Int J Clin Exp Pathol 2016;9(2):684-694
www.ijcep.com /ISSN:1936-2625/IJCEP0019215

Original Article

Wogonin attenuates high glucose-induced human breast cancer cell MCF-7 viability, migration and invasion via the expression of AKT, PKCδ and p38 MAPK

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Received November 5, 2015; Accepted January 1, 2016; Epub February 1, 2016; Published February 15, 2016

Abstract: Wogonin, a major flavonoid extracted from the root of scutellaria baicalensis, has been shown to antidiabetic and antitumor effects in vitro and in vivo. However, the mechanism of wogonin on high glucose-induced human breast cancer cell viability, migration and invasion remains poorly understood. In our study, we investigate the effects of wogonin on high glucose-induced MCF-7 human breast cancer cell viability, migration and invasion in vitro experiments. High glucose induces activation of cell viability, migration and invasion in a time and concentration-dependent manner, while wogonin suppress those effects in a concentration-dependent way. The mechanism reveals that wogonin significantly inhibits high glucose-induced the phosphorylation of AKT and protein kinase Cδ (PKCδ) and activates p38 MAPK that could be attenuate by SB203580 (p38 MAPK inhibitor) and p38 shRNA. These results suggest that wogonin can attenuate high glucose-induced human breast cancer cell MCF-7 viability, migration and invasion via the expression of AKT, PKCδ and p38 MAPK.

Keywords: Wogonin, High Glucose, MCF-7, AKT, PKCδ, p38 MAPK

Introduction

Breast cancer is the most commonly occurring tumor among women worldwide. Approximately one in every ten women will develop the disease in their lifetime, and it is the first leading cause of cancer-related death in women [1]. Type 2 diabetes (T2DM) is characterized by a chronic hyperglycemic state caused by insulin resistance in skeletal muscle, adipose tissue, the liver, and/or impaired insulin secretion [2]. It is now well-established that T2DM is linked to increasing breast cancer incidence and mortality [3-5]. According to recent studies, T2DM conferred as much as 20%-40% increased risk of breast cancer in women [6] and both T2DM and breast cancer incidence are increasing at alarming rates worldwide. Even prediabetes may also increase the risk of breast cancer [7-9]. The detailed mechanisms remains unknown, however, the hyperglycemia in T2DM patients may promote cancer progression is a possible mechanism.

Wogonin (5,7-dihydroxy-8-methoxyflavone, PubChem CID: 5281703) is a major flavonoid extracted from the root of Scutellaria baicalensis Georgi that has long been used as a traditional medicine in East Asian countries [10]. It has been reported that wogonin has several biological properties, including anti-inflammatory, antidiabetic and antitumor effects in several studies [11-17]. Importantly, wogonin showed no organ toxicity in a subchronic study [18].

The central role of AKT in the PI3Ks pathway makes it one of the most activated downstream effectors, the AKT kinase family includes three members AKT1, AKT2, and AKT3. It is becoming increasingly clear that AKT isoforms underline their distinct functional role in cancer development and progression [19-21]. Therefore hyperglycemia enhances the viability of non-tumorigenic and malignant mammary epithelial cells through increased leptin/IGF1R signaling and activation of AKT/mTOR [22]. In cancer, PKCs have been shown to contribute to the progres-

sion of malignant phenotype [23-25]. In diabetes, PKCδ regulates hepatic insulin sensitivity and hepatosteatosis in mice and humans [26]; meanwhile, hyperglycemia causes vascular cell apoptosis and diabetic retinopathy by activation of PKCδ [27].

The mitogen-activated protein kinase (MAPK) pathway is an important signaling pathway in living beings in response to extracellular stimuli. There are three main subgroups manipulating by a set of sequential actions: ERK1/2, JNK/SAPK, p38 MAPK. p38 MAPK that mediates various cellular functions such as apoptosis, cell growth and differentiation are activated by inflammatory cytokines and a variety of environmental stresses. p38 MAPK activity plays an important role in tumor progression (Galliher and Schiemann 2007; Shin et al. 2005). Meanwhile the high glucose modulates proliferation in MCF-7 via MAPK pathway [28].

Taken together, these evidences suggest that wogonin may be beneficial for attenuating high glucose-induced human breast cancer cell viability, migration and invasion, but it is possible mechanism have not been investigated. To our knowledge, this is the first study demonstrating that wogonin attenuate high glucose-induced human breast cancer cell MCF-7 viability, migration and invasion via the expression of AKT, PKC and p38 MAPK.

Materials and methods

Materials

Wogonin was purchased from Sigma (USA). Wogonin was dissolved in dimethyl sulfoxide (DMSO) at a maximum concentration of 0.1%. β-actin, PKCδ and the phospho-PKCδ antibody, AKT and the phospho-AKT (Ser473) antibody, the MAPK family antibody sampler kit, and the phospho-MAPK family antibody sampler kit were purchased from Cell signaling Technology (USA). A p38 MAPK inhibitor (SB203580), D-Mannitol was purchased from Sigma (USA).

