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Thyroxine treatment stimulated ovarian follicular angiogenesis in immature hypothyroid rats

Jin-Yi Jiang[1,4], Kanako Miyabayashi[1] Stefania A. Nottola[2], Motoaki Umezu[5], Sandra Cecconi[3], Eimei Sato[1] and Guido Macchiarelli[3]

[1]Laboratory of Animal Reproduction, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [2]Laboratory of Electron Microscopy "Pietro M. Motta", Department of Anatomy, University La Sapienza, Rome, Italy, [3]Department of Health Sciences, University of L'Aquila, L'Aquila, Italy, [4]Ottawa Health Research Institute, The Ottawa Hospital, Ottawa, Ontario, Canada and [5]Faculty of Agriculture, Utsunomiya University, Utsunomiya, Japan

Summary. The development of mature ovarian follicles is greatly dependent on healthy thecal angiogenesis. Recent experimental evidence showed that thyroxine (T4) treatment promoted ovarian follicle development in immature hypothyroid (*rdw*) rats. However, an involvement of thyroid hormone in ovarian follicular angiogenesis has not yet been demonstrated. By morphological and molecular approaches, the present studies demonstrated that antral follicles in untreated, T4- or equine chorionic gonadotropin (eCG)-treated *rdw* rats were mainly small and/or atretic, and presented a poorly developed thecal microvasculature with ultra-structural evidence of diffuse quiescent or degenerative thin capillaries. However, T4 together with eCG increased the number of large antral and mature follicles with numerous activated capillaries and ultra-structural evidence of rich and diffuse angiogenesis in the theca layer. While T4 alone significantly increased mRNA expression of vascular endothelial growth factor (VEGF) and tumor necrosis factor alpha (TNFα), it decreased that of fetal liver kinase compared with those in the untreated group. Combined treatment of T4 and eCG markedly increased mRNA abundance of not only VEGF and TNFα, but also basic fibroblast growth factor. These data suggest that T4 may promote ovarian follicular angiogenesis in *rdw* rats by up-regulating mRNA expression of major angiogenic factors.

Key words: Thyroid hormone, Ovarian follicle, Angiogenesis, Angiogenic factor, Angiogenic receptor, SEM, TEM, Hypothyroid rat

Offprint requests to: Jin-Yi Jiang, 3rd Floor, Box 926, Centre for Cancer Therapeutics, Ottawa Health Research Institute, The Ottawa Hospital (General Campus), 501 Smyth Road, Ottawa, Ontario, Canada K1H 8L6. e-mail: jjiang@ohri.ca

Introduction

The thecal microvascular architecture plays a key role in ovarian folliculogenesis (Redmer and Reynolds 1996; Motta et al., 2003). It changes dynamically and continuously during follicular development and atresia (Macchiarelli et al., 1993; Macchiarelli, 2000; Jiang et al., 2001). Indeed, the maintenance of the follicular microvasculature is essential for follicle health (Mattioli et al., 2001). When a follicle grows from the primary to the preovulatory stage, its thecal microvasculature develops from a sparse capillary wreath into a single-layer and basket-like plexus in rats (Murakami et al., 1988; Miyamoto et al., 1996; Jiang et al., 2001; Iijima et al., 2005). In other species, it becomes a well-developed multi-layer vascular network as a result of active angiogenesis, while regressive vascular networks are observed in atretic follicles (Yamada et al., 1995; Kanzaki et al., 1982; Kitai et al., 1985; Kikuta et al., 1991; Ferrara and Davis-Smyth, 1997; Macchiarelli, 2000). Indeed, capillaries in atretic follicles are thin and decreased in number. They vary in size with poor or no angiogenesis (Macchiarelli et al., 1993; Jiang et al., 2003b).

Gonadotropins stimulate ovarian follicular angiogenesis by regulating the expression of many growth factors and cytokines, which include epidermal growth factor (EGF), transforming growth factor (TGF), fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) (Shimizu et al., 2002). VEGF is the key angiogenic factor in the regulation of ovarian vascularization via its two receptors (fms-like tyrosine kinase, Flt-1 and fetal liver kinase, Flk-1) (Ferrara and Davis-Smyth, 1997; Ferrara et al., 1998; Jiang et al., 2001; Stouffer et al., 2001).

In addition to gonadotropins, other hormones, such as thyroid hormones, play an important role in the

regulation of follicular development and atresia. Follicle development is markedly hampered in hypothyroidism, but thyroxine (T4) therapy improves follicle development, especially in the presence of gonadotropin (Maruo et al., 1992; Jiang et al., 2000; Cecconi et al., 2004). While thyroid hormone receptors are expressed in several follicular cell types (Wakim et al., 1987, 1993, 1994, 1995; Zhang et al., 1997), the cellular and molecular mechanisms by which thyroid hormone is involved are poorly understood. In addition, studies have demonstrated that equine chorionic gonadotropin (eCG) treatment enhances the angiogenic activity in ovaries (Sato et al., 1982; Koos and LeMaire, 1983) by up-regulating the expression of TGF-ß1 and VEGF (Dissen et al., 1994). However, nothing is known about whether and how thyroid hormone is involved in the regulation of ovarian follicular angiogenesis.

Our previous studies in a well-established infertile immature hypothyroid rodent model (*rdw* rat) showed that T4 alone, or together with eCG, markedly increased the number of healthy small or larger antral follicles, respectively (Jiang et al., 2000; Sato and Jiang, 2001). The combined treatment also significantly increased the number of ovulated eggs following human chorionic gonadotropin stimulation (Jiang et al., 1999a). In the present study, using morphological and biochemical assessments of vascular development, we demonstrated that thyroid hormone stimulated the development of follicular microvasculature by regulating gene expression of angiogenic factors and their receptors.

Materials and methods

Preparation of Animals

Immature hypothyroid *rdw* rats and normal Wistar-Imamichi rats were produced and maintained as described previously (Jiang et al., 1999a,b, 2000). *rdw* rats were distinguished according to low body weight and retarded development of the ears at about two weeks of age. The hypothyroidism was confirmed with a marked decrease in serum T4 concentrations as described previously (Jiang et al., 1999a). The studies were approved by the Ethics Committee for Care and Use of Laboratory Animals for Biomedical Research in the Faculty of Agricultural Sciences, Tohoku University, Japan, and conform to related European Community regulations.

