Cinnamic acid inhibits cell viability, invasion, and glycolysis in primary endometrial stromal cells by suppressing NF-κB-induced transcription of PKM2

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Running title: Cinnamic acid targets PKM2 in endometriosis

Abstract

Background: Endometriosis is a painful disorder characterized by the growth of endometrial tissue outside the uterine cavity. Here, we investigated the effects of the cinnamic acid isolated from the Chinese medicinal plant *Cinnamomum cassia Presl* on primary endometrial stromal cells.

Methods: Immunohistochemistry was used to examine protein expression and cell purity. Quantitative RT-PCR was conducted to assess mRNA expression, and Western blot was performed to determine protein level. Cell viability was assessed using cell counting kit-8 (CCK-8) assay. Glycolysis and mitochondrial function were evaluated by measuring the extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR) of cells, respectively. Lastly, plasmid transfection and inhibitor treatment were used for overexpression and inhibition studies.

Results: Cinnamic acid inhibited cell viability and cell invasion, as well as decreased ECAR and OCR, in primary endometrial stromal cells. Cinnamic acid suppressed the effects of PKM2 overexpression, and inhibition of PKM2 using Compound 3k mimicked the effects of cinnamic acid. Treatment with Compound 3k and cinnamic acid did not lead to additive effects, but rather displayed effects similar to those of Compound 3k alone, suggesting that cinnamic acid elicits its effects on primary endometrial stromal cells by targeting PKM2.

Conclusions: Our study identified cinnamic acid as a novel compound from *Cinnamomum cassia Presl* that displays potent effects on primary endometrial stromal cell viability, invasion, and glycolysis, suggesting its potential use for endometriosis treatment.

Keywords: Endometriosis; cinnamic acid; PKM2; glycolysis; invasion

Introduction

Endometriosis is a gynecological disorder that affects at least 10% of women of reproductive age worldwide [1, 2] and features abnormal growth of endometrial tissues and stroma-like lesions outside the uterus. Endometriosis can result in severe pelvic pain, as well as subfertility, with a significant impact on quality of life [3]. While there is no definitive etiology of endometriosis, it is generally accepted that retrograde menstruation and subsequent implantation of the endometrial tissue on different tissue surfaces in the pelvic cavity play major roles in endometriosis pathophysiology [3, 4].

The establishment and progression of endometriosis as a disease involves a number of key biological processes, including cell adhesion to and invasion into the peritoneum [5]. Recent findings have shown that women with endometriosis exhibit a shift in cell metabolism from mitochondrial oxidative phosphorylation to aerobic glycolysis in the peritoneal microenvironment [6, 7]. Transforming growth factor beta (TGF-β) has been implicated in the etiology of peritoneal endometriosis [8]. In tumors, TGF-β is known induce the conversion of glucose to lactate via glycolysis, a process known as the "Warburg effect" [9-11], resulting in cell invasion and angiogenesis, both of which are crucial steps in endometriosis pathophysiology. Indeed, it is increasingly becoming recognized that TGF-β-induced Warburg-like metabolic reprogramming may underlie the initiation of endometriosis [8, 12].

A notable feature of the Warburg effect is a shift in the expression of pyruvate kinase (PK) isoform M1 to isoform M2 [13]. Pyruvate kinase isoform 2 (PKM2) functions as a cytosolic receptor for thyroid hormone and plays an important role in the epigenetic regulation of gene transcription [14]. Upon oncogenic stimulation, PKM2 enters the nucleus, where it phosphorylates its target proteins, including histones. Moreover, PKM2 regulates glycolysis and mitochondrial function. As a central point of regulation in metabolism, PKM2 is widely associated in cancer [15]. Interestingly, PKM2 has been implicated as a potential metabolic biomarker that distinguishes endometrial cancer associated with poor prognosis from endometrial precancer [16].

Nuclear factor-κB (NF-κB), a transcription factor that plays a crucial role in cell proliferation, apoptosis, invasion, inflammation, and immunity, is involved in the development of endometriosis [17]. It has been demonstrated that NF-κB is activated in endometriotic lesions and blocking NF-κB is effective at reducing endometriosis-associated symptoms [18]. NF-κB inhibitors therefore seem promising for the treatment of endometriosis since they could act in a wide range of key processes in endometriosis development. Previous studies have shown that NF-κB transcriptionally induces PKM2 [19] and favors the survival of the ectopic endometrial tissue [20]. However, the role of PKM2 in regulating endometriosis is still unknown.

