Am J Transl Res 2019;11(2):865-874
www.ajtr.org /ISSN:1943-8141/AJTR0086459

Original Article

Herbacetin treatment remitted LPS induced inhibition of osteoblast differentiation through blocking AKT/NF-κB signaling pathway

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Received June 21, 2018; Accepted December 29, 2018; Epub February 15, 2019; Published February 28, 2019

Abstract: Inflammation, a common situation during the process of bone healing, is reported to play a negative role in bone regeneration. Up to date, therapeutic strategies for inflammation triggered inhibition of osteoblast differentiation are still limited. The aim of this study was to explore the potential roles and molecular mechanisms of Herbacetin in the process of osteoblast differentiation under LPS-mediated inflammatory environment. By using MC3T3-E1, C2C12 and primary mouse calvarial osteoblast (PMCO) cells as experimental models, we observed that LPS stimulation suppressed osteoblast differentiation via inhibiting alkaline phosphatase (ALP) activity and the expression of several osteoblastic genes (osterix, runx2 and osteocalcin). However, the negative role of LPS during osteoblast differentiation could be restored by Herbacetin treatment. Mechanistical studies revealed that Herbacetin treatment suppressed AKT activation and in turn blocked NF-κB signaling pathway. Furthermore, reactivating AKT by a selective PTEN inhibitor SF1670 suppressed the effect of Herbacetin. These data suggested that Herbacetin might play a protective role in osteoblast differentiation in MC3T3-E1/C2C12/PMCO cells under LPS stimulation.

Keywords: Herbacetin, LPS, osteoblast differentiation, AKT/NF-κB

Introduction

The recovery process of bone fracture involves in the reprograming of multiple gene expression as well as the microenvironment [1]. Despite bone formation during fetal skeletal development and postnatal fracture repair share similar processes such as differentiation from mesenchymal stem cells (MSCs) to osteoblasts, fracture healing is initiated by an inflammatory phase during which cellular activities could be regulated by multiple inflammatory factors [2, 3]. Lipopolysaccharide (LPS), the main pathogenic component of gram-negative bacteria, is a commonly used stimulator of inflammatory response in cell experiments. LPS can increase the synthesize and release of IL-6 and other inflammatory factors, activate pro-inflammatory signaling pathways and thus inhibit osteoblast differentiation [4, 5]. Therefore, the research based on LPS-mediated inhibition of osteoblast differentiation might provide novel opportunity to enhance osteoblastic differentiation and bone formation.

Herbacetin (3,4,5,7,8-pentahydroxyflavone), an active flavonol compound extracted from plants, exhibited anti-cancer efficacy and strong antioxidant capacity [6-8]. Recently, the anti-inflammatory role of Herbacetin was reported in RAW264.7 macrophage cells. In their study, Li et al. demonstrated that pretreatment of Herbacetin decreased nitric oxide (NO) production in LPS-induced RAW264.7 cells and primary macrophages derived from mouse bone marrow [9]. However, whether Herbacetin had anti-inflammatory role during osteoblast differentiation was still elusive. The aim of this study was to evaluate the potential roles and mechanisms of Herbacetin on osteoblast differentiation under LPS-mediated inflammatory environment.

Materials and methods

Cell culture

Animal care and the experimental protocol in this study were approved by the ethics commit-

Table 1. Primers for osterix, runx2, osteocalcin, and GAPDH

Name	Sequence (5'-3')
Runx2 (Foward)	GCTTGATGACTCTAAACCTA
Runx2 (Reverse)	AAAAAGGGCCCAGTTCTGAA
Osterix (Foward)	AGGCACAAAGAAGCCATAC
Osterix (Reverse)	AATGAGTGAGGGAAGGGT
Osteocalcin (Foward)	CTCACTCTGCTGGCCCTG
Osteocalcin (Reverse)	CCGTAGATGCGTTTGTAGGC

tees of Henan Provincial People's Hospital. Primary mouse calvarial osteoblasts (PMCO) were obtained from 3-day-old NIH mice calvarias following the sequential enzymatic digestion method. In brief, skulls were dissected, and the endosteum and periosteum were stripped off. Then the bone was cut into approximately 1-2 mm^2 pieces and subjected to five sequential digestions at 37°C in 0.1% dispase and 0.1% collagenase P solution. Finally, the Second to Fifth digestions were collected following centrifuge. Preosteoblastic cell line MC3T3-E1 and mouse myoblast cell line C2C12 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). MC3T3-E1 and PMCO cells were maintained in a-minimum essential medium (a-MEM) containing 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Sigma-Aldrich). C2C12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin (Sigma-Aldrich). Herbacetin (purity: >90% by HPLC) was obtained from Sigma-Aldrich.

Quantitative real time polymerase chain reaction (qPCR)

RNA was extracted by using the GenElute™ Total RNA Purification Kit (Sigma-Aldrich). Reverse transcription reaction was conducted by ReverTra Ace qPCR RT Kit (TOYOBO). The PCR primers for osterix, runx2, osteocalcin, and GAPDH was listed in **Table 1**. Gene expression was quantitated by using the $2^{-\Delta\Delta CT}$ method [10].