Cell culture and treatment

Human breast cancer cell line, MCF-7(ATCC, Manassas, USA) were cultured in RPMI 1640 (Gibco BRL, GrandIsland, NY, USA) containing 10% fetal bovine serum(FBS) (HyClone, Logan, UT, USA) and 1% penicillin/streptomycin (Invitrogen, Grand Island, NY) at 37°C in a humidified atmosphere of 5% CO_2. Cells were cultured in normal-glucose (5.5 +16.5 mM D-Mannitol for osmotic balance) and high-glucose (11+11 mM D-Mannitol for osmotic balance or 22 mM) conditions. At 80-90% confluence, cells were subjected to serum starvation in serum-free RPMI 1640 overnight and then stimulated with wogonin under normal- and high-glucose conditions for 48 h.

MTT assay

The thiazolyl blue tetrazolium bromide (MTT) (Amresco, Solon, OH, USA) was dissolved in phosphate buffered saline (PBS) at a concentration of 5 mg/ml, filtered, and stored at 4°C. Cells were seeded into a 96-well plate, washed three times with PBS and starvation in serum-free RPMI 1640 overnight. Cells were treated with wogonin under normal- and high-glucose conditions for 24 h, 48 h, and 72 h. For the viability assay, 20 μl MTT was added into each well. An ELISA plate reader (Biotek, Winooski, Vermont, USA) was used to measure the optical density at 490 nm. The viability of control cells was 100%.

TUNEL assay

The TUNEL assay was performed using an In Situ Cell Death Detection Kit (Roche, Basel, Switzerland) following the manufacturer's instructions. Cells that were cultured in serum-free RPMI 1640 containing 5.5 mM glucose was used as a negative control. Images were taken with the Olympus FluoView FV1000 Confocal Microscope.

Wound healing assay

MCF-7 cells were grown to confluent monolayers on 6-well plates and a pipette tip was used to create linear scratch wounds. Mitomycin C (Amresco) was used to inhibit cell viability. 1% FBS also was used in the assay. Cells migrated into the wound surface and the relative wound closure was determined under an inverted microscopy at various times, five randomly chosen fields were analyzed for each well. The percentage of inhibition was expressed using control wells at 100%. Wound images were taken with a digital camera mounted on light microscope. The wound gap widths were measured using Image J software.

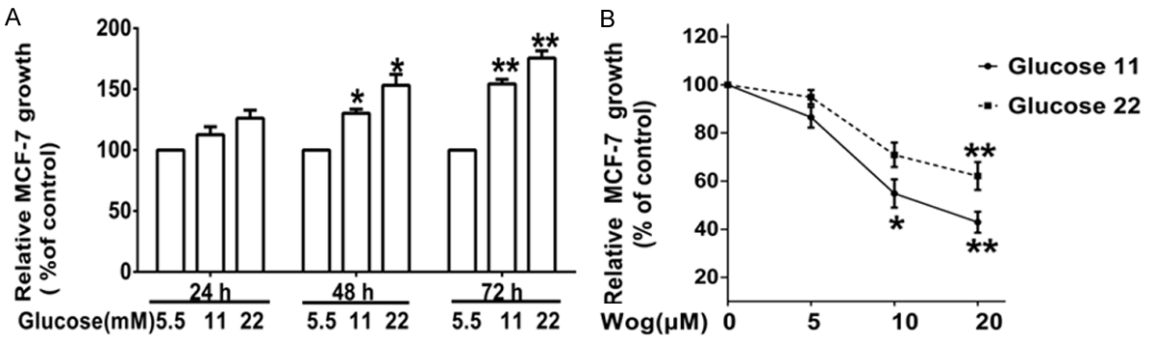

Figure 1. Effect of wogonin and high glucose on cell viability in MCF-7 cells in a concentration- and time-dependent manner. A. Effects of high glucose on MCF-7 cell viability. Cell viability was analyzed using the MTT assay. Cells were incubated with at the concentrations indicated for 24, 48 and 72 h. B. Effects of wogonin on high glucose-induced MCF-7 cell viability. Cells were incubated with at the glucose 11 mM and 22 mM concentrations indicated for 0, 5, 10 and 20 μM after 48 h. Viability was calculated as the percent of control. The bars represent the mean values ± SD triplicate (n = 3). *P < 0.05, **P < 0.01 versus control values.

Transwell assay

The upper chamber of each 8.0 μm pore size Transwell apparatus (Corning, NY, USA) was coated with Matrigel (BD Biosciences, San Jose, CA). MCF-7 cells were added to the upper chamber at a density of $2*10^6$ cells/ml (100 μl per chamber) in serum-free RPMI 1640 and incubated for 48 h with 10% FBS and wogonin under normal- and high-glucose conditions in the lower compartment. Cells on the upper surface were removed by a cotton swab. Cells that penetrated to the lower membrane surface were fixed in 4% paraformaldehyde, stained with crystal violet, and quantified by manual counting and ten randomly chosen fields were analyzed for each group.

Plasmids and virus infection

p38 MAPK shRNA lentiviruses were obtained from GeneChem Biotechnology (Shanghai, China), target sequences of human p38 MAPK (GenBank accession no. NM_002745).

