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Lysyl oxidase interacts with AGE signalling to modulate collagen synthesis in polycystic ovarian tissue

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Abstract

Connective tissue components – collagen types I, III and IV – surrounding the ovarian follicles undergo drastic changes during ovulation. Abnormal collagen synthesis and increased volume and density of ovarian stroma characterize the polycystic ovary syndrome (PCOS). During the ovulatory process, collagen synthesis is regulated by prolyl hydroxylase and lysyl oxidase (LOX) activity in ovarian follicles. LOX catalyzes collagen and elastin cross-linking and plays essential role in coordinating the control of ovarian extracellular matrix (ECM) during follicular development. We have recently shown accumulation of advanced glycation end products (AGEs), molecules that stimulate ECM production and abnormal collagen cross-linking, in ovarian tissue. However, the possible link between LOX and AGEs-induced signalling in collagen production and stroma formation in ovarian tissue from PCOS remains elusive. The present study investigates the hypothesis of AGE signalling pathway interaction with *LOX* gene activity in polycystic ovarian (PCO) tissue. We show an increased distribution and co-localization of LOX, collagen type IV and AGE molecules in the PCO tissue compared to control, as well as augmented expression of AGE signalling mediators/effectors, phospho(p)-ERK, phospho(p)-c-Jun and nuclear factor κB (NF-κB) in pathological tissue. Moreover, we demonstrate binding of AGE-induced transcription factors, NF-κB and activator protein-1 (AP-1) on *LOX* promoter, indicating a possible involvement of AGEs in *LOX* gene regulation, which may account for the documented increase in *LOX* mRNA and protein levels compared to control. These findings suggest that deposition of excess collagen in PCO tissue that induces cystogenesis may, in part, be due to AGE-mediated stimulation of LOX activity.

Keywords: AGE signalling • lysyl oxidase • ovaries • collagen IV • polycystic ovary syndrome

Introduction

Polycystic ovary syndrome (PCOS) affects approximately 6% of reproductive age women and is the leading cause of anovulatory infertility [1]. It is characterized by excessive ovarian androgen production and polycystic ovaries with a thickened, fibrotic *tunica albuginea* and a subcortical band consisting of many cystic follicles in various stages of growth and atresia [2]. Despite intense research, the local mechanisms underlying anovulation in PCOS remain obscure.

Collagen fibres in the ovarian follicles change during ovulation because of the preovulatory increase of collagenolytic activities. The collagenous components of the ovarian stroma are interstitial collagen types I, III and IV in the extracellular matrix (ECM) of stroma and in *tunica albuginea* [3]. They provide the structural strength to the ovaries and support the matrix where follicular maturation, ovulation and formation of *corpus luteum* take place [3–5].

Physiological collagen cross-linking in the ECM requires the enzyme lysyl oxidase (LOX). LOX is a copper-dependent amine

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oxidase that catalyzes the oxidative deamination of peptidyl lysine and hydroxylysine to peptidyl-α-aminoadipic-δ-semialdehyde into elastin and collagen chains. The consequent aldehydes lead to a spontaneous condensation, forming inter- and intra-chain cross-links within these important ECM components [6].

This post-translational modification of the ECM molecules appears to have a crucial role both for collagen and elastin structural properties and in triggering some still unresolved signal transduction pathways [7]. Because of its action on ECM, deficit of normal LOX expression has been described in many polygenic and monogenic disorders such as atherosclerosis, type IX Ehlers–Danlos syndrome and Menkes disease [7, 8]. Controversially, an enhanced level of LOX also seems to be involved in human pathology. Several reports have recently suggested a clear association between organ fibrosis and increased LOX activity. This has been described in several chronic human liver diseases [9], in idiopathic and experimental lung fibrosis [10], in experimental hepatic fibrosis [11] and adriamycin-induced kidney fibrosis in rats [12], as well as in other pathologies resulting in fibrosis [13–17].

In the ovulatory process, collagen synthesis regulated by prolyl hydroxylase and LOX activities in the ovarian follicles of rabbits is induced after follicle rupture, resulting in reconstruction of collagen fibre [18]. There is evidence from *in vivo* studies that LOX is expressed in rat and bovine ovarian tissue and specifically in the granulosa cell layer [19, 20]. These studies further indicate control of LOX at endocrine, paracrine and autocrine levels within the ovary and suggest coordinated regulation of ovarian ECM during follicular development [21]. Additionally, a study investigating expression of LOX in dehydroepiandrosterone (DHEA)-induced PCOS in rats showed that DHEA treatment increased *LOX* mRNA expression to more than three times the control levels, suggesting that increased LOX expression may be one of the causes of PCOS cystogenesis [22]. However, the presence of LOX in human ovarian tissue has not been explored, and furthermore, the mechanisms and/or the signalling molecules implicated in *LOX* gene regulation remain unknown.