Cell viability assay

MC3T3-E1/C2C12 cells were seeded into a 96-well plate (500 cells per well per 200 μl) and incubated with indicated concentrations of Herbacetin (0 μM, 10 μM, 20 μM, 30 μM, 40 μM) for 8 days. At the same time point of each day, 20 μl CCK-8 (Dojindo laboratories) were added into each well and OD 450 was measured by spectrophotometry (BioTek) 2 h after incubation.

Protein extraction and western blot assay

Total cellular protein was lysed using RIPA buffer (Solarbio, Beijing, China) containing 1% PMSF (Solarbio, Beijing, China), protease inhibitors and phosphatase inhibitors (Selleckchem). Nuclear/cytoplasm protein was extracted using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Haimen, China). Proteins were separated by 10% SDS-polyacrylamide gels and electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Invitrogen). The membranes were blocked with 5% non-fat milk, incubated with primary antibodies at 4°C overnight, and then incubated with secondary antibodies. Purpose bands were visualized using enhanced chemiluminescence (ECL) system (Pierce, Thermo Fisher Scientific, Inc.). Primary antibodies used in this study were anti-NF-κB p65 (8242, Cell Signaling Technology), anti-Lamin B (13435, Cell Signaling Technology), anti-AKT (2920, Cell Signaling Technology), anti-$pAKT^{Thr308}$ (13038, Cell Signaling Technology), anti-$pAKT^{Ser473}$ (4060, Cell Signaling Technology) and anti-GAPDH (60004, Proteintech). Secondary antibodies were purchased from Cell Signaling Technology.

Pull-down assay

MC3T3E1/C2C12 cell lysates were incubated with herbacetin-Sepharose 4B or Sepharose 4B beads (50 μl, 50% slurry, GE Healthcare, Piscataway, NJ) in reaction buffer (50 mM Tris pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% NP40, 2 μg/mL bovine serum albumin) at 4°C overnight. Then the beads were washed with elution buffer (50 mM Tris pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% NP40) and binding was visualized by Western blotting assay.

Osteogenic induction

MC3T3-E1 cells were cultured in α-MEM supplemented with 10 mM β-glycerophosphate (G6251, Sigma-Aldrich), 10 nM dexamethasone (D1756, Sigma-Aldrich), and 50 μg/ml ascorbic acid (A5960, Sigma-Aldrich). C2C12 cells were

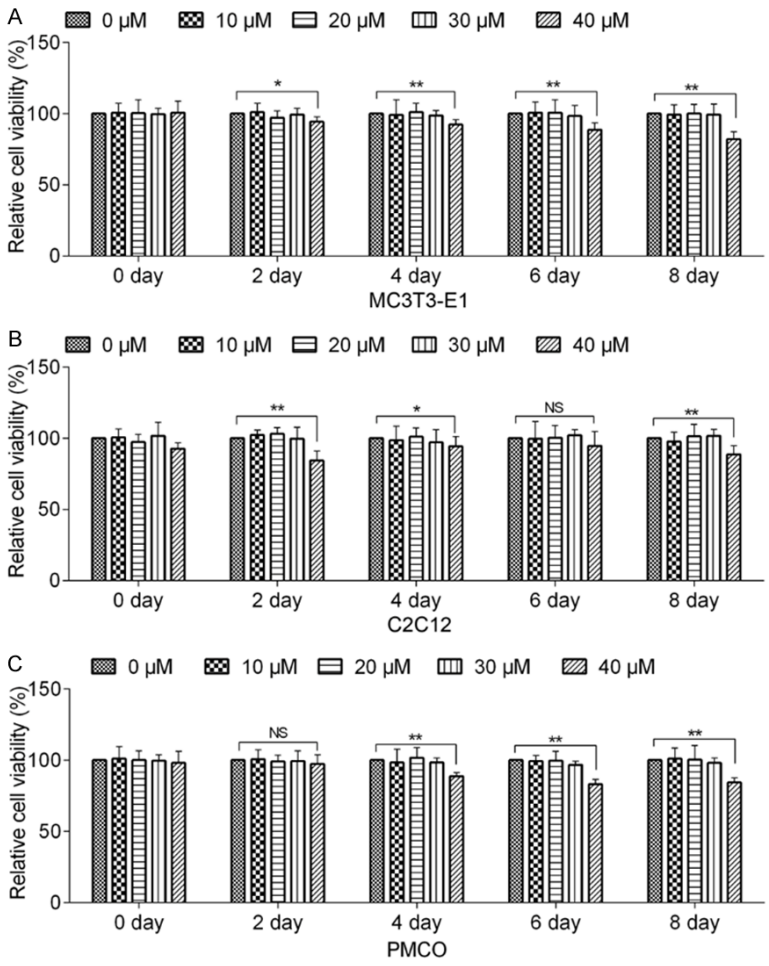

Figure 1. Cytotoxicity of Herbacetin treatment on MC3T3-E1/C2C12/PMCO cells. A. MC3T3-E1 cells were treated with various concentrations of Herbacetin for 0 day, 2 days, 4 days, 6 days and 8 days. Cell viability was detected by CCK-8 assay. B. Effect of Herbacetin treatment on C2C12 cells. C. Effect of Herbacetin treatment on the viability of PMCO cells. Representative data from at least 3 independent experiments are shown. Data are shown as mean ± SD. NS, not significant; *P<0.05; **P<0.01.