MCF-7 cells were cultured at a density of 5×10^5 cells per 6 well plate. One day later, the cells were transfected with p38 MAPK shRNA and control sequences using CON077 (GeneChem Biotechnology, China) following the manufacturer's instructions. Cell lysates were collected and western blots were performed to detect protein expression using specific antibodies.

Western blots

Cells were collected with lysis buffer after being washed three times with ice-cold PBS. Lysates were boiled in SDS loading buffer for 10 min then cleared by centrifugation (14,000 rpm, 10 min, 4°C). The proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and detected with specific antibodies.

Statistical analysis

All experiments were done independently at least three times. Results are represented as the mean ± SD. The quantification of the relative increase in protein expression and phosphorylation was performed using NIH Scion Image software and was normalized with the control protein expression in each experiment. Significant differences between groups were analyzed by using a r the paired t-test. A *P*-value of < 0.05 was considered statistically significant.

Results

Effect of wogonin and high glucose on cell viability of MCF-7 cells in a concentration-dependent and time-dependent manner

The overall effect of wogonin and high glucose in MCF-7 cells was assessed using an MTT assay. As shown in **Figure 1A**, high glucose promoted the viability of MCF-7 cells in a concentration-dependent and time-dependent manner when compared to the control. In 22 mM glucose group, cell viability after 48 h of treatment was promoted by 153.2% compared to 5.5 mM group. While, as shown in **Figure 1B**, wogonin inhibited high glucose-induced MCF-7 cell viability in a concentration-dependent man-

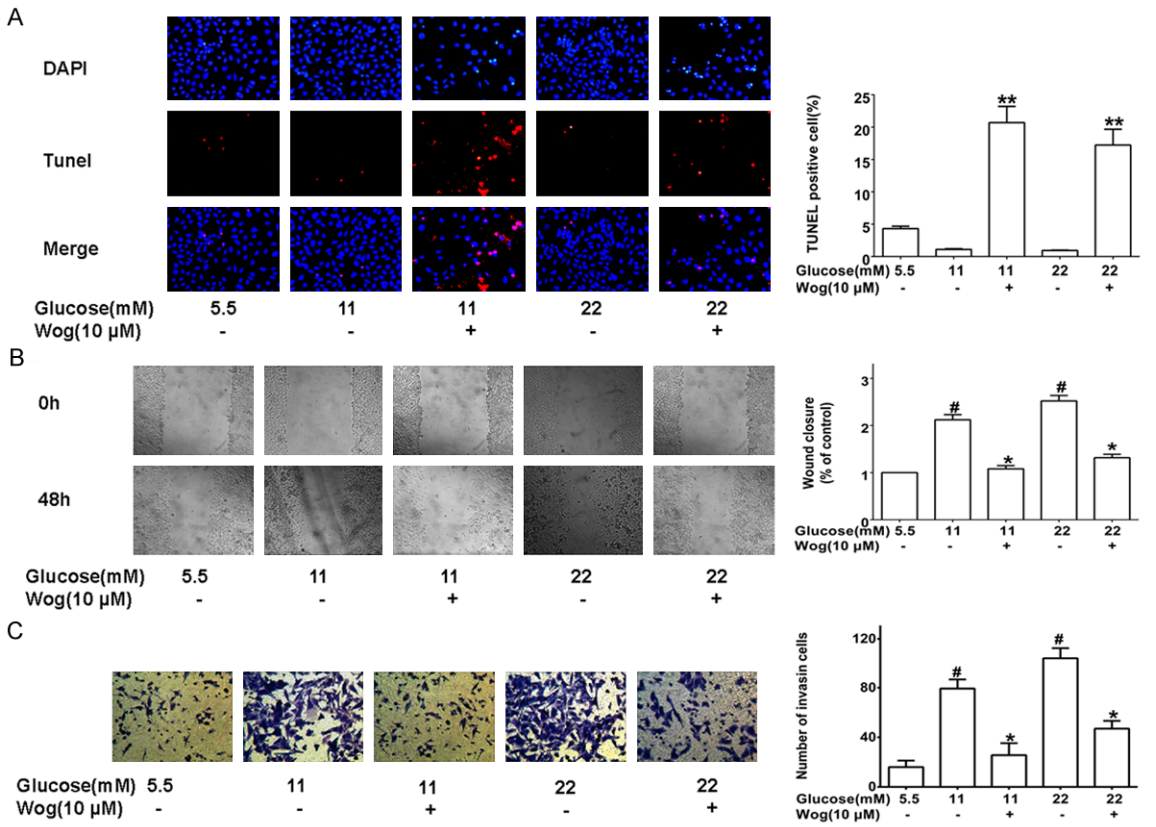

Figure 2. Wogonin inhibits MCF-7 cells viability, migration, invasion and triggers cell apoptosis under high glucose concentrations in vitro. Cells were exposed to different glucose concentrations with or without wogonin (10 μM) for 48 h in 5% CO_2 incubator at 37°C. A. Wogonin triggers high glucose-induced cell apoptosis. TUNEL assay was used to exam the effects of high glucose and wogonin on cells apoptosis. B. Wogonin inhibits high glucose-induced cell migration. Confluent monolayer was scraped with a pipette tip and cells migrated into the wounded monolayer was assessed by microscope equipped with a camera. C. Wogonin inhibits high glucose-induced cell invasion. Cells were seeded in the upper chamber of transwell coated with matrigel, after 48 h incubation, invasive cells passed through the membrane and were measured by crystal violet staining. The bars represent the mean values ± SD triplicate (n = 3). #P < 0.05 the 11 mM group or the 22 mM group vs. the 5.5 mM group; *P < 0.05, **P < 0.01 the non-wogonin group vs. the wogonin group.

