Original Article

Anti-osteoporotic roles of protein kinase C in Kang-shu Jian-gu granules

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Abstract: Purpose Objective: The aim of this study was to explore the ability of Kang-shu Jian-gu granules to enhance bone density in a rat model of osteoporosis, to determine the molecular mechanisms of the anti-osteoporotic effects of Protein Kinase C (PKC) in vivo, and to provide reference information for the clinical treatment of osteoporosis. Methods: A rat model of osteoporosis was established and treated with Kang-shu Jian-gu (KSJG) granules (1.428 g/kg). Bone mineral density (BMD), serum calcitonin (CT), osteocalcin (BGP), serum calcitonin (CT), osteocalcin (BGP), and bone calcium values were monitored. Protein expression of PRKCA and PRKCB of kidneys and bones was evaluated by Western blotting. Biological functions, signaling pathways, and key proteins involved with PRKCA and PRKCB proteins in reversing osteoporosis were identified using the STRING database. Results: Treatment with KSJG granules significantly increased levels of CT, BGP, BMD, bone calcium, and bone-specific alkaline phosphatase (BALP) \((P < 0.05)\). Compared with the sham-surgery group (controls), expression of PRKCA and PRKCB proteins was significantly lower than that in the ovariectomy group (OVX) \((P < 0.05)\). Relative expression levels of PRKCA and PRKCB, in kidneys and bones, were positively correlated with bone calcium levels \((r^2 = 0.7762, r^2 = 0.6033, \text{respectively})\), but negatively correlated with BALP \((r^2 = 0.8746, r^2 = 0.8166, \text{respectively})\) \((P < 0.05)\). There was direct interaction of PRKCA with PLD1, ARHGDIA, PLD2, RHOA, RAC1, MAP2K1, BRAF, PLCB1, and PICK1. PRKCB interacted directly with PIK3CG, RPS6KB1, and MTOR. PRKCA and PRKCB were involved principally in estrogen receptor signaling pathways. Conclusion: Kang-shu Jian-gu granules can enhance bone mineral density in a rat model of osteoporosis. Protein expression of PRKCA and PRKCB can be regulated by Kang-shu Jian-gu granules, which mediate bone cell differentiation and enhance bone density.

Keywords: Protein kinase C, anti-sparse bone granule, molecular mechanisms, bioinformatics

Introduction

Osteoporosis (OP) is a generalized skeletal disease characterized by low bone mass and degradation of bone tissue microstructure. Clinically, approximately 25%-50% of postmenopausal women are diagnosed with osteoporosis [1-5]. Protein kinase C (PKC) is one family in a group of protein kinases. PKC in mammalian cells can be divided into three categories in terms of structure and cofactors [6-8]. PRKCA and PRKCB belong to classical PKC. Studies have reported that a positive correlation exists between expression of PKC in kidney tissues and bone mineral density (BMD), suggesting that the manifestation of osteoporosis may be related to the PKC family and related pathways [9-11]. However, the molecular mechanisms by which PKC affects bone density remain unclear. Kang-shu Jian-gu is a typical Chinese patent drug, applied clinically to treat OP by practitioners of Traditional Chinese Medicine. Its mode of action is “kidney-based, combined with invigoration of the spleen”. Several studies have shown that it provides positive treatment effects on OP. However, its mechanisms of action are not fully understood [12-15].

Previous in vitro studies have reported that the number of MC3T3-E1 osteoblast-like cells increased significantly and the membrane and cytoplasm protein kinase C (PRKC) activity improved significantly following treatment with the drug. Results suggest that PKC may be involved in osteoblast differentiation [16].
The current study first treated rats with Kang-shu Jian-gu granules after ovariectomies, observing protein expression of PRKCA and PRKCB in bone and renal tissue. Comprehensive bioinformatics analysis was conducted, concentrating on the biological functions of PRKCA and PRKCB, aiming to explore the possible mechanisms through which Kang-shu Jian-gu granules potentially exert changes to PRKCA and PRKCB expression. In combination with bioinformatics analysis, this study also investigated the molecular networks and possible osteoblast-related differentiation pathways associated with PRKCA and PRKCB. This current study identified molecular targets that Kang-shu Jian-gu granules potentially influence, providing perspective for further exploration of the mechanisms involved in the treatment of postmenopausal OP.

