

Collagen and prostaglandin E₂ regulate aromatase expression through the PI3K/AKT/IKK and the MAP kinase pathways in adipose stromal cells

TING TAN^{1*}, LIE WANG² and BING WANG^{2*}

Departments of ¹Breast Surgery and ²General Surgery, Fuzhou General Hospital of Nanjing Military Region, Fuzhou, Fujian 350025, P.R. China

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Abstract. Excessive local estrogen production in the breast promotes estrogen-dependent breast cancer. Aromatase is a key enzyme in estrogen biosynthesis. Aromatase inhibitors used in the treatment of breast cancer are very effective, but indiscriminately reduce estrogen synthesis in all tissues, causing major side-effects. It is thus desirable to develop inhibitors that selectively block aromatase and estrogen production in breast cancer. To this end, it is important to identify the mechanisms by which aromatase is activated in the tumor microenvironment. Prostaglandin E₂ (PGE₂) and collagen are two important factors in the tumor microenvironment, which contribute to tumor development and progression. In this study, we show that collagen-induced aromatase expression in adipose stromal cells (ASCs) was significantly reduced by inhibitors of phosphatidylinositol 3-kinase (PI3K), I κ B kinase (IKK), mitogen-activated protein kinase kinase (MEK), c-Jun NH₂-terminal kinase (JNK), protein kinase A (PKA), and by the knockdown of the *JunB* and *AKT2* genes. In addition, PGE₂-induced aromatase expression was significantly inhibited by inhibitors of IKK, MEK, JNK, p38 and PKA. These results indicate that the PI3K/AKT/IKK and the mitogen-activated protein (MAP) kinase pathways are involved in collagen- and PGE₂-induced aromatase expression, and also suggest that collagen and PGE₂-induced signaling pathways may crosstalk in regulating aromatase expression. This study enhances our understanding on the mechanism of regulation of aromatase expression by collagen and PGE₂. Furthermore, this study provides a theoretical foundation for the development of specific inhibitors

of aromatase by exploiting the signaling pathways identified herein in the context of breast cancer.

Introduction

Excessive estrogen exposure is a critical risk factor for breast cancer (1-7). While the ovary is the major site of estrogen biosynthesis in premenopausal women, adipose stromal cells (ASCs) in the breast are an important source of locally produced estrogen. Estrogens produced in distal adipose tissue and within the breast tissue influence the growth of breast epithelial cells (8). Especially, excessive local estrogen production in the breast promotes estrogen-dependent breast cancer. At the molecular level, tumor cells secrete factors such as prostaglandin E₂ (PGE₂). PGE₂ stimulates stromal expression of aromatase, a key enzyme in estrogen biosynthesis (9). The breast quadrant bearing a malignant tumor shows consistently high levels of aromatase activity (10), and breast adipose tissue adjacent to the tumor shows a marked increase in aromatase expression and activity (11-13). The clinically proven efficacy of aromatase inhibitors (AIs) in treating estrogen receptor-positive (ER⁺) post-menopausal breast cancer patients highlights the important role of excessive local estrogen production in breast cancer development. However, AIs indiscriminately reduce estrogen synthesis throughout the body, causing major side-effects including bone loss, increased fracture rates, and abnormal lipid metabolism (14-16). Specific inhibitors that selectively inhibit aromatase expression in the tumor microenvironment would considerably benefit patients, by reducing side-effects. In addition, the aromatase promoters I.3 and PII have been reported to be activated in tumor, but not in healthy tissues (8). This implies that specific inhibition of pathways that lead to the activation of these promoters may specifically inhibit aromatase expression in the tumor tissue. In order to identify these pathways, the mechanism of regulation of aromatase expression needs to be elucidated.

