Rhein Promotes the Proliferation of Keratinocytes by Targeting Oestrogen Receptors for Skin Ulcer Treatment

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Research Article

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Abstract

Background

The Sheng-ji Hua-yu (SJHY) formula is a quite effective Traditional Chinese Medicines (TCM) in the treatment of delayed diabetic wounds. Previous research has shown that the SJHY formula has significant anti-inflammatory and wound-healing effects, but the precise mechanism remains unknown. The purpose of this study was to evaluate the effects of rhein, a compound extracted from SJHY formula, in keratinocytes and to investigate the underlying mechanisms.

Methods

Microscale thermophoresis (MST) technology was used to confirm that rhein binds directly to oestrogen receptors (ERs). Rhein was then used to treat keratinocytes in vitro. Cell cycle and proliferation analysis, Real-time polymerase chain reaction (RT-PCR) and Western-blot were conducted.

Results

Rhein increased the proportion of cells in the S phase of the cell cycle and promoted keratinocyte proliferation. ICI 182780, an ER inhibitor, was also used to treat keratinocytes. The expression of c-myc mRNA and protein induced by rhein was antagonized by ICI 182780, indicating that this induction is ER dependent. Intervention with ICI 182780 had no effect on the upregulation of FosB and JunD, indicating that activator protein 1 (AP-1) members (FosB and JunD) are involved in rhein-induced c-myc mRNA and protein expression but does not require the ER.

Conclusion

The present study found that rhein stimulates keratinocyte proliferation by activating the oestrogen signalling pathway via the oestrogen receptor, which induces the expression of c-myc in collaboration with FosB and JunD, thereby accelerating the process of re-epithelialization.

Background

Skin ulcers are the most common complication among patients with skin diseases caused by various factors. Bacterial infection, trauma, radiation, psychological spirit, immune deficiency, and other factors are common pathogenic factors of skin ulcers [1]. Because there are many pathogenic factors of skin ulcers, the pathological mechanism involved in wound healing is complex and has not been clarified, which greatly restricts the further development of effective treatment methods and drugs. At the treatment level, in addition to the basic treatment according to the primary disease, the treatment of local wounds can be summarized as follows: improving the wound microenvironment; actively controlling the

inflammatory reaction; promoting angiogenesis; promoting proliferation and migration of keratinocytes; late stage collagen remodelling; and accessory regeneration. Therefore, effective targeted treatment schemes mainly include wound debridement, dressing change, systemic application of antibiotics, hyperbaric oxygen therapy, surgery, and phototherapy [2]. The above treatment is helpful to alleviate the condition of wound rupture, but it does not show a significant effect on long-term refractory wounds and wound closure in the later stage. At present, traditional Chinese medicine has become a global alternative medicine and is gradually being used to treat skin ulcers.

The Sheng-ji Hua-yu (SJHY) formula is one of the most effective Traditional Chinese Medicines (TCMs) in the treatment of delayed diabetic wounds. SJHY consists of *Astragalus membranceus* (Fisch) Bunge, *Salvia miltiorrhiza* Bunge, *Rheum palmatum* L., *Daemonorops draco* (Willd.) Blume, *Arnebia euchroma* (Royle) I.M. Johnst., *Angelica dahurica* (Hoffm.) Benth. & Hook.f. ex, *Hyriopsis cumingii* (Lea), and Calamine. Our previous clinical and basic studies have demonstrated that SJHY has significant anti-inflammatory and wound-healing effects [3, 4]. To clarify the effective components of SJHY, we extracted, separated, and analysed them in the early stage and obtained 15 components. Furthermore, with the help of network pharmacology and computer model-assisted molecular docking technology, we found that rhein has oestrogen-like effects and potential binding ability with oestrogen receptors. The present study was based on microscale thermophoresis (MST) technology to verify the direct binding ability of rhein to the target protein, i.e., oestrogen receptor. Subsequent experiments confirmed the proliferation promoting effect of rhein on keratinocytes (HaCaT cells), and further explained that rhein activate estrogen signaling pathway through estrogen receptors. The molecular pharmacological mechanism of CO inducing c-myc expression with AP-1 members (FosB, JunD).

Rhein is a natural anthraquinone derivative derived from the rhizomes of several traditional medicinal plants, including Rheum palmatum (also known as “da huang”) and Polygonum multiflorum (also known as “he shou wu”) [5, 6]. Rhein and its derivatives have gained increasing attention in scientific research and clinical treatment as modern drug extraction, separation, and synthesis technology has matured. Recent research has revealed that rhein has a wide range of pharmacological effects, including liver protection [7], kidney protection [8, 9], anti-inflammatory effects [10], antioxidant effects [11], antibacterial effects, antitumour effects, glucose metabolism regulation, and lipid metabolism regulation. Because of its significant antiproliferative and apoptosis-promoting effects in a variety of tumour cells via various mechanisms, rhein has become one of the hotspots of antitumour research [12–15]. Previous studies have focused on the prominent anti-proliferation and pro-apoptosis ability of rhein, and research on the effect of rhein on the cell cycle has also focused on its blocking effect. However, few studies have reported that rhein promotes DNA synthesis and has an effect on the cell cycle by increasing the proportion of cells in S phase. Rhein inhibits the proliferation of keratinocytes, specifically colo-16 cells (a squamous cell carcinoma cell line), in the G1 phase by blocking the cell cycle, implying that rhein has a two-way regulatory effect on keratinocyte proliferation. As a result, rhein, as an active component of Rheum palmatum, has significant developmental potential in the field of skin diseases.

