行政院國家科學委員會專題研究計畫　成果報告

桔皮苷對應力所誘發血管內皮細胞基因表現的作用以及細胞內的分子機轉

計畫類別：個別型計畫
計畫編號：NSC92-2320-B-038-034-
執行期間：92年08月01日至93年07月31日
執行單位：臺北醫學大學醫學系

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報告類型：精簡報告
報告附件：出席國際會議研究心得報告及發表論文
處理方式：本計畫可公開查詢

中　華　民　國　93年11月8日

中文摘要

桔皮苷對應力所誘發血管內皮細胞內皮素基因表現的作用

桔皮苷(hesperidin)是傳統中藥”陳皮”中所富含具強力抗氧化能力的物質，於動物實驗上，證實可抑制動脈硬化的發生以及降低血膽固醇，臨床上已有商品名為 Daflon 500 mg 的含桔皮苷之藥品，普遍運用於改善慢性靜脈閉鎖不全。然而，目前對於其在血管系統的細胞作用與分子生物機轉仍不明確。（1）內皮細胞(endothelial cells; ECs)是血管系統內層的細胞，具有調節血管舒張或收縮及其他重要功能。內皮細胞可受到機械力的作用而產生一些血流動力學上(hemodynamic)的變化。在實驗上，常以循環性應力(cyclic strain)的模式模擬血管收縮，舒張的作用，來探究培養中內皮細胞的變化。近來實驗發現；循環性應力可增加內皮細胞素-1(endothelin-1；ET-1)的基因表現。有此種作用與細胞內活性氧族群(reactive oxygen species; ROS)有關。實驗發現，循環性應力可誘發 ROS 的產生，ROS 進一步激活 Ras/Raf 的訊息傳導路徑並活化 nuclear factor-KB 和 activator protein-1 (AP-1)等轉錄因子來促進 ET-1 的基因表現。因桔皮苷為抗氧化劑可減少 ROS 的產生。所以本研究即以循環性應力的模式來探究桔皮苷對於培養中內皮細胞分泌 ET-1 的影響及其分子生物機轉。桔皮苷有抑制應力增加內皮素基因表現的作用。

關鍵詞：桔皮苷；應力；內皮素；訊息傳遞；內皮細胞；活性氧族群

英文摘要

Effect of hesperidin on cyclic strain-induced endothelin-1 gene expression in endothelial cells

The pure polyphenol hesperidin is common in juices, ascorbic acid, and the citrus juices and has beneficial properties such as being strong antioxidants. In a hamster model of atherosclerosis, the juices were able to significantly inhibit atherosclerosis and lowered cholesterol and triglycerides. Furthermore, hesperidin is clinically used in the formular of purified micronized flavonoid fraction (S5682 or registered as Daflon; containing 90% diosmin and 10% hesperidin). It is concluded that Daflon is of benefit to patients with chronic venous insufficiency. However, the cellular and molecular mechanisms of the beneficial effect of hesperidin in the vascular system remain to be elucidated.

Endothelial cells (ECs) are constantly under the influence of mechanical forces, including cyclic strain, as a consequence of vessel contraction and relaxation. Activation of several signal transduction systems has been demonstrated by hemodynamic forces in ECs. Recently, it has been proposed that the reactive oxygen species (ROS) function as second messengers in cells exposed to cyclic strain. Activation of nuclear factor-kB and activator protein-1 (AP-1), which can be regulated by ROS, are believed to be involved in the upregulation of certain genes by hemodynamic forces. Cyclic strain-induced ROS appear to be involved in the cyclic strain-induced gene expression of plasminogen activator inhibitor-1,

monocyte chemotactic protein-1, and endothelin-1 (ET-1). ROS are also involved in the cyclic strain-induced expression of the ET-1 gene through modulation of Ras/Raf/extracellular signal-regulated kinase pathway in ECs. However, the effect of hesperidin on cyclic strain-induced ET-1 gene expression and the intracellular mechanism remains to be determined.

In this project, we investigate whether hesperidin affects cyclic strain-induced ET-1 gene expression and explore its molecular mechanism in culture system. Hesperidin inhibited the strain-induced ET-1 gene expression as revealed by Nothern blotting and promoter activity assay. Hesperidin also inhibited strain-increased intracellular ROS generation as measured by a redox sensitive fluorescent dye, 2' 7'-dichlorofluorescin diacetate, and ERK phosphorylation. In summary, our results suggest that Hesperidin inhibits strain-induced ET-1 gene expression, partially by interfering with the ERK pathway *via* attenuation of ROS generation.

Keywords：hesperidin; cyclic strain; endothelin-1; proliferation; signal transduction; endothelial cells；reactive oxygen species

報告內容：

前言

It is well known that eating fruits and vegetables lowers the risk of chronic diseases such as cardiovascular disease and cancer. Recently the polyphenols have been investigated since they have been found to have beneficial properties such as being strong antioxidants(Vinson et al., 2002). The pure polyphenol hesperidin is common in juices, ascorbic acid, and the citrus juices. In a hamster model of atherosclerosis, the juices were able to significantly inhibit atherosclerosis and lowered cholesterol and triglycerides(Vinson et al., 2002). Furthermore, hesperidin is clinically used in the formular of purified micronized flavonoid fraction (S5682 or registered as Daflon; containing 90% diosmin and 10% hesperidin). However, the cellular and molecular mechanisms of the beneficial effect of hesperidin as a rational basis to explain its clinical efficacy in the vascular system remain to be elucidated.