ner when compared to the control. In 11 mM and 22 mM glucose groups, cell viability was separately inhibited by 45.1% and 29.1% after 48 h of treatment by 10 μM of wogonin compared to untreated cells.

Wogonin inhibits MCF-7 cells viability, migration, invasion and triggers cell apoptosis under high glucose concentrations in vitro

MCF-7 cells were incubated with wogonin under normal- and high-glucose conditions for 48 h to investigate the effects of wogonin. MTT and TUNEL assays were performed to measure cell viability and apoptosis. As demonstrated in **Figure 2A**, wogonin triggered high glucose-induced MCF-7 cell apoptosis. We used wound healing and transwell assays as described in the materials and methods section to test the effect of wogonin on high glucose-induced MCF-7 cell migration and invasion. As shown in **Figure 2B**, after 48 h the wound was almost covered due to the influx of highly migratory cells in high glucose groups, whereas wogonin-treated cells remained close to the initial state. As shown in **Figure 2C**, compared with the control, a dose-dependent augment in the number of invasive cells was seen in high glucose groups. Meanwhile, wogonin-treated cells respectively reduced.

Wogonin inhibits high glucose-induced the phosphorylation of AKT and PKCδ

AKT and PKCδ, which are both important glucose effectors in tumor progression, are

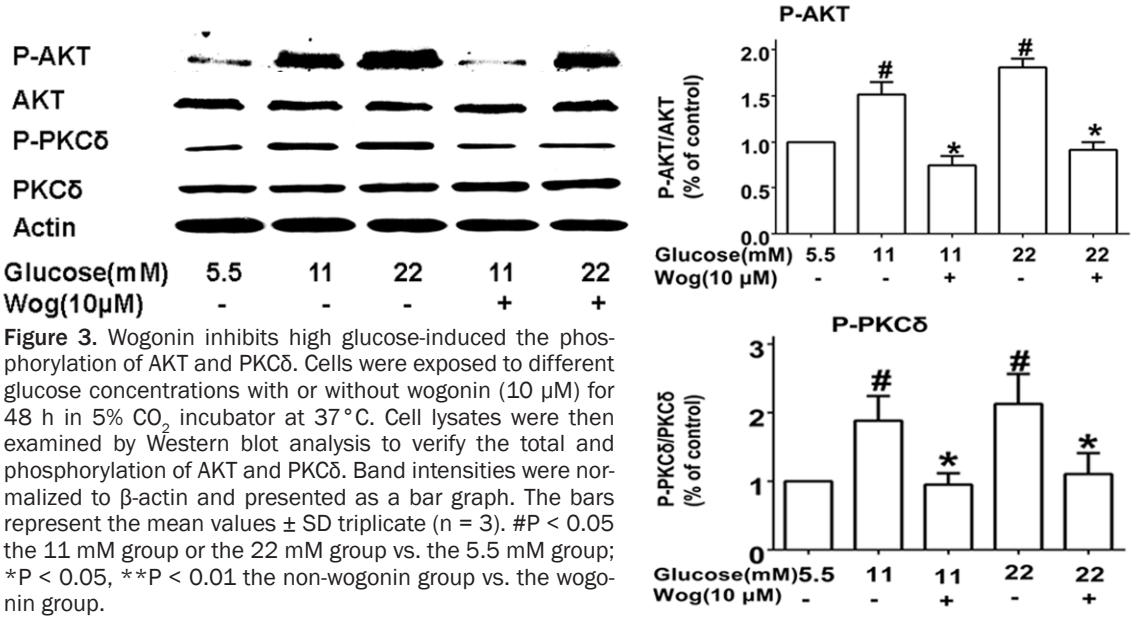

Figure 3. Wogonin inhibits high glucose-induced the phosphorylation of AKT and PKCδ. Cells were exposed to different glucose concentrations with or without wogonin (10 μM) for 48 h in 5% CO_2 incubator at 37°C. Cell lysates were then examined by Western blot analysis to verify the total and phosphorylation of AKT and PKCδ. Band intensities were normalized to β-actin and presented as a bar graph. The bars represent the mean values ± SD triplicate (n = 3). #P < 0.05 the 11 mM group or the 22 mM group vs. the 5.5 mM group; *P < 0.05, **P < 0.01 the non-wogonin group vs. the wogonin group.