Materials And Methods

Microscale Thermophoresis Assay

ERα full-length recombinant protein was obtained from Thermo Fisher Technology Co., Ltd. (Shanghai, China, No. A15674). ERβ full-length recombinant protein was obtained from Thermo Fisher Technology Co., Ltd. (Shanghai, China, No. A15664). Rhein was obtained from the National Institutes for Food and Drug Control (Beijing, China, No. 110757–201607).

MST was used to determine the binding affinity of ERs for rhein. ERα and ERβ proteins were labelled with fluorescence dye using the Monolith Protein Labelling Kit RED-NHS 2nd Generation (NanoTemper Technologies, München, Germany, Cat. No. MO-L011). The assays were performed in a buffer (pH 7.4) containing 20 mM Tris, 0.3 M NaCl, 5% glycerol, 3% DMSO, and 0.05% Tween-20. After a 30-minute incubation, the samples were loaded into Monolith NT.115 (NanoTemper Technologies) standard glass capillaries. During the MST experiments, the concentration of the labelled ERs was kept constant at 6.74 μM, while the concentration of rhein was serially diluted at a ratio of 1:2. In total, 16 titration series of rhein from the maximal final concentration of 0.1 mM to the minimal concentration of 3.05 nM were prepared and mixed with the labelled ERs. Fluorescence was analysed in the Monolith NT.115 device. The MST power and excitation power used were 20% and 75%, respectively.

Cell Culture

HaCaT cells were obtained from Ruijin Hospital, Shanghai Jiaotong University School of Medicine (Shanghai, China). HaCaT cells were cultured in DMEM (Gibco, USA) supplemented with 10% foetal bovine serum (Gemini, USA) and 1% antibiotics (Thermo Fisher Scientific, USA). All cells were incubated at 37°C in a 5% CO_2 humidified incubator. Cells passaged under oestrogen-free culture conditions did not exceed 4 generations.

Cell Viability Assay

HaCaT cells were seeded into 96-well plates (5×10^3 cells/well) to allow attachment, incubated overnight, and then treated with phenol-free DMEM with various concentrations of rhein for 24 h. Next, 10 μL of CCK-8 solution (Bimake, USA) was added to each well, and the plates were incubated for an additional 70 min at 37°C in a 5% CO_2 incubator. The absorbance at 450 nm was measured using a spectrophotometer (Tecan, Switzerland).

Cell Cycle and Proliferation Analysis

HaCaT cells were seeded into 60-mm culture dishes (1×10^8 cells/dish). After a 12 h incubation, cells were exposed to various concentrations of rhein for 24 h. For proliferation assays, cells were labelled with BrdU (10 μM) for 1 h using the BrdU Flow Kit (BD Science, USA, Cat. No. 552598). Cells were fixed and permeabilized with Cytofix/Cytoperm buffer, and cells were then stained using DNase (300 μg/mL)

staining buffer. For cell cycle assays, cells were stained with 7-Aminoactinomycin D (BD Science, USA). The cell cycle and proliferation were analysed by flow cytometry (Beckman Coulter, USA).

Quantitative Real-Time Polymerase Chain Reaction

HaCaT cells were seeded into 6-well plates (6×10^5 cells/well) to allow attachment, incubated overnight, and then treated with phenol-free DMEM with 50 μM rhein for 3 h. Next, 300 μL of TRIzol reagent (Thermo Fisher Scientific, USA) was added to each well for extraction of total RNA. The suspension was transferred to 1.5 mL EP tubes and allowed to rest at room temperature for 5 min, and 60 μL of chloroform was then added followed by vigorous shaking for 30 s and incubation at room temperature for 20 min. The mixture was centrifuged at 12,000 r/min for 10 min at 4°C. The obtained water phase was transferred to another EP tube, and equal volumes of isopropanol, 75% ice ethanol, and DEPC water solution were added to measure the OD value and estimate the purity of RNA. RNA values from 1.8 to 2.0 were used for reverse transcription. The Reverse Transcription System First Strand cDNA Synthesis Kit (PrimeScript® RT reagent Kit, Takara, Japan, Cat No. RR036A) was used with a 10 μL reaction volume. Quantitative real-time polymerase chain reaction (qRT–PCR) was performed using a PowerUp SYBR Green Master Mix Kit (Thermo Fisher Scientific, USA, Cat No. A25742) on a Thermo Fisher Scientific Real-Time System. GAPDH was amplified as an internal control. The following primer sequences were used: c-myc F, CGAGGAGAATGTCAAGAGGCGAAC; c-myc R, TCGGCGAACTCCTGCTCCTC; FosB F, TCTGTCTTCGGTGGACTCCTTCG; FosB R, TGGAGGTCCTGGCTGGTTGTG; JunD F, CGCCTCATCATCCAGTCCAACG; JunD R, GCTTGGACGGACAGGATGTATGC; GAPDH F, AGAAGGCTGGGGCTCATTTG; and GAPDH R, AGGGGCCATCCACAGTCTTC.

