Diosgenin inhibits tumor necrosis factor-induced tissue factor activity and expression in THP-1 cells via down-regulation of the NF-κB, Akt, and MAPK signaling pathways

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[ABSTRACT] AIM: To investigate whether diosgenin could modulate tissue factor (TF) procoagulant activity, expression, and related signal transduction pathways. METHODS: Human THP-1 monocytic cells were exposed to tumor necrosis factor-α (TNF-α, 10 ng·mL⁻¹) with or without diosgenin (0.01, 0.1, and 1 μmol·L⁻¹) for 2 h or 5 h to induce TF procoagulant activity and expression, which were determined by the simplified chromogenic assay, reverse transcription-polymerase chain reaction (RT-PCR), real-time quantitative PCR, and Western blotting assays. In addition, the activation of the NF-κB, Akt, and MAPK signaling pathways were also measured by Western blotting. RESULTS: Diosgenin significantly inhibited TNF-α-induced TF procoagulant activity at concentrations of 0.01 to 1 μmol·L⁻¹ with IC50 of 0.25 μmol·L⁻¹. It also reduced protein expression and mRNA accumulation of TF dose-dependently in activated THP-1 cells. TNF-α stimulated significantly phosphorylation on Ser536 of NF-κB/p65, Ser473 of Akt at 5–15 min, and activations of IKK-β and ERK at 15–30 min. Diosgenin (1 μmol·L⁻¹) could inhibit the phosphorylation of NF-κB/p65, IKK-β, Akt, ERK, and JNK, but had no remarkable effects on IκB and p38 phosphorylation in THP-1 cells. CONCLUSION: Diosgenin inhibits TNF-α-induced TF activity and expression in monocytes, partly due to its down-regulation of the phosphorylation of NF-κB/p65, IKK-β, Akt, ERK, and JNK.

[KEY WORDS] Diosgenin; Tissue factor; Monocytes; TNF-α; Cardiovascular diseases

1 Introduction

Cardiovascular disease is the leading cause of death worldwide, and preventive approaches, particularly achievable dietary changes, have major public health implications. Tissue factor (TF), or thromboplastin, is a 47-KD membrane-bound glycoprotein with structural homology to class II cytokine receptors. As the key initiator of the coagulation cascade, TF binds to factor Va and thereby activates factor IX and factor X, resulting in thrombus formation [1]. Accumulating evidence indicates that TF plays an important role in cardiovascular diseases, especially in thrombosis caused by coronary arteriosclerosis [2-3]. TF is widely expressed in both vascular and non-vascular cells. Blood monocytes represent the predominant source of TF in the circulation. Similar to endothelial cells, monocytes constitutively express little TF under basal conditions, but its expression can be further enhanced by specific stimuli including C-reactive protein, oxidized low density lipoprotein, tumor necrosis factor-α (TNF-α), and lipopolysaccharide [4-6]. Monocytes play very crucial roles in the pathogenesis of atherosclerosis. They turn to macrophages in the blood vessel, and highly cross link with foam cells, which finally leads to atherosclerosis. Studies also reveal that under the stimulation of TNF-α, monocytes generate TF to activate the coagulation protease cascade, which leads to fibrin deposition and the activation of platelets [7-8].

Many cardiovascular risk factors, including hypertension, lipid metabolic disorders, diabetes, smoking, obesity and hyperhomocysteinaemia exert elevated plasma levels of TF,
which may contribute to the pro-atherosclerotic effect of such risk factors. Moreover, TF expression is up-regulated in atherosclerotic plaques, and large amounts of TF are released into the bloodstream during plaque rupture, leading to acute thrombus formation in patients with unstable angina or acute coronary syndromes [9]. Interfering with the TF pathway appears to be an attractive target for the treatment of cardiovascular diseases [10].

It has been reported that many natural products, such as resveratrol, curcumin, and green tea could modulate TF expression and related signal pathways so as to provide benefit for the prevention and treatment of cardiovascular diseases [11-13]. In a previous study some interesting compounds were found which could inhibit TF expression [14]. Diosgenin ((25R)-spirost-5-en-3β-ol; 3β-hydroxy-5-spirostene), Dio, an important steroidal sapogenin, was one such compound and is widespread in Dioscoreacea, Liliaceae, Rosaceae, Caryophyllaceae and other plants. Many studies have shown that diosgenin has various significant activities, such as anti-tumor [15], cardiovascular-protection [16-17], anti-inflammation [18-19], and anti-endothelial dysfunctions [20] etc. There are also a variety of medicines in the clinic in China for atherosclerosis, coronary heart disease, and other cardiovascular diseases, whose major active component is diosgenin [21-22]. Despite many studies about its activity and molecular mechanism, there are few reports about its modulation on TF and related signaling pathways.

