAGE. Shown is an electrophoretic separation of pooled fractions obtained from DEAE chromatography. 28- and 43-kDa bands were excised, trypsinized, and subjected to analysis by MALDI mass spectroscopy as described under "Experimental Procedures." The mass spectrum obtained for the protein in the 43-kDa band is shown. *Asterisk*, tryptic peptides derived from the sequence P06727 for apoA-IV; *O*, oxidized methionine; *E*, esterified glutamic acid.

directly examine angiogenic effects of follicular fluid using an assay for new vessel formation. When placed on growth factor-reduced Matrigel in the absence of angiogenic factors or follicular fluid, HUVEC formed very few incomplete and narrow tube-like structures (Fig. 1*D*, *Control*). Untreated follicular fluid induced the development of expansive tubes organized by a high number of endothelial cells (Fig. 1*D*, *FF*). Similar although less pronounced effects were observed in the presence of heat-treated follicular fluid (*ht-FF*) and follicular fluid after immunoneutralization of bFGF or VEGF (Fig. 1*D*, *ht-FF*, *FF + anti-bFGF*, *FF + anti-VEGF*). Total sprouted area amounted to 458.3 ± 24 and 15,230 ± 238 pixels in the absence or presence of native follicular fluid ($n = 3$, $p < 0.01$), 7826 ± 84 pixels in the presence of heat-treated follicular fluid ($n = 3$; $p < 0.01$), and 13,773 ± 239 and 15,133 ± 832 pixels in the presence of follicular fluid pretreated with antibodies against VEGF and bFGF ($n = 3$; not significant), respectively. Collectively, these results point to the presence of a heat-stable mitogenic and angiogenic activity distinct from bFGF and VEGF in follicular fluid.

Purification and Identification of the Mitogenic Activity of Human Follicular Fluid—Heat-stable mitogenic activity from human follicular fluid was partially purified in a two-step procedure. Heat treatment resulted in an approximately 2-fold increase of the relative mitogenic activity, whereas protein content decreased from 48.3 to 2.0 mg/ml. This indicates that the heat pretreatment is an effective initial step in the purification of mitogenic activity. Ion exchange chromatography on DEAE-Sepharose was applied as a second step. As indicated in Fig. 2*A* (*arrows*), three protein-rich fractions with retention times between 65 and 80 min could be eluted from the DEAE-Sepharose column using a linear NaCl gradient (0.1–0.4 mol/liter). All eluted fractions were subsequently pooled, desalted, and tested for their mitogenic activity in the MTT assay. As shown in Fig. 2*B*, the protein-rich DEAE fraction markedly increased endothelial cell proliferation. The subsequent SDS-PAGE of the mitogenically active fraction obtained by DEAE chromatography revealed five protein bands with molecular masses of approximately 14, 28, 43, 47, and 50 kDa (Fig. 2*C*). Dominant bands were excised from the gel, trypsinized, and subjected to the MALDI mass spectrometric analysis. The mass spectrograms of protein digests and the sequences of derived tryptic peptides are shown in Fig. 2*D*. Apolipoproteins A-I (SwissProt P02647) and A-IV (SwissProt P06727) were identified as major constituents of 28- and 43-kDa bands. Although a large part of the sequence was detected, signals for some terminal peptides were missing. Whereas the possibility cannot be entirely excluded that apolipoproteins present in follicular fluid are shorter, it is also possible that they were not detected with mass spectrometry due to suppression effects. In addition to bands corresponding to apoA-I and apoA-IV, several weak bands with lower molecular masses were observed that may represent the products of apolipoprotein cleavage occurring at aspartic acid residues at high temperature. Modifications of several amino acid residues were observed, such as the oxidation of methionine residues 164, 245, and 322 in apoA-IV and 118 and 154 in apoA-I. In addition, a large signal suggests ester formation at glutamic acid residue 96 of apoA-IV.