Western Blotting

HaCaT cells were seeded into 6-well plates (4×10^5 cells/well), allowed to attach overnight, and then treated with phenol-free DMEM containing 50 μM rhein for 24 h. Cells were collected, and RIPA lysis buffer (Beyotime, Shanghai, China) was used to extract total protein. A BCA protein analysis kit (Thermo Fisher Scientific, USA) was used to determine the protein concentration, and total protein (50 μg per sample) was separated and transferred to a polyvinylidene fluoride membrane. The membranes were then incubated with the following primary antibodies overnight at 4°C: anti-c-myc antibody (Cell Signaling Technology, 5605S, 1:1000), anti-FosB antibody (Cell Signaling Technology, 2251S, 1:1000), anti-JunD antibody (Cell Signaling Technology, 5000S, 1:1000), and GAPDH antibody (Cell Signaling Technology, 2118S, 1:5000). The membranes were then incubated with appropriate secondary antibodies (MedChemExpress, 7074S, 1:10000) for 1 h at room temperature.

Results

Binding Validation

The potential target of rhein was investigated using a computer model-assisted molecular docking technique, and the binding target of rhein was discovered to be the oestrogen receptor. We performed

MST assays to examine whether rhein binds to ERs. The results of the MST assays showed that the binding affinity of PA to ERα was 407.01 ± 75.97 μM and that the binding affinity of PA to ERβ was 289.44 ± 18.59 μM, suggesting that rhein maintains specific binding to ERs (Fig. 1).

Rhein Promotes the Proliferation of Keratinocytes

To confirm the effects of rhein (Fig. 2A) on keratinocytes, HaCaT cells were cultured in HG media to mimic hyperglycaemia. As shown in Fig. 2B, treatment with 100 μM rhein had no effect on the viability of HaCaT cells after 24 hours, but rhein significantly increased the viability of HaCaT cells at concentrations ranging from 6.25 to 50 μM after 24 hours compared to the DMSO control (Fig. 2C). We next determined whether rhein affects the cell cycle of HaCaT cells. Rhein increased the proportion of S phase cells in the cell cycle and promoted keratinocyte proliferation at concentrations of 25 μM and 50 μM (Fig. 2D), and the effect was more significant at a concentration of 50 μM ($P < 0.05$).

Rhein Activates the Oestrogen Signalling Pathway and Induces C-myc Levels

mRNA expression was assessed using qRT–PCR. Consistent with the protein levels, the mRNA expression levels of c-myc, FosB, and JunD in HaCaT cells were higher in the rhein group compared to the DMSO group (Fig. 3A). The protein expression of c-myc, FosB, and JunD after rhein treatment was detected by Western blot analysis. Compared to the DMSO group, c-myc, FosB, and JunD were enhanced significantly in the rhein group (Fig. 3B). To further explore the mechanism by which rhein regulates c-myc in HaCaT cells, we utilized an oestrogen receptor inhibitor (ICI 182780). The oestrogen receptor inhibitor had no cytotoxicity at 1 μM and did not affect the proliferation of HaCaT cells ($P > 0.05$) (Fig. 4A). In addition, the upregulation of c-myc was blocked by the oestrogen receptor inhibitor ($P < 0.05$) but had no significant effect on the mRNA and protein expression levels of FosB and JunD ($P > 0.05$) (Fig. 4B–4E). These results indicated that rhein activates the oestrogen signalling pathway and cooperates with FosB and JunD to induce the levels of c-myc.

Discussion

Previous research has shown that the SJHY formula has significant anti-inflammatory and wound-healing effects, but the precise mechanism remains unknown. Traditional Chinese medicine primarily interacts with a wide range of natural products with many targets in a variety of ways, making it difficult to study its mechanism. Nonetheless, we used network pharmacology and computer model-assisted molecular docking data to focus on oestrogen receptors and oestrogen signalling pathways.

Diabetic ulcers have a variety of causes, and the pathogenesis is complicated. The chronic inflammatory microenvironment created by inflammatory factors further impedes wound healing. Many of the ulcers that do not heal are caused by these factors [16]. It has been established that the skin is a hormone-sensitive organ [17]. Exogenous oestrogen treatment may reverse the effects of oestrogen deficiency on

wound healing, particularly during the stages of inflammation and tissue remodelling [18]. In addition, clinical practice has discovered that the healing rate of acute skin injury in elderly male patients is significantly slower than that of female patients of the same age and that elderly males more easily develop chronic skin ulcers. The wound-healing ability of postmenopausal women is significantly improved after receiving oestrogen replacement therapy [19–21]. The oestrogen receptor system is involved in the entire skin defect healing process, influencing granulation tissue formation, re-epithelialization, and remodelling after healing [22].