Hormone administration

Female immature *rdw* rats were divided randomly and treated as follows: (1) untreated *rdw* rats [i.e., *rdw* rats treated with vehicle]; (2) T4-treated *rdw* rats; (3) eCG-treated *rdw* rats; and (4) T4/eCG-treated *rdw* rats [i.e., *rdw* rats given T4 and eCG treatment]. Their normal littermates without (untreated normal) or with eCG treatment (eCG-treated normal) were used as controls. The *rdw* rats were administered T4 (L-thyroxine, Sigma Chemical Company, St Louis, MO) intraperitoneally each day at a dose of 10 µg per 100 g of body weight from day 21 to day 29 as described previously (Jiang et al., 2000). The eCG (10 IU) (Sankyo Kabu Company, Tokyo) was injected subcutaneously on day 28 into both *rdw* and normal rats. The animals were sacrificed 48 hours after eCG administration to prepare ovarian vascular casts for scanning electron microscopy (SEM) study, to collect ovaries for light microscopy (LM) and transmission electron microscopy (TEM) observation, and for the assessment of mRNA levels of angiogenic factors and their related receptors by RT-PCR, as described in detail hereafter.

SEM study of ovarian vascular corrosion casts

The ovarian vascular corrosion casts were prepared according to our previous reports (Macchiarelli et al., 1991, 1993, 1995; Jiang et al., 2002; Shimizu et al., 2002). Briefly, five to six animals in each group were anaesthetized with pentobarbital sodium (Abbot Laboratories, Illinois), and perfused with heparinized saline and a solution of Mercox (Okenshoji Co. Ltd., Tokyo, Japan) resin through the left ventricle until polymerization started (Murakami, 1971; Murakami et al., 1988). Casted ovaries were then removed and placed in hot water for complete polymerization. The samples were corroded in 10% NaOH solution, washed in hot water and dried in a hot oven. Dried samples were mounted on aluminium stubs and coated with platinum.

Observations were made with a scanning electron microscope (Hitachi S-4200, Tokyo, Japan) with criteria as reported previously (Jiang et al., 2004). Briefly, vessels were classified according to their diameters and shape of endothelial cell nuclei. Budding, sprouting and splitting of capillaries from pre-existing blood vessels were considered angiogenic figures. Incompletely filled or thinned capillaries were considered degenerative.

LM and TEM study of ovarian follicular microvasculature

To assess the cellular morphological changes of ovarian microvasculature in eCG- and eCG/T4-treated *rdw* rats, LM and TEM studies were conducted as described previously (Motta et al., 2003). Briefly, after animals (n=4 in each group) were perfused with heparinized saline and then 2.5% glutaraldehyde, ovaries were excised and fixed further in 2.5% glutaraldehyde, postfixed with 1% osmium tetroxide, dehydrated in a graded ethanol series, and embedded in Agar resin. Sections were cut with an ultramicrotome (Ultracut E-Reichert-Jung, Vienna, Austria). For LM, 1 µm-thick sections were stained with aqueous methylene blue, examined and photographed with a light microscope (Zeiss Axioskop 40, Gottingen, Germany). For TEM observation, ultra-thin sections (0.11-0.13 µm in thickness) were prepared on unsupported copper grids and stained with uranyl acetate and lead citrate. Specimens were observed and photographed with a

transmission electron microscope (Zeiss EM 10A, Oberkochen, Germany).

Healthy and atretic follicles were examined according to the criteria (i.e. pyknotic and floating granulosa cells, irregular thickness of the follicular wall, ooplasm fragmentation and zona pellucida alterations) reported previously (Hirshfield and Midgley, 1978; Braw and Tsafriri, 1980; Jiang et al., 2000) in LM studies. In TEM studies of ultra-thin sections, activated (angiogenic) or quiescent thecal capillaries were evaluated according to endothelial cell ultrastructural morphology (shape, plasma membrane specialization acquisition, organular pattern) and the presence of capillary buds and sprouts (Rhodin and Fujita, 1989; Hansen-Smith et al., 1996; Zhou et al., 1998b).

Detection of mRNA levels of angiogenic factors and their receptors by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from frozen ovaries (5 replicates in each group) with the RNeasy Mini Kit (QIAGEN K.K., Tokyo, Japan) treated with DNase I and quantified using an ultraviolet (UV)-visible recording spectrophotometer (UV-160, Shimadzu Corporation, Tokyo, Japan). To assess mRNA levels of VEGF, TGF-ß1, bFGF, EGF, tumor necrosis factor alpha (TNFα), TNFα receptors 1 and 2 (TNFR-1 and TNFR-2), Flt-1 and Flk-1, semi-quantitative RT-PCR was performed using Ready-To-Go RT-PCR Beads (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) following the method provided by the manufacturer with 1 µg of total RNA. The RT reaction was carried out at 42°C for 15 min, and samples were incubated for reaction at 95°C for 5 min to inactivate the reverse transcriptase and to denature completely the template. The oligonucleotide primers for angiogenic factors and related receptors, as well as PCR cycles, are listed in Table 1. The PCR cycles were determined in the linear range in preliminary experiments (data not shown). The amplification cycle consisted of 95°C for 1 min, 60°C for 1 min, and 72°C for 2 min. After amplification, each PCR product was resolved in a 2% agarose gel and visualized by ethidium bromide staining. The bands were quantified by densitometry using the NIH Image 1.63 analysis program (National Institute of Health, Bethesda, MD). Each gene mRNA level was normalized against its respective 18S mRNA and expressed as fold difference.

Statistical analysis

Data in Figs. 4 and 5 were analyzed by one way ANOVA. Fisher's PLSD multiple-range tests were used for further comparisons. Differences with $P<0.05$ were considered statistically significant.

Results

SEM study of ovarian vascular corrosion casts

The surfaces (cortical region) of ovarian casts were arranged in rounded structures made of arterioles, venules and capillaries, corresponding to the vascular plexuses of follicles (FVP). These structures varied in

Table 1. Primer pairs used for detection of mRNAs.