There is mounting evidence that advanced glycation end products (AGEs) are engaged in the pathogenesis of PCOS. Increased AGE levels are present in serum of lean, normoglycaemic PCOS women and accumulate in their ovaries, possibly interfering with normal ovarian structure and function [23, 24]. AGEs are the end products of a chemical process called Maillard reaction in which the carbonyl group of carbohydrates reacts non-enzymatically with protein primary amino groups such as lysine or arginine [25–27]. Excessive AGE levels are known to stimulate the production of ECM and inhibit its degradation [28]. AGEs are capable of forming covalent cross-links with long-lived structural proteins such as collagen and elastin [28] and in doing so lead to modification of protein function by reducing their enzymatic activity, altering their biophysical properties and changing the protein interactions with other enzymes [29, 30]. Exposure of cultured mesangial cells to AGEs results in a receptor-mediated up-regulation of mRNA and protein secretion of collagen IV α1, one of the major components of ECM, through activation of the activin receptor-like kinase 1 [31].

AGEs induce a variety of cellular events through several functional AGEs receptors, thereby modulating disease processes. The signalling mechanisms behind formation of cross-links partly involve the multiligand receptor (receptor for AGE, RAGE) that mediates the up-regulation of type IV collagen by AGEs [32]. The interaction between AGE and RAGE has been shown to ignite generation of intracellular oxidative stress and subsequent activation of the extracellular signal-regulated kinase (ERK)1/2 mitogen-activated protein kinases (MAPKs) and c-Jun, leading to potentiation of the transcription factors NF-κB and AP-1 [33–36]. A previous study from our group has shown that RAGE and AGE-modified proteins are expressed in human ovarian tissue and activated NF-κB is localized at the same sites [24].

A possible – direct or indirect – role of AGEs in the regulation of LOX expression and activity in the ovary cannot be excluded and has never been investigated in ovarian tissue from PCOS. The present study was undertaken to examine the putative involvement of AGE signalling pathway in the regulation of *LOX* gene activity in polycystic ovarian (PCO) tissue. Our results support a possible contribution of AGE signalling to LOX expression and activity in PCO tissue, which could mediate interactions that may 'catalyze' deregulation of folliculogenesis in PCOS.

Materials and methods

General

Chemical reagents were supplied from Sigma (Sigma, St. Louis, MO, USA) unless otherwise stated. The human *LOX* promoter cDNA (nucleotides 1–2241) cloned into a pGL2 vector plasmid (Promega Inc., Madison, WI, USA) was kindly provided by A. Di Donato (Laboratory of Nephrology, Institute G. Gaslini, Genova, Italy). Restriction enzymes were obtained from New England Biolabs (Beverly, MA, USA). Antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA): anti-LOX goat polyclonal (sc-32410), anti-p-ERK mouse monoclonal (sc-7383), anti-p-c-Jun (sc-822x), anti-collagen type IV (sc-59814) and anti-NF-κB p65 (sc-8008x). Another monoclonal antibody against NF-κB p65 suitable for immunohistochemistry was purchased from Zymed Laboratories (San Francisco, CA, USA). Monoclonal antibody against AGE was obtained from Research Diagnostics (Cocord, MA, USA). Rabbit polyclonal anti-collagen type IV antibody (AB748MI) used in immunocytochemistry was obtained from Chemicon (Temecula, CA, USA). Secondary goat anti-mouse (sc-2005) and rabbit anti-goat (sc-2768) antibodies were obtained from Santa Cruz Biotechnologies.

Ovarian specimens

Ovarian tissues from six women diagnosed with PCOS, who had undergone laparoscopic ovarian wedge resection performed previously and independently of this project [37], were partly kept frozen in liquid nitrogen and the rest 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 [37]. All

patients with PCOS (mean age 25.6 ± 3.0 years; body mass index (BMI) 26.36 ± 7.86 kg/m^2) were diagnosed according to the following criteria: (1) oligo- and/or anovulation (less than eight menses per year); (2) clinical and/or biochemical signs of hyperandrogenism; and (3) exclusion of other endocrine disorders (non-classical 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 [24].

Immunohistochemistry

Sections of formalin-fixed ovarian tissue were treated with 3% hydrogen peroxide in phosphate-buffered saline (PBS), rinsed in PBS, placed in 0.01 M citric acid-buffered solution (pH 7.0) and microwaved at 500 W for 5 min. Following thorough washing, the sections were incubated with rabbit serum (20 min, room temperature) and left overnight at 4°C with the anti-AGE monoclonal antibody (dilution 1:50) or anti-collagen type IV polyclonal antibody (dilution 1:50) or anti-LOX polyclonal antibody (sc-32410, dilution 1:50), respectively, in PBS-bovine serum albumin (BSA) (PBS + 1% BSA).

The same tissue sections were incubated overnight at 4°C with the anti-p-ERK (sc-7383, dilution 1:50), the anti-p-c-Jun (sc-822, dilution 1:100) or the anti-NF-κB p65 (Zymed, dilution 1:100) antibodies, respectively. 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 (DAB) tetrahydrochloride (LSABplus detection kit, Dako, Carpinteria, CA, USA) in the case of anti-AGE, NF-κB p65 and anti-LOX antibodies, and with the Dako REAL™ EnVision™ Detection System (Dako, Carpinteria, CA, USA) in the case of collagen type IV, p-ERK, p-c-Jun. Brain tissue sections from a patient with Alzheimer's disease and glioblastoma multiform sections were employed as positive controls for AGE and NF-κB p65 immunostaining, respectively. Skin epidermis tissue sections were used as positive controls for collagen type IV and LOX immunostaining. Sections from breast carcinoma were used for p-c-Jun immunostaining. Colon carcinoma sections were used for p-ERK immunostaining. Negative controls (*e.g.* normal and PCO tissue in which the primary antibody was substituted with non-immune 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; +++, strong.

