Insulin Receptor Expression in Follicular and Stromal Compartments of the Human Ovary over the Course of Follicular Growth, Regression and Atresia

TAKASHI SAMOTO, TAKESHI MARUO, CECILIA A. LADINES-LLAVE, HIROYA MATSUO, JUN DEGUCHI, EYTAN R. BARNEA*, AND MATSUTO MOCHIZUKI

Department of Obstetrics and Gynecology, Kobe University School of Medicine, Kobe 650, Japan, and *Department of Obstetrics and Gynecology, Cooper Hospital/University Medical Center, the University of Medicine and Dentistry of New Jersey, Camden, New Jersey 08103, U.S.A.

Abstract. The cytologic localization and cellular levels of insulin receptors in the human ovary during follicular growth, regression and atresia were examined by the avidin/biotin immunoperoxidase techniques with a monoclonal antibody to insulin receptor. In primordial follicles, only the oocyte showed a weak immunostaining for insulin receptor, whereas the stromal cells surrounding primordial follicles were moderately immunostained. The earliest stage of follicular growth at which immunostaining for insulin receptor in granulosa cells and theca interna cells became apparent was the preantral stage. With the increase in the size of the follicles, the immunostaining of the oocyte and follicular elements intensified, whereas the staining intensity of the stromal cells surrounding growing follicles was reduced compared to those surrounding primordial follicles. The immunostaining in granulosa and theca interna cells persisted in the corpus luteum, and further intensified during the midluteal phase. In the regressing corpus luteum, the immunostaining was present only in the peripheral lutein cells adjacent to the central scar tissue. The corpus albicans was negative for the immunostaining, but the surrounding stromal cells exhibited predominant staining. In atretic follicles, the theca interna cells exhibited intense staining for insulin receptor without appreciable staining in the scattered granulosa cells, whereas the surrounding stromal cells were moderately immunostained.

This is the first study to demonstrate notable changes in insulin receptor expression in the oocyte, granulosa cells, theca cells, lutein cells and surrounding stromal cells during follicular growth, regression and atresia. The results obtained indicate insulin participation in oocyte maturation, follicular growth and stromal cell function. The increased expression of insulin receptors in theca interna cells of atretic follicles and in stromal cells surrounding the corpora albicaps raise the intriguing possibility of insulin involvement in the transformation of theca interna cells into stromal cells. This implies that insulin may participate in remodelling ovarian local tissues following follicular atresia and luteolysis in the human ovary.

Key words: Insulin receptor, Human ovary, Follicular growth, Corpus luteum, Atresia.

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follicular maturation followed by ovulation is well established [1]. However, a growing body of evidence indicates that various factors other than gonadotropins participate in the regulation of the growth and differentiation of ovarian follicles. Particularly, there is increasing interest in the regulatory role of growth factors in ovarian follicular growth and function [2, 3]. Insulin is among these regulators [4–7]. Insulin has been demonstrated to act synergistically with FSH to regulate aromatase activity in granulosa cells [8–10] and with LH to regulate progesterone production by luteal cells [11] and androgen production by thecal stromal cells [12, 13].

Insulin possibly initiates its action on the ovary by binding to its own receptors or to the structurally similar insulin-like growth factor-I (IGF-I) receptors [14, 15]. Indeed, it has been noted that insulin competes for IGF-I binding to granulosa cells with about 100-fold less activity compared with IGF-I [16]. Radioligand binding studies with [125I] insulin have demonstrated the presence of specific binding sites for insulin in follicular and stromal compartments of the human [17–20] and porcine [7, 16] ovaries. There is, however, little information available on the changes in the cellular levels of insulin receptors in the follicular and stromal compartments of the human ovary during follicular growth and regression throughout the menstrual cycle. Thus, in the present immunohistochemical study we examined human ovaries at different stages of follicular growth and regression for cytologic localization and cellular levels of insulin receptors.