cultured in DMEM supplemented with 200 ng/mL BMP-2 (120-02, Peprotech, NJ, USA). The medium was changed every other day. Alizarin Red S staining was used to analyze calcium accumulation after 21 days of osteogenic differentiation. After removing the medium, cells were washed with PBS and fixed with 10% formalin solution for 15 min. Then cells were stained with 2% Alizarin Red S (pH 8.3, A55-33; Sigma-Aldrich) for 30 min in the dark, and then rinsing extensively with PBS. After pictures of Alizarin Red S staining were taken, the stained nodules were dissolved with 10% cetylpyridinium chloride (C0732, Sigma-Aldrich) and absorbance 570 nm was examined. ALP staining was conducted using the BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime, Haimen, China) according to the manufacturer's protocol.

ALP activity assay

ALP enzymatic activity was examined by the ALP activity assay kit (Beyotime, Haimen, China). The cells were seeded in 96-well plates at a density of 5 × 10^3 cells/well. ALP activity was analyzed on culture days 3, 7, 14 and 21, according to the manufacturer's instructions. The results were measured at 405 nm by spectrophotometry (BioTek).

Immunofluorescence assay

MC3T3-E1/C2C12 cells were fixed with 4% paraformaldehyde for 15 min. After washed with PBS, cells were permeabilized with 0.5% NP-40 (Sigma-Aldrich, St Louis, MO, USA) for 10 min. Then cells were blocked with 10% normal goat serum for 30 min, incubated with anti-NF-κB p65 at 4°C overnight. The second day, sections were incubated with Alexa Fluor® 555 goat anti-rabbit IgG (H+L) (Invitrogen, Carlsbad, CA) at 37°C for 30 min. Cell nuclei were stained with DAPI (Beyotime, Haimen, China). All samples were then analyzed by an Olympus BX50 microscope (Olympus, Tokyo, Japan).

Statistical analysis

The data are expressed as mean ± S.D. from at least three independent experiments. Differences between two groups were analyzed by Student's t test. Statistical analyses used to analyze the total differences among multiple groups were performed by analysis of variance (ANOVA) and the subsequent statistical test between any two groups were performed by Least Significant Difference (LSD) using the SPSS 20.0 software (SPSS Inc., Chicago, IL,

