Original Article
Xanthohumol inhibits PDGF-BB-induced vascular smooth muscle cell proliferation and migration through suppression of PDGF signaling pathway

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Abstract: Proliferation and migration of vascular smooth muscle cells (VSMCs) contribute to the development of atherosclerosis. Xanthohumol (XN) is a prenylatedchalcone derived from hops (Humuluslupulus L.) that decreases atherosclerotic lesion area. However, the underlying molecular mechanism of the anti-atherosclerosis effects of XN is still unclear. Therefore, in the present study, we investigated the effects of XN on human aortic smooth muscle cells (HASMCs) proliferation and migration, the potential mechanism was also explored. Our results showed that XN significantly inhibited platelet-derived growth factor-BB (PDGF-BB)-induced HASMC proliferation and migration. It also induced the arrest of cell cycle progression at G0/G1 phase. In addition, XN significantly inhibited the phosphorylation of PDGF receptor (PDGF-R) β, AKT and ERK1/2 in PDGF-BB-stimulated HASMC. In conclusion, this study demonstrated that XN inhibits PDGF-BB-induced HASMC proliferation and migration through suppression of PDGF signaling pathway. Therefore, XN may be a potential candidate for preventing or treating atherosclerosis.

Keywords: Xanthohumol (XN), platelet-derived growth factor (PDGF)-BB, human aortic smooth muscle cells (HASMCs), proliferation, migration

Introduction
Atherosclerosis is a worldwide disease that induces acute cardiocerebrovascular events, causing serious damage to human health [1]. It is characterized by the development of an arterial occlusion containing lipid and cellular deposits. In the progression of atherosclerosis, the proliferation and migration of vascular smooth muscle cells (VSMCs) play important role in causing stenosis or intimal thickening [2].

Platelet-derived growth factor-BB (PDGF-BB), one of the most potent mitogens and chemoattractants for vascular smooth muscle cells (VSMCs), initiates a multitude of biological effects through the activation of intracellular signal transduction pathways that play critical roes in the proliferation and migration of VSMC [3]. Therefore, inhibiting PDGF-BB-mediated VSMC proliferation and migration may be an important therapeutic approach for atherosclerosis.

Xanthohumol (XN) is a prenylatedchalcone derived from hops (Humuluslupulus L.). A growing body of evidence indicates that XN possesses many physiological properties, such as anti-tumor [4-6], anti-inflammatory [7, 8] and antioxidant potential [9]. Most notably, several studies have reported that XN significantly reduced plasma cholesterol concentrations, decreased atherosclerotic lesion area, and attenuated plasma concentrations of the pro-inflammatory cytokine monocyte chemoattractant protein 1 [10]; XN also decreased accumulated cholesterol in the aortic arch and increased HDL cholesterol (HDL-C) in cholesteryl ester transfer protein (CETP)-transgenic mice [11]. However, the underlying molecular mechanism of the anti-atherosclerosis effects of XN is...
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still unclear. Therefore, in this study, we investigated the effects of XN on PDGF-BB-induced proliferation and migration of human aortic smooth muscle cells (HASMCs).

Materials and methods

Cell culture and reagents

HASMCs were purchased from Bio-Whittaker (California, USA). HASMCs were cultured in DMEM (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 5% CO₂ at 37°C. XN (≥99% pure) was purchased from Sigma (Saint Louis, MO, USA).

Cell proliferation assay

Cell proliferation was analyzed by using the MTT assay. In brief, HASMCs were seeded at a density of 1×10⁵ cells/well in 96-well culture plates, and then pretreated with various concentrations (2.5, 5 and 10 μg/ml) of XN for 1 h prior to stimulation of the cells with PDGF-BB (20 ng/ml) for additional 24 h. Then, 10 μl of 5 mg/ml MTT (Sigma, St. Louis, MO, USA) was added to each well. After incubation for 4 h at 37°C in the dark, the supernatant was discarded, and 100 μl DMSO was added to solubilize the formazan crystals. Cell proliferation was assessed by measuring the absorbance at 450 nm using a microplate reader.

Cell migration assay

HASMCs were seeded in 6-well plates and treated with various concentrations (2.5, 5 and 10 μg/ml) of XN for 1 h prior to stimulation of the cells with PDGF-BB (20 ng/ml) for additional 24 h. The cells were harvested and resuspended in 200 μl ice-cold phosphate-buffered saline (PBS) and added to 4 ml ice-cold ethanol and incubated on ice for 45 min. After an additional washing, cells were incubated with RNase A (20 μg/ml) at 37°C for 30 min, stained with propidium iodide (100 μg/ml; Sigma Aldrich) for 10 min, and analyzed with flow cytometry.