**Materials and Methods**

**Tissue preparation**

Ovarian tissues were obtained from women with regular menstrual cycles who underwent abdominal hysterectomy with uni- or bilateral salpingo-oophorectomy for a variety of gynecological conditions including leiomyomata, endometriosis and early cervical neoplasia. The collection of these tissues has been approved by the Institutional Review Board. These patients ranged in age from 32 to 41 years with a mean age of 35 years. Informed consent was obtained from each patient before surgery for the use of ovarian tissues for immunohistochemical studies. Each ovarian specimen was examined by a pathologist for histological evaluation and dating of the endometrium. Endometrial tissues were obtained either from the extirpated uteri or from endometrial biopsies, and the criteria for the day of the menstrual cycle were determined by endometrial histological dating according to the method of Noyes et al. [21]. Ovaries were graded into six categories: early follicular, mid follicular, late follicular, early luteal, mid luteal, and late luteal phases of the menstrual cycle. On histological examination, individual follicles were categorized as primordial, preantral, antral or preovulatory, and atretic follicles. Of the 36 patients included in this study, 9 had corpora lutea at early (cycle days 14–18, n=3), mid (cycle days 19–24, n=3) and late (cycle days 25–28, n=3) stages of the luteal phase.

Ovarian tissues obtained were fixed in 4% buffered neutral formalin, dehydrated and embedded in paraffin. Sections, 5 to 6 µm in thickness, were deparaffinized and followed by standard histologic techniques. Immunohistochemical staining was performed by avidin/biotin immunoperoxidase techniques with the use of a polyvalent immunoperoxidase kit (Omnitags, Lipshaw, Michigan) as previously described by Maruo and Mochizuki [22]. A mouse monoclonal antibody to human insulin receptor (Oncogene Science, Manhasset, NY), which specifically binds to an internal epitope of human insulin receptors, was used as the primary antibody in this study. The anti-insulin receptor antibody (100 µg IgG/ml) was diluted 1:100 before use. The first incubation with primary antibody was followed by the second incubation with biotinylated polyvalent antibody, and by the third incubation with avidin-horseradish peroxidase. Chromogenic reaction was then developed by incubating with freshly prepared solution of tetrahydrochloride-diaminobenzidine and hydrogen peroxide. The sections were then counterstained with Harris hematoxylin, mounted with glycerine phosphate buffer solution, and examined microscopically.

The following control procedures were undertaken to assure the specificity of the immunologic reactions. Adjacent control sections were subjected to the same immunoperoxidase method with the exception that the primary antibody to the insulin receptor was replaced by nonimmune murine IgG (Miles, Erkhart, Indiana) at the same dilution as
the specific primary antibody. In the abovementioned controls, no positive staining was observed. Furthermore, preincubation with human recombinant insulin (Sigma, St. Louis, MO: 1 µg/ml) before incubating with the monoclonal antibody to insulin receptor resulted in the abolishment of positive immunostaining of the ovarian compartments for insulin receptor, but preincubation with human recombinant EGF (Amersham, Arlington Heights, IL: 1 µg/ml) before incubating with the monoclonal antibody to insulin receptor did not alter immunostaining of ovarian tissues for insulin receptor.

The intensity of immunostaining was evaluated by repeated staining of the same specimens and by more than two observers. It was graded as (−) for no immunostaining, (+) for weak but definitely detectable immunostaining, (++) for moderate immunostaining, and (+++) for intense immunostaining.

Results

For the follicular phase of the menstrual cycle, the cytologic localization and cellular levels of insulin receptors in the follicular and stromal compartments of the human ovaries at the various stages of follicular growth are given in Table 1. In primordial follicles, positive immunostaining for insulin receptor was restricted only to the oocyte, whereas the stromal cells surrounding the primordial follicles were moderately immunostained for insulin receptor (Fig. 1). The earliest stage of follicular growth at which insulin receptor expression in granulosa and theca cells became apparent was the preantral follicular stage. In preantral follicles during the early follicular phase, not only the oocyte but also the granulosa and theca cells were immunostained for insulin receptor (Fig. 2). As the size of the follicle increased, the staining intensity of the oocyte, granulosa and theca cells increased, whereas the staining intensity of the stromal cells surrounding the growing follicles decreased compared with the stromal cells.