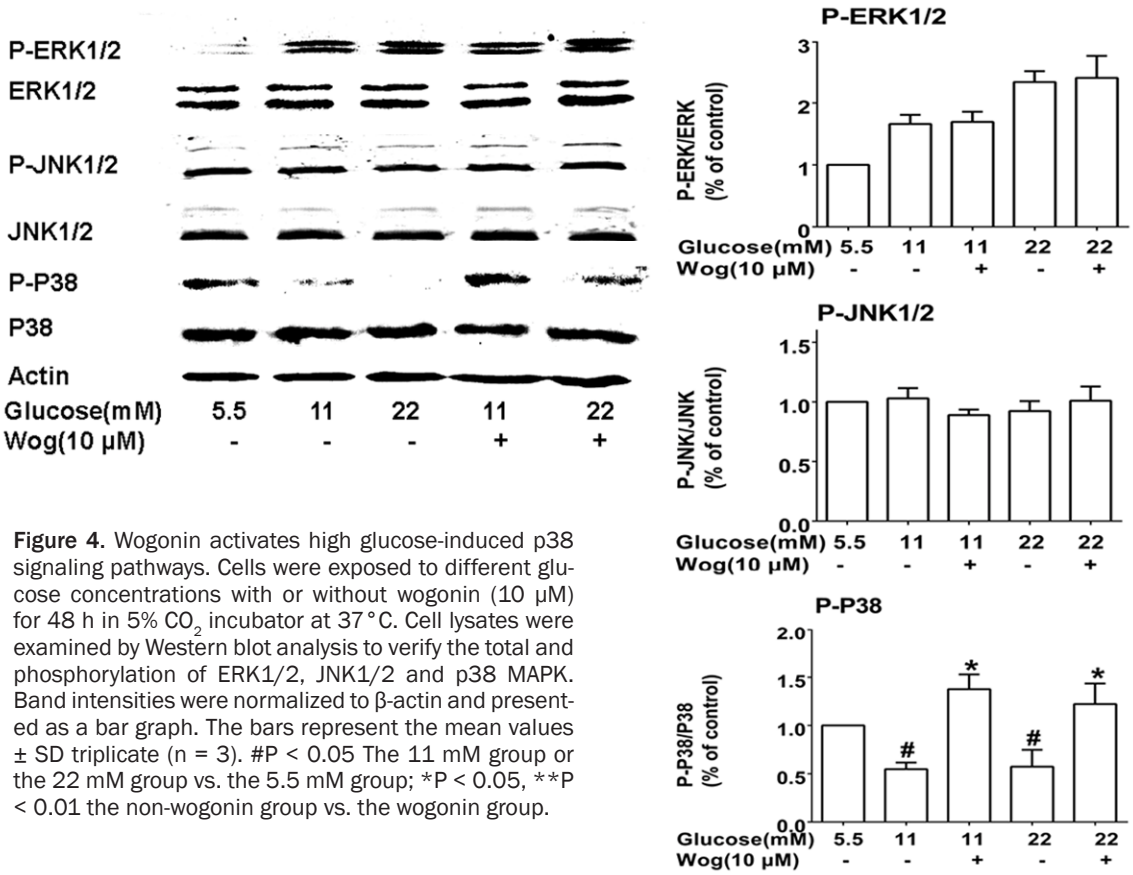

Figure 4. Wogonin activates high glucose-induced p38 signaling pathways. Cells were exposed to different glucose concentrations with or without wogonin (10 μM) for 48 h in 5% CO_2 incubator at 37°C. Cell lysates were examined by Western blot analysis to verify the total and phosphorylation of ERK1/2, JNK1/2 and p38 MAPK. Band intensities were normalized to β-actin and presented as a bar graph. The bars represent the mean values ± SD triplicate (n = 3). #P < 0.05 The 11 mM group or the 22 mM group vs. the 5.5 mM group; *P < 0.05, **P < 0.01 the non-wogonin group vs. the wogonin group.

involved in the regulation of MCF-7 growth, migration, invasion. Western blotting assay was also used to find out the mechanism of wogonin on high glucose-induced MCF-7 cells growth, migration, invasion. We tested the total and phosphorylation of AKT and PKCδ expres-