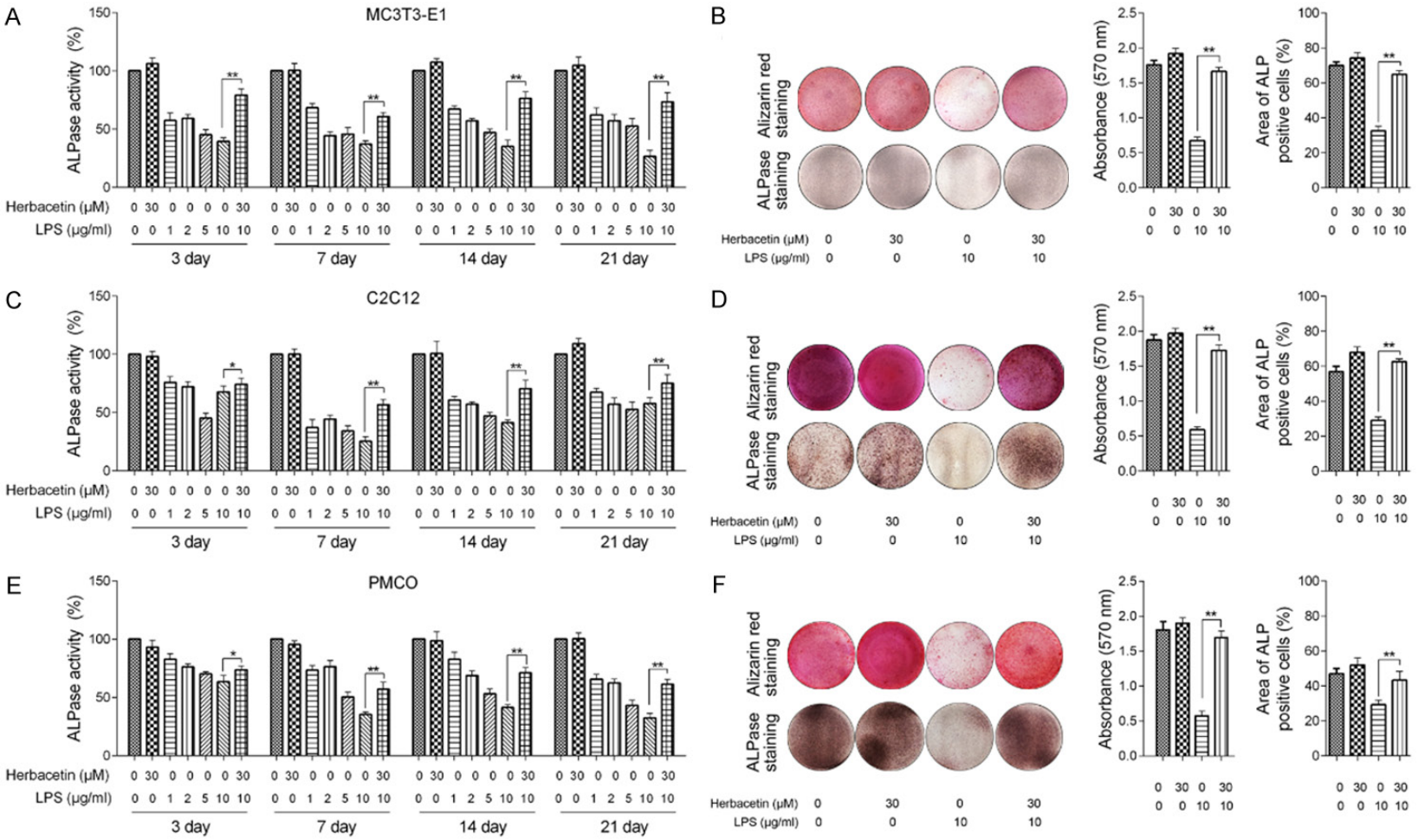

Figure 2. Herbacetin treatment remitted LPS induced inhibition of osteoblast differentiation. MC3T3-E1/C2C12/PMCO cells were treated with various concentrations of LPS in the presence or absence of Herbacetin in a time-course experiment. Quantification assay of ALPase activity was presented in (A) (MC3T3-E1), (C) (C2C12) and (E) (PMCO). ALP staining and Alizarin Red S staining was presented and quantified in (B) (MC3T3-E1), (D) (C2C12) and (F) (PMCO). Representative data from at least 3 independent experiments are shown. Data are shown as mean ± SD. *P<0.05; **P<0.01.