Figure 1. Effects of xanthohumol on proliferation of PDGF-BB-stimulated HASMCs. A. HASMCs (1×10⁵ cells/well) in 96-well plates were treated with various concentrations (2.5, 5 and 10 μg/ml) of XN for 24 h, and the MTT assay was performed to detect cell proliferation. B. HASMCs (1×10⁵ cells/well) in 96-well plates were pretreated with various concentrations (2.5, 5 and 10 μg/ml) of XN for 1 h prior to stimulation of the cells with PDGF-BB (20 ng/ml) for additional 24 h, and the MTT assay was performed to detect cell proliferation. All experiments were repeated at least three times. Data are means ± SD. *P<0.05 compared with the control group; #P<0.05 compared with the PDGF-BB group.
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Enhanced chemiluminescence reagents (GE Healthcare, Buckinghamshire, UK). The signals were quantified by densitometry using Sion Image software (Scion Corporation, Frederick, MD, USA).

Statistical analysis

Results were presented as mean ± SD. Statistical analysis was performed using One-way ANOVA analysis. *P<0.05 was considered statistically significant.

Results

Effects of xanthohumol on proliferation of PDGF-BB-stimulated HASMCs

We first examined the effect of XN on HASMCs proliferation. As indicated in Figure 1A, XN treatment in the absence of PDGF-BB did not decrease the viability of the HASMCs compared with the untreated cells. In addition, the effect of XN on PDGF-BB-stimulated HASMCs was evaluated. As shown in Figure 1B, PDGF-BB treatment significantly increased the proliferation of HASMCs, as compared with the untreated cells. However, XN significantly inhibited the proliferation of PDGF-BB, exhibiting a dose-dependent manner.

Effects of xanthohumol on PDGF-BB-induced cell cycle progression

We investigated the effect of XN treatment on the cell cycle stage distribution of PDGF-BB-stimulated HASMCs using flow cytometry analysis. As indicated in Figure 2, treatment with PDGF-BB markedly decreased the percentage of HASMCs at G0/G1 phase and correspondingly increased their percentage at the S phase compared to the control group. However, XN significantly blocked this event. Furthermore, we investigated the effects of XN on cell cycle regulatory proteins expression. As indicated in Figure 3, PDGF-BB markedly increased in the cellular levels of cyclin D1 and CDK4 in HASMCs. However, XN treatments attenuated the expression of cyclin D1 and CDK4 in PDGF-BB-stimulated HASMCs.

Western blot

Total protein was extracted from the HASMCs using RIPA lysis buffer (Beyotime, Nantong, China) according to the manufacturer’s instructions. Protein concentrations were determined by the BCA method. Equal amounts of protein (30 μg) were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Whatman Schleicher & Schuell, Middlesex, UK). Immunoblots were probed with primary antibodies [anti-cyclin D1, anti-CDK4, anti-PDGFRβ, anti-phospho-PDGFRβ (Tyr 751), anti-AKT, anti-phospho-AKT (Ser473), anti-p44/42 MAPK (ERK1/2), antiphospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (Cell Signaling, MA, USA), and anti-GAPDH (Santa Cruz, CA, USA)] at 4°C overnight. Membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized using enhanced chemiluminescence reagents (GE Healthcare, Buckinghamshire, UK). The signals were quantified by densitometry using Sion Image software (Scion Corporation, Frederick, MD, USA).
Effects of xanthohumol on PDGF-BB-induced migration

Then, we evaluated the effect of XN on HASMCs migration using the Transwell assay. As indicated in Figure 4, the cell migration was induced by PDGF-BB that increased the basal migration of HASMCs by 3.7-fold compared to PDGF-BB non-treated cells. However, XN prevented PDGF-BB-stimulated HASMC migration in a dose-dependent manner.

Xanthohumol suppressed PDGF signaling pathway

To further elucidate the mechanism of XN-inhibited HASMCs proliferation and migration induced by PDGF-BB, we investigated the effect of XN on PDGF signaling pathway. As indicated in Figure 5, treatment with PDGF-BB significantly increased the phosphorylation of PDGFRβ, as well as the phosphorylation of AKT and ERK1/2 in HASMCs. However, XN obviously inhibited the phosphorylation of PDGFRβ, AKT and ERK1/2 by PDGF-BB in HASMCs, exhibiting in concentration-dependent manner.