Genes[a]	PCR primers[b]	PCR cycles	Fragment size (bp)	GenBank accession No.
VEGF	(*)	30	322	M95200
TGF-ß1	for: 5'-GGAGCTGGTGAAACGGAAGC-3' rev: 5'-ACGTCAAAAGACAGCCACTC-3'	30	486	X52498
bFGF	for: 5'-AGGATCCCAAGCGGCTCTAC-3' rev: 5'-GGAAGAAACAGTATGGCCTT-3'	30	370	M22427
EGF	for: 5'-AAGCTCTTTCTGGCCGCAGT-3' rev: 5'-CACTTTCTCCTCACTTTCCA-3'	30	573	L05489
TNFα	for: 5'-GGTACCCCAGCAAACAGAAT-3' rev: 5'-GTGCTGGGGCTGAAGTGTAA-3'	30	385	L00981
Flt-1	for: 5'-GCAGCACCTTGACCTTGAAC-3' rev: 5'-AGGATTGTATTGGTCTGCCG-3'	26	424	D28498
Flk-1	for: 5'-GCTCTGTGGTTCTGCGTGGA-3' rev: 5'-CATGGGATCACCACAGTTTT-3'	24	422	U93306
TNFR1	for: 5'-CTAAGCCCCTAACTCCAGCC-3' rev: 5'-GGAGCCGCATGAACTCCTTC-3'	26	407	M37394
TNFR2	for: 5'-AAACGTGATATGCAGTGCCT-3' rev: 5'-GGATGAAGCAGGTCGTTAGT-3'	26	363	M63122
18S	(*)	#	488	

[a]: See text for definitions. [b]: for, forward; rev, reverse. #: The PCR cycles for 18S were the same as those for corresponding genes. (*) VEGF and 18S were purchased from Ambion, Inc. (Cat. No. 5442)

number, size and type of capillaries, according to different treatments. FVP were considered as undergoing active angiogenesis (normal developing follicles) when the number of angiogenic figures was higher than that of degenerative figures in a defined area (0.0378 mm^2) in three different regions, whereas plexuses were considered degenerative (atretic follicles) when an avascular area was present or the number of capillaries with degenerative figures was greater than that of angiogenic figures.

In ovaries of untreated (Fig. 1A) and T4-treated *rdw* rats (Fig. 1B), a few FVP with a diameter ranging from 100 to 400 µm were present in the cortical region. In these groups, most of the FVP were atretic and presented avascular areas (Fig. 1A, B) with thin or degenerative capillaries (Fig. 1C). A few focal areas with sprouting capillaries, often associated with degenerative figures, were also recognized (Fig. 1D). Atretic FVP were slightly more abundant in untreated than T4-treated *rdw* rats. The average ratio of normal/atretic FVP in both groups was 1:2.5 (21:53) and 2:1 (69:35), respectively. The ovaries of eCG-treated *rdw* rats showed more FVP (100-600 µm in diameter) with active angiogenesis (normal developing follicles) than those with degeneration (atretic follicles) (ratio 3:1 = 36:12). Numerous FVP, either normal or atretic, presented a well-developed vascular supplying apparatus made of arterioles and venules (Fig. 1E). Angiogenic figures consisting of budding, sprouting and dilated capillaries were also present (Fig. 1F). In T4/eCG-treated *rdw* rats, numerous FVPs (100-600 µm in diameter) were present. Many more angiogenic (normal follicles) than degenerative (atretic follicles) FVPs were observed with a ratio of 5:1 (45:9). Normal FVPs were always supplied by arterioles and venules (Fig. 1G). The angiogenic figures, consisting of dilated capillaries rich in budding and sprouting, were diffusely observed and characterized normal developing FVP (Fig. 1H).

Correlated LM and TEM study

LM and TEM studies were performed in T4/eCG-treated *rdw* group, which presented SEM signs of diffuse angiogenesis, and compared with eCG-primed *rdw* rats, in order to reveal the fine morphological features of newly formed capillaries in the follicle.

LM of eCG-treated *rdw* rat ovaries showed that numerous antral follicles (100-550 µm in diameter) were present in the cortical region. Some of these follicles showed morphological signs of atresia (Fig. 2A). The ratio of healthy and atretic antral follicles was 3:1 (16:5). Healthy antral follicles had a regular circular or oval profile and fully structured theca layers, which were thick (50-80 µm) with numerous capillaries. Arterioles and venules were recognized at the boundary line between the theca interna and externa. Atretic antral follicles appeared irregularly shaped or collapsed, and encircled by irregular theca layers (Fig. 2B). In these follicles the theca interna was often thin (15-25 µm) and displayed few capillary vessels (Fig. 2B).

In eCG-treated *rdw* rat ovaries TEM showed a quiescent morphology in thecal capillaries of healthy antral follicles (Fig. 2C). These capillaries were characterized by a regularly shaped round or oval lumen in cross sections. Endothelial cells were flat and had oval regular nuclei and smooth plasma membranes. Few organelles were present in their cytoplasm (Fig. 2D). Few capillaries with morphological signs of activation were recognized in the theca interna of these follicles (Fig. 2E). These vessels showed an irregularly shaped lumen, and endothelial cells with irregular and short plasma membrane processes. Pericytes were observed and closely associated with activated capillaries (Fig. 2D).

T4/eCG-treated *rdw* rat ovaries (Fig. 3A-D) had more healthy antral (larger than 550 µm in diameter) than atretic antral follicles (100-400 µm), with a ratio of healthy/atretic follicles of 5:1 (36:7). Atretic follicles were characterized by a thin theca interna (15-30 µm) with very few capillaries (Fig. 3C,D).

Further studies by TEM on T4/eCG-treated *rdw* rat ovaries showed the presence of numerous activated capillaries in the theca interna of healthy antral follicles. Capillaries often showed a flat vascular lumen (pocket-like lumen), sometimes with a sinusoidal profile, and a remarkable endothelial cell heteromorphism (Fig. 3E,F). These cells often showed cytoplasmic protrusions towards the lumen or the interstitium (Fig. 3E-G). Capillary vessels also originated lateral buds or sprouts (Fig. 3F,G). A polarized, amoeboid endothelial cell (leading cell) generally guided bud or sprout formation. The endothelial cells localized at the tail of the bud/sprout were particularly flat and elongated (Fig. 3E-G). Filopodia and cytoplasmic spurs were often recognized on the leading cell plasma membrane (Fig. 3E). Leading cells could also be distinguished by the presence of an irregularly shaped nucleus that often protruded into the cytoplasmic spurs (Fig. 3E-G). By means of cytoplasmic protrusions, the leading cells often gave rise to newborn secondary vascular lumina (Fig. 3G). Activated capillaries were often closely associated with pericytes. These cells were mainly localized in correspondence of sprout origin (Fig. 3E,F). Pericytes appeared as voluminous cells with a regular oval shaped nucleus, embracing capillary vessels with long cytoplasmic processes. Some fibrocyte cells with a few lipid droplets were recognized and closely associated with activated capillaries (Fig. 3G).

