

In vitro mechanism of luteolin suppresses enhanced endothelial permeability

Research paper

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Received 25 June, 2024, accepted 7 August, 2024

Abstract Luteolin, a flavonoid, has been reported to inhibit the enhanced endothelial permeability *in vitro*. The purpose of this study was to determine the mechanism of luteolin affected the *in vitro* suppression of elevated endothelial permeability involves protein kinase C activity and intracellular calcium concentration. The inducer bradykinin was tested using the *in vitro* vascular permeability assay in endothelial cells obtained from human umbilical vein endothelial cells (HUVECs). Protein kinase C assay test and the intracellular calcium concentration were also determined. Results revealed luteolin (5, 10, and 25 μ M) significantly suppressed increased endothelial permeability ($P < 0.001$). The increased activity of protein kinase C was significantly suppressed by luteolin at the dose of 5 μ M ($P < 0.05$), 10 μ M ($P < 0.01$), and 25 μ M ($P < 0.01$). The increased concentration of intracellular calcium was significantly suppressed by luteolin at the dose of 10 μ M ($P < 0.01$) and 25 μ M ($P < 0.001$). Collectively, these results showed luteolin suppressed the activity of protein kinase C and suppressed the increased concentration of intracellular calcium when HUVECs were induced by bradykinin, leading to the suppression of increased endothelial permeability as the nitric oxide-cyclic guanosine monophosphate (NO-cGMP) pathway was being inactivated. This may explain the pharmacologic properties of luteolin, which is anti-inflammatory, antioxidant, and neuroprotective. These results also revealed the potential use of luteolin in treating many other diseases involve endothelial permeability.

Keywords Luteolin – Endothelial Permeability – Protein Kinase C – Intracellular Calcium

INTRODUCTION

Numerous human diseases have inflammatory component to their pathogenesis. When inflammation arises, leukocytes are drawn to the site of injury and cytokines, chemokines, free radicals, and other mediators are formed (Chen et al. 2018). The change in endothelial permeability comes next, and prolonged increase in endothelial permeability may lead to an abnormal extravasation of blood components and accumulation of fluid in the extravascular space, which will result in multiorgan dysfunction as well as compromising the normal pharmacokinetics of therapeutic drugs (Hu et al., 2007). Hence, by suppressing the abnormal increase of endothelial permeability, certain related diseases can be prevented (Bates et al., 2002). Luteolin, which is also known as 3',4',5,7-tetrahydroxyflavone, is a common flavonoid (Figure 1) that can be found in many

types of plants. It has been used in Chinese Traditional Medicine for treating all kinds of diseases including inflammatory disorders, cancer and hypertension (Li et al, 2023; Jia et al., 2015, Lin et al., 2008). Specific anti-inflammatory effects at micromolar concentrations were displayed by luteolin, which are only partly explained by its antioxidant capacities. Suppression of the nuclear factor kappa B (NF κ B) pathway (Chen et al., 2007), activation of anti-oxidative enzymes, and inhibition of proinflammatory substances are some of its anti-inflammatory actions (Ashokkumar and Sudhandiran, 2008). Luteolin was shown to reduce the increased endothelial permeability *in vivo* and was effective in animal models of inflammation after parenteral and oral application (Jia et al., 2015, Li and Zhang, 2023). Thus, the present study was undertaken to elucidate its mechanism of activity.

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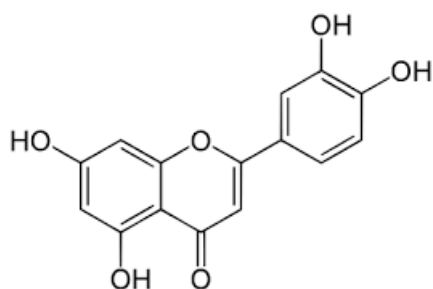


Figure 1. Chemical Structure of Luteolin.

MATERIALS AND METHODS

Chemicals and Reagents

The following reagents were used: Cascade Biologics Medium 200 phenol red-free cell culture medium, trypsin-Ethylenediaminetetraacetic acid (EDTA) solution (Sigma-Aldrich Co.), phosphate-buffered saline (PBS), trypan blue stain, luteolin (10004161, Cayman Chemical Co.), dimethyl sulfoxide (DMSO), bradykinin acetate (B3259, Sigma-Aldrich Co.), HOE140 (H157 Sigma-Aldrich Co.), GF109203X hydrochloride (B6292, Sigma-Aldrich Co.), 2-aminoethyl diphenylborinate (D9754, Sigma-Aldrich Co.), Fura-2AM (F0888, Sigma-Aldrich Co.), *in vitro* vascular permeability assay kit (ECM640, Chemicon® Int.), protein kinase C (PKC) assay kit (539484, Calbiochem®).

