The Protective Effect of Eupatilin against Hydrogen Peroxide-Induced Injury Involving 5-Lipoxygenase in Feline Esophageal Epithelial Cells

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In this study, we focused to identify whether eupatilin (5,7-dihydroxy-3',4',6-trimethoxyflavone), an extract from Artemisia argyi folium, prevents H_2O_2-induced injury of cultured feline esophageal epithelial cells. Cell viability was measured by the conventional MTT reduction assay. Western blot analysis was performed to investigate the expression of 5-lipoxygenase by H_2O_2 treatment in the absence and presence of inhibitors. When cells were exposed to 600 μM H_2O_2 for 24 hours, cell viability was decreased to 40%. However, when cells were pretreated with 25 ∼ 150 μM eupatilin for 12 hours, viability was significantly restored in a concentration-dependent manner. H_2O_2-treated cells were shown to express 5-lipoxygenase, whereas the cells pretreated with eupatilin exhibited reduction in the expression of 5-lipoxygenase. The H_2O_2-induced increase of 5-lipoxygenase expression was prevented by SB202190, SP600125, or NAC. We further demonstrated that the level of leukotriene B_4 (LTB_4) was also reduced by eupatilin, SB202190, SP600125, NAC, or nordihydroguaiaretic acid (a lipoxygenase inhibitor) pre-treatment. H_2O_2 induced the activation of p38MAPK and JNK, this activation was inhibited by eupatilin. These results indicate that eupatilin may reduce H_2O_2-induced cytotoxicity, and 5-lipoxygenase expression and LTB_4 production by controlling the p38 MAPK and JNK signaling pathways through antioxidative action in feline esophageal epithelial cells.

Key Words: 5-lipoxygenase, Esophageal epithelial cell, Eupatilin, Flavonoid, Hydrogen peroxide

INTRODUCTION

Reactive oxygen species (ROS) are forms of oxygen that are produced by the incomplete reduction of molecular oxygen [1]. ROS participate and regulate diverse downstream signaling pathways leading to specific cellular functions such as growth, metabolic rate, cell division, necrosis, apoptosis and the aging process [2,3]. Although low levels of ROS play an important role in physiological functions, several studies have reported that high concentrations of ROS result in oxidative damage and induce cytotoxic effects in cells [4,5]. Since hydrogen peroxide, one type of ROS, has remarkable membrane permeability [6], exposure of cells to exogenous hydrogen peroxide (H_2O_2) can induce detrimental effects [7]. From these processes, further oxidative stimulation will be propagated, damaging cellular mole-
diseases such as inflammatory disorders [18,19]. Werz and Steinhilber [17] observed that p38 MAPK activation by cell stress is required for efficient leukotriene synthesis in B-lymphocytes. MAPK pathways are critical for converting diverse extracellular signals, including ROS, to biological responses. MAPKs modulate many cellular processes, such as gene induction, cell survival/apoptosis, as well as cellular stress and inflammatory responses [20]. ERK1/2 behave mainly as mitogen-activated proliferation/differentiation factors [21], whereas JNK and p38 MAP kinase are mainly stress-activated proteins related to apoptotic cell death [22]. Based on these evidences, in the present study, we tested the roles of 5-LOX and MAPKs in external H2O2 stimulation with esophageal epithelial cells.

On the other hand, flavonoids, which are secondary metabolites in plants, are considered relatively non-toxic bioactive substances and have diverse biological effects, such as anti-inflammatory, anti-oxidant, anti-allergic, hepatoprotective, anti-thrombotic, anti-viral, and anti-carcinogenic activities [23,24]. Thus, these activities may explain the beneficial effects of flavonoid intake in different human pathologies, such as hypertension, inflammatory conditions, and even cancer [25]. In the present study, we used eupatilin (5,7-dihydroxy-3’4’,6-trimethoxyflavone) being one of the pharmacologically active flavonoid components of Stillen to test the protective potential of flavonoids. Stillen (Artemisia herba extract) is a quality-controlled compound extracted from Artemisiae argyi folium, a traditional Korean herbal medicine for the treatment of abdominal tenderness, bloody diarrhea, and gynecological disorders. It shows muco-protective activity against noxious agents and exhibits favorable effects in experimental models of gastrointestinal disease such as gastritis, peptic ulcer, inflammatory bowel disease, and pancreatitis. Eupatilin has a potent antiagastisic effect [26]. Eupatilin is reported to exert strong anti-inflammatory, and anti-oxidative activity as well as cytoprotective effects against experimentally induced gastrointestinal, hepatic, and pancreatic damage in vivo and in vitro [27-30].

