EFFECTS OF LUTEOLIN ON VASCULAR ENDOTHELium EXPOSED TO INFLAMMATORY STIMULI

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Abstract
The adhesion of leukocyte to endothelium under inflammatory situation plays an important role in the development of various disease; arteriosclerosis, cancer. Previously, our group showed the suppressive effect of several flavonoids including luteolin on the cell surface expression of adhesion molecule induced by tumor necrosis factor (TNF)-α in human umbilical vein endothelial cells (HUVECs). This study aims to clarify the effects of luteolin on vascular endothelium exposed to inflammatory stimuli in more detail. Using ELISA system, we investigated the effects of luteolin on cell surface adhesion molecule-1 (ICAM-1) in HUVECs. Luteolin (2-40 µM) significantly suppressed the cell surface expression of ICAM-1 induced by oxidized low-density lipoprotein (ox-LDL) (200 µg/ml) and TNF-α (0.1 ng/ml). HUVECs exposed to TNF-α (0.1 ng/ml) adhered to THP-1 monocytes. Luteolin inhibited the adhesion of human THP-1 monocyte to HUVECs induced by TNF-α. These results suggest the possibility that intake of natural products including luteolin suppressed inflammatory responses in vascular endothelium via adhesion of leukocyte to endothelium.

Keywords: Luteolin, inflammation, ICAM-1, endothelium

INTRODUCTION
Luteolin is a flavone contained in many herbs including parsley, celery, perilla, chamomile tea, rosemary, oregano as well as olive oil, carrots and peppermint, and possesses various pharmacological activities (Figure. 1). Our group and other groups reported that luteolin had anti-oxidant (Shimo et al., 1994; Van Hoyweghen et al., 2012), anti-cancer (Chen et al., 2013; Pandurangan et al., 2014), anti-diabetic (Deqiu et al., 2011; Liu et al., 2013) and anti-inflammatory (Wölfle et al., 2011; Park et al., 2013) activities. By using HPLC and liquid chromatography/mass spectrometry analyses, we showed that the main metabolite after oral administration of luteolin was luteolin monoglucuronide and that free luteolin, the aglycone of luteolin, was also presented in rat plasma. Free luteolin were also detected in human serum after ingestion of luteolin (Shimo et al., 1998).

An important factor regulating leukocyte traffic into and within inflammatory tissues is the expression of various adhesion molecules on the cell surface, and this facilitates the appropriate cell-cell interactions (Springer, 1990). For example,
intercellular adhesion molecule-1 (ICAM-1), one of cell surface molecules, expressed in not only leukocyte but also endothelial cells and extra vascular resident cells. This molecule plays important roles in leukocyte adhesion during inflammatory responses, resulting in deterioration of atherosclerosis and cancer (Galkina et al., 2007 and Kobayashi et al., 2007). Thus, the inhibition of expression of adhesion molecules induced by inflammatory stimuli is valid for amelioration of inflammation and inflammatory diseases.

Previously, our group investigated the suppressive effect of several flavonoids on the cell surface expression of adhesion molecule induced by tumor necrosis factor (TNF)-α in human umbilical vein endothelial cells (HUVECs), and among nine flavonoids (40 µM) examined, chrysin, apigenine, quercetin, galangin and luteolin demonstrated significant suppressive effects on it (Shimoi et al., 2000). However, the detail function of them remains unclear. Therefore, we focused this inhibitory effect of luteolin. This study aims to clarify the effects of luteolin on vascular endothelium exposed to inflammatory stimuli in more detail.

![Figure 1. Chemical structure of Luteolin](image)

**MATERIALS AND METHODS**

**Chemicals.** Luteolin was isolated from perilla seed, purified by HPLC, and confirmed by mass spectrometry and NMR spectral data at the Oryza Oil and Fat Chemical Co., Ltd. (Ichinomiya, Japan) as reported previously (Shimoi et al., 2000). All other chemicals were of reagent grade, and purchased from Wako Pure Chemicals (Osaka, Japan) unless specified otherwise.

