J Food Sci Nutr
Vol. 8, p. 365~371 (2003)

J Food Science and Nutrition

Induction of Quinone Reductase and Glutathione S-Transferase in Murine Hepatoma Cells by Flavonoid Glycosides

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Abstract

The potential of seven flavonoid glycosides to induce quinone reductase (QR), an anticarcinogenic marker enzyme, in murine hepatoma cells (hepa1c1c7) and its mutant cells (BPRc1) was evaluated. Among test compounds, kaempferol-3-*O*-glucoside, luteolin-6-*c*-glucoside, and quercetin-3-*O*-glucoside (Q-3-G) induced QR in hepa1c1c7 cells in a dose-dependent manner. However, in BPRc1 cells lacking arylhydrocarbon receptor nuclear translocator (ARNT), only Q-3-G caused a significant induction of quinone reductase at the concentration range of 0.5 to 8 ug/mL, suggesting that it is a monofunctional inducer. Q-3-G induced not only phase 2 enzymes, including QR and glutathione-S-transferase, but also nitroblue tetrazolium reduction activity in HL-60 cells, a biochemical marker for cell differentiation promoting agents. In conclusion, Q-3-G merits further study to evaluate its cancer chemopreventive potential.

Key words: quinone reductase, cancer prevention, flavonoid glycosides, phase 2 enzymes

INTRODUCTION

A high intake of vegetables and fruits is associated with a reduced risk of many cancers and of coronary heart disease (1). Major bioactive components in edible plants including vegetables and fruits appear to be flavonoids (2). In fact, there are several reports showing an inverse association of the intake of flavonols and flavones with the incidence of cancer and coronary heart disease in prospective epidemiological studies (3,4). Flavonoids are categorized into flavonols, flavones, catechins, flavanones, anthocyanidins, and isoflavonoids. They may have beneficial health effects because of their antioxidant properties and their inhibitory role in various stages of tumour development in animal studies. Flavonoids present in foods were previously considered non-absorbable because they are bound to sugars as β-glycosides. However, Hollman and Katan (2) reported that human absorption of the quercetin glycosides from onions is far better than that of the pure aglycone.

It is widely agreed that induction of phase 2 detoxification enzymes such as NAD(P)H:quinone oxidoreductase (NQO, QR), glutathione S-transferase (GST) and elevation of glutathione is a major strategy for protecting cells against a variety of endogenous and exogenous toxic components, such as reactive oxygen species and chemical carcinogens (5). Cellular phase 2 enzymes are induced by a wide variety of seemingly unrelated chemical agents. Compounds acting as inducers of anticarcinogenic enzymes are divided into two classes: (1) bifunctional inducers, which induce phase 1 xenobiotic-metabolizing enzymes, such as the cytochome P450 1A1 system (e.g. aryl hydrocarbon hydroxylase), which act through a process involving an A*h* receptor-dependent mechanism, and subsequently generate intermediates which transcriptionally activate genes encoding phase 2 enzymes, including QR; (2) monofunctional inducers, which induce phase 2 enzymes directly without inducing the phase 1 enzymes, and operate independently of A*h* receptors (6-8). Bifunctional inducers appear less likely to show anticarcinogenic activity since they also induce phase 1 enzymes. In fact, some bifunctional inducers may activate pro-carcinogens into ultimate carcinogens. Monofunctional inducers, therefore, are considered to be more promising as anticarcinogenic agents since they induce QR, and other protective phase 2 enzymes, without promoting the activation of carcinogens (9).

Previous studies confirmed that some flavonoids induce QR in cultured cell systems (10,11). Some flavonoids are bi-functional and others are monofuctional

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inducers. Many studies have examined the biological effects of aglycone forms of flavonoids, while the forms of flavonoids that are most abundant in edible plants are the glycosides. Therefore, these glycoside derivatives are more relevant in a dietary context than the widely studied aglycone counterparts.

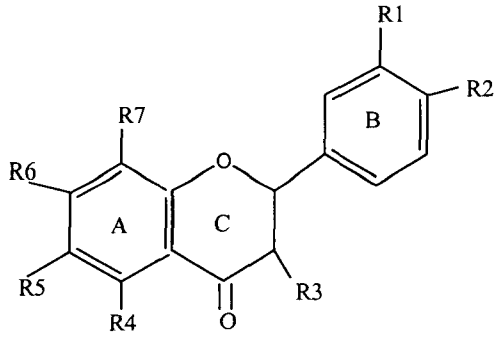
This study was therefore performed to identify naturally occurring flavonoid glycosides that induce QR, an anticarcinogenic biomarker, in a cultured cell model system.

