Baicalein Enhances Migration and Invasion of Extravillous Trophoblasts via Activation of the NF-κB Pathway

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Background: Baicalein, one of the major flavonoids in the plant Scutellaria baicalensis, can regulate the invasive ability of cancer cells. The invasion of trophoblasts is similar to the invasion of tumor cells into host tissues. The appropriate invasion of trophoblast cells into the endometrium is an important factor for successful embryo implantation. In this research, we investigated the effect of baicalein on the invasion and migration of trophoblast cells and its possible molecular mechanism.

Material/Methods: We treated HTR-8/SVneo cells with different concentrations (0, 0.05, 0.1, and 0.5 µM) of baicalein. The invasion and migration abilities of HTR-8/SVneo cells were studied. Protein levels and gene expression related to invasion and migration were analyzed by Western blot analysis and reverse transcription-quantitative polymerase chain reaction, respectively.

Results: Baicalein enhanced the migration and invasion of HTR-8/SVneo cells. In addition, gene expression and protein levels of MMP-9 in HTR-8/SVneo cells changed in the presence of baicalein. Moreover, the data show that baicalein activated the NF-κB pathway. Baicalein was also able to rescue effects of an NF-κB-specific inhibitor (JSH-23) on the migration and invasion of HTR-8/SVneo cells.

Conclusions: In conclusion, our results indicate that baicalein enhances migration and invasion of HTR-8/SVneo cells, which is important for successful pregnancy.

MeSH Keywords: Baicalein • HTR-8/SVneo Cell • Invasion • Migration • NF-κB Pathway

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Background

Baicalein is a major active ingredient of *Radix scutellariae*, a commonly used Chinese herbal medicine for anti-inflammatory and anti-cancer therapy [1]. Research shows that baicalein can suppress adhesion, migration, and invasion of human breast cancer cells [2], hepatocellular carcinoma cells [3], and osteosarcoma cells [4]. However, the effects of baicalein on trophoblast cells are unknown.

Successful embryo implantation requires effective proliferation, differentiation, and invasion into the endometrium of villous trophoblast cells [5]. There are many similarities between trophoblastic diseases and tumor cells in invasion of host cells. However, the invasion of human trophoblast cells is strictly controlled by the endometrium and the upper third of the myometrium, which is different from that of tumor cells [6].

The invasion of trophoblast cells is regulated by many factors. Matrix metalloproteinases 2 and 9 are closely related to the invasion of trophoblast cells [7,8]. Many signaling cascades are involved in regulating the invasive function of trophoblast cells. The PI3K/AKT/NF-κB pathway is a major signaling pathway used to study the function of trophoblast proliferation, migration, and invasion [9].

The expression of MMP is often mediated by NF-κB activation [10]. NF-κB is an important transcription factor that regulates inflammation, apoptosis, and invasion [11,12]. Studies have shown that NF-κB activation can increase the invasiveness of carcinoma cells by regulating the expression of MMP-2 and MMP-9 [13,14]. However, the effect of baicalein on these signaling cascades and key molecules in trophoblast cells has not been reported yet.

In this study, we used HTR-8/SVneo cells with the abilities of adhesion, migration, and invasion [15]. Thus, the study was designed to investigate the effects and mechanisms of baicalein on the invasion of extravillous trophoblasts. The purpose of this research was to elucidate the molecular mechanisms underlying the reproductive protection effect of baicalein.

Material and Methods

Baicalein was purchased from Sigma-Aldrich (St. Louis, MO, USA). JSH-23 was obtained from Merck, Germany. Anti-MMP-2, anti-MMP-9, anti-NF-κB/p65, and GAPDH antibodies were purchased from Cell Signaling Technology (CST). Unless otherwise specified, all other reagents were of analytic grade.

Cell culture

HTR-8/SVneo cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 100 μg/mL streptomycin, and 100 U/mL penicillin. All cells were incubated at 37°C with 5% CO₂.

Cell viability

The cell counting kit-8 (CCK-8) assay was used to determine the cell viability. HTR-8/SVneo cells were seeded into 96-well plates with serum-free medium with 2.0×10⁵ cells in each well. After 24 h, cells were treated with different doses of baicalein (0, 0.01, 0.05, 0.1, 0.5, and 1 μM) for 24 h. Cells were them incubated in 10 μL CCK-8 at 37°C for 2 h. Absorbance in each well was measured at 450 nm with a Synergy 2 Absorbance Microplate Hybrid Reader (Biotek, USA). Cell growth promotion rate was expressed as the fraction of absorbance compared to control.