Table 1. Immunostaining for insulin receptors in follicular and stromal compartments in the different stages of follicular growth

<table>
<thead>
<tr>
<th>Follicular stage</th>
<th>Oocyte</th>
<th>Granulosa cells</th>
<th>Theca interna cells</th>
<th>Surrounding stromal cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primordial (n=8)</td>
<td>+</td>
<td>−</td>
<td></td>
<td>++</td>
</tr>
<tr>
<td>Preantral (n=8)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Antral (n=8)</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Preovulatory (n=3)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

Grading by intensity; −, not detectable; +, weak but definitely detectable; ++, moderate staining; ++++, intense staining. The n represent the number of ovaries examined. The results shown in this table were obtained by examining more than fifteen follicles for each of the primordial, preantral and antral stages as well as three follicles for the preovulatory stage.

Table 2. Immunostaining for insulin receptors in luteal tissues and the surrounding stromal cells over the course of the luteal phase

<table>
<thead>
<tr>
<th>Luteal phase</th>
<th>Luteal cells</th>
<th>Surrounding stromal cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early phase</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>(n=3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mid phase</td>
<td>+++</td>
<td>±</td>
</tr>
<tr>
<td>(n=5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Late phase</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>(n=5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corpus albicans</td>
<td>−</td>
<td>+++</td>
</tr>
<tr>
<td>(n=13)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Footnotes as in Table 1. The results shown in this table were obtained by examining three corpora lutea for each of the early luteal, mid luteal and late luteal phases as well as more than twenty corpus albicans.
surrounding the primordial follicles. The immunostaining in the granulosa cells and theca cells was pronounced in antral follicles during the mid follicular phase (Fig. 3), and further intensified in preovulatory follicles during the late follicular phase (Fig. 4A). Replacement of the primary antibody with nonimmune murine IgG showed a lack of positive immunostaining of the granulosa,
theca cells and stromal cells (Fig. 4B). Furthermore, preincubation with human recombinant insulin before incubating with the monoclonal antibody to insulin receptor resulted in the abolishment of positive immunostaining of the ovarian elements for insulin receptor (data not shown).

Table 2 summarizes the immunohistochemical localization and cellular levels of insulin receptors in luteal tissues and the surrounding stromal cells over the course of the luteal phase of the menstrual cycle. In corpora lutea during the early luteal phase, the lutein cells were immunostained for insulin receptor with a similar intensity as observed in the granulosa cells and theca cells in preovulatory follicles. The staining intensity of the lutein cells increased remarkably during the mid luteal phase of the menstrual cycle (Fig. 5A), while the stromal cells surrounding the corpora lutea during the mid luteal phase showed weak immunostaining for insulin receptor. Replacement of the primary antibody with nonimmune murine IgG showed a lack of positive immunostaining of the luteal cells and stromal cells (Fig. 5B). In regressing corpora lutea during the late luteal phase, the staining intensity remarkably declined and the positive immunostaining for insulin receptor was restricted only to the peripheral theca lutein cells adjacent to the central core of scar tissue (Fig. 6). Corpora albicans were totally negative for immunostaining for insulin receptor, whereas only the stromal cells surrounding the corpora albicans exhibited notably intensified immunostaining for insulin receptor (Fig. 7).

In atretic follicles, the theca interna cells exhibited prominent immunostaining for insulin receptor, while the degenerating granulosa cells had negligible staining for insulin receptor. The stromal cells surrounding the atretic follicles were moderately immunostained for insulin receptor (Fig. 8).

Discussion

The purpose of this study was to determine the possible changes in the cytologic localization and cellular levels of insulin receptors in the follicular and stromal compartments of the human ovary over the course of follicular growth and regression. Our interest in insulin receptor expression in the ovary derives from increasing evidence that insulin regulates ovarian endocrine function either alone or in synergy with gonadotropins. The immunohistochemical data obtained in the present study demonstrate for the first time that...
insulin receptor expression in the follicular and stromal compartments of the human ovary notably changes during follicular growth and regression.