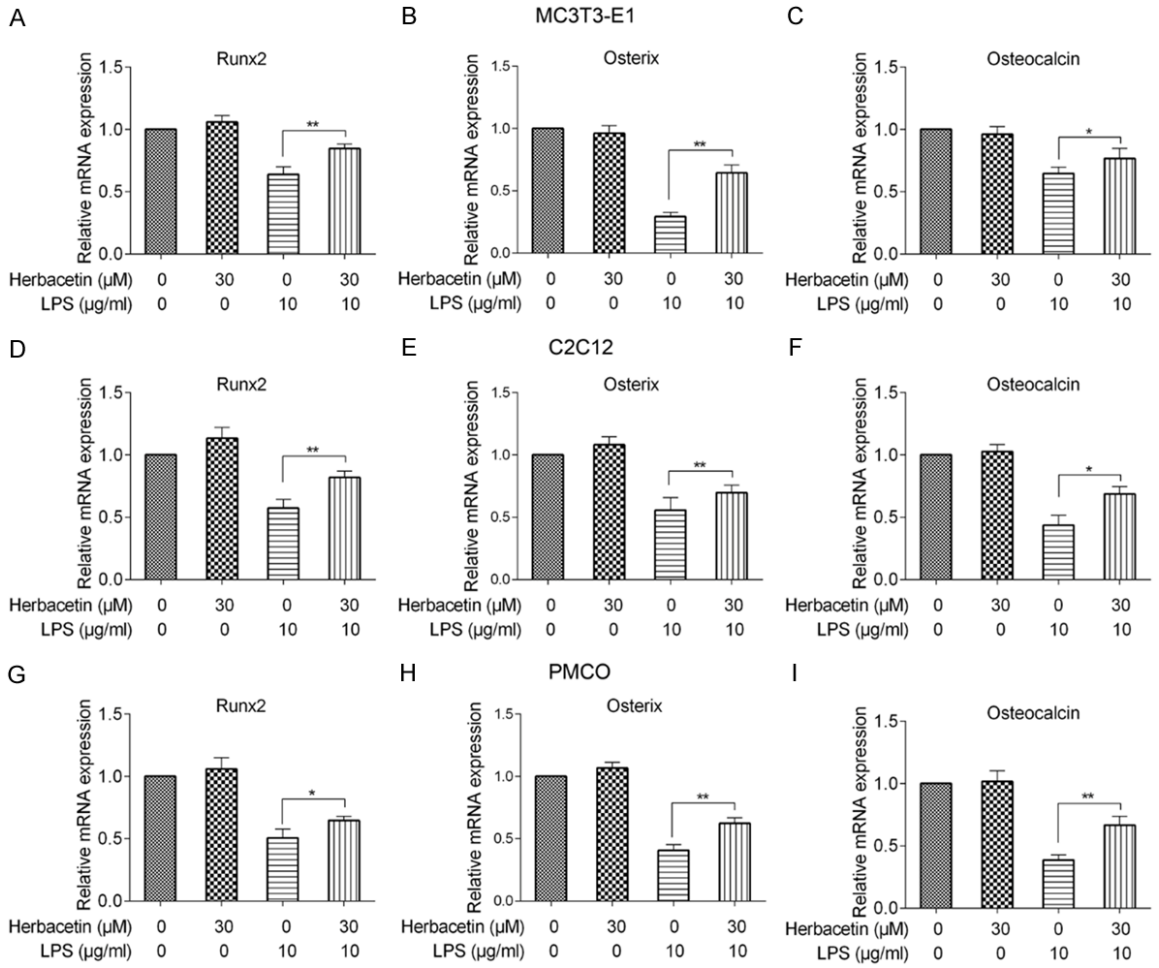

Figure 3. Effect of Herbacetin on the expression of osteoblastic genes in MC3T3E1/C2C12/PMCO cells. A-C. MC3T3E1 cells were treated with LPS or/and Herbacetin for 7 days. Total RNA was extracted and the expression of Runx2, osterix, and osteocalcin was quantified by qPCR. D-F. C2C12 cells were treated with LPS or/and Herbacetin for 7 days. The mRNA levels of Runx2, osterix, and osteocalcin was quantified by qPCR. G-I. Herbacetin restored the expression of Runx2, osterix, and osteocalcin in PMCO cells. Representative data from at least 3 independent experiments are shown. Data are shown as mean ± SD. *P<0.05; **P<0.01.

USA). *P* value less than 0.05 indicated a significant difference between groups.

Results

Cytotoxicity of Herbacetin treatment on MC3T3-E1/C2C12 cells

To determine a suitable concentration for Herbacetin treatment, we first evaluated the cytotoxicity of Herbacetin by CCK-8 assay. MC3T3-E1 cells were treated with various concentrations of Herbacetin (0 μM, 10 μM, 20 μM, 30 μM and 40 μM) for 0 day, 2 days, 4 days, 6 days and 8 days respectively. After examining the cell viability, we noticed that MC3T3-E1 cells treated with 40 μM Herbacetin exhibited significantly decreased viability (**Figure 1A**). Consistently, 40 μM Herbacetin treatment also significantly deceased the viability of C2C12 cells (**Figure 1B**) and PMCO cells (**Figure 1C**). To avoid biased results caused by reduced cell viability, we intended to use 30 μM as the final concentration in osteoblast differentiation assay.

Herbacetin restored LPS induced inhibition of osteoblast differentiation

We first evaluated the effect of Herbacetin single treatment on osteoblast differentiation. As indicated in **Figure 2A**, **2C** and **2E**, Herbacetin (30 μM) treatment only slightly promoted the ALPase activity in MC3T3-E1/C2C12/PMCO