Transcription of the aromatase gene is controlled by a number of tissue- and cell type-specific promoters that are located upstream of the aromatase coding region. The coding region of the aromatase transcripts is identical in different tissues, but a noncoding exon 1 is transcribed in a tissue-specific manner and spliced with the common coding

Correspondence to: Dr Bing Wang, Department of General Surgery, Fuzhou General Hospital of Nanjing Military Region, 156 Xi Er Huan Bei Lu, Fuzhou, Fujian 350025, P.R. China
E-mail: wangbing20131031@163.com

*Contributed equally

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exons (9,17-19). In cancer-free breast adipose tissue, the aromatase gene is mainly transcribed under control of the relatively weak I.4 promoter, with a small amount of aromatase mRNA coming from ovary-specific promoters I.3 and PII. However, in ASCs adjacent to the breast tumor, aromatase expression is activated via the proximally-located promoters I.3 and PII (8). The switch in promoter utilization from the weak I.4 to the stronger I.3 and PII results in elevated aromatase expression and excessive production of local estrogen (20,21).

Several lines of evidence indicate that PGE₂ is involved in breast cancer development and progression (22-24). In the tumor microenvironment, PGE₂ activates the protein kinase A and C (PKA and PKC) pathways and induces aromatase expression via promoters I.3 and PII in adjacent ASCs (25). In PGE₂-treated ASCs, the aromatase mRNA level was found to be markedly increased, and phosphorylation of a number of signaling proteins was observed. These signaling proteins include PKA, PKC, transforming growth factor- β -activated kinase-1 (TAK1), mitogen-activated protein kinase kinase 4 (MMK4), c-Jun NH₂-terminal kinase 1 (JNK-1) and mitogen-activated protein kinase (MAPK) p38 (26). This leads to phosphorylation of members of the Jun family of transcription factors and of the activating transcription factor (ATF) 2 protein. The phosphorylated Jun and ATF transcription factors directly bind to the promoter I.3 and PII to regulate aromatase gene expression (26,27). Inhibition of p38 or JNK1 by respective inhibitors effectively blocked PGE₂-induced aromatase expression. Consistently, knockdown of p38, JNK1, JunB or JunD by small interfering RNA (siRNA) also blocked PGE₂-induced aromatase expression (28). Therefore, PGE₂-induced pathways play important roles in regulation of aromatase expression in the tumor microenvironment.

It has been shown that mechanical changes, including elevated extracellular matrix (ECM) stiffness and increased interstitial pressures, are associated with epithelial carcinomas. Moreover, mechanical force due to altered architecture in the tissue microenvironment can affect gene expression patterns (29-34). In breast cancer, mechanical force significantly affects the invasive behavior of tumor cells, as well as breast cancer incidence and mortality (35-39).

In this study, we used a collagen 3D culture system to investigate the signaling pathways involved in regulation of aromatase expression. Mammographically dense breast tissue is linked to increased risk of breast carcinoma (40,41). Areas of increased breast density are not only associated with increased epithelial and stromal cellularity, but also significantly increased fibrillar collagen deposition (42-45). It was also shown that increased stromal collagen in mouse mammary tissue significantly increases tumor formation and results in a significantly enhanced invasive phenotype (46). However, the mechanisms driving collagen-related breast tumor formation and progression remain largely unknown. Initiation of collagen-induced signals is mediated by a batch of cell-surface receptors, including integrins, discoidin domain receptors, glycoprotein VI, leukocyte-associated immunoglobulin-like receptor-1, and members of the mannose receptor family (47). We are interested in two collagen receptors, $\alpha 2\beta 1$ integrin and discoidin domain receptor 1. These two receptors propagate signals through separate, as well as shared pathways. One pathway is initiated by the

integrin-linked kinase (ILK), which passes the signal to the I κ B kinase (IKK) β via phosphatidylinositol 3-kinase (PI3K)/AKT (48-51). Another pathway is the MAPK pathway, including extracellular-signal-regulated kinase (ERK)1/2, JNK and p38 (48-55). It has been shown that IKK β is involved in cell shape-induced aromatase expression, while MAPK pathways are involved in PGE₂-induced aromatase expression (26,28,56). Based on this, we hypothesized that collagen may induce aromatase expression through these pathways and that there may be a crosstalk between collagen- and PGE₂-induced signaling pathways. In this study, we investigated the signaling pathways involved in collagen- and PGE₂-induced aromatase expression. This study will contribute to the understanding of the mechanism of regulation of aromatase expression by collagen and PGE₂. Our study provides useful insights for the design of selective inhibitors of aromatase in the context of breast cancer via the inhibition of specific signaling pathways.

