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ORIGINAL PAPER

Immunohistochemical localization of advanced glycation end-products (AGEs) and their receptor (RAGE) in polycystic and normal ovaries

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Abstract The aim of the present study was to investigate the localization/immunohistochemical distribution of AGEs and RAGE, as well as their putative signalling mediator NF-κB in ovaries of women with polycystic ovary syndrome (PCOS) compared to normal. Archival ovarian-tissue samples from biopsies of six women with PCOS and from six healthy of similar age women, were examined immunohistochemically with monoclonal anti-AGEs, anti-RAGE and anti-NF-κB(p50/p65) specific antibodies. In healthy women, AGE immunoreactivity was observed in follicular cell layers (granulosa and theca) and luteinized cells, but not in endothelial cells. PCOS specimens displayed AGE immunoexpression in theca interna and granulosa cells as well as in endothelial cells, but staining of granulosa cells was stronger than in that of normal ovaries. RAGE was highly expressed in normal and PCOS tissues. Normal tissue exhibited no staining differences between granulosa cell layer and theca interna. However, in PCOS ovaries, granulosa cells displayed stronger RAGE expression compared to theca interna cells in comparison to controls. NF-κB(p50/p65) was expressed in the cytoplasm of theca interna and granulosa cells of both normal and PCOS ovaries; whereas the NF-κB p65 subunit was only observed in granulosa cells nuclei in PCOS tissue. In conclusion, these findings demonstrate for the first time that RAGE and AGE-modified proteins with activated NF-κB are expressed in human ovarian tissue. Furthermore, a differential qualitative distribution of AGE, RAGE and NF-κB p65 subunit was observed in women with PCOS compared to healthy controls, where a stronger localization of both AGE and RAGE was observed in the granulosa cell layer of PCOS ovaries.

Keywords Advanced glycation end-products · NF-κB · Ovaries · PCOS · RAGE

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Introduction

Polycystic ovary syndrome (PCOS) is the commonest endocrinopathy in women of reproductive age (Dia-

manti-Kandarakis et al. 1999) and is characterized by hyperandrogenism, hyperinsulinemia and insulin resistance. Women with PCOS have recently been reported to exhibit endothelial dysfunction and, interestingly, the magnitude of the dysfunction was related to androgen levels as well as insulin resistance (Diamanti-Kandarakis et al. 1996, 2005a; Conway et al. 1992).

Plasma or serum advanced glycation end-products (AGEs) levels are high in patients with hyperglycemia/diabetes and conditions associated with increased oxidative stress, such as rheumatoid arthritis, renal insufficiency, or cigarette smoking (Cerami et al. 1997; Rodriguez-Garcia et al. 1998; Nicholl and Bucala 1998; Ligier et al. 1998; Mohamed et al. 1999; Schmidt et al. 1999; 2000; Singh et al. 2001).

Recently, we have shown increased levels of serum AGEs and their receptor (RAGE) in a group of young normoglycemic women with PCOS (Diamanti-Kandarakis et al. 2005b) compared to healthy controls.

AGEs are reactive, cross-linking molecules, formed from non-enzymatic reaction of reducing sugars with the amino groups of proteins and lipids. They may cause tissue injury both directly, through phenomena such as trapping and cross-linking and indirectly, by binding to specific receptors for AGE (RAGE) on the surface of various cells (endothelium, mononuclear phagocytes, neurons and smooth muscle cells, Schmidt et al. 1999, 2000; Singh et al. 2001).

The pathological crosslink formation induced by AGEs affects usually the stable and long-lived proteins such as collagen, leading to increased stiffness of the protein matrix and increased resistance to removal by proteolytic means, which in turn affects the process of tissue remodeling. Furthermore, the interaction of AGE-RAGE results in generation of intracellular oxidative stress and subsequent activation of the redox-sensitive transcription factors such as NF-κB (Mohamed et al. 1999; Schmidt et al. 1999, 2000; Singh et al. 2001).

NF-κB, a heterodimer comprised of p65 and p50 subunits, exists in most cells in an inactive form predominantly in the cytoplasm, as a result of its binding to the inhibitory protein IκB (Siebenlist et al. 1994; Baldwin et al. 1996). Various inducing stimuli such as cytokines [e.g. tumor necrosis factor-α (TNF-α)] trigger protein kinases, which phosphorylate IκB. Once phosphorylated at specific sites (Ser32 and Ser36), IκB is subject to degradation by ubiquitin-dependent proteasomes. This releases active NF-κB protein from cytoplasmic retention signals and allows it to translocate to the nucleus and activate numerous target genes (Karin and Ben Neriah 2000; Read et al. 2000).