研究目的

The goal of this project was to evaluate of the effect of hesperidin on cyclic strain-induced ET-1 gene expression in ECs.

文獻探討

It is well known that eating fruits and vegetables lowers the risk of chronic diseases such as cardiovascular disease and cancer. Recently the polyphenols have been investigated since they have been found to have beneficial properties such as being strong antioxidants(Vinson et al., 2002). The pure polyphenol hesperidin is common in juices, ascorbic acid, and the citrus juices. In a hamster model of atherosclerosis, the juices were able to significantly inhibit atherosclerosis and lowered cholesterol and triglycerides(Vinson et al., 2002). Furthermore, hesperidin is clinically used in the formular of purified micronized flavonoid fraction (S5682 or registered as Daflon; containing 90% diosmin and 10% hesperidin). In the cheek pouch preparation of diabetic hamsters, at reperfusion, after 30 min ischemia, S 5682 significantly decreased the observed macromolecular permeability(Bouskela and Donyo, 1995). In total, 183 patients were treated with Daflon 500 mg versus a control group of equal

number of patients. Daflon 500 mg produced a significant decrease in venous capacitance, venous distensibility, and venous emptying time. In addition, these changes were accompanied by improvement in clinical symptoms and a decrease in the supramalleolar circumference. It is concluded that Daflon 500 mg is of benefit to patients with chronic venous insufficiency(Geroulakos and Nicolaides, 1994). However, the cellular and molecular mechanisms of the beneficial effect of hesperidin as a rational basis to explain its clinical efficacy in the vascular system remain to be elucidated.

Endothelial cells (ECs) are constantly under the influence of hemodynamic forces, including cyclic strain, as a consequence of vessel contraction and relaxation. Activation of several signal transduction systems has been demonstrated by cyclic strain in ECs (Chien et al., 1998). Recently, it has been proposed that the reactive oxygen species (ROS) function as second messengers in cells exposed to cyclic strain (Cheng et al., 1996; Wung et al., 1997). Activation of nuclear factor-kB (NF-kB) (Khachigian et al., 1995) and activator protein-1 (AP-1) (Sumpio et al., 1994), which can be regulated by ROS (Collins, 1993; Li and Jaiswal, 1994), is believed to be involved in the upregulation of certain genes by hemodynamic forces. Cyclic strain-induced ROS appear to be involved in the cyclic strain-induced gene expression of plasminogen activator inhibitor-1 (PAI-1) (Cheng et al., 1996) and monocyte chemotactic protein-1 (MCP-1) (Wung et al., 1997) and endothelin-1 (ET-1) (Cheng et al., 2001). ET-1 is a potent vasopressor consisting of 21-amino acids. It was originally isolated from a culture of porcine endothelial cells and it has a prolonged duration of action (Yanagisawa et al., 1988). ROS are also involved in the cyclic strain-induced expression of the ET-1 gene through modulation of Ras/Raf/extracellular signal-regulated kinase (ERK) pathway in ECs (Cheng et al., 2001). However, the effect of trilinolein on cyclic strain-induced ET-1 gene expression and the intracellular mechanism remains to be determined.

In this project, we will investigate whether hesperidin affects cyclic strain-induced ET-1 gene expression and explore its molecular mechanism in culture system.

研究方法

Materials

Imubind ET-1 ELISA kits are purchased from Biochemica (ENDOTHELIN, Biomedica, Germany). ET-1 cDNA is obtained from a human endothelial cell cDNA library as previously described (Wang et al., 1993). This ET-1 cDNA is cloned into pGEM4 then excised with restriction endonuclease EcoRI and Ban HI. The ET-1 cDNA probe (652 nucleotides) is labeled with a random primer for Northern hybridization. A 4.4-kilobase (kb) or 204-bb EcoRI-BglII human ET-1 genomic fragment was cloned upstream of the chloramphenol acetyltransferase gene in p0CAT1 plasmid as previously described (Cheng et al., 2001). Other chemicals of reagent grade are obtained from Sigma (St. Louis, MO).

Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVECs) are isolated from human umbilical cords according to the procedures previously described (Cheng et al., 2001). After 3 days of

growth in medium 199 (GIBCO BRL) containing 20% fetal calf serum, endothelial cells (2.0×10^5 cells per well) are seeded on the flexible membrane base of a culture well (Flex 1, Flexcell Co. Mckeesport, PA), they are grown for 3 more days until the monolayer becomes confluent. The medium for the cultured endothelial cells is then changed to the same medium containing 2% fetal calf serum and the cells are incubated overnight before the experiment. Bovine aortic endothelial cells (BAECs) are grown for 2 or 3 days in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum, 100U penicillin, and 100 μg of streptomycin/ml before they are subcultured.

