Original Article
17β-estradiol activates mTOR in chondrocytes by AKT-dependent and AKT-independent signaling pathways

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Abstract: To confirm whether 17β-estradiol (E2) activates mammalian target of rapamycin (mTOR) signaling pathway in chondrocytes and in what way activates mTOR. Human immortalized chondrocytes cell lines TC28a2 and C28/I2 were subjected to incubate with or without E2, LY294002 (the inhibitor of PI3K), rapamycin (the inhibitor of mTOR), or E2 in combination with LY294002 or rapamycin. Thereafter, protein levels of S6K1, p-S6K1, protein kinase B (AKT), and p-AKT were determined by Western blot analysis. Matrix metallopeptidase (MMP) 3 or MMP13 mRNA levels were evaluated by quantitative real-time PCR (qRT-PCR). Co-immunoprecipitation and Western blot analysis were performed to verify the interaction between ERα and mTOR. Both p-S6K1 and p-AKT protein levels in TC28a2 and C28/I2E2 cells were significantly increased by incubation with E2 (0.5 h and 1 h) ($P < 0.05$). Rapamycin did not affect the levels of p-AKT, but were significantly reduced by LY294002 or E2 in combination with LY294002. The levels of p-S6K1 were significantly decreased by incubation with LY294002, but the effect could be reversed by E2 in combination with LY294002. Rabbit anti-mTOR antibody was able to immunoprecipitate ERα after incubation with E2. Moreover, E2 inhibited the mRNA levels of MMP3 and MMP13 by mTOR pathway. E2 actives mTOR in chondrocytes through AKT-dependent and independent ways.

Keywords: 17β-estradiol, mTOR, chondrocytes, AKT-dependent and AKT-independent pathways

Introduction

Osteoarthritis (OA) is a chronic, degenerative, skeletal and progressive joint disorder that commonly affects the knee joint, leading to joint tissue damage and physical disabilities [1]. The degeneration of articular cartilage caused by the death of chondrocytes and the loss of extracellular matrix is the major pathological hallmark of OA. Chondrocytes are involved in the synthesis, organization and homeostatic maintenance of cartilage matrix by producing diverse cytokine and chemokine receptors, matrix-anabolic/catabolic enzymes or inhibitors such as matrix metalloproteinases (MMPs), tissue inhibitor of metalloproteinases (TIMPs) and aggrecanases [2].

Multiple signaling pathways have been reported to be responsible for the pathogenesis of OA. Thus, increased emphasis is now being given to obtain a better understanding of the regulatory mechanism of major signaling pathways in OA chondrocytes, which will facilitate the prevention and design of targeted therapies of OA. The mammalian target of rapamycin (mTOR) pathway, a serine-threonine protein kinase, is a downstream target of the phosphatidylinositol 3-kinase-related kinase (PI3Ks) and protein kinase B (AKT) pathway [3-5]. It has been demonstrated that mTOR plays significant roles in controlling many critical cellular processes, including cell growth, motility, survival, proliferation, protein synthesis, and transcription [6, 7]. Recent studies have shown that
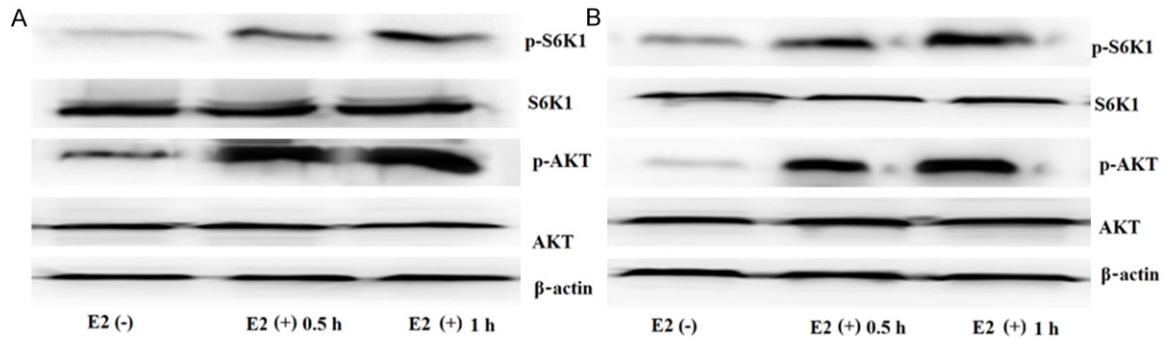
Figure 1. The effects of E2 on the activity of mTOR signaling pathways. A. E2 activates the activity of mTOR signaling pathway in TC28a2 cells at different time points (0.5 h and 1 h); B. E2 activates the activity of mTOR signaling pathway in C28/I2E2 cells at different time points (0.5 h and 1 h). E2, 17β-estradiol; mTOR, mammalian target of rapamycin; AKT, protein kinase B.