MATERIALS AND METHODS

Materials

The seven flavonoids (Fig. 1) tested in the study were apigenin-8-c-glucoside, apigenin-6-c-glucoside, kampferol-3-*O*-galactoside, kampferol-3-*O*-glucoside, luteolin-6-c-glucoside, quercetin-3-*O*-glucosyl galactoside, and quercetin-3-*O*-glucoside (Q-3-G). These compounds were isolated from *Atractylodes japonicus* (Korean name: sabju) and *Ligusticum jeholense* (Korean name: Tocheongung) using solvent fractionation and column chromatography (Chung et al., in preparation). The chemical structures of flavonoid derivatives were confirmed by by FAB-MS and ^{13}C-NMR. Tertiary butylhydroquinone (tBHQ, Sigma) was used as a positive control of QR induction.

All cell culture reagents and fetal bovine serum (FBS) were obtained from Gibco BRL (Gaitherburg, MD, USA). Hepa1c1c7 and BPRc1 cells were from American Type Culture Collection (Rockville, MD, USA). All other chemicals were of reagent grade.



Cell culture

Hepa1c1c7 and its mutant (BPRc1) cells were plated at a density of 3×10^5 and 5×10^5 cells per 100 mm plate in 10 mL of α-minimal essential medium (MEM) supplemented with 10% FBS, respectively. The plates were normally incubated for 3~4 days in a humidified incubator in 5% CO_2 at 37°C. Cells were cultured for 48 hrs, exposed to various concentrations of flavonoids for another 24 hrs, followed by biochemical assays.

Biochemical assays

QR activity was measured by a spectrophotometric assay in which the rate of reduction of 2,6-dichlorophenolindophenol was monitored at 600 nm (12). Glutathinone *S*-transferase activity was assayed by the method described by Habig (13), with 1-chloro-2,4-dinitrobenzene as a substrate. Total cellular glutathione (GSH) was determined by rate measurements in a recycling assay (14). In brief, cells were grown for 24 hrs in 96-well plates (10,000 per well for hepa1c1c7 and BPRc1 cells), exposed to serial dilutions of flavonoids for 24 hrs, and finally lysed in 50 μL of 0.08% digitonin. One half of the wells were used for protein determination. The other half received 50 μL of ice-cold metaphosphoric acid (50 g/liter) in 2 mM EDTA to precipitate cellular protein. After 10 min at 4°C, plates were centrifuged at $1,500\times g$ for 15 min and 50 μL of the resulting supernatant fractions was transferred to the corresponding wells of a parallel plate. To each of these wells, 50 μL of 200 mM sodium phosphate buffer, pH 7.5, containing 10 mM EDTA, was added and GSH content was measured by rate measurements in a recycling assay.

Measurement of intracellular concentration of Q-3-G

Hepa1c1c7 and its mutant (BPRc1) cells plated at density of 3×10^5 and 5×10^5 cells per 100 mm plate were treated with 8 ug/mL Q-3-G in 10 mL of α-MEM supplemented with 10% FBS for different periods, and were subjected to HPLC analysis. Cells were disrupted by sonication (50W, Kontes, Vineland, NJ, USA) and filtered through 0.2 u syringe nylon filter (Nunc, USA),

	R1	R2	R3	R4	R5	R6	R7	C_2-C_3
Apigenin-6-c-glucoside	H	OH	H	OH	Glucose	OH	H	Double
Apigenin-8-c-glucoside	H	OH	H	OH	H	OH	Glucose	Double
Kaempferol-3-*O*-glucoside	H	OH	Glucose	OH	H	OH	H	Double
Kaempferol-3-*O*-galactoside	H	OH	Galactose	OH	H	OH	H	Double
Luteolin-6-c-glucoside	OH	OH	H	OH	Glucose	OH	H	Double
Quercetin-3-O-glucoside	OH	OH	Glucose	OH	H	OH	H	Double
Quercetin-3-O-glucosyl-galactose	OH	OH	Glucosyl-galactose	OH	H	OH	H	Double

Fig. 1. Structures of flavonoid glycosides used in the study.

injected into Jasco HPLC (PU-1580, Tokyo, Japan) and detected at 254 nm using a UV-visible detector (Young-In Scientific Co., Seoul, Korea). The mobile phases were solvent A, 1% acetic acid in acetonitrile; and solvent B, 1% acetic acid in water (10 : 90), and eluted isocratically. The flow rate was 0.8 mL/min. The Shiseido capcell C18 ODS 4.6×250 mm column was used. The volume of sample injected was 20 uL.

