Luteolin Inhibits Angiotensin II-Induced Human Umbilical Vein Endothelial Cell Proliferation and Migration Through Downregulation of Src and Akt Phosphorylation

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Background: The proliferation and migration of vascular endothelial cells (VECs) plays a vital role in angiogenesis, a process that influences plaque vulnerability in human atherosclerosis. Luteolin is a type of flavonoid that has shown a positive effect on the morbidity and mortality of cardiovascular diseases. However, it remains unclear whether this compound has a protective effect against the proliferation and migration of human umbilical vein endothelial cells (HUVECs) induced by angiotensin II (AngII).

Methods and Results: HUVECs were treated with different concentrations of luteolin for varying lengths of time. Analysis using methyl thiazolyl tetrazolium and 5-ethynyl-2'-deoxyuridine revealed that 25 μmol/L luteolin had a particularly inhibitory effect on the AngII-induced proliferation of HUVECs. A Transwell chamber was then used to assay the migration of HUVECs in the presence of 12.5 μmol/L luteolin. The results showed that the migration of AngII-induced HUVECs was also inhibited by luteolin. Further investigations showed that the phosphorylation levels of Src, p-Akt (308), and p-Akt (473) in the group treated with both luteolin and AngII were significantly lower than those of the group treated with only AngII.

Conclusions: The inhibitory effects of luteolin on the proliferation and migration of VECs stimulated by AngII are mediated through the downregulation of the PI3K/Akt signaling pathway. (Circ J 2013; 77: 772–779)

Key Words: Angiogenesis; Luteolin; Migration; Proliferation; Vascular endothelial cells

Atherosclerosis (AS) is a chronic inflammatory disease of the arteries that is complicated by cardiovascular events, which usually occur when a plaque ruptures or erodes. Usually, inflammation contributes to the formation of lesions and rupturing of the plaque. Angiotensin II (AngII), one of the most important factors in the renin-angiotensin-aldosterone system, regulates blood pressure and the volume of circulating blood. In addition, this peptide hormone is involved in chronic inflammation, as it mediates various processes that are implicated in inflammation, such as cell activation, proliferation, migration, and apoptosis. Beyond its hemodynamic actions, AngII is also implicated in atherogenesis (plaque formation).

The main cause of coronary artery disease is atherosclerotic plaques, and cardiovascular events are usually triggered by the rupturing or erosion of plaques. It is believed that angiogenesis is mainly responsible for the rupture of an unstable atherosclerotic plaque. Essentially, angiogenesis involves the proliferation of vascular endothelial cells (VECs) for the development of new vasculature. The proliferation and migration of VECs greatly influences angiogenesis.

Protein tyrosine kinases of the Src family regulate signaling transduction between surface receptors and cellular communication. A non-receptor protein tyrosine kinase, Src, plays necessary roles in the regulation of angiogenesis, as well as cell survival, proliferation, adherence, and movement. As an upstream regulator of Akt kinase, Src can activate the PI3K signaling pathway to mediate intracellular signal transduction. Akt/protein kinase B (PKB) is a serine/threonine kinase that plays a key role in a variety of biological responses, including cell proliferation, survival, migration, and angiogenesis. Akt is activated by phosphorylation on 2 critical residues, thrreo-
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nine 308 (Thr308) and serine 473 (Ser473), and is regulated by upstream second messengers, as well as other enzymes.\textsuperscript{15} 3-phosphoinositide-dependent kinase-1 (PDK-1) is responsible for the phosphorylation of Thr308, whereas the phosphorylation of Ser473 has been suggested to be regulated by PKB/Akt autophosphorylation.\textsuperscript{16}

Luteolin is a common dietary flavonoid that has been reported to have a wide range of pharmacological effects, including antiinflammatory and antiproliferative activities, as well as cardioprotective effects against ischemia/reperfusion (I/R) injury.\textsuperscript{17} At present, there are many studies on the antitumor effects of luteolin and its mechanisms of action.\textsuperscript{18} However, only a few studies have focused on luteolin with respect to its antiatherosclerotic properties.

Because the proliferation and migration of VECs play a vital role in angiogenesis, thereby contributing to the focal distribution of atherosclerotic lesions, we carried out a preliminary study to determine whether luteolin could inhibit the proliferation and migration of VECs. Further, we sought to identify this molecule’s mechanisms of action. The purpose of this study was to elucidate the mechanism by which luteolin protects against cardiovascular diseases, particularly those caused by AS.