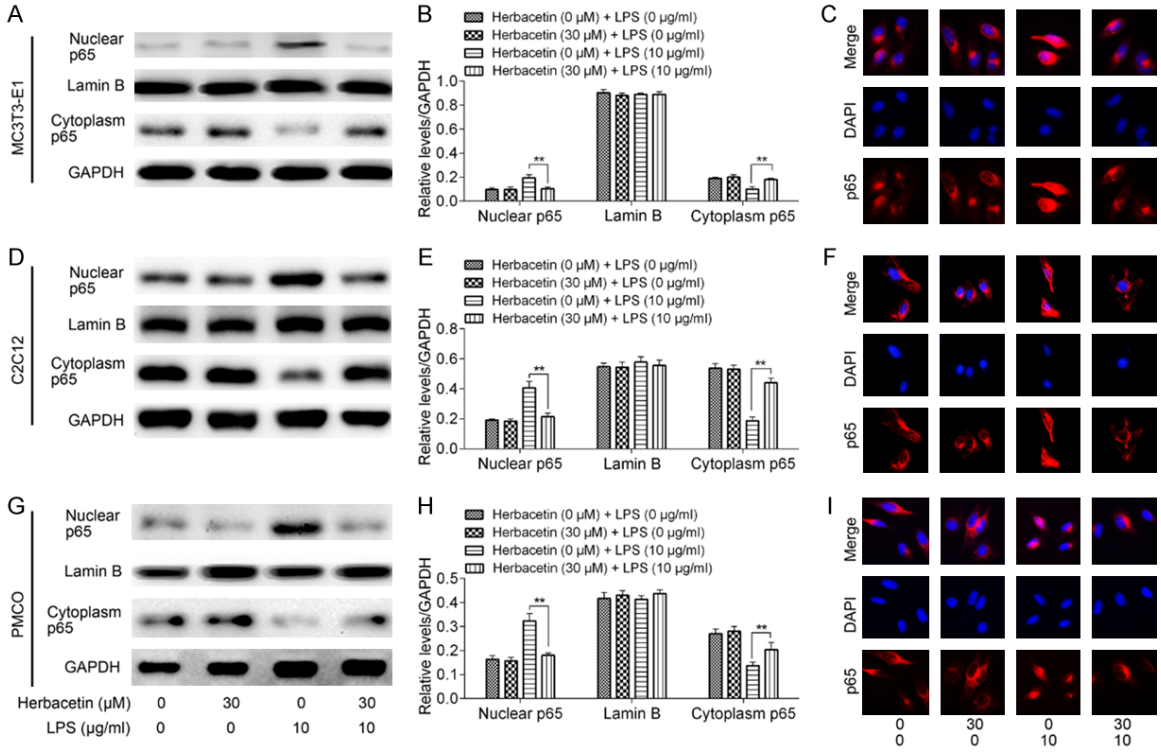

Figure 4. Effect of Herbacetin on NF-κB signaling pathway. (A, B) MC3T3E1 cells were treated with LPS or/and Herbacetin for 7 days. Protein was extracted and the nuclear/cytoplasmic NF-κB p65 was detected by western blot assay. (C) Cytoplasmic-nuclear translocation of NF-κB p65 was detected by immunofluorescence assay. (D, E) Nuclear/cytoplasmic protein was extracted after C2C12 cells were treated with LPS or/and Herbacetin for 7 days. NF-κB p65 in subcellular fractions was detected by western blot assay. (F) Subcellular localization of NF-κB p65 in C2C12 cells. (G, H) Nuclear/cytoplasmic NF-κB p65 in PMCO cells was detected by western blot assay and the result was confirmed by immunofluorescence assay (I). Representative data from at least 3 independent experiments are shown. Data are shown as mean ± SD. *P<0.05; **P<0.01.

cells. LPS stimulation induced inflammatory environment was a well-known inhibitory factor for osteoblast differentiation. To examine the effect of LPS on ALPase activity, MC3T3-E1/C2C12/PMCO cells were treated with LPS at different concentrations. Our data indicated that LPS stimulation suppressed ALPase activity in a dose-dependent manner (**Figure 2A**, **2C** and **2E**). Importantly, LPS (10 μg/ml) induced decrease of ALP activity could be restored by simultaneous treatment of LPS (10 μg/ml) and Herbacetin (30 μM). As to ALP and Alizarin Red S staining, single addition of Herbacetin showed no difference compared to the negative control with vehicle. However, Herbacetin (30 μM) treatment increased ALPase expression and calcium deposition in MC3T3-E1/C2C12/PMCO cells under LPS stimulation (**Figure 2B**, **2D** and **2F**). To further confirm the protective role of Herbacetin on MC3T3-E1/C2C12/PMCO cell differentiation, we investigated the transcriptional levels of osteogenic genes such as Runx2, Osterix and Osteocalcin. As the result, gene expression of Runx2 (**Figure 3A**, **3D** and **3G**), osterix (**Figure 3B**, **3E** and **3H**) and osteocalcin (**Figure 3C**, **3F** and **3I**) was significantly downregulated by LPS in a dose-dependent manner, while dual treatment of Herbacetin and LPS restored the expression of these genes. Again, the single addition of Herbacetin did not significantly change the expression of osteogenic genes as compared to the negative control. These results suggested that Herbacetin restored osteoblast differentiation under LPS stimulation.

Herbacetin abrogated LPS induced NF-κB p65 nuclear translocation

LPS induced activation of NF-κB signaling pathway is a well-known mechanism for blocking osteoblast differentiation, while the anti-inflammatory role of Herbacetin under LPS stimulation has not been reported yet. To this end, we

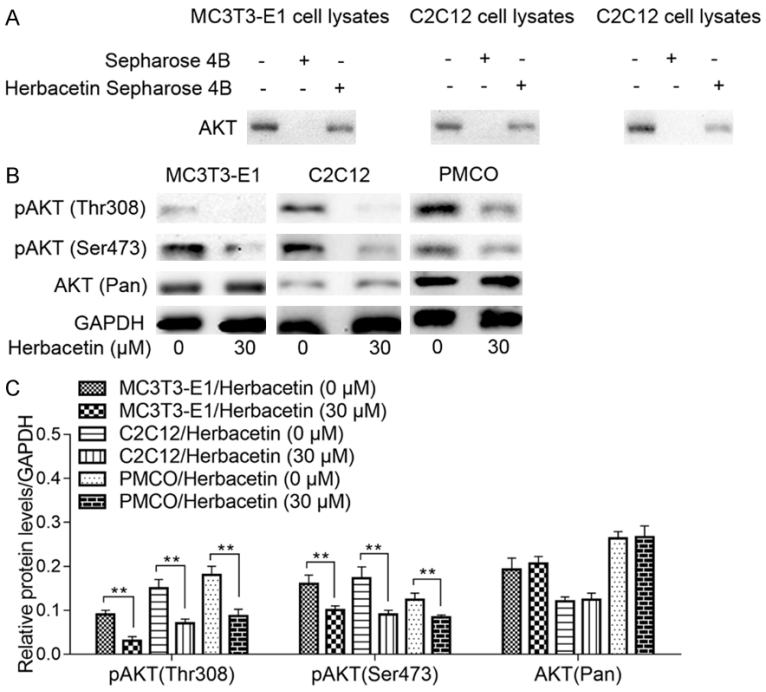

Figure 5. Effect of Herbacetin on AKT signaling pathway. A. MC3T3E1/C2C12/PMCO cell lysates were incubated with herbacetin-conjugated Sepharose 4B beads or Sepharose 4B beads alone. Proteins were pulled down and detected by anti-AKT. B. MC3T3E1/C2C12/PMCO cells were treated with Herbacetin for 7 days. Protein was extracted and the phosphorylation level of AKT was detected by western blot assay. C. The result of western blot assay was quantified. Representative data from at least 3 independent experiments are shown. Data are shown as mean ± SD. *P<0.05; **P<0.01.