Materials and methods

Animals

A total of 30 unfertilized female SPF level Sprague-Dawley (SD) rats, weighing 150 g-210 g, were provided by the Experimental Animal Center of the Fourth Military Medical University (license number SCXK (Shaan) 2015-008). They were housed in a standard environment (indoor temperature: 23 ± 4°C, humidity 60%-70%) with ad libitum access to food and water.

Experimental model

The postmenopausal osteoporosis model was established using the classic bilateral ovariectomy technique. After 2 weeks of adaptive feeding, ovariectomies were performed on the rats after fasting for 24 hours. The rats were anesthetized with 10% chloral hydrate at a dosage of 0.3 mL/100 g. Bilateral dorsal incisions were then made along both sides of the lumbar vertebrae on the dorsolateral surfaces to expose the dorsolateral abdominal muscles. Both ovaries were identified, ligated, and removed. The abdominal cavity was washed with saline and the incisions in the muscle and skin were sutured. Sham surgery was performed on control animals involving visualization of the ovaries without ligation or removal. Following surgery, the animals were treated with iodine at the incision site to prevent postoperative infections. The rats were injected intramuscularly with penicillin (200,000 units) once a day for 3 days. All experimental rats were housed in a standard environment at an indoor temperature of 23 ± 4°C in well-ventilated conditions. They were given ad libitum access to food and water for 12 weeks.

Experimental groups and administration of the drug

Thirty SD female rats were randomly divided into a sham-surgery group (controls), ovariectomy group (OVX), and an ovariectomy plus Kang-shu Jian-gu granule group (OVX+KSJG), with ten rats per group. All groups were provided with appropriate treatment for a total of 12 weeks. They were under weekly continuous drug administration lasting 6 days. This was followed by a one-day break.

Drug treatment

Dry extract powder of Kang-shu Jian-gu was provided by Shaanxi Provincial Key Laboratory of Traditional Chinese Medicine and new drugs. Each gram of the purified drug, composed of Epimedium, Achyranthes, Eucommia, Drynaria, Atractylodes, and Salvia, was equivalent to 2.83 g of crude drug granule. Animals in the KSJ group were treated with Kang-shu Jian-gu granules, dissolved in distilled water, through oral gavage at a dosage of 1.428 g/kg. Rats in the control and OVX groups were treated with 2 mL daily saline infusion through oral gavage. All experimental groups were treated for 6 contiguous days. This was followed by 1 day of rest, for a total of 12 weeks.

Methods

Sample collection

After 12 weeks of treatment, the rats were sacrificed. They were sacrificed after 24 hours of fasting following the final administration of drug or saline. Rats were anesthetized with 10% chloral hydrate under aseptic conditions. Renal and femoral tissues were removed, including muscle and surrounding tissue on the femurs which were bluntly removed using sterile surgical gauze. The resulting specimens were placed in EP tubes and stored in a -70°C freezer.

Measurement of indicators related to bone development

Bone mineral density (BMD): Bone tissue was placed under the probe of a dual-energy X-ray
Absorptiometry (DEXA) instrument to measure BMD.

**Serum calcitonin (CT) and osteocalcin (BGP):** After 12 weeks of KSJG treatment, rats in each group were decapitated and the serum was separated from blood. Levels of serum calcitonin (CT) and osteocalcin (BGP) were determined by radioimmunoassay (Biosharp, Shanghai, China).

**Measurement of bone Calcium and BALP (Bone Alkaline Phosphatase):** The bones were treated in an oven at 100°C for 24 hours. The resultant bone ash was diluted with deionized water, then tested for calcium concentrations. BALP was measured by enzyme-linked immunosorbent assay (ELISA) (Shanghai, China).