Mitogenic Activity of HDL Isolated from Follicular Fluid—Because apoA-I and apoA-IV are major protein constituents of HDL, we assumed that the observed heat-stable mitogenic activity of human follicular fluid was induced by this lipoprotein fraction. As shown in Fig. 3*A*, FF-HDL induced proliferation of HUVEC in a dose-dependent manner with effective stimulation at a concentration of ~0.4 mg/ml, which is close to the physiological level of HDL in follicular fluid (23). Fig. 3*B* demonstrates that the heat-treated HDL displayed no reduction in the mitogenic activity as compared with the native FF-HDL. Furthermore, more than 90% of HDL estimated as apoA-I concentration could be recovered from heat-treated follicular fluid. We conclude that the mitogenic activity of FF-HDL is heat-stable.

To further examine the contribution of HDL to the mitogenic activity exerted by follicular fluid, the effect of HDL depletion on follicular fluid-induced endothelial growth was investigated. The removal of an HDL fraction from follicular fluid by a discontinuous gradient centrifugation reduced its apoA-I content from 23.2 ± 0.05 to 4.8 ± 0.06 mg/dl ($n = 4$) and the HDL-cholesterol content from 12.2 ± 2.4 to 2.25 ± 1.5 mg/dl. Fig. 3*C* (*inset*) demonstrates a marked reduction of the electrophoretic HDL fraction after ultracentrifugation. Follicular fluid was previously demonstrated to contain two HDL subfractions, α and pre-β, characterized by distinct migration patterns in two-dimensional electrophoresis. As shown in Fig. 3*D*, the reduction of HDL content in the follicular fluid after ultracentrifugation was largely accounted by the depletion of phospholipid and cholesterol-rich α-HDL subfraction. The preferential removal of the α-HDL subfraction is also reflected by a disproportional reduction of HDL-cholesterol concentration in comparison with the apoA-I concentration in follicular fluid after ultracentrifugation. Fig. 3*E* demonstrates that HDL-depleted follicular fluid exhibited a markedly reduced ability to stimulate endothelial cell proliferation as compared with its native counterpart.

HDL is known to be a complex lipoprotein fraction comprising several distinct protein and lipid entities. To discriminate which component of HDL accounts for its mitogenic activity, the endothelial proliferation-inducing effects of proteins or lipid fraction derived from HDL were investigated. As shown in Fig. 4*A*, purified apolipoproteins A-I and A-IV were not able to substitute the native FF-HDL in its ability to stimulate endothelial cell proliferation. Similarly, neither apoA-I com-