Cell Culture

Endothelial cells used the from human umbilical vein endothelial cells (HUVECs) from Millipore. HUVECs between passages 2 and 5 were used. Cascade Biologics Medium 200 phenol red-free cell culture medium purchased commercially was used to grow the HUVECs.

Luteolin

Luteolin was purchased commercially from Cayman Chemical Company. A concentrated stock solution (10 mM) was prepared by dissolving the crystalline luteolin in DMSO and stored at -20°C (Tawornchat et al. 2021). Serial dilutions of the concentrated stock solution with cell culture medium were prepared at room temperature to obtain luteolin solutions with a concentration of 5, 10, and 25 μM .

In Vitro Vascular Permeability Assay Test

The *in vitro* vascular permeability assay kit (ECM640, Chemicon® Int.) was used to determine the permeability of HUVECs. Protocols of this test were optimized from the assay instructions provided by Chemicon® International. Briefly, 200 μl of the HUVECs suspension (1.0×10^6 cells/ml) was seeded into the insert of the 24-well plate and incubated for 72 h in a 37°C CO_2 tissue culture incubator. Two hundred microliters of luteolin at a concentration of 5, 10, and 25 μM were added to

the treatment groups, while the antagonist, HOE140 (10 μM) was added to the positive control group after the incubation period. The plate was then incubated for 1 h. The inserts were transferred to the permeability detection plate, where the HUVECs were then induced with 100 μl of bradykinin (1 μM) followed by addition of FIT-C dextran (Gifford et al., 2004, Terzuoli et al., 2018). The plate was incubated for 5 min at room temperature. One hundred microliters of the plate well solution was then transferred to a 96-well plate. The plate was read at 485/530 nm (excitation/emission). The reading was directly proportional to the permeability of HUVECs.

PKC Assay Test

An assay kit (539484, Calbiochem®) was used to determine the concentration of PKC in HUVECs. Protocols of this test were modified and optimized with the assay instructions provided by Calbiochem®. Briefly, 200 μl of HUVECs suspension (1.0×10^7 cells/ml) was transferred into six centrifuge tubes. Then, 200 μl luteolin at a concentration of 5, 10, and 25 μM was added to the treatment groups, while the antagonist, GF109203X hydrochloride (3 μM), was added to the positive control group. One hundred microliters of bradykinin (1 μM) was added to induce the HUVECs after the addition of luteolin and the antagonist. The tubes were then incubated for 60 min. After the incubation period, the tubes were sonicated on ice for four or five times and then centrifuged at 2500 rpm for 60 min at 4°C . Supernatant of the cell was then collected. The mixture provided by the kit was added in designated wells of a polyvinyl plate. The plate was then preincubated at 25°C for 5 min. Twelve microliters of the supernatant was added to the designated well and mixed. Next, 100 μl of this mixture was transferred to designated wells of peptide pseudosubstrate-coated plate. Stop solution provided by the kit was added to each well after an incubation period of 5–20 min at 25°C in a water bath. One hundred microliters of horseradish peroxidase-conjugated streptavidin was then added to each well and incubated at 25°C for 60 min. After the incubation period, the wells were washed five times using the wash solution. One hundred microliters of substrate solution provided by the kit was added to each well and incubated at 25°C for 3–5 min. The absorbance was read at 492 nm in a microplate reader, and the absorbance is directly proportional to the activity of PKC.