Ovarian mRNA levels of angiogenic factors

The ovarian VEGF mRNA expression was significantly lower in untreated *rdw* rats than untreated normals ($P<0.01$), its expression was significantly increased in T4-treated and eCG-treated *rdw* rats ($P<0.001$) while still markedly lower than eCG-treated normals ($P<0.01$, Fig. 4A). The combined treatment of T4 with eCG in *rdw* rats markedly increased its

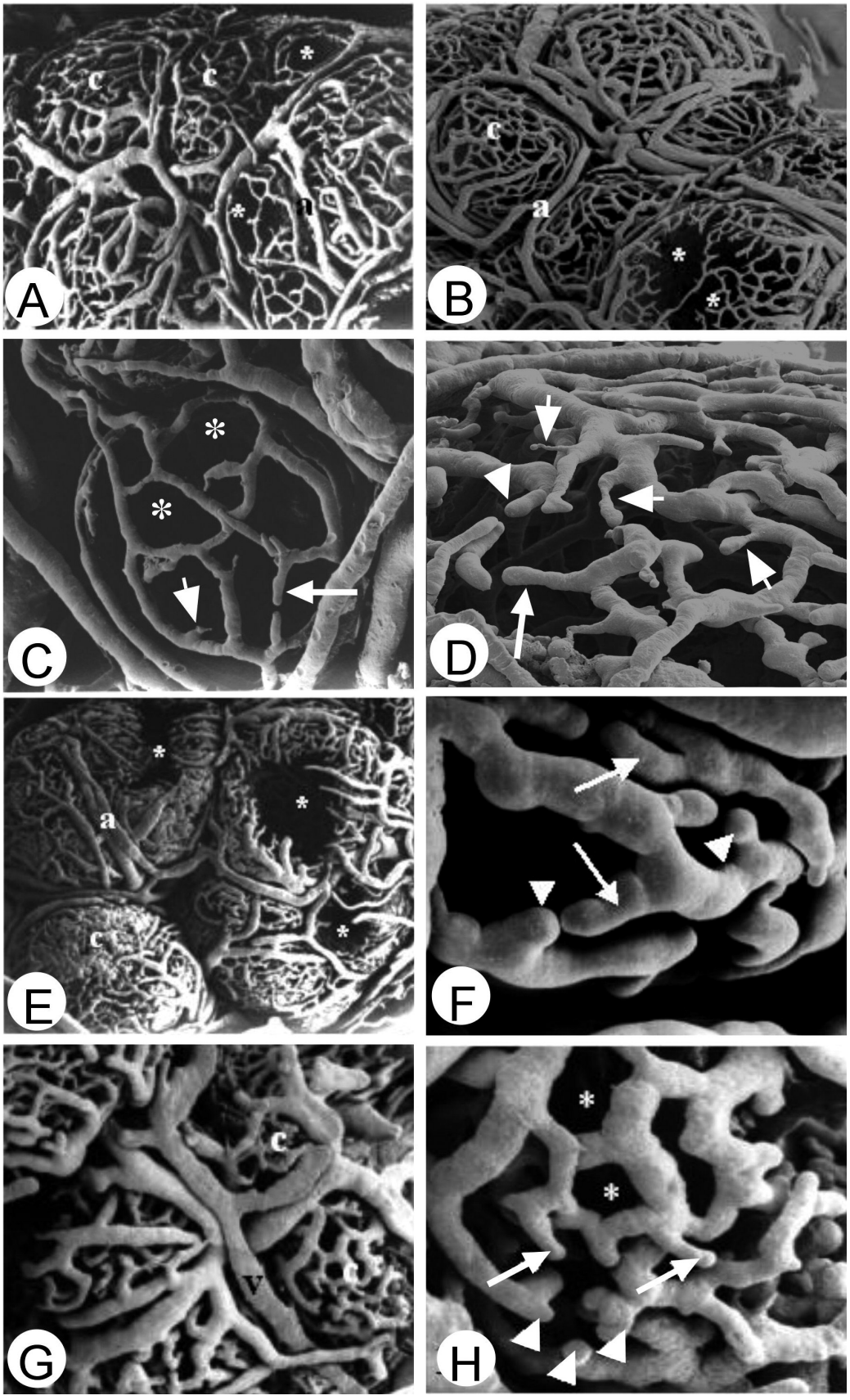

Fig. 1. SEM of outer surface of FVP of untreated **(A, C)**, T4-treated **(B, D)**, eCG-treated **(E, F)** and T4/eCG-treated **(G, H)** *rdw* rats. Higher magnification views of selected areas in **A, B, E and G** are shown in **C, D, F and H**, respectively. Budding and sprouting are indicated by arrowhead and long arrow, respectively. Degenerative capillaries are indicated by short arrow. Avascular areas are indicated by asterisk. a: arterioles; v: venules; c: capillaries. Original magnifications: A, B, E, x 130; C, D, x 330; F, x 380; G, x 230; H, x 345

expression (P<0.01) to an extent comparable to that in eCG-treated normal littermates.

There were no significant differences in the ovarian TGF-ß mRNA expression between *rdw* groups and untreated normal rats (P>0.05). Its expression was markedly higher in eCG-treated normals than untreated and T4-treated *rdw* rats (P<0.05) while no significant difference was observed between untreated and eCG-treated normals (P>0.05, Fig. 4B).

The ovarian bFGF mRNA expression was two- to three-fold higher in T4/eCG-treated *rdw* rats and eCG-treated normals (P<0.05), while its expression was not different in untreated, T4-treated and eCG-treated *rdw* rats, or in untreated normals (P>0.05, Fig. 4C).

There were no significant differences in ovarian EGF mRNA expression among any *rdw* groups and the untreated normals, while eCG treatment increased its expression 7-fold in normal rats (P<0.001, Fig. 4D).