Given that TNF-α is an important mediator in inflammatory reactions and cardiovascular diseases [23-24], which could induce TF expression via the MAPK and NF-kB pathways in endothelial cells, monocytes and other cells [9, 25]. In the present study, whether diosgenin could regulate TNF-α-induced TF procoagulation activity, expression and related signal pathways in THP-1 monocytic cells was investigated. It was expected that these findings would allow some new insights for its molecular mechanism in the treatment of cardiovascular diseases, and also provide some pharmacological basis for expanding its clinical applications.

2 Materials and Methods

2.1 Cell culture and drug treatment

The THP-1 cells were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The cell pellet was suspended in RPMI 1640 medium (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% heat-inactivated newborn calf serum, and then incubated at 37 °C in 5% CO2. The THP-1 cells were inoculated into 96-well plates treated with 0.01–1 μmol·L−1 diosgenin (Shanxi Huike Botanical Development Co. Ltd., Shanxi, China) or control vehicle (dehydrated alcohol, 0.01% vol/vol) for 1 h, and then treated with TNF-α (10 ng·mL−1, Sigma, St. Louis, MO, USA) for 2 h or 5 h to induce TF procoagulation activity or expression.

2.2 Measurement of TF procoagulation activity

The cell lysates were frozen and thawed three times before they were used in the assay. Based on previous work [26], cell lysates (45 μL) were incubated with a reagent mixture (5 μL, pH 7.3) containing 10 g·L−1 prothrombin complex (Hualan Bioengineering Company, Xinzhuang, China) and 100 mmol·L−1 CaCl2 in a 96-well plate at 37 °C for 15 min. Then, 50 μL factor Xa chromogenic substrate (0.5 mmol·L−1, Sigma, St. Louis, MO, USA) containing 100 mmol·L−1 EDTA (pH 8.4) was added, and the absorbance was measured at 405 nm. Purified reconstituted human TF was used to generate a standard curve. When testing the effects of compounds, TF activity obtained in cells pretreated with a control vehicle (0.1% of DMSO) and then stimulated with TNF-α (10 ng·mL−1, 5 h) was taken as 100%, and the inhibition rate of tested compounds was calculated as (TF activity control – TF activity sample)/(TF activity control – TF activity normal) × 100%.

2.3 Cell viability assessment by the MTT assay

Briefly, 10 μL of 0.5 mg·mL−1 MTT (3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide, SunShine Bio Co. Ltd., Nanjing, China) in phosphate buffered saline was added to the media after 6 h of drug treatment. The plates were cultured for 4 h at 37 °C, followed by the addition of 150 μL of dimethyl sulfoxide. The 96-well plates were shaken for 10 min, and the OD values were detected by a microplate reader using a detection wavelength of 570 nm, with a reference wavelength of 650 nm. Cell viability was expressed as a percentage, with the control cells treated with vehicle as 100%.

2.4 RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared by the acid phenol method using Trizol reagent (Invitrogen Corporation, Carlsbad, CA, USA). RT-PCR was performed as described in a previous study [27], and the reverse-transcribed cDNA was then amplified by PCR using the following primers: TF, 5'-GAACCCCAACCCCGTCAAT-3' (sense) and 5'-TCTCATTACAGAGGCCTCCC-3' (antisense); β-actin, 5'-AGCGGGGAAATCGTG CGTGAC-3' (sense) and 5'-TCTCATACAGAGGCTCCC-3' (antisense). The thermal cycling conditions included 5 min at 94 °C, then 32 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 45 s. After amplification, PCR products were separated by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide dying.

2.5 Real-time quantitative polymerase chain reaction

Quantitative real-time polymerase chain reaction (PCR) was performed using a FastStart DNA Master SYBR Green I kit. The FastStart Taq DNA polymerase was activated by incubation at 95 °C for 10 min. This was followed by 40 cycles at 95 °C for 5 s and 60 °C for 34 s. Fluorescence was measured at 86 °C after the extension step at 72 °C. The PCR primers used for the amplification of TF and β-actin were as follows. The TF forward primer was 5'-TGATGTGGATAACAGAGGCTCCC-3' and the reverse primer was 5'-TCTCATTACAGAGGCTCCC-3'.
AAGGAGAAA ACT CTG T-3', and the reverse primer was 5'-CTACCC GGTCTGCTACTTCTTCC-3'. β-actin forward primer was 5'-CCCAGCTGGCC CATCTACG-3', and the reverse primer was 5'-AGGA TCTCATGAGGT AGTCAGTCAG-3'.