Therefore, the purpose of this study is to investigate the anti-inflammatory potential and the cytoprotective mechanism of eupatilin on the H2O2-activated 5-LOX and LTB4 production in feline esophageal epithelial cells.

**METHODS**

**Materials**

Eupatilin was graciously provided by Dong-A Pharmaceutical Co., Ltd. (Yong-In, Korea) and dissolved in 0.1% dimethyl sulfoxide (DMSO). H2O2, bovine serum albumin (BSA), 4-(2-hydroxyethyle)-1-piperazine-N,N,N',N'-tetraacetic acid (HEPES), leupeptin, aprotinin, β-mercaptoethanol, ethylene glycol-bis-(β-aminooxyethyl) N,N,N',N'-tetraacetic acid (EGTA), ethylenediamine tetra acetic acid (EDTA), phenylmethyl-sulfonylfluoride (PMSF), thizoyl blue tetrazolium bromide, Hank’s Balanced Salt Solution-Modified (HBSS), NAC and NDGA were purchased from Sigma Chemical Co. (St. Louis, MO, USA);SB202190 and SP600125 from Calbiochem (San Diego, CA, USA); 5-LOX, phospho-SAPK/JNK, SAPK/JNK, phospho-p38 MAPK, and p38 MAPK antibodies from Cell Signaling Technology (Beverly, MA, USA); Actin antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA); goat anti-rabbit IgG-HRP, goat anti-mouse IgG-HRP, and rabbit anti-goat IgG-HRP from Zymed Laboratories Inc. (Eccles Avenue, CA, USA); Rainbow prestained molecular weight marker from Amersham (Arlington Heights, IL, USA); Enhanced Chemiluminescence (ECL) agents from PerkinElmer Life Sciences (Boston, MA, USA); Ammonium persulfate, N,N,N’,N’-tetramethylethylene diamine (TEMED), nitrocellulose (NC) membrane, Tris/Glycine/SDS buffer, Tris/Glycine buffer, and 30% acrylamide/its solution from BioRad (Richmond, CA, USA); RestoreTM Western Blot Stripping Buffer from Pierce (Rockford, IL, USA); LTB4 ELISA kit from Cayman Chemical Company (Ann Arbor, MI, USA).

Preparation of feline esophageal epithelial tissue squares

All animal experiments were approved by the Institutional Animal Care and Use Committee of Chung-Ang University, in accordance with the guide regarding the Care and Use of Laboratory Animals in Seoul, Korea. Adult cats of either sex weighing between 2.5 and 3.5 kg were anesthetized with Zoletil 50 (12.5 mg/0.25 ml/kg), which was composed of tiletamine and zolazepam, and euthanized with an overdose of 25% urethane (Aldrich, St. Louis, MO, USA). After the abdomen was opened with a midline incision, the esophagus was excised, cleaned and maintained in Krebs buffer composed of 116.6 mM NaCl, 2.19 mM NaHCO3, 1.2 mM Na2HPO4, 3.4 mM KCl, 2.5 mM CaCl2, 5.4 mM glucose and 1.2 mM MgCl2. The esophagus was opened along the lesser curvature. The location of the squamocolumnar junction was identified and the mucosa was then peeled off. The submucosal connective tissues were then removed by microspring scissors. The mucosa from esophagus was sliced into 0.5-mm-thick sections with a Studie Riggs tissue slicer (Thomas Scientific Apparatus, Philadelphia, PA, USA). The last slices were cut into 2×2 mm tissue squares using scissors.

Cultures of feline esophageal epithelial cells

The sliced tissue was placed into DMEM supplemented with 10% FBS containing 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.025 µg/ml amphotericin B and incubated in a humidified atmosphere of 5% CO2 and 95% air at 37°C [31]. After 10 days, the medium was exchanged with fresh DMEM containing 10% FBS. After reaching confluence, the cells were detached using 1% trypsin in HBSS with bicarbonate. Afterwards, the cells were then counted, seeded at 2×10³ cells/ml on 100-mm culture dishes and maintained in DMEM containing 10% FBS. The medium was changed every 48 hours until the cells reached confluence. Experiments were performed on cells at passage 3 or 4.