mTOR is involved in the growth, development, homeostasis of articular cartilage, as well as the process of cartilage degeneration [6, 8-10]. Pharmacological inhibition and cartilage-specific deletion of mTOR have been shown to protect from OA [11, 12]. Moreover, several studies suggest that estrogen deficiency is associated with the onset or progression of OA and both endogenous and exogenous estrogens have a potential protective effect against OA [13, 14]. Previous studies have showed that 17β-estradiol (E2) and its receptor could promote cell proliferation [15], decrease the cartilage damage in experimental arthritis models [16, 17] and prevent spontaneous or substance-induced apoptosis in chondrocytes [18]. However, the mechanism of E2 in OA chondrocytes remains unclear. Whether E2 and its receptor activate mTOR signaling pathway and in what way E2 and its receptor can activate mTOR are unclear.

Therefore, the purpose of our study was to investigate the effect of E2 and its receptor on chondrocytes, as well as the possible underlying mechanism. This new molecular mechanism might reveal the physiological effects of estrogen on the chondrocytes. Our study might provide insight into mTOR as a potential target for OA therapy.

Material and methods

Cell culture and treatments

Human immortalized chondrocytes cell lines TC28a2 and C28/I2 were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). They were cultured in Dulbecco Modified Eagle Medium (DMEM)/F12 (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO), L-glutamine (Life Technologies, Rockville, MD), 100 U/mL penicillin (Life Technologies), 100 µg/mL streptomycin (Life Technologies) in 5% CO_2 at 37°C. After culture for 24 h, TC28a2 and/or C28/I2 cells were subjected to incubate with or without 10 ng E2 (Sigma-Aldrich, St Louis, MO, USA) 0.5 h or 1 h, 10 µm LY294002 (Sigma-Aldrich), 20 nm rapamycin (Sigma-Aldrich), E2 in combination with LY294002, or E2 in combination with rapamycin.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using TRIZOL Reagent (Life Technologies) according to the manufacturer's instruction. First strand complementary DNA (cDNA) was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). MMP3 or MMP13 mRNA transcription was evaluated by qRT-PCR on the Bio-Rad Connet Real-Time PCR platform (Bio-Rad) with SYBR Green PCR core reagents using the following conditions: initial denaturation at 95°C for 1 min, 40 cycles of denaturation at 95°C for 5 s, annealing extension at 60°C for 20 s. Data of selected genes was analyzed using $2^{-\Delta\Delta Ct}$ statistical method. GAPDH was used as an internal reference control.

Co-immunoprecipitation

For co-immunoprecipitation analysis, protein G Sepharose Fast Flow beads (GE Healthcare, Uppsala, Sweden) were incubated with anti-
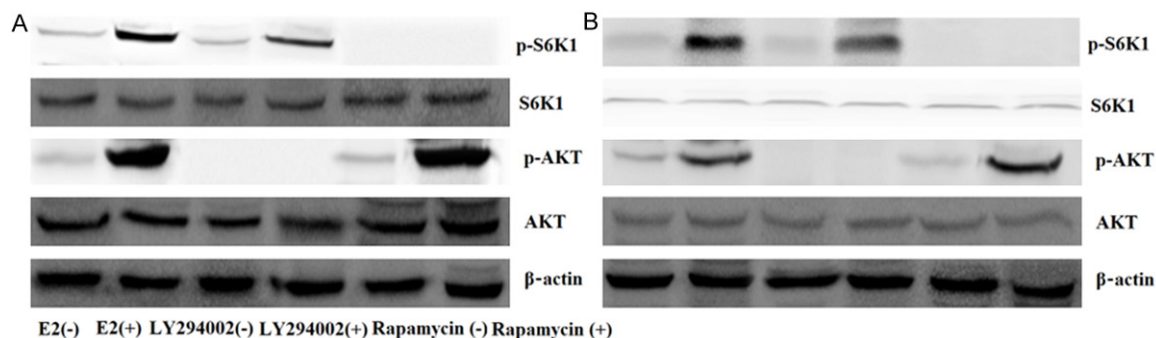
Figure 2. E2 activates the activity of mTOR independently of AKT. A. E2 activates the activity of mTOR independently of AKT in TC28a2 cells; B. E2 activates the activity of mTOR independently of AKT in C28/I2E2 cells. E2, 17β-estradiol; mTOR, mammalian target of rapamycin; AKT, protein kinase B.
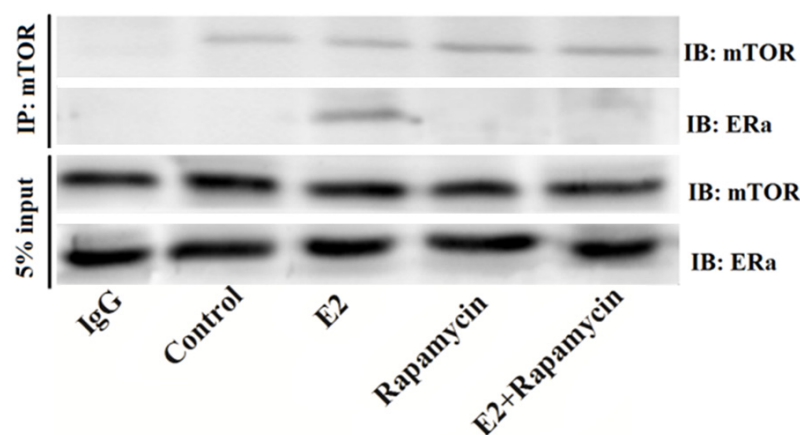