Chinese medicine has been traditionally used to treat a variety of diseases, including cancer, and many current anti-cancer drugs are derived from natural products. For instance, oleanolic acid, a

triterpenoid component in plants, has been shown to reduce the viability and proliferation of cancer cells [21]. Chinese medicine has also been gaining attention for the treatment of a number of disorders, including endometriosis [22]. For example, a classic Chinese medicinal formula consisting of *Gui-Zhi-Fu-Ling* capsules has shown promising result in treating endometriosis in rat mouse model [23]. In this study, we examined the effects of different compounds such as cinnamaldehyde, cinnamic acid, coumarin, or protocatechuic acid isolated from ramulus of *Cinnamomum cassia Presl*, a medicinal plant from the Lauraceae family known to possess antioxidant and antimicrobial activities [24], on primary endometrial stromal cells. Cinnamaldehyde is an active compound of Guizhi Fuling Pills that the mechanisms in the treatment of endometriosis mainly include acesodyne, anti-inflammation and improvement of hemodynamics [25]. Coumarin derivatives as selective nonsteroidal inhibitors of 17β-Hydroxysteroid dehydrogenase type 1 have therapeutic potential in endometriosis [26]. Cinnamic acid, a natural precursor of the coumarin, is molecularly docked with proteins associated with endometriosis and is a Quality Marker of Guizhi Fuling Prescription for endometriosis treatment [27]. Protocatechuic acid is a major ingredient of Wenshen Xiaozheng Tang that induces apoptosis and inhibits migration of ectopic endometriotic stromal cells [17]. Although these different compounds have been studied in previous reports, their functions in regulating endometriosis have not been clearly elucidated. We discovered that cinnamic acid inhibited cell viability, invasion, and glycolysis of primary endometrial cells, and these effects were regulated by suppressing NF-κB-induced transcription of PKM2.

Materials and methods

Tissue collection and cell culture

Twelve active peritoneal endometriotic red, chocolate, and blue lesions were obtained from 12 women with endometriosis who underwent laparoscopic treatment in the Shanghai Municipal Hospital of Traditional Chinese Medicine Affiliated to Shanghai TCM University. Control endometrial samples were collected from 12 women without endometriosis who underwent laparoscopy and hysteroscopy surgery for benign gynecological diseases. Patients that received hormonal treatment and birth control method prior to enrollment served as exclusion criteria. Primary endometrial stromal cells were obtained from ectopic endometria of endometriosis patients or from normal endometria of women without endometriosis. Cells isolation and culture

were performed as follow: endometrial tissues were minced, isolated by 4% collagenase digestion at 37°C for 60 min, and centrifugation at 500 × g for 5 min; cell suspension were further centrifugation at 3000 × g for 10 min and the cell deposition were then resuspended in Dulbecco modified Eagle medium (Gibco) containing 10% fetal bovine serum (Gibco) and in 5% CO_2 atmosphere at 37°C. Immunocytochemistry using anti-cytokeratin (CK) 19 (Abcam, ab52625) and anti-vimentin (Abcam, 92547) antibodies was performed to determine cell purity as previous described [28]. Over 95% purity of stromal cells was achieved after 2-3 passages. Immunofluorescence using anti-PKM2 (Affinity, AF5234) was performed to determine the PKM2 expression levels in primary endometrial stromal cells as previous described [29]. The Ethics Committee of Shanghai Municipal Hospital of Traditional Chinese Medicine Affiliated to Shanghai TCM University approved this study, with written informed consent provided by the patients.

PKM2 overexpression

Full-length human PKM2 cDNA sequence was inserted into the pcDNA3.1(+) vector. For negative control, an empty pcDNA3.1(+) vector was used. Lipofectamine 2000 (Invitrogen) was used to transfect pcDNA3.1(+) or pcDNA3.1(+)-PKM2 vector into primary endometrial stromal cells according to the instructions of the manufacturer.

Experiment groups

Group 1: cells were treated with 50 μM of cinnamaldehyde, cinnamic acid, coumarin, or protocatechuic acid (all from Selleck Chemicals, Houston, TX, USA) for 48 hr. Group 2: cells were treated with different concentrations of cinnamic acid (20, 50 and 100 μM) for 48 hr. Group 3: cells were transfected with PKM2 expression vector or empty vector as control and treated with 100 μM of cinnamic acid for 48 hr. Group 4: cells were treated with different concentrations of Compound 3k (1 and 2 μM; Selleck Chemicals) for 48 hr. Group 5: cells were treated with 100 μM of cinnamic acid or 2 μM of Compound 3k alone for 48 hr, or with 2 μM of Compound 3k for 24 hr, followed by 100 μM of cinnamic acid for another 24 hr.