MST is a new technology that has been used to study the interaction of biomolecules in the last ten years. In recent years, MST technology has been successfully applied to the interactions of proteins with small molecules, proteins, and nucleic acids, providing an important method to study biological transduction pathways, regulatory mechanisms, structure, function, physiological pathways, and biochemical metabolic pathways of biomolecules [23–26]. The present study utilized MST technology to confirm the direct binding ability of rhein to the oestrogen receptor target protein. The results revealed that rhein directly bind to the ERα and ERβ target proteins, providing direct evidence to study the molecular pharmacological mechanism of rhein.

Based on the above findings, we used keratinocytes (HaCaT cells) as the cell model, performed cell oestrogen removal culture, and examined the effect of rhein on keratinocytes. Our findings indicated that rhein improves the viability of HaCaT cells. We discovered that rhein increased the proportion of cells in the S phase of the cell cycle and promoted keratinocyte proliferation. As a result, we hypothesized that rhein promotes keratinocyte proliferation and re-epithelialization.

The skin has been demonstrated to be a hormone-sensitive organ. The ER is highly expressed in various cells of the epidermis, dermis, and blood vessels, indicating that oestrogen has an action target in the skin [27]. In fact, the ER system plays a role in the entire skin defect healing process, influencing granulation tissue formation, re-epithelialization, and remodelling after healing [22]. Oestrogen passes through the cell membrane and interacts with nuclear ERα, and ERβ binding leads to changes in its conformational structure. The complex then translocates into the nucleus and binds to oestrogen receptor elements (EREs) in the promoter region of the target gene to activate or inhibit gene transcription, which is the classical oestrogen signalling pathway [28]. Many oestrogen-regulated genes, such as c-myc, have no ERE in their promoters. Oestrogen regulates gene expression in these genes via other mechanisms. Recent research has indicated that FosB and JunD, which are ER and AP-1 members, do not bind to the distal enhancer region in the absence of oestrogen, and c-myc is expressed at a low level. When oestrogen is introduced into cells, the ER-JunD-FosB complex forms to act as a transactivator, which interacts with the c-myc promoter region, recruits RNA polymerase II (Pol II), and finally induces c-myc gene expression [29]. Thus, oestrogen can also induce c-myc gene expression via its receptor and the AP-1 members, FosB and JunD.

Although c-myc is a member of the proto-oncogene myc family, its role is not limited to tumorigenesis. c-myc is involved in a variety of physiological processes, such as cell growth, proliferation, and

differentiation, and it encodes receptors, growth factors, protein kinases, signal molecules, and transcriptional regulators [30]. Interestingly, studies have shown that there is a subtle internal relationship between the occurrence and development of tumours and the repair and healing of wounds [31–34]. In short, the intersection of the two processes covers the following stages: inflammatory response, cell proliferation, differentiation, angiogenesis, and tissue remodelling. Based on these similarities, the overlap on the material basis is not accidental; that is, some key genes, proteins, and even signal pathways not only stimulate tumour formation but also participate in the corresponding process in wound healing [35]. Thus, c-myc is important not only in skin wound repair and healing but also in tumour transformation. However, the distinction is that the outcomes are diametrically opposed. Wound healing is relatively conservative and controllable in comparison to the absolute uncontrollable excess of tumour growth and infiltration. External intervention and stimulation induce c-myc expression and promote the cell cycle from G0/G1 to S phase, resulting in cell division and proliferation [36]. Although cell proliferation is not good when combating tumours, cell proliferation is necessary for wound healing. Furthermore, the upregulation of c-myc expression during the healing process of diabetic ulcers can be regarded as a proliferation marker of skin cells at a certain level.

Previous microarray results have highlighted the significant upregulation of the c-myc gene after SJHY intervention in HaCaT cells. In vitro experiments also confirmed that SJHY has a proliferation-promoting effect on HaCaT cells at a specific concentration of 10 mg/L, and this effect is accompanied by an increase in c-myc protein expression. However, SJHY has no effect on the phosphorylation level of c-myc, suggesting that the increase in its expression level is regulated at the level of translation and transcription. However, it remains unknown how SJHY regulates the expression of c-myc.

In the present study, we discovered that rhein directly binds to ERs, implying that rhein may play an oestrogenic role in the process of healing diabetic ulcers via oestrogen receptors. Further experiments confirmed that rhein intervention in HaCaT cells for 24 hours promoted cell proliferation and accelerated the re-epithelialization process by increasing the proportion of S phase cells in the cell cycle. Previous research has shown that oestrogen rapidly and steadily induces c-myc expression in oestrogen receptor-positive breast cancer cells (MCF-7). The induction of c-myc mRNA occurs as early as 30 minutes and lasts for 6 hours after treatment [29]. As a result, we treated HaCaT cells with rhein for 3 hours, which resulted in increased mRNA expression levels of c-myc, FosB, and JunD. After 24 hours, rhein increased the protein expression of c-myc, FosB, and JunD in HaCaT cells. The ER inhibitor, ICI 182780, was used to treat cells to study the ER dependence of c-myc expression. The expression of c-myc mRNA and protein induced by rhein was antagonized by ICI 182780, indicating that this induction is ER dependent. Intervention with ICI 182780 had no effect on the upregulation of FosB and JunD, indicating that AP-1 members (FosB and JunD) are involved in rhein-induced c-myc mRNA and protein expression but do not require the ER.