Figure 3. ERα interacts with mTOR complex. E2, 17β-estradiol; ERα, estrogen receptor α; mTOR, mammalian target of rapamycin; IB, immunoblotting ERα.

tion. Total protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as a standard. Then the samples were separated by 10-12% SDS-PAGE as procedure, transferred onto nitrocellulose membranes, and blocked with 5% (w/v) skimmed milk powder in PBS/0.1% Tween 20 for 2 h, and incubated with the following primary antibodies at 4°C overnight: anti-S6K1 antibody, anti-p-S6K1 antibody, anti-AKT antibody, and anti-p-AKT antibody. All antibodies were purchased from Cell Signaling Technology Inc (Beverly, MA). β-actin was used as a control. Membranes were then incubated with appropriate secondary antibodies for 2 h. Immunoreactive proteins were visualized with enhanced chemiluminescence and densitometric analysis according to the manufacturer's instructions.

mTOR (Cell Signaling Technology Inc, Beverly, MA) or normal rabbit IgG (Sigma-Aldrich) antibodies in phosphate buffered saline (PBS) for 1 h at room temperature. The cells were lysed in 250 µl lysis buffer containing protease inhibitors (Complete mini protease inhibitors, Roche Applied Science). Immune complexes were precipitated with Protein G Sepharose beads for 1 h at 4°C, washed with lysis buffer, and then incubated with loading buffer at 65°C for 5 min, and separated on 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed as previous described.

Western blot analysis

Chondrocytes were rinsed three times with Hank's solution, seeded into 6-well tissue culture plates, and treated with E2, LY294002, or rapamycin and followed by cell protein extrac-

Statistical analysis

The collected data were expressed as mean ± standard deviation (SD). All statistical analyses were performed using statistical package for the social sciences (SPSS, 13.0 Inc., Chicago, IL) software. The differences between groups were compared using Student's t-test or one-way analysis of variance (ANOVA). Statistically significant difference was accepted at $P < 0.05$.
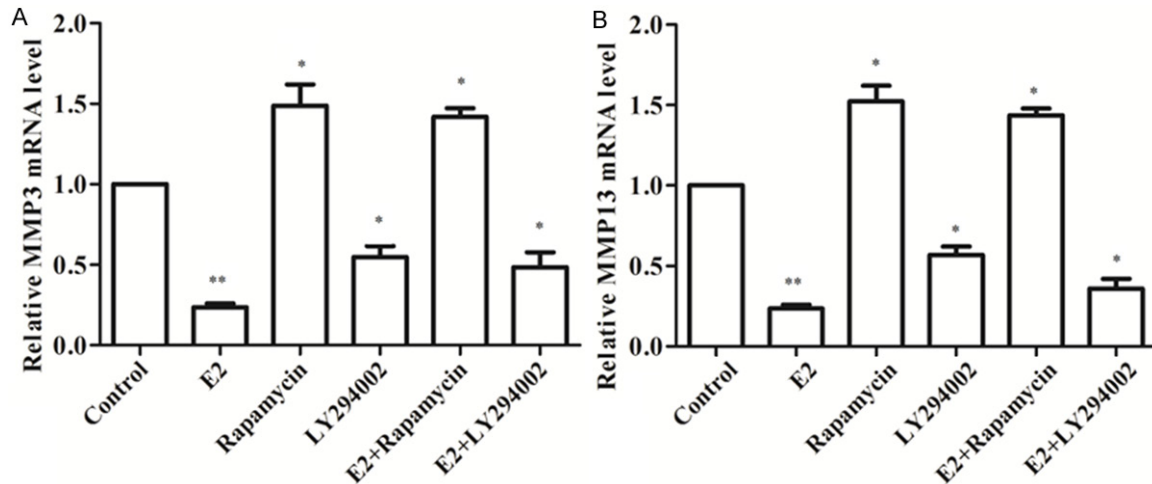
Figure 4. E2 inhibits the mRNA levels of MMP3 and MMP13 by mTOR pathways. A. Relative MMP3 mRNA level; B. Relative MMP13 mRNA level. E2, 17β-estradiol, MMP, matrix metallopeptidase; mTOR, mammalian target of rapamycin. **$P < 0.01$ compared to the control group. *$P < 0.05$ compared to the control group.