CCK-8 assay

Cells were trypsinized and counted under a microscope. A cell suspension of $3x10^3$ cells/well was prepared, and 100 μL was seeded in each well of 96-well plates to culture overnight. After incubation for 0, 24, 48, 72, and 96 hr, 100 μL of Cell Counting Kit-8 (Dojindo) solution in serum free media (1:10) was added to each well and then incubated at 37ºC for 1 hr. Thereafter, the absorbance value (OD) at 450 nm, which indicates cell viability, was determined on a microplate reader (Wellscan MK3, Thermo/Labsystems).

Transwell assay

After 48 hr treatment, cells were grown in serum-free media for 24 hr, after which cells were trypsinized and 300 μL cell suspension containing $6x10^4$ cells was seeded into Matrigel-coated (BD Biosciences) 24-well Transwell chambers (Costar). Then, 700 μL of DMEM media containing 10% FBS were added to the lower chamber, and cells were incubated for 48 hr in a 37ºC incubator. Subsequently, cells were fixed in 4% formaldehyde (Jinsan Chemical Reagent Co. Ltd., Chengdu, China) for 10 min, then stained with 0.5% crystal violet (Aladdin Chemical Reagent Co., Ltd., Shanghai, China) for 30 min, after which cells were examined under the microscope and the number of invading cells was counted.

Extracellular flux evaluation

After 48 hr treatment, Seahorse Extracellular flux 24 Extracellular Flux Analyzer was used to assess mitochondrial function and glycolysis by measuring the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), which are indicative of respiration and glycolysis, respectively, as previously described [30].

Quantitative PCR (qPCR) assay

Trizol Reagent (Invitrogen) was used to extract total RNA from primary endometrial stroma cells following the manufacturer's instruction. For reverse transcription, iScript TM cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) was used. Quantitative PCR was performed using SYBR Green PCR Master Mix (QIAGEN, Hilden, Germany) on a 7500 Real-Time PCR System (Applied Biosystems). $2^{-\Delta\Delta Ct}$ method was used to calculate relative quantification. GAPDH was used as a reference gene and the primer sequences used in this study include the following: PKM2

(5’-GCTTCTGACCCCATCCTCTACC-3’ and 5’-GCGTTATCCAGCGTGATTTTG-3’); GAPDH (5’-AATCCCATCACCATCTTC-3’ and 5’-AGGCTGTTGTCATACTTC-3’).

Western blotting

Primary endometrial stromal cells were lysed in RIPA buffer containing protease and phosphatase inhibitor cocktail (P8340 and P2850; Sigma). Then, 25 μg of total protein was separated on a 10% SDS-PAGE gel and transferred onto nitrocellulose membranes (Whatman®, GE Healthcare) for 30 min at 4°C, after which membranes were incubated with anti-PKM2 (Abcam; ab137852) or anti-GAPDH (CST; 5174) antibodies for 12 hr at 4°C. After washing, membranes were incubated with secondary antibodies (Beyotime Institute of Biotechnology, A0208 and A0216) for 1 hr at 37°C with. An enhanced chemiluminescence substrate kit (Amersham Biosciences) was used to quantify protein signal. Target protein expression relative to GAPDH was quantified using ImageJ software (National Institutes of Health, USA).

Dual luciferase assay

Cells were transfected with pGL3-basic plasmid containing PKM2 promoter or pRL-TK plasmid using Lipofectamine 2000 (Invitrogen) and incubated with a vehicle or cinnamic acid at 37°C for 6 h. A Luciferase Assay System (Promega) was applied to measure the luciferase activity according to the manufacturer protocols.

Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed as previously described [31]. The antibody used was NF-κB p65 (Cell Signaling Technology; 3034). Purified ChIP DNA was confirmed by PCR (PKM2 promoter primer sequences: F, 5’-TTTCTCCCAGGGCGACTTT-3’ and R, 5’-GACGACAGAAGCGTCCAGAG-3’).

Statistical analysis

Results were presented as mean $\pm$ SD of at least three samples in triplicates. Statistical analysis was conducted using GraphPad Prism 8.02 (GraphPad Software Inc.). Mean comparisons were performed using unpaired t-test for two groups or analysis of variance (ANOVA) for multiple comparisons among groups. A p-value <0.05 was considered statistically significant.