Materials and methods

Cell culture. Primary human ASCs were isolated from individuals undergoing elective surgical procedures at the Department of Breast Surgery, General Hospital of Nanjing Military Region (Fuzhou, China). Informed consent was obtained and this study was approved by the Ethics Committee of the General Hospital of Nanjing Military Region (Fuzhou, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/nutrient mixture F-12 (Gibco Industries, Inc., Big Cabin, OK, USA), supplemented with 10% fetal bovine serum (FBS; Hyclone, Lawrenceville, GA, USA) and 1% antibiotic-antimycotic solution (Gibco Industries, Inc.). The 3D culture was performed in 24-well plates using collagen (collagen bovine type I; BD Biosciences, San Jose, CA, USA). Briefly, 2×10^5 cells were suspended in 125 μ l medium and mixed with 125 μ l collagen. Following the formation of a gel-like 3D structure, additional medium was added to the top.

Chemicals and treatment. PGE₂ was purchased from Cayman Chemicals (Ann Arbor, MI, USA). The PI3K inhibitor LY-294002 was purchased from Sigma-Aldrich (St. Louis, MO, USA). The mitogen-activated protein kinase kinase (MEK)1/2 inhibitor U0126, JNK inhibitor AS601245, p38 inhibitor SB202190, and IKK inhibitor BAY11-7082 were purchased from Calbiochem (La Jolla, CA, USA). The PKA inhibitor H89 dihydrochloride was purchased from Cell Signaling Technology (Danvers, MA, USA). ASCs were serum-starved for 16 h and exposed to collagen or PGE₂ with and without inhibitors for 12 h. Samples were harvested for RNA analysis. Chemical concentrations used in this study are shown in the figure legends.

Quantitative (q) and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Cells in collagen were released by using collagenase (Sigma-Aldrich). Total RNA was isolated using the Invitrogen™ TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The RNA concentration was measured using a NanoDrop2000 (Thermo Fisher Scientific), and RNA was reverse-transcribed using the ImPrompII™ kit

