Regulation of aromatase P450 expression by puerarin in endometrial cell line RL95-2

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Objective: To study the effects of puerarin on the aromatase P450 (P450arom) mRNA expression and the effects of low-dose puerarin on transcription factors of the P450arom gene (P II) 5'-flanking region.

Methods: The effects of puerarin on the P450arom mRNA expression were determined by real-time polymerase chain reaction (RT-PCR). The 5'-flanking region was amplified by PCR using human genomic cDNA as a template. By means of the restriction sites and sequence confirmation, the PCR product was cloned into reporter vector. Series of sequential deletion reporter constructs were transiently transfected into RL95-2 cells which were treated with or without puerarin. Luciferase activity was measured by Dual-Luciferase Reporter Assay System and Luminoskan Ascent luminometer. Furthermore, by using web-based search program, the most possible cis-acting elements and transcription factors were evaluated.

Results: The data demonstrated that low-dose puerarin treatment could decrease P450arom expression at mRNA level compared to dimethyl sulfoxide (DMSO) treatment (P<0.01), and puerarin (10^-7 mol/L) had a time-course effect on P450arom mRNA expression, which reached the bottom at 12 h (P<0.01). Cells transfected with the 763+/-879 bp constructs showed decrease in relative luciferase activity after puerarin (10^-7 mol/L) treatment compared to DMSO treatment (P<0.05), indicating an essential regulatory site between -763 bp and -543 bp responsible for the transcription suppression by puerarin. Furthermore, the most possible transcription factors, which turned out to be AP-1 (c-jun/c-fos) at -410/-401 bp were also evaluated. The activity of exogenous AP-1 was reduced after 12 hours of puerarin treatment (P<0.05). The inhibition of c-jun mRNA also showed a time-course effect, which bottomed out at 12 h in parallel with that of P450arom (P<0.01). The protein level of c-jun was also down-regulated by puerarin (10^-7 mol/L) treatment at 12 h.

Conclusion: The suppression of P450arom expression and activity may be associated with the down-regulation of transcription factor AP-1/c-jun. This partially explains the mechanisms whereby puerarin treats endometriosis.

Keywords: puerarin, aromatase P450, endometriosis

葛根素对子宫内膜 RL95-2 细胞 P450 芳香化酶表达的调控作用

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目的：探讨葛根素对子宫内膜RL95-2细胞P450芳香化酶（aromatase P450，P450arom）表达的影响及其对RL95-2细胞P450arom基因表达的调控作用。

方法：将梯度稀释的葛根素在不同时间点加入RL95-2细胞中，分别采用实时聚合酶链反应（real-time polymerase chain reaction, RT-PCR）检测药物对RL95-2细胞P450arom mRNA表达水平的影响。以人类基因组DNA为模板扩增P450arom组织特异性启动子2（P II）转录起始位点上游序列。PCR产物经克隆到报告基因载体pGL3-Basic中，构建人P450arom P II系列报告基因质粒，并受限制性内切酶消化鉴定和基因测序证实。瞬时转染系列重组质粒至RL95-2细胞，加入葛根素刺激，分析相关转录因子，利用RT-PCR及Western blotting方法检测相关转录因子在葛根素作用下mRNA及蛋白表达的变化。

结果：与溶剂对照组比较，低浓度葛根素会抑制P450arom mRNA表达（P<0.01），抑制作用在6、12小时显著（P<0.01）。酶酶切鉴定，基因测序，转录因子分析及启动子活性测定，成功构建了人P450arom P II系列重组质粒（-1,017，-763，-543，-234～+8 bp）。瞬时转染系列质粒至细胞中，于12小时后加入葛根素（10⁻⁷ mol/L）刺激，又12小时后检测荧光蛋白水平。结果表明，-763～-543 bp间的活性被抑制（P<0.05），通过转录因子分析找到了-410～-401 bp的AP-1（c-jun/c-fos）。利用RT-PCR，Western blotting及荧光素酶活性检测发现葛根素（10⁻⁷ mol/L）对AP-1（c-jun/c-fos）的抑制作用平行于对P450arom的作用。