Results

Cinnamic acid decreased ECAR and OCR in primary endometrial stromal cells

As shown by immunocytochemistry, primary endometrial stromal cells isolated from patients with or without endometriosis displayed positive vimentin expression and negative CK19 expression (Figure 1A). Using primary normal and endometriosis-derived ectopic endometrial stromal cells, we evaluated the effects of different compounds on the cell viability, glycolysis and mitochondrial function by determining the CCK-8 assay, extracellular acidification rate (ECAR) and oxygen consumption rate (OCR), respectively. These compounds included cinnamaldehyde, cinnamic acid, coumarin, and protocatechuic acid. Protocatechuic acid promotes the cell viability, ECAR and OCR in primary normal endometrial stromal cells isolated from patients without endometriosis (Figure S1A-C) and primary endometrial stromal cells from ectopic endometria of endometriosis patients (Figure 1B-D). However, cinnamaldehyde and cinnamic acid inhibited cell viability, ECAR and OCR in primary ectopic endometrial stromal cells (Figure 1B-D), suggesting that this compound can modulate glycolytic activity and mitochondrial functions. Moreover, the effects of cinnamic acid were more effective than cinnamaldehyde. Therefore, cinnamic acid was used for following study.

Cinnamic acid inhibited cell viability, invasion, ECAR, and OCR in primary endometrial stromal cells

To probe further the cellular effects of cinnamic acid, primary endometrial stromal cells from ectopic endometria of endometriosis patients were treated with varying concentrations of cinnamic acid. We assessed cell viability at 0, 24, 48, 72 and 96 hours after treatment by cell counting kit-8 (CCK-8) assay, and found that cinnamic acid inhibited cell viability in a dose-dependent manner (Figure 2A). Similarly, treatment with increasing concentrations of cinnamic acid strongly blocked cell invasion of primary endometrial stromal cells (Figure 2B-C). Furthermore, cinnamic acid dose-dependently decreased ECAR and OCR in primary endometrial stromal cells from ectopic endometria (Figure 2D-E). Interestingly, PKM2 mRNA and protein levels were increased in primary ectopic endometrial stromal cells compared with primary normal endometrial stromal cells (Figure S2A and B). Moreover, cinnamic acid treatment also resulted in reductions of PKM2 mRNA and protein levels (Figure 2F-G). It has been shown that NF-κB transcriptionally induces

PKM2 [19] and favors the survival of the ectopic endometrial tissue [20]. However, cinnamic acid inhibits NF-κB activation [32]. Therefore, we suggest that cinnamic acid may regulate PKM2 expression through NF-κB-dependent transcription. Indeed, a Luciferase Reporter assay demonstrated that the activity of the PKM2 promoter in primary ectopic endometrial stromal cells was suppressed by cinnamic acid and NF-κB inhibitor PDTC (Figure 2H). ChIP analysis revealed that NF-κB could bind to PKM2 promoter, which was inhibited by cinnamic acid and PDTC (Figure 2I). Together, these data demonstrate that cinnamic acid negatively regulates viability, invasion, glycolysis, and PKM2 expression in primary ectopic endometrial stromal cells.

Cinnamic acid suppressed PKM2 overexpression-induced effects on cell viability, invasion, ECAR, and OCR in primary endometrial stromal cells

We next investigated whether cinnamic acid played a role in mediating the effects of PKM2 on primary endometrial stromal cells from ectopic endometria. To do this, primary normal endometrial stromal cells from women without endometriosis were transfected with either pcDNA3.1 vector or PKM2 cDNA, and then treated with 100 μM of cinnamic acid. As indicated in Figure 3A, PKM2 overexpression elevated both the mRNA expression and protein level of PKM2. We then assessed the effects of PKM2 overexpression on cell viability and invasion, as well as on ECAR and OCR. As shown in Figure 3B, PKM2 overexpression increased viability of primary endometrial stromal cells from normal endometria (Figure 3B), and treatment with cinnamic acid strongly suppressed PKM2-induced increase in cell viability (Figure 3B). Likewise, PKM2 overexpression promoted cell invasion, which was reduced upon cinnamic acid treatment (Figure 3C-D). Furthermore, PKM2 overexpression elevated ECAR and OCR (Figure 3E-F), as well as PKM2 protein expression (Figure 3G), in primary endometrial stromal cells, and these effects were suppressed by cinnamic acid treatment. Collectively, these data demonstrate that cinnamic acid suppressed PKM2-induced effects on cell viability, invasion, and glycolysis in primary endometrial stromal cells.