Assay of differentiation

The potential of compounds to promote cell differentiation was evaluated by determining the nitro blue tetrazolium (NBT)-reducing activity as reported by Suh and coworkers (15). In brief, HL-60 cells (1.2×10^5 cells/well) were plated onto 24-well plate with 1 mL Eagle's modified minimum essential medium (EMEM) containing 10% FBS. After 18 hrs of incubation, an agent dissolved in dimethylsulfoxide (DMSO) was added into each well, followed by 98 hrs of incubation at 37°C, 5% CO_2. After incubation, the cells were mixed with an equal volume of freshly prepared solution containing 1 ug/mL phorbol-12-myristate-13-acetate (Sigma) and 2 mg/mL NBT (Sigma), and incubated for 60 min at 37 °C. A minimum of 100 cells was counted and the percentage of NBT-positive cells was assessed under the inverted microscope.

Statistical analysis

Statistical significance of enzyme activity, GSH content, and degree of cell differentiation data was tested by analysis of variance, followed by Duncan's multiple range test, using SPSS software (SPSS Inc, Chicago, IL, USA). The probability of $p \leq 0.05$ means statistical difference among treatment groups.

RESULTS

Among the seven compounds tested in this study, kaempferol-3-*O*-glucoside, luteolin-6-*O*-glucoside, and Q-3-G caused a significant induction of quinone reductase (QR) in hepa1c1c7 cells at the concentration range of 0.5 to 8 ug/mL (Fig. 2). That is, kaempferol-3-*O*-glucoside, luteolin-6-*O*-glucoside, and Q-3-G induced QR activity by 2.2-, 1.5-, and 1.8-fold, respectively, compared to the enzyme activity of the control cells exposed to the vehicle (DMSO). The other flavonoid derivatives did not cause a dose-dependent induction in the enzyme activity in the concentration range of flavonoid glycosides used.

In the experiment where BPRc1 cells were used, only Q-3-G caused a significant induction of quinone reductase at the concentrations of 4 and 8 ug/mL while the other flavonoids were ineffective (Fig. 3). Q-3-G increased cellular QR activity by 1.2- and 1.6-fold of the control at the concentrations of 4 and 8 ug/mL, respectively. The potential of Q-3-G to induce QR activity in BPRc1 cells was comparable to that of tertiary butyl hydroquinone (tBHQ), a well-known QR inducer, which caused 1.6-fold induction of QR activity at the concentration of 20 uM.

Glutathione S-transferase (GST) activity, one of the phase 2 enzymes, was also induced by Q-3-G in a dose-dependent manner (Fig. 4). Specifically, Q-3-G caused a significant increase in GST activity at the concentrations of 4 and 8 ug/mL. However, treatment with Q-3-G did not affect the cytosolic GSH level in BPRc1 cells.

The cell differentiation-promoting activity of flavonoid glycosides was evaluated by determining the potential to reduce nitroblue tetrazolium inherent in granulocytic cells differentiated from HL-60 human promyelocytic leukaemia cells. As shown in Table 1, only Q-3-G out of 7 flavonoids resulted in the statistically significant differentiation of HL-60 cells. Q-3-G appeared to be transported into the cells intact, without being converted into the aglycone form since its intracellular concentration reached a maximum 7 and 1.8 ug/mg protein within 60 min in hepa1c1c7 and BPRc1 cells, respectively. The concentration of Q-3-G rapidly decreased to almost undetectable levels immediately after the maximum concentration was attained.