Cell Invasiveness

The Transwell assay was conducted to investigate invasive ability of HTR-8/SVneo cells. Transwell chambers with 10-mm diameter and 8-μm pore size polycarbonate membrane coated with 60 μL of Matrigel extracellular matrix (Corning, Tewksbury, MA, USA) were used. Cells were trypsinized and suspended with 60 μL of Matrigel extracellular matrix (Corning, Tewksbury, MA, USA) were used. Cells were trypsinized and suspended at a final concentration of 1.0×10³/mL in serum-free medium. Cell suspension was added to each upper chamber of the Transwell device. The bottom chamber contained medium with 10% FBS to serve as a chemoattractant. Various concentrations of baicalein (0.01, 0.05, 0.1, 0.5, and 1 μM) were given to cells for 24 h prior to invasion assay. For the rescue experiment, cells were pretreated with 0.5 μM baicalein plus 10 μM of a specific inhibitor of NF-κB (JSH-23; Merck, Germany), for 24 h before the start of the invasion assay. After culturing for 24 h, all of the non-invaded cells were removed and invasive cells were fixed and stained. The percent invasive rate is expressed as a percentage of control.

Western blot analysis

Total cellular proteins of HTR-8/SVneo cells were extracted by lysis buffer. Then, equal amounts of proteins were separated by Sodium Dodecyl Sulfate (SDS) – Polyacrylamide Gel Electrophoresis (PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The blots were incubated overnight at 4°C with anti-MMP-2, anti-MMP-9, and anti-GAPDH antibodies. Bound antibodies were incubated at room temperature for 2 h with horse-radish peroxidase-conjugated secondary antibody. The intensity of selected bands was captured and analyzed using the Odyssey Infrared Imaging System (Li-COR, USA).
Total RNA extraction and qRT–PCR

Total RNA from cultured HTR-8/SVneo cells were extracted using TRIzol reagent (Invitrogen, USA) following the manufacturer’s recommended protocol, and then reverse-transcribed using the PrimeScript RT reagent kit (Takara Bio Inc., Japan). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using SYBR Green II, as indicated in the PrimeScript RT-PCR kit protocol. Quantification of gene expression relative to β-actin was performed using the 2−ΔΔCT method. The primers are listed in Table 1.

Indirect Immunofluorescence and confocal laser scanning microscopy

After incubation of HTR-8/SVneo cells with baicalein in the presence or absence of inhibitors, cells were fixed with 4% paraformaldehyde dissolved in PBS solution at room temperature for 30 min. They were made to react with anti-NF-kB/p65 antibody for 24 h at 4°C, and then with secondary anti-rabbit Alexa Fluor-labeled antibody (Life Technologies, USA) at room temperature for 2 h. Slide were observed under a fluorescence microscope (LSM710, ZEISS, Germany).

Gelatin zymography

Gelatin zymography technique was used to assess the activation status of MMP-9. It was performed on pre-made 10% polyacrylamide gels. After electrophoresis, gels were washed with 2.5% Triton X-100 and we renatured the MMP-9 species in the gels. Then, the gels were stained with 0.1% Coomassie blue and then destained with 10% acetic acid. MMP-9 concentration was analyzed using the FluorChem E Chemiluminescence Gel Imaging System (Protein Simple, USA).

Results

Cell Viability

CCK-8 assays revealed that viability of HTR-8/SVneo cells increased in a dose-dependent manner with 0.01 to 0.05 µM exposure to baicalein, but viability then decreased with concentrations higher than 0.1 µM (Figure 1).

Invasiveness

HTR-8/SVneo cell invasiveness increased when exposed to 0.01 µM baicalein and continued to increase with increasing baicalein concentration (0.05, 0.1, 0.5 µM) (Figure 2A). Invasion ability of HTR-8/SVneo cell increased by 24.1%, 30.1%, 40.9%, and 57.4% compared to the DMSO control (Figure 2B).

MMP

Comparison of the baicalein-treated groups with the control revealed that expression of MMP-9 protein, as measured on Western blots, increased with baicalein treatment (Figure 3). In contrast, no difference in MMP-2 protein expression level was observed. Further, baicalein increased the abundance of MMP-9 mRNAs in trophoblast cells in a concentration-dependent manner (Figure 4), indicating that MMP-9 gene expression was upregulated. Again, MMP-2 mRNA levels did not change significantly.

Table 1. Primers for quantitative reverse transcription-quantitative polymerase chain reaction.

| Gene     | Forward sequence | Reverse sequence |
|----------|------------------|------------------|
| MMP-2    | 5’-AAGTGCGTAGACGTACTC-3’ | 3’-AGAAGTCCCACGGATAATGGACTT-5’ |
| MMP-9    | 5’-CCCTCTCGTGTCCACCTG-3’ | 3’-TACCAATGTGAAGCCACCG-5’ |
| β-actin  | 5’-TAAACCCACCTGCTACC-3’ | 3’-TTGTCTACTCTACGGTAC-5’ |

MMP-2 – matrix metalloprotein 2; MMP-9 – matrix metalloprotein 9.