Measurement of cell viability

The cell viability was determined by the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) reduction assay using the method previously described [32]. Briefly, cells were made quiescent at confluence by incubation in serum-free DMEM for 24 hours to arrest cell growth and silence gene activity, followed by treatment
with each indicated agent for the designated time periods. After incubation, the cells were rapidly washed twice with ice-cold PBS and incubated with MTT solution (final concentration, 5 mg/ml) for 4 hours at 37°C. Then, the supernatant was removed and the formazan crystals were dissolved with DMSO. Absorbance at 570 nm was measured with a microplate reader (Molecular Devices, Sunnyvale, CA, USA), and ECC Cell image were observed and acquired with Leica DM IL LED fluorescence microscopy (Leica Microsystems, Wetzlar, Germany).

Preparation of cell extracts

When the cells reached confluence, they were serum starved by incubation in serum-free DMEM for 24 hours. The cells were then stimulated with each compound for the indicated time periods or at the specified concentrations. After incubation, the cells were rapidly washed twice with ice-cold PBS and lysed with an ice-cold lysis buffer (20 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 1% (w/v) Triton X-100, 0.01% (w/v) SDS, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, and 0.7 μg/ml β-mercaptoethanol) for 5 min. The lysates were scraped with a cell scraper and collected in Eppendorf tubes. They were then sonicated for 6 seconds, 3× and centrifuged for 10 min at 13,000 rpm at 4°C to remove cellular debris; the supernatants were collected and stored at −70°C for protein assay and Western blot analysis.

Western blot analysis

Equal amounts of protein from each sample were subjected to electrophoresis on a 10% SDS-polyacrylamide gel and transferred to a NC membrane using the Power Pac 1,000 (Bio-Rad, Melville, NY, USA) power supply. To block any nonspecific binding, the NC membrane was incubated in 5% nonfat dry milk in PBS for 60 min followed by three rinses in milk-free PBS. The membranes were incubated overnight at 4°C with primary antibodies raised against 5-LOX, phospho-SAPK/JNK, or phospho-p38 MAP kinase followed by three washes with PBS containing 0.05% Tween 20. This was followed by 60 min incubation in a horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were detected with an ECL agent. Molecular mass markers were estimated by comparison with a prestained molecular mass marker. To confirm the uniformity of protein loading, the same blots were subsequently stripped with Western blot stripping buffer and reprobed with actin, SAPK/JNK, or p38 MAPK antibodies [33]. The results were analyzed by Quantity One analysis software (Bio-Rad Chemical Division, Richmond, CA, USA). The percentage of p38 MAPK, SAPK/JNK activation or the 5-LOX expression was calculated as the ratio of phosphorylated p38 MAPK to total p38 MAPK, phosphorylated SAPK/JNK to total SAPK/JNK or 5-LOX to Actin, respectively.

Measurements of LTB₄ production from EECs

Cells were pretreated with each indicated agent for the designated time periods. EECs were then stimulated with H₂O₂. Regarding experiments designed to measure the production of LTB₄, the medium was collected, centrifuged, and stored at −70°C until assayed [34]. The level of LTB₄ released into the culture medium was quantified using a LTB₄ ELISA kit. Assays were then performed according to the manufacturer’s instructions.

Statistics

Differences among the groups were determined using Student’s t-test. Data were expressed as the means±S.E.M. of 4~6 experiments and differences between groups were considered significant at p<0.05.

RESULTS

The cytotoxic effect of external H₂O₂ in cultured EECs

To investigate the cytotoxic effects concerning the external addition of H₂O₂, we performed MTT assays in cultured EECs. Cells were incubated with H₂O₂ at the indicated concentration for 24 hours, and then cell viability was measured using the MTT assay (Fig. 1A). As a result, cell viability was significantly decreased by greater than 300 μM H₂O₂ in a concentration-dependent manner. Moreover, cell viability after exposure to 600 μM H₂O₂ was reduced to 40% of the control. In addition, morphologic observation of EECs treated with H₂O₂ was performed to identify the H₂O₂-induced morphologic change (Fig. 1B). After H₂O₂ treatment, the number of cells was reduced and a high fraction of cells exhibited cytoplasmic condensation.

The identification of cytotoxicity of eupatilin

To study the cytotoxic effect of eupatilin, we employed the MTT assay in EECs (Fig. 1C). We treated EECs with various concentrations of eupatilin for 24 hours. The cell viability did not show significant changes until 200 μM of eupatilin was used.