Western blot analysis

Whole-cell protein extracts (20 μg) were resolved on SDS 4–12% polyacrylamide gels and electrotransferred onto nitrocellulose membrane (Hybond ECL, Amersham Biosciences, Buckinghamshire, UK). Membranes were blocked for 2 hrs at room temperature with 5% non-fat dry milk in Tris-buffered saline with Tween (TBST; 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) and probed with the primary antibody (anti-LOX, anti-collagen type IV, anti-p-ERK, anti-p65 or anti-p-c-Jun; see above), overnight at 4°C. Immunoreactive bands were visualized by the enhanced SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL, USA).

Recombinant human *LOX* promoter: purification and labelling of double-stranded oligonucleotide probes

Cultures of *Escherichia coli* (strain DH5a) cells transfected with part of the cDNA of the human *LOX* promoter ($LOX_{1–2241}$, nucleotides 1–2241) were grown in complete LB medium (50 mg/ml ampicillin), at 37°C in a shaking incubator (250–280 rpm) for 16–18 hrs, and purification of the plasmid DNA was performed according to the manufacturer's instructions (Macherey-Nagel, Duren, Germany). Analysis of the promoter with sequence analysis software revealed binding sites for transcription factors NF-κB and AP-1. Specified double-stranded oligonucleotide fragments of the pGL2-LOX plasmid that harboured those transcription factor-binding sites were isolated by restriction digestion with appropriate enzymes (see Fig.1 and Fig. 3). The fragments were as follows: (*i*) NF-κB probe: enzymes ApaI and MscI were used to digest, at nucleotide sites 5 and 94 respectively, a 89-nt fragment containing the 5′-GGGGATCCAC-3′ NF-κB-binding sequence; (*ii*) AP-1 probe: enzymes BsgI and BpmI were used to digest, at nucleotide sites 2094 and 2190 respectively, a 96-nt fragment containing the 5′-CTGGAGTCACC-3′ AP-1-binding sequence. The fragments were separated by agarose electrophoresis, purified from the agarose gel (Macherey-Nagel, Duren, Germany), 3′-end labelled with the Biotin 3′-End DNA Labeling Kit (Pierce Biotechnology, Rockford, IL, USA) and subsequently used as probes or (unlabelled) competitors in the following experiments.

Electrophoretic mobility-shift assay

Nuclear extracts from control or PCOS ovarian tissue (30–40 μg) were mixed with 50 fmol of biotin-labelled probe in a total reaction volume of 20 μl containing10 mM Tris, 170 mM KCl (0.5 mM $MgCl_2$, in the reactions with the AP-1 probe), 2.5% glycerol, 0.04% NP40, 50 ng/μl poly(dI·dC), 2 mM EDTA, 2.6 mM DTT, 50 mM NaF, 1 mM PMSF, 1 mM Na_3VO_4 and protease inhibitor cocktail. For competition experiments, a 100- or 200-fold molar excess of unlabelled NF-κB or AP-1 oligonucleotide was added to the binding reaction together with the other components, whereas in another set of experiments antiserum (2 μg of anti-NF-κB p65 or anti-p-c-Jun antibody) was incubated with the reaction mixture for 30 min at room temperature prior to the addition of the labelled probe. Reactions were allowed to proceed for 25 min at room temperature. DNA–protein complexes were resolved on 5% native polyacrylamide gels in 1 × Tris-borate-EDTA (TBE) buffer (125 V, room temperature) and electrotransferred onto nylon membrane at 380 mA (~100 V) for 60 min. The biotin-labelled DNA complex was detected by chemiluminescence using the LightShift Chemiluminescence EMSA (electrophoretic mobility-shift assay) kit (Pierce Biotechnology, Rockford, IL, USA).

Real-time PCR

Total RNA from 100 mg of tissue was extracted using the monophasic solution of phenol and guanidine isothiocyanate in the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Reverse transcription was performed using 200 units of AMV reverse transcriptase (Promega, Madison, WI, USA). Real-time quantitative PCR of the *LOX* gene was carried out and relative quantification was established with the ΔΔCt method against an internal control (*GAPDH*). The primers used for the amplification of *LOX* were forward primer, 5′-ATATAGGGGCGGATGTCAGAG-3′, and reverse primer, 5′-CGAATGTCACAGCGTACAAC-3′. The