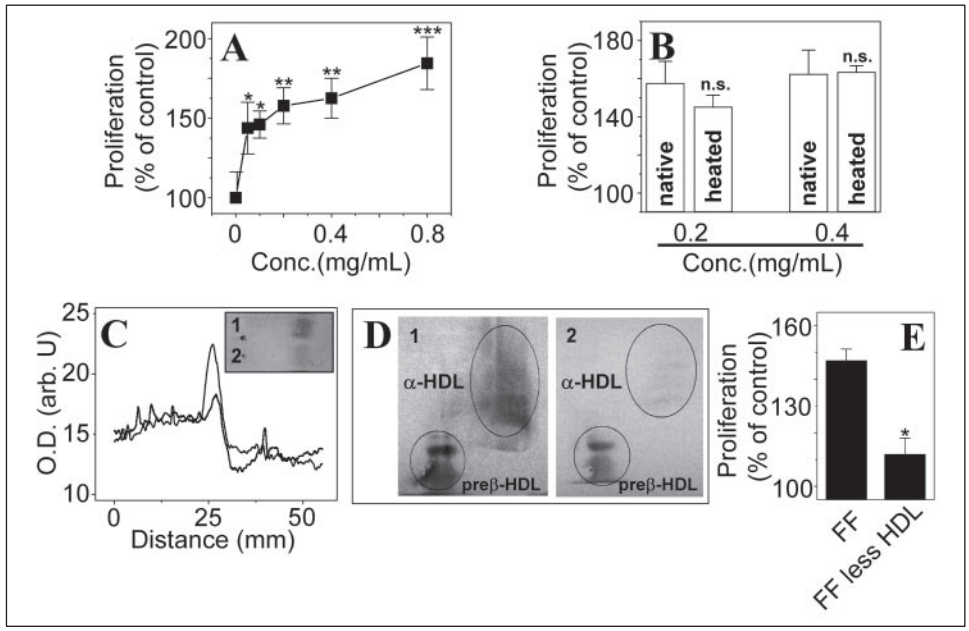

FIGURE 3. **Characterization of FF-HDL.** *A*, HUVEC were seeded in a 96-well plate at a density of 5×10^3 cells/well and exposed for 48 h to FF-HDL at the indicated concentrations. The proliferation rate was determined with an MTT assay as described under "Experimental Procedures." Data represent mean ± S.D. from three separate determinations. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.002$ (control *versus* FF-HDL). *B*, follicular fluid was heat-treated at the indicated temperature and added to HUVEC for determination of proliferation using an MTT assay. Data represent mean ± S.D. from three separate determinations. *n.s.*, not significant (heat-treated *versus* native). *C*, follicular fluid before (*1*) and after (*2*) removal of HDL was subjected to electrophoresis on agarose gel as described under "Experimental Procedures." Shown is a representative densitogram of a separating gel. *Inset*, presentation of an agarose gel stained with Sudan black. Note the complete absence of lipoprotein fractions other than HDL in follicular fluid. *D*, follicular fluid before (*1*) and after (*2*) removal of HDL was subjected to a two-dimensional electrophoresis and Western blotting using antibodies against apoA-I as described under "Experimental Procedures." Note the complete absence of HDL fraction with α-mobility after centrifugation. *E*, HUVEC were exposed to follicular fluid before (*FF*) and after (*FF less HDL*) the removal of HDL for 48 h, and the proliferation rate was determined with an MTT assay. Data represent mean ± S.D. from three separate determinations. *, $p < 0.02$ (*FF versus FF less HDL*).

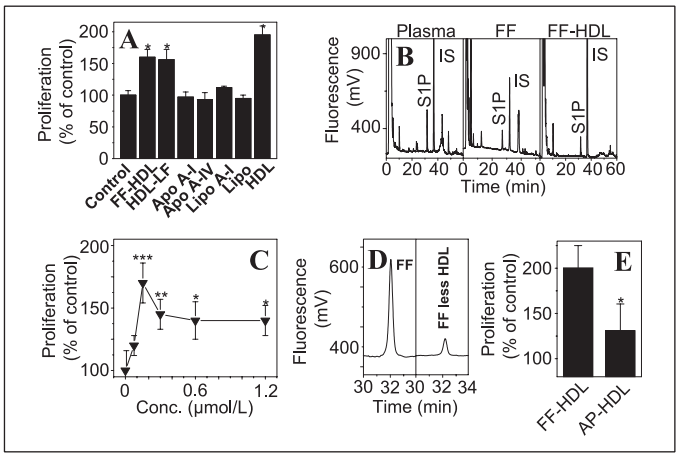

FIGURE 4. **Demonstration of S1P in follicular fluid.** *A*, HUVEC were seeded in a 96-well plate at a density of 5×10^3 cells/well and exposed for 48 h to FF-HDL (0.5 g/liter), an HDL-lipid fraction equivalent to 0.5 g/liter FF-HDL (*HDL-LF*), apoA-I (50 μg/ml), apoA-IV (50 μg/ml), apoA-I-phosphatidylcholine complexes (*Lipo A-I*; 20 μg/ml protein, 50 μg/ml phospholipid), phosphatidylcholine vesicles (*Lipo*; 50 μg/ml phospholipid), or HDL isolated from plasma (*HDL*; 0.5 g/liter). The proliferation rate was determined with an MTT assay as described under "Experimental Procedures." Data represent mean ± S.D. from three separate determinations. *, $p < 0.01$ (agonist *versus* control). *B*, HPLC profile of S1P and dihydro-S1P (internal standard, *IS*) separated on a reverse-phase C18 column after a two-step lipid extraction and derivatization with *o*-phthaldialdehyde. Shown are representative separations of plasma, follicular fluid, and FF-HDL. *C*, HUVEC were seeded in a 96-well plate at a density of 5×10^3 cells/well and exposed for 48 to S1P at the indicated concentrations. The proliferation rate was determined with an MTT assay as described under "Experimental Procedures." Data represent mean ± S.D. from three separate determinations. *, $p < 0.05$; **, $p < 0.02$; ***, $p < 0.001$ (S1P *versus* control). *D*, S1P content was analyzed in follicular fluid before and after the removal of FF-HDL by ultracentrifugation. Shown are representative chromatograms. *E*, FF-HDL was treated with alkaline phosphatase for S1P degradation. HUVEC were exposed to FF-HDL and to alkaline phosphatase-treated HDL (*AP-HDL*) for 48 h, and the proliferation rate was determined with an MTT assay. Data represent mean ± S.D. from three separate determinations. *, $p < 0.05$ (FF-HDL *versus* alkaline phosphatase-treated HDL).