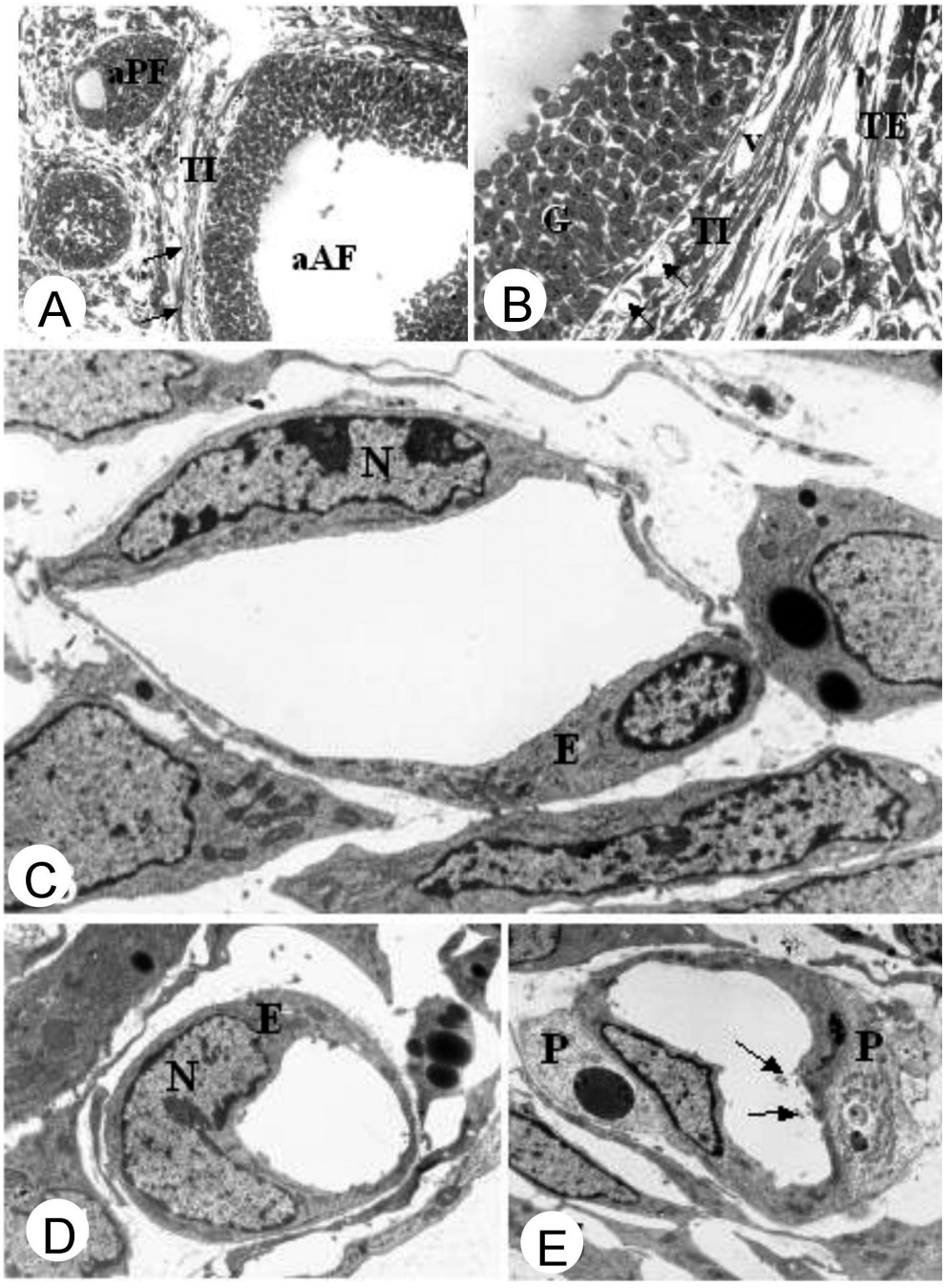

Fig. 2. LM (**A and B**) and TEM (**C-E**) micrographs of eCG-treated *rdw* rat ovaries. Atretic follicles are shown in **A and B**. Quiescent and activated (angiogenic) capillaries are shown in **C-E**, respectively. aPF: atretic preantral follicle; aAF: atretic antral follicle; TI: theca interna; Arrows: capillaries; TE: theca externa; G: granulosa cells; E: endothelial cell; P: pericytes; N: nucleus; v: venule. Arrowheads: short cytoplasmic protrusions. Original magnifications: A, x 100; B, x 400; C, x 2,500; D, E, x 6,500

The ovarian TNFα mRNA expression was significantly increased by eCG treatment in normal rats ($P<0.05$), but not in *rdw* rats ($P>0.05$). Its expression was markedly increased by T4 treatment ($P<0.001$) while this increment was attenuated by a combined treatment with eCG in *rdw* rats ($P<0.001$, Fig. 4E).

Ovarian mRNA levels of angiogenic factor-related receptors

The Flk-1 mRNA expression was markedly decreased by T4 treatment in *rdw* rats when compared with untreated *rdw* rats and eCG-treated normals

