Paeoniflorin Inhibits Proliferation of Fibroblast-Like Synoviocytes through Suppressing G-Protein-Coupled Receptor Kinase 2

Abstract

Paeoniflorin (Pae) is a monoterpenoid glucoside and the main component of the total glucosides of paeony (TGP) extracted from the roots of *Paeonia lactiflora*. Its anti-inflammatory effect is associated with regulating G-protein-coupled receptors (GPCRs) signaling. The aim of this study was to explore the expression change of G-protein-coupled receptor kinase 2 (GRK2) in fibroblast-like synoviocytes (FLS) and the effect of Pae. Pae was obtained and purified from the roots of *Paeonia lactiflora*. We investigated the expression of GRK2 in synovium during the inflammatory process and assessed the effects of a specific GRK2 inhibitor and Pae on proliferation, cAMP level, and protein kinase A (PKA) activity of FLS in vitro. Additionally, the effect of Pae on GRK2 expression in FLS was detected in vitro. Expression of GRK2 in synovium from CIA rats increased during the inflammatory process. The specific GRK2 inhibitor suppressed proliferation and increased the cAMP level as well as PKA activity of FLS, and Pae had the same effects. Furthermore, Pae decreased GRK2 expression in FLS in vitro. Our results indicate that a chronic inflammatory process in CIA induces upregulation of GRK2 expression in FLS, and Pae can reverse this change, which might be one of the important mechanisms for Pae regulating GPCRs signaling and suppressing the proliferation of FLS in CIA.

Introduction

Rheumatoid arthritis (RA) is an autoimmune and inflammatory disease with chronic synovial inflammation and progressive articular damage in multiple joints [1]. Collagen-induced arthritis (CIA) exhibits similar symptoms as RA including uncontrolled proliferation of synoviocytes, pannus formation, erosion of cartilage and bone, and ultimately, loss of articular function [2,3]. Fibroblast-like synoviocytes (FLS), the key effector cells in the inflamed joint, release a lot of pro-inflammatory and matrix-degrading effector molecules, which have the capacity to provide chemotactic and activation signals to resident parenchymal cells and infiltrating immunocytes and the destruction of the involved joints [4–6]. G-protein-coupled receptors (GPCRs) constitute a family of seven-transmembrane domain proteins that transmit signals to the interior of cells by activating a variety of signaling pathways [7]. More and more studies suggest that alterations of GPCRs, G proteins, and their signaling are involved in RA. Our previous studies have shown that GPCRs signaling is associated with the pathogenesis of CIA and adjuvant arthritis (AA) rats. In synoviocytes from CIA rats, the cyclic adenosine monophosphate (cAMP) level and mRNA expression of Gαi decreases, while mRNA expression of Gαs increases [8]. In synovium of AA rats, the expression of prostaglandin E2 receptor subtypes EP2 and EP4 decrease significantly [9].

Paeoniflorin (Pae), a monoterpenoid glucoside, is one of the main bioactive components of the total glucosides of paeony (TGP) extracted from the roots of *Paeonia lactiflora* (Paeoniaceae). TGP has both anti-inflammatory and immune-regulatory effects and is used in Chinese clinics to treat RA. TGP contains more than 90% of Pae. The anti-inflammatory activity of Pae and TGP has been extensively studied in our previous studies. We have previously reported that Pae and TGP significantly ameliorate the symptoms of CIA and AA rats. In CIA rats, TGP suppresses the synoviocytes proliferation and increases the cAMP level [10]. In vivo and in vitro, Pae leads to an increase in the cAMP level and protein kinase A (PKA) activity...
and a decrease in Gai expression in FLS from CIA rats [11]. These findings provide support for the concept that Pae is involved in regulation of GPCRs signaling of FLS.

As a key regulator of GPCRs signaling, G-protein-coupled receptor kinases (GRKs) terminate GPCRs signaling by phosphorylating the agonist-occupied receptors and blocking G-proteins from further interaction with the receptors [12]. Changes of GRKs levels have been reported in a number of inflammation diseases including RA.