AGE-RAGE signaling through NF-κB activation has been reported to regulate expression of inflammatory cytokines (such as TNF-α and interleukin-1) (Singh et al. 2001), PAI-1 (Diamanti-Kandarakis et al. 2004) and endothelin-1 (Diamanti-Kandarakis et al. 2001), molecules that have also been found to be elevated in women with PCOS and are linked with endothelial dysfunction and vascular damage as well as with dysregulation of the ovulatory process (Glueck et al. 1999; Liu 1999).

Progressive AGE crosslinking of collagen, as well as the endothelial dysfunction can alter the ovarian structure, its hormonal milieu and the whole reproductive function, possibly contributing to the infertility underlying the pathogenesis of PCOS.

To date, we are not aware of histological studies evaluating the AGE and RAGE localization in the human ovary. Moreover, to our knowledge, the possible role of the AGE/RAGE system interaction in PCOS pathology has not been investigated. Therefore, the aim of the present study was to examine the immunohistochemical localization of AGE-modified proteins and their receptor in normal ovarian tissue in comparison with ovaries from women with PCOS.

Materials and methods

Ovarian specimens

Ovarian tissue from six women diagnosed with PCOS, who had undergone laparoscopic ovarian wedge resection performed previously and independently of this project (Duleba et al. 2003), were fixed in formalin and embedded in paraffin. The study of ovarian wedge resection has been approved by the local ethics committee and written informed consent was obtained from all patients (Duleba et al. 2003). All subjects with PCOS (mean age: 25.6 ± 3.0 years; BMI: 26.36 ± 7.86 kg/m^2) were diagnosed according to the following criteria: (1) oligo- and/or anovulation (8 ≤ menses per year), (2) clinical and/or biochemical signs of hyperandrogenism and (3) exclusion of other endocrine disorders (nonclassical congenital adrenal hyperplasia, androgen-secreting neoplasms, thyroid disease and hyperprolactinemia).

In parallel, normal ovarian tissue was obtained from four normally menstruating women. These women (mean age: 28.80 ± 5.47 years; BMI: 25.85 ± 6.73 kg/m^2) had undergone oophorectomy for benign disease, had regular menstrual cycles every 25–30 days and normal plasma androgen levels and histologically normal ovaries.

Immunohistochemical staining

Paraffin-embedded sections of formalin-fixed ovarian tissue were deparaffinized by xylene and dehydrated in graded ethanol. Sections were treated in 3% hydrogen peroxide in phosphate-buffered saline (PBS) for 15 min and then rinsed in PBS. To increase the immunoreactivity of AGEs, the sections were placed in 500 ml of 0.01 M citric acid-buffered solution (pH 7.0) and microwaved at 500 W for 5 min. After thorough washing, the sections were incubated with normal rabbit serum for 20 min at room temperature, to avoid nonspecific binding of the antibodies. The sections were then incubated overnight at 4°C with the anti-AGE monoclonal antibody, 6D12 (0.25 mg/ml stock, dilution 1:50; Research Diagnostics, Concord, MA, USA) or goat monoclonal anti-RAGE-antibody (0.25 ml, dilution 1:400; RDI-RAGEabG, Research Diagnostics) in PBS containing 1% bovine serum albumin. The same tissue sections were also incubated overnight at 4°C with the mouse anti-phospho-IκB-α antibody (specific for Ser32 phosphorylated p- IκB-α of human origin; 200 μg/ml, dilution 1:50; Santa Cruz Biotechnology Inc., CA, USA), the anti-NF-κB p50 (specific for the p50 subunit of NF-κB; dilution 1:100; Santa Cruz Biotechnology Inc.) and the anti-NF-κB p65 (specific for the p65 subunit of NF-κB; dilution 1:100; Zymed, USA). Immunoreactivity was detected by the streptavidin–biotin–peroxidase method according to the manufacturer's protocol. The final reaction product was visualized with 3,3′-diaminobenzidine tetrahydrochloride (LSAB detection kit; Dako, Carpinteria, CA, USA). Brain tissue sections from a patient with Alzheimer disease were used as positive controls for AGE and RAGE antibodies and sections from breast carcinoma were used for phospho-IκB antibody and glioblastoma multiforme sections were used for NF-κB p50/p65 antibodies. Negative controls (e.g. normal and polycystic ovarian tissue in which the primary antibody was substituted with nonimmune mouse or goat serum) were also stained in each run. The percentage of positive cells was estimated using light microscopy. The staining intensity was also assessed semi-qualitatively as +, weak; ++, moderate; and +++, strong.

