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Flavonoids Are Potential Inhibitors of Glucose Uptake in U937 Cells

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Flavonoids are a group of polyphenolic compounds ubiquitously found in plants including fruits, and vegetables. Broad ranges of the biological activities of flavonoids have been reported using *in vitro* studies. I report that several natural flavonoids blocked glucose uptake in myelocytic U937 cells. Although there were some variations in the blocking activity of individual flavonoids, approximately half of the glucose uptake was blocked by flavonoids at the concentrations of 8–50 μM. The decreasing order of the blocking activity was fisetin ≥ myricetin ≥ quercetin ≥ apigenin > genistein > cyanidin > daidzein ≥ hesperetin > naringenin > catechin. Fisetin showed approximately 50% inhibition of glucose uptake at a concentration of 8 μM. Similar patterns of the inhibition were observed in lymphocytic Jurkat cells. Fisetin and quercetin inhibited glucose transport in a competitive manner. K_i values for fisetin and quercetin were proximately 9 and 12 μM, respectively. This study showed that some types of natural flavonoids block glucose uptake in U937 cells and that natural flavonoids could be used as alternative blockers of glucose uptake *in vitro*.

Flavonoids are widely distributed in plant-derived foods including fruits and vegetables (1). The primary structure of flavonoids consists of two aromatic carbon groups; benzopyran (A and C rings) and benzene (B ring) (Fig. 1). The variation in the heterocyclic C-ring of flavonoids and the interlinkage between benzopyran and benzene groups are the basis for the classification of flavonoids into the flavone, flavonol, flavonone, isoflavone, anthocyanidin, and catechin groups (2).

Flavonoids have been proposed to have a variety of biological effects on human health (3). For example, *in vitro*, flavonoids were reported to reduce low-density lipoprotein oxidation (4) and quench reactive oxygen radicals, (5) thus decreasing the risk of chronic diseases (e.g., cardiovascular diseases and cancer) (6), and to inhibit or induce enzymes (7). Although the biological effects of flavonoids on humans have been documented, their biological mechanisms have not yet been defined. Previously, genistein, an isoflavone, has been reported to be an inhibitor of a glucose transporter (8). However, this study examined the inhibitory effect of only two isoflavonoids (genistein and daidzein) on glucose uptake. Also, some conflicting data were reported regarding the inhibitory effects of flavonoids on glucose transport (9, 10). Due to the inconsistency of an ability to inhibit the glucose uptake in cells, and the use of limited numbers of flavonoids in the studies, the chemical structure of flavonoids responsible for blocking the transport of glucose has not been described, and the potential of other flavonoids to block glucose transport has not been studied.

In this paper I tested more than 10 flavonoids to identify potent blockers for glucose transport. Apigenin, quercetin, rutin, fisetin, myricetin, naringenin, naringin, hesperetin, genistein, daidzein, catechin, and cyanidin were selected for study on the basis of flavonoid group, degree of hydroxylation, and interlinkage between benzopyran and benzene groups. The diversity of flavonoids and its analogues made it possible to identify flavonoids as inhibitors for glucose uptake and to characterize a moiety of flavonoid structure as a responsible site for the inhibition of the glucose transporter. Data indicated that in addition to genistein, other flavonoids also showed an inhibitory effect on glucose uptake. The possible involvement of flavone structure and hydroxylation of flavonoids in blocking glucose uptake was also discussed.

MATERIALS AND METHODS

Materials. Apigenin, quercetin, rutin, fisetin, myricetin, naringenin, naringin, hesperetin, genistein, daidzein, catechin, and catechol were purchased from Sigma (St. Louis, MO). Cyanidin coumarin, chromone, 4-chromanone, flavone, 3-hydroxyflavone, 5-hydroxyflavone, 7-hydroxyflavone, and 5,7-dihydroxyflavone were obtained from Indofine Chemical Company, Inc (Somerville, NJ). U937 and Jurkat cells were purchased from ATCC (Rockville, MD).

Cell culture condition. U937, and Jurkat, cells were cultured in RPMI medium supplemented with 10% fetal bovine serum. Cell viability was determined microscopically by trypan blue exclusion

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(11). The cells were grown to a concentration of 5×10^6/ml for the uptake experiments. The number of cells was counted by hematocytometer.