Results

E2 activates the activity of mTOR signaling pathway

To explore the effects of E2 on chondrocytes, human immortalized chondrocytes cell lines TC28a2 and C28/I2E2 were incubated without or with E2 (0.5 h and 1 h). The protein levels of p-S6K1, S6K1, p-AKT, and AKT were evaluated after incubation with or without E2. The results showed that both p-S6K1 and p-AKT protein levels were significantly increased by incubation with E2 (0.5 h and 1 h) compared to without incubation with E2 in both TC28a2 and C28/I2E2 cells (all $P < 0.05$). In addition, the levels of p-S6K1 and p-AKT were both statistically higher when incubated with 1 h than those when incubated with 0.5 h (**Figure 1A** and **1B**).

E2 activates the activity of mTOR independently of PI3K-AKT-mTOR

To investigate in what way E2 activates the activity of mTOR, we incubated TC28a2 and C28/I2E2 cells with or without E2, LY294002 (the inhibitor of PI3K) or rapamycin (the inhibitor of mTOR). As shown in **Figure 2A** and **2B**, there was no significant differences in the protein levels of p-AKT between incubation with E2 and E2 in combination with rapamycin, indicating that rapamycin did not affect the levels of p-AKT. However, the protein levels of p-AKT were significantly reduced by LY294002 or E2 in combination with LY294002, suggesting that inhibition of PI3K could significantly affect the

expression of downstream target protein. Although the protein levels of p-S6K1 were significantly decreased by incubation with LY294002, the effect could be reversed by E2 in combination with LY294002, demonstrating that E2 activates mTOR not dependently through PI3K-AKT-mTOR, there might be other pathways.

ERα interacts with mTOR

The observation of co-localization of ERα and the mTOR complex increased the possibility that these proteins may directly interact. Co-immunoprecipitation experiment using whole cell lysates of TC28a2 cells was performed to probe for ERα and mTOR complex interactions. Normal rabbit IgG did not immunoprecipitate ERα, demonstrating the co-immunoprecipitation of ERα was not due to nonspecific antibody binding. Rabbit anti-mTOR antibody was able to immunoprecipitate ERα after incubation with E2, but was unable to immunoprecipitate ERα after incubation with rapamycin or incubation with E2 in combination with rapamycin, suggesting that E2 activates mTOR partly due to the direct interactions between ERα and mTOR complex (**Figure 3**).

E2 inhibits the mRNA levels of MMP3 and MMP13 by mTOR pathway

To further elucidate the effect of E2 on chondrocytes by mTOR signaling pathway, the mRNA levels of MMP3 and MMP13 were assessed

after treatment with E2, LY294002, rapamycin, E2 in combination with LY294002, or E2 in combination with rapamycin. As indicated in **Figure 4A** and **4B**, both the mRNA levels of MMP3 and MMP13 were significantly decreased by E2 ($P < 0.01$), LY294002 ($P < 0.05$), and E2 in combination with LY294002 ($P < 0.05$), while were statistically increased by rapamycin ($P < 0.05$) and E2 in combination with rapamycin ($P < 0.05$). These results suggested that E2 inhibited the mRNA levels of MMP3 and MMP13 by mTOR pathway.

Discussion

The chondrocytes play an important role in the normal function of the articular cartilage, as well as in the repair of joint cartilage defects. Estrogen has been reported to provide protection against OA. However, the molecular mechanism of estrogen on chondrocytes is still unclear. In the present study, we find that mTOR could be activated by at least two different mechanisms by E2 in chondrocytes. In addition to the classical AKT-mTOR signaling pathway, there might be a direct function on mTOR, which can inhibit the expression of MMP3 and MMP13. This new molecular mechanism reveals the physiological effects of estrogen on chondrocytes. It is also suggests that mTOR might be as a potential target for the therapy of OA.