DISCUSSION

Some naturally occurring flavonoid glycosides were evaluated for their potential to modulate quinone reductase and glutathione S-transferase, two major phase 2 enzymes involved in detoxification of environmental carcinogens. Among seven compounds examined in the study, three flavonoids including kaempferol-3-*O*-glucoside, luteolin-6-*O*-glucoside, and Q-3-G showed a significant induction of QR activity at concentrations of 4 ug/mL or higher. The ability of these glycoside forms to induce QR suggests that the compounds can be transported into the cell and/or nuclear compartment to regulate the expression.

QR inducers are classified into two groups, monofunctional and bifunctional (16). Our study showed that quercertin-3-*O*-glucoside is a monofunctional inducer, since it induced QR in BPRc1 cells lacking ARNT. The QR gene promoter contains an antioxidant response element, xenobiotic response element, and activator protein 2 elements, which are responsible for regulating gene expression. In fact, several antioxidants have been reported to increase QR gene expression (17-20). Q-3-G,

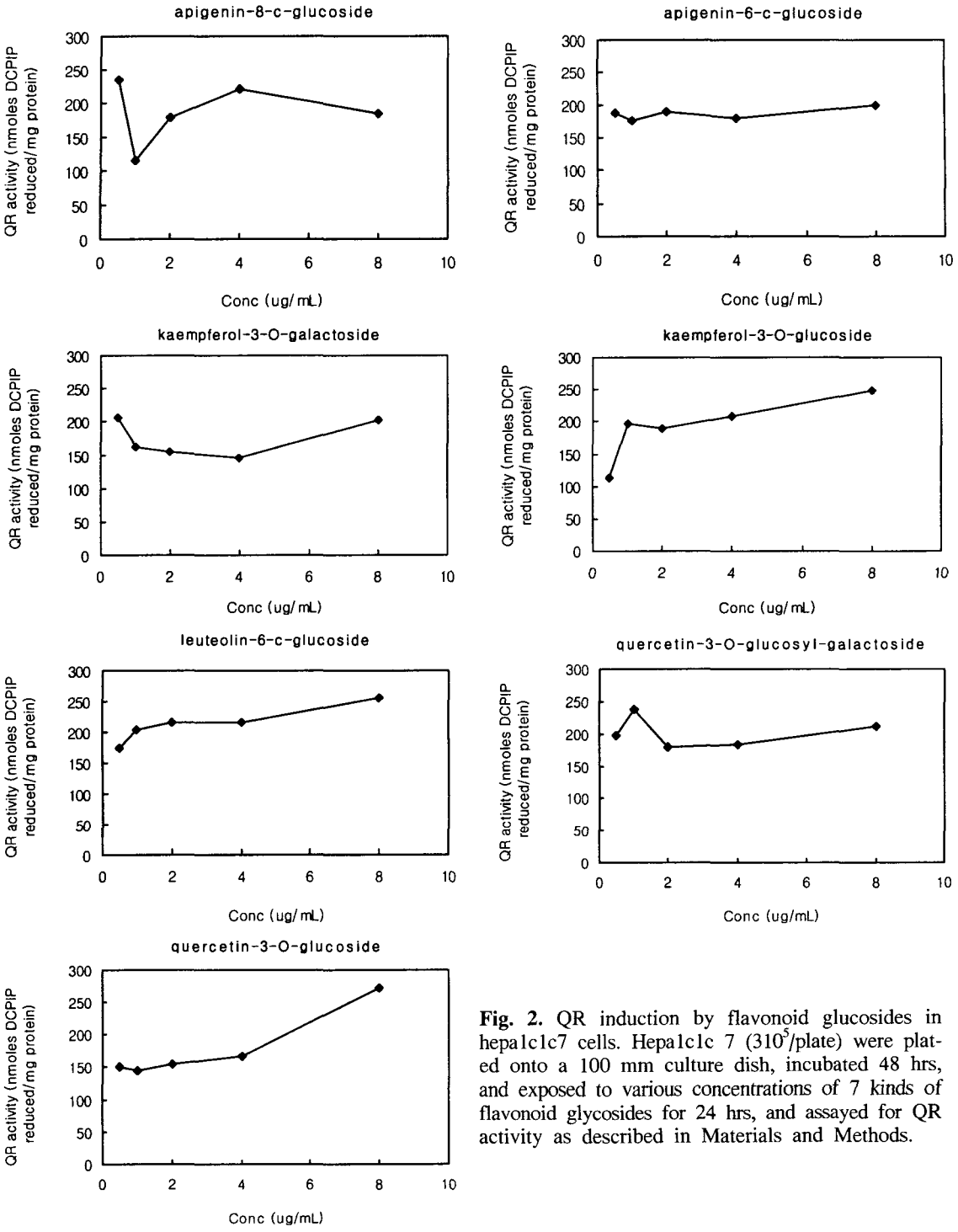


Fig. 2. QR induction by flavonoid glucosides in hepa1c1c7 cells. Hepa1c1c 7 (310^5/plate) were plated onto a 100 mm culture dish, incubated 48 hrs, and exposed to various concentrations of 7 kinds of flavonoid glycosides for 24 hrs, and assayed for QR activity as described in Materials and Methods.

which has potent antioxidative activity, appears to cause a significant induction of QR activity through the antioxidant response element in the promoter of the QR gene.

Meanwhile, Williamson and coworkers (10) reported that quercetin-4′-glucoside and quercetin were effective inducers of QR, while phase 2 enzyme inducers had a strong antioxidant activity. Most compounds with antioxidant activity appear to have the potential to induce QR activity because they are usually electrophiles promoting the transcriptional activation of phase 2 enzymes. Meanwhile, the number of hydroxyl groups in the flavone B-ring seems to have an effect on QR induction activity since quercetin and luteolin glycosides, both with two hydroxyl groups, appeared to have a stronger QR inducing activity than apigenin and kaempferol glycosides which contain one hydroxyl group.