**Methods**

**Main Reagents and Antibodies**

Luteolin (Figure 1) was purchased from Sigma (St Louis, MO, USA) and dissolved in absolute alcohol. AngII was supplied by the Beijing Union Pharmaceutical Factory (Beijing, China) and dissolved in deionized water. Human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection (ATCC, USA). Fetal bovine serum (FBS) and 0.25% trypsin were purchased from Gibco (Grand Island, NY, USA). Endothelial cell growth factor was obtained from ScienCell (San Diego, CA, USA). Methyl thiazolyl tetrazolium (MTT) was purchased from Sigma. The Transwell chambers were obtained from Corning Incorporated Life Sciences (Lowell, MA, USA). The phosphospecific polyclonal antibodies against Src, Akt (308), and Akt (473), as well as the anti-Akt polyclonal antibody and anti-Src polyclonal antibody, were obtained from Cell Signaling Technology (Danvers, MA, USA). 5-Ethynyl-2’-deoxyuridine (EdU) was obtained from RiboBio (Guangzhou, China).

**Cell Culture**

HUVECs were grown in Endothelial Cell Medium (ECM) basal medium containing 5% FBS (ScienCell, Carlsbad, CA, USA). In addition to penicillin/streptomycin solution, the media was supplemented with endothelial cell growth supplement (ScienCell), and incubated at 37°C in 5% CO₂.

**Cell Viability Assay**

MTT was used to measure the viability of HUVECs. HUVECs in the logarithmic growth phase were digested, centrifuged, and inoculated in 96-well flat-bottomed plates. Each well contained 1×10⁴ HUVECs in suspension. After being initially cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 10% FBS for 24 h, cells were placed in serum-free media for another 24 h to achieve cell cycle synchronization at G₀/G₁. After pretreatment with various concentrations of luteolin for 12 h, HUVECs were stimulated with AngII for 24 h. MTT solution (5 mg/ml) was added to each well. Following a 4-h incubation at 37°C, the cell culture medium was removed and 100μl of Dimethyl sulfoxide (DMSO) was added to each well. The absorbance of each well was measured with an ELx800 Universal Microplate Reader (Bio-Tek, Winooski, VT, USA) with the detection wavelength set at 570nm. The viability of the experimental groups was expressed as a percentage of the viability of control cells (which was taken to be 100%).

**Cell Proliferation Assay**

The proliferation of HUVECs was measured using a DNA-labeling assay. Following pretreatment with different concentrations of luteolin for 12 h, HUVECs were stimulated with AngII for 24 h and then incubated with 50μmol/L EdU for 2 h at 37°C. The cells were then harvested, fixed, and incubated with Apollo fluorescent dye for 30 min. HUVECs were washed in phosphate-buffered saline (PBS) for 10 min and analyzed by flow cytometry. All incubations were carried out at room temperature. The proliferation of cells in the experimental groups was expressed as a percentage of the proliferation of control cells (which was taken to be 100%).

**Cell Migration Assay**

The migration of the cultured cells was examined using a Transwell chamber. The HUVECs were cultured in the upper chamber, with 10% FBS was placed in the lower chamber in the absence of cells. The cells were incubated at 37°C in 5% CO₂ for 8 h. The HUVECs were cultured with AngII and different concentrations of luteolin (depending on experimental group) for 12 h. The HUVECs migrated through the micropores, and the migrated cells attached to the lower surface of the Transwell filter. The upper chamber was removed from the 24-well plate and the residual cells on the inner side of the Transwell filters were wiped with a cotton swab. Subsequently, the Transwell filters were flushed with 0.01 mol/L PBS and fixed with 4% paraformaldehyde. Following this step, the Transwell filters were washed for 2 min with distilled water. After hematoxylin-eosin staining was performed, 5 visual fields were randomly selected from each of the Transwell filters, and the average number of cells that migrated through the Transwell filters was counted under a microscope.

**Western Blotting Analysis**

The cell culture medium was removed, and the culture plate was washed twice using PBS cooled to 4°C. Cells were lysed on ice in the lysis buffer for 30 min; during this incubation, the culture plate was intermittently shaken to ensure that the lysis buffer completely covered the cells. The cell lysate was centrifuged at 12,000 g and maintained at 4°C for 15 min. Equal
amounts of soluble protein were subjected to electrophoresis on 12% SDS-polyacrylamide gels. The separated protein in the SDS-polyacrylamide gels was then electrophoretically transferred to a polyvinylidene difluoride membrane. The membranes were immunoblotted with the indicated primary and secondary antibodies. The membranes were photographed after the bands were visualized with the BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) method. The greyscale values of the acquired images were analyzed with NIH Image J 3.0 software (National Institutes of Health, MD, USA).