**Western blotting**

Tissue lysate was prepared by grinding 200 mg renal tissue in 2 mL cold lysis buffer. Homogenized tissue was lysed twice, for 12 seconds each time, then centrifuged. The resulting supernatant was tested using a bicinchoninic acid (BCA) assay to measure protein concentrations, as follows. Protein standards or samples (100 μL) and sample buffer (40 μL) were mixed in 1.5 mL micro-centrifuge tubes. They were then incubated in a water bath for 5 minutes at 100°C. After separation by SDS-PAGE (in either a concentrated gel at a concentration of 5% or separation gel at a concentration of 10%), the protein was transferred to PVDF membranes using the wet-tank method. They were then incubated with the appropriate diluted primary antibody overnight. The following day, membranes were washed with 1XTBST 3 times, 8 minutes each time. They were then incubated with diluted secondary antibody for one hour. The membranes were again washed in 1XTBST 3 times, then stained with DAB. An X-ray film was placed on the membranes and exposed for 5 minutes before development.

**Hematoxylin and eosin (HE) staining**

Paraffin-embedded bone tissues were sequentially sliced into sections of 4 μm and heated to 60°C overnight. After dewaxing with xylene (Biosharp, Beijing, China) and dehydration through a gradient of ethanol concentrations, the bone sections were stained with hematoxylin and eosin (HE) for 3 minutes. This was followed by dehydration and clearing prior to finally being sealed with a neutral resin. Image-Pro Plus IPP (Mediaplayer, NY, USA) was used to analyze the images. Four tissue sections from each rat were selected and a total of 20 fields (5 fields for each section) were analyzed.

**Bioinformatics analysis**

Network analyst software (http://www.networkanalyst.ca/) was used to analyze the target networks of PRKCA and PRKCB, the therapeutic drugs, and their associated protein-protein interaction networks. Gene Ontology (GO) Enrichment Analysis and Protein-Protein Interaction Network by STRING 9.02 (http://string-db.org/) online analysis software and R software were used to determine the related biological functions of PRKCA, PRKCB, and signal enrichment analysis.

**Statistical analysis**

Experimental data were processed using SPSS 23.0 statistical software. Results are expressed as mean ± standard deviation (X ± s). One-way ANOVA was used for statistical comparisons for single-factor variance. The least significant difference (LSD) method was used for pairwise comparisons, when necessary. P < 0.05 indicates statistical significance.

**Results**

**General condition of animals**

During the experiment, the control group diet was normal. However, rats in the OVX group suffered appetite loss, had rough fur, and an irritable mood. The state of the KSJG-treated group was no different from the control group.

**Effects of KSJG granules on bone morphology**

After 12 weeks of treatment, the trabecular meshwork of the femurs in the rats in the control group and the OVX group became thinner, with decreased trabeculae. There was a decrease in the number of osteoporotic tracts which were discontinuous, exhibiting typical osteoporotic bone morphology. Compared with the OVX group, trabecular bone in the KSJG-treated group was thicker and denser and the continuity of the bone was better, with a regular arrangement that formed a network structure (Figure 1).
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Detection of CT and BGP in serum

As shown in Table 1, ovariectomies decreased levels of CT and BGP, compared to the control group (P < 0.05). KSJG granules increased the content of CT and BGP, compared to the OVX group (P < 0.05). There were no significant differences observed, compared to the control group (P > 0.05).

Evaluation of Bone Mineral Density (BMD)

Table 1 presents the degree of bone mineral density (BMD) in the excised femurs. Ovariectomies induced a significant decrease in BMD (P < 0.05). KSJG granules increased BMD (P < 0.05) but did not restore it to control levels.

Measurement of bone calcium and BALP

The OVX group suffered a significant reduction in femur calcium content, compared to the control group (P < 0.01). KSJG-treatment reversed the effects of ovariectomies, causing a significant increase in femur calcium content, compared to the OVX group (P < 0.01). There was a significant increase in BALP in the OVX group, compared to the control group (P < 0.05). KSJG granules were able to decrease levels of BALP, compared to the OVX group (P < 0.05) (Table 1).

