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RESEARCH NOTE

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Inhibitory Effects of Naringenin, Kaempherol, and Apigenin on Cholesterol Biosynthesis in HepG2 and MCF-7 Cells

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Abstract The inhibitory effects of naringenin, kaempherol, and apigenin on the production of cholesterol in HepG2 KCLB 88065 and MCF-7 KCLB 30022 cells were evaluated. In this study, quercetin was used as a reference reagent. After incubation for 3 days, fat-soluble contents of both cell types were extracted by using the Folch method and the cholesterol contents in both cultured cells were determined by high performance liquid chromatography. The concentration of cholesterol in untreated each tissue cells was 12.2±0.11 and 8.83±0.12 mg/g of lipid, respectively. The total concentration of each flavonoid was adjusted to 0, 35, or 350 μM in the culture broth. As the results, the addition of 2% methanol and dimethyl sulfoxide (DMSO) to the media (control for flavonoid solvents) did not significantly affect cell growth; however, DMSO caused an increase in the production of cholesterol. Each flavonoid inhibited the production of cholesterol in both HepG2 and MCF-7 cells at the concentration of 35 μM above. In addition, the inhibitory effect of kaempherol on the production of cholesterol in these cells was greater than the other flavonoids tested and HepG2 cells are more sensitive to flavonoids than MCF-7. From the results, the inhibitory effects of flavonoids on cholesterol production are different depending on the cell type.

Keywords: flavonoid, cholesterol biosynthesis, naringenin, kaempferol, apigenin, HepG2, MCF-7

Introduction

Cholesterol is an indispensable component of many biological membranes, in which it regulates membrane fluidity and activates enzymes and transport mechanisms indirectly. However, excessive concentrations of cholesterol in the body result in the stifling of membrane and physiological functions and the development of atherosclerosis and emboli. That is, hypercholesterolemia is a major risk factor for cardiovascular diseases such as atherosclerosis, myocardial infarction, heart attacks, and cerebrovascular diseases, some of the leading causes of death in industrialized countries (1). Recently, it has been actively studied on lowering cholesterol by using probiotics (2-4).

Flavonoids comprise a large group of naturally occurring compounds widely distributed in the plant kingdom. Some of these compounds have been reported to contain various and potent biological activities including antioxidative, tissue-protective, and tumoristatic effects as well as the inhibition of hepatic cholesterol biosynthesis (5,6). In special, it has been known that quercetin has the inhibitory effect on cholesterol biosynthesis of HepG2. Therefore, the consumption of plant foods such as fruits, vegetables, red wines, and juices provides protection against various diseases, including cancer and cerebrovascular disease (7,8).

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These functions have been demonstrated most convincingly using appropriate *in vitro* assays that provide insight into the underlying molecular mechanisms (5). In addition, the lipid accumulation process in animal cells has been thoroughly studied and it has been proposed that phenomenon results from scavenger receptor-mediated internalization of modified low-density lipoprotein (LDL) (9). This reinforces the idea that free radicals play an important role in the development of a variety of human diseases, particularly atherosclerosis. Animal studies have also been used to evaluate the inhibitory effects of flavonoids on cholesterol production, however the inhibitory mechanism is not known, possibly due to the presence of many other factors in animal tissues. However, it appeared that the inhibitory effects of flavonoids on cholesterol biosynthesis may be different according to tissue species (5).

Therefore, the purpose of this study is to evaluate naringenin, kaempherol, and apigenin (which are representative flavonoids in plants) as inhibitors of cholesterol production in HepG2 cell (a cancer cell of hepatocytes) and MCF-7 cell (a breast cancer cell) culture. This will provide important information relevant to the pharmaceutical industry with regard to hypercholesterolemia and hypertension.

Materials and Methods

Materials HepG2 KCLB 88065 and MCF-7 KCLB 30022 cells were obtained from the Korean Cell Line Bank (KCLB; Seoul, Korea). Dulbecco's modified Engle's medium (DMEM) and Roxwell Park Memorial Institute (RPMI) 1640 medium was purchased from Gibco Lab.