plexed to phosphatidylcholine nor phosphatidylcholine-containing liposomes exerted any mitogenic activity. By contrast, the lipid fraction isolated from HDL exhibited a strong mitogenic effect toward endothelial cells. Both the mitogenic effects of FF-HDL and the lipid fraction isolated from FF-HDL were comparable with effects of HDL obtained from plasma. Recently, several lysophospholipids with potent mitogenic properties such as S1P were identified in the HDL fraction of human plasma. Therefore, we next examined whether follicular fluid and FF-HDL contain detectable amounts of S1P. As shown in Fig. 4*B*, S1P could be detected in both native follicular fluid and FF-HDL. The concentration of S1P in follicular fluid was estimated at 0.87 ± 0.11 ng/mg protein ($n = 3$; ~170 nmol/liter). At this concentration, S1P was found to efficiently stimulate endothelial cell proliferation as determined by an MTT assay (Fig. 1*C*). Furthermore, the S1P content of the follicular fluid was considerably reduced after the removal of FF-HDL by performing discontinuous gradient centrifugation (Fig. 4*D*), indicating that this lysosphospholipid is associated with FF-HDL. To examine the contribution of S1P to mitogenic effects exerted by follicular fluid, we performed a digestion with alkaline phosphatase, which is known to degrade S1P (18). As shown in Fig. 4*E*, alkaline phosphatase pretreatment of FF-HDL reduced the initially observed increase in endothelial cell proliferation as compared with the native FF-HDL.

Contribution of Various Signal Transduction Pathways to Follicular Fluid-induced Proliferation—One consequence of the predicted role of S1P and FF-HDL in the follicular fluid-induced endothelial proliferation is that these agonists share common signal transduction pathways leading to mitogenicity. In order to examine this proposition, we investigated the mitogenic effects of native follicular fluid, FF-HDL, and S1P in the presence of pharmacological inhibitors of proliferation-relevant signaling pathways previously shown to be activated in the presence of plasma HDL (24–27). As shown in Fig. 5*A*, preincubation of endothelial cells for 30 min with 25 μmol/liter PD98059, an inhibitor of MEK1-