2.6 Western blot analysis

THP-1 cells were harvested in lysis buffer. Western blotting was performed as previously described [28] and the following antibodies were used for the procedure: anti-TF monoclonal antibody (R&D Systems, Minneapolis, MN, USA), anti-p65 and anti- phospho-NF-B/p65 antibodies (Kangchen Biotechnology, Shanghai, China), anti-JNK and anti-phospho-JNK antibodies, anti-p38MAPK and anti-phospho-p38 MAPK antibodies, and anti-Akt and anti-phospho-Akt antibodies (Bioworld Technology, St. Louis Park, MN, USA). The samples (20–40 μg) were treated with 5 × sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer, followed by heating at 100 °C for 5 minutes, and were then subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The proteins were then transferred to PVDF membranes (Millipore Corp, Bedford, MA, USA). The membranes were blocked with 3% bovine serum albumin (BSA), incubated with the primary antibodies for a night and incubated with the second antibodies for 1 h. The antigen–antibody complexes were then detected with ECL reagent and visualized by Quantity One software 4.6.2.

2.7 Statistical analyses

Results were expressed as the \( \bar{x} \pm s \). Data were analyzed by Student's two-tailed-t-test for comparison between two groups and one-way ANOVA, followed by Dunnett’s test when the data involved three or more groups. A probability value of less than 0.05 was considered statistically significant.

3 Results

3.1 Diosgenin reduces TNF-α-induced TF procoagulation activity in THP-1 cells

To study whether diosgenin regulated TF, the effect of diosgenin on TF procoagulation activity in THP-1 cells was tested. THP-1 cells were incubated with various concentrations of diosgenin for 1 h, and then cells were stimulated with TNF-α (10 ng·mL\(^{-1}\)) for 5 h to induce TF activity. As shown in Fig. 1A, TF activity was significantly increased by TNF-α (10 ng·mL\(^{-1}\), 5 h) in THP-1 cells. Diosgenin (0.01, 0.1, and 1 μmol·L\(^{-1}\)) suppressed TF procoagulation activity in THP-1 cells in a dose-dependent manner. The inhibition rates of diosgenin at 0.1 and 1 μmol·L\(^{-1}\) were approximately 42.78% and 70.43%, respectively, and the IC\(_{50}\) value was calculated as about 0.25 μmol·L\(^{-1}\). Curcumin, as positive control, also had significantly reduced TF procoagulation activity. Meanwhile, the MTT assay showed that diosgenin (0.01, 0.1 and 1 μmol·L\(^{-1}\)) did not affect the viability of THP-1 cells (data not shown), which suggested that the inhibition of TF procoagulation activity was not related to cytotoxicity of diosgenin.

3.2 Diosgenin down-regulates TNF-α-induced TF protein expression in THP-1 cells

In order to investigate whether diosgenin inhibited TF activity linked with suppressing TF induction, its effects on TF expression in THP-1 cells were observed. As shown in Fig. 1B, TNF-α (10 ng·mL\(^{-1}\), 5 h) markedly induced TF expression by more than three-fold in THP-1 cells. Diosgenin at three concentrations could tend to down-regulate TF expression induced by TNF-α in THP-1 cells. Curcumin (1 μmol·L\(^{-1}\)) significantly down-regulated TNF-α-induced TF protein expression. In addition, there was no obvious effect on TF expression when diosgenin was treated without TNF-α induction (data not shown).

3.3 Diosgenin suppresses TNF-α-induced TF mRNA expression in THP-1 cells

Whether diosgenin modulated TF gene expression was then examined. THP-1 cells were pre-incubated with various concentrations of diosgenin (0.01, 0.1 and 1 μmol·L\(^{-1}\)) for 1 h, and then treated with TNF-α (10 ng·mL\(^{-1}\)) for 2 h. The RT-PCR results showed that TF mRNA was almost not expressed in un-stimulated THP-1 cells, but TNF-α markedly induced TF mRNA expression. Diosgenin (0.01, 0.1, and 1 μmol·L\(^{-1}\)) suppressed TNF-α induced TF mRNA expression in a concentration-dependent manner in THP-1 cells (Fig. 2A), which suggested that diosgenin regulated TF expression at the gene transcriptional level. These findings were further confirmed by real-time PCR assay (shown in Fig. 2B).