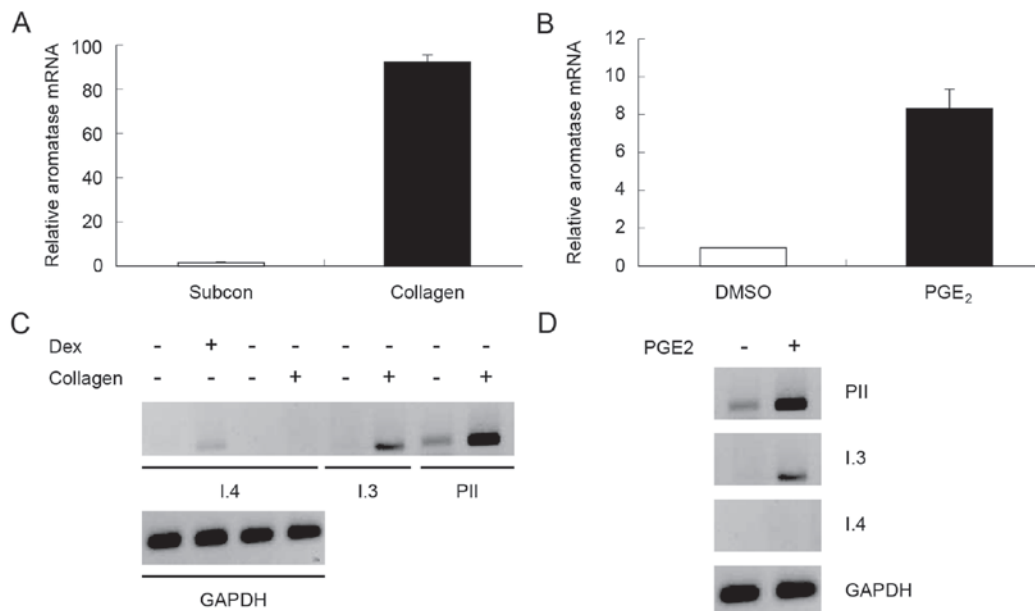


Figure 1. Collagen and prostaglandin E₂ (PGE₂) induce aromatase expression in adipose stromal cells. (A) Relative aromatase mRNA levels measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (A) in the presence of collagen and (B) following PGE₂ (1 μ M) treatment. Data come from three independent measurements for each condition, with bars denoting standard error (SE). * $P < 0.005$ compared to subcon and DMSO, respectively. Subcon, subconfluent culture without collagen; DMSO, dimethylsulfoxide. Promoter utilization was tested by semi-quantitative RT-PCR in the presence of (C) collagen and (D) PGE₂. Dex, dexamethasone, positive control for I.4 activity. It has been shown that Dex activates aromatase expression through the I.4 promoter (49-50).

(Promega, Madison, WI, USA). qPCR was carried out using the fluorescent dye SYBR-Green (Applied Biosystems, Foster City, CA, USA) on an ABI 7900 Real-Time PCR system (Applied Biosystems). The primers for amplification of aromatase were: forward, 5'-TGGAATTATGAGGGCACATCC-3', and reverse, 5'-GTCCAATTCCCATGCAGTAGC-3'. Semi-quantitative RT-PCR was performed using primer pairs that are specific to the promoters I.4, I.3 and PII of the aromatase transcripts. The glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) served as an internal control. The forward primers used were: promoter I.3, 5'-CCTTGTTTTGACTTGTAACCA-3'; promoter I.4, 5'-GTAGAACGTGACCAACTGG-3'; and promoter PII, 5'-GCAACAGGAGCTATAGAT-3'. The reverse primer for all three promoters was 5'-ATTCCCATGCAGTAGCCAGG-3'. The *GAPDH* primers were: forward, 5'-CCATCAATGACCCCTTCATTG-3', and reverse, 5'-GACGGTGCCATGGAATTT-3'. The PCR reaction was performed using reagents from the PCR Master Mix kit (Promega). The PCR cycling conditions were as follows: 94°C for 2 min (1 cycle), 94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min (35 cycles), 72°C for 10 min (1 cycle), and hold at 4°C.

siRNA knockdown. Gene-specific knockdown by siRNA oligonucleotides was conducted using the Invitrogen™ Lipofectamine® RNAiMAX reagent (Thermo Fisher Scientific). Briefly, cells at an approximate density of 60% were transfected with siRNA oligos at a final concentration of 10 nM. Cells were trypsinized 24 h after transfection using 0.25% Trypsin-EDTA (Gibco Industries, Inc.), plated in subconfluent condition, and cultured in collagen. Cells were harvested for RNA analysis 12 h after plating. *c-Jun*, *JunB*, *AKT2* and control siRNA oligos were purchased from Dharmacon, Inc. (Lafayette, CO, USA).

Statistics and data analysis. A paired t-test was used for pair-wise comparisons of chemical-treated cells to control cells. Aromatase mRNA levels from independent measurements were collected for data analysis. The data were analyzed using the SPSS 18.0 software (International Business Machines, Armonk, NY, USA).

Results

Collagen and PGE₂ induce aromatase expression in adipose stromal cells. Collagen is a major component of the ECM and contributes to the formation of mechanical force in the tissue microenvironment. Mechanical force influences cell shape, as well as gene expression patterns (35,53,57,58). Furthermore, IKK β is a downstream signaling molecule of collagen-induced signaling pathways that may play the same role in regulating aromatase expression. Based on these findings, we decided to test whether collagen can induce aromatase expression in ASCs.

Collagen-induced aromatase expression was revealed to be nearly 100-fold higher in ASCs compared to control ASCs that grew on 2D dishes (Fig. 1A). Data from semi-quantitative RT-PCR showed that collagen-induced aromatase expression involves the promoters I.3 and PII (Fig. 1C). PGE₂-treated ASCs also showed induced aromatase expression through promoters I.3 and PII (Fig. 1B and D), in accordance with a previous study (26). In conclusion, both collagen and PGE₂ can induce aromatase expression via promoters I.3 and PII in adipose stromal cells.