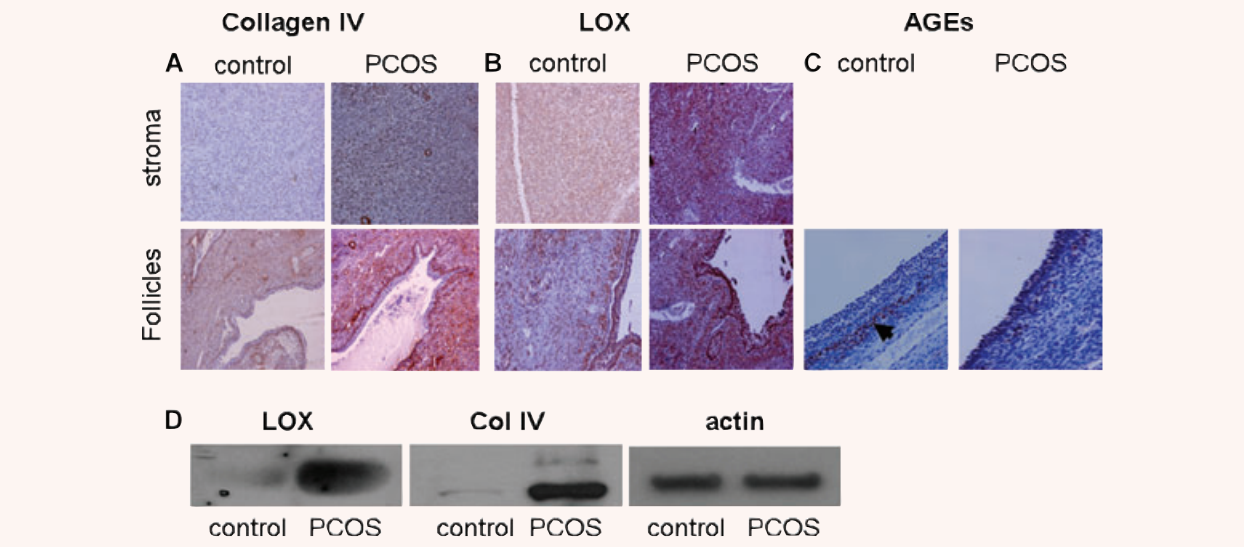

Fig. 1 Immunodetection of collagen type IV and LOX in PCO and control samples. Immunohistochemistry was performed on sections from stromal (top panels) or follicular (bottom panel) ovarian tissue, which were stained for collagen type IV (**A**) and LOX (**B**). Increased selective staining is observed in PCO sections (magnification ×100). Pictures are representative of 20 sections. (**C**) Immunohistochemical detection of AGEs in polycystic ovaries [24]. Positive staining is observed in the follicular cell layers (granulosa and theca layers), luteinized cells and in endothelial cells of polycystic ovaries, with stronger staining intensity observed in granulosa cells compared to normal tissue ($P = 0.036$). AGE immunoreactivity positively correlates with LOX staining in granulosa cells ($R = 0.285$, $P = 0.041$). (**D**) Western immunoblotting of 40 μg of whole-cell extract from control and PCO tissue against anti-collagen type IV and anti-LOX antibodies. Collagen type IV protein expression is increased by 3-fold and LOX protein expression by approximately 4-fold in the PCO tissue relative to control.

PCR protocol consisted of 30 cycles each of 94°C for 30 sec, 55°C for 1 min and 72°C for 30 sec, followed by an extension at 72°C for 5 min.

Statistical methods

The expression of AGE, p-ERK, p-c-Jun, NF-κB p65, LOX and collagen type IV was treated as categorical or ordinal variables. Comparisons between the expression levels among patients with PCOS and controls were performed using Fisher's exact test. Correlations between different markers were tested using Kendall's *t* coefficient analysis. Statistical calculations were performed using statistical package STATA 9.0 for Windows. All results with a two-sided *P*-value ≤ 0.05 were considered significant.

Results

Immunohistochemical detection of collagen type IV and LOX in polycystic ovaries

Previous studies have shown variations in the production and/or distribution of collagen type IV in polycystic ovaries, suggesting a possible involvement of basement membranes in the pathogenesis of PCOS [38]. In agreement with this observation, in our samples collagen type IV was detected mainly in the stroma of polycystic ovaries where both the extent ($P = 0.036$) and the intensity ($P = 0.036$, Fig. 1) of positive staining were higher compared to normal ovaries (Fig. 1A). Increased collagen type IV staining was also observed in the theca cell layer of polycystic ovary compared to normal ($P = 0.048$) and less intense staining was observed in the granulosa follicular layer.

Because physiological collagen cross-linking requires LOX, we investigated LOX distribution in normal and PCO tissue. LOX staining exhibits a similar tissue distribution to collagen type IV, demonstrating intense positive staining in the stroma of polycystic ovary compared to normal (Fig. 1B). Both the extent ($P = 0.004$) and the intensity of LOX staining ($P = 0.009$) are higher in polycystic ovaries than in controls. Increased LOX staining was observed in the theca interna of polycystic ovaries (extent, $P = 0.009$; intensity, $P = 0.011$) as well as in the granulosa cell layer (extent, $P = 0.009$; intensity, $P = 0.004$) compared to normal ovaries. Finally, LOX immunoreactivity was also higher in the vessels of PCO tissue compared to normal (extent, $P = 0.002$; intensity, $P = 0.002$).

Immunohistochemical detection of AGEs in polycystic ovaries

AGE immunoreactivity in the same sections of polycystic and control ovarian tissue has been previously shown [24]. In those