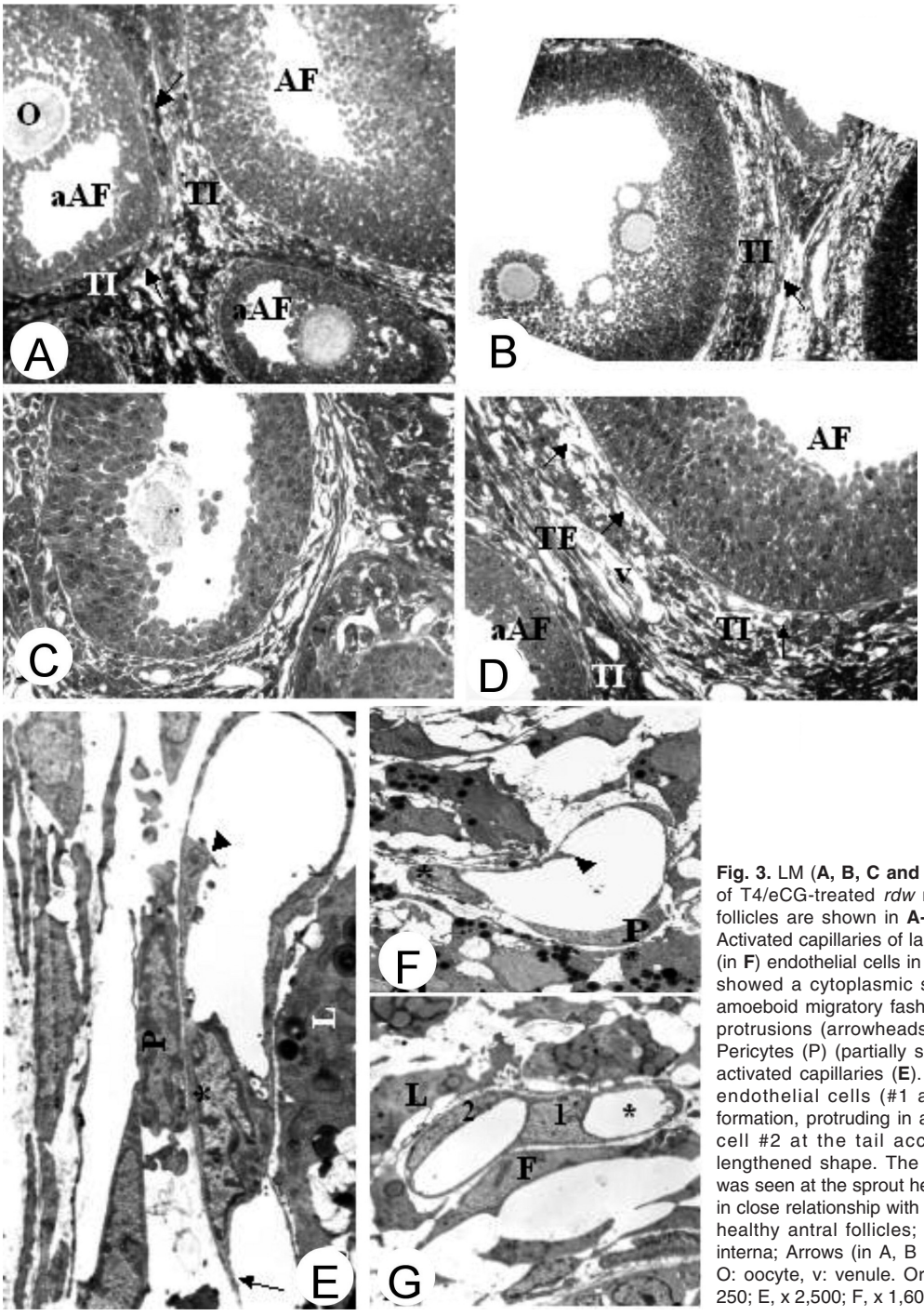

Fig. 3. LM (**A, B, C and D**) and TEM (**E, F and G)** micrographs of T4/eCG-treated *rdw* rat ovaries. Healthy and atretic antral follicles are shown in **A-D**. A multi-ovular follicle is seen in **B**. Activated capillaries of large antral follicles had one (in **E**) or two (in **F**) endothelial cells in which the leading cell (asterisk in E, F) showed a cytoplasmic spur (arrow) and was polarized in an amoeboid migratory fashion (in **E**). Short and large cytoplasmic protrusions (arrowheads) were also seen in the luminal side. Pericytes (P) (partially sectioned) closely contacted with these activated capillaries (**E**). In **G**, the sprout was made up of two endothelial cells (#1 and #2). The cell #1 led the sprout formation, protruding in an amoeboid migratory movement. The cell #2 at the tail accompanied the sprout formation in lengthened shape. The newly-formed sprout lumen (asterisk) was seen at the sprout head. An activated fibrocyte (**F**) was seen in close relationship with the sprout. L: luteinized theca cells. AF: healthy antral follicles; aAF: atretic antral follicles; TI: theca interna; Arrows (in A, B and D): capillaries, TE: Theca externa; O: oocyte, v: venule. Original magnifications: A, x 100; B-D, x 250; E, x 2,500; F, x 1,600; G, x 2,000

(P<0.05, Fig. 5A). There were no differences in the mRNA expression of Flt-1 and TNFR1 among any treatment groups in either *rdw* or normal rats (P>0.05, Fig. 5B,C). The TNFR2 expression was markedly decreased by eCG treatment in *rdw* rats when compared with other groups (P<0.05, Fig. 5D).

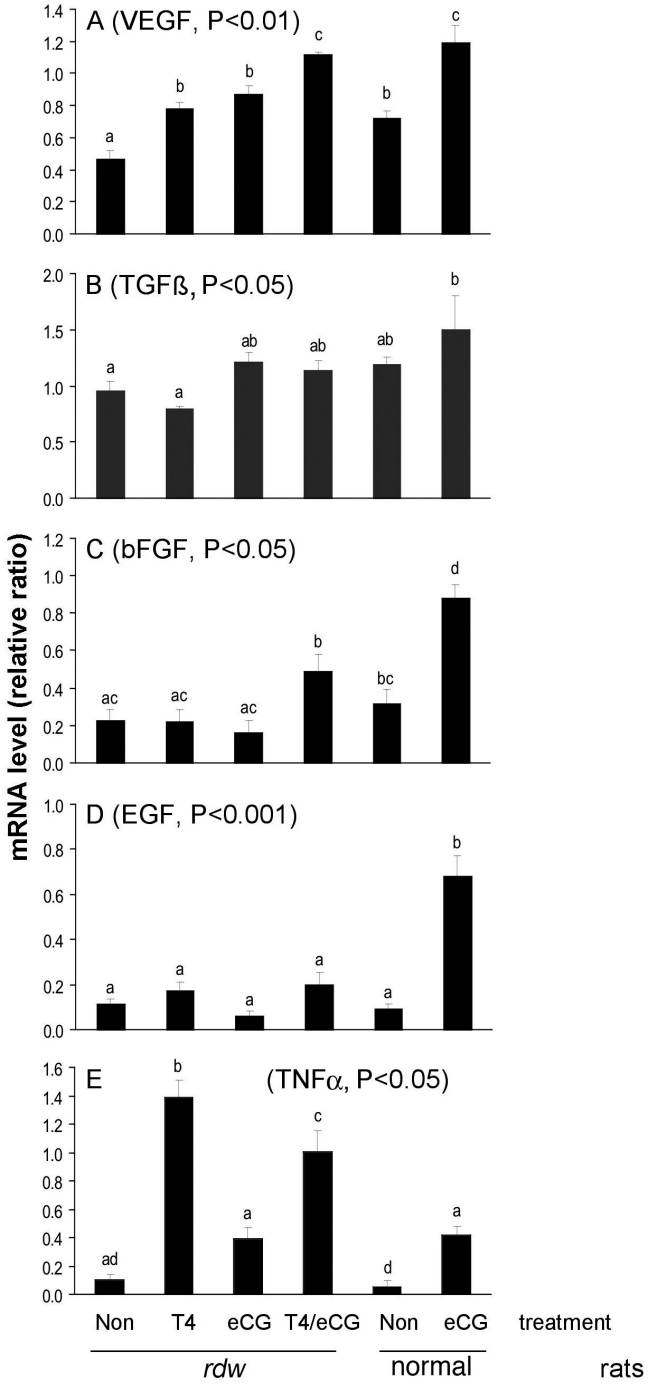

Fig. 4. Ovarian mRNA expression of VEGF **(A)**, TGFß **(B)**, bFGF **(C)**, EGF **(D)** and TNFα **(E)** in untreated, T4-, eCG- and T4/eCG-treated *rdw* rats, and untreated and eCG-treated normal rats (5 replicates in each group). Different letters indicate significant differences at P<0.01, 0.05, 0.05, 0.001 and 0.05 in A, B, C, D and E, respectively.