The protective effect of eupatilin on the H₂O₂-induced cell death

To study the cytoprotective effect of eupatilin against H₂O₂-induced cell death, cells were pre-incubated with 25~150 μM eupatilin for 12 hours and then exposed to 600 μM H₂O₂ for 24 hours (Fig. 1D). H₂O₂ treatment alone significantly decreased cell viability to about 40%. However, when cells were pretreated with 25~150 μM eupatilin for 12 hours, the cell viability was restored to roughly 65% of the control at a concentration of 150 μM. Morphologic observation of EECs treated with H₂O₂ in the absence or presence of eupatilin was also performed. (Fig. 1E). H₂O₂ induced cytoplasmic condensation of EECs, whereas the morphology of cells incubated with H₂O₂ in the presence of 150 μM eupatilin was shown to maintain similar to control.

Effect of eupatilin on H₂O₂-induced 5-LOX expression

To examine whether H₂O₂ causes 5-LOX expression in cultured EECs, the cells were exposed to H₂O₂ at the indicated concentrations, and then 5-LOX expression was measured by western blotting analysis. When the cells were treated with 100~400 μM H₂O₂ for 24 hours, 5-LOX expression peaked at 300 μM H₂O₂ (Fig. 2A). Next, to assess whether eupatilin affects H₂O₂-induced 5-LOX expression in EECs, western blotting analysis was performed (Fig. 2B). After pre-treatment with the indicated concentration of eupatilin for 12 hours, EECs were further exposed to 300 μM
Fig. 1. Effect of H₂O₂ on the cell viability of feline EECs and Effect of eupatilin on the H₂O₂-induced cell viability. Serum-starved EECs were incubated with H₂O₂ for 24 hours at the indicated concentration. (A) The cell viability was estimated using MTT assay. (B) The morphologic changes of EECs were observed (magnification: 100×). (C) Serum-starved EECs were incubated in the presence of eupatilin alone for 12 hours at the indicated concentration. (D) the cells were incubated in the 600 μM H₂O₂ with or without eupatilin 12 hours before and during 24 hours, and then their survival was estimated using the MTT assay and the morphologic changes of cells were observed (E) (magnification: 100×). Data are expressed as Means±S.E of four experiments (*; p < 0.05 vs. control, **; p < 0.01 vs. control, #; p < 0.05 vs. H₂O₂ alone, ##; p < 0.01 vs. H₂O₂ alone).

H₂O₂ in the presence of eupatilin for 24 hours. Moreover, pretreatment with 100~150 μM eupatilin significantly reduced the H₂O₂-induced 5-LOX protein expression. 5-Lox expression by H₂O₂ was reduced 10% by eupatilin.

Effect of eupatilin, MAPK inhibitors, ROS scavenger or LOX inhibitor on H₂O₂-induced 5-LOX expression and LTB₄ production

Serum-starved EECs were treated with or without 150 μM eupatilin for 12 hours, 5 mM NAC, 30 μM SB202190 or 30 μM SP600125 for 1 hours prior to 300 μM H₂O₂ stimulation for 24 hours. As shown in Fig. 3A, pretreatment of the cells with SB202190, SP600125 or NAC significantly reduced H₂O₂-induced the 5-LOX expression. These results indicated that p38 MAPK, JNK and ROS scavenging action may mediate the inhibitory effect of eupatilin on the 5-LOX expression by H₂O₂. In parallel experiments, the inhibitory effect of eupatilin on H₂O₂-induced LTB₄ production was determined using LTB₄ EIA kit (Fig. 3B). Fig. 3B showed that treatment of cultured EECs with H₂O₂ caused a significant increase in the production of LTB₄. However, when EECs were treated with eupatilin, SB202190, SP600125, NAC or NDGA (a lipoygenase inhibitor), the levels of LTB₄ production was significantly reduced by all of them. Eupatilin and inhibitors reduced 10~12 pg/ml of LTB₄ production. These data were similar to the results of the 5-LOX expression by H₂O₂ with or without inhibitors.