结论：低浓度葛根素对子宫内膜RL95-2细胞P450芳香化酶基因表达的抑制作用很可能受上游调控转录因子AP-1（c-jun/c-fos）的影响。

关键词：葛根素；芳香化酶P450；子宫内膜异位症


1 Introduction

Aromatase P450 (P450arom) is a key enzyme for biosynthesis of estrogen which is an essential hormone for the establishment and growth of endometriosis. Endometriosis tissue contains very high levels of aromatase, which leads to produce significant quantities of estrogen[12]. Apart from endometriosis, endometrial cancers and uterine fibroids also overexpress aromatase and produce local estrogen that exerts paracrine and intracrine effects. This may partially result from the molecular alterations in stromal cells, which is aromatase promoter's favor of binding transcriptional enhancers versus inhibitors to initiate transcription[13-14]. So the aromatase inhibitors (AI) have successfully been used to treat endometriosis and uterine leiomyomatia and underlined the clinical significance of these molecular studies of AI[14-15].

Flavonoids and isoflavones, as phytoestrogens, exert various biological effects, including anti-estrogenic effects, modulation of sex hormone homeostasis, anticarcinogenic and antioxidant effects[32]. They modulate enzyme activities as well as signal transduction, so their effects on P450arom and interaction with estrogen receptor are of interest to investigate. A recent study showed that higher levels of urinary genistein and daidzein were associated with decreased risk of advanced endometriosis but not early endometriosis[36]. There was also evidence that phytoestrogens and their low dose combinations inhibited mRNA expression and activity of aromatase in human granulosa-luteal cells[37].

Puerarin is a major isoflavonoid compound isolated from Pueraria lobata, a popular Chinese herb known as gyno. It has been suggested to be useful to treat various disorders, such as coronary artery diseases, hypercholesteremia, liver fibrosis, neurotoxicity and hydrogen peroxide-induced pancreatic islet damage[8-13]. According to our clinical experience, Pueraria lobata in the treatment of endometriosis can relieve pain and treat infertility.

Based on the above rationale, it was hypothesized that puerarin, the main component of Pueraria lobata has effects on estrogen or aromatase inhibition as other isoflavonoids. Since levels of enzyme activity and cell responsiveness is highly variable in different patients and samples[37], also considering that endometrial cancer is an estrogen dependent disease and may have the same molecular mechanisms in overexpressing P450arom[27, 31-33], RL95-2 cells, a well-defined human endometrial epithelial cell line, were selected as a model to study[15]. Therefore, this study was carried out with a view to elucidating the inhibitory potential and mechanisms whereby puerarin acts on RL95-2 cells with P450arom overexpression.
2 Material and methods

2.1 Materials and cell culture Pueraarlin was purchased from Sigma (St. Louis, MO). Luciferase Assay Systems and pGL3-Basic Vector were from Promega (Madison, WI). Four AP-1-luciferase plasmids were gifts from Prof. Nancy Colburn (Laboratory of Cancer Prevention, National Cancer Institute-Frederick, Frederick, MD). All chemicals and enzymes were obtained from Sigma (St. Louis, MO). Phenol red-free (PRF) DMEM/F12, and charcoal-stripped fetal bovine serum (CS-FBS) were purchased from Gibco (Grand Island, NY). RL95-2 (ATCC CRL-1671TM, USA) human endometrium carcinoma cells were cultured in PRF DMEM/F12, supplemented with 10 % CS-FBS in a humidified atmosphere of 95% air and 5% CO₂, at 37 °C.

2.2 RNA isolation and quantitative RT-PCR analysis RL95-2 cells were plated in 24-well plates and allowed to grow to approximately 70% to 80% confluence prior to the commencement of each experiment. Total RNA was isolated by Trizol (Invitrogen, Carlsbad, USA) reagent. And cDNA was synthesized from 2 μg of total RNA with random primers following the manufacturer’s protocol (MBI Fermentas, Vilnius, Lithuania). The single-stranded cDNA was then used in a quantitative real-time PCR to evaluate the relative expression levels of P450_200 (5-AGAGACATAGAAGACGACT-TG and 5'-GACGACCTTCTCCTAGTTCTCA) and human c-jun (5'-CCCCCGGCTTCTATATGGAA and 5'-GCTGTCTCCCTCCACTGCA) compared with human β-actin (5'-CTGAAAGCTGAGTGTT- GACA and 5'-AAGGGACTTTCCGTAAACATGCA). RT-PCR was performed according to the ABI manufacturer’s protocols (Applied Biosystems, CA). All samples were examined in triplicate. PCR-specific amplification was performed in the Applied Biosystems (ABI 7300) quantitative RT-PCR machine. The reactions were running for 40 cycles (96 °C 15 s, 60 °C 60 s). Fluorescence was analyzed by the Light Cycler Software version 3.5 (Roche Diagnostics, Switzerland). Quantitative analysis of target genes expression was done by the comparative CT (ΔCT) method[15].