MAP kinase pathways are involved in collagen- and PGE₂-induced aromatase expression. It has been shown that collagen activates MAP kinases, including ERK1/2, JNK and p38 (48-55). In addition, MAP kinases are induced by

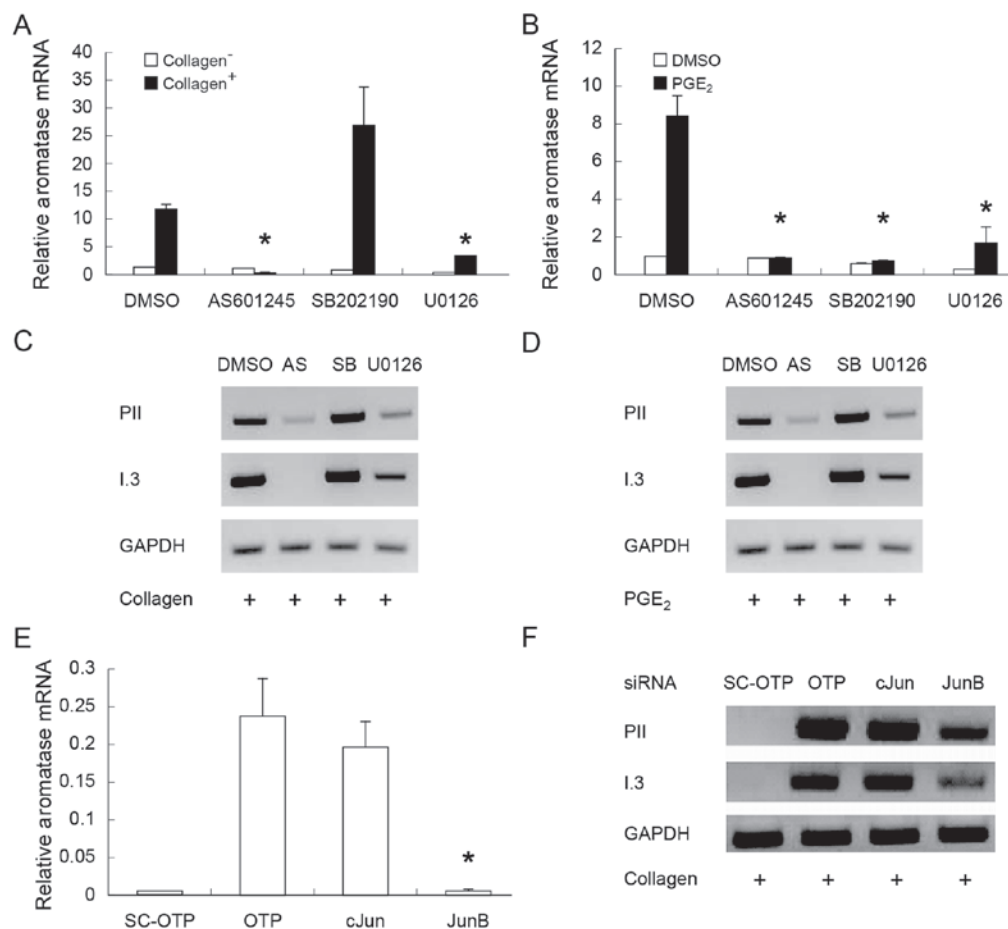


Figure 2. Collagen- and prostaglandin E₂ (PGE₂)-induced aromatase expression is inhibited by mitogen-activated protein (MAP) kinase inhibitors. (A and B) Relative aromatase mRNA level was measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) after drug treatment. AS601245 (or AS; 7.5 μ M), c-Jun NH₂-terminal kinase (JNK) inhibitor; SB202190 (or SB; 5 μ M), p38 inhibitor; U0126 (10 μ M), mitogen-activated protein kinase kinase (MEK) inhibitor. Data come from three independent measurements for each condition. (C and D) Promoter utilization was tested by semi-quantitative RT-PCR. (E) siRNA knockdown of *JunB* significantly reduces collagen-induced aromatase expression, measured by RT-qPCR. The knockdown experiment was performed in the presence of collagen. SC-OTP, subconfluent control; OTP, collagen control; c-Jun, knockdown of *c-Jun*; JunB, knockdown of *JunB* in adipose stromal cells. (F) Semi-quantitative RT-PCR was performed using the knockdown samples. I.3, aromatase promoter I.3; PII, aromatase promoter II. Bars in (A, B and E) denote standard error (SE). * $P < 0.005$ compared to the controls collagen⁺, dimethylsulfoxide (DMSO) and PGE₂, respectively.

PGE₂ in ASCs (26). Furthermore, PGE₂ induces aromatase expression in ASCs through JNK and p38 (26). Therefore, we hypothesized that collagen may induce aromatase expression by activating MAP kinase pathways. Collagen-induced aromatase expression was found significantly inhibited by the MEK inhibitor U0126, which leads to reduced ERK phosphorylation (Fig. 2A). Collagen-induced aromatase expression was also significantly inhibited by the JNK inhibitor AS601245, but not by the p38 inhibitor SB202190. PGE₂-induced