Lombardi et al. have reported a decreased expression and activity of GRKs in peripheral blood mononuclear cells of patients with RA; furthermore, AA induces downregulation of GRKs in splenocytes and mesenteric lymph node cells [13, 14]. Our previous studies have shown a decrease in the expression of β2-adrenalin receptor and GRK2 in mesenteric lymph node lymphocytes from AA and CIA rats, and Pae can reverse the changes [15, 16]. All these studies about GRK2 focus on immune organs and leukocytes, while the expression of GRK2 in FLS is not clear. Furthermore, it is still unknown whether the effects of Pae on regulating GPCRs signaling in FLS are associated with GRK2. Thus, the aim of this study was to investigate the expression change of GRK2 in FLS from CIA rats and the effect of Pae.

Materials and Methods

Drugs and reagents

The following reagents were obtained commercially: chicken type II collagen (CII) (Institute of Bencao Biological Medicine in Shanghai, China), anti-GRK2 and anti-β-actin antibodies (Santa Cruz Biotechnology, Inc.), SuperSignal West Femto Maximum Sensitive Substrate (Pierce Biotechnology, Inc.), [125I]cAMP radioimmunoassay (RIA) kit (Shanghai University of Traditional Chinese Medicine, China), luminescent kinase assay kit (Promega, Inc.), a specific GRK2 inhibitor (methyl 5-[2-(5-nitro-2-furyl) vinyl]-2-furoate, C12H9NO6, purity 100%; Calbiochem, Inc.), a specific GRK2 inhibitor (methyl 5-[2-(5-nitro-2-furyl) vinyl]-2-furoate, C12H9NO6, purity 100%; Calbiochem, Inc.), and tritiated thymidine ([3H]-TdR) (Shanghai Institute of Applied Physics, China). Other chemicals used in these experiments were of analytical grade from commercial sources.

Plant material

The roots of Paeonia lactiflora were collected from plants growing under controlled conditions in Bozhou (latitude 33° 47′ 59.64 north and longitude 115° 50′ 15.90 east), Anhui Province, China. A voucher specimen was deposited at the herbarium of the Anhui Medical University (No. 08274). Pae (C23H28O11, MW: 480.45) was extracted from the roots of Paeonia lactiflora and purified by methods of solvent extraction and column chromatography in the Chemistry Lab of the Institute of Clinical Pharmacology of the Anhui Medical University (Hefei, Anhui Province, China). The dried and powdered roots of Paeony lactiflora were extracted with 70% ethanol under reflux. The concentrated extract was dissolved in water and then eluted with EtOAc/MeOH (20/1). The pure compound was yielded after the concentration of the collected eluate, which contained only Pae (17). The purity of Pae determined by HPLC assay (LC-10AD; Shimadzu Co.) is above 95%. Its structure was identified by physicochemical properties and spectroscopic analysis (Fig. 1). Pae was suspended in distilled water at the required concentration before use.

Animals

This study was approved by the Ethical Committee on Animal Research at the Institute of Clinical Pharmacology, Anhui Medical University (approval number 037426, January 25, 2008). Sprague-Dawley (SD) rats (male, 180 ± 20 g, Grade 11, Certificate No. 006) were purchased from the Animal Department of the Anhui Medical University (Hefei, Anhui Province, China). All animals were housed under standard laboratory conditions. They were given commercial food and tap water ad libitum and kept on sawdust in plastic-bottomed cages in groups of no more than six. They were bred in a temperature-controlled room at 23°C (± 1) with lighting from 6 a.m. to 6 p.m.