2.3 Western blot analysis Total protein was isolated from whole cells using ice-cold protein lysis buffer [1% Triton X-100, 50 mmol/L Tris-Cl, pH 7.4; 150 mmol/L NaCl; 0.1% sodium dodecyl sulfate (SDS); 1 mmol/L phenylmethyl sulfonyl fluoride (PMSF); 1 mmol/L ethylenediamine tetraacetic acid (EDTA)]. This was followed by 30 min incubation on ice and centrifugation at 10 000×g for 10 min at 4 °C. Protein concentrations were determined by a modified Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA). Protein extracts were separated on a SDS-polyacrylamide gel, blotted onto a nitrocellulose membrane (Millipore Corp, Bedford, USA), and incubated with anti-P450_200 (Biovision, USA) and anti-β-actin (Cell Signaling Technology, Beverly, USA). Immunoblots were developed using horseradish peroxidase-conjugated goat-anti mouse immunoglobulin G (IgG), followed by detection with enhanced chemiluminescence. Western blot analysis was performed for each protein of interest derived at three times.

2.4 Estradiol assay Estradiol in the culture medium was measured in duplicate by a direct RIA kit (ICN Pharmaceuticals Ltd., United Kingdom) according to the manufacturer’s instructions.

2.5 Isolation of the 5’-flanking of the P450_200 gene PII region and deletion constructs The 5’-flanking region of the P450_200 gene was amplified by PCR using human genomic cDNA as a template and using the following primers: 5’-GGATACGTAATGACTACTCTTCC (underlined nucleotides indicate a KpnI site) and 5’-CTCGAGTTAGAGCTCTGAGAATC (Underlined nucleotides indicate an Xhol site). Primers were designed based on a published sequence of the P450_200 PII sequence (Version; S85356.1 Accession No. S85356). After sequence confirmation, the 1 026 bp PCR product was cloned into pGL3 basic luciferase reporter vector (Promega, Madison, WI) using KpnI and Xhol restriction sites. Putative transcription factor binding sites within the full-length P450_200 PII were identified using the web-based search program Transcription Element Search System (TESS, http://www.cbil.upenn.edu/tess/). To determine the putative regulatory site(s) necessary for P450_200 promoter activity, the truncated fragments −763/+8 bp, −543/+8 bp and −234/+8 bp were obtained by PCR.

2.6 Transient transfection and luciferase activity assay For reporter gene assays, RL95-2 cells were plated in 48-well plate at a density of 0.5 × 10⁵ cells/well so that the cells would be 80% confluent at the time of transfection. After the growth medium was replaced with Opti-MEM (Invitrogen, USA), cells were cotransfected with pGL3-basic or recombinant pGL3-basic luciferase reporter
plasmids that contained serial deletion mutant of P450 arom P II and pRL-CMV (Renilla luciferase; Promega) as an internal control. The transfection medium was changed after 6 h of incubation. The cells were then incubated with or without 10−7 mol/L puerarin for 12 hours. Luciferase activity was measured by Dual-Luciferase Reporter Assay System (Promega, Madison, WI) and Luminoskan Ascent luminometer (Biotek, USA). Relative luciferase was calculated by normalizing luciferase activity to Renilla luciferase activity. Experiments were repeated in triplicate.

2.7 Statistical analysis The data shown are the mean ± SD of the triplicate experiments. Statistical significance was estimated with Student’s t-test for unpaired observations. A P value of less than 0.05 was considered significant.

3 Results

3.1 Effects of puerarin on P450 arom expression and activity To find out whether puerarin (Figure 1A) inhibited aromatase and led to its function of endometriosis treatment, P450 arom expression in RL95-2 cells treated with puerarin (10−7 mol/L) or DMSO was estimated by quantitative RT-PCR and Western blot method. A significant decrease of P450 arom expression at mRNA level by 10−7 mol/L puerarin treatment in RL95-2 compared with dimethyl sulphoxide (DMSO) treatment was demonstrated by our group (Figure 1B). Furthermore, there was a time-course effect on P450 arom mRNA expression at 10−7 mol/L puerarin, which was significantly reduced at 6 h, reached the bottom (>70%) at 12 h and returned to normal at 24 h (Figure 1C).