Expression of PRKCA and PRKCB protein levels in renal tissue

Compared to controls, relative expression levels of PRKCA and PRKCB in the renal tissue of the OVX group decreased significantly (P < 0.05). Relative expression of PRKCA and PRKCB in the OVX+KSJG group was significantly increased in renal tissue (P < 0.05) (Figure 2A, 2B).

Expression of PRKCA and PRKCB protein levels in femoral tissue

Relative expression levels of PRKCA and PRKCB in the femurs of the OVX group were significantly lower than controls (P < 0.05). Compared to the OVX group, relative expression of PRKCA and PRKCB in the femoral tissues was significantly higher in rats from the OVX+KSJG group (P < 0.05) (Figure 2C, 2D).

Correlation between expression of PRKC proteins in renal tissue and bone calcium

Figure 3A-D shows the correlation between expression of PRKC proteins in renal tissue and bone calcium. Results indicate that relative expression levels of PRKCA and PRKCB were positively correlated with bone calcium levels (r² = 0.7762, r² = 0.6033, respectively), but negatively correlated with BALP levels (r² = 0.8746, r² = 0.8166, respectively), both showing statistical significance (P < 0.05).

Correlation between expression of PRKC proteins in femoral tissue and bone calcium

Figure 3E-H presents the correlation between expression of PRKC proteins in femoral tissue and bone calcium. Results indicate that relative expression levels of PRKCA and PRKCB were positively correlated with bone calcium levels (r² = 0.7762, r² = 0.6033, respectively), but negatively correlated with BALP levels (r² = 0.8746, r² = 0.8166, respectively), both showing statistical significance (P < 0.05).

Table 1. Levels of CT, BGP, BMD, Bone calcium, BALP in serum (x ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>CT (ng/mL)</th>
<th>BGP (ng/mL)</th>
<th>BMD (g/cm²)</th>
<th>Bone calcium (mg/cm³)</th>
<th>BALP (U/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td>161.362 ± 5.124</td>
<td>1.550 ± 0.347</td>
<td>0.304 ± 0.019</td>
<td>290.37 ± 10.23</td>
<td>20.69 ± 0.82</td>
</tr>
<tr>
<td>OVX</td>
<td>8</td>
<td>120.452 ± 3.478*</td>
<td>0.874 ± 0.187*</td>
<td>0.203 ± 0.016*</td>
<td>191.56 ± 20.63**</td>
<td>26.47 ± 0.88*</td>
</tr>
<tr>
<td>OVX+KSJG</td>
<td>9</td>
<td>159.251 ± 4.785*</td>
<td>1.389 ± 0.310*</td>
<td>0.248 ± 0.013*</td>
<td>287.94 ± 20.59**</td>
<td>22.51 ± 0.64*</td>
</tr>
</tbody>
</table>

*P < 0.05 compared to the controls; **P < 0.01 compared to the controls; †P < 0.05 compared to the OVX group; ‡P < 0.01 compared to the OVX group. CT: Serum calcitonin, BGP: osteocalcin; BMD: Bone mineral density; BALP: Bone Alkaline Phosphatase.
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Results indicate that relative expression levels of PRKCA and PRKCB in femoral tissue were also positively correlated with bone calcium levels ($r^2 = 0.5898$, $r^2 = 0.3459$, respectively) and negatively correlated with BALP levels ($r^2 = 0.8290$, $r^2 = 0.6315$, respectively) ($P < 0.05$). Compared to the OVX group, relative expression of PRKCA and PRKCB was significantly higher in rats in the OVX+KSJG group ($P < 0.05$).

Protein-protein interaction network

To fully understand the relationship between PRKCA and PRKCB protein expression in patients with osteoporosis, the corresponding protein-protein interaction network was searched, using PRKCA and PRKCB as seed proteins (Figure 4). The PPI network, in which PRKCA and PRKCB are key proteins, contains 3 sub networks. There was direct interaction between PRKCA and PLD1, ARHGDIA, PLD2, RHOA, RAC1, MAP2K1, BRAF, PLCB1, and PICK1. Additionally, PRKCB interacted directly with PIK3CG, RPS6KB1, and MTOR (Figure 4).