Specificity of anti-AGE antibody (6D12)

6D12 is a mouse anti-human monoclonal antibody with immunospecificity to a common structure among AGE-proteins (Horiuchi et al. 1991). The epitope of 6D12 is N3-carboxymethyllysine (CML)-protein adduct. This antibody also recognizes carboxyethyllysine and does not recognize the early products, Schiff bases and Amadori products (Ikeda et al. 1996). However, it shows positive reaction to AGE-samples obtained either from proteins, lysine derivatives, or monoamino-carboxylic acids. Immunological studies using 6D12 demonstrated the presence of AGE-modified proteins in several human tissues, indicating its validity in the biochemical quantification of AGE-modified proteins (Nakayama et al. 1997; Yoshida et al. 1998; Matsuse et al. 1998; Sakata et al. 1998).

Specificity of anti-RAGE antibody

The goat anti-human monoclonal RAGE antibody is an antiserum prepared to a synthetic peptide that corresponds to amino acids 42–59 of the N-terminus of human RAGE protein ($_{42}$PKKPPQRLEWKLNTGR TE$_{59}$) and not the splice variant form esRAGE (in the C-terminal amino acids $_{332}$EGFDKVREAEDSPQH M$_{347}$). This antiserum has shown immunoreactivity with the unconjugated immunizing peptide by ELISA, and has been previously used for immunolabeling of vessels from diabetic tissues and neurons from the brains of Alzheimer disease patients in formalin-fixed, paraffin-embedded tissues (based on the manufacturer's indications).

Statistical methods

Data are presented as means ± standard deviation (SD) and staining expression as median (range, %).

Results

Clinical data

The mean age did not differ between the two groups (controls: 28.80 ± 5.47 years versus PCOS: 25.6 ± 3.01 years, $P = 0.06$) as well as BMI (controls: 25.85 ± 6.73 kg/m^2 versus PCOS: 26.36 ± 7.86 kg/m^2, $P = 0.07$). Total testosterone levels were higher in PCOS compared to controls (controls: 0.42 ± 3.04 versus PCOS: 0.76 ± 0.2 ng/ml), SHBG levels were 87.4 ± 3.63 for controls and 58.03 ± 45.65 nmol/l for PCOS, whilst insulin was 4.3 ± 3.2 for controls and 12.76 ± 5.11 μU/ml for PCOS.

Immunohistochemical localization of AGE in ovarian tissue of control and PCOS patients

AGE expression was detected in all cases of normal tissue, the percentage of positive cells ranging from 5 to 10% (median: 7.2%). AGE immunoreactivity was

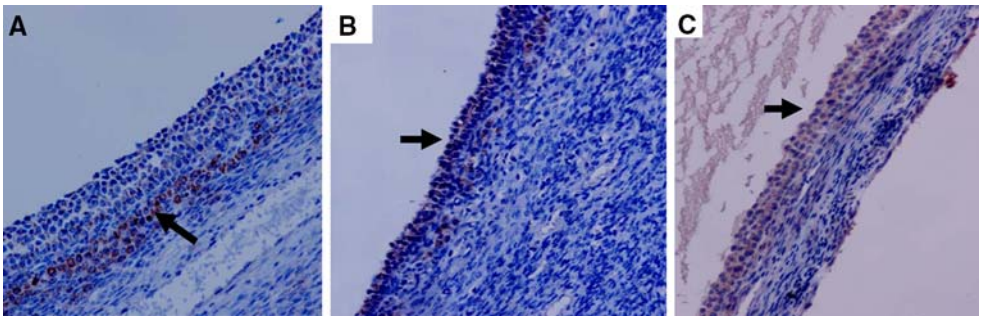

Fig. 1 **a** Immunohistochemical localization of AGE expression in normal ovary. AGE staining is stronger (++) in theca interna cells compared with granulosa cell layer (+). **b** Immunohistochemical localization of AGE expression in polycystic ovary. AGE immunoreactivity is observed in theca interna and granulosa cells. **c** AGE staining displaying stronger AGE localization in granulosa cells of polycystic ovaries (positive staining is indicated by *arrows*, ×100 magnification)