Figure 1. Effect of baicalein concentration on HTR-8/Svneo cell proliferation. Cells were treated with different concentrations of baicalein for 24 h and cell proliferation was measured using the CCK-8 cell proliferation assay kit. Data are presented from 3 independent experiments. * P<0.05.
Role of NF-κB p65 protein

To explore the potential molecular mechanism by which baicalein increases the invasiveness of trophoblast cells, we further examined changes in the abundance of NF-κB p65 protein obtained from whole-cell and nucleus extracts. Expression of nuclear NF-κB p65 protein increased in a dose-dependent manner with baicalein (Figure 5), while whole-cell extracts were unaffected.

Immunofluorescence staining indicates that with baicalein stimulation (0.1 μM), NF-κB p65 was translocated to the nucleus. The addition of 0.5 μM baicalein clearly promoted NF-κB p65 translocation (Figure 6).
**Figure 4.** Cells cultured in monolayer were treated with different concentrations of baicalein for 24 h, and then mRNA levels of MMP-2/MMP-9 were quantified by qRT-PCR. The mRNA levels were normalized to the expression of β-actin. Values are mean ±SD from 3 different experiments. * P<0.05 compared to the control cells not cultured with baicalein.

**Figure 5.** Nuclear and cellular proteins were extracted separately and protein levels of cytosolic NF-kB and nuclear NF-kB detected using Western blots. Nuclear translocation activity of NF-kB was determined by the ratio of p65(nucleus)/p65(cytosol). * P<0.05 compared to control cells without baicalein treatment.

**Figure 6.** Immuno-staining analysis of baicalein effects on NF-kB nuclear translocation. After 24 h, HTR-8/Svneo cells on coverslips were blocked with PBS containing 4% BSA and immuno-stained with anti-p65 antibody, and then stained with FITC-conjugated secondary antibody (green). Nuclei were stained with DAPI (blue). The stained cells were imaged on a confocal laser scanning microscopy system using a 400× magnification and the fluorescence intensity of p65 in nuclei of each sample was analyzed. * P<0.05 compared to control cells without baicalein treatment.
Mediation of NF-κB in MMP-9 expression and cell Invasion

To verify that NF-κB p65 was involved in baicalein-induced invasion ability, NF-κB p65 specific inhibitor (JSH-23) was used. Cell invasion ability decreased with JSH-23 (10 µM) incubation but was markedly restored by baicalein (0.5 µM) treatment (Figure 7).

Nucleus NF-κB p65 was significantly reduced in cells treated with 10 µM of JSH-23 inhibitor compared to cells given inhibitor plus baicalein (JSH-23 vs. 10 µM JSH-23+ plus 0.5 µM baicalein) (Figure 8). In addition, immunofluorescence revealed that, with 10 µM JSH-23, NF-κB p65 was predominately found in the cytoplasm, but in the combined JSH-23 plus 0.5 µM baicalein treatment, NF-κB p65 was translocated to the nucleus (Figure 9). These findings indicate that the JSH-23 changes both baicalein-induced expression of nuclear NF-κB p65 protein and its translocation activity in HTR-8/SVneo cells.

The rescue experiment results revealed that the gene expression of MMP-9 decreased in the JSH-23-treated group compared to the baicalein-treated group (Figure 10), consistent with the invasion assay results.

**Figure 7.** Role of NF-κB in baicalein-induced MMP-9 expression and enhancement of cell invasiveness analyzed by Transwell invasion assay. a: baicalein–, JSH-23–; b: baicalein + (0.5 µM), JSH-23–; c: baicalein–, JSH-23+ (10 µM); d: baicalein+ (0.5 µM), JSH-23+ (10 µM). Invading cells were stained with crystal violet and 3 randomly selected regions were captured under light microscopy (100×). One representative of 3 independent experiments is shown. OD at 570 nm was analyzed for the stained cells of each sample. * P<0.05 compared to control cells.