**Statistical Analysis**

Data are expressed as mean±SEM. Statistical analysis was performed using GraphPad Prism 4.0 (Graphpad prism, Inc, CA, USA). Statistical significance was determined using ANOVA, followed by the Bonferroni correction for post-hoc t tests. The threshold for statistical significance was set at P<0.05.

**Results**

**Luteolin Inhibits the Proliferation of HUVECs Induced by AngII**

To assay the cytotoxic and antiproliferative effects of luteolin on HUVECs, the cells were pretreated with luteolin for 12 h.
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Luteolin Suppresses AngII-Stimulated HUVEC Migration

The migration of HUVECs stimulated by AngII was examined using the Transwell chamber method. As shown in Figure 3, 1 μmol/L AngII markedly promoted the migration of HUVECs from the upper chamber to the lower chamber in comparison with the control group (P<0.05). We discovered that pretreatment with luteolin for 12h significantly suppressed AngII-stimulated migration in a dose-dependent manner (P<0.05).

After incubation, MTT was used to measure the viability and proliferation of the HUVECs. As shown in Figure 2A, a cytotoxic effect was observed at a concentration of up to 100 μmol/L (P<0.05). In addition, EdU staining was also used to measure the proliferation of the HUVECs. As shown in Figure 2B and Figure 2C, the results indicated that although treatment with 1 μmol/L AngII significantly increased the proliferation of HUVECs compared with the control group (P<0.05), the action of AngII was inhibited by luteolin (P<0.05; Figure 2B).

Figure 3. Inhibitory effects of luteolin on the migration of human umbilical vein endothelial cells (HUVECs) induced by angiotensin II (AngII). After being incubated with the indicated concentrations of luteolin, HUVECs were stimulated by 1 μmol/L AngII. The migration rate of HUVECs was examined using Transwell assays. Bright-field images (×100) of randomly selected squares of each group are shown. The cell migration rate of the control group was taken as 1. *P<0.05 vs. control group; #P<0.05 vs. AngII-treated group.
Luteolin Inhibits the Activation of Src Tyrosine Kinase by AngII

HUVECs were treated with different concentrations of luteolin for varying lengths of time, and were then stimulated with 1 μmol/L AngII for 12 h. As shown in Figure 4, AngII caused a statistically significant increase in Src activation compared with the control group (P<0.05). Luteolin was able to significantly suppress the phosphorylation of Src stimulated by AngII in a time-dependent manner (P<0.05). As shown in Figure 5, luteolin also significantly suppressed the AngII-induced phosphorylation of Src in a dose-dependent manner (P<0.05).

Effect of Luteolin on the Stimulation of Akt Tyrosine Kinase by AngII

After HUVECs were incubated with different concentrations of luteolin for varying lengths of time, they were then stimulated with 1 μmol/L AngII for 12 h. As shown in Figure 6, p-Akt (308) and p-Akt (473) were significantly activated by...
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In the present study, the experimental results indicate that luteolin has particularly inhibitory effects on AngII-induced proliferation and migration of HUVECs. Our data indicate that this result is probably mediated through downregulation of the phosphorylation of Src and Akt.

As a contributor to inflammation, AngII plays a crucial role in the pathogenesis of cardiovascular disease, as it can stimulate the proliferation and migration of VECs. The migration and proliferation of VECs are critical processes involved in angiogenesis, atherogenesis, post-angioplasty restenosis, and other inflammatory vascular diseases.

In our study, the proliferation of HUVECs was measured by MTT and EdU incorporation. As shown in Figures 2B and 2C, luteolin could inhibit the proliferation of HUVECs induced by AngII. Because angiogenesis contributes to the development and progression of AS, effective inhibition of the proliferation and migration of VECs has great significance for the therapeutic management of AS. Hence, this may become a new means of reducing plaque vulnerability and preventing acute coronary syndrome.

Discussion

Because of changes in lifestyle and socioeconomic status, cardiovascular diseases have become leading causes of death, with AS being one of the most prominent. Consequently, in-depth study of the molecular and cellular mechanisms of AS development and progression is necessary for the development of therapeutic strategies. There are many cells that participate in AS, with the action of VECs being particularly important. Because of its complex etiology and pathology, there are currently very few safe and effective drugs available for the treatment of AS. Therefore, the search for inexpensive and highly effective natural antiatherosclerosis drugs that cause minimal adverse reactions has intensified.