While Q-3-G induced GST activity at 4 ug/mL or

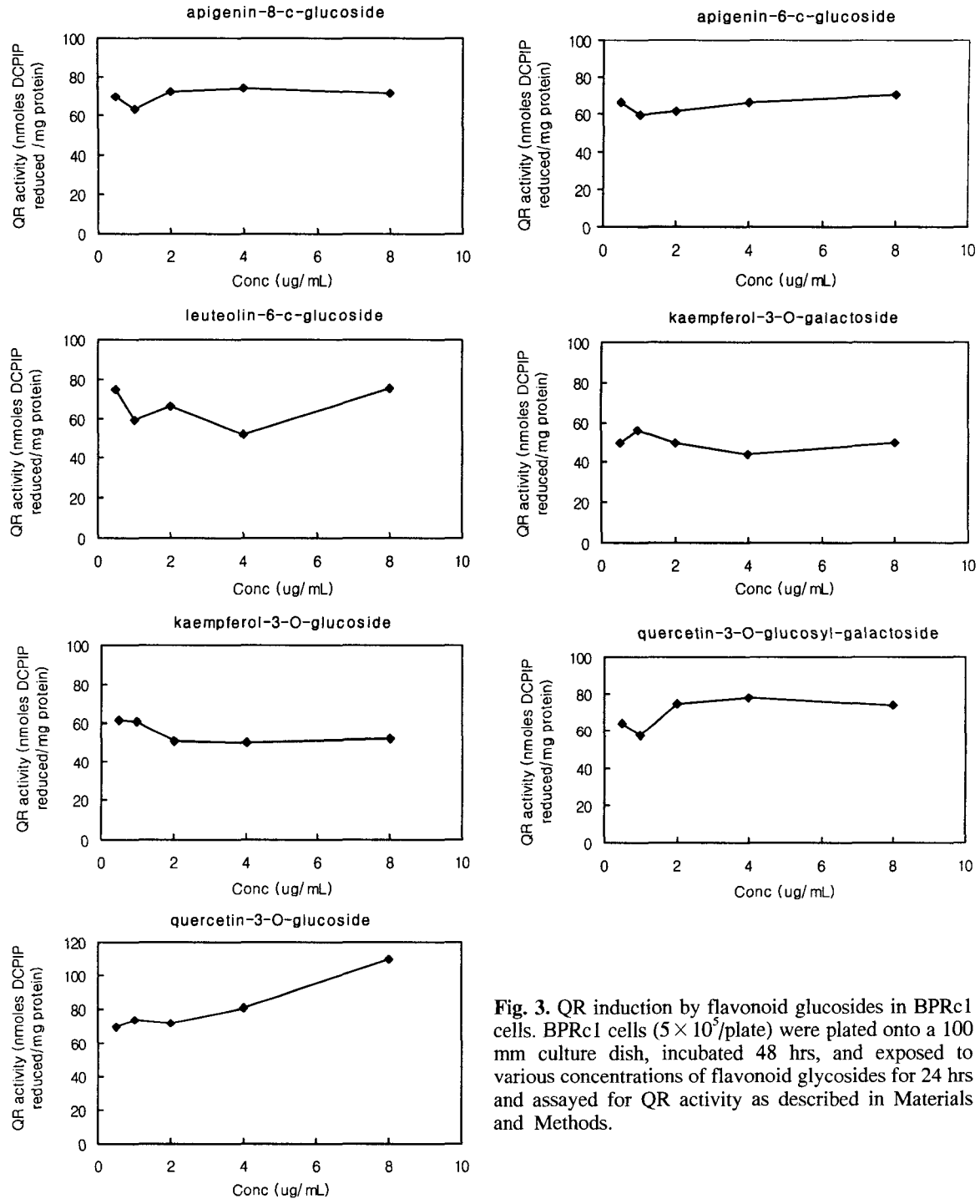


Fig. 3. QR induction by flavonoid glucosides in BPRc1 cells. BPRc1 cells (5×10^5/plate) were plated onto a 100 mm culture dish, incubated 48 hrs, and exposed to various concentrations of flavonoid glycosides for 24 hrs and assayed for QR activity as described in Materials and Methods.

above, it did not have a significant effect on the cellular level of GSH. The compound, therefore, appeared to have little effect on the activity of L- γ -glutamyl- L-cysteine synthase, a rate-limiting enzyme in GSH synthesis.

Hollman and coworkers (21) reported that flavonoid glycosides were preferentially absorbed compared to the flavonoid aglycone. Our study also showed that a significant amount of Q-3-G but no aglycone form of quercetin was accumulated in hepa1c1c7 cells incubated in the presence of Q-3-G (4 ug/mL) in the cell culture medium, suggesting the lack of β-glucosidase activity in the cell membrane (Fig. 5). Although the transport mechanism of flavonoid glycosides is not clear, the study by Holman and coworkers suggested that the glucose transporter (SGLT-1) is responsible for the transport