Discussion

The present study demonstrated for the first time that T4 markedly improved the development of thecal microvasculature by stimulating follicular angiogenesis, especially in the presence of eCG via regulating gene expression of angiogenic factors and their receptors. T4 treatment significantly increased mRNA levels of VEGF and TNFα, but decreased that of Flk-1. The combined treatment of T4 with eCG markedly increased mRNA expression of not only VEGF and TNF-α, but also bFGF. It is clear that follicular angiogenesis is under the

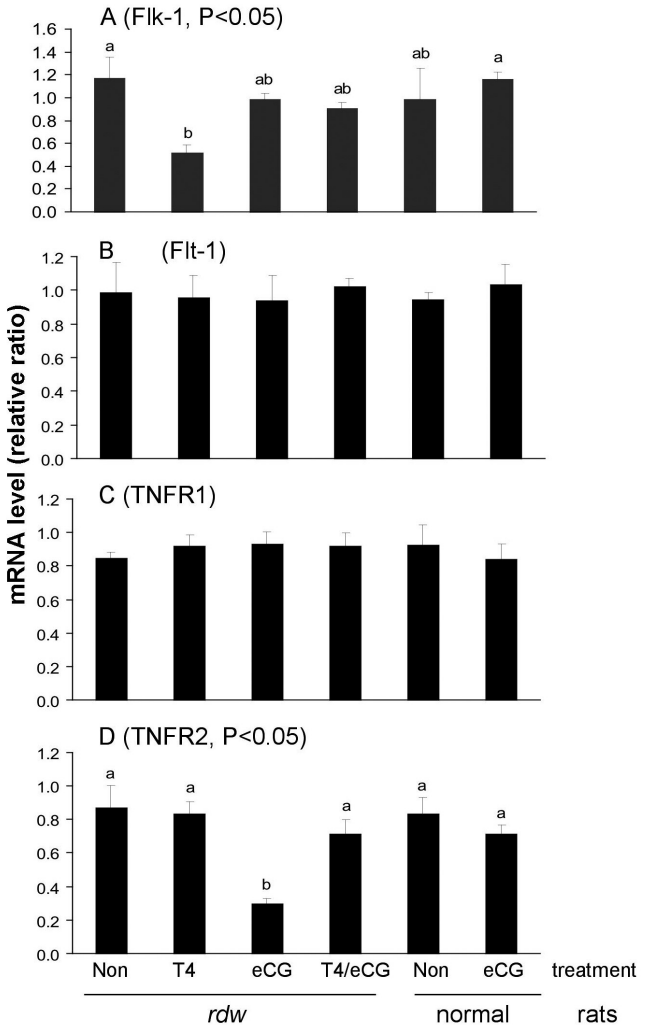

Fig. 5. Ovarian mRNA expression of Flk-1 (**A**), Flt-1 (**B**), TNFR1 (**C**) and TNFR2 (**D**) in untreated, T4-, eCG- and T4/eCG-treated *rdw* rats, and untreated and eCG-treated normal rats (5 replicates in each group). Different letters indicate significant differences at P<0.05 in A and D.

control of VEGF, TNF-α and bFGF, and thyroid hormone has an important role in regulating the expression of these important angiogenic factors.

Although several studies in rats (Chilian et al., 1985; Hudlicka et al., 1995; Tomanek et al., 1995, 1998) and pigs (Breisch et al., 1989) demonstrated that T4 is involved in coronary angiogenesis, to our knowledge, the effect of T4 on ovarian follicular angiogenesis has not yet been addressed. The SEM study of ovarian vascular corrosion casts in the present study revealed that hormonal treatment with T4, eCG or T4/eCG are able to influence in a different way capillary growth in the theca interna of *rdw* rat ovarian follicles. eCG and T4/eCG treatments also influenced thecal arteriolar and venular distribution of healthy follicles. In untreated *rdw* rats, antral follicle FVP was shown with major capillary degeneration. T4 alone lightly influenced thecal angiogenesis of *rdw* rat ovarian follicles, and promoted only sporadic and focal areas of capillary growth (presence of buds and sprouts of the resin) in healthy antral follicles. eCG administration, which is currently used to induce folliculogenesis in normal rats, also lightly stimulated antral follicular angiogenesis in *rdw* rats. On the contrary, T4/eCG administration strongly promoted not only thecal angiogenesis in antral and preovulatory FVPs, but also vasodilation and sinusoidalization which represent the capillary morphological adaptation to thecal steroidogenic activation and upcoming ovulation (Macchiarelli et al., 1991, 1992, 1993, 1995, 1998; Macchiarelli, 2000). Thus, SEM results indicate that in eCG-primed *rdw* rats, quiescent thecal capillaries could be rescued and develop when T4 was also given. This actually led to a decrease in the number of atretic antral follicles and to an increase in the number of healthy antral and preovulatory follicles, as reported previously (Jiang et al., 2000; Sato and Jiang, 2001).