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As a contributor to inflammation, AngII plays a crucial role in the pathogenesis of cardiovascular disease as it can stimulate the proliferation and migration of VECs. The migration and proliferation of VECs are critical processes involved in angiogenesis, atherogenesis, post-angioplasty restenosis, and other inflammatory vascular diseases. In our study, the proliferation of HUVECs was measured by MTT and EdU incorporation. As shown in Figures 2B and 2C, luteolin could inhibit the proliferation of HUVECs induced by AngII. Because angiogenesis contributes to the development and progression of AS, effective inhibition of the proliferation and migration of VECs has great significance for the therapeutic management of AS. Hence, this may become a new means of reducing plaque vulnerability and preventing acute coronary syndrome.

AngII can activate a series of cellular proteins and kinases, including Src and Akt/PKB. This in turn activates signal transduction pathways and redox-sensitive transcription factors, which induce a gene expression pattern that contributes to the pathogenesis of AS. Previous research has shown that tyrosine

Figure 6. Luteolin inhibits the activation of Akt induced by angiotensin II (AngII) in a time-dependent manner. After pretreatment with luteolin for different lengths of time, human umbilical vein endothelial cells (HUVECs) were incubated with 1 μmol/L AngII for 12 h. Equal protein loading was confirmed by probing for β-actin. The cells were lysed and analyzed with antibodies against p-Akt (308), p-Akt (473), and Akt. *P<0.05 vs. control group; #P<0.05 vs. AngII-treated group.
kinases play an important role in transducing the signal initiated by AngII. AngII receptors are G-protein coupled receptors that do not possess intrinsic tyrosine kinase activity; however, intracellular signal transduction induced by AngII requires protein tyrosine phosphorylation. As a result, tyrosine kinases, which participate in the intracellular signal transduction induced by AngII, have become the focus of intense investigation. Src is the upstream regulator of Akt; hence, the reduction of Src phosphorylation may effectively block the Src-PDK1 signaling pathway. The Src-PDK1 signaling pathway plays an important role in regulating the proliferation and migration of VECs. Our experiments showed that luteolin could effectively reduce the elevated levels of p-Src.

Many growth factors regulate the PI3K/Akt signal transduction pathways. Akt is a critical kinase in the body that promotes the proliferation and migration of cells. Hence, the activation of Akt is an important prerequisite for the promotion of cell survival and migration. The phosphorylation of Akt at both Thr308 and Ser473 is essential for full activation of the kinase. It has been reported that through interactions with the PI3K/Akt signaling pathway, luteolin may play a role in the inhibition of cell proliferation and migration. Our study also indicated that luteolin can significantly reduce the level of phosphorylation at Akt residues Thr308 and Ser473. Hence, the downregulation of p-Akt levels may effectively attenuate the proliferation and migration of VECs.

Our previous studies showed that luteolin treatment has a protective effect against I/R injury. However, it is still unknown if luteolin has other cardioprotective effects. In this study, we have shown that luteolin can inhibit HUVEC proliferation and migration. In another study by our group, we investigated the inhibitory effects of luteolin on the proliferation and migration of vascular smooth muscle cells (VSMCs) that were stimulated by hydrogen peroxide (H2O2). Abnormal VSMC proliferation and migration are strongly implicated in the process of vessel wall remodeling, which influences the progression of AS.

Therefore, there is evidence suggesting that luteolin could stabilize atherosclerotic plaques and play a useful role as an antiatherosclerosis agent. However, this remains to be proven by additional in vivo studies, which is the present concentration of our research group.

Based on existing literature, published by groups in China and other countries, luteolin has various pharmacological properties, including anticancer, antioxidation, and antiinflammatory activities, as well as facilitating the removal of oxygen free radicals. The etiology and pathology of AS are very complex, and understanding of its pathogenesis remains incomplete. However, the flavonoid, luteolin, has a positive influence on human health. Previous studies have shown that this molecule may inhibit the survival and proliferation of endothelial cells, as well as VEGF-induced in vivo angiogenesis.

**Conclusion**

The results obtained from this study indicate that luteolin inhibits the proliferation and migration of VECs stimulated by AngII. The effect of luteolin is partially associated with the PI3K/Akt pathway. Further study on the detailed mechanisms of this process is now in progress. We anticipate that this study
will provide a solid foundation for the application of luteolin in the clinical treatment of cardiovascular diseases in the near future.

Acknowledgments

We gratefully acknowledge the excellent technical assistance of Yasong Lang.

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