3.2 P450 arom P II from −417 bp to −336 bp responsible for the transcription suppression by puerarin To identify the regions that might involve in regulating P450 arom gene transcription by puerarin, a series of sequential deletion reporter constructs (−1017, −763, −543 and −234 to +8 bp) were cloned into the luciferase-containing vector pGL3-basic. Constructs were transiently transfected into RL95-2 cells which were treated with or without puerarin (10−7 mol/L). Later on relative luciferase activity was determined. Cells transfected with the −543/+8 bp constructs with puerarin showed approximately 30% decrease in relative luciferase activity compared with DMSO treatment group (Figure 2A), denoting an essential regulatory site existed between −234 bp and −543 bp responsible for the transcription suppression by puerarin. Furthermore, TESS job was used to evaluate the most possible cis-acting elements and transcription factors, and it turned out to be AP-1 (c-jun/c-fos) or c-jun at −410/−401 bp (Figure 2B).

3.3 Suppression of AP-1 activity and c-jun mRNA expression by puerarin As indicated above, AP-1 or c-jun might be the key link for puerarin to regulate P450 arom. AP-1-luciferase activity and c-jun mRNA expression were tested to find out whether they varied along with P450 arom by puerarin. In Figure 3A, the activity of exogenous AP-1 was reduced after 12 hours of puerarin treatment. A time-course effect of the inhibition of c-jun mRNA was shown, which began at 6 h, reached the minimum at 12 h and returned to basal level at 24 h in parallel with that of P450 arom (Figure 3B). And the protein level of c-jun was also obviously downregulated by 10−7 mol/L puerarin treatment at 12 h (Figure 3C).
Figure 2  P450_{com} P II responsible for the transcription suppression by puercarin

A1: RL95-2 cells were transfected with −374 bp, −763 bp, −763/+8 bp, −543/bp and −234+8 bp constructs and were then treated with puercarin or DMSO for 12 hours. Luciferase activity was normalized by cotransfected pRL-CMV and these results represented the mean ± SE of three different experiments. *P<0.05 with respect to the DMSO-treated samples; B1, Sequence of the cloned 5’-flanking region of P450_{com} P II from −417 bp to −336 bp. Putative transcription factor binding sites were identified using the TESS job and cis-acting elements were indicated in boxes. Two potential AP-1/c-jun binding sites with the highest scores were observed.

Figure 3  Suppression of 4×AP-1-luciferase activity and c-jun mRNA expression by puercarin

A1: To find out whether AP-1 varies along with P450_{com} by puercarin, the activity and mRNA levels of AP-1 were assayed. RL95-2 cells were transfected with 4×AP-1-luciferase plasmid and treated with DMSO (control) or puercarin (10^-6 mol/L) for 12 hours. Cells were collected at indicated time point after treated with puercarin (10^-6 mol/L) or DMSO and then used for RNA extraction and quantitative RT-PCR. C1, Western blot analysis showed a significant decrease of c-jun protein expression by puercarin (10^-5 mol/L) treatment for 12 hours, compared with the amount of β-actin. The target gene mRNA levels were normalized by the amount of β-actin mRNA levels. These results represented the mean ± SE of three separate experiments. *P<0.05, **P<0.01, vs DMSO line.

4 Discussion

Isoflavones are selectively incorporated in certain tissues like the breast and ovaries, and are able to bind to ER-alpha and ER-beta [18]. With weak estrogen agonists/antagonists and some other enzymatic activities, isoflavones are increasingly advocated as a natural alternative to estrogen replacement therapy and are available as dietary supplements. They are providing a useful model for the actions of endocrine disruptors [19]. Puercarin, an isoflavonoid derived from the Chinese medicinal herb Radix puerariae, has been proved to be practical in the management of various cardiovascular disorders, alcoholism and neurological disease [20, 21]. However, its role in dealing with endocrine disorders has not been well elucidated.

There is evidence shown in the clinical study that puercarin can be used in the treatment of endometriosis, relieving pain and treating infertility. Concerning the overexpression pattern of P450_{com} in both endometriosis and endometrial cancer, the mechanistic studies were exerted on an endometrium carcinoma epithelial cell line RL95-2, which also overexpressed aromatase. The effects of puercarin hypothesized in the treatment of endometriosis may partially result from P450_{com} inhibition, the pivotal enzyme of estrogen production.