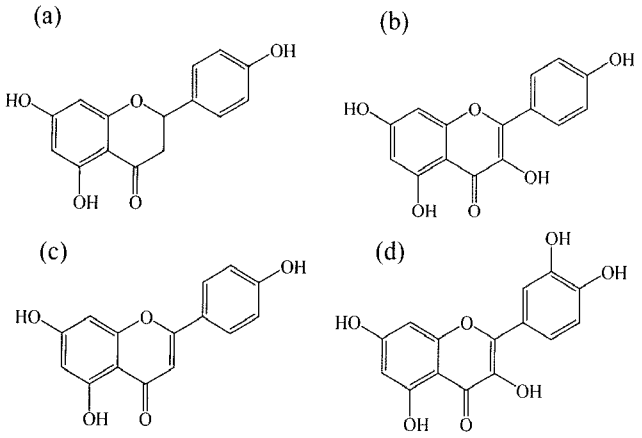

Fig. 1. Chemical structure of flavonoids. (a) Naringenin, (b) kaempferol, (c) apigenin, and (d) quercetin.

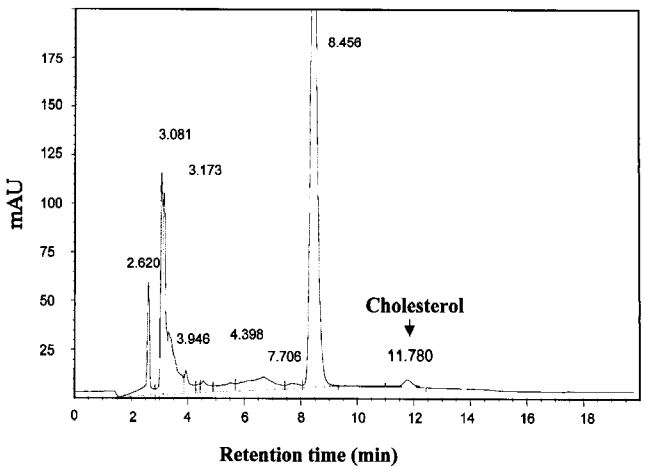

Fig. 2. HPLC chromatogram of cholesterol in HepG2 cells.

(North Andover, MA, USA) and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Both culture media were supplemented with 10% FBS and penicillin/streptomycin (100 U/mL each, Sigma-Aldrich). Naringenin, kaempferol, apigenin, and quercetin (Fig. 1) and cholesterol (free form, 5-cholesten-3β-ol) as a standard reagent for high performance liquid chromatography (HPLC) also were purchased from Sigma-Aldrich.

Cell culture HepG2 and MCF-7 cells were seeded in DMEM and RPMI containing various flavonoids. Flavonoids dissolved in 99% methanol or dimethyl surfoxide (DMSO, Sigma-Aldrich) were diluted 1:50 in culture medium such that the total concentration of flavonoids was 0 (control), 35, or 350 μM. Cultures were incubated in a humidified incubator (MCO-18AIC; Sanyo, Osaka, Japan) with a 5% CO_2 atmosphere at 37°C for 2 days (10-12).

Extraction of crude cholesterol in cells Lipids were recovered by using a modified method of Folch (13). That is, 100 mL of cell culture was added to 100 mL of solvent (chloroform:methanol=3:1), stirred rigorously, and extracted in a separatory funnel. The solvent layer was filtered and the filtrates were evaporated in a rotary vacuum evaporator (N-1000 series; Rikakikai Co., Tokyo, Japan). The concentrates were weighed, dissolved in methanol, and filtered with a 0.45-μm membrane filter (MFS-25; Advantec MFS, Inc., Dublin, CA, USA) for further analysis.

Cholesterol analysis HPLC consisted of an Agilent 1100 pump and UV detector series (1100 series; Agilent Technologies, Inc., Palo Alto, CA, USA). Cholesterol as a standard reagent was purchased from Sigma-Aldrich. Twenty μL was injected into a μ-Bondapak C_{18} column (3.9×300 mm, Waters, Milford, Ireland) (14). The mobile phase was acetonitrile-methanol (3:1) and the flow rate was 1.0 mL/min. Detection was done at 202 nm.

Statistical analysis Analysis of variance was performed for each group of 3 samples using the SAS program. Duncan's test also was used to verify the significance of differences for each treatment.

Results and Discussion

Cholesterol contents of cultured cells The HPLC pattern of cholesterol in HepG2 and MCF-7 cells is shown in Fig. 2. The concentration of cholesterol in untreated HepG2 and MCF-7 without the treatments was 12.2±0.11 and 8.83± 0.12% in total lipid of cells, respectively (Table 1 and 2). In addition, the effects of 2.0% methanol and 2.0% DMSO itself in broth as controls for the flavonoid treatments on cholesterol biosynthesis were tested (DMSO also was used as a solvent because apigenin was insoluble in methanol at this concentration range). As the results, it appeared that both 2.0% methanol and 2.0% DMSO in the broth did not significantly affect the cell growth rate (data not shown). However, in case of cholesterol production in cells, 2.0% DMSO resulted in a slight increase in the production of cholesterol in cells, although the reason for these phenomena is still unknown.