Intracellular Calcium Concentration Determination Test

Two hundred microliters of HUVECs suspension (1.0×10^6 cells/ml) was transferred into six centrifuge tubes. After that, 200 μl of luteolin at a concentration of 5, 10, and 25 μM was added to the treatment groups, while the antagonist, 2-aminoethyl diphenylborinate (75 μM), and was added to the positive control group. Two hundred microliters of Fura-2AM was then added into the respective tubes and incubated

at room temperature for 60 min. The centrifuge tubes should be covered with aluminium foil before incubation. After the incubation period, the tubes were centrifuged at 1000 rpm for 10 min at 4°C. The cell suspension was centrifuged again for the second time at 1000 rpm for 10 min at 4°C. Finally, 1 ml of PBS was used to resuspend the cell pellets and the cell suspension was transferred to cuvettes, to which 1 ml of bradykinin (1 µM) was added to induce the HUVECs. The fluorescence intensity from the HUVEC suspensions was recorded in cuvettes with a Ca²⁺ analyzer (at excitation wavelengths of 340 and 380 nm and at an emission wavelength of 510 nm). The concentration of intracellular calcium is proportional to the ratio of fluorescence at 340/380. The Grynkiewicz equation describes this relationship (Maravall et al., 2000).

Intracellular calcium = $K_d \times [(R - R_{min}) / (R_{max} - R)] \times S_{fb}$

$K_d = 135$

$R = 340/380$ ratio

$R_{max} = 340/380$ ratio under calcium-saturating conditions

$R_{min} = 340/380$ ratio under calcium-free conditions

S_{fb} = ratio of fluorescence under calcium-free and -bound conditions

Statistical Analysis

The results were expressed as mean ± standard error of the mean (SEM). The statistical analysis was performed by analysis of variance (ANOVA). Differences between each group were further analyzed by post hoc test, which is the Tukey's Honest Significant Difference (HSD) test. *P*-values of 0.05 or less were considered as indicative of significance.

RESULTS

In Vitro Vascular Permeability Assay

The results obtained from the *in vitro* vascular permeability assay test are shown in Table 1. Positive control with 2.638 ± 3.089 showed a significant suppression of increased HUVECs' permeability (*P* < 0.001) with suppression of 94.05%. A significant reduction was found for all the three treatment groups, 5, 10, and 25 µM, at 13.62 ± 3.763, 5.690 ± 4.210, and 0.8780 ± 3.763, respectively (*P* < 0.001). The treatment groups of 5 and 10 µM showed a suppression of 69.27% and 87.16%, respectively, which were slightly lower compared to the positive control. However, the treatment group of 25 µM showed a significantly higher suppression compared to the positive control, with suppression of 98.02%.

PKC Assay Test

The results obtained from the PKC assay test are shown in Table 2. Positive control with 0.03693 ± 0.001637 showed a significant reduction of PKC activity (*P* < 0.01) with suppression of 104.38%. A significant reduction was found for all the three

Table 1. In Vitro Vascular Permeability Assay Test: Mean of Permeability Index.

Group	Mean ± S.E.M.	Suppression (%)
Basal Cells	-	-
Negative Control	44.32 ± 4.803	-
Positive Control	2.638 ± 3.089***	94.05
Luteolin (5 µM)	13.62 ± 3.763***	69.27
Luteolin (10 µM)	5.690 ± 4.210***	87.16
Luteolin (25 µM)	0.8780 ± 3.763***	98.02

*** *P* < 0.001 significantly different from the negative control

Table 2. Protein Kinase C Assay Test: Mean of Protein Kinase C Activity.

Group	Mean ± S.E.M.	Suppression (%)
Basal Cells	0.03747 ± 0.002667	-
Negative Control	0.04980 ± 0.002458	-
Positive Control	0.03693 ± 0.001637**	104.38
Luteolin (5 µM)	0.03817 ± 0.001506*	94.32
Luteolin (10 µM)	0.03550 ± 0.001646**	115.98
Luteolin (25 µM)	0.03437 ± 0.001713**	125.14

**P* < 0.05 significantly different from the negative control

***P* < 0.01 significantly different from the negative control

treatment groups, 5, 10, and 25 µM, at 0.03817 ± 0.001506 (*P* < 0.05), 0.0355 ± 0.001646 (*P* < 0.01), and 0.03437 ± 0.001713 (*P* < 0.01), respectively. The treatment group of 5 µM showed suppression of 94.32%, which was slightly lower compared to the positive control. However, the treatment groups of 10 and 25 µM showed a significantly higher suppression compared to the positive control, with suppression of 115.98% and 125.14%, respectively.