There are still some controversies in recent studies. A study showed significant inhibition of P450_{com} mRNA expression at a dose of 10^-7 mol/L after 48-hour exposure to a combination of isoflavonoids in patients [22]. But the data indicated that the inhibiting effect of puercarin was more obvious after 12-hour treatment. The mechanism whereby different isoflavones decrease P450_{com} mRNA expression remains unclear. Moreover it has been reported that phytoestrogens had estrogenic or antiestrogenic effects, which was determined by the levels and the timing of exposure to these
compounds\(^{[21]}\). Some studies reported that genistein increased P450 arom activity and promoter specific P450 arom transcripts in H295R cells, and increased P450 arom activity in isolated immature rat follicles at doses between 10\(^{-7}\) and 10\(^{-5}\) mol/L\(^{[25,26]}\). A significant decrease of P450 arom expression at both mRNA and protein levels by 10\(^{-7}\) mol/L was first demonstrated by our group. So the data showed that 10\(^{-7}\) mol/L puerarin had an antiestrogenic effect in RL95-2. In other words, 10\(^{-7}\) mol/L puerarin can reduce the mRNA and protein levels of P450 arom. In Figures 3B and 3C, the mRNA and protein levels of c-jun was significantly down-regulated by puerarin treatment. So the diminished c-jun and estrogen production may contribute to the treatment of endometriosis to some extent.

Later on, the transcription regulation of P450 arom by puerarin was focused on. Tissue-specific promoters distributed over a long regulatory region upstream alternatively control P450 arom expression\(^{[21]}\). It has been demonstrated that promoter II (P II) was the major promoter which drives P450 arom expression in endometrial cancer, leiomyoma and endometriosis tissues\(^{[23]}\). So we cloned P II (\(-543/\ + 8\) bp) to a luciferase-containing vector and examined the sequential deletion reporter constructs derived from the whole construct. Our results suggested that the essential regulatory site by puerarin might exist between \(-234\) bp and \(-543\) bp (Figure 2A) and the full length \(-1 \ 017/\ + 8\) bp constructs showed low activity. TESS job was used to further analyze the cis-acting elements and transcription factors of \(-234/\ -543\) bp. Two high scored binding sites (overlapped with each other at GAGTGA) were all associated with c-jun (Figure 2B). It should be mentioned that puerarin decreased only 30% luciferase activity but 70% mRNA levels compared with the control. Thus there must be other ways involved in regulating the transcription activity of P450 arom by puerarin, namely somewhere upstream of P II and some other transcription factors.

In this experiment, the function of c-jun was only investigated, since c-fos, the other component of AP-1, was missing or poorly expressed in RL95-2 cells, as indicated by an early study\(^{[27]}\). The inhibition of c-jun mRNA by puerarin showed a time-course effect and paralleled that of P450 arom. As Figure 3A showed, luciferase assay further confirmed the DNA binding deficiency of AP-1 by puerarin treatment, which might result from c-jun reduction. Another study done in glomerular mesangial cells found that puerarin (10\(^{7}\) mol/L) significantly ameliorated the high-glucose effects on c-fos, c-jun and Co\(^{\text{II}}\) expression. This effect was accompanied by a reduced protein kinase C (PKC) activity in these cells\(^{[28]}\), which supported the hypothesis that puerarin could down-regulate AP-1 (c-fos/c-jun dimer). However, it was not proved whether it acted through PKC signaling in this experiment. Work on the mechanism of AP-1 regulation by puerarin treatment will be carried out.

Last but not the least, it was demonstrated that in untreated RL95-2 cells, when c-jun was knocked down, the expression of P450 arom exhibited >50% decrease at both mRNA and protein levels, which meant c-jun was essential for P450 arom overexpression. Therefore, when puerarin was used, c-jun was down-regulated and so was P450 arom.

In conclusion, the suppression of P450 arom expression and activity may be associated with the down-regulation of transcription factor AP-1/c-jun. c-jun is also essential for the active transcription and overexpression of P450 arom in endometrial cancer cells RL95-2. This partially explains the mechanisms of puerarin in treating endometriosis, and it also provides us with a new target and an efficient product in dealing with endometrial cancer and endometriosis.

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