Induction and evaluation of CIA

CII was dissolved in 0.1 M acetic acid at 2 mg/mL overnight at 4°C and emulsified with an equal volume of Freund’s incomplete adjuvant to a final concentration of 1 mg/mL. Rats were injected intradermally twice with 100 µL of the emulsion (containing 100 µg of CII). The first injection was made in the left hind footpad; the second, 7 days later, into the base of the tail and 3–5 sites on the back. The control group rats were injected with 0.1 M acetic acid. The day of the first immunization was defined as day 0 [18]. The onset and severity of disease were monitored daily and evaluated by two observers blinded to treatment. At day 0, 14, 18, 22, 26, 28, 30, 35, 46 after immunization, the right hind paw volume was determined with a YLS-7A toe volume meter (equipment station of the Academy of Medical Sciences, Shandong, China). Paw swelling (△ mL) was calculated by taking away the paw volume at day 0 from the related one at day 14, 18, 22, 26, 28, 30, 35, and 46.

Synovium

Rats were anesthetized and sacrificed on day 0, day 14, day 28, or day 46 after immunization, and the synovium from rats knees were excised and stored at –20°C for Western blot analysis. For each time point, four rats were anesthetized and sacrificed to excise the synovium to assess GRK2 expression.
Synoviocytes culture and stimulation
Rats were anesthetized and sacrificed on day 28 after immunization, and the synovium from the rats’ knee joints were excised. Then the synovium were minced and digested with type IA collagenase for 3 h, filtered, thoroughly washed, and then cultured in DMEM containing 20% heat-inactive FBS at 37°C in a humidified atmosphere of 5% CO2. At confluence, adherent cells were trypsinized, split in a 1 : 3 ratio and re-cultured in medium. The spindle-like synoviocytes of passages 3 were used in these experiments, during which time they were a homogeneous population of FLS [19].

Western blot analysis
Synovium and synoviocytes prepared above were lysed in lysis buffer [50 mM Tris-HCl, pH 7.4, 1% Tween 20, 2 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 10 µg/mL aprotinin, 10 µg/mL leupeptin] followed by homogenization at 4°C for 20 to 30 min on ice. The homogenate was centrifuged at 2000 g for 20 min at 4°C, and the supernatants obtained were diluted into 4 mg protein/mL and kept frozen at −80°C before use. A total of 50 µg of denatured protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrotheroretically to polyvinylidene fluoride membranes (Immun-Blot PVDF membrane, 0.2 µm; Bio-Rad) using a Mini Protean II system (Bio-Rad). A SDS–PAGE protein standard (Institute of Shanghai Biochemistry) was used to check transfer efficiency and as a molecular weight marker. PVDF membranes were incubated with blocking buffer (0.05% Tween 20-PBS with 5% nonfat milk) for 2 h. A primary antibody to GRK2 was used at a final dilution of 1 : 1000 overnight at 4°C. The blot was washed three times with 0.05% Tween 20-PBS and incubated with the appropriate goat anti-rabbit antibody for 2 h. Immunodetection was carried out using enhanced chemiluminescence reagent according to the manufacturer’s instructions (Pierce Biotechnology, Inc.). Equivalent protein loading and transfer efficiency were verified by staining for β-actin.

Proliferation assay by 3H-TdR
[3H] thymidine incorporation was used for detecting the FLS proliferation. FLS (1 × 10^4 cells/L) were added to 96-well flat-bottomed culture plates at 1 × 10^4 cells/well in DMEM with 20% FBS and incubated at 37°C, 5% CO2 for 24 h. 100 µL DMEM containing GRK2 inhibitor (50 µM) or Pae (1 × 10−5 mol/L, 1 × 10−6 mol/L, 1 × 10−7 mol/L, 1 × 10−8 mol/L, 1 × 10−9 mol/L) were added to each well. Subsequently, the cells were incubated at 37°C, 5% CO2 for 42 h and then with [3H]Tdr (1 µCi/well) at 37°C and 5% CO2 for 6 h prior to harvest. Afterwards, the cells were trypsinized, washed, and harvested onto filter paper. Radioactivity on the filters was determined by liquid scintillation counting (Beckman multipurpose scintillation counter; Beckman Coulter, Inc.).

