Luteolin Inhibits Endothelin-1 Secretion in Cultured Endothelial Cells

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We discovered that luteolin, a typical flavonoid contained in various kinds of plants, inhibits the secretion and gene expression of endothelin-1 (ET-1), a potent vasoconstrictor regulating blood pressure, in porcine aortic endothelial cells. Its ED₅₀ was about 10 μM. In addition, the inhibition of ET-1 by a glycoside compound of luteolin (luteolin-6-C-glucoside) was weak.

Key words: endothelin; luteolin; flavonoid; secretion; gene expression

Overproduction of Endothelin-1 (ET-1) is seen as an important etiological factor in the development of vascular disease and atherosclerosis. For example, the coronary blood supply of patients with coronary heart disease is severely perturbed by local ET-1 production.¹,² It has been reported that wine and grape products containing polyphenols such as quercetin and tannic acid can cause an endothelium-dependent relax-ation in the rat thoracic aorta.³ In addition, it has been reported that red wine extract and some polyphenols present in it cause a decrease in ET-1 synthesis by cultured endothelial cells.⁴,⁵ But the effects of luteolin, a typical flavonoid contained in various kinds of plants, on the secretion and gene expression of ET-1 are not understood. Here we show that luteolin can potently inhibit the secretion and gene expression of ET-1 in porcine aortic endothelial cells.

Primary porcine aortic endothelial cells (Cell Systems, Kirkland, WA) were cultured in a 24-well plate (5 x 10⁵ cells/well) for ET-1 release experiments, and in a 10 cm²-dish (1 x 10⁶ cells/ml) for ET-1 gene expression studies, in a medium (D-MEM:Ham F12 = 1:1) containing 10% fetal bovine serum, 15 mM HEPES, 5 ng/ml FGF-1, and 10 μg/ml Heparin, at 37 ºC. After 4 days cultivation, the cells were washed in Krebs-Ringer’s solution (pH 7.4), and then incubated for 0.5 to 8 h at 37 ºC in Krebs-Ringer’s solution containing luteolin (Sigma, St. Louis, MO) and luteolin-6-C-glucoside (Extrasynthese, Genay, France). Luteolin and luteolin-6-C-glucoside were dissolved in dimethyl sulfoxide (DMSO) to make high-concentration stock solutions. Stock solution (0.1 ml) was added to Krebs-Ringer’s solution (9.9 ml) to adjust the final concentration. To measure ET-1 concentration, Krebs-Ringer’s solution was removed from each well. The remaining cells were collected for measurement of ET-1 gene expression. The samples at the concentrations used in this experiment showed no inhibitory effect on cell proliferation by MTT assay. The cell viability at control treatment (Krebs-Ringer’s solution containing 1% DMSO) was 102 ± 3% when compared with non-treatment (n = 12). We used primary epithelial cells within passage 2 because the inhibitory effect of high luteolin concentrations on cell proliferation occurred at more than passage 4.

ET-1, released into the cell culture supernatant of endothelial cells, was measured with a commercial ELISA kit (Amersham Biosciences, Piscataway, NJ). After the experiment, the cell culture supernatant was frozen at −80 ºC until measurement. ET-1 concentration was measured by sandwich ELISA using peroxidase-labelled ET-1 first antibody. Results were read at 450 nm by micro-plate reader. The first antibody had 100% cross-reactivity for ET-1, more than 100% cross-reactivity for ET-2, less than 0.001% for ET-3, and 0.07% cross-reactivity for big ET-1.

Sample cDNA for real-time PCR was obtained by reverse transcriptase reaction of total RNAs prepared from cultured cells. Amplification was carried out by two-step PCR using the TaqMan PCR kit (PE Applied Biosystems, Foster City, CA). The oligonucleotide primers for porcine ET-1 (Genbank Accession no. X07383) were 5’-TGCTGTTTTGTGGGCCTTCCA-3’ (sense) and 5’-TCCGGGCGTGGAGCTCG-3’ (anti-sense). The oligonucleotide primers for porcine ACTIN (Genbank accession no. AY550069) were 5’-CTCCTT...
The ET-1 concentration in the control was significantly decreased ET-1 release (50 μM, 79.7%; 100 μM, 68.6%). But the inhibition of ET-1 by luteolin-6-glucoside was weak as compared with the nonglycoside compound. These results indicate that luteolin but not its glycoside has strong inhibitory activity on ET-1 release from endothelial cells.