Analysis of PRKCA and PRKCB-related pathways

KEGG pathway enrichment analysis was performed for PRKCA and PRKCB. As shown in Table 2, PRKCA and PRKCB were mainly involved in estrogen receptor signaling pathways, followed by the phosphatidylinositol signaling system, the mTOR signaling pathway.

Discussion

Osteoporosis is a worldwide health problem. The WHO has identified it as the second leading disease threatening human health, after cardiovascular disease. Research shows that, with age, the risk of osteoporosis in women rises dramatically. According to relevant statistics, the prevalence of osteoporosis in Chinese women, aged 50-59 years, is 10.6%. It is 42.7% in women aged 60-69 and 67% in women aged 70-79. In women over 80 years of age, the prevalence of osteoporosis is more than 90%. These percentages are higher than those for men of the corresponding age group [5, 17, 18]. Reports from the International Osteoporosis Foundation show that the probability of a woman’s lifetime risk of osteoporosis is 40%, higher than even the sum of the probabilities of breast, endometrial, and ovarian cancer.

In the past, it was suggested that OP was mainly caused by decreasing levels of estrogen, leading to the lowering of bone mineral content and subsequent changes in fine bone structure and bone strength, including increased bone fragility [19]. However, as a typical disease with a complicated etiology, the pathogenesis of OP remains poorly understood. Factors such as
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insufficient sex hormones, age, genetic composition, race, malnutrition, lack of physical exercise, smoking, excessive alcohol consumption, and oral medication of bone metabolism-related drugs are associated with OP [20]. Currently, a more representative point of view is as follows. Menopause-induced ovarian failure and body function decline. This, in turn, leads to decreased estrogen, resulting in hyperparathyroidism, abnormal changes in the activity of vitamin D products, and lack of calcitonin secretion. As a result, the kidneys reduce the absorption of calcium and that in the gut, while increasing its excretion. It decreases the number of osteoblasts, but on the other hand, increases that of osteoclasts. Besides, it may decrease levels of bone mineral plus other degenerative changes. Therefore, postmenopausal women are typically a high-risk group for OP [16].

There are certain advantages of Chinese Medicine in the treatment of OP. Kidney deficiency is the main pathogenesis of osteoporosis and blood stasis is a promoting factor for osteoporosis. Chinese herbal compound treatments for osteoporosis have been shown to be multi-component, multi-target, and multi-channel, with good safety and fewer side effects observed. They can inhibit bone resorption in addition to the promotion of bone formation. Therefore, research and development of comprehensively-targeted Traditional Chinese Medicine preparations is likely to benefit osteoporosis treatment. Kang-shu Jian-gu granules are a typical Chinese patent drug, applied clinically to treat OP using Traditional Chinese Medicine. Based on the classical principle of Chinese Medicine, which is “syndrome differentiation” and “treatment aiming at pathogenesis”, Kang-shu Jian-gu granules are effective in enhancing kidney function, accompanied by nourishment of the spleen [12, 21, 22].

Additional treatment effects of the whole formula of this drug include invigorating the kidneys and enhancing strong bones, benefiting Qi and nourishing the spleen, activating blood circulation, and comprehensively enhancing whole body function. These effects help to prevent a reduction in bone mineral density. However, the mechanisms remain unclear. Previous reports have suggested that Chinese medication aims to invigorate kidney function, enhance bone strength, and also increase bone mass.
Effects in the treatment of OP in rats are through modification of the activity of PKC and other enzymes on the membranes of red blood cells [23]. It has not been reported whether Kang-shu Jian-gu granules play a role in the treatment of OP by regulating expression of PKC proteins.