first determined the influence of LPS or/and Herbacetin on NF-κB signaling pathway by western blot assay. As indicated in **Figure 4A**, **4B**, **4D**, **4E**, **4G** and **4H**, single addition of Herbacetin showed no influence on the cytoplasmic/nuclear levels of NF-κB in MC3T3-E1/C2C12/PMCO cells, while single addition of LPS significantly increased the level of nuclear NF-κB p65 and deceased the level of cytoplasmic NF-κB p65. The levels of nuclear NF-κB p65 were restored after Herbacetin treatment. We then conducted immunofluorescence assay to further evaluate the influence of LPS/Herbacetin on NF-κB p65 translocation. Consistently, cell membrane fluorescence signal of NF-κB p65 was significantly decreased by LPS stimulation and restored by Herbacetin treatment (**Figure 4C**, **4F** and **4I**).

Herbacetin suppressed AKT activation

Kim et al. demonstrated that Herbacetin could bind to AKT1/2 and suppressed the kinase activity in lung cancer cell line A431 and melanoma cell line SK-MEL-5, which provided a reasonable explanation for Herbacetin mediated AKT inhibition [6]. In this study, we confirmed that Herbacetin could also bind to AKT in MC3T3-E1/C2C12/PMCO cells by pull-down assay (**Figure 5A**). To further clarify the effect of Herbacetin on AKT signaling pathway in MC3T3-E1/C2C12/PMCO cells, we then detected the phosphorylation levels of AKT under Herbacetin treatment. The result of western blot assay revealed that Herbacetin treatment could significantly suppress the phosphorylation of $pAKT^{Thr308}$ and $pAKT^{Ser473}$ (**Figure 5B** and **5C**). Taken together, Herbacetin induced AKT inactivation might be involved in the repression of NF-κB signaling pathway and osteoblast differentiation.

Reactivation of AKT abrogated the effect of Herbacetin on osteoblast differentiation

To confirm the protective effect of Herbacetin during osteoblast differentiation was indeed mediated by AKT signaling pathway, we then observed the osteoblast differentiation after activating AKT pathway. We used a small molecular inhibitor (SF1670) targeting PTEN (a well-reported AKT inhibitor) to activate AKT and the result showed that protein levels of p-AKT were significantly up-regulated (**Figure 6A**). The ALP and Alizarin Red S staining experiment showed that SF1670 treatment significantly suppressed the osteoblast differentiation in all of the three cell lines (**Figure 6B** and **6C**). These results suggested that the function of Herbacetin in osteoblast differentiation was mediated by the repression of AKT signaling pathway.

Discussion

MC3T3-E1 cells, which were derived from the calvaria of C57/BL mouse, could differentiate into osteoblasts and produce mineralized matrix [11]. MC3T3-E1 cells were the most

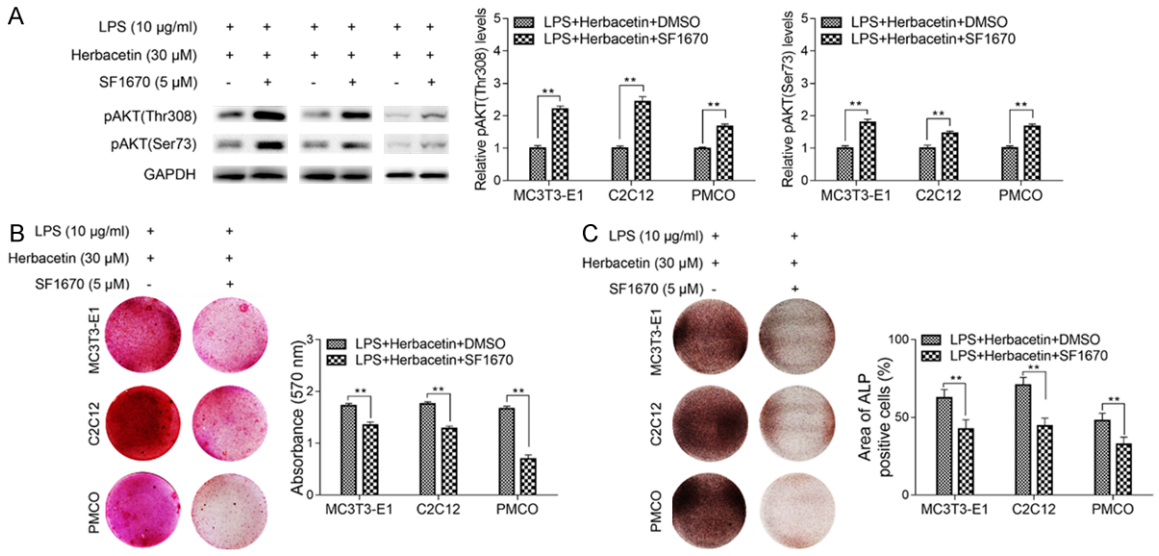

Figure 6. Reactivation of AKT signaling pathway abrogated the effect of Herbacetin in osteoblast differentiation. MC3T3-E1/C2C12/PMCO cells were treated with LPS (10 μg/ml), Herbacetin (30 μM) together with/without SF1670 (5 μM). A. The protein levels of pAKT was detected by western blot and the result showed SF1670 treatment promoted the levels of pAKT. B. Alizarin Red S staining showed lower absorbance at 570 nm in cells treated with SF1670. C. ALP staining showed significantly decreased proportion of ALP positive cells in SF1670 treated group. Representative data from at least 3 independent experiments are shown. Data are shown as mean ± SD. *P<0.05; **P<0.01.