Analysis of cAMP level and PKA activity in FLS
FLS were cultured in 24-well flat-bottomed culture plates at 5 × 10^5 cells/well in 20% FBS-DMEM. They were then incubated with GRK2 inhibitor (50 µM) for 1 h or with Pae (1 × 10−5 mol/L, 1 × 10−6 mol/L, 1 × 10−7 mol/L, 1 × 10−8 mol/L, 1 × 10−9 mol/L) at 37°C, 5% CO2 for 48 h. After centrifugation at 2000 rpm for 10 min, supernatants were quickly removed and the cells were resuspended in 250 µL Tris-EDTA (50 mM) buffer at pH 7.5. The samples were placed in boiling water for 5 min and then frozen. After thawing, samples were sonicated. After centrifugation, the supernatants were collected and stored at −80°C for measuring the cAMP level and PKA activity. The level of cAMP was determined by RIA according to procedures offered in [15]cAMP kit. PKA activity was assessed by a luminescent kinase assay kit. Briefly, 50 µL supernatants were placed in each well of a 96-well plate. An equal volume of Kinase-Glo® reagent was added and mixed for 10 min. Finally, 1 µM ATP was added to each well. The kinase reactions were mixed at room temperature, and luminescence was measured on a GLOMAXTM 96 microplate luminometer (Promega) 10 minutes later.

Statistical analysis
Data are expressed as mean ± standard deviation (SD). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnett’s test. Values of p less than 0.05 were considered to be significant.

Results
The paw swelling and increased expression of GRK2 during the articular process in synovium are shown in Fig. 2. To evaluate the secondary arthritis of CIA rats, paw swelling was used as an apparent indicator of arthritis. The onset of secondary arthritis appeared on day 14 after injection of CCII, with a peak onset on day 28 (Fig. 2A). During the inflammatory process (d14, d28, d46 after immunization), we observed a profound upregulation of GRK2 in synovium from CIA rats (Fig. 2B and C). The scatter plot of the paw swelling and the protein expression of GRK2 revealed a strong positive correlation (R^2=0.983, p<0.01) (Fig. 2D). This result indicates that the expression of GRK2 is related to the severity of CIA inflammation.

The proliferation increased in FLS from CIA rats as compared with that in normal rats. The GRK2 inhibitor and Pae (10−5 mol/L, 10−6 mol/L, 10−7 mol/L, 10−8 mol/L) had significant inhibitory effect on proliferation in FLS from CIA rats (Fig. 3A). The cAMP level and PKA activity were reduced in FLS from CIA rats compared to those in normal rats and increased after administration of the GRK2 inhibitor and Pae (10−5 mol/L, 10−6 mol/L, 10−7 mol/L) (Fig. 3B and C). The expression of GRK2 in FLS from CIA rats and effect of Pae in vitro are shown in Fig. 4. As compared with the normal rats, the expression of GRK2 increased significantly in FLS from CIA rats. After treatment with Pae (10−5, 10−6, 10−7, and 10−8 mol/L), the expression of GRK2 in FLS decreased evidently.

Discussion
GRKs play a crucial role in the regulation of a vast array of GPCRs involved in neurotransmission, cardiovascular function, and immune response, including inflammatory processes [20]. Although GRKs can play multiple functions in cells, little is known about their participation in the modulation of the proliferation of synoviocytes in mammals. To date, the role of GRKs in arthritis has not been investigated. In the present study, we observed that the increased expression of GRK2 in synovium was related to the severity of CIA inflammation. Inhibition of GRK2 led to an increase in the cAMP level and PKA activity and a decrease in proliferation capacity in FLS. Pae suppressed the expression of GRK2 and proliferation of FLS. Our results indicate that a chronic inflammatory process in CIA induces upregulation of GRK2 expression in FLS, and Pae can reverse this change, which might be one of the im-
important mechanisms for Pae regulating GPCRs signaling and suppressing the proliferation of FLS in CIA.

Hundreds of different GPCRs are regulated by seven subtypes of GRKs (GRK1–GRK7), of which four members are expressed at particularly high levels in cells of the immune system (GRK2, 3, 5, and 6) [21]. It has been reported that inflammation is associated with a decrease of GRK2 expression during several inflammatory pathologies in human and animal models. Studies in humans show that in peripheral blood mononuclear cells and leukocytes from patients with RA or multiple sclerosis, the GRK2 level is significantly reduced [13, 22]. Similarly, GRK2 and GRK6 protein expressions were markedly reduced in immune organs and immune cells from rats with chronic relapsing experimental autoimmune encephalomyelitis or AA [23].