of quercetin glucosides across intestinal epithelial cells (21). However, there is also a possibility that some cells contain β-glucosidase activtiy and uptake the flavonoids

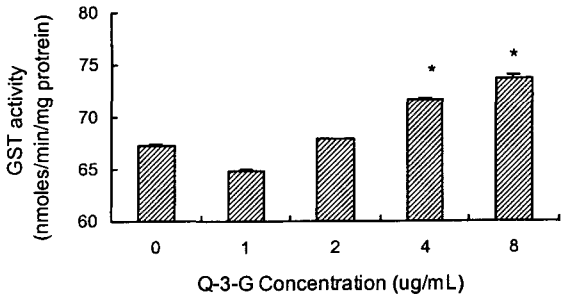


Fig. 4. Effect of quercetin-3-glucoside on glutathione-S-transferase (GST) in BPRc1 cells. BPRc1 cells (5×10^5/plate) were plated onto a 100 mm culture dish, incubated 48 hrs, and exposed to various concentrations of flavonoid glycosides for 24 hrs, and assayed for GST activity as described in Materials and Methods. Values are mean ± SD (n=3). The asterisk above bars represents 'significantly different' from the control (vehicle).

Table 1. NBT-reducing activities in HL-60 cells treated with flavonoid glycosides

Samples	NBT reducing activity (%)
Vehicle (DMSO)	20.6 ± 5.2[1)]
Apigenin-6-c-glucoside	17.7 ± 2.8
Apigenin-8-c-glucoside	24.0 ± 10.7
Kaempferol-3-*O*-glucoside	24.8 ± 7.8
Kaempferol-3-*O*-galactoside	21.6 ± 4.1
Quercetin-3-*O*-glucoside	31.3 ± 2.8*
Quercetin-3-*O*-glucosyl-galactoside	26.4 ± 3.2
Leuteolin-6-c-glucoside	26.3 ± 2.3

[1)]Data represent the mean ± SD (n=3).
*Value is significantly different from the control (vehicle) ($p < 0.05$).