In primordial follicles, in spite of the appreciable expression of insulin receptors in the oocyte, there was no insulin receptor expression detectable in the flattened granulosa cells, whereas the stromal cells surrounding the primordial follicles moderately expressed insulin receptors. Primordial follicles are known to remain at a resting stage for a long period of time. Thus, the fact that the follicular element of primordial follicles was negative for insulin receptor expression suggests that insulin might not participate in the selection of primordial follicles that are set in motion in the initial stage of follicular maturation during each

![Fig. 4. Immunohistochemical localization of insulin receptor in preovulatory follicle. Insulin receptor immunostaining was predominant in the granulosa cells and theca cells of the preovulatory follicle, while the surrounding stromal cells were immunostained weakly (A). Replacement of primary antibody with nonimmune murine IgG showed a lack of positive immunostaining of the granulosa, theca cells and stromal cells for insulin receptor (B). G, Granulosa; T, Theca. Bar represents 5 \( \mu m \) (Original magnification \( \times 400 \)).]
menstrual cycle. On the other hand, the fact that the stromal cells surrounding the primordial follicles were moderately positive for insulin receptor expression supports the notion that insulin may participate in the stromal cell proliferation and androgen production by those cells throughout the resting stage.

The earliest stage of follicular growth, at which insulin receptor expression in the granulosa cells and theca cells became apparent, was the preantral stage during the early follicular phase. We found that the immunohistochemically detected cellular levels of insulin receptors in the oocyte, granulosa and theca cells, augmented as the size of the oocyte and follicle increased. These findings imply a possible role for insulin in the modulation of

Fig. 5. Immunohistochemical localization of insulin receptor in the corpus luteum of the midluteal phase. Almost all luteal cells in the corpus luteum exhibited intense immunostaining for insulin receptor (A), whereas the stromal cells surrounding the corpus luteum were faint for the immunostaining. Replacement of primary antibody with nonimmune IgG showed a lack of positive immunostaining of the luteal cells for insulin receptor (B). Bars represent 10 μm (Original magnification × 200).
oocyte maturation. Indeed, insulin has been shown to stimulate maturation of pig oocytes beyond the first metaphase and extrusion of the first polar body [23] and to facilitate germinal vesicle breakdown in leopard frogs [24]. Insulin receptor expression in granulosa cells and theca cells was more pronounced in preovulatory follicles. Since it is known that the steroidogenic ability of ovarian follicles is weak in the preantral stage and gradually increases as the follicle grows and matures, the changes in insulin receptor expression in the granulosa cells and theca cells observed during follicular maturation seem to be consistent with the changes in the steroidogenic ability of growing follicles throughout the follicular phase. Therefore, the data obtained support the idea of insulin participation in regulating the steroidogenic ability of ovarian follicles throughout the follicular phase.

After the oocyte has been discharged from the mature follicle, the granulosa layer undergoes pronounced folding and the granulosa cells become hypertrophied and are converted into granulosa lutein cells, while the cells of the theca interna undergo hypertrophy and become theca lutein cells. During this luteinization process, capillaries invade the granulosa layer, together with connective tissue elements. Thereafter, the two types of lutein cells reach a stage at which they are virtually indistinguishable, and the invasive process marks the stage of full maturity of the corpus luteum during the midluteal phase, associated with the maximal ability to produce progesterone and other steroid hormones. In the absence of fertilization, the functional capacity of the corpus luteum only persists for about 10 days before degenerative changes set in. During the late luteal phase, the lutein cells undergo lipolysis and atrophy, associated with a reduction in steroidogenic capacity. Eventually, the corpus luteum is converted into a hyaline body, the corpus albicans. The increase in immunohistochemically detected cellular levels of insulin receptors in lutein cells during the midluteal phase followed by the subsequent decline in insulin receptor expression in degenerated luteal cells during the late luteal phase therefore appears to be consistent with the change in the steroidogenic capacity of the corpus luteum throughout the luteal phase. This supports the theory of insulin participation in the regulation of the steroidogenic function of the corpus luteum. Indeed, insulin has been demonstrated to act synergistically with LH to regulate progesterone production by luteal cells [11].