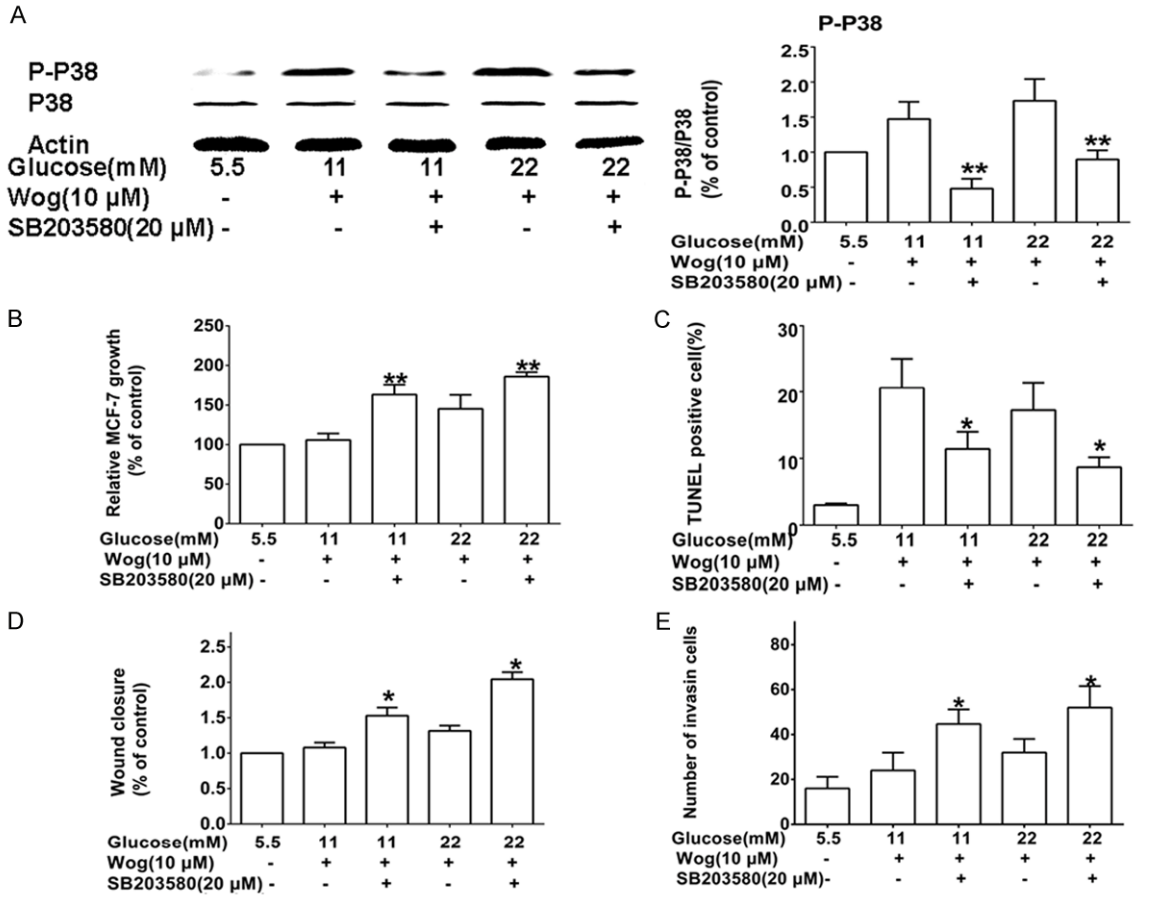

Figure 5. Effects of p38 MAPK inhibition on MCF-7 cells viability, migration, invasion. Cells were incubated for 48 h with different glucose concentrations and/or wogonin (10 μM) and/or treated with 20 μM of SB203580 (p38 MAPK inhibitor). A. The effect of p38 MAPK inhibition on the expression of total and phospho-p38 MAPK. The total and phosphorylation of p38 MAPK were examined by Western blot. Band intensities were normalized to β-actin and presented as a bar graph. B. The effect of p38 MAPK inhibition on cell viability. MTT assay was used to exam the cells viability. C. The effect of p38 MAPK inhibition on cell apoptosis. TUNEL assay was used to exam the cells apoptosis. D. The effect of p38 MAPK inhibition on cell migration. Confluent monolayer was scraped with a pipette tip and cells migrated into the wounded monolayer was assessed by microscope equipped with a camera under the conditions. E. The effect of p38 MAPK inhibition on cell invasion. Cells were seeded in the upper chamber of transwell coated with matrigel, after 48 h incubation, invasive cells passed through the membrane and were measured by crystal violet staining. The bars represent the mean values ± SD triplicate (n = 3). *P < 0.05, **P < 0.01 vs. the control values.

sion in MCF-7 cells exposed to wogonin under normal- and high-glucose conditions for 48 h. As shown in **Figure 3**, the phosphorylation of AKT and PKCδ was increased in high glucose groups and decreased by wogonin.

Wogonin activates high glucose-induced P38 signaling pathways

Since activated MAPKs play a critical role in viability, migration, invasion, we analyzed the total and phosphorylation of MAPK family members including ERK1/2, JNK1/2 and p38 by Western blotting assay. As shown in **Figure 4**, the amount of phosphorylation p38 reduced in high glucose groups and increased by wogonin, while the phosphor-ERK1/2 increased in high glucose groups but was not influenced by wogonin. The phosphorylation JNK1/2 was not significantly influenced under high-glucose conditions with/without wogonin treatment.