Next we examined the time-course of endothelin-1 release when the luteolin concentration was increased from zero to 50 μM in Krebs-Ringer’s solution (Fig. 1B, n = 3–7). The values of vehicle-treated cells 3 h after changing the culture medium to Krebs-Ringer’s solution were used as the control. The ET-1 concentration in the control was 214.11 ± 16.73 fmol/ml. Luteolin (from 12.5 to 50 μM) significantly inhibited ET-1 release from 37.7% to 22.4% of the control treatment, and its ED50 was about 10 μM. On the other hand, a high concentration of luteolin-6-C-glucoside significantly decreased ET-1 release (50 μM, 79.7%; 100 μM, 68.6%). But the inhibition of ET-1 by luteolin-6-glucoside was weak as compared with the nonglycoside compound. These results indicate that luteolin but not its glycoside has strong inhibitory activity on ET-1 release from endothelial cells.

The effect of luteolin might occur not only by the process of ET-1 release, but also by the down regulation of ET-1 gene expression, because luteolin did not inhibit ET-1 release after 0.5 h of treatment (the action of luteolin was not fast). Hence, we measured ET-1 gene expression at 2 and 4 h after exposure of cells to luteolin by quantitative real-time PCR. As shown in Fig. 2 (n = 3), 10 μM luteolin caused a reduction in ET-1 gene expression at 4 h but not 2 h. These results show that luteolin has different pathways for the inhibitory effect on ET-1 release and gene expression, because 10 μM luteolin decreased ET-1 release (Fig. 1B) but not ET-1 gene expression (Fig. 2) at 2 h. On the other hand, 50 μM luteolin almost completely inhibited ET-1 gene expression at 2 and 4 h. A high concentration of luteolin has a strong inhibitory effect on ET-1 gene expression (Fig. 2) and ET-1 release (Fig. 1B).

It has been reported that luteolin can bind estrogen receptors and has estrogenic activity in human breast cancer MCF-7.10 In addition, 17β-estradiol inhibits ET-1 gene expression via estrogen receptors in vascular endothelial cells and smooth muscle cells.7,8 In the present study, a decrease in ET-1 gene expression by luteolin might occur via estrogen receptors binding nonglycoside luteolin in the intracellular mechanism.

The reaction conditions were 95 °C for 10 min followed by 40 cycles of the amplification step (95 °C for 15 sec and 58 °C for 30 sec). The amplification products from mRNAs were predicted to be 65 pairs (bp) for ET-1 and ACTIN. The gene expression rate was obtained by normalizing the amount of ET-1 with that of ACTIN.

Figure 1A shows the dose-dependent manner of inhibition by luteolin and its glycoside on ET-1 release 3 h after changing the cell culture medium to Krebs-Ringer’s solution containing luteolin and luteolin-6-C-glucoside (n = 4–7). The values of vehicle-treated cells 8 h after changing the culture medium to Krebs-Ringer’s solution were used as the control. Results (% of control values) are shown as mean ± SEM, Bonferroni’s test). Asterisks indicate a significant difference between vehicle-treated and luteolin- or luteolin’s glucoside-treated cells (\(P < 0.05\), \(***P < 0.001\), Bonferroni’s test). B, time-course of ET-1 release of Krebs-Ringer’s solution containing luteolin (0 to 50 μM). Values of vehicle-treated cells were used as the control. Results (% of control values 8 h after changing the culture medium to Krebs-Ringer’s solution) are shown as the mean ± SEM (n = 3–7). Asterisks indicate a significant difference between vehicle-treated and luteolin- or luteolin’s glucoside-treated cells (\(P < 0.05\), \(**P < 0.01\), Bonferroni’s test).

**Fig. 1.** Inhibitory Effects of Luteolin on ET-1 Release in Primary Porcine Aortic Endothelial Cells.

The culture medium was changed to Krebs-Ringer’s solution containing luteolin or luteolin-6-C-glucoside. A, inhibitory effects of luteolin and luteolin-6-C-glucoside at 3 h after changing the culture medium to Krebs-Ringer’s solution. Values of vehicle-treated cells were used as the control. Results (% of control values) are shown as the mean ± SEM (n = 4–7). Asterisks indicate a significant difference between vehicle-treated and luteolin- or luteolin’s glucoside-treated cells (\(P < 0.05\), \(***P < 0.001\), Bonferroni’s test). B, time-course of ET-1 release of Krebs-Ringer’s solution containing luteolin (0 to 50 μM). Values of vehicle-treated cells were used as the control. Results (% of control values 8 h after changing the culture medium to Krebs-Ringer’s solution) are shown as the mean ± SEM (n = 3–7). Asterisks indicate a significant difference between vehicle-treated and luteolin- or luteolin’s glucoside-treated cells (\(P < 0.05\), \(**P < 0.01\), Bonferroni’s test).
From these results, we conclude that luteolin can potently inhibit the secretion and gene expression of ET-1 in porcine aortic endothelial cells.

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References