Effects of flavonoids on cholesterol production in cells In the pharmaceutical industry, chemicals such as lovastatin and simvastin have been used as inhibitors of cholesterol synthesis and to treat hypercholesterolemia (15) because these compounds inhibit 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, which catalyzes the conversion of HMG-CoA to mevalonic acid as a rate-limiting step in cholesterol biosynthesis. In this pathway, it has been known that HMG-CoA reductase is regulated by phosphorylation/ dephosphorylation (16).

Flavonoids as the natural materials also have been suggested to lower blood cholesterol levels by 2 kinds of mechanisms. Initially, it was suggested that flavonoids enhance the phosphorylation of HMG-CoA reductase indirectly and thus diminish endogenous cholesterol production (16). Secondly, flavonoids also probably exert their influence on steroid metabolism at other pivotal points. That is, flavonoids bind to the cytoplasmic steroid receptor due to the hydrophobicity of their aglycone portion, and this complex is likely to interact with steroid regulatory elements. Alternatively, the flavonoid may intercalate itself between the bases of this DNA segment and affect the transcription of genes involved in lowering the blood cholesterol level.

Table 1. Effects of flavonoids in methanol on the cholesterol contents of HepG2 and MCF-7 cells

Flavonoid	Conc. (μM)	Cholesterol content[1] (%)	
		HepG2	MCF-7
Control	Media	12.2±0.11	8.83±0.12
	Methanol	11.5±0.23	8.60±0.05
Naringenin	35	0.34±0.07	4.77±0.11
	350	0.06±0.02	4.06±0.03
Kaempferol	35	2.27±0.18	3.16±0.20
	350	Trace	Trace
Quercetin[2]	35	1.48±0.23	7.35±0.15
	350	0.52±0.11	4.30±0.23

[1]mg/g of total lipid.
[2]Used as a reference reagent.

Table 2. Effects of flavonoids in DMSO on the cholesterol contents of HepG2 and MCF-7 cells

Flavonoid	Conc. (μM)	Cholesterol content[1] (%)	
		HepG2	MCF-7
Control	Medium	12.2±0.11	8.83±0.12
	DMSO	20.0±0.23	15.4±0.20
Naringenin	35	3.65±0.15	4.15±0.02
	350	2.50±0.12	2.96±0.13
Kaempferol	35	3.70±0.04	4.26±0.11
	350	Trace	Trace
Apigenin	35	9.18±0.14	4.85±0.20
	350	2.31±0.09	0.77±0.11
Quercetin[2]	35	8.23±0.02	6.77±0.07
	350	Trace	3.16±0.23

[1]mg/g of total lipid.
[2]Used as a reference reagent.

In this study, quercetin was used as a reference because Gläβer *et al.* (5) explained that quercetin has inhibitory effects on cholesterol biosynthesis in HepG2 cells. According to the data in Table 1 and 2, it appeared that naringenin, kaempherol, apigenin, and quercetin affected the production of cholesterol in both HepG2 and MCF-7 cells at the concentration of 35 μM above. As shown from Table 1, HepG2 cells were more sensitive to the inhibitory effects of flavonoids than MCF-7 cells. At a 35 μM concentration in the methanol solvent in HepG2 cell cultures, naringenin was the most effective. However, kaempherol at a 350 μM concentration was the most effective of the 4 flavonoids tested at lowering cholesterol in both tissues. In case of MCF-7 cells, kaempherol had the greatest inhibitory effects on cholesterol production in both methanol and DMSO solvents (Table 1 and 2). Overall, the flavonoids negatively affected the production of cholesterol in both cell lines at the concentration of 35 μM above. These results support that the inhibitory effects of flavonoids toward the production of cholesterol vary depending on the flavonoids and the cell type. From these results, it appeared that the inhibitory effect of kaempherol on cholesterol production in cells is greater than that of the other flavonoids tested.

Based on these studies, it appears that the inhibitory effects of flavonoids on the synthesis of cholesterol differ depending on the type and size of molecular groups and the positions of their side chains in the flavonoid backbone structure, and the cell type being treated. The detailed mechanisms and kinetics of the inhibitory effects of flavonoids are as yet unknown. However, the results of this study suggest that flavonoids can be used clinically to treat patients with hypercholesterolemia and hypertension. In future studies, the effective concentration and synergistic effects of flavonoids used in combination will be determined.

Acknowledgments

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