Effects of p38 MAPK inhibition on MCF-7 cells viability, migration, invasion

Since wogonin could activate p38 signaling pathway, SB203580 was used as an inhibitor was used for knocking down p38 to further testify this effect. The data showed that SB203580 suppressed wogonin-induced the phosphoryla-

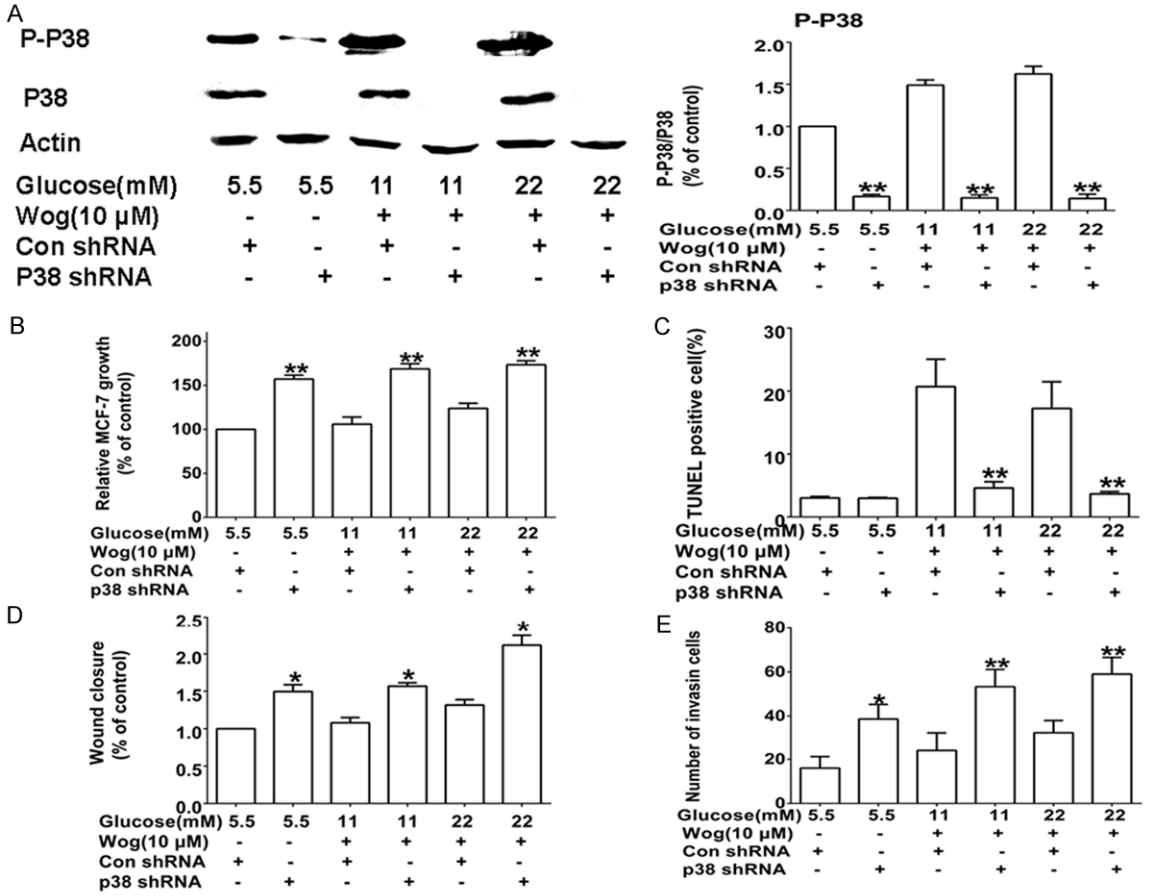

Figure 6. Effects of p38 MAPK Gene Silencing on MCF-7 cells viability, migration, invasion. After transfected with p38 MAPK shRNA or control sequences,cell were incubated for 48 h with different glucose concentrations and/or wogonin (10 μM). A. The effect of p38 MAPK gene silencing on the expression of total and phospho-p38 MAPK. Then, the total and phosphorylation of p38 MAPK were examined by Western blot. Band intensities were normalized to β-actin and presented as a bar graph. B. The effect of p38 MAPK gene silencing on cell viability. MTT assay was used to exam the cells viability. C. The effect of p38 MAPK gene silencing on cell apoptosis. TUNEL assay was used to exam the cells apoptosis. D. The effect of p38 MAPK gene silencing on cell migration. Confluent monolayer was scraped with a pipette tip and cells migrated into the wounded monolayer was assessed by microscope equipped with a camera under the conditions. E. The effect of p38 MAPK gene silencing on cell nvasion. Cells were seeded in the upper chamber of transwell coated with matrigel, after 48 h incubation, invasive cells passed through the membrane and were measured by crystal violet staining. The bars represent the mean values ± SD triplicate (n = 3). *P < 0.05, **P < 0.01 vs. the control values.

tion of P38 on high glucose conditions (**Figure 5A**). SB203580 could ameliorate wogonin-induced MCF-7 cells growth on high glucose conditions (**Figure 5B**), decrease the apoptotic cells (**Figure 5C**), increase the wounded closure (**Figure 5D**) and the more invasive cells through the matrigel (**Figure 5E**).