Intracellular Calcium Concentration Determination Test

The results obtained from the intracellular calcium concentration determination test are shown in Table 3. Positive control with 412.2 ± 17.15 showed a significant reduction of intracellular calcium concentration (*P* < 0.05) with suppression of 63.61%. A significant reduction was found for two treatment groups, 10 and 25 µM, at 373.5 ± 13.48 (*P* < 0.01) and 354.7 ± 16.06 (*P* < 0.001), respectively. There was no significant difference in the treatment group of 5 µM. The treatment groups of 10 and 25 µM showed a significantly higher suppression compared to the positive control, with suppression of 90.90% and 104.16%, respectively.

Table 3. Intracellular Calcium Concentration Determination Test: Mean of Intracellular Calcium Concentration.

Group	Mean $\pm$ S.E.M.	Suppression (%)
Basal Cells	360.6 $\pm$ 11.85	-
Negative Control	502.4 $\pm$ 22.05	-
Positive Control	412.2 $\pm$ 17.15*	63.61
Luteolin (5 μ M)	439.4 $\pm$ 12.43	44.43
Luteolin (10 μ M)	373.5 $\pm$ 13.48**	90.90
Luteolin (25 μ M)	354.7 $\pm$ 16.06***	104.16

* $P < 0.05$ significantly different from the negative control

** $P < 0.01$ significantly different from the negative control

*** $P < 0.001$ significantly different from the negative control

DISCUSSION AND CONCLUSION

Effect of Luteolin on Bradykinin-induced HUVECs' Permeability

These experiments investigated the action of luteolin in suppressing the increased endothelial permeability induced by bradykinin. Increased endothelial permeability is due to disruption of endothelial cell barrier, which is induced by the actions of various inflammatory mediators such as bradykinin, histamine, and growth factors (Serhan, 2005; Terzuoli et al., 2018). The ability of luteolin to suppress the increase in endothelial permeability was evaluated here with *in vitro* vascular permeability assay test, PKC assay test, and intracellular calcium concentration determination test.

The *in vitro* vascular permeability assay is an effective test for evaluating compounds that can either increase or reduce the endothelial permeability (Mehta and Malik, 2006). This assay has frequently been used to assess the effects of chemicals and drugs on endothelial cell adsorption, transport, and permeability. The present study has shown that HUVECs that were pretreated with luteolin suppress the increased endothelial permeability induced by bradykinin. Luteolin at the dose of 5, 10, and 25 μ M significantly reduced increased HUVECs' permeability, with a maximum suppression of 98.02%. This reveals the ability of luteolin to suppress increased endothelial permeability when bradykinin was used as the inducer. Pretreatment of HUVECs with luteolin resulted in a significant suppression of increased HUVECs' permeability. It has been suggested that multiple intracellular messengers that lead to endothelial permeability, such as PKC and intracellular calcium, were being suppressed by luteolin, leading to the suppression of increased HUVECs' permeability (Mehta et al., 2006). These will be beneficial in vascular and inflammatory diseases. Luteolin has been reported to minimize ischemia-reperfusion injury-induced microvascular damage by regulating Wnt/ β -catenin signaling in animal models (Qin et al., 2022).

Effect of Luteolin on the Activity of PKC in Bradykinin-Induced HUVECs

PKC is being referred as the key mediator of endothelial permeability under stimulated conditions (Pan et al., 2022). Flux of fluid and macromolecules across endothelial cell monolayers will increase with the direct activation of PKC with diacylglycerol (DAG) or phorbol esters (Wolf et al., 1990). This eventually will lead to increase in endothelial permeability. PKC assay test, which is based on enzyme-linked immunosorbent assay (ELISA) detection method, was carried out. This study has shown that the activity of PKC in induced HUVECs was being suppressed when the induced HUVECs were pretreated with luteolin. A significant reduction of PKC activity was found for all the three treatment groups. The treatment groups of 5, 10, and 25 μ M showed suppression of 94.32%, 115.98%, and 125.14%, respectively. Indeed, the exact effects of PKC downstream remain unclear. However, Byun et al. (2010) reported the luteolin may possess anticarcinogenic effects against UVB-induced skin cancer mainly by targeting PKC. Luteolin is also reported to be a potent inhibitor of human mast cell activation through the inhibition of Ca^{2+} influx and PKC activation (Kimata et al., 2000).