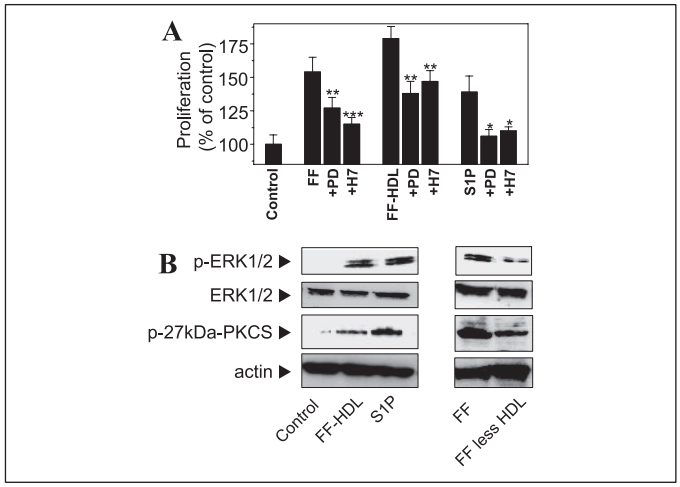

FIGURE 5. **The involvement of intracellular signaling pathways in follicular fluid-induced proliferation.** A, HUVEC were preincubated for 30 min with PD98059 (25 μmol/liter) or H7 (12.5 μmol/liter) and exposed for 48 h to follicular fluid, FF-HDL (0.5 g/liter), and S1P (0.2 mmol/liter) in the continuous presence of inhibitors. The proliferation rate was determined with an MTT assay as described under "Experimental Procedures." Data represent means from three separate determinations. *, $p < 0.02$; **, $p < 0.01$; ***, $p < 0.001$ (FF + inhibitor, FF-HDL + inhibitor, or S1P + inhibitor *versus* FF, FF-HDL, or S1P). *B*, HUVEC were stimulated with FF-HDL (0.5 g/liter) or S1P (0.2 μmol/liter) for 10 min (*left panel*) or with follicular fluid before and after the removal of FF-HDL (*right panel*). Cell lysates were subjected to Western blotting and probed with phosphospecific antibodies against ERK1/2 or PKC substrate containing the phosphorylation consensus sequence (R/K)*X*(S)Hyd(R/K), where Hyd indicates the hydrophophic residue. The loading equality was controlled using antibody against ubiquitously expressed protein (α-actin) or antibodies against unphosphorylated isoforms of ERK1/2. Shown are blots representative for 3–5 experiments. *p*, phosphorylated isoform.

mediated ERK1/2 activation, significantly reduced the mitogenic effects exerted by follicular fluid, FF-HDL, and S1P. Similar inhibitory effects were observed after a 30-min pretreatment of endothelial cells with 12.5 μmol/liter H7, an inhibitor of protein kinase C (PKC).

Since FF-HDL and S1P-induced mitogenic signaling appeared to involve ERK1/2 and PKC, we next sought to determine the direct effect of both compounds on the activation of kinases. Using antibodies specifically reacting with phosphorylated isoforms of proteins, we next examined the phosphorylation of ERK1/2, which is a prerequisite of ERK1/2 activation, as well as the phosphorylation of PKC substrates containing a phosphorylation consensus sequence (R/K)*X*(S)Hyd(R/K), where Hyd indicates the hydrophobic residue. As shown in Fig. 5*B* (*left*), exposure of endothelial cells to FF-HDL (0.5 mg/ml) and S1P (0.2 μmol/liter) led to phosphorylation of ERK1/2 and the 27-kDa PKC substrate within 10 min after the addition of the agonist. In addition, we found that both the phosphorylation of the ERK1/2 and the 27-kDa PKC substrate induced by follicular fluid were markedly attenuated when the stimulation was performed after the removal of HDL from follicular fluid (Fig. 5*B*, *right*).

The Effect of FF-HDL and S1P on New Vessel Formation—In the final step, we directly examined the angiogenic effects of FF-HDL and S1P using an assay for new vessel formation. As shown in Fig. 6*A*, placement of HUVEC on the growth factor-reduced Matrigel in the presence of 0.5 g/liter FF-HDL or 0.2 μmol/liter S1P led to the formation of elongated tube-like structures that were organized by a much larger number of cells compared with the control ($n = 3$; $p < 0.01$). Direct quantification of total sprout length demonstrated that both HDL and S1P induced tubuli formation that was about as effective as by native follicular fluid (Fig. 6*B*) ($n = 3$; not significant). The ability of follicular fluid to induce tubuli formation was markedly reduced after the removal of FF-HDL ($n = 3$; $p < 0.01$) by centrifugation and in the presence of LY294002 (25 μmol/liter) ($n = 3$; $p < 0.01$), an inhibitor of protein kinase Akt, which was previously postulated to mediate proangiogenic effects of plasma HDL (23). In addition, both the HDL- and S1P-induced tubuli formation were markedly reduced in HUVEC pretreated with LY294002 ($n = 3$; $p < 0.05$). The direct effect of FF-HDL and S1P on AKT activation is shown in Fig. 6*C*. The exposure of HUVEC to both compounds led to Akt phosphorylation. However, the Akt phosphorylation was considerably less pronounced, when HUVEC were exposed to follicular fluid deprived of FF-HDL (Fig. 6*C*, *right*).