Table 1 Staining intensity/localization of AGE and RAGE in normal and PCOS ovaries

Cell types	AGE staining intensity	RAGE staining intensity
Normal theca interna	++	+++
PCOS theca interna	++	++
Normal granulosa	+	+++
PCOS granulosa	++	+++
Normal endothelial	–	+++
PCOS endothelial	+	+++
Normal stromal	–	+++
PCOS stromal	–	+++

+ weak; ++ moderate; +++ strong

observed in follicular cell layers (granulosa and theca layers), luteinized cells, but not in endothelial cells. Furthermore, AGE staining was stronger (++) in theca interna cells compared with granulosa cell layer (+) (Fig. 1).

In all PCOS tissues, AGE expression was ranging from 8 to 15% (median: 11.0%). AGE immunoreactivity was observed in theca interna and granulosa cells as well as in some endothelial cells. By contrast to controls, AGE staining in theca interna and granulosa cells was of similar intensity (++, respectively). However, the staining of granulosa cells was stronger (++) compared to that observed in normal tissue (+) (Table 1, Fig. 1).

Immunohistochemical localization of RAGE in ovarian tissue of control and PCOS patients

RAGE was highly expressed in normal ovary, the percentage of positive cells ranging from 40 to 50% (median: 45%). RAGE immunoreactivity was stronger (+++) and more extensive than that of AGE (++), being observed in granulosa cells, theca interna cells, as well as endothelial and stromal cells. RAGE staining in follicular cells displayed both cytoplasmic and nuclear localization. In normal ovarian tissue there was no difference in RAGE staining between granulosa cell layer and theca interna (+++, respectively; Fig. 2).

PCOS ovarian tissue exhibited also an increased percentage of immunopositive cells for RAGE, ranging from 30 to 60% (median: 40%). RAGE immunoreactivity revealed the same distribution as in normal ovaries. However, immunoexpression was stronger in granulosa cells (+++) compared to theca interna cells of PCOS ovaries (++; Table 1, Fig. 2).

Immunohistochemical localization of NF-κB in ovarian tissue of control and PCOS patients

The NF-κB transcription factor is present in the cytoplasm in an inactive state, complexed with the inhibitory IκB proteins. NF-κB activation occurs via signal-initiated phosphorylation of IκB at specific sites, resulting in the release and nuclear translocation of active NF-κB (Figs. 3, 4). Phosphorylated—hence induced—IκB was investigated in normal and PCOS ovarian tissue and it was found strongly expressed in the nuclei of theca interna cells. Phospho-IκB staining was much stronger in theca interna cells of PCOS ovaries compared to healthy tissues (Fig. 5).

NF-κB p50 staining was observed in the cytoplasm of theca interna cells of normal tissue, as well as in the cytoplasm of granulosa cells (Fig. 3). In PCOS ovaries, p50 staining was observed in the cytoplasm of theca interna cells at the same intensity as in healthy tissue, and it was also obtained in the cytoplasm of granulosa cells.

NF-κB p65 staining was observed in the cytoplasm of theca interna cells in normal ovaries and in the cytoplasm of granulosa cells. In PCOS ovarian tissue, p65 staining was observed in the cytoplasm of theca interna cells. In granulosa cells, p65 staining was observed in

