Betulin inhibits pro-inflammatory cytokines expression through activation STAT3 signaling pathway in human cardiac cells

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Abstract. – OBJECTIVE: Signal transducer and activator of transcription 3 (STAT3) is an important regulator of cardiac survival pathways. Decreased expression or activity of STAT3 in patients with end-stage heart failure demonstrated a clinical relevance of STAT3 in cardiac diseases. Betulin, a pentacyclic triterpene, has drawn extensive attention towards its beneficial effects. However, little is known about its roles in cardiac cells.

MATERIALS AND METHODS: We investigated the effects of betulin on the pro-inflammatory processes in human cardiac AC16 cells. Genes expression of pro-inflammatory cytokines and activation of NF-κB signaling were analyzed. Besides, levels of phosphorylated STAT3 and its down-stream target genes were measured to evaluate the activation of STAT3. Finally, STAT3 inhibitor and small interfering RNA (siRNA) oligos were used to determine the roles of STAT3 in AC16 cells treated with betulin.

RESULTS: Our results revealed that betulin inhibited pro-inflammatory cytokines expression and NF-κB signaling activation through STAT3 signaling. Besides, betulin treatment also induced the expression of Bcl-xL, an anti-apoptotic downstream effector of STAT3.

CONCLUSIONS: Our results, for the first time, uncovered the cardioprotective roles of betulin, which may be useful to reduce the occurrence of adverse cardiovascular events.

Key words:
Betulin, Inflammation, STAT3, NF-κB, Cardiac cells.

Introduction

Under pathological stress or stimuli, the myocardium expresses and secretes several pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, and monocyte chemoattractant protein-1 (MCP-1)1–3. These cytokines usually exert several autocrine effects in cardiac cells, leading to the activation of NF-κB signaling pathway. Subsequently, NF-κB signaling activation contributes to cardiac hypertrophy, ischemic myocardial injury and chronic heart failure4,5.

Signal transducer and activator of transcription 3 (STAT3) is a member of a family of transcription factors transmitting signals from the cell membrane to the nucleus6. STAT3 is activated by several cytokines and growth factors. For example, interleukin-6 (IL-6) or epidermal growth factor (EGF), induces phosphorylation of STAT3 by janus kinase 2 (JAK2) or growth factor receptor tyrosine kinases, respectively7,8. STAT3 was shown to regulate a variety of biological processes, including cell proliferation, differentiation, apoptosis, and development9,10. In addition, growing evidence indicate that STAT3 plays an important role in cardiac cells. Multiple studies have revealed that STAT3 improves cardiomyocyte survival and promotes cardiac angiogenesis, indicating that activation of STAT3 is beneficial for the heart11,12. Indeed, cardiomyocyte-specific deficiency of STAT3 leads to higher sensitivity to inflammation, cardiac fibrosis, and heart failure in mice13.

Betulin, a compound found in birch tree bark, can be converted to betulinic acid, an important pharmacological substance14. Recently, betulin was reported to serve as a cytotoxic agent for several tumor cell lines and as an apoptotic inducer15,16. Besides, betulin could improve hyperlipidemia and insulin resistance in obese rodents, through inhibition of sterol regulatory element-binding protein (SREBP)17. However, its roles in cardiac cells have not been investigated. In the present study, our aim was to determine the effects of betulin in cardiac cells and examine whether betulin could serve as a therapeutic target in the heart.
Materials and Methods

Cell culture and regents

Human AC16 cells were purchased from the ATCC Cell Biology Collection (Manassa, VA, USA), and cultured in Dulbecco modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin and 100 g/ml streptomycin.

siRNA, RNA extraction and Real-time Analysis

siRNA oligos targeting STAT3 were purchased from GenePharma (Shanghai, China). A scramble siRNA was used as a negative control. Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was conducted from 1 \( \mu \)g total RNA using Oligo-dT primers and M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Real-time PCR (polymerase chain reaction) assays were performed on a MiniOpticonTM Real-Time PCR system (BioRad, Hercules, CA, USA). Thermal cycling conditions were as follows: activation of DNA polymerase at 95 °C for 5 min, followed by 45 cycles of amplification at 95 °C for 10 s and at 60 °C for 45 s. mRNA expression of \( \beta \)-actin gene was used as an internal control.