PKM2 inhibition displayed similar effects as cinnamic acid treatment on cell viability, invasion, ECAR, and OCR

Next, we used a PKM2 inhibitor, Compound 3k, to inhibit PKM2 function. Primary endometrial stromal cells from ectopic endometria of endometriosis patients were treated with 1 μM or 2 μM of

Compound 3k, and effects on cell viability, invasion, ECAR, and OCR level were determined. As shown in Figure 4A, treatment with Compound 3K reduced PKM2 mRNA and protein levels, with 2 μM displaying a more robust effect. Inhibition of PKM2 resulted in decreased cell viability and invasion (Figure 4B-D), as well as reduced ECAR and OCR (Figure 4E-F). Together, these data reinforce the functions of PKM2 in viability, invasion, and glycolysis of primary endometrial stromal cells.

Cinnamic acid inhibited cell viability, invasion, ECAR, and OCR by targeting PKM2

Lastly, to determine whether cinnamic acid elicits its effects by targeting PKM2, we treated primary endometrial stromal cells with vehicle, cinnamic acid and/or Compound 3k. We then analyzed the effects of the different treatments on cell viability, invasion, ECAR and OCR. We found that cinnamic acid and Compound 3k both reduced cell viability and invasion, with Compound 3k having the more robust effect (Figure 5A-C). Cinnamic acid did not display additive effects under the condition of Compound 3k treatment but rather showed almost similar effects as Compound 3k alone (Figure 5A-C), suggesting that inhibition of PKM2 drives the observed cellular effects of cinnamic acid. Similarly effects of cinnamic acid on ECAR and OCR in primary endometrial cells were also found (Figure 5D-E). Collectively, these data demonstrate that cinnamic acid modulates cell viability, invasion, and glycolysis by reducing PKM2.

Discussion

In this study, we found that cinnamic acid, a compound isolated from ramulus of *Cinnamomum cassia Presl*, inhibited cell viability, cell invasion, and glycolysis in primary endometrial stromal cells, highlighting the potential of traditional medicine in the treatment for endometriosis. Cinnamic acid is a well-known naturally occurring compound that has low toxicity and a broad spectrum of biological activities [24], and may be used to bring therapeutic benefits for women with endometriosis.

Our study uncovered that cinnamic acid elicited its effects on primary endometrial stromal cells by targeting PKM2, a protein involved in glycolysis and cancer. PKM2 expression has been suggested as a potential metabolic biomarker in endometrial carcinoma [16]. The frequency of $PKM2^{high}$ tumor cells in endometrial carcinoma was also found to be associated with worse

prognosis [16]. Given the reported role of PKM2 in endometrial carcinoma, our findings that cinnamic acid can regulate PKM2 expression, as well as cell viability, invasion, and glycolysis in primary endometrial stromal cells are particularly important and may be used as a therapeutic avenue for endometriosis, but may also help control potential progression to endometrial cancer.

It is curious how PKM2 affects cell viability, invasion, and glycolysis in primary endometrial cells. In cancer cells and immune cells, such as macrophages and T cells, PKM2 has been noted to support the function of transcription factors, including HIF1-α and STATs [33-35]. Interestingly, in natural killer (NK) cells, PKM2 expression does not significantly alter the expression of HIF1-α or STAT target genes but instead regulate the glycolytic flux of NK cells toward anabolic or catabolic processes, conferring these cells metabolic plasticity [36]. Consistent with this finding, PKM2 expression in liver cancer cells affects the flux of glucose metabolism [37]. Another potential mechanism is also suggested by findings in prostate cancer, where PKM2 promotes metastasis by modulating the extracellular-regulated protein kinase-cyclooxygenase pathway [38]. It will be interesting to explore whether any of these regulators are affected by PKM2 in primary endometrial stromal cells to understand its function and mechanism in these cells.

Of note, cinnamic acid inhibited the expression of PKM2 at the mRNA and protein levels, suggesting that cinnamic acid may affect a transcription factor that regulates PKM2 transcription. It also been previously reported that under physiological conditions, PKM2 expression was upregulated by epidermal growth factor receptor, and this upregulation is important for EGF-induced activation of cyclin D1 and c-Myc [39]. NF-κB transcriptionally induces PKM2 [19] and favors the survival of the ectopic endometrial tissue [20]. However, cinnamic acid inhibits NF-κB activation [32]. In the present study, cinnamic acid inhibited the transcription of PKM2 induced by NF-κB. These data suggest that cinnamic acid may reduce PKM2 expression through inhibiting NF-κB-induced transcription to modulate ectopic endometrial stromal cells (Figure 5F). It will be interesting to explore the potential relationships among these proteins in endometriosis as a potential future study.