**Cells.** HUVECs were cultured on 0.1% gelatin (Sigma, St. Louis, MO) coated dishes in MCDB-104 (Nihon Pharmaceutical Co., Ltd.) supplemented with 10% FBS (Moregate, Australia and New Zealand), 100 ng/ml endothelial cell growth factor (ECGF), 10 ng/ml epidermal growth factor (EGF, Collaborative Research Inc., Bedford, MA) and 100 µg/ml heparin (Sigma) at 37°C in an incubator with 5% CO2 and 95% air. THP-1 cells were grown in Roswell Park Memorial Institute medium (RPMI-1640, Sigma) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Sekisui Medical Co., Ltd., Tokyo, Japan), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C under a humidified atmosphere of 95% air and 5% CO2.
Cell ELISA of ICAM-1 on HUVECs. After HUVECs reached confluence in 96-well microplates, the cells were treated with TNF-α (0.1 ng/ml) or Cu2+-Oxidized low-density lipoprotein (Cu2+-ox-LDL) (200 µg/ml) for 6 hours in the presence and absence of luteolin (2-40 µM), and then fixed with 1% (w/v) paraformaldehyde. The cells were incubated with mouse anti-human ICAM-1 monoclonal antibody (Wako Pure Chemicals) and then treated with peroxidase-conjugated goat anti-mouse IgG (Seikagaku kogyo, Tokyo, Japan). The absorbance of the reaction mixture was determined at 492 nm using a microplate reader.

Adhesion assay of THP-1 monocyte to HUVECs. After HUVECs reached confluence in 96-well microplates, the cells were incubated with TNF-α (0.1 ng/ml) and luteolin (2-40 µM). After 6 hours, THP-1 cells (2 x 10⁵ cells/well) were added and incubated for 10 minutes. These cells were washed with PBS and treated with 0.25% Rose Bengal in PBS for 5 minutes at room temperature, and fixed with 50% ethanol in PBS for 30 minutes. Absorbance was measured at 550 nm.

Statistical Analysis. Statistical analysis was performed using Student’s t-test. Any difference between the two groups with a value of P < 0.05 was considered significant.

RESULTS AND DISCUSSION
As shown in Figure 2A, treatment with TNF-α (0.1 ng/ml) and Cu²⁺-ox-LDL for 4 hours enhanced the expression of ICAM-1 in the surface of HUVECs. When the cells were treated with TNF-α and luteolin (2-40 µM) simultaneously, luteolin significantly inhibited TNF-α-induced ICAM-1 at the concentration of 20 and 40 µM by 83% and 93% (P < 0.01), respectively. Luteolin also suppressed Cu²⁺-ox-LDL-induced ICAM-1 expression by 71% (P < 0.05). Our group previously reported that TNF-α (0.5 ng/ml)-induced ICAM-1 expression was inhibited by luteolin at the concentration of 40 µM by 48% (Shimoi et al., 2000). It showed nearly the same results in this study.

To assess the effects of luteolin on the adhesion of leukocyte to endothelium exposed to inflammatory stimuli, we investigated the effects of luteolin on the adhesion of THP-1 monocytes treated with TNF-α (Figure 2B). Incubation of THP-1 monocytes for 10 minutes with HUVECs exposed to TNF-α (0.1 ng/ml) and luteolin (2-40 µM), the adhesion of THP-1 to HUVECs was significantly suppressed by 82% and 100% (P < 0.01), respectively. To our knowledge, there is no report that demonstrated the inhibitory effects of luteolin on the adhesion of leukocytes to endothelium.

Luteolin suppressed ICAM-1 expression in HUVECs and adhesion of THP-1 monocytes to HUVECs. In addition, the production of ICAM-1 in the plasma was enhanced in the mice after injection of LPS, and administration of
luteolin significantly decreased this production (Yasuda et al., unpublished data). Collectively, these results indicate that luteolin may be a food phytochemical capable of anti-inflammatory activities by its ability to modulate the adhesion of leukocyte to endothelium. As one of natural products including luteolin, we focus on the Rooibos tea, produced from the endemic South African shrub *Aspalathus linearis*, and to clarify the effects of Rooibos tea in mice, the investigation is now in progress.

**Figure 2.** The effects of luteolin on ICAM-1 expression (A) and adhesion of leukocyte (B)

**CONCLUSIONS**

Luteolin significantly inhibited TNF-α-induced ICAM-1 at the concentration of 20 and 40 µM by 83% and 93%, respectively. Luteolin also suppressed Cu^{2+}-ox-LDL-induced ICAM-1 expression by 71%. In conclusion, the present study results led to the hypothesis that intake of natural products including luteolin suppressed inflammatory responses in vascular endothelium via adhesion of leukocyte to endothelium. Further investigation to identify this underlying molecular mechanism may lead to development of this flavonoid with pronounced anti-inflammatory activities.

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REFERENCES