Western Blot

Cells were harvested and lysed with lysis buffer [(50 mM Tris-HCl, pH 7.4, 100 mM DTT (dithiothreitol), 2% w/v SDS (sodium dodecyl sulphate), 10% glycerol)]. After centrifugation at 20,000 g for 10 min at 4°C, proteins in the supernatants were quantified and separated by 12% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis), transferred to PVDF (polyvinylidene fluoride) membrane. After blocking with 5% nonfat milk, membranes were immunoblotted with indicated antibodies, followed by HRP (horse-radish peroxidase)-linked secondary antibodies. The signals were detected by Millipore SuperSignal®. Rockford, IL, USA HRP Substrate kit according to manufacturer’s instructions.

ChIP Assays

Chromatin immunoprecipitation (ChIP) assay kits were used following its instructions (Upstate Biotechnology Inc., Lake Placid, NY, USA). In short, AC16 cells were fixed with 1% formaldehyde to cross-link the proteins and DNA, followed by sonication in an ultrasound bath on ice. DNA was sheared to fragments at 500-1000 bp using sonication. The chromatin was then incubated and precipitated with p65 antibody or IgG control. The immunoprecipitated DNA fragments were analyzed using Real-time PCR analysis.

Statistical Analysis

Data were analyzed with the PRISM 5.0 software package (GraphPad, San Diego, CA, USA). Results are expressed as the mean S.E. and were compared by Student’s t test or analysis of variance. A value of \( p < 0.05 \) was considered significant.

Results

Betulin inhibits the inflammatory gene expression profile in human cardiac cells

As a first approach, we determined whether betulin treatment led to a decreased expression of pro-inflammatory genes in AC16 cells. As expected, addition of TNF-\( \alpha \) markedly enhanced the mRNA levels of IL-6, MCP-1, and IL-1 (Figure 1A). Interestingly, Betulin co-treatment significantly repressed these genes expression levels (Figure 1A). Consistent with their mRNA expression levels, betulin also abrogated the effects of TNF-\( \alpha \) on the secretion of IL-6 and MCP-1 (Figure 1B).

Betulin inhibits the activation of NF-\( \kappa \)B signaling

Previous studies have proved that pro-inflammatory cytokines are under the transcriptional regulation of the ubiquitous transcription factor named nuclear factor-\( \kappa \)B (NF-\( \kappa \)B) in the heart. Therefore, we further performed luciferase reporter and ChIP assays to assess the effects of betulin on the transcriptional activity of NF-\( \kappa \)B in AC16 cells. As shown in Figure 2A, Betulin blocked the transcriptional activity of p65 using a NF-\( \kappa \)B luciferase reporter. Besides, the recruitment of p65 on the promoter regions of pro-inflammatory cytokines, including IL-6 and MCP-1 was also attenuated by betulin (Figure 2B). Moreover, we observed that p65 contents in the nucleus were significantly decreased in AC16 cells with betulin (Figure 2C). In addition, abundance of phosphorylation of p65, an active form of p65, was also largely reduced in cells treated with betulin (Figure 2D). Together, our results suggest that betulin could inhibit the activation of NF-\( \kappa \)B signaling in human cardiac cells.
Betulin inhibits pro-inflammatory cytokines expression through activation STAT3 signaling pathway

Figure 1. Betulin inhibits the inflammatory gene expression in AC16 cells. A, Relative mRNA expression of IL-6, MCP-1 and IL-1 was assessed by Real-time PCR in human cardiac AC16 cells treated as indicated. AC16 cells were pre-treated with vehicle control (C) or Betulin (B, 10 µM) for 4 hours. Cells were then treated with TNF (T, 15 ng/ml) for another 8 hours. B, Determination by ELISA of IL-6 and IL-1β secretion into the culture media in AC16 cells. AC16 cells were pre-treated with vehicle control (C) or Betulin (B, 10 µM) for 4 hours. Cells were then treated with TNF (T, 15 ng/ml) for another 20 hours.

*p < 0.05 vs Control (C), & p < 0.05 vs TNF (T).

Figure 2. Betulin inhibits the activation of NF-κB signaling. A, Luciferase assays using NF-κB reporter plasmids in AC16 cells. Cells were treated as Fig.1A. B, ChIP assays showing the recruitment of p65 on the promoter regions of IL-6 and MCP-1. Data were shown as a fold compared to IgG. C, Nuclear p65 contents were determined using western blot from nuclear lysates. D, Phosphorylation of p65 (p-p65) was analyzed in AC16 cells treated as indicated. Total p65 (t-p65) and β-actin were used as an internal control.