DISCUSSION

Ovarian angiogenesis plays an important role in the sequence of events leading to the development of the ovarian follicle and the formation of the corpus luteum (1). Several angiogenic factors including VEGF and bFGF have been identified in follicular fluid, and their concentrations were in most studies positively correlated with gonadotrophin concentrations, oocyte maturation, and high fertilization rates (28–31). In the previous study, however, we observed that the ability of follicular fluid to induce endothelial proliferation exceeded that of equimolar concentrations of purified VEGF or bFGF (12). Here, we extend this observation to show that the neutralization of VEGF and bFGF with specific antibodies or their thermal inactivation substantially reduced but did not completely eliminate the proliferation-stimulating activity of follicular fluid. We interpret these findings to mean that follicular fluid is a source of other angiogenic activities distinct from heat-sensitive proteins or peptides. However, it is important to note that the immunoneutralization might not lead to complete elimination of VEGF and bFGF in terms of biological activity.

The existence of a heat-stable angiogenic activity in follicular fluid has been proposed before, but its molecular identity has never been clarified (32–34). Several lines of evidence in this study point to HDL as a new angiogenic factor derived from follicular fluid. First, the chromatographic analysis of follicular fluid revealed that HDL was the main component of three fractions most potently promoting endothelial growth. Second, HDL isolated from follicular fluid by discontinuous ultracentrifugation stimulated both the proliferation of endothelial cells and the angiogenesis as determined with new vessel formation assay. Conversely, follicular fluid deprived of HDL displayed much reduced ability to stimulate the endothelial cell proliferation and the new vessel formation. Third, similar patterns of intracellular signals relevant to cell proliferation such as activation of PKC and ERK1/2 or to vessel formation such as Akt could be recorded in endothelial cells stimulated with follicular fluid or HDL. The intensities of these signals were diminished after the removal of HDL from follicular fluid. Nevertheless, it has to be emphasized that HDL-deprived follicular fluid retained a significant portion of its capacity to stimulate endothelial growth, angiogenesis, and intracellular signaling. It seems, therefore, that several independent but complementary activities present in follicular fluid are responsible for the proper execution of angiogenic processes in the ovary. This redundancy may reflect the importance of maintaining key processes in reproduction intact.

HDL has been long recognized as a sole lipoprotein class present in follicular fluid until ovulation (13, 15). Whereas HDL in plasma is believed to protect against atherosclerosis by virtue of transporting cholesterol from peripheries to the liver, little is known about the physiological role of FF-HDL. HDL isolated from plasma has been shown to deliver cholesterol into human granulosa-lutein cells through a selective uptake pathway utilizing the scavenger receptor class B, type 1 (SR-B1), and, in addition, to stimulate progesterone synthesis in a process involving increased generation of luteotropic prostaglandins and cAMP (35, 36). It is unclear to what extent FF-HDL is capable of stimulating ovar-