Conclusion

In conclusion, our findings showed that rhein activates the oestrogen signalling pathway via the oestrogen receptor, induces c-myc expression synergistically with AP-1 members (FosB and JunD), and promotes keratinocyte proliferation, thereby accelerating the process of re-epithelialization (Fig. 5).

Abbreviations

SJHY
Sheng-ji Hua-yu
TCM
Traditional Chinese Medicine
MST
Microscale Thermophoresis
ER
Oestrogen Receptors
RT-PCR
Real-time polymerase chain reaction
AP-1
Activator protein 1
ERE
Oestrogen Receptor Elements
Pol II
RNA polymerase II.

Declarations

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Authors' contributions

Conceptualization, FL and NX; methodology, NX; validation, NX, YC and WG; formal analysis, DG; investigation, XL; writing—original draft preparation, NX; writing—review and editing, YD and JZ; visualization, YW and HL; supervision and project administration, FL. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

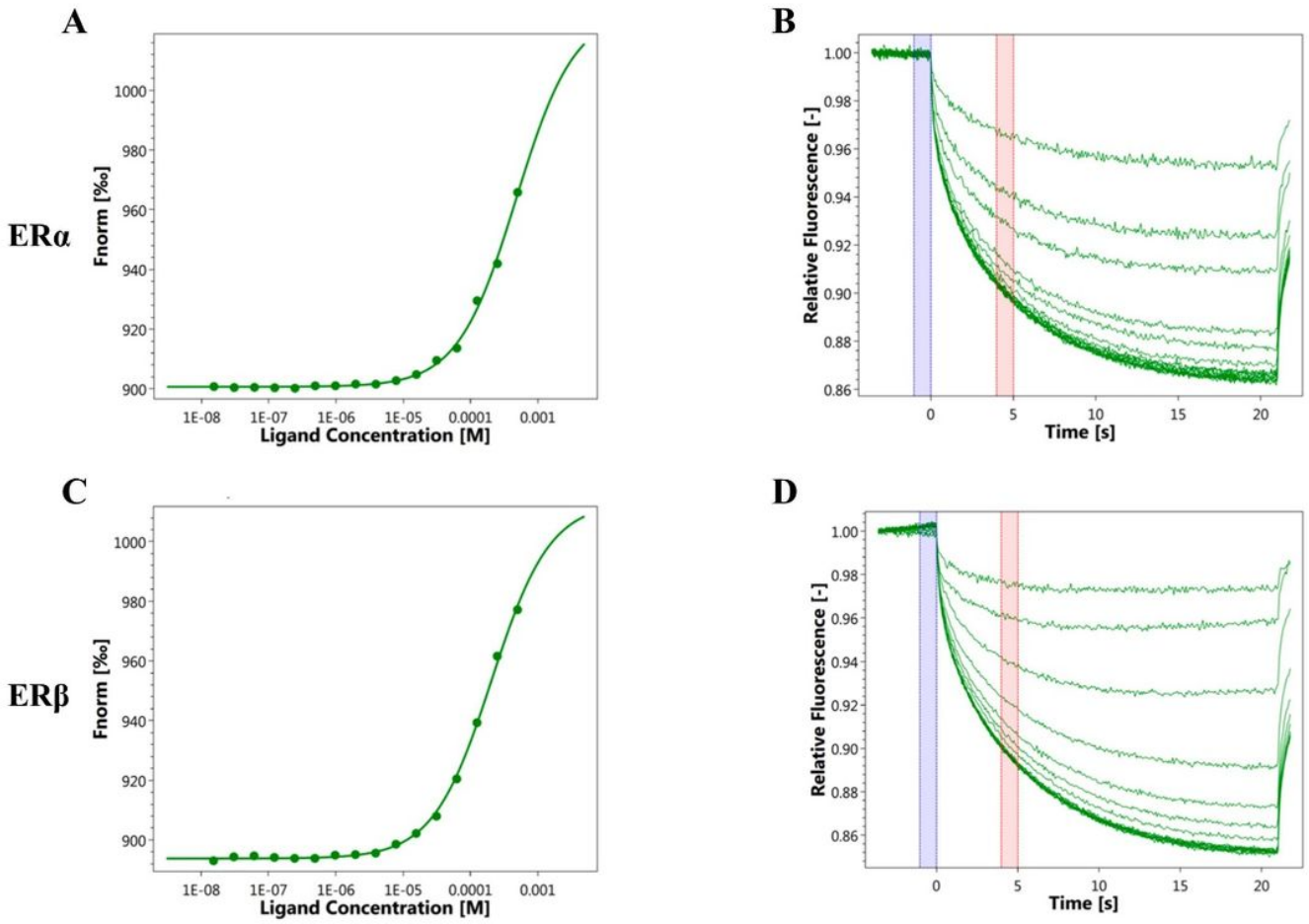

Figure 1

Rhein directly binds to ERs. Quantification of the binding affinity of rhein to ERs using a microscale thermophoresis assay. **A** and **B** The binding affinity of rhein for ERα was 407.01 ± 75.97 μM (shown in the upper panel). **C** and **D** The binding affinity of rhein for ERβ was 289.44 ± 18.59 μM (shown in the lower panel).