aromatase expression was significantly inhibited by all three inhibitors (Fig. 2B). Semi-quantitative RT-PCR confirmed these results (Fig. 2C and D). Therefore, we conclude that the MAP kinases ERK1/2 and JNK are involved in collagen-induced aromatase expression, while ERK1/2, JNK and p38 are all involved in PGE₂-induced aromatase expression.

Since transcription factors of the Jun family act downstream of the MAP kinase pathways, and previous data from our laboratory, as well as other studies showed that the Jun family plays important roles in regulating aromatase expression (26,28,59,60), we knocked down *c-Jun* and *JunB* by siRNA to determine whether these molecules have

an effect on aromatase expression. Knockdown of *JunB* significantly reduced collagen-induced aromatase expression. Knockdown of *c-Jun* did not reduce collagen-induced aromatase expression in our experiments (Fig. 2E). However, we observed that the *c-Jun* knockdown was not as efficient as the *JunB* one in terms of protein expression (data not shown). Semi-quantitative RT-PCR confirmed these results and showed that the *JunB* knockdown affects aromatase expression via both promoters I.3 and PII (Fig. 2F). In conclusion, JunB may be involved in collagen-induced aromatase expression by regulating the aromatase promoters I.3 and PII.

Cell-ECM communications are predominantly mediated by integrins, and integrin-mediated signaling events can activate PI3K/AKT (61,62). On the other hand, AKT is physically associated with IKK β and can trigger phosphorylation of IKK in response to inflammatory factors such as tumor necrosis factor- α (63,64). We therefore hypothesized that AKT may play an important role in passing signals from ECM to downstream IKK, and thus, in activating aromatase expression. There are three *AKT* genes in the human genome: *AKT1*, *AKT2* and *AKT3*. These genes code for enzymes that belong to the serine/threonine-specific protein kinase