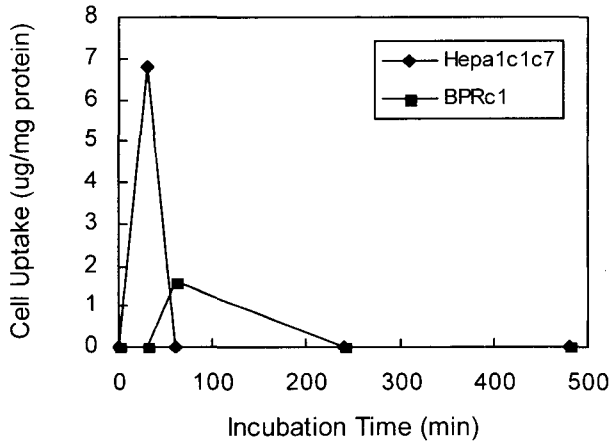


Fig. 5. Intracellular accumulation of Q-3-G. Hepa1c1c7 cells (3×10^5/plate) and BPRc1 cells (5×10^5/plate) were plated onto a 100 mm culture dish, incubated 48 hrs, and exposed to 8 ug/mL of Q-3-G for different periods, followed by the measurement of intracellular Q-3-G by HPLC as described in Materials and Methods.

after being converted into aglycone forms. Recently, Nemeth and coworkers (22) showed that the absorption of dietary flavonoid glycosides in humans involves a critical deglycosylation step that is mediated by epithelial β-glucosidases, and the significant variation in β-glucosidase activity between individuals might be a factor determining variation in flavonoid bioavailability. The negligible concentration of intracellular Q-3-G after 60 min incubation indicates either rapid metabolism of the compound in the cells or the mobilization of fast transport system which pumps the compound out of cells. Walgren and coworkers (23) emphasized that even if quercetin glucosides are capable of entering the cell, they are easily excreted from the cell via the multidrug resistance-associated protein-2 (MRP-2).

The induction of differentiation of HL-60 human myelocytic leukemia cells is a common biomarker of cancer preventive potential. For instance, all-*trans*-retinoic acid, a potent inducer of the granulocytic differentiation of HL-60 cells has been used to treat acute promyelocytic leukemia. This study showed that Q-3-G promoted the differentiation of HL-60 cells. However, the mechanism by which Q-3-G induces the granulocytic differentiation remains to be clarified (24). There are many ways to induce cell differentiation, and the inducers of cell differentiation may act by different mechanism to produce more or less identical end stage cells, reflecting the flexibility in the differentiation program.

Taken together, our data suggest that Q-3-G has significant anti-carcinogenic potential since it induced the activities of detoxifying phase 2 enzymes and stimulated the differentiation of malignant cells.

ACKNOWLEDGEMENT

This work was supported by a grant from BioGreen 21 Program, Rural Development Administration, Republic of Korea.

REFERENCES

1. Kris-Etherton PM, Hecker KD, Bonanome A, Coval SM, Binkoski AE, Hilpert KF, Griel AE, Etherton TD. 2002. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. *Am J Med* 13 (Suppl 9B): 71S-88S.
2. Hollman PCH, Katan MB. 1999. Dietary flavonoids: Intake, health effects and bioavailability. *Food Chem Toxicol* 37: 937-942.
3. Formica JV, Regelson W. 1995. Review of the biology of quercetin and related bioflavonoids. *Food Chem Toxicol* 33: 1061-1080.
4. Cook NC, Saman S. 1996. Flavonoids- Chemistry, metabolism, cardioprotective effects, and dietary sources. *Nutr Biochem* 7: 66-76.
5. Ye L, Zhang Y. 2001. Total intracellular accumulation levels of dietary isothiocyanates determine their activity in elevation of cellular glutathione and induction of Phase 2 detoxification enzymes. *Carcinogenesis* 22: 1987-1992.