Conclusion

In sum, our study uncovered a function of cinnamic acid in inhibiting PKM2 and highlights the promising benefit of traditional Chinese medicine for endometriosis treatment.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QWY, GYJ, XWZ and MLL were involved in experimental designs and drafting of the manuscript. QHY, QWY and LHW performed the experiments. GYJ, XWZ and MLL confirm the authenticity of all the raw data. QHY and LHW acquired, analyzed and interpreted the data and involved in writing, review and editing the manuscript, as well as supervision. All authors read and approved the final version of the manuscript

Ethics approval and consent to participate

The Ethics Committee of Shanghai Municipal Hospital of Traditional Chinese Medicine Affiliated to Shanghai TCM University approved this study, with written informed consent provided by the patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figure Legends

Figure 1. Effects of compounds on cell viability, ECAR and OCR in primary endometrial stromal cells. (A) Immunocytochemistry of cytoskeleton proteins vimentin and CK19 in primary endometrial stromal cells from endometriosis patients or patients without endometriosis. Scale bar: 50 μm. The primary endometrial stromal cells from endometriosis patients were treated with 50 μM of the compound cinnamaldehyde, cinnamic acid, coumarin, or protocatechuic acid. The (B)

cell viability, (C) ECAR, and (D) OCR were measured. Data were expressed as mean ± SD (n=3). ***P<0.001 compared with vehicle.

Figure 2. Cinnamic acid inhibited cell viability, invasion, ECAR, and OCR in primary endometrial stromal cells. Primary endometrial stromal cells from endometriosis patients were subjected to cinnamic acid treatment of varying concentrations (20, 50, and 100 μM). (A) Cell viability, (B, C) invasion, (D) ECAR, (E) OCR, and (F, G) PKM2 expression were measured. Scale bar: 50 μm. (H) Luciferase Reporter assay was performed to evaluate the activity of the PKM2 promoter in primary ectopic endometrial stromal cells treated with cinnamic acid, PDTC (10 μM) or vehicle. (I) The ChIP assay showed that NF-κBp65 bound to PKM2 promoter in primary ectopic endometrial stromal cells treated with cinnamic acid (100 μM), PDTC (10 μM) or vehicle. Data were expressed as mean ± SD (n=3). *P<0.05, ***P<0.001 compared with vehicle.

Figure 3. PKM2 overexpression suppressed the effects of cinnamic acid on cell viability, invasion, ECAR, and OCR in primary endometrial stromal cells. (A) Primary normal endometrial stromal cells isolated from patients without endometriosis were transfected with pcDNA3.1(+)-PKM2 vector and PKM2 expression was measured. Primary normal endometrial stromal cells isolated from patients without endometriosis were transfected with pcDNA3.1(+)-PKM2 vector and treated with cinnamic acid (100 μM), and (B) cell viability, (C, D) invasion, (E) ECAR, (F) OCR, and (G) PKM2 expression were measured. Scale bar: 50 μm. Data were expressed as mean ± SD (n=3). ***P<0.001 compared with vector, $^{\#\#\#}P$<0.001 compared with PKM2.

Figure 4. PKM2 inhibition displayed similar effects as cinnamic acid treatment on cell viability, invasion, ECAR, and OCR. Primary endometrial stromal cells from endometriosis patients were treated with different concentrations of PKM2 inhibitor, Compound 3k (1 and 2 μM). (A) PKM2 expression, (B) cell viability, (C, D) invasion, (E) ECAR, and (F) OCR were measured. Scale bar: 50 μm. Data were expressed as mean ± SD (n=3). *P<0.05, ***P<0.001 compared with vehicle.

Figure 5. Cinnamic acid inhibited cell viability, invasion, ECAR, and OCR by targeting PKM2. Primary endometrial stromal cells from endometriosis patients were treated with cinnamic acid (100 μM) and/or PKM2 inhibitor Compound 3k (2 μM). (A) Cell viability, (B, C) invasion, (D) ECAR, and (E) OCR were measured. Scale bar: 50 μm. (F) Schematic representation of the regulation of endometriosis by cinnamic acid through inhibition of NF-κB-induced PKM2 transcription. Data were expressed as mean ± SD (n=3). ***P<0.001 compared with vehicle, $^{\#}P$<0.05, $^{\#\#\#}P$<0.001 compared with cinnamic acid.