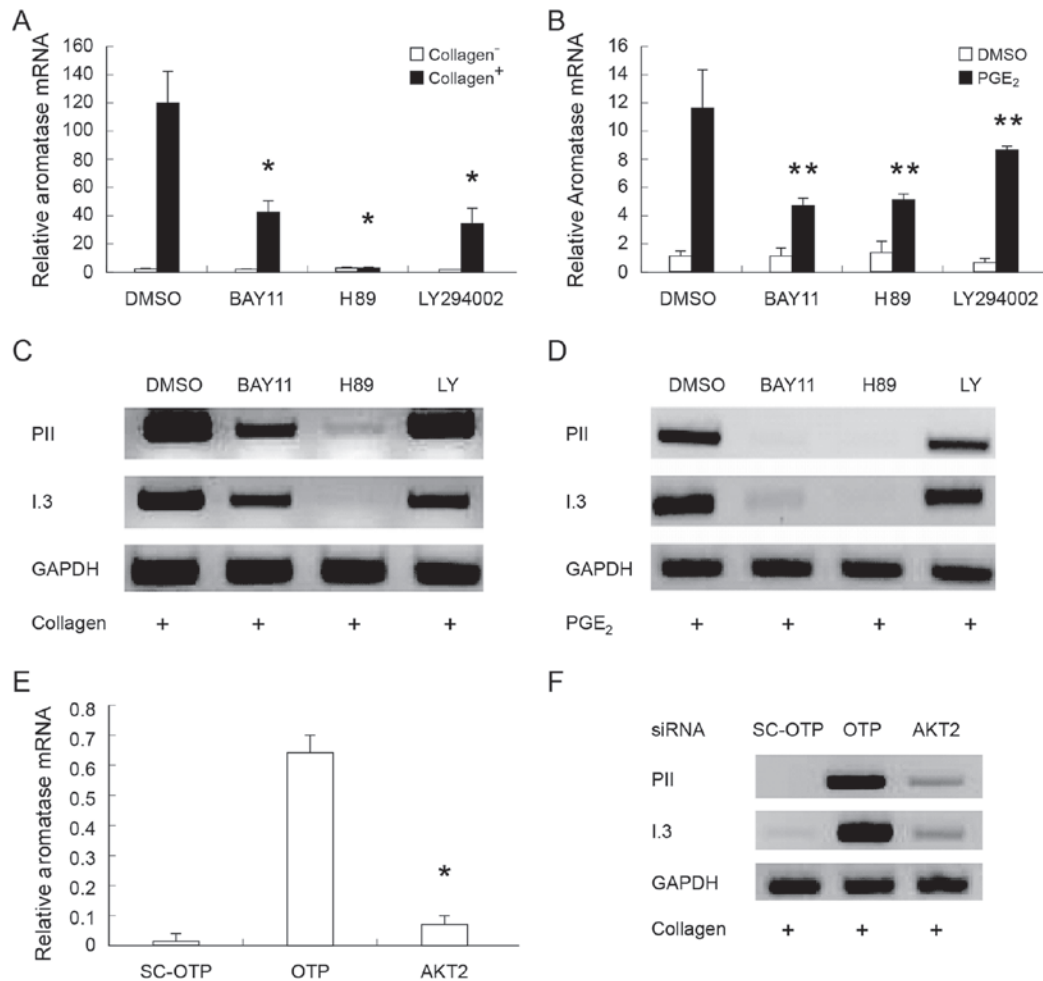


Figure 3. Collagen- and prostaglandin E₂ (PGE₂)-induced aromatase expression is inhibited by the IκB kinase (IKK), protein kinase A (PKA) and phosphatidylinositol 3-kinase (PI3K) inhibitors. (A and B) The relative aromatase mRNA level was measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) after drug treatment. Data come from from three independent measurements for each condition. BAY11-7082 (5 μM), IKK inhibitor; LY294002 (or LY; 20 μM), PI3K inhibitor; H89 (20 μM), PKA inhibitor. (C and D) Promoter utilization was tested by semi-quantitative RT-PCR. (E) *AKT2* knockdown significantly reduces collagen-induced aromatase expression, measured by RT-qPCR. The knockdown experiment was performed in the presence of collagen. SC-OTP, subconfluent control; OTP, collagen control; AKT2, knockdown of *AKT2* in adipose stromal cells. (F) Semi-quantitative RT-PCR was performed using the knockdown samples. I.3, aromatase promoter I.3; PII, aromatase promoter II. Bars in (A, B and E) denote standard error (SE). *P<0.005 and **P<0.05, compared to the controls collagen⁻, dimethylsulfoxide (DMSO) and PGE₂, respectively.

family (65-67). Knockdown of *AKT2* significantly reduced collagen-induced aromatase expression (Fig. 3E). Data from semi-quantitative RT-PCR confirmed these results and showed that knockdown of *AKT2* affects both promoters I.3 and II (Fig. 3F). These data indicates that collagen induces aromatase expression at least partly via the PI3K/AKT/IKK pathway.