In the current study, a rat model of bilateral ovariectomy was employed to investigate treatment with Kang-shu Jian-gu granules on protein expression of PRKCA and PRKCB in renal tissue. Based on present in vivo data, this study further investigated the mechanisms of these changes in protein expression using bioinformatics technology, aiming to explore the molecular mechanisms of Kang-shu Jian-gu granules in the treatment of OP. Present in vivo data demonstrates that expression of PRKCA and PRKCB in the kidneys of OP model rats was significantly downregulated. Treatment with Kang-shu Jian-gu granules significantly enhanced protein expression of PRKCA and PRKCB in OP rats. Results suggest that KSJG played a key role in PRKC activation in renal cells, enhancing expression of PRKCA and PRKCB and promoting the differentiation of osteoblasts, ultimately enhancing bone mineral density.

However, one question remains. What were the key links triggered by Kang-shu Jian-gu granules after activating PRKC in renal cells? This is a core question which could explain the relationship between Kang-shu Jian-gu granules.
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Table 2. KEGG pathway enrichment related to PRKCA and PRKCB

<table>
<thead>
<tr>
<th>Pathway ID</th>
<th>Pathway description</th>
<th>Count in gene set</th>
<th>False discovery rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>4012</td>
<td>ErbB signaling pathway</td>
<td>2</td>
<td>0.00022</td>
</tr>
<tr>
<td>4070</td>
<td>Phosphatidylinositol signaling system</td>
<td>2</td>
<td>0.00022</td>
</tr>
<tr>
<td>4150</td>
<td>mTOR signaling pathway</td>
<td>2</td>
<td>0.00022</td>
</tr>
<tr>
<td>4370</td>
<td>VEGF signaling pathway</td>
<td>2</td>
<td>0.00022</td>
</tr>
<tr>
<td>4540</td>
<td>Gap junction</td>
<td>2</td>
<td>0.00022</td>
</tr>
<tr>
<td>4644</td>
<td>Fc epsilon RI signaling pathway</td>
<td>2</td>
<td>0.00022</td>
</tr>
<tr>
<td>4666</td>
<td>Fc gamma R-mediated phagocytosis</td>
<td>2</td>
<td>0.00022</td>
</tr>
<tr>
<td>4713</td>
<td>Circadian entrainment</td>
<td>2</td>
<td>0.00022</td>
</tr>
<tr>
<td>4720</td>
<td>Long-term potentiation</td>
<td>2</td>
<td>0.00022</td>
</tr>
<tr>
<td>4727</td>
<td>GABAergic synapse</td>
<td>2</td>
<td>0.00022</td>
</tr>
</tbody>
</table>

and PRKC signaling, answering whether PRKCA and PRKCB were targeted using this treatment. Therefore, this study analyzed PRKCA and PRKCB-related drug targets. Data demonstrates that PRKCA and PRKCB are both the drug targets of Tamoxifen and Vitamin E. Tamoxifen is an antiestrogen and Vitamin E is a liposoluble vitamin also associated with levels of estrogen in women. The main active ingredients of Tamoxifen, Vitamin E, and Kang-shu Jian-gu granules have a similar drug composition [11]. Therefore, it was hypothesized that, with the cooperation of estrogen, PRKCA and PRKCB can be targeted by Kang-shu Jian-gu granules, subsequently exerting sequential biological effects [24].

Analysis of signaling pathways further confirmed the above speculation. Signaling pathway analysis also indicated that the estrogen receptor-signaling pathway is the most closely related to PRKCA and PRKCB. Therefore, it was speculated that Kang-shu Jian-gu granules might exert its treatment effects on OP through modification of the estrogen receptor-related signaling pathways, which subsequently upregulate protein expression of PRKCA and PRKCB.

In summary, there is a potential drug-targeted relationship between Kang-shu Jian-gu granules and PRKCA and PRKCB. Under the guidance of the estrogen receptor signaling pathway, Kang-shu Jian-gu granules regulate protein expression of PRKCA and PRKCB. However, these assumptions should be verified by more comprehensive, independent, and rigorous experiments.

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Disclosure of conflict of interest

None.

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