3.4 Diosgenin inhibits TNF-α-induced activation of NF-κB pathways in THP-1 cells

The NF-κB pathway was reported to be involved in TF mRNA accumulation in stimulation of TNF-α [29]. Thus whether diosgenin could influence the phosphorylation of NF-xB/p65 and IKK in THP-1 cells was examined. As shown in Figs. 3A & 3B, in un-stimulated THP-1 cells, NF-xB/p65 and IKKβ were phosphorylated at a very low level. After stimulation with TNF-α (10 ng·mL\(^{-1}\)), NF-xB/p65 and IKKβ were phosphorylated, peaking at 5–15 min or 15–30 min, respectively. Pre-incubation with diosgenin (1 μmol·L\(^{-1}\)) for 1 h prior to TNF-α stimulation, and the following co-incubation blocked the phosphorylation of NF-xB/p65 induced by TNF-α at 5 min, 15 min, and 30 min, with the highest inhibition of 55.6%. However, diosgenin had no obvious effect on IkB phosphorylation (data not shown). These results indicated that diosgenin might inhibit the activation of NF-xB/p65 and IKKβ induced by TNF-α in THP-1 cells.

3.5 Diosgenin inhibits TNF-α-induced activation of Akt and MAPK in THP-1 cells

MAP kinases and PI3-kinase are also involved in TF mRNA accumulation in the stimulation of TNF-α [30], thus
whether diosgenin could influence the phosphorylation of Akt, ERK, and JNK was examined. As shown in Figs. 4A-C, in un-stimulated THP-1 cells, Akt, ERK, and JNK were phosphorylated at a very low level. After stimulation with TNF-α (10 ng·mL⁻¹), Akt, ERK, and JNK were phosphorylated, peaking at 15 min–30 min. Pre-incubation with diosgenin (1 μmol·L⁻¹) for 1 h prior to TNF-α stimulation and the following co-incubation blocked the phosphorylation of Akt, ERK, and JNK at 15 min. However, diosgenin had no obvious effect on p38 phosphorylation (data not shown). These results indicated that diosgenin might inhibit the MAPK pathways induced by TNF-α in THP-1 cells.

4 Discussion

In this study, it was shown that diosgenin suppressed TNF-α-induced TF procoagulant activity, expression, and related signaling pathways in THP-1 monocyctic cells. The results indicate that modulating TF could be the possible explanation of the activity of diosgenin for the treatment of cardiovascular diseases.

TF is an initiator of extrinsic coagulation cascade, which is expressed in the peripheral blood monocytes and on macrophage of atherosclerotic plaques. It was recently found that up-regulation of monocyte tissue factor activity is significantly associated with low-grade chronic inflammation and insulin resistance in patients with metabolic syndrome, which is a risk of cardiovascular disease [31]. The involvement of TNF-α in the pathogenesis of atherosclerosis is supported by its presence in human atherosclerotic plaques. Furthermore, circulating TNF-α
levels are associated with increased risk of recurrent myocardial infarction, atherosclerotic thickening of carotid intima-media, disturbances in triglyceride and glucose homeostasis, and with age-related atherosclerosis [32]. TF expression in response to TNF-α in endothelial cells, monocytes, and arterial smooth muscle cells has been suggested to participate in thrombus formation in animal models [33]. All of the evidence presented supports the key role of TNF-α induced TF in cardiovascular diseases.

In the present study, THP-1, a human monocytic cell line, was used [34]. It was confirmed that TNF-α induced TF procoagulation activity in THP-1 cells by improved chromogenic substrate method. Against this, diosgenin (0.01, 0.1 and 1 μmol·L⁻¹) suppressed TF procoagulation activity in THP-1 cells in a dose-dependent manner, whose inhibition rate of diosgenin at 1 μmol·L⁻¹ was similar to that of curcumin (Fig. 1A). Meanwhile, diosgenin (0.01, 0.1, and 1 μmol·L⁻¹) had no obvious effect on the viability of THP-1 cells (data not shown), which indicated that the inhibition of TF procoagulation activity was not related to the cytotoxicity of diosgenin. It was further proved that TF procoagulation activity was related to the expression of TF at both the protein and gene levels by Western blotting, RT-PCR, and real-time PCR. The results revealed that diosgenin (0.01, 0.1, and 1 μmol·L⁻¹) suppressed the TNF-α-induced TF protein and mRNA expression so as to inhibit TF procoagulation activity in a concentration-dependent manner in THP-1 cells (Fig. 1B and Figs. 2A & 2B), which provided the first evidence of an association between diosgenin and TF expression. These findings also suggested that the interference of TF pathway might be the important mechanism of diosgenin for cardiovascular diseases.