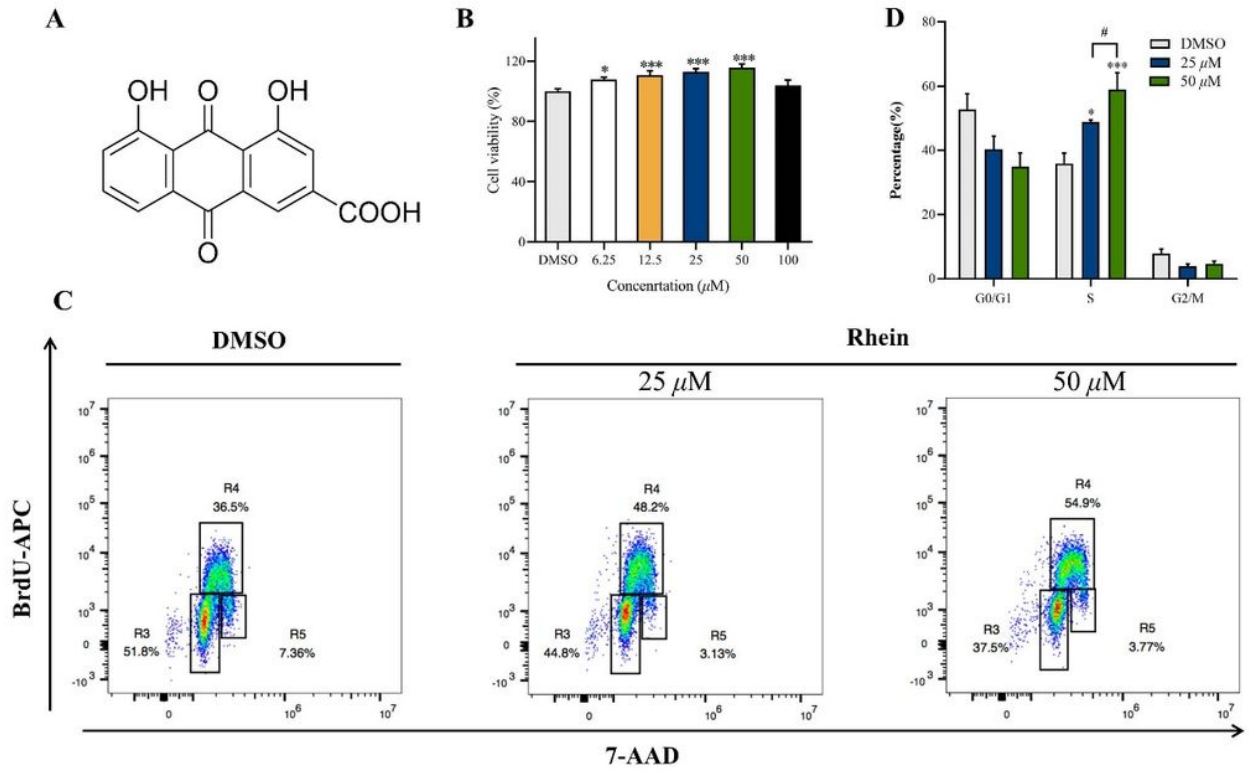

Figure 2

Rhein promotes the proliferation of keratinocytes. **A** Structure of rhein. **B** Cell viability of keratinocytes was tested using the CCK-8 assay. The results showed that rhein at concentrations ranging from 6.25–50 μM increased the viability of keratinocytes. **C** and **D** Flow cytometry showed that rhein increased the proportion of S phase cells in the cell cycle and promoted the proliferation of keratinocytes at concentrations of 25 μM and 50 μM, and the effect was more significant at a concentration of 50 μM. Data are presented as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus DMSO group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus the 25 μM rhein group.