Glucose uptake assays. For inhibition study on uptake, $1–2 \times 10^6$ cells were suspended in 1 ml HEPES/phosphate buffer containing 147 mM NaCl, 5 mM KCl, 1.9 mM KH_2PO_4, 1.1 mM Na_2HPO_4, 5.5 mM glucose, 0.3 mM $MgSO_4$:$7H_2O$, 1 mM $MgCl_2$:$6H_2O$, 0.3 mM $CaCl_2$:$2H_2O$, and 10 mM HEPES, pH 7.4. Individual flavonoids were added to the cell suspension, as indicated in Figs. 2–5. The reaction was initiated by adding 1.0 μCi 2-[1,2-^{3}H(N)] deoxy-D-glucose (specific activity 25 mCi/mmol). After 3 min, the reaction was terminated by washing the cells twice in cold phosphate-buffered saline (pH 7.4) (11). Uptake activity was measured in whole cells using scintillation spectrometry. To test the sodium dependence of the blocking activities of flavonoids, the sodium free buffer was prepared by replacing, NaCl and Na_2HPO_4 in HEPES buffer with choline chloride and K_2HPO_4.

Kinetic analysis. Kinetics for all substrates were determined in the linear range of the transport activity. Analysis of the inhibition of the glucose uptake by flavonoids was performed using a non-linear regression program (12). K_i was determined using an Eadie-Hofstee plot. Data points in all figures represent the mean ± SD of more than 3 samples.

RESULTS

Effect of flavonoids on glucose uptake in U937 cells. To measure the blocking activities of flavonoids, the flavonoids were divided into 6 groups; flavone (apigenin), flavonol (fisetin, myricetin, quercetin), flavonone (naringenin, hesperetin), isoflavone (genistein, daidzein), catechin, and anthocyanidin (cyanidin) (Fig. 1). As shown in Fig. 2, except for catechin, all flavonoids inhibited glucose uptake in U937 cells in a dose-dependent manner. Approximately half of the uptake activity was blocked at 8–50 μM concentrations of the tested flavonoids. The decreasing order of the effectiveness was fisetin ≥ myricetin ≥ quercetin ≥ apigenin > genistein > cyanidin > daidzein ≥ hesperetin > naringenin > catechin. For the most effective flavonoid, fisetin, 50% inhibition occurred at approximately 8 μM. Similar results were obtained with a different cell line (Jurkat cells, data not shown). Although there were some variations among flavonoids in the concentration to achieve 50% inhibition of glucose uptake in U937 and Jurkat cells, the general pattern of the inhibition was similar. 16–60 μM concentrations of the tested flavonoids were required to obtain 50% inhibition in Jurkat cells. All the flavonoids tested contain benzopyran (A and C rings) and a phenyl group (B ring) (Fig. 1). Since there is a slight difference between flavone (apigenin) and isoflavones (genistein and daidzein) in the efficacy of blocking glucose uptake (Fig. 2A and 2D), the interlinkage between the B and C rings may have a slight effect on the blocking efficacy. The additional hydroxylations at the 3 position of benzopyran and 3′-position of the phenyl group seem dispensable for the blocking activity, because flavonols (fisetin, myricetin, quercetin) and flavone (apigenin) showed no difference in the blocking effect (Figs. 2 and 3). However, saturation of a 2,3 double bond in the C ring seems to decrease the blocking effect on glucose uptake, since the inhibitory effects of naringenin and hesperetin were not as strong as those of quercetin and fisetin (Fig. 2C). In Fig. 2E, Cyanidin and catechin were tested for their blocking activities of glucose uptake. Cyanidin containing 1,2 and 3,4 double bonds, and no ketone at the 4 position exhibited a similar, but smaller inhibitory effect on glucose uptake than that of quercetin. Surprisingly, catechin which lacks a 2,3 double bond and 4 position ketone in C ring showed no