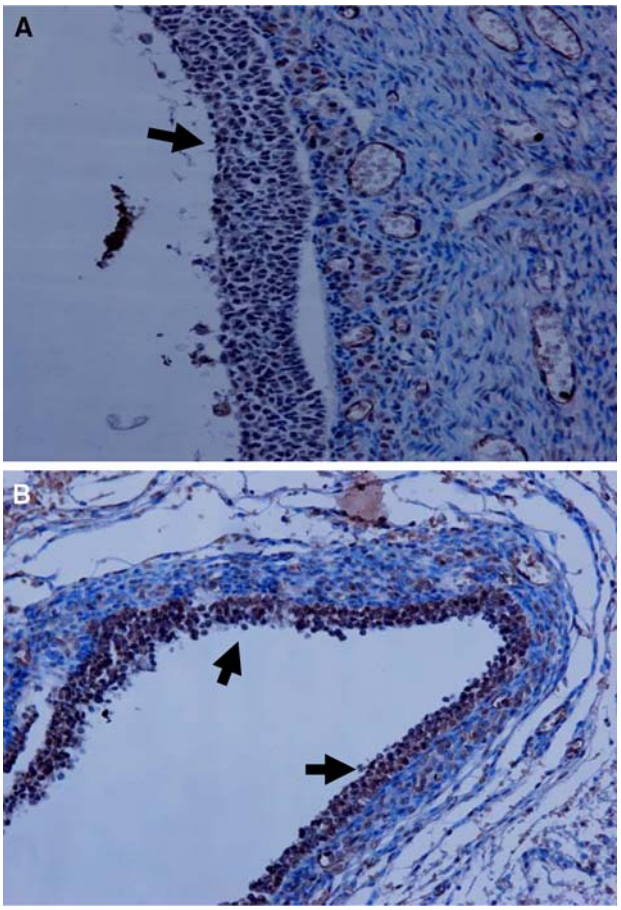

Fig. 2 **a** Immunohistochemical localization of RAGE expression in normal ovary. RAGE immunoreactivity is observed in granulosa cells, theca interna cells, as well as endothelial and stromal cells. No difference in RAGE staining is observed between granulosa cell layer and theca interna (+++, respectively). **b** Immunohistochemical localization of RAGE expression in polycystic ovary. RAGE immunoreactivity shows the same distribution as in normal ovary (staining granulosa cells, theca interna cells, as well as endothelial and stromal cells). However, RAGE expression is stronger in granulosa cells (+++) of PCOS ovaries compared to theca interna cells (++) (positive staining is indicated by *arrows*, ×100 magnification)

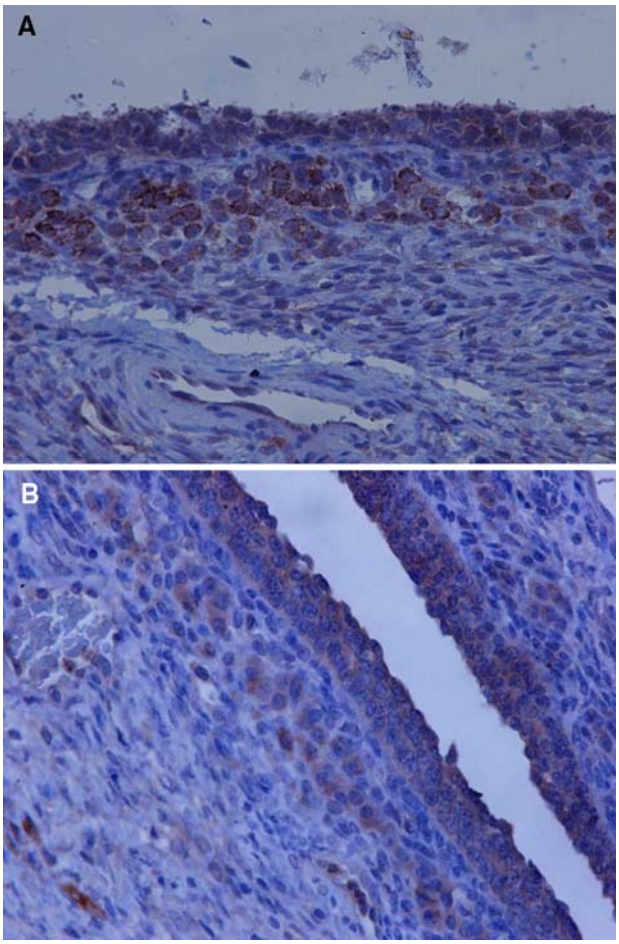

Fig. 3 Immunohistochemical localization of NF-κB p50 expression in normal (**a**) and polycystic ovary (**b**). NF-κB p50 staining is observed in the cytoplasm of theca interna cells and granulosa cells in both tissues (×200 magnification)

the cytoplasm (100%) and in a substantial number of nuclei (30%, Fig. 4).

Discussion

The present study demonstrates for the first time the localization of AGE-modified proteins, their receptor RAGE as well as the putative signalling mediator NF-κB in human ovaries. It is possible that AGEs may affect ovarian functions via RAGE. Binding of AGEs to RAGE leads to oxidative stress, which could be of importance at sites of inflammation (Schmidt et al. 1999, 2000; Singh et al. 2001; Bierhaus et al. 1997; Loske et al. 1998; Neumann et al. 1999; Loske et al. 2000). RAGE is also known to be expressed during physiological embryonic development (Hori et al. 1995).