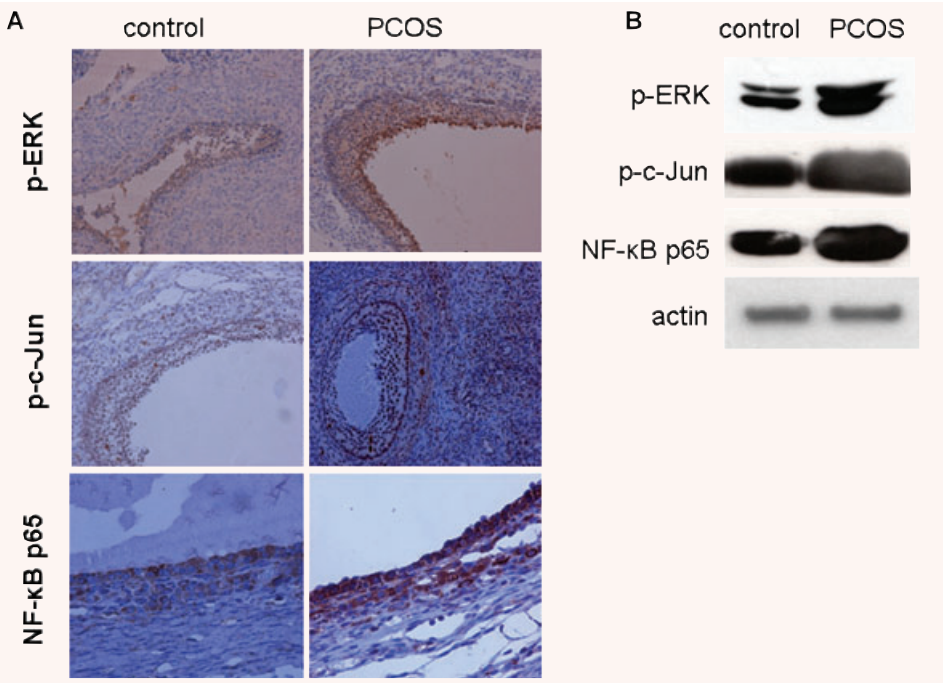

Fig. 2 Immunodetection of p-ERK, p-c-Jun and NF-κB p65 in PCO and control samples. (**A**) Sections of ovarian tissue were immunostained for p-ERK, p-c-Jun and NF-κB p65. Increased staining is observed on the pathological tissue. Pictures are representative of 20 sections (magnification ×100). (**B**) Western immunoblotting of 40 μg of whole-cell extract from control and PCO tissue incubated with anti-p-ERK, anti-p-c-Jun and anti-NF-κB p65 antibodies. Protein levels of p-ERK are increased by 3-fold, of p-c-Jun 1.5-fold and of NF-κB p65 2-fold relative to control.

samples, positive staining was observed in the follicular cell layers (granulosa and theca layers), luteinized cells and in endothelial cells of polycystic ovaries, with stronger staining intensity observed in granulosa cells compared to normal tissue ($P = 0.036$) (see Fig. 2A, upper panel). In the present study, AGE immunoreactivity was found to positively correlate with LOX staining in granulosa cells based on Kendall's *t* coefficient analysis ($R = 0.285$, $P = 0.041$) (Fig. 1B and C).

Expression of collagen type IV and LOX in polycystic ovaries

In order to verify the immunostaining data and assess the protein levels of both collagen type IV and LOX in PCO tissue, we performed Western immunoblotting analysis in protein extracts derived from human ovarian tissue. The assay revealed enhanced expression of both proteins in the pathological tissue (Fig. 1D). Collagen type IV protein expression is increased by 3-fold and LOX protein expression by approximately 4-fold in the PCO tissue.

Immunohistochemical detection of phospho(p)-ERK, phospho(p)-c-Jun and NF-κB p65 in polycystic ovaries

To investigate the activation of AGE signalling pathway in PCO tissue, we stained both control and PCO samples for the known AGE signalling mediator/effector molecules, p-ERK, p-c-Jun and NF-κB p65.

P-ERK staining was higher in the cytoplasm of granulosa cells of PCO tissue compared to normal ovaries ($P = 0.048$) as well as in the nuclei of theca interna ($P = 0.048$) and theca externa cells ($P = 0.048$) compared to normal (Fig. 2A, upper panel).

P-c-Jun expression was higher in the cytoplasm of granulosa cells of PCO tissue compared to normal ovaries ($P = 0.046$) as well as in the stroma of PCO tissue. P-c-Jun staining was increased in the nuclei of stroma cells of polycystic ovaries compared to controls ($P = 0.048$) (Fig. 2A, middle panel).

NF-κB p65 staining was increased in the nuclei of granulosa cells of polycystic ovaries compared to normal ($P = 0.036$) as well as in the nuclei of theca interna ($P = 0.048$) and theca externa cells ($P = 0.083$) compared to normal tissue. NF-κB p65 staining displayed a positive correlation with LOX staining in granulosa cell layer based on Kendall's *t* coefficient analysis ($R = 0.2857$, $P = 0.0412$) (Fig. 2A, lower panel).

Protein expression of p-ERK, p-c-Jun and NF-κB p65 in polycystic ovaries

In order to quantitate the protein levels of the active (*i.e.* phosphorylated) species p-ERK, p-c-Jun and NF-κB p65, Western immunoblotting analysis was performed in whole-cell extracts isolated from healthy and PCO tissue for the presence of these molecules using appropriate antibodies. The expression levels of p-ERK, p-c-Jun and NF-κB p65 were increased 2.5 to 3-fold in the PCO tissue (Fig. 2B).