Differently from previous studies, in the present study, the expression of GRK2 was increased during the arthritic process in synovium and FLS. Changes of GRK2 can affect various GPCRs signaling pathways including cAMP signaling. Agonist-stimulated cAMP production decreases in human melanoma cells enriched with GRK6 after stable transfection [24], while in cells transfected with GRK2 or GRK3 siRNA, cAMP accumulation is significantly elevated [25]. cAMP and its principal target, the cAMP-dependent PKA, constitute an important signaling pathway involved in cell growth and proliferation. In fibroblasts such as NIH3T3 cells, Rat-1 cells, and a number of other cell lines [26], cAMP antagonizes proliferation due to the ability to inhibit ERK signaling. We have previously reported that in synoviocytes from AA rats, the cAMP level decreases, while proliferation of synoviocytes increases [9]. In the present study, inhibition of GRK2 led to an increase of the cAMP level and PKA activity and a decrease in proliferation capacity in FLS. Our data suggests that GRK2 is an important molecular pathway involved in regulating the GPCRs-cAMP-PKA signaling in FLS, and upregulation of GRK2 expression during chronic inflammation may inhibit cAMP-PKA signaling, then induce the proliferation of FLS in CIA. cAMP does not only inhibit cell proliferation, it can also stimulate cell proliferation by stimulating ERKs in diverse cell types [27, 28]. So the change of GRK2 expression in inflammatory pathologies may be different in different tissues and cells. That may be the reason why expression change of GRK2 is different in lymphocytes and FLS from CIA.

The anti-inflammatory activity of Pae has been extensively studied. We have previously reported that Pae significantly ameliorates the symptoms and suppresses inflammatory response by inhibiting FLS from production of inflammatory mediators in CIA rats. The results of the present study showed that Pae could suppress the expression of GRK2 and proliferation of FLS. The anti-inflammatory effects of Pae may be mediated by regulating G-protein-cAMP signaling. Pae shows inhibition of the cAMP-phosphodiesterase activity [29]. In vivo and in vitro, administra-
tion of Pae leads to a decrease in Gαi expression and an increase in the cAMP level and PKA activity in synoviocytes from CIA rats [11]. Our previous studies have shown a decrease in the expression of the β2-adrenalin receptor and GRK2 in mesenteric lymph node lymphocytes from AA and CIA rats, and Pae can reverse the changes [15, 16]. An inflammatory process in vivo induces an up-regulation of β-arrestins in synoviocytes from CIA rats while TGP can inhibit this change [30]. In combination with our correlated research, we presumed that the effect of Pae on modulating FLS function may be partly through affecting GRK/β-arrestin desensitization, then regulating G-protein-cAMP signaling.

In conclusion, our results suggest that GRK2 may be an important molecular target in the pathogenesis of FLS in RA, but the precise mechanism responsible for increased GRK2 in FLS from CIA rats...
remains undefined. Further studies to evaluate the activity and distribution variation of GRK2 are needed. Furthermore, the present study indicates that Pae can regulate GPCRs-cAMP-PKA signaling by affecting GRK2 and then suppress the proliferation of FLS in CIA. Therefore, researches on regulation of GRK2 may provide a new therapeutic strategy to control inflammatory diseases, such as RA.

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Conflict of Interest
None.

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Fig. 4 The expression of GRK2 in FLS from CIA rats and effect of Pae in vitro. A Western blot image of GRK2 in FLS. B Quantification of GRK2 expression as an arbitrary density of β-actin. Each data point represents the mean ± SD from at least four independent experiments. Statistical significance as indicated (ANOVA followed by Dunnett’s test); * p < 0.05, ** p < 0.01 compared with CIA FLS; ** p < 0.01 compared with normal group.


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