Next, we tested whether PKA is involved in collagen-induced aromatase expression, since it has been shown to be involved in PGE₂-induced aromatase expression (26,68). Our data confirmed the important role of PKA in regulating PGE₂-induced aromatase expression (Fig. 3B). In addition, our data showed that the PKA inhibitor H89 significantly inhibits collagen-induced aromatase expression (Fig. 3A). The RT-qPCR results were confirmed by semi-quantitative RT-PCR (Fig. 3C and D). Taken together, these findings strongly suggest that PKA plays an important role in PGE₂-, as well as in collagen-induced aromatase expression.

Discussion

Excessive exposure to estrogen is a critical risk factor for breast cancer, while aromatase is a key enzyme controlling estrogen production. Currently, AIs are the most effective endocrine treatment for breast cancer (8,69). However, AIs indiscriminately reduce estrogen synthesis throughout the body, causing major side-effects (14-16). To address this issue, new drug targets need to be identified, allowing to selectively block aromatase expression in the tumor microenvironment. To this end, it is important to dissect the mechanisms by which aromatase expression is regulated. Data from this study showed that collagen-induced aromatase expression in ASCs is significantly reduced by inhibitors of PI3K, IKK, MEK, JNK, PKA and the knockdown of the *JunB* and *AKT2* genes. These findings indicate that the PI3K/AKT/IKK, as well as the MAP kinase pathways play important roles in collagen-induced aromatase expression. On the other hand, PGE₂-induced aromatase expression was significantly

reduced by inhibitors of IKK, MEK, JNK, p38 and PKA. Data from semi-quantitative RT-PCR showed that collagen and PGE₂ induce aromatase expression through the aromatase promoters I.3 and PII. In addition, the semi-quantitative data were consistent with quantitative RT-PCR data in the drug treatment and the knockdown experiments.

Data from this study also indicated that there is a crosstalk between collagen and PGE₂-induced signaling pathways in regulating aromatase expression. Signaling molecules that are responsible for this crosstalk could serve as potential drug targets. Signals induced by both PGE₂ and collagen could be blocked by hitting the targets that are common to both pathways involved in regulating collagen and PGE₂-induced expression, which may effectively silence the aromatase promoters. In addition to these shared-between-pathways molecules, our data also highlighted a number of proteins that are unique to one of the two aromatase-activating pathways. For example, p38 plays a critical role in PGE₂-induced but not in collagen-induced aromatase expression. PI3K is another example, since this protein plays a critical role in collagen-induced, but not in PGE₂-induced aromatase expression.

In the present study, PGE₂-induced aromatase expression was inhibited by a MEK inhibitor. This indicates that the ERK pathway may be involved in regulating aromatase expression in response to PGE₂ induction. Data from another study showed that the same MEK inhibitor has no effects on PGE₂-induced aromatase expression (26). This discrepancy could be due to the use of different cells in the two studies. Since regulation of gene expression is complex, the signaling pathways involved in regulating expression are only one facet of the process. Other factors likely contribute to aromatase expression as well. For instance, the DNA methylation status of the aromatase promoter region also plays important roles in regulating aromatase expression. In another project of our research team, a DNA methylation assay was performed to test the methylation status of aromatase promoters in a batch of adipose stromal cells. Our data showed that the highest DNA methylation load of certain CpG sites corresponds to the lowest aromatase activation. Reduction of the DNA methylation load by drug treatment restored aromatase responsiveness to forskolin activation (unpublished data). Another study also showed that CpG dinucleotide methylation of the aromatase promoter modulates cAMP-stimulated aromatase activity (70).

This study elucidated the signaling pathways involved in collagen- and PGE₂-induced aromatase expression in adipose stromal cells. It is thus expected to shed light on the mechanisms of aromatase activation in response to collagen, as well as PGE₂. This study provides an opportunity to test whether breast cancer can be effectively treated by selectively blocking aromatase expression via the inhibition of the specific signaling pathways identified herein. Further investigation is however needed to identify the downstream transcription factors and additional molecular targets for selective inhibition of aromatase expression.

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