Effect of H₂O₂ on activation of MAPKs

To determine the effect of H₂O₂ on activation of MAPKs, the phosphorylation of p38 MAPK and JNK was investigated. The concentration-dependence of p38 MAPK and JNK phosphorylation was investigated by Western blot analysis (Fig. 4). p38 MAPK and JNK phosphorylation levels were significantly increased by 300 μM H₂O₂. P38 MAPK expression after exposure to 300 μM H₂O₂, in Fig. 4A, was increased to 40% of the control and JNK activation after exposure to 300 μM H₂O₂ in Fig. 4B, was increased to 30% of the control.
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Fig. 3. The effect of eupatilin, SB202190, SP600125 or NAC on the H$_2$O$_2$-induced 5-LOX expression and LTB$_4$ production. Serum-starved EECs were preincubated in the presence of eupatilin (150 μM, 12 hours), SB202190 (30 μM, 1 hour), SP600125 (30 μM, 1 hour), or NAC (5 mM, 1 hour). The cells were then stimulated with H$_2$O$_2$ (300 μM, 24 hours). (A) The change level of 5-LOX expression was estimated by Western blot analysis. (B) The production level of LTB$_4$ was estimated by LTB$_4$EIA kit. Data are expressed as Means±S.E of three experiments (*; p < 0.05 vs. control, #; p < 0.05, ##; p < 0.01 vs. H$_2$O$_2$ alone).

Fig. 4. p38 MAPK and JNK phosphorylation by H$_2$O$_2$. Serum-starved EECs were stimulated with H$_2$O$_2$ for 24 hr at each dose. (A) The change in the level of phosphorylated p38 MAPK was estimated by Western blot analysis. (B) The change of phosphorylated JNK level was estimated by Western blot analysis. Data are expressed as Means±S.E of three experiments (*; p < 0.05 vs. control, **; p < 0.001 vs. control).

Effect of eupatilin, MAPK inhibitors, and ROS scavenger on H$_2$O$_2$-induced p38 MAPK and JNK phosphorylation

Serum-starved EECs were treated in the presence or absence of 150 nM eupatilin for 12 hr and with NAC, SB202190 or SP600125 for 1 hr prior to 300 μM H$_2$O$_2$ treatment for 24 hr. p38 MAPK and JNK phosphorylation levels in EECs were estimated by Western blot analysis (Fig. 5). Pretreatment with eupatilin, NAC, SB202190, or SP600125 inhibited the expression of H$_2$O$_2$-induced p38 MAPK and JNK phosphorylation.

The group with H$_2$O$_2$ treatment as well as Eupatilin, in Fig. 5A, decreased 21% when compared to that of the H$_2$O$_2$ treatment alone. The ROS scavengers presented similar effect to Eupatilin, and MAPK inhibitors showed further decrease down to 30%, similar to that of the non-treated group. In Fig. 5B, the effect of drugs and reagents on P-JNK/T-JNK cell were shown. Eupatilin, MAPK inhibitors, and ROS scavenger treated groups showed diminishment as well.

DISCUSSION

In this study, the addition of external H$_2$O$_2$ to esophageal epithelial cells exhibited significant cytotoxicity. The cell viability was decreased and the shapes of cells were remarkably altered. However, eupatilin enhanced the reduction of cell viability by H$_2$O$_2$. Previously, we identified that the cytoprotective properties of eupatilin could be attributed to the induction of the antioxidant protein heme oxygenase-1 (HO-1) in ileal smooth muscle cells or esophageal epithelial cells [30,35]. We also confirmed that eupatilin induced HO-1 expression in esophageal epithelium of rats in vivo [35]. Cytoprotective roles for HO-1 in ileal smooth muscle cells or esophageal epithelial cells [30,35]. We also confirmed that eupatilin induced HO-1 expression in esophageal epithelium of rats in vivo [35]. Cytoprotective roles for HO-1 in ileal smooth muscle cells or esophageal epithelial cells [30,35]. Eupatilin is also a flavonoid compound isolated from a traditional Korean herbal medicine, Artemisiae argyi folium. In the present study, although we did not test for the role of eupatilin-induced HO-1 in cell death by H$_2$O$_2$, we expect that the ability of eupatilin regarding HO-1 induction may be involved in cytoprotection against H$_2$O$_2$-induced cytotoxicity. In addition, the cytotoxicity of H$_2$O$_2$ could be asso-
cipated with its ability to induce the expression of 5-LOX. As one study previously demonstrated, methyl jasmonate which is a plant stress hormone, induced apoptosis in human prostate carcinoma cells via 5-LOX dependent pathway [40]. In our study, co-treatment of eupatilin with H₂O₂ inhibited the increase of the H₂O₂-stimulated 5-LOX expression and LTB₄ production. Therefore, it is possible that the cytoprotective effect of eupatilin could involve its ability to decrease the 5-LOX expression.