Discussion

In the present study, we found that XN significantly inhibited PDGF-BB-induced HASMC proliferation and migration. It also induced the arrest of cell cycle progression at G0/G1 phase. In addition, XN significantly inhibited the phosphorylation of PDGFRβ, AKT and ERK1/2 by PDGF-BB-stimulated HASMC.

It has been reported that PDGF stimulated VSMCs proliferation through the activation of cell cycle when the vascular injury occurred [12]. Here, we used PDGF-BB as a proliferative agent. Consistent with the results, in this study, we found that PDGF-BB increased HASMC proliferation. However, XN inhibited the proliferation of PDGF-BB-stimulated HASMCs. And, XN induced the arrest of cell cycle progression at G0/G1...
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Abnormal migration of VSMCs is regarded as the essential step leading to atherosclerosis [13]. In addition, PDGF-BB has been reported to induce an increase in VSMC migration [14]. In this study, we found that PDGF-BB increased HASMC migration. However, XN inhibited the migration of PDGF-BB-stimulated HASMCs.

Several studies showed that PDGF and its beta receptor subtype (PDGFR) are significantly upregulated and activated at sites of vascular injury [15,16]. PDGF-BB binds to PDGF receptor (PDGFR)-Binding the auto-phosphorylation on its tyrosine residues and resulting in the recruitment and activation of specific signaling molecules including ERK and AKT, which are supposed to play important roles in PDGF-induced VSMC migration and proliferation [17]. Therefore, the modulation of PDGF signaling pathway in VSMCs can be a good pharmacological strategy for the prevention of atherosclerosis. Castro et al reported that pharmacological inhibition of ERK suppresses of PDGF-BB-induced downregulation of p27kip1 in rabbit VSMCs [18]. Many studies have reported that PDGFR signaling pathway is implicated in the PDGF-BB-induced proliferation, migration and the changes of cytoskeleton of VSMC [19,20]. In addition, previous reports indicate that PDGF-R beta antagonists or protein kinase inhibitors reduced VSMC proliferation and migration in vitro [21,22]. Recent reports have demonstrated that natural foods or compounds can inhibit VSMCs proliferation and migration. Apamin, a component of bee venom, inhibits PDGF-BB-induced vascular smooth muscle cell proliferation and migration through suppression of activated PDGF signaling pathway [23]. Gonio-lactone C, a styryl lactone derivative, inhibits PDGF-BB-induced VSMC migration and proliferation via PDGFR/ERK signaling [24]. Consistent with these results, in the present study, we found that treatment with PDGF-BB significantly increased the phosphorylation of PDGFRB, as well as the phosphorylation of AKT and ERK1/2 in HASMCs. However, XN obviously inhibited the phosphorylation of PDGFRB, AKT and ERK1/2 by PDGF-BB in HASMCs. These results suggest that XN inhibits PDGF-BB-induced HASMC proliferation and migration through suppression of PDGF signaling pathway.

In conclusion, this study demonstrated that XN inhibits PDGF-BB-induced HASMC proliferation and migration through suppression of PDGF signaling pathway. Therefore, XN may be a potential candidate for preventing or treating atherosclerosis.

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Disclosure of conflict of interest

None.

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Figure 4. Effects of XN on migration of PDGF-BB-stimulated HASMCs. Cell migration was assessed by Transwell assay. XN prevented PDGF-BB-stimulated HASMC migration in a dose-dependently manner. All experiments were repeated at least three times. Data are means ± SD. *P<0.05 compared with the control group; #P<0.05 compared with the PDGF-BB group.
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Figure 5. Xanthohumol suppressed PDGF signaling pathway. A. HASMCs were stimulated with PDGF-BB for 30 min with or without pretreatment by various concentrations (2.5, 5 and 10 μg/ml) of XN. Western blots analysis was performed with antibodies specific for p-PDGFRβ, PDGFRβ, p-AKT, AKT, p-ERK1/2 and ERK1/2. GAPDH was used as an internal control. XN suppresses PDGFRβ, AKT and ERK1/2 signaling pathways activated by PDGF-BB in VSMC. B. The graph represents the relative expression of p-PDGFRβ, p-AKT and p-ERK1/2. All experiments were repeated at least three times. Data are means ± SD. *P<0.05 compared with the control group; #P<0.05 compared with the PDGF-BB group.

References

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