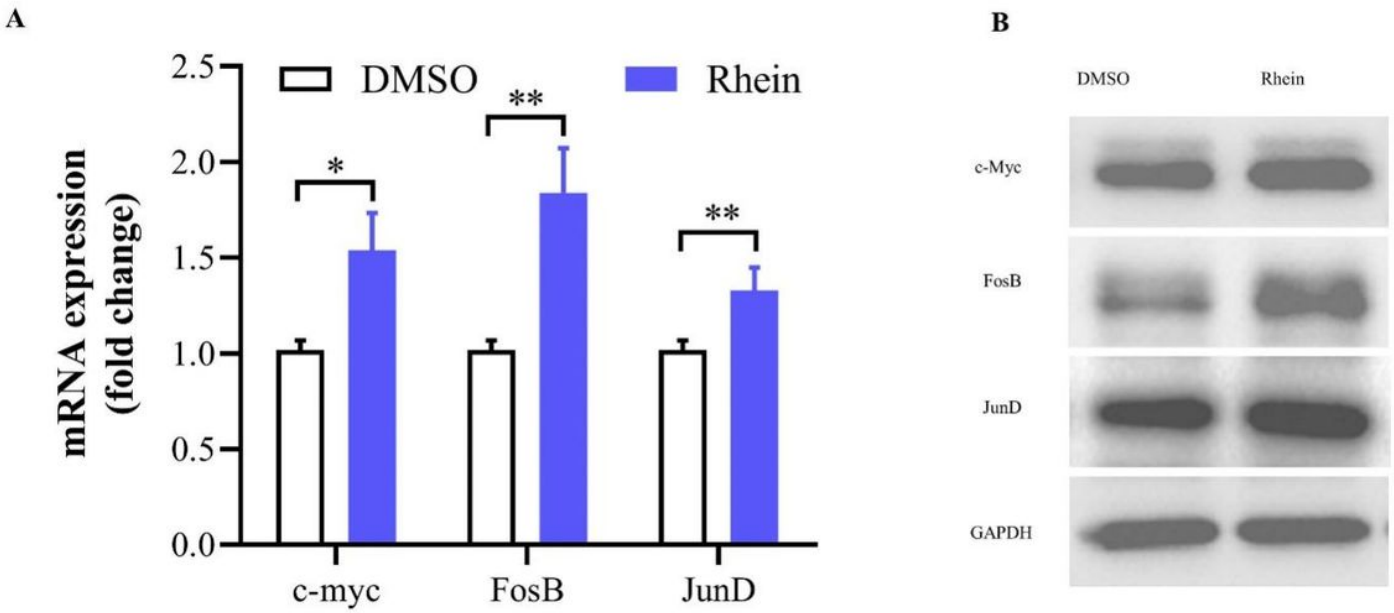

Figure 3

Rhein upregulates c-myc, FosB, and JunD expression in HaCaT cells. **A** Rhein at a concentration of 50 μM upregulated c-myc, FosB, and JunD mRNA expression following treatment for 3 hours. **B** Rhein at a concentration of 50 μM upregulated c-Myc, FosB, and JunD protein levels following treatment for 24 hours. Full-length blots are shown in Supplementary Fig. S1. Data are presented as the mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the DMSO group.