ROS act as second messengers to stimulate intracellular signaling pathways including MAPK [41]. Modulation of the MAPK signaling pathways by H₂O₂ is distinctive, depending on the cell type, concentration and duration of exposure. For example, exogenous H₂O₂ activates ERK and JNK but not p38 MAPK in human gastric epithelial cells [42], while endogenous H₂O₂ production by ethanol treatment in EECs activates ERK, but not JNK and p38 MAPK. As shown in our results, the H₂O₂-induced 5-LOX expression and LTB₄ production were mediated by activation of p38 MAPK and JNK. Eupatilin inhibited H₂O₂-induced p38 MAPK and JNK activation. Considering the inhibitory effect of SB202190 and SP600125 on the 5-LOX expression in EECs. Eupatilin also inhibits the H₂O₂-induced 5-LOX expression and LTB₄ production through the inactivation of p38 MAPK and JNK pathways. Considering the 5-LOX-inhibiting effect of eupatilin in the present study, eupatilin might act as a dual inhibitor with regards to COX and 5-LOX. Taken together, the present study provides evidence that eupatilin has a protective effect against H₂O₂-induced cell damage in cultured feline EEC. Eupatilin also inhibits the H₂O₂-induced 5-LOX expression and LTB₄ production through the inactivation of p38 MAPK and JNK pathways.

ACKNOWLEDGEMENTS

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011-0012139).

REFERENCES

1. Lee SH, Heo JS, Lee MY, Han HJ. Effect of dihydrotestosterone on hydrogen peroxide-induced apoptosis of mouse embryonic stem cells. J Cell Physiol. 2008;216:269-275.
2. de Magalhães JP, Church GM. Cells discover fire: employing reactive oxygen species in development and consequences for aging. Exp Gerontol. 2006;41:1-10.
3. Menon SG, Goswami PC. A redox cycle within the cell cycle: riding in the old with the new. Oncogene. 2007;26:1101-1109.
4. Lee MN, Lee SH, Lee MY, Kim YH, Park JH, Ryu JM, Yun SP, Lee YJ, Kim MO, Park K, Han HJ. Effect of dihydrotestosterone on mouse embryonic stem cells exposed to H2O2-induced oxidative stress. J Vet Sci. 2008;9:247-256.
5. Lee SY, Na SI, Heo JS, Kim MH, Kim YH, Lee MY, Kim SH, Lee YJ, Han HJ. Arachidonic acid release by H₂O₂ mediated proliferation of mouse embryonic stem cells: involvement of Ca²⁺/PKC and MAPKs-induced EGFR transactivation. J Cell Biochem. 2009;106:787-797.
6. Fischer S, Wiesnet M, Renz D, Schaper W. H2O2 induces paracellular permeability of porcine brain-derived microvascular endothelial cells by activation of the p44/42 MAP kinase pathway. Eur J Cell Biol. 2005;84:687-697.
7. Lee WC, Chai CH, Cha SH, Oh HL, Kim YK. Role of ERK in hydrogen peroxide-induced cell death of human glioma cells. Neurochem Res. 2005;30:263-270.
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8. Farinati F, Cardin R, Degan P, Rugge M, Mario FD, Bonvicini P, Naccarato R. Oxidative DNA damage accumulation in gastric carcinogenesis. Gut. 1998;42:351-356.

9. Martindale JL, Holbrook NJ. Cellular response to oxidative stress: signaling for suicide or survival. J Cell Physiol. 2002;192:1-15.

10. Kang KA, Lee KH, Zhang R, Piao MJ, Kang MY, Kwak YS, Yoo BS, You HJ, Hyun JW. Protective effects of Castanopsis cuspidata through activation of ERK and NF-kappaB on oxidative cell death induced by hydrogen peroxide. J Toxicol Environ Health A. 2007;70:1319-1328.

11. Olyae M, Sontag S, Salmon W, Schnell T, Moharan S, Elizazhan D, Keshavarzian A. Mucosal reactive oxygen species production in oesophagus and Barrett's oesophagus. Gut. 1995;37:168-173.

12. Stein HJ, Esplugués J, Whittle BJ, Bauerfeind P, Hinder RA, Blum AL. Direct cytotoxic effect of oxygen radicals on the gastric mucosa. Surgery. 1989;106:318-323.

13. Stein HJ, Hinder RA, Oosthuizen MM. Gastric mucosal injury caused by hemorrhagic shock and reperfusion: protective role of the antioxidant glutathione. Surgery. 1990;108:467-473.