Effects of p38 Gene Silencing on MCF-7 cells viability, migration, invasion

Since wogonin could activate p38 signaling pathway, p38 shRNA lentivirus was used for knocking down p38 to further testify this effect. The data showed that p38 shRNA could block both of the phosphorylation and the total protein level of p38 (**Figure 6A**). p38 shRNA could ameliorate wogonin-induced MCF-7 cells growth on high glucose conditions (**Figure 6B**), decrease the apoptotic cells (**Figure 6C**), increase the wounded closure (**Figure 6D**) and the more invasive cells through the matrigel (**Figure 6E**).

Discussion

Wogonin is a major flavonoid extracted from the root of Scutellaria baicalensis that has sev-

eral biological properties including anti-inflammatory, antidiabetic and antitumor effects. As cancer cells use glucose as the source of energy for their viability, high glucose provides a favorable environment for the growth and survival of breast cancer cells [29]. Furthermore, it has been reported that high glucose confers resistance to chemotherapy in malignant cancer cells but not in non-malignant cell [30, 31]. We demonstrate that wogonin effectively attenuate high glucose-induced MCF-7 viability, migration and invasion, and the mechanism of the effect is associated with inhibiting the phosphorylation of AKT, PKCδ and p38 MAPK.

Viability, migration and invasion of tumor is the major cause of morbidity and mortality. The mechanism is a multistep and complex process involving the extracellular signaling, tumor microenvironment and stimulating factors. It is reported that activation of AKT and PKCδ plays a critical role in high glucose-induced cancer cell lines [22, 25, 32-35]. It is well-established that hyperglycemia enhances the viability of non-tumorigenic and malignant mammary epithelial cells through increased leptin/IGF1R signaling and activation of AKT/mTOR [22]. Moreover, high glucose increases PKCδ-phosphorylation to enhance MCF-7 viability, migration and invasion [36]. The tyrosine phosphorylation of PKC by phorbol 12-myristate 13-acetate (PMA) and various growth factors [34, 37, 38]. It is defined that Y64 and Y155 as critical residues for proapoptotic signaling by PKCδ and suggest that phosphorylation of PKCδ at these residues regulates nuclear translocation and hence cell survival [39]. Barbara et. Al [40] shows that active PKCδ is a proproliferative factor in estrogen-dependent breast cancer cells. Activation of PKCδ by TPA resulted in activation and nuclear translocation of ERα and in an increase of ER-dependent reporter gene expression. Grossoni et al [24] report that PKCδ overexpression show enhance resistance to apoptotic stimuli, such as serum deprivation or doxorubicin treatment, an effect that correlates with activation of the Akt survival pathway. PKCδ can interact positively with Akt/mTOR [41], which has also been implicated in survival signaling [20]. It remains to be determined whether survival signals generated by PKCδ in MCF-7 cells are dependent on Akt/mTOR. Meanwhile, suppression of AKT and translocation of PKCδ have been associated with regulation of cell fate by wogonin [17, 42]. Consistent with these findings, we observe that suppression of AKT and PKCδ are involved in effects of wogonin on high glucose-induced MCF-7 viability, migration and invasion.

p38 kinases are members of MAPK family that transduce signals from various environmental stresses, growth factors and steroid hormones. p38 has recently gained attention as a tumor suppressor. This effect is reported that increased tumorigenicity of the lack of p38 MAPK cells is caused mainly by a decrease in the apoptosis rate indicating that the lack of the p38 MAPK caused an imbalance to increase the ERα:ERβ ratio and a reduction in the activity of the p53 tumor suppressor protein [43]. Further, there is much evidence to support a role for p38α as a tumor suppressor, and this function of p38α is mostly mediated by both negative regulation of cell cycle progression and the induction of apoptosis, although the induction of terminal differentiation also contributes to tumor suppressive function [44]. The study [45] reveals that the activation of MAPK by estrogen is mediated through a HER-2/PKCδ/Ras. These data suggest that ER(+) MCF-7 has used PKCδ to activate MAPK and this may prevent apoptosis in MCF-7. It is reported that wogonin induces apoptosis by activation of ERK1/2 and p38 MAPKs signaling pathways and generation of reactive oxygen species in MCF-7 [13]. Consistent with these findings, we deduced that wogonin attenuate high glucose-induced MCF-7 viability, migration and invasion by activating the phosphorylation of p38 MAPK.

Conclusion

This is the first study to show that wogonin could inhibit high glucose-induced MCF-7 viability, migration and invasion in vitro via suppressing the phosphorylation of AKT and PKCδ and activating the phosphorylation of p38 MAPK. It is further demonstrated that the activation of p38 potentially associating with the suppression of AKT and PKCδ proteins phosphorylation. Nevertheless, we find that wogonin could attenuate high glucose-induced MCF-7 viability, migration and invasion just by the experiments in vitro, and those need to be further investigated in vivo.

Acknowledgements

This work was partially supported by a NSFC grant to Dr. Shengrong Sun (Grant NO: 3070-

1032), and a NSFC grant to Prof. Changhua Wang (Grant NO: 30770758/H0178). This work was also supported by a NSFC grant to Dr. Juanjuan Li (Grant NO: 81302314/H1622) and the Fundamental Research Funds for the Central Universities of China to Shan Zhu (Grant NO: 2042014kf0189).

Disclosure of conflict of interest

None.

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