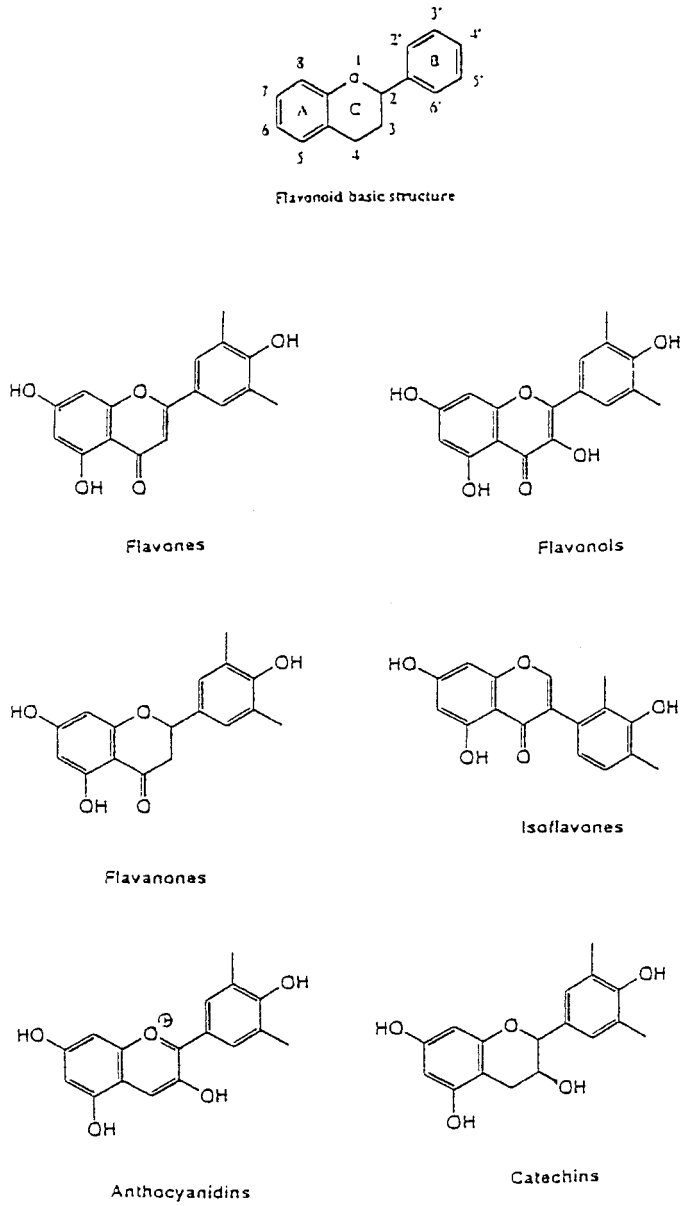

FIG. 1. Structures of flavonoids. The basic structure of flavonoids is composed of benzopyran and benzene groups. Apigenin is classified as flavones. Fisetin, myricetin, and quercetin are flavonols. Naringenin and hesperetin are flavononones. Genistein and daidzein are isoflavones. Cyanidin belongs to anthocyanidin group.