Estrogen is an important regulator of bone formation. When the levels of estrogen are increased, impaired periosteal expansion is observed [19]. Conversely, when estrogen levels are decreased, radial expansion restarts, indicating that apposition of periosteal bone could be prevented by estrogen [20]. Also, estrogen has been reported to inhibit exercise-induced periosteal expansion [21, 22]. The effective mechanisms for estrogen on preventing bone formation are through distinct receptor isoforms. The biological actions of estrogen are regulated by two receptor subtypes, ERα and ERβ, which belong to the superfamily of nuclear receptors [23]. They are both located mainly in the cytoplasm and expressed in many tissues of the body with different proportions in each tissue. In joint tissues, ERα and ERβ are both expressed by the chondrocytes [24], synoviocytes [25], subchondral bone cells [26], myoblasts [27], and ligament fibroblasts [28].

However, ERα predominates in cortical bone and while ERβ is mainly seen in cartilage, synovium, and cancellous bone [29]. In addition to reproductive system, estrogen produces pleiotropic effects on non-sexual tissues through direct and indirect mechanisms, and estrogen deletion is associated with a variety of age-related conditions [30]. OA is a multifactorial disease with gender and age as risk factors, suggesting that OA increases with age and the prevalence of OA is higher among women than men, especially among women in menopause [31]. In fact, women older than 50 years demonstrate a significantly higher prevalence compared to men at the same age, and those who are treated by an estrogen replacement therapy (ERT) show reduced cartilage loss [32].

The impact of E2 on chondrocytes is still unclear. Previous studies have suggested that the direct chondro-protective role of estrogen. For example, E2 decreases mechanical injury-related cell death and proteoglycan degradation in mature cartilage [33], increases glycosaminoglycan synthesis [34], inhibits cyclooxigenase-2 mRNA expression [35], and stimulates expression of osteoprotegerin (OPG) via ERα [36]. In addition, many signaling pathways including mTOR have been reported to be involved in the progression and development of OA, and now considered as a potential therapeutic target for OA [6]. mTOR signaling is also implicated in the proliferation of estrogen-induced osteoblast [37]. Therefore, we speculated that mTOR signaling is activated by estrogen-induced chondrocytes. To confirm the assumption, we first incubated immortalized chondrocytes cell lines TC28a2 and C28/I2 without or with E2 and determined the related protein levels of AKT-mTOR signaling pathway (p-S6K1, S6K1, p-AKT, and AKT). Our results showed that AKT-mTOR signaling pathway was significantly activated by incubation with E2, with time-dependent potentials (0.5 h and 1 h). The results were similar with Huang *et al.* who found that chondrocyte proliferation could be induced by E2, and the expression levels of AKT, especially the expression levels of p-AKT were increased by E2 [15]. Then we further investigated the possible mechanism for activation of mTOR by E2. In addition to incubation with E2, the inhibitor of PI3K (LY294002) or the inhibitor of mTOR (rapamycin) were incubated with TC28a2 and C28/I2. The results showed

that rapamycin had no effect on the levels of p-AKT, but inhibition of PI3K could only partly influence the activation of mTOR by E2, demonstrating that E2 activates mTOR independently through PI3K-AKT-mTOR, there might be other involved pathways. Co-immunoprecipitation and Western blot were then performed to probe for ERα and mTOR complex interactions. The results showed that anti-mTOR antibody was able to immunoprecipitate ERα by incubation with E2, indicating that E2 could stimulate the activation of mTOR partly because of the direct interactions between ERα and mTOR complex.

An important characteristic of OA is the deterioration of articular cartilage, which is featured by the breakdown of matrix proteins via MMPs. Several extracellular matrix (ECM) molecules such as cartilage proteoglycan and type II collagen can be degraded by MMPs. MMP1 (collagenase 1) and MMP13 (collagenase 3) play key roles in OA. Previous study showed that 17β-estradiol significantly reduced the expression of MMP1, MMP3, and MMP 13 in human primary articular chondrocytes [38]. In line with the above research, our study also showed that both the mRNA levels of MMP3 and MMP13 were significantly decreased by E2, LY294002, and E2 in combination with LY294002, and while were statistically increased by rapamycin. The results indicated that the mRNA levels of MMP3 and MMP13 could be inhibited by E2 through mTOR pathway.

In conclusion, mTOR could be activated by E2 in chondrocytes via at least two different mechanisms: AKT-dependent and AKT-independent signaling pathways. However, further studies should be performed to confirm the specific mechanism that how ER interplays with mTOR and that how ER transfers and combines with mTOR.

Disclosure of conflict of interest

None.

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