The effects of AGEs are mediated by growth factors, cytokines and other bioactive molecules, which modulate in a paracrine/autocrine fashion cell proliferation, extracellular matrix accumulation, hemodynamics, permeability and hemorheological changes (Singh et al. 2001; Kirstein et al. 1990; Yui et al. 1994; Tsuchida et al. 1999).

Increased formation and deposition of collagen characterizes the stroma area in polycystic ovaries, which also contributes to dysregulation of ovarian hormonal milieu and reproductive function in these young women. A possible negative role of AGEs in the ovary as in other tissues, direct or indirect, cannot be excluded and has never been investigated in ovarian tissue from PCOS. It could also be speculated that

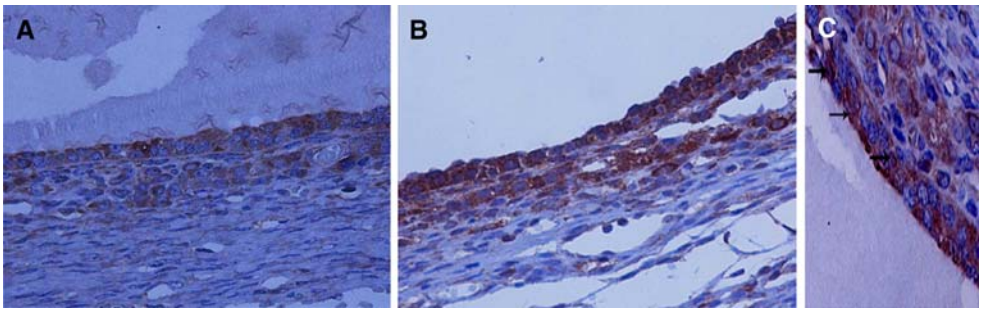

Fig. 4 Immunohistochemical localization of NF-κB p65 expression in normal (**a**) and polycystic ovary (**b, c**). NF-κB p65 immunoreactivity is observed in the cytoplasm of theca interna cells and granulosa cells in both tissues. NF-κB p65 subunit localization is observed in nuclei of granulosa cells from polycystic ovaries (**c**) (×200 magnification)

since women with this syndrome have elevated AGEs in serum, the environmental fortification, either by food intake (Diamanti-Kandarakis et al. 2006) or smoking, would have additionally detrimental effects on fertility as well as on general health issues, in women with this multifaceted disorder. Furthermore, it could be an aggravating factor contributing to ovarian dysfunction in young women with PCOS and a role of these glycotoxins in general female population with fertility problems cannot be excluded.

The possible accumulation of endogenous and exogenous AGEs may have deleterious effects on the endothelium, as it happens in diabetes and via compromised circulation, to put in danger not only the reproductive function of these women but also affect general health issues, like cardiovascular disease.

A recent study by Rice et al. demonstrated that abnormal glucose metabolism is present with significantly impaired insulin-stimulated lactate production by granulosa-lutein cells from women with anovulatory PCOS, compared to women with either normal ovaries or ovulatory women with polycystic ovaries (Rice et al. 2005). This finding suggests that the granulosa cells appear—whilst maintaining insulin regulation of steroidogenesis—to have resistance in insulin-mediated glucose metabolism, as it has been observed in other insulin-target tissues in women with PCOS (Rice et al. 2005; Wu et al. 2003). This observation implies abnormal insulin intracellular signalling in carbohydrate metabolism and activation or deactivation of key factors like PI-3K or mitogen-activated protein kinases (MAPKs), which are known to be involved in several aspects of intracellular pathways including AGE metabolism and RAGE activation (Sano et al. 1998; Cai et al. 2004; Vlassara et al. 1985; Dunaif et al. 2001). Interestingly, some clinical and experimental studies bring these molecules closer to ovarian function in PCOS. The clinical studies have shown a positive correlation of serum AGEs with androgens in PCOS women (Diamanti-Kandarakis et al. 2005b) and also the stimulatory effect of AGEs in collagen type IV production (Abe et al. 2004; Dan et al. 2004; Mott et al. 1997; Pugliese et al. 1997; Tsuji et al. 1998), which appears to be present in excess in polycystic ovary (Puistola et al. 1986; Kruk et al. 1994; Yamada et al. 1999; Oksjoki et al. 2004), in conjunction with the increased activity of lysyl oxidase by androgens which stimulated the same type of collagen in the polycystic ovary (Krawetz 1994; Harlow et al. 2003; Henmi et al. 2001), indicating a possible interaction between these factors and bringing together metabolic and steroidogenic dysregulation, environmental and genetic interactions.