6. Prestera T, Zhang YS, Spencer SR, Wilczak CA, Talalay P. 1993. The electrophile counterattack response-Protection against neoplasia and toxicity. *Adv Enzyme Regul* 33: 281-296.
7. Rushmore TH, Pickett CB. 1990. Transcriptional regulation of the rat glutathione S-transferase Ya subunit gene-Characterization of a xenobiotic-responsive element controlling inducible expression by phenolic antioxidants. *J Biol Chem* 265: 14648-14653.
8. Talalay P, Delong MJ, Prochaska HJ. 1988. Identification of a common chemical signal regulating the induction of enzymes that protect against chemical carcinogenesis. *Proc Natl Acad Sci USA* 85: 8261-8265.
9. Talalay P. 1989. Mechanisms of induction of enzymes that protect against chemical carcinogenesis. *Adv Enzyme Regul* 28: 237-250.
10. Williamson G, Plumb GW, Uda Y, Price KR, Rhodes MJC. 1996. Dietary quercetin glycosides: Antioxidant activity and induction of the anticarcinogenic phase II marker enzyme quinone reductase in Hepalclc7 cells. *Carcinogenesis* 17: 2385-2387.
11. Yanaida Y, Kohno H, Yoshida K, Hirose Y, Yamada Y, Mori H, Tanaka T. 2002. Dietary silymarin suppresses 4-nitroquinoline 1-oxide-induced tongue carcinogenesis in male F344 rats. *Carcinogenesis* 23: 787-794.
12. Benson AM, Hunkeler MJ, Talalay P. 1980. Increase of NAD(P)H:quinone reductase by dietary antioxdants; Possible role in protection against carcinogenesis and toxicity. *Proc Natl Acad Sci USA* 77: 5216-5220.
13. Habig WH, Pabst MJ, Jakoby WB. 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* 249: 7130-7139.
14. Anderson ME. 1985. Determination of glutathione and glutathione disulfide in biological samples. *Methods Enzymol* 113: 548-555.
15. Suh N, Luyengi L, Fong HH, Kinghorn AD, Pezzuto JM. 1995. Discovery of natural product chemopreventive agents utilizing HL-60 cell differentiation as a model. *Anticancer Res* 15: 233-239.
16. Zhang YS, Talalay P, Cho CG, Posner GH. 1992. A major inducer of anticarcinogenic protective enzymes from broccoli-Isolation and elucidation of Structure. *Proc Natl Acad Sci USA* 89: 2399-2403.
17. Kwon CS, Kim J-S. 1999. Induction of quinone reductase, an anticarcinogenic marker enzyme, by vitamin E in both hepalclc7 cells and mice. *J Food Sci Nutr* 4: 122-124.
18. Valerio LG Jr, Kepa JK, Pickwell GV, Quattrochi LC. 2001. Induction of human NAD(P)H:quinone oxidoreductase (NQO1) gene expression by the flavonol quercetin. *Toxicol Lett* 119: 49-57.
19. Okada K, Wangpoengtrakul C, Tanaka T, Toyokuni S, Uchida K, Osawa T. 2001. Curcumin and especially tetrahydrocurcumin ameliorate oxidative stress-induced renal injury in mice. *J Nutr* 131: 2090-2095.
20. Joseph P, Long DJ 2nd, Klein-Szanto AJ, Jaiswal AK. 2000. Role of NAD(P)H:quinone oxidoreductase 1 (DT diaphorase) in protection against quinone toxicity. *Biochem Pharmacol* 60: 207-214.
21. Hollman PC, de Vries JH, van Leeuwen SD, Mengelers MJ, Katan MB. 1995. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *Am J Clin Nutr* 62: 1276-1282.
22. Nemeth K, Plumb GW, Berrin JG, Juge N, Jacob R, Naim HY, Williamson G, Swallow DM, Kroon PA. 2003. Deglycosylation by small intestinal epithelial cell beta-glucosidases is a critical step in the absorption and metabolism of dietary flavonoid glycosides in humans. *Eur J Nutr* 42: 29-42.
23. Walgren RA, Walle UK, Walle T. 1998. Transport of quercetin and its glucosides across human intestinal epithelial Caco-2 cells. *Biochem Pharmacol* 55: 1721-1727.
24. Pae HO, Oh H, Choi BM, Oh GS, Paik SG, Jeong S, Hwang KM, Yun YG, Chung HT. 2002. Differentiation-inducing effects of verticinone, an isosteroidal alkaloid isolated from the bulbus of *Fritillaria ussuriensis*, on human promyelocytic leukemia HL-60 cells. *Biol Pharm Bull* 25: 1409-1411.

(Received August 8, 2003; Accepted October 18, 2003)