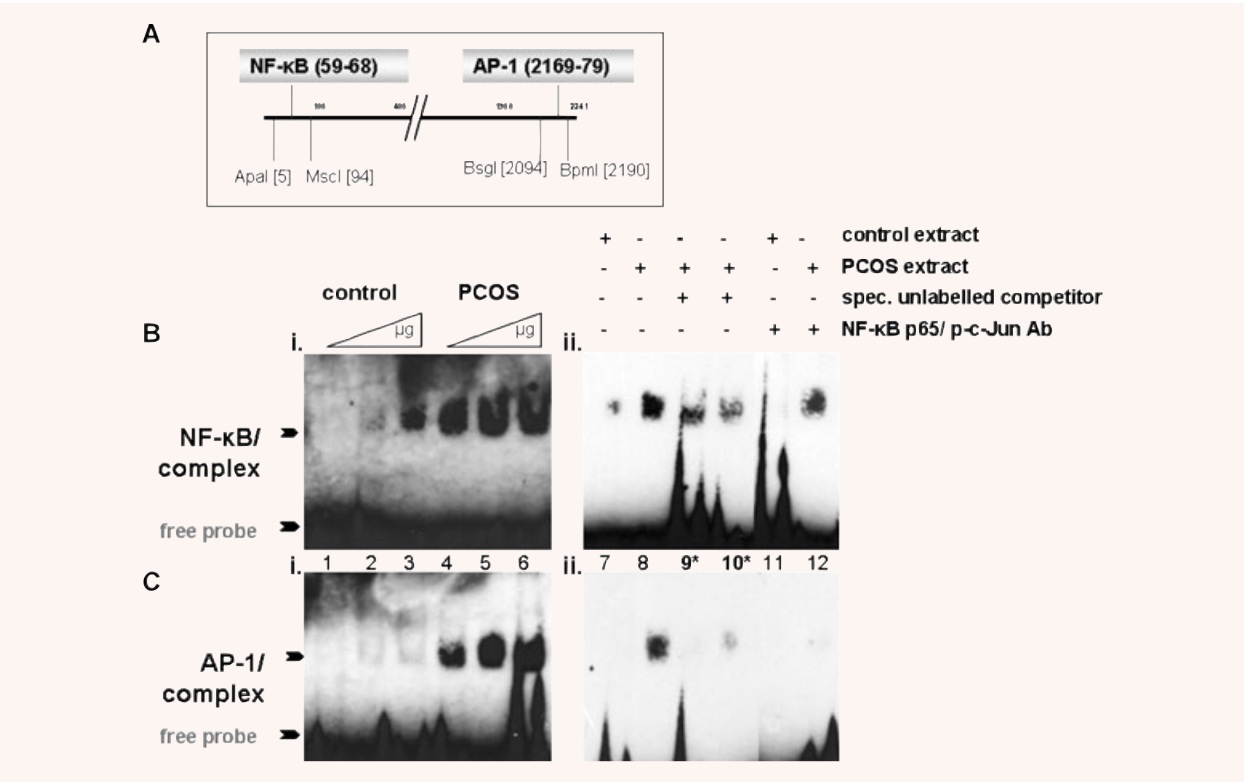

Fig. 3 Induced NF-κB and AP-1 binding to *LOX* promoter in PCO tissue. (**A**) Line diagram of part of the *LOX* promoter. The binding sites for the NF-κB and AP-1 transcription factors are indicated, as well as the restriction enzymes are used to isolate the respective DNA fragments from *E. coli* transfected cells. (**B**, **C**) EMSA with the NF-κβ p65 DNA biotin-labelled probe (**Bi**) or the AP-1/c-Jun biotin-labelled probe (**Ci**) and whole-cell ovarian protein extract from human samples of healthy (control) and PCO tissue. Increasing concentrations (10, 20, 40 μg) of protein were used (lanes 1–3 for control and 4–6 for PCO tissue protein extract) and the LOX promoter – NF-κB and *LOX* promoter – AP-1 complex is indicated in the two panels, respectively. (**Bii**, **Cii**) Binding competition assays with 100-fold (lane 9) and 200-fold (lane 10) molar excess of specific unlabelled probe reacting with protein extract from PCO tissue. Note that in the lower panel (AP-1 probe, **Cii**), lane 9* contains 200-fold molar excess and lane 10* contains 100-fold molar excess of specific unlabelled probe. Lanes 11 and 12 correspond to reactions in which pre-incubation of control and PCO tissue protein extract with anti-NF-κB p65 or anti-p-c-Jun antibody was performed, before the addition of the labelled probe.

NF-κB and AP-1 binding to *LOX* promoter

Co-expression of LOX and AGEs in the ovary as well as activated AGE signalling in this tissue prompted us to investigate the existence of a possible interaction between the AGE-induced transcription factors, NF-κB and AP-1 and *LOX* promoter region.

Towards this end, biotin-labelled double-stranded oligonucleotides derived from the human *LOX* promoter encompassing the NF-κB- and AP-1-binding motifs, respectively, were used as probes in a standard EMSA employing whole-cell extracts from control and PCO tissue. A concentration-dependant DNA–protein complex was obtained for both binding reactions (Fig. 3Bi and Ci). The specificity of this complex formation was verified by binding competition analyses with 100- to 200-fold excess of the corresponding unlabelled probe (Fig. 3Bii and Cii).