14. Li CT, Zhang WP, Lu YB, Fang SH, Yuan YM, Qi LL, Zhang LH, Huang XJ, Zhang L, Chen Z, Wei EQ. Oxygen-glucose deprivation activates 5-lipoxygenase mediated by oxidative stress through the p38 mitogen-activated protein kinase pathway in PC12 cells. J Neurosci Res. 2009;87:991-1001.

15. Funk CD. Prostaglandins and leukotrienes: advances in eicosanoid biology. Science. 2001;294:1871-1875.

16. Koshihara Y, Neichi T, Murata S, Lao A, Fujimoto Y, Tatsuno T. Selective inhibition of 5-lipoxygenase by natural compounds isolated from Chinese plants, Artemisia rubrae Nakai. FEBS Lett. 1983;158:41-44.

17. Werz O, Steinhilber D. Therapeutic options for 5-lipoxygenase inhibition. Pharmacol Rev. 2006;58:701-718.

18. Charlier C, Michaux C. Dual inhibition of cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) as a new strategy to provide safer non-steroidal anti-inflammatory drugs. Eur J Med Chem. 2005;38:645-659.

19. Brain SD, Williams DJ. Leukotrienes and inflammation. Pharmacol Ther. 1990;46:57-66.

20. Thallamers T, McGrath MA, Harnett MM. MAPKs and their relevance to arthritis and inflammation. Rheumatology (Oxford). 2008;47:491-502.

21. Tamura S, Hanuda M, Ounishi M, Katsura K, Sasaki M, Kobayashi T. Regulation of stress-activated protein kinase signaling pathways by protein phosphatases. Eur J Biochem. 2002;269:1060-1066.

22. Ichijo H, Nishida E, Icie K, ten Dijke P, Saitoh M, Morishita T, Takakura K, Mano J. Itoh Y. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates JNK and p38 signaling pathways. Science. 1997;275:90-94.

23. Kiddle EJ. Effect of plant flavonoids on immune and inflammatory cell function. Adv Exp Med Biol. 1998;439:175-182.

24. Moreira AJ, Fraga C, Alonso M, Collado PS, Zettler C, Marroni C, Marroni N, González-Gallego J. Quercetin prevents oxidative stress and NF-kappaB activation in gastric mucosa of portal hypertensive rats. Biochem Pharmacol. 2004;68:1939-1946.

25. Middleton E Jr, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmaco 2000; 52:673-751.

26. Ryu BK, Ahn BO, Oh TY, Kim SH, Kim WB, Lee EB. Studies on protective effect of DA-9601, Artemisia asiatica extract, on acetaminophen- and CCl4-induced liver damage in rats. Arch Pharm Res. 1998;21:505-513.

27. Ahn BO, Ko KH, Oh TY, Cho H, Kim WB, Lee KJ, Cho SW, Hahn KB. Efficacy of use of colosonol in dextran sulfate sodium induced ulcerative colitis in rats: the evaluation of the effects of antioxidant by colosonol. Int J Colorectal Dis. 2001;16:174-181.

28. Hahn KB, Kim JH, You BM, Kim YS, Cho SW, Yim H, Ahn BO, Kim WB. Induction of apoptosis with an extract of Artemisia asiatica attenuates the severity of cerulein-induced pancreatitis in rats. Pancreas. 1998;17:153-157.

29. Oh TY, Lee JS, Ahn BO, Cho H, Kim WB, Kim YB, Suh YJ, Cho SW, Lee KM, Hahn KB. Oxidative stress is more important than acid in the pathogenesis of reflux esophagitis in rats. Gut. 2001;49:364-371.

30. Bang HJ, Shin CY, Oh TY, Sohn UD. The protective effect of eupatilin on indomethacin-induced cell damage in cultured feline ileal smooth muscle cells: involvement of HO-1 and ERK. J Ethnopharmacol. 2008;118:94-101.

31. Kim do T, Song HJ, Jeong JH, Suh JS, Sohn UD. Regulation of lysophosphatidic acid-induced COX-2 expression by ERK1/2 activation in cultured feline esophageal epithelial cells. Arch Pharm Res. 2008;31:1331-1338.

32. Park SY, Sohn UD. Inhibitory effect of rosiglitazone on the acid-induced intracellular generation of hydrogen peroxide in cultured feline esophageal epithelial cells. Naunyn Schmiedebergs Arch Pharmacol. 2011;383:191-201.