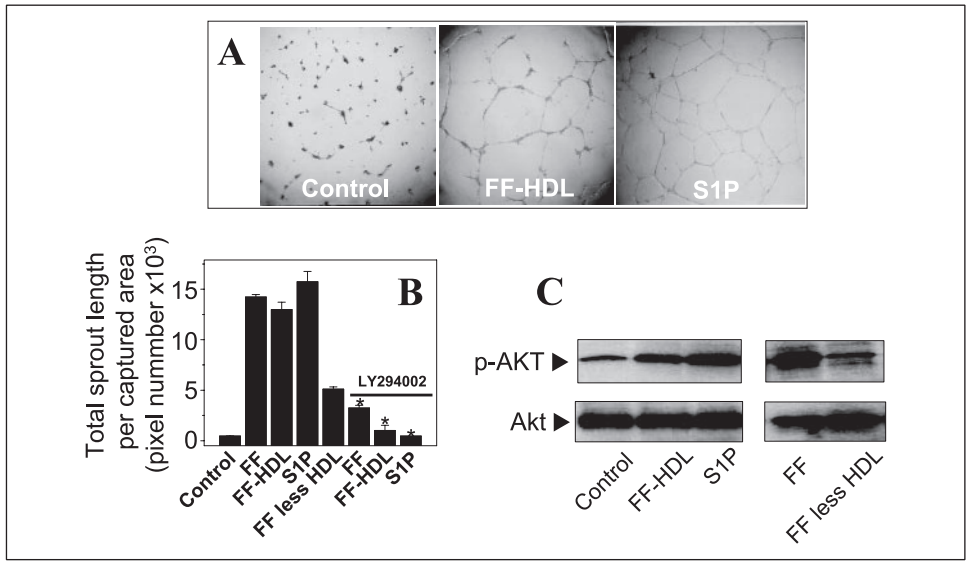

FIGURE 6. **Demonstration of FF-HDL- and S1P-induced angiogenesis.** *A*, HUVEC were seeded on Matrigel in a 96-well plate at a density of 1×10^5 cells/well for 18 h in the absence (control) or presence of FF-HDL (0.5 g/liter) or S1P (0.2 mmol/liter). Sprouting tubuli were observed under a light microscope at × 40 magnification. Shown are pictures representative of one experiment of three. *B*, HUVEC plated on Matrigel were exposed to follicular fluid, FF-HDL, and S1P in the presence or absence of Akt inhibitor, LY294002 (25 μmol/liter), or after the removal of FF-HDL from follicular fluid by centrifugation (*FF less HDL*). The extent of tube formation was quantified using image processing software as described under "Experimental Procedures." Shown are means ± S.D. from three independent measurements. *, $p < 0.001$ (FF + LY294002, FF-HDL + LY294002, or S1P + LY294002 *versus* FF, FF-HDL, or S1P). *C*, HUVEC were stimulated with FF-HDL (0.5 g/liter) or S1P (0.2 μmol/liter) for 10 min (*left panel*) or with follicular fluid before and after the removal of FF-HDL (*right panel*). Cell lysates were subjected to Western blotting and probed with phosphospecific antibodies against Akt. The loading equality was controlled using antibody against the unphosphorylated isoform of Akt. Shown are blots representative for 3–5 experiments. *p*, phosphorylated isoform.

ian steroidogenesis. However, it is worth noticing that FF-HDL contains about 3–4 times less cholesterol than plasma HDL (23) and is thus likely to be a less effective cholesterol donor. The results of the present study suggest that promoting ovarian angiogenesis may be an important physiological function of FF-HDL. In this respect, FF-HDL resembles plasma HDL, which was previously shown to support endothelial cell proliferation, to counteract endothelial apoptosis, and to stimulate formation of new vessels (27, 37–39). Furthermore, similar pathways of intracellular signaling, such as PKC, ERK1/2, and Akt, are utilized by both FF-HDL and plasma HDL to trigger cell growth and to promote angiogenesis. In contrast to plasma HDL, which primarily contains spheroid α-HDL particles, FF-HDL is composed of roughly equal amounts of α-HDL and discoid pre-β-HDL particles. Our results demonstrate that the removal of α-HDL from follicular fluid is sufficient to substantially reduce its capacity to stimulate endothelial proliferation and new vessel formation. Thus, α-HDL seems to be the angiogenically active subfraction in case of both HDL derived from follicular fluid and plasma.