**Figure 8.** Effects of NF-κB p65 protein-specific inhibitor (JSH-23) on baicalein-induced change in NF-κB p65 protein activity in HTR-8/SVneo cells. Nuclear and cellular proteins were extracted separately before the protein levels of cytosolic NF-κB and nuclear NF-κB were detected by Western blot. The nuclear translocation activity of NF-κB was estimated as the ratio of p65(nucleus)/p65(cytosol). a: baicalein–, JSH-23–; b: baicalein+ (0.5 µM), JSH-23–; c: baicalein–, JSH-23+ (10 µM); d: baicalein+ (0.5 µM), JSH-23+ (10 µM). * P<0.05 compared to control cells.
Figure 9. Effect of NF-κB p65 protein-specific inhibitor (JSH-23) on baicalein-induced change in NF-κB p65 protein nucleus translocation in HTR-8/Svneo cells. After 24 h, HTR-8/Svneo cells on coverslips were blocked with PBS containing 4% BSA and immunostained with anti-p65 antibody and then stained with FITC-conjugated secondary antibody (green). Nuclei were stained with DAPI (blue). Stained cells were captured by a confocal laser scanning microscopy system using 400× magnification. The fluorescence intensity of p65 in the nuclei of each sample was calculated and analyzed. a: baicalein–, JSH-23–; b: baicalein+ (0.5 µM), JSH-23–; c: baicalein–, JSH-23+ (10 µM); d: baicalein+ (0.5 µM), JSH-23+ (10 µM). * P<0.05 compared to control cells.

Figure 10. Effect of NF-κB p65 protein-specific inhibitor (JSH-23) on baicalein-induced change of invasion-related gene expression. The mRNA levels of MMP-9 were quantified by qRT-PCR. The mRNA levels were normalized to the expression of β-actin. Values are mean ±SD from 3 independent experiments. * P<0.05 compared to control cells.

Figure 11. Effect of NF-κB p65 protein-specific inhibitor (JSH-23) on baicalein-induced change of invasion-related protein activity. MMP-9 activity was analyzed by gelatin zymography. After 48-h treatment, HTR-8/SVneo cells were removed and supernatants were used for SDS-PAGE gelatin zymography analysis of MMP-9. Experiments were conducted in triplicate. * P<0.05 compared to control cells.
with the gelatin zymography analysis (Figure 11). This demonstrates that the NF-κB p65 signaling pathway is involved in baicalein-induced MMP-9 gene expression and protein activity.

**Discussion**

Trophoblast cells exhibit invasive ability. Proper invasion of trophoblast is crucial for successful pregnancy [16]. Insufficient invasion of trophoblast results in early spontaneous abortion, preeclampsia, and intrauterine growth restriction [17]. Our study demonstrates that baicalein enhances HTR-8/Svneo trophoblast cell invasion, especially at concentrations of 0.5 μM. The ability of baicalein to increase trophoblast invasion may allow it to reduce the occurrence of early spontaneous abortion.

MMP-2 and MMP-9 are invasion-related molecules. A decrease in the expression of MMP-2 and MMP-9 reduces trophoblast invasiveness [18]. In our results, baicalein increased MMP-9 but not MMP-2 expression, which is consistent with the change of trophoblast cell invasion ability.

After examining the effect of baicalein on trophoblast cell invasiveness and the expression pattern of individual molecules, we performed pathway analysis. PI3K/AKT/NF-κB signaling promotes cell-invasive phenotype, potentially via modulation of the expression of MMP-9 and other pro-invasive genes [19]. However, the involvement of the pathway in the essential physiological process of trophoblast cells is not yet known. MMP-9 is regulated by activating NF-κB [13,14]. Therefore, the PI3K/AKT/NF-κB pathway is a likely regulatory candidate of baicalein for trophoblast cell invasion through MMP-9. Western blot assays showed, and immunofluorescence confirmed, that baicalein treatment induces nuclear translocation of NF-κB.

Further evidence for the proposed molecular mechanism came from JSH-23 suppression assays. Increased MMP-9 activity and invasiveness were inhibited by JSH-23 in HTR-8/Svneo trophoblast cells with baicalein, suggesting that NF-κB plays a crucial role in MMP-9 expression and invasiveness enhancement. Since some researchers suggest that MMPs can be regulated by the PI3K/AKT signaling pathway through the NF-κB activation [20], we measured the activity of PI3K and phosphorylating AKT. Unexpectedly, we found no significant difference between the baicalein treatment group and the control group in signaling pathway activity (data not shown). Thus, more experiments are required to resolve the mechanisms underlying MMP-9 activity and invasive ability.

**Conclusions**

The results of this study suggest that baicalein treatment promotes HTR-8/Svneo trophoblast cell invasion by stimulating MMP-9 mRNA and protein expression. Baicalein activates NF-κB by accelerating its translocation into the nucleus and enhancing binding activity. NF-κB activation may be responsible for enhanced MMP-9 expression and HTR-8/Svneo trophoblast cell invasiveness. This is the first study to reveal that NF-κB mediates the stimulation effects of baicalein on MMP-9 expression and HTR-8/Svneo trophoblast cell invasion. Since different flavonoids may have synergic or cooperative effects in clinical administration, a natural next step is to include other flavonoids of *Radix scutellariae*, to better explore how this traditional herbal Chinese medicine might provide clinical benefits in reducing risk of miscarriage.

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