To demonstrate the presence of NF-κB p65 and p-c-Jun in the above specific DNA–protein complexes, anti-NF-κB p65 and anti-p-c-Jun antibodies were included in the corresponding reaction mixtures prior to addition of the labelled probe (Fig. 3Bii and Cii). Pre-incubation with the anti-NF-κB p65 antibody hampered complex formation in the control tissue (Fig. 3Bii, lane 11) and drastically reduced the abundance of complex in the PCO tissue (Fig. 3Bii, lane 12). Concurrently, pre-incubation with the anti-p-c-Jun antibody inhibited complex formation in both tissues (Fig. 3Cii, lanes 11 and 12).

These data provide evidence that NF-κB and AP-1 could have an impact on collagen metabolism in PCO tissue by affecting *LOX* gene expression.

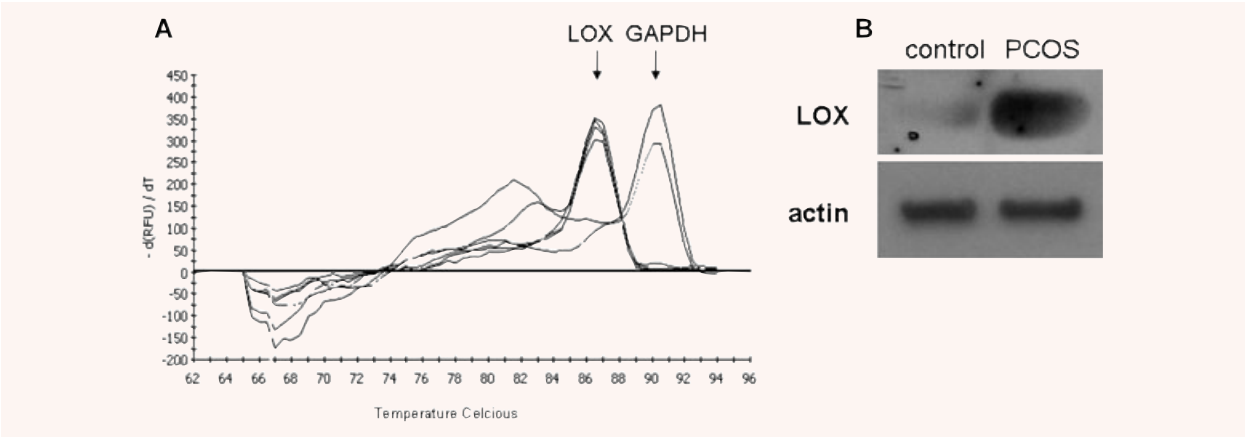

Fig. 4 Induction of the *LOX* mRNA and LOX protein levels in the pathological ovarian tissue. (**A**) Real-time PCR was employed to achieve relative quantification of *LOX* mRNA in control and PCO tissue. Using the Pfaffl equation increase in *LOX* mRNA expression was calculated as 3-fold relative to control. Representative graph of the melting curves of the *LOX* and *GAPDH* PCR products. (**B**) LOX protein levels are increased by almost 4-fold in PCO tissue as revealed by Western immunoblotting.

LOX mRNA levels in ovarian tissue

To strengthen the above-mentioned observations, we investigated the *LOX* mRNA expression profile in control and PCO tissue. In support of the EMSA findings, relative quantification of the *LOX* gene using real-time PCR revealed a 3-fold increase in the amount of *LOX* mRNA in the PCO tissue compared to control (Pfaffl equation, Fig. 4).

Discussion

LOX plays a critical role in the organization of ECM, stabilizing the molecules of collagen and elastin [39–42]. In ovaries of rat models with PCOS, induced by administration of DHEA, increased *LOX* mRNA expression has been implicated to be one of the causes of the cystogenesis in PCOS [21].

Intracellular AGE signalling has been shown to stimulate the production of ECM and up-regulate collagen type IV expression in various cell types such as mesangial cells [31]; however, its role in ovarian cells is unclear. Furthermore, the effect of AGE signalling in *LOX* gene regulation has never been explored.

In this study we present evidence that LOX protein is overexpressed in PCO tissue compared to normal and that the transcriptional activity of *LOX* promoter is regulated by AGE signalling mediator/effector molecules. We documented that LOX expression co-localizes with collagen type IV in the stroma of the ovaries as well as in the follicular cell layers, being more abundant in the case of PCO tissue. In the ovarian follicles, intense LOX immunostaining was observed both in the theca and granulosa cell layers, whereas collagen type IV stained strongly only the theca cells. The finding that LOX expression is present in both stroma and follicular layers of the ovaries suggests its possible involvement in the cross-linking and deposition of collagen in ovarian ECM during follicular development. Nevertheless, the differential distribution of LOX in the follicular layers compared to collagen type IV poses a possible additional role of LOX in granulosa cells.

Although the cellular origins of the type IV collagen-rich basement membrane of the follicle are a matter of controversy [43], there is evidence that granulosa cells have the capacity to produce and secrete many of their components [44]. Previous studies have shown that *LOX* mRNA expression being restricted to the granulosa cell compartment of the ovary, pointing to a local mechanism for assembly of the basement membrane ECM collagen component [19]. However, expression of collagen type IV has been demonstrated in the theca cells of human follicles [45], which is in accordance with our observations. We have also demonstrated high levels of LOX expression in human theca cells suggesting that transportation of LOX into the theca may occur. The increased LOX immunostaining in granulosa cells is in concert with previous studies where expression of the *LOX* gene was detected in rat granulosa cells and it was found that *LOX* gene was negatively regulated by follicle-stimulating hormone (FSH) in pre-/early antral follicles [19]. These studies further indicate that LOX is regulated at endocrine, paracrine and autocrine levels by FSH, androgen and transforming growth factor-β superfamily members emanating from the oocyte, thecal cells and the granulosa compartment itself.