These results of PKC revealed the ability of luteolin to suppress the increased activity of PKC when HUVECs were induced by bradykinin. Similar to other cells (Dempsey et al., 2000), pretreatment of HUVECs with luteolin causes a significant reduction in PKC activity, which in turn reduces enhanced cellular permeability. When the inducer bradykinin attached to the receptor, the G protein was triggered. The enzyme, phosphatidylinositol-specific phospholipase C (PLC) will be activated by the stimulated G protein. Phosphatidylinositol 4,5-bisphosphate (PIP_2) will split into DAG and inositol 1,4,5-trisphosphate (IP_3) (Gutterman, 2002). DAG recruits PKC to the membrane, and this enzyme is being activated (Wu et al., 1999; Sandoval et al., 2001). Direct activation of PKC by DAG increases the flux of fluid and macromolecules across the endothelial cell monolayers as well as the microvascular wall (Johnson et al., 1990). This eventually leads to increase in endothelial permeability. However, PKC activity was decreased in HUVECs that had received luteolin pretreatment. Consequently, there was a suppression of the enhanced fluid and macromolecule flux across the endothelial cell monolayers. As a result, elevated endothelial permeability was suppressed (Mehta et al., 2001).

Effect of Luteolin on the Concentration of Intracellular Calcium in Bradykinin-Induced HUVECs

Bradykinin can also cause an increase in the concentration level of intracellular calcium by activating a sequence of events. This involves generation of IP_3 , which will induce the endoplasmic reticulum store to release calcium. The calcium release will in turn activate the calcium entry into

endothelial cells, which eventually causes an increase in endothelial permeability (Gerald, 2005). Intracellular calcium was measured with Fura-2 AM ester as described in detail by Grynkiewicz *et al.* (1985). This test was carried out to determine the concentration of intracellular calcium in induced HUVECs that were pretreated with luteolin at the dose of 5, 10, and 25 μ M. This study has shown that the concentration of intracellular calcium in induced HUVECs was being suppressed when the induced HUVECs were pretreated with luteolin at the dose of 10 and 25 μ M, with the maximum suppression being 104.16%.

The results of intracellular calcium assay test reveal the ability of luteolin to suppress the increased concentration of intracellular calcium when HUVECs were induced by bradykinin. HUVECs that were pretreated with luteolin (10 and 25 μ M) resulted in a significant suppression of increased concentration of intracellular calcium, which led to suppression of the increased permeability of HUVECs. When bradykinin bound to the receptor, G protein was activated making the PLC enzyme was activated by the stimulated G protein. PIP_2 was split into DAG and IP_3 . IP_3 induced the endoplasmic reticulum store to release calcium. The calcium release in turn activates the calcium entry into endothelial cells, which eventually causes an increase in endothelial permeability (Glass and Bates, 2003). Recent studies have shown that the store depletion-activated intracellular calcium that enters through the cation channels of the plasma membrane is an important determinant of the increase in endothelial permeability (Krul and Maleth, 2021). However, HUVECs that were pretreated with luteolin suppressed the increased concentration of intracellular calcium. This led to the suppression of increased endothelial permeability as the NO-cGMP pathway was inactivated with the low concentration of intracellular calcium concentration.

The Interrelationship of Increased Endothelial Permeability and Inflammation

Vasodilatation of arterioles and increased endothelial permeability are the initial processes of inflammation. Changes in endothelial permeability are the hallmarks of inflammatory conditions. Heat, redness, and swelling are the three symptoms produced by the dilation of arterioles and increased endothelial permeability. Increased endothelial permeability allows the release of histamine that will activate the blood clotting factors in the tissues. The clotting cascade is set into motion, and insoluble fibrin that is produced localizes and traps the invading microbes and blocks their spread. Nevertheless, prolonged increase in endothelial permeability leads to an abnormal extravasation of blood components and accumulation of fluid in the extravascular space, which will result in multiorgan dysfunction as well as compromise the normal pharmacokinetics of therapeutic drugs (Hu *et al.*, 2007). Hence, by suppressing the abnormal increase of endothelial permeability, certain related diseases can be prevented.

Experimental evidences obtained in this study indicate that luteolin possesses the ability to suppress increased endothelial permeability as it produced significant suppression effect in the assays. Further studies are needed to determine the minimal effective dosage and to deduce the benefits that this flavonoid offers as a suppressant of increased endothelial permeability (Luo *et al.*, 2004). Indeed, Jia *et al.*, (2015) reported potential luteolin protects vascular inflammation in mice. An extensive *in vivo* mechanistic investigation into reduction of endothelial permeability induced by luteolin in inflammatory diseases is required.

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