reported commercialized cells lines for observing osteoblast differentiation. C2C12 is a myoblast cell line with differentiation potential and often used as an in vitro system to study skeletal muscle development and osteoblast differentiation [12, 13]. To induce differentiation into osteoblast-like cells, the medium should be replaced with DMEM supplemented with BMP-2 [12], while myotube formation was induced by replacing the medium with DMEM supplemented with FBS [14, 15]. The differentiation potential of these two cell lines could be detected by dyeing their metabolites such as ALPase and deposition of calcium. In this study, we observed that LPS stimulation induced inflammation microenvironment suppressed MC3T3-E1 and C2C12 cells differentiation. When cells were pre-treated with Herbacetin, the inhibitory effect on osteoblast differentiation by LPS stimulation was largely restored. Additionally, we also observed Herbacetin treatment restored the expression of osteoblastic genes osterix, runx2 and osteocalcin in both cell lines. To generate more data with better cell model besides commercialized cell lines (C2C12 and MC3T3-E1), we've generated primary mouse calvarial osteoblasts and conducted the functional studies to observe the effect of Herbacetin during osteoblast differentiation. The results in primary cells also indicated a protective role of Herbacetin during osteoblast differentiation. Based on our results in three cell lines, we suggested that Herbacetin had an anti-inflammatory role during osteoblast differentiation.

Activation of NF-κB pathway, by the phosphorylation and nuclear translocation of the cytoplasmic complexes, plays critical roles in inflammation via transcriptionally activating various proinflammatory genes [16-18]. The activation of NF-κB signaling pathway could induce upregulation of adhesion molecules and chemokines, following by the recruitment of effector cells such as neutrophils, macrophages and leukocytes [18, 19]. It is demonstrated that NF-κB mediated inflammation exerts negative role on osteoblast differentiation and postnatal fracture repair through suppression of BMP/Smad signaling [5, 20, 21]. A well-known activated form of NF-κB is a heterodimer consisting of a p50 and p65 translocates into nuclear, which could be evaluated by detecting the cytoplasmic and nuclear levels of p65 subunit. To explore the mechanisms through which Herbacetin regulated osteoblast

differentiation, we first screened Herbacetin-related articles. We noticed that the anti-inflammatory role of Herbacetin was tightly correlated with the activation of NF-κB signaling pathway. In RAW264.7 and mouse bone marrow-derived macrophages, LPS induced activation of the JNK and NF-κB pathway could be blocked by Herbacetin treatment [9]. Meanwhile, previous literatures also revealed that Herbacetin suppressed AKT pathway in breast cancer, melanoma, hepatocellular carcinoma and lung cancer cells [6-8]. Thus, we first extracted cytoplasm and nuclear proteins respectively to confirm LPS stimulation could induce p65 nuclear translocation in MC3T3-E1/C2C12/PMCO cells. The results of western blot assay suggested that LPS stimulation upregulated nuclear p65 level, which could be minified by Herbacetin treatment. These results were further confirmed by immunofluorescence assay. Interestingly, we also observed that Herbacetin treatment suppressed the phosphorylation and activation of AKT, while re-activation of AKT abrogated the effect of Herbacetin in MC3T3-E1/C2C12/PMCO cells. As is well reported that AKT was an upstream regulator of NF-κB [22, 23], we concluded that Herbacetin exhibited the anti-inflammatory effect in MC3T3-E1/C2C12 cells through inhibiting AKT/NF-κB pathway.

It should be noted that there are some limitations of our work. Firstly, we didn't explore the role of Herbacetin on osteoblast differentiation *in vivo* due to lack of suitable animal models. Our future study will be focused on seeking for a suitable animal model to explore the possibility of developing Herbacetin as a therapeutic drug. Additionally, the biological process of osteoblast differentiation involved in the crosstalk of complicated signaling pathways. Lu et al. concluded that LPS-mediated inflammatory environment activated the intrinsic association of BMP/MAPK and Smad signaling [4]. In another study, Huang et al. reported that crosstalk between TLR4/MyD88/NF-κB and BMP/smad Signaling activated by LPS stimulation inhibited osteoblastic differentiation [5]. Thus, the crosstalk between AKT/NF-κB signaling pathway and these reported pathways during osteoblast differentiation still need further investigation.

In conclusion, our current work uncovered the anti-inflammatory role and mechanism of Herbacetin in MC3T3-E1/C2C12/PMCO cells. Herbacetin exhibited great biocompatibility with osteoblasts, and have the potential to be developed as a drug for bone and tissue engineering in the future.

Disclosure of conflict of interest

None.

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