33. Kim JS, Song HJ, Ko SK, Whang WK, Sohn UD. Quercetin-3-O-beta-d-glucuronopyranoside (QGC)-induced HO-1 expression through ERK and PI3K activation in cultured feline esophageal epithelial cells. Fitoterapia. 2010;81:85-92.

34. Kim N, Cao W, Song IS, Kim CY, Sohn UD, Harnett KM, Biancani P. Leukotriene D4-induced contraction of cat esophageal and lower esophageal sphincter circular smooth muscle. Gastroenterol Res Pract. 2010:90-94.

35. Song HJ, Shin CY, Oh TY, Min YS, Park ES, Sohn UD. Eupatilin with heme oxygenase-1-inducing ability protects cultured feline esophageal epithelial cells from cell damage caused by indomethacin. Biol Pharm Bull. 2009;32:589-596.

36. Amsess F, Bienew R, Kat M, Ko EJ, Goto AJ, Shen XD, Zhao D, Zaky J, Melinek J, Lassman CR, Kolls JK, Alam J, Ritter T, Volk HD, Farmer DG, Ghoebrial RM, Busuttil RW, Kupiec-Weglinski JW. Upregulation of heme oxygenase-1 protects genetically fat Zucker rat livers from ischemia/reperfusion injury. J Clin Invest. 2009;119:1631-1639.

37. Otterbein LE, Kolls JK, Mantell LL, Cook JJ, Alam J, Choi AM. Exogenous administration of heme oxygenase-1 by gene transfer provides protection against hyperoxia-induced lung injury. J Clin Invest. 2007;119:1181-1191.

38. Wang RS, Hsu MC, Wu CC, Hsieh CW. Piceatannol upregulates endothelial heme oxygenase-1 expression via novel protein kinase C and tyrosine kinase pathways. Pharmacol Res. 2006;53:113-122.

39. Chen CY, Jiang JH, Li MH, Such YJ. Resveratrol upregulates heme oxygenase-1 expression via activation of NF-E2-related factor 2 in PC12 cells. Biochem Biophys Res Commun. 2005;331:993-1000.

40. Ezekwudo DE, Wang RC, Elegbede JA. Methyl jasmonate induced apoptosis in human prostate carcinoma cells via 5-lipoxygenase dependent pathway. J Exp Ther Oncol. 2007;6:267-277.

41. Li SY, Li Q, Shen JJ, Dong F, Sigmun VK, Liu Y, Ren J. Attenuation of aldehyde dehydrogenase-induced cell injury by overexpression of aldehyde dehydrogenase-2 (ALDH2) transgene in human cardiac myocytes: role of MAP kinase signaling. J Mol Cell Cardiol. 2006;40:293-294.

42. Lee S, Lee M, Kim SH. Eupatilin inhibits H(2)O(2)-induced apoptotic cell death through inhibition of mitogen-activated protein kinases and nuclear factor-kappaB. Food Chem Toxicol. 2006;44:2863-2870.

43. Nieves D, Moreno JJ. Role of 5-lipoxygenase pathway in the regulation of RAW 264.7 macrophage proliferation. Biochem Pharmacol. 2006;72:1022-1030.

44. Werz O, Bünzelt F, Fischer L, Sirellas D, Dishart D, Sammeldson B, Rádmak O, Steinhilber D. Extracellular signal-regulated kinases phosphorylate 5-lipoxygenase and stimulate 5-lipoxygenase production formation in leukocytes. FASEB J. 2002;16:1441-1443.
45. Hanaka H, Shimizu T, Izumi T. Stress-induced nuclear export of 5-lipoxygenase. *Biochem Biophys Res Commun.* 2005;338:111-116.

46. Fiorucci S, Meli R, Bucci M, Cirino G. Dual inhibitors of cyclooxygenase and 5-lipoxygenase. A new avenue in anti-inflammatory therapy? *Biochem Pharmacol.* 2001;62:1433-1438.

47. Choi ED, Lee S, Chae JR, Lee HS, Jun CD, Kim SH. Eupatilin inhibits lipopolysaccharide-induced expression of inflammatory mediators in macrophages. *Life Sci.* 2011;88:1121-1126.

48. Min SW, Kim NJ, Baek NI, Kim DH. Inhibitory effect of eupatilin and jaceosidin isolated from Artemisia princeps on carrageenan-induced inflammation in mice. *J Ethnopharmacol.* 2009;125:497-500.