In our study LOX expression in the human ovarian follicles co-localizes with AGE immunostaining. Previously, we have shown expression of AGEs in the follicular layers of PCO tissue, especially

in the granulosa cells [24]. In the present study, a positive correlation was observed between AGE staining and LOX in the granulosa cells of the ovarian follicles, leaving the possibility of an interaction between intracellular AGE signalling and LOX open. Indeed, we sought to investigate the possible AGE–RAGE-mediated regulation of LOX in PCOS, given the increased RAGE expression in PCOS ovaries compared to control shown before [24]. The AGE–RAGE signalling pathway was found activated in the PCO tissue compared to normal ovary. The pathway's key molecules, ERK1/2 and c-Jun, were found activated in the follicular cell layers of PCO tissue and the same was true for transcription factors NF-κB p65 and AP-1. Notably, NF-κB p65 immunostaining exhibited a positive correlation with LOX staining in granulosa cell layer of PCO follicles. Up-regulation of AP-1 is in accord with studies that show potentiation of AP-1 in response to oxidative stress [36]. Various *in vitro* studies demonstrate that treatment with AGEs enhances AP-1 activation [46].

Given the above-mentioned data, the next step was to investigate the possible regulation of the transcriptional activity of *LOX* promoter by AGEs. Using homology analysis, we identified in the functional part of *LOX* promoter highly conserved sequences for NF-κB binding (nt 59–68) and AP-1 binding (nt 2169–2179). EMSAs revealed that these sites bear functional role. DNA–protein complexes on these sites were detected, whose specificity and composition was verified by binding competition assays and use of specific antibodies to NF-κB p65 and AP-1/p-c-Jun. Recognition and binding of both transcription factors to *LOX* promoter region indicate that it is highly likely for AGE molecules to regulate *LOX* gene expression using at least one of the two signalling pathways. In fact, for many types of stress a concurrent activation of NF-κB and AP-1 has been observed, albeit the signal transduction pathways differ for the two transcription factors [47, 48]. It is therefore reasonable to postulate that accumulation of AGEs in the ovaries may lead to activation of both NF-κB and AP-1 and further stimulation of LOX activity.

The importance of NF-κB and AP-1 sites in the transcriptional activation of *LOX* gene could be further verified by functional studies on the *LOX* promoter, using site-directed mutagenesis of the sequences identified by EMSA as binding sites of AP-1/c-Jun and NF-κB p65. However, several difficulties involving the efficient isolation and culture of human primary ovarian cells arise in performing this kind of experiments [49–52]. Current challenges for follicle culture are numerous and include optimization of culture media and the tailoring of culture environments to match the physiological needs of the cell *in vivo*; the maintenance of cell–cell communication and signalling during culture; and the evaluation of the epigenetic status of *in vitro* derived mature oocytes [53–58]. In large animals and human beings, the complete *in vitro* growth and maturation of oocytes is only likely to be achieved following the development of a multistage strategy that closely resembles the ovary *in vivo* [59].

Furthermore, transfection of isolated primary ovarian cells has been shown to be rather difficult because in mature follicles the oocyte is surrounded by the *zona pellucida*, a glycoprotein layer that presents a barrier for transfection of human oocytes [60]. On the other hand, cell-line transfection experiments have raised serious questions regarding the regulation of *LOX* gene expression. These studies have demonstrated that *LOX* promoter is controlled by negative and positive *cis*-acting regions, which are differentially active in various cells and thus expression of LOX depends largely on cellular context and varied extracellular cues [61, 62].

Quantitative assays of *LOX* mRNA levels in PCO tissue compared to normal revealed an increase in the levels of *LOX* mRNA (3-fold) and protein (4-fold), which is in symphony with data from experimental animals with androgen-induced PCOS phenotype [22]. *In vitro*, primary ovarian cultures have detectable LOX enzymatic activity (data not shown), further establishing the functional role of the enzyme in the ovarian tissue.

In an attempt to explore the implication of AGE signalling in the control of *collagen type IV* gene activity, we employed homology search analysis to identify binding sites for NF-κB and AP-1 in its promoter region. However, no binding sites for these transcription factors were identified indicating that the effect of AGEs in ovarian stroma is mainly mediated through *LOX* gene.

Our findings suggest that LOX, under the regulation of the intracellular activities of AGEs, may be responsible for the formation of cysts in the PCO tissue. The absence of AGE-specific transcription factor-binding sites and the differential localization of collagen type IV and LOX in the ovarian follicles indicate that AGE–RAGE signalling targets specifically *LOX* gene and not collagen. Although presently difficult to define the exact mechanism by which AGEs would increase *LOX* gene activity, the evidence that AGEs might be involved in LOX regulation is completely novel.

Obviously, further studies are needed to fully elucidate the regulatory mechanism that links AGEs to LOX in PCOS. Oxidative stress and increased androgen levels, which are a hallmark of the syndrome, are known to stimulate LOX activity in other cell types and have been shown to positively correlate with increased AGE levels in our previous studies. Future work will aim at characterizing the complete signal transduction pathway of AGEs–LOX interaction.

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