In Vitro Cyclic Strain on Cultured

Endothelial Cells

The strain unit Flexcell FX-2000 (Flexcell International Co.) consists of a vacuum unit linked to a valve controlled by a computer program. Endothelial cells cultured on the flexible membrane base are subjected to cyclic strain produced by this computer-controlled application of sinusoidal negative pressure. These flexible membranes supporting cultured cells are deformed with a pressure of -20 kPa (~25% of maximum strain) at a frequency of 1 Hz (60 cycles/min) for various time periods. After the experiments, culture supernatants, total RNA and total protein from unstrained or strained cells are collected for analysis of ET-1, ET-1 mRNA or CAT activity.

Measurement of ET-1 Concentration

ET-1 concentrations in culture media are assayed with Imubind-ET-1 ELISA kits. Briefly, the supernatant is mixed with an assay buffer, and the samples are processed according to the manufacturer's instructions. The samples are measured in duplicate and the concentration is obtained by comparing the results with a standard curve.

Detection of intracellular ROS

ROS is measured by previously described method (Shih et al., 2001). Prior to the cyclic strain treatment, ECs are incubated in culture medium containing DCFH-DA of 30 μM for 1 hour to establish a stable intracellular level of the probe. The same concentration of DCFH-DA is maintained during the cyclic strain treatment. Subsequently, the cells are washed with PBS, and removed from petri dishes by scraping, then measured for the 2',7'-dichlorofluorescein (DCF) fluorescence intensity. DCFH-DA penetrating the cells is initially converted into DCFH by cellular esterase, and DCFH is in turn oxidized to DCF in the presence of ROS. The DCF fluorescence intensity of the cells is an index of intracellular levels of ROS; and it can be determined by fluorescence spectrophotometry with excitation and emission wavelengths at 475 and 525 nm, respectively. The cell number in each sample is counted and utilized to normalize the fluorescence intensity of DCF.

RNA Isolation and Northern Blot Analysis

Total RNA is isolated from ECs by the guanidine isothiocyanate/phenochloroform method as previously described(Cheng et al., 1999). The RNA (10 μg/lane) is separated by electrophoresis on a 1% agarose formaldehyde gel and transferred onto a nylon membrane (Nytran, Schleicher & Schuell, Inc) by a vacuum blotting system (VacuGene XL, Pharmacia). After hybridization with the

^{32}P-labeled cDNA probes(Cheng et al., 2001), the membrane is washed with 1xSSC containing 1% SDS at 42°C for 30 min and then exposed to x-ray film at -70°C. Autoradiographic results are analyzed by using a densitometer (Computing Densitometer 300S, Molecular Dynamics). Blots are stripped and reprobed for 18S cDNA probe (obtained from American Type Culture Collection) to control for loading. Expression of ET-1 mRNA is quantitated and is normalized to the 18S signal.

Transfection and Chloramphenicol Acetyltransferase Assays

For the transient transfections, ECs are transfected with different expression vectors by the calcium phosphate method(Cheng et al., 1999). DNA concentration for all samples is adjusted to equal amount in each experiment. Briefly, ECs are maintained in culture for 48 h prior to transfection. The indicated expression vectors are mixed with calcium phosphate and immediately added to the EC cell culture. After incubation for 5 h, cells are then washed three times with PBS and incubated with 10 % serum DMEM. After 12 h, cells are washed with serum-free medium and incubated in serum-free medium for an additional 48 h. Cells are then treated with different agents. To correct for variability in transfection efficiency, 5 µg of pSV-β'-galactosidase plasmid DNA is cotransfected in all the experiments. The CAT and β'-galactosidase assays are performed as previously described(Cheng et al., 1999). The relative CAT activity is corrected by normalizing the respective CAT value to that of β-galactosidase activity. Cotransfected β-galactosidase activity varied less than 10% within a given experiment and is not affected by any of the experimental manipulations described. As positive and negative controls, pBLCAT2 (with thymidine kinase promoter) and pBLCAT3 (without promoter) are included in every assay.

Western Blot Analysis

Rabbit polyclonal anti–phospho-specific anti–phospho-specific ERK1/2 antibodies are purchased from New England Biolabs (Beverly, MA). Anti-ERK1/2 antibodies are purchased from Santa Cruz Biotechnology. Western blot analysis is performed as previously described(Cheng et al., 2001).

結果與討論

We found that hesperidin inhibits strain-induced ET-1 secretion, ET-1 mRNA level, and ET-1 promoter activity. Trilinolein also inhibits strain-increased reactive oxygen species (ROS) formation, and the extracellular signal-regulated kinases1/2 (ERK1/2) phosphorylation. In summary, we demonstrate for the first time that hesperidin inhibits strain-induced ET-1 gene expression, partially by interfering with the ERK1/2 pathway via attenuation of ROS formation. Thus this study provides important new insights in the molecular pathways that may contribute to the proposed beneficial effects of hesperidin in the vascular system

計畫成果自評 :研究內容與原計畫完全相符、順利達成預期目標、研究成果之學術價值甚高、適合在學術期刊發表。