The previous studies have shown that many signaling pathways modulate TF expression. NF-κB, as a kind of transcription factor, is widespread in monocytes, endothelial cells, and vascular smooth muscle cells. Inflammatory mediators such as TNF-α can activate the NF-κB pathway rapidly in human monocytes and endothelial cells. IκB is an inhibition protein of NF-κB. The N-terminal of IκB has the site for Ser phosphorylation and polyubiquitination, which is very important for the degradation of IκB. Most NF-κB proteins are related to the degradation of IκB [35]. So the IκB kinase (IKK) complex is a main modulator for the NF-κB pathway. The IKK complex is composed of three subunits, and the most important subunit for activation of the classical NF-κB signalling pathway is IKKβ [34]. In this experiment, it was found that after stimulation with TNF-α (10 ng·mL⁻¹), IKKβ and IκB were phosphorylated, peaking at 15–30 min and NF-κB/p65 was phosphorylated, peaking at 5–15 min in THP-1 cells, which suggested that TNF-α activated IKK-β, phosphorylated...
p65 and IκB, made p65 enter the nucleus to connect with a DNA specific site, and finally regulated the transcription of the target gene. In addition, this research showed that diosgenin significantly inhibited the phosphorylation of p65 and IKK-β (Fig. 3A & 3B), while it had no obvious effect on the phosphorylation of IκB (data not shown), which indicated that diosgenin mainly inhibited the activation of IKK-β and the phosphorylation of p65 to suppress TF expression in THP-1 cells.

On the other hand, phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) also does contribute much to the cell signal transduction pathway to modulate TF expression, which is also an indispensable pathway in the activation of NF-κB [29]. This study showed that after stimulation with TNF-α, Akt was phosphorylated at 15–30 min significantly, and that diosgenin could inhibit Akt activation (Fig. 4A). Thus, it was supposed that TNF-α activated the NF-κB pathway via the phosphorylation of Akt, and that diosgenin could block this process to interfere with the expression of TF. Mitogen-activated protein kinase (MAPK) exists in most cells, and is another important pathway for cellular response to external stimulus, including TNF-α [30]. MAPK has three concurrent pathways: p38MAPK, JNK/SAPK, and ERK. The results showed that after stimulation with TNF-α, the phosphorylation of p38MAPK, JNK/SAPK, and ERK was enhanced at different levels. These data also dem
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References
薯蓣皂苷元对肿瘤坏死因子诱导的单核细胞组织因子表达的抑制作用研究

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【摘要】目的：研究薯蓣皂苷元对肿瘤坏死因子（TNF-α）诱导的THP-1细胞中组织因子（TF）促凝活性及表达的作用，并探讨其可能的作用机制。方法：采用TNF-α（10 ng·mL−1）诱导THP-1细胞活化，采用改良的发色底物法测定TF的促凝活性；应用逆转录聚合酶链式反应（RT-PCR）及荧光定量聚合酶链式反应（qPCR）测定TF的mRNA表达；Western blotting分析TF蛋白及相关信号通路激酶表达水平。结果：薯蓣皂苷元（0.01，0.1和1 μmol·L−1）预处理，可浓度依赖性地抑制TNF-α诱导的TF促凝活性，经计算其IC50为0.25 μmol·L−1；薯蓣皂苷元明显抑制TNF-α诱导的TF mRNA和蛋白表达。同时，薯蓣皂苷元1 μmol·L−1对于TNF-α诱导的5–30 min NF-κB/p65、IKKβ、Akt、ERK和JNK的激活，也有一定的抑制作用。结论：薯蓣皂苷元可明显抑制TNF-α诱导的THP-1细胞TF的活性及表达，其机制可能与下调NF-κB/p65、IKKβ、Akt、ERK和JNK的磷酸化有关。

【关键词】薯蓣皂苷元；组织因子；单核细胞；肿瘤坏死因子；心血管疾病

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