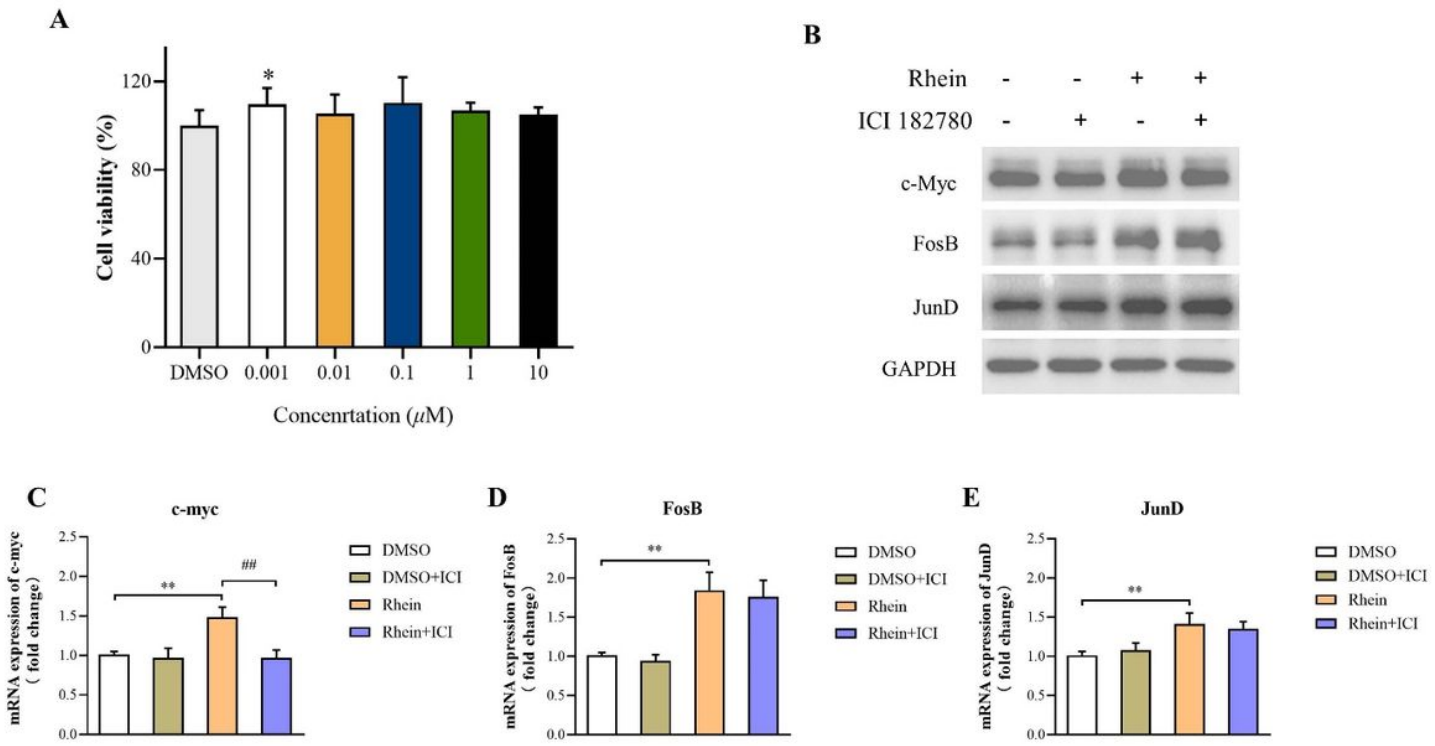

Figure 4

Rhein activates the oestrogen signalling pathway through the oestrogen receptor and cooperates with FosB and JunD to induce c-myc expression. A The oestrogen receptor inhibitor had no cytotoxicity at 1 μM and did not affect the proliferation of HaCaT cells. B-E Rhein regulated c-myc, FosB, and JunD expression at a concentration of 50 μM. The oestrogen receptor inhibitor blocked the rhein-induced upregulation of c-myc, but the inhibitor had no significant effect on the mRNA and protein expression levels of FosB and JunD. Full-length blots are shown in Supplementary Fig. S1. Data are presented as the mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the DMSO group, $^{\#\#}P < 0.01$ versus the rhein group.

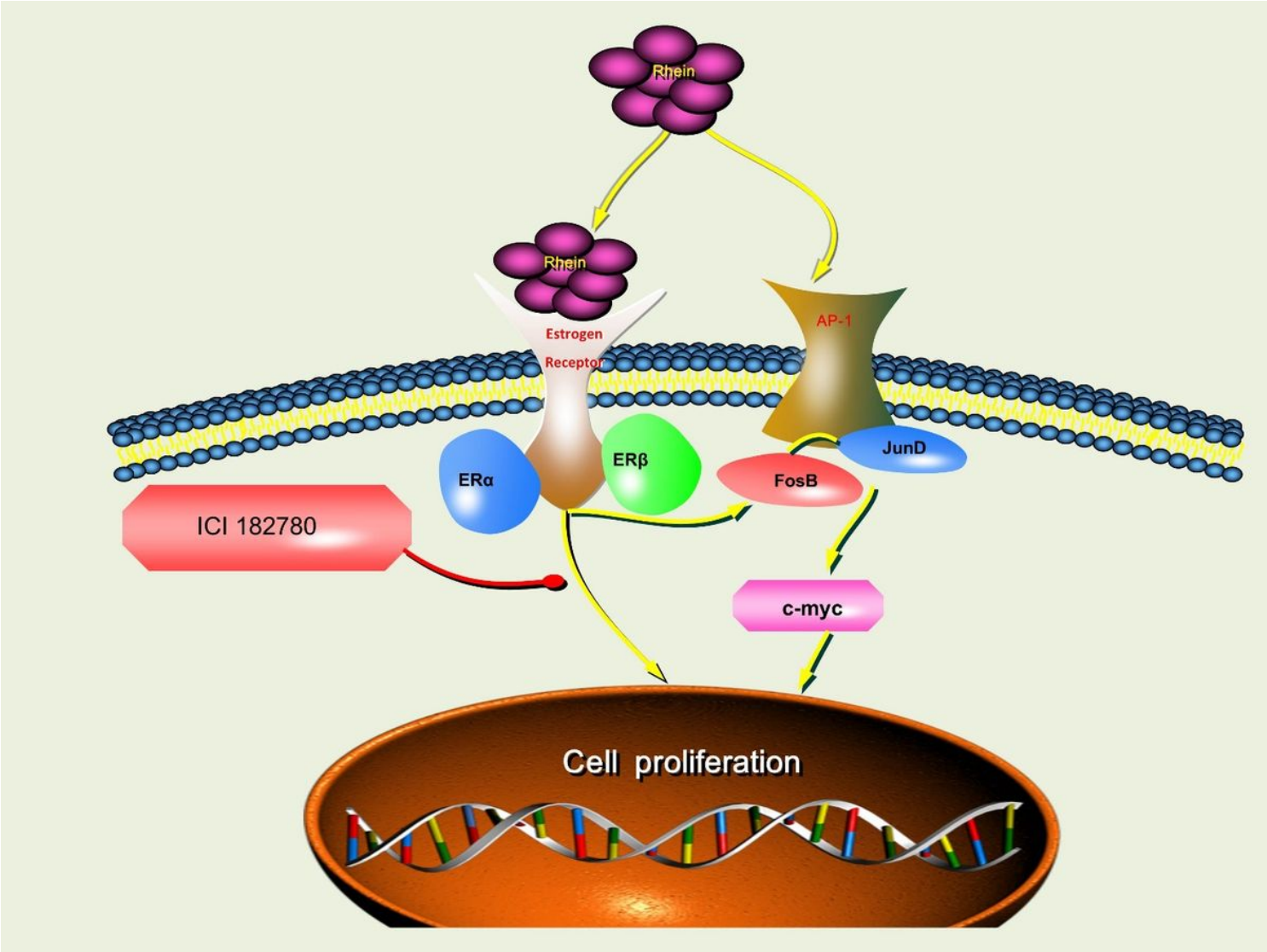

Figure 5

Schematic illustration of the underlying mechanism of the oestrogenic activity of rhein via the oestrogen receptor in keratinocytes.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigureS1.docx