As widely demonstrated previously (Murakami, 1971; Macchiarelli, 2000, 2006), SEM of vascular corrosion casts allows the clear study of the course and extent of microvasculature within a tissue, and to precisely evaluate the presence of angiogenesis and/or capillary regression in the ovarian follicle (Macchiarelli et al., 1993; Shimizu et al., 2002). Indeed, correlated LM/TEM analysis, selectively performed in eCG- and T4/eCG-treated *rdw* groups, allowed us to adequately investigate T4 effects on the morphology of thecal angiogenesis. The endothelium of growing capillaries, in fact, showed, by TEM, typical angiogenic ultrastructural parameters, such as highly variable shape, increment of plasma membrane specializations, presence of irregularly shaped nuclei, and an increase of cytoplasmic organelles (Rhodin and Fujita, 1989; Hansen-Smith et al., 1996; Zhou et al., 1998b). Capillary lumen restriction (pocket-like lumen) as well as the occurrence of lateral buds and sprouts of capillaries are, in particular, considered morphological features of sprouting angiogenesis (Chilian et al., 1985; Zhou et al., 1998a). On the contrary, regularly shaped capillary vessels provided with circular or oval lumen and flattened, smooth endothelial cells, are generally considered quiescent. Healthy antral follicles in eCG-primed *rdw* rats, showed few activated capillaries. Some budding and sprouting capillaries were observed. However, healthy antral and preovulatory follicles in *rdw* rats treated with eCG/T4 showed very numerous activated capillaries, as well as budding and sprouting capillaries. Additionally, TEM analysis revealed an interesting positive correlation between activated/angiogenic capillaries and pericytes in both groups. This agrees with what is reported in the literature about the crucial role of pericytes in the angiogenesis in several organs, including the ovary (Nehls et al., 1992; Nicosia and Villaschi, 1995, 1999; Watanabe et al., 1997; Egginton et al., 2000; Redmer et al., 2001). Pericytes, in both processes of sprouting and non-sprouting (intussusceptive) angiogenesis, in fact, are likely able to cooperate with growing capillaries, helping endothelial cell migration, as well as interendothelial interaction (breakage/reconstruction). Recently, experimental evidence also demonstrated pericyte involvement in angiogenesis and vascular remodeling which occurred during ectopic ovarian transplants, revealing the importance of these cells for the maintenance of ovarian graft vascular growth (Israely et al., 2003).

VEGF is the key angiogenic factor and plays an important role in stimulating follicular angiogenesis during ovarian follicular development (Shimizu et al., 2002). VEGF expression is high in healthy large antral follicles that are associated with active angiogenesis (Shimizu et al. 2002). In contrast, its expression and protein levels are low in atretic follicles (Jiang et al., 2003b). In the present study, eCG markedly increased VEGF expression in normal rats. However, in *rdw* rats, although either T4 or eCG significantly increased their expression when compared to the untreated group, its level was similar to that in untreated normal rats and significantly lower than that in eCG-primed normal rats. This observation suggested that eCG treatment alone was sufficient to induce VEGF expression, which promotes active follicular angiogenesis and normal follicular development in normal rats. However, T4 or eCG alone in *rdw* rats was not sufficient to induce VEGF expression to the extent which was observed in eCG-primed normal rats. The combined treatment of both T4 and eCG (T4/eCG) in *rdw* rats markedly increased VEGF mRNA to levels comparable to that in eCG-primed normal rats. These data indicated that T4/eCG, but neither T4 nor eCG alone, are required to induce VEGF expression and therefore stimulate follicular angiogenesis, which was observed by SEM, LM, and TEM. The signaling cascades in which T4 and eCG cross talk in the regulation of VEGF expression is unclear and needs to be further investigated.

It has been demonstrated that T4 administration stimulated coronary angiogenesis by up-regulating bFGF (Tomanek et al., 1998). In our study, T4 alone did not increase the expression of bFGF in *rdw* rat ovaries.

However, T4/eCG treatment significantly up-regulated bFGF mRNA levels. These findings suggested that the mechanism of action of thyroid hormone in regulating angiogenesis in ovarian follicles is different from that in other tissues. In the chick chorioallantoic membrane model, the regulation of FGF by thyroid hormone is MAPK dependent and initiated at the plasma membrane (Davis et al., 2004). Whether this regulatory pathway also functions in the ovary remains to be determined.

TNF-α has been shown to be anti-angiogenic in vitro (Sato et al. 1987), but proangiogenic in vivo (Frater-Schroder et al., 1987; Leibovich et al., 1987; Curiel et al., 2004), possibly through stimulation of the production of VEGF and other angiogenic factors (Ryuto et al., 1996; Lehmann et al., 2005; Scott et al., 2005) and enhanced expression of cell-associated proteases (van Hinsbergh et al., 1990; Hanemaaijer et al., 1993; Koolwijk et al., 1996). In the male, TNF has been shown to promote angiogenesis during testicular recrudescence (Pyter et al. 2005). In the female, it was shown that both TNF-α mRNA and polypeptide are expressed within several ovarian cells (oocyte, granulosa cells, theca interna cells, endothelial cells and macrophages) and follicular fluid (Terranova, 1997; Prange-Kiel et al., 2001; Jiang et al., 2003a). However, there is little evidence in the literature concerning the role of TNF-α in the context of thyroid hormone-driven angiogenesis in ovarian follicles. In the present study we showed that thyroid hormone treatment up-regulated TNF-α mRNA expression and facilitated angiogenesis. Although it remains unknown how thyroid hormone regulates this cytokine and what may be the detailed function of TNF-α in stimulating follicular angiogenesis, it is possible that thyroid hormone enhances TNF-α mRNA expression by activating NF-κB as observed in rat liver (Tapia et al. 2003). TNF-α may enhance follicular angiogenesis by up-regulating other angiogenic factors, such as VEGF and/or promoting proliferation and differentiation of mesenchymal cells into pericytes or smooth muscle cells that support newly formed vessels during angiogenesis, which were observed in the present study and as reported in other tissues (Distler et al. 2003).

Since we demonstrated in the present study that T4 treatment is greatly involved in regulating the expression of VEGF and TNF-α, we further analyzed the mRNA expression of their receptors in rat ovaries. It has been shown that the expression of Flt-1 and Flk-1 mRNA increased in the theca of medium and large porcine follicles after eCG treatment (Shimizu et al. 2003). However, our results showed that T4 and eCG did not have any effects on the expression of Flt-1 and TNFR1. T4 markedly decreased the expression of Flk-1, although eCG alone or T4/eCG did not have any effects on its expression. In addition, eCG markedly decreased the expression of TNFR2, although T4 alone or T4/eCG did not have any effects on its expression.

In conclusion, ultrastructural as well as biomolecular data clearly indicated that T4 plays an important role in stimulating ovarian follicle angiogenesis, especially in the presence of eCG, by up-regulating the expression of VEGF, bFGF and TNF-α, although the molecular pathways involved in this regulating process need to be further investigated.

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