Additionally, given the role of NF-κB in apoptotic processes of granulosa cells (Xiao et al. 2001, 2002; Tamm et al. 2003; Krueger et al. 2001), its activation may be implicated in the mechanisms of follicular maturation and in the pathobiology of granulosa cells' dysfunction. The concomitant presence of AGE, RAGE and activated NF-κB in granulosa cells implies a putative interaction among them and a possible role in the pathophysiology of ovarian dysregulation of the syndrome should be persuaded.

In conclusion, these are preliminary imumohistochemical findings, demonstrating for the first time localization of AGEs, their receptor RAGE and activated NF-κB in normal and polycystic ovaries. Moreover, the presence of differential localization of AGEs, RAGE and their putative signalling mediator NF-κB in granulosa cells in polycystic ovary compared to normal ovaries, requires further investigation and molecular studies to clarify their role in metabolic and steroidogenic pathways.

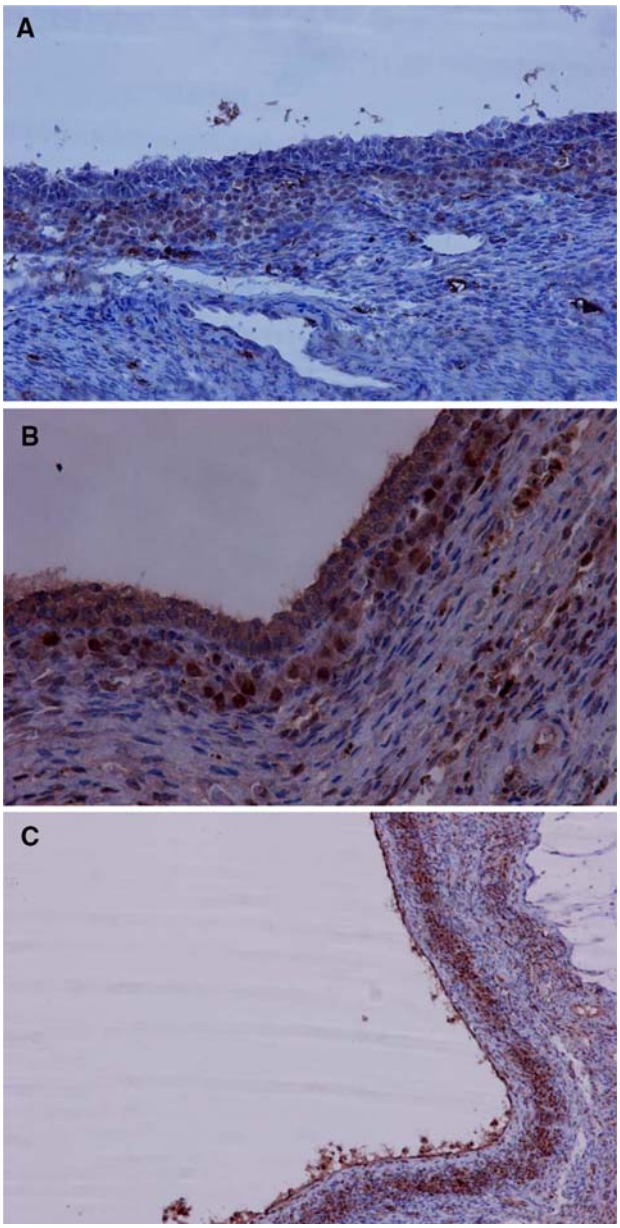

Fig. 5 Immunohistochemical localization of phospho-IκB expression in normal (**a**; ×100 magnification) and polycystic ovary (**b**; ×200 magnification). Phospho-IκB immunoreactivity is observed in the nuclei of theca interna cells in both tissues. Phospho-IκB staining is much stronger in theca interna cells of PCOS ovaries (**c**) compared to healthy tissues (×20 magnification)

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