HDL is a complex molecule known to exert a multitude of physiological function, for which its different components have been made responsible. Previously, we failed to detect any growth-stimulating activities of apoA-I and apoA-II, two major protein constituents of HDL, toward fibroblasts and smooth muscle cells (21, 40), and the present study extends these observations to apoA-IV and to endothelial cells. By contrast, endothelial proliferation could be induced by a lipid fraction isolated from FF-HDL. Previous studies shown that plasma HDL acts as a carrier of biologically active lysophospholipids such as S1P, sphingosylphosphorylcholine, and lysosulfatide (22, 40, 41). Furthermore, we and others demonstrated that several physiological functions exerted by plasma HDL, such as stimulation of cell proliferation and migration, inhibition of apoptosis, induction of NO-dependent vasorelaxation, and, in particular, stimulation of angiogenesis, can be attributed to HDL-associated lysophospholipids (22, 27, 34, 37–40, 42). In the present study, we report for the first time that one of the biologically active lysophospholipids, S1P, is present in follicular fluid and that its significant portion is associated with FF-HDL, since much lower amounts of S1P could be detected in follicular fluid after the removal of FF-HDL. The S1P concentration determined in follicular fluid was ~170 nmol/liter. This is less than the concentration estimated by us in serum (~900 nmol/liter),[4] but nevertheless well within the range shown in this and other studies to induce growth and other responses in endothelial cells, such as angiogenesis and migration (38, 39). We further demonstrate that S1P fully mimicked FF-HDL in its capacity to stimulate endothelial proliferation and new vessel formation, that similar intracellular signaling pathways were used by FF-HDL and S1P to promote cell proliferation, and that S1P-induced endothelial growth was not affected by heat treatment. By contrast, enzymatic degradation of S1P in FF-HDL suppressed the growth-supporting effects of FF-HDL toward endothelial cells. Taken together, these findings strongly support the contention that S1P represents the HDL-associated angiogenic activity derived from follicular fluid. However, it is crucial to emphasize that, based on our results, the presence of other heat-stable growth-promoting factors in follicular fluid cannot be dismissed. Whether sphingosylphosphorylcholine and lysosulfatide are associated with FF-HDL, has not been specifically addressed in this study. If so, these compounds could additionally contribute to the angiogenic potential of follicular fluid. Other bioactive lipids, such as fatty acid esters of pregnenolone, were found to be carried by FF-HDL (43), but their ability to support endothelial growth and angiogenesis has not been investigated to date.

The identification of S1P in the follicular fluid may have far reaching consequences for the understanding of key regulatory processes in reproduction. S1P and other lysophospholipids were previously identified both intracellularly and in the extracellular space, where they appear to modulate a dazzling array of physiological processes, including proliferation and apoptosis, cell motility and migration, angiogenesis, connective tissue synthesis, wound healing, and immune response (44, 45). Each of these processes is known to contribute to follicle

[4] J. R. Nofer, unpublished observations.

growth and the formation of the corpus luteum, and their coordinate execution is of crucial importance during ovulation and in the course of early embryonic development. Bioactive lysophospholipids mediate various physiological processes by binding to their cognate G-protein-coupled receptors from the lysophospholipid receptor family. Whereas the expression of S1P receptors, including $S1P_1$ (EDG-1), $S1P_2$ (EDG-5), and $S1P_3$ (EDG-3), has been demonstrated in ovarian tissues (46) and granulosa cells,[5] their exact contribution to the regulation of the reproductive system remains obscure. In this regard, however, it is of great interest that decreased fertility was observed in $S1P_2/S1P_3$ double-deficient animals (47). Furthermore, recent data show that S1P increases germ cell survival against cytotoxicity induced by drugs used in cancer therapy (48). Moreover, the same group demonstrated that oocyte loss in adult wild-type female mice, the event that drives premature ovarian failure and infertility in female cancer patients, was completely prevented by *in vivo* therapy with S1P (49).

The origin of S1P in preovulatory follicular fluid is currently unknown. Sphingosine kinase, an enzyme primarily responsible for S1P synthesis, is ubiquitously expressed (44, 45) and may represent an important source of S1P in follicular fluid. It is of interest, however, that thrombocytes, which due to the absence of S1P lyase fail to degrade S1P, were shown to represent a considerable source of S1P in plasma. Thrombocytes are present in the follicular cavity during the formation of a blood clot around the time of ovulation, albeit their exact role in the formation of the emerging corpus luteum remains obscure. It would be tempting to speculate that by the provision of S1P, thrombocytes specifically support local vasculogenesis and thereby promote the development of corpus luteum.

In conclusion, the present study demonstrates that HDL and associated lysophospholipid S1P are important mitogenic and angiogenic factors present in follicular fluid. Further studies are necessary to fully elucidate the cellular source and the specific role that might be played by follicular HDL and lysophospholipids in the physiology of reproduction.

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