The fate of the remaining theca cells after luteolysis is not clear. However, it is worth noting
that cytologic localization of insulin receptor is observed not only in theca interna cells from the time of theca layer formation in the preantral follicle stage up to the time of the atretic follicle stage, but also in theca lutein cells of the corpus luteum. Furthermore, in contrast to reduced expression of insulin receptors in the peripheral theca lutein cells adjacent to the central core of scar tissue in regressing corpora lutea during the late luteal phase, the stromal cells on the periphery of the corpora albicans demonstrated remarkably increased expression of insulin receptors. These findings imply that insulin may participate in the dedifferentiation of remnant theca cells into stromal cells. On the other hand, it is known that in atretic follicles the death of the ovum is the immediate cause of the death of the follicles, being followed by degeneration of the satellite follicular
epithelium. The cystic stage of the atretic follicle lined by degenerating granulosa is followed by cicatricial obliteration [25]. A layer of cicatrical tissue develops just within the theca, and with increasing cicatrization the cavity of the atretic follicle becomes completely obliterated, the cicatrix being surrounded by a hyalinized layer of connective tissue. Taken together, the fact that the theca interna cells of atretic follicles exhibited notably increased expression of insulin receptors also supports the intriguing possibility of insulin involvement in the transformation of theca cells into stromal cells. Our data therefore imply that insulin may participate in remodelling the ovarian local tissue following follicular atresia and luteolysis.

In this context, our recent study [26] revealed that EGF receptor expression becomes apparent in the oocyte in preantral follicles and in the granulosa cells and theca interna cells in antral follicles. EGF receptor expression in the granulosa and theca cells persisted in the preovulatory follicle and early corpus luteum, and further intensified in the lutein cells during the midluteal phase. In the regressing corpus luteum, EGF receptor expression was restricted to theca luteal cells peripheral to the central core of scar tissue. Although the corpus albicans showed no expression of EGF receptors, the surrounding stromal cells exhibited increased expression of EGF receptors. In atretic follicles, only the theca interna cells exhibited prominent expression of EGF receptors. The pattern of expression of EGF receptors in the follicular elements of the human ovary during the growth and regression of follicles appears to be similar to that of insulin receptors in the human ovary observed in the current study. However, appreciable differences between the features of expression of insulin receptors and EGF receptors in the human ovary are noted on the following two points. One is that insulin receptor expression not only in the oocyte but also in granulosa cells and theca cells becomes apparent in more immature follicles than EGF receptor expression. The other is that in the stromal cells surrounding primordial follicles and atretic follicles there is prominent expression of insulin receptors in the absence of appreciable expression of EGF receptors. These findings suggest that although both insulin and EGF are involved inocyte maturation as well as follicular growth and regression, insulin rather than EGF may participate more primarily in those events. In addition, the presence of insulin receptors in the ovarian stromal cells suggests insulin
participation in stromal cell proliferation and androgen production particularly by those cells surrounding primordial follicles and atretic follicles.

Because follicles are exposed to a large number of substances (i.e. growth factors, cytokines and hormones), it is possible that the presence or withdrawal of these factors, either alone or in combination, modulates the induction of follicular growth, atresia and luteolysis [27]. Recently Hughes and Gorospe [28] and Tilly et al. [29, 30] have analyzed molecular events underlying the progression of ovarian follicular degeneration during atresia, and demonstrated that apoptotic cell death is initiated in both granulosa and theca cells during atresia. These observations and ours provide a new and meaningful insight relevant to the understanding of the possible interaction between apoptosis and growth factors during the initiation and progression of follicular atresia and luteolysis in the human ovary.

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