*p < 0.05 vs Control (C), & p < 0.05 vs TNF (T).
Betulin activates STAT3 signaling pathway

Next, we tried to seek the molecular mechanisms of inhibition of NF-kB signaling by betulin. As shown in Figure 3A, treatment of human cardiac AC16 cells with betulin activated the phosphorylation at Tyr705 residues of STAT3 (Figure 3A). Besides, betulin treatment also increased mRNA and protein levels of the suppressor of cytokine signaling (SOCS3) (Figure 3B and 3C), whose expression is induced by STAT3. Interestingly, betulin induced the expression of the anti-apoptotic Bcl-xL gene (Figure 3D and E), another target of STAT3 signaling involved in the cell survival and proliferation. In summary, our results suggested that betulin could activate STAT3 signaling pathway.

Knockdown of STAT3 abolished anti-inflammatory roles of Betulin

Moreover, AC16 cells were infected with small interfering RNA targeting STAT3, which significantly inhibits endogenous STAT3 expression (Figure 4B and C). The inhibition of endogenous STAT3 also significantly inhibited the ability of betulin to expression levels of proinflammatory cytokines (Figure 4D), demonstrating that STAT3 plays an indispensable role in betulin-inhibited NF-kB activation.

Discussion

Following myocardial infarction, quiescent cardiac fibroblasts are transformed into a proliferative and invasive myofibroblast phenotype, which accounts for a major source of pro-inflammatory cytokines, leading to the activation of NF-kB signaling pathway. Persistent NF-kB activation promotes remodeling, apoptosis and endoplasmic reticulum stress in cardiomyocytes. Besides, it contributes to decreased cardiac function, and faster progression to heart failure and cardiac hypertrophy. NF-kB activity is also regulated by cytoplasmic degradation of the IκB inhibitor and its subsequent nuclear translocation.

Several studies have shown not only that STAT3 may activate NF-kB, but also that it may be re-

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**Figure 3.** Betulin activates STAT3 signaling pathway. (A) Phosphorylation of STAT3 (p-STAT3) was analyzed in AC16 cells treated as indicated. Total STAT3 (t-STAT3) and β-actin were used as an internal control. (B-C) mRNA (B) and protein (C) levels of SOCS3 were determined in AC16 cells treated with control or Betulin for 12 or 24 hours, respectively. (D-E) mRNA (D) and protein (E) levels of BCL-xL were determined in AC16 cells treated as Figure 3B.

*p < 0.05 vs Control (C)
required to maintain NF-κB activity\textsuperscript{21}. For instance, it has been reported that STAT3 may form a complex with the p65/p50 heterodimer, which then translocates to the nucleus, binds to DNA, and activates NF-κB-regulated genes\textsuperscript{22}. Especially in tumors, STAT3 was shown to positively regulate p65 activity through enhancement of its acetylation by p300\textsuperscript{22}. The interconnection of STAT3 with NF-κB signaling has been recognized as a major pathway responsible for both inflammation-induced carcinogenesis and anti-tumor immunity\textsuperscript{23}. However, our studies found that STAT3 might negatively regulate p65 transcriptional activity. Although the consistency remains unclear, the cell types used in these studies are different. Besides, whether betulin could activate other signaling pathways remains to be investigated in the future studies.

Conclusions

We demonstrated that betulin reduced NF-κB activity in human cardiac cells, in a process that coincides with the activation of STAT3 and anti-apoptotic downstream effectors. Therefore, activation of the STAT3 pathway by betulin might be involved in the cardioprotective effects of this compound and, since it may improve cardiac performance, it might be useful to reduce the occurrence of adverse cardiovascular events.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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Figure 4. STAT3 inhibition abolished anti-inflammatory roles of Betulin. A, Relative mRNA expression of IL-6 and MCP-1 was assessed by Real-time PCR in human cardiac AC16 cells treated as indicated. AC16 cells were pre-treated with STAT3 inhibitor AG490 (5 µM) for 2 hours. B-C, mRNA (B) and protein (C) levels of STAT3 in AC16 cells transfected with siRNA oligos targeting STAT3 or control. D, mRNA levels of IL-6 and MCP-1 in AC16 cells. Cells were transfected with control siRNA or STAT3 siRNA oligos (S) for 24 hours. Then, cells were treated with vehicle control (C), TNF. (T), or Betulin (B) as indicated. *p < 0.05 vs Control (C), & p < 0.05 vs TNF. (T), # p < 0.05 vs TNF plus Betulin (T+B).

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