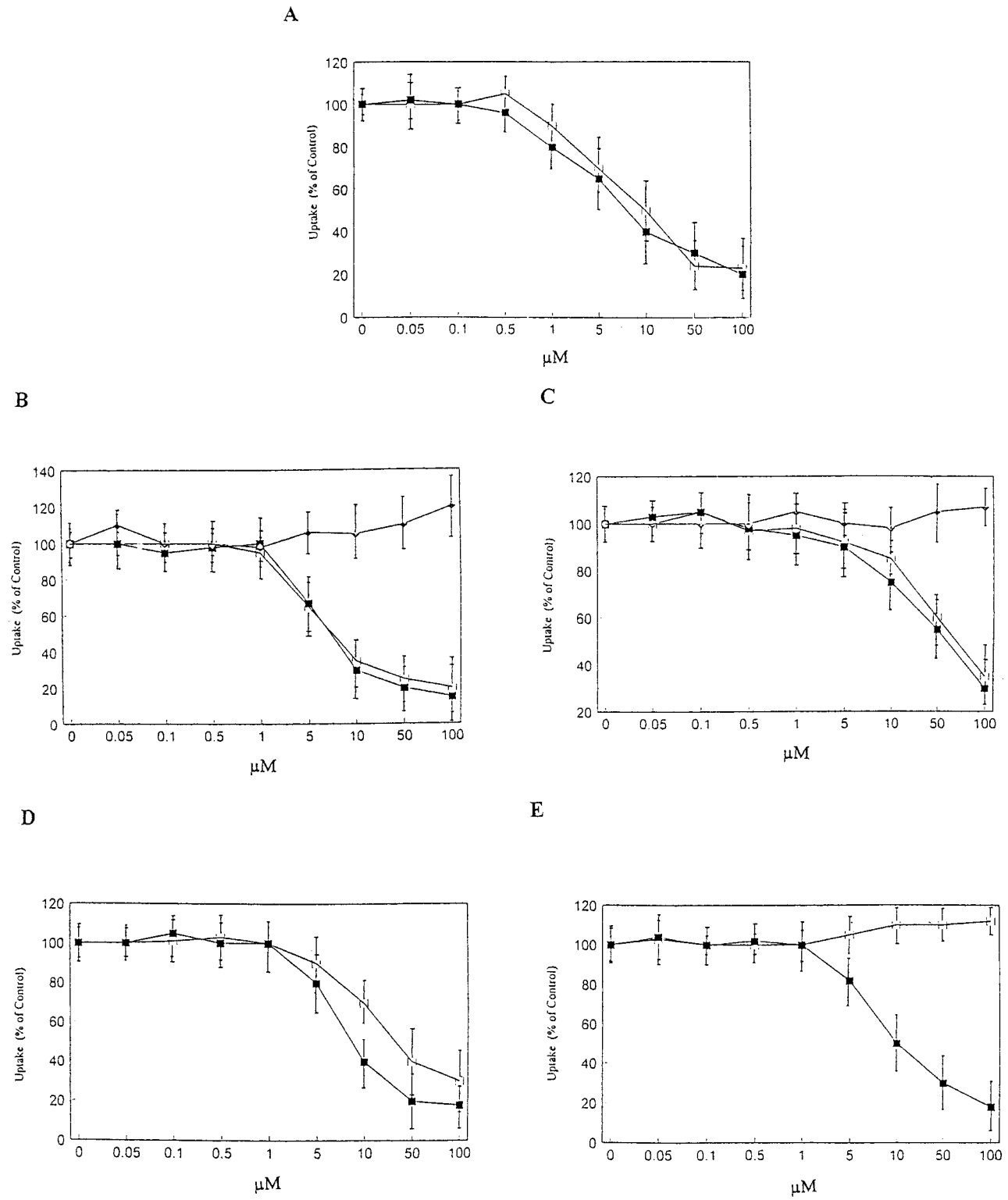

FIG. 2. The effect of flavonoids on glucose uptake in U937. Described in "Materials and Methods", the transport of 2-[1,2-^{3}H(N)] deoxy-D-glucose was measured in the presence of the indicated concentrations of flavonoids. (A) myricetin (open squares) and fisetin (closed squares); (B) rutin (diamonds), apigenin (open squares), and quercetin (closed squares); (C) naringenin (diamonds), naringenin (open squares), and hesperetin (closed squares); (D) daidzein (open squares), and genistein (closed squares); (E) catechin (open squares) and cyanidin (closed squares).

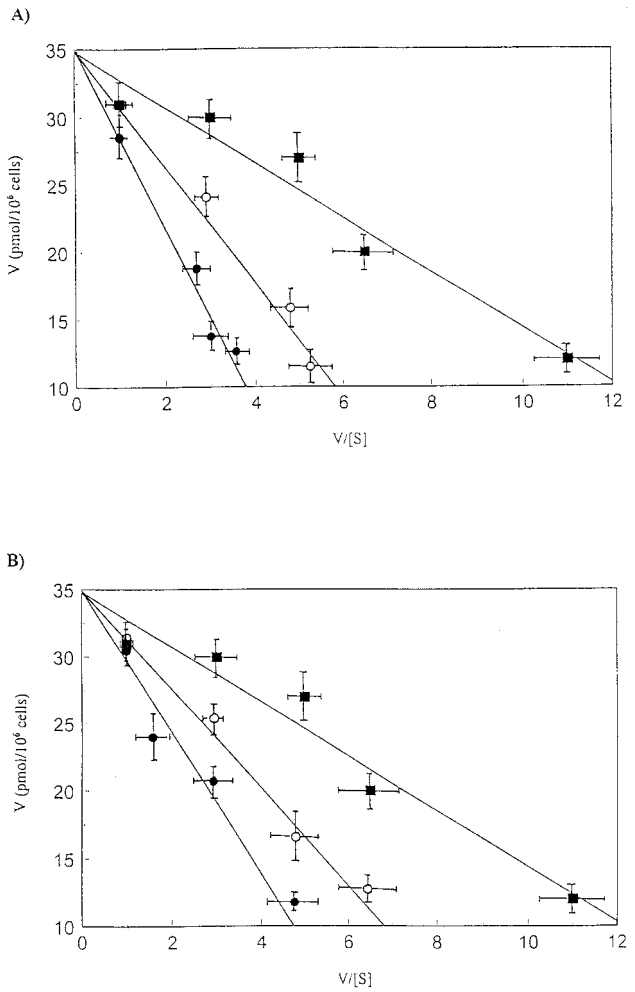

FIG. 3. Glucose uptake was inhibited by fisetin and quercetin in a competitive manner in U937. K_i was determined using an Eadie-Hofstee Plot. Data points in all figures represent the mean ± SD of more than 3 samples. Transport of deoxyglucose at 1.25, 2.5, 5, and 10 mM was measured in the absence of or in the presence of 10, and 20, μM of fisetin or quercetin, respectively.

inhibitory effect but did show a stimulatory effect on glucose uptake (Fig. 2E). These data imply that a moiety of benzopyran structure of flavonoids may be important for blocking glucose uptake. However, it is not certain that only benzopyran structure of flavonoids is necessary for delivering the blocking effect. I discussed more about the relationship between the structure of flavonoids and their blocking activities in the last section of results.

Determination of K_i value for flavonoids. To further define the pattern of the inhibition by flavonoids and determine the K_i values of flavonoids, inhibition experiments were performed. Two flavonoids (fisetin and quercetin) were used for the experiments, because of their strong inhibition of glucose uptake. As shown in Fig. 3, fisetin and quercetin inhibit the transport of glucose competitively. K_i values were calculated using an Eadie-Hofstee plot. The K_i values for fisetin and quercetin were approximately 9 μM and 12 μM, respectively. These data indicated that flavonoids (fisetin and quercetin) are competitive inhibitors of glucose transport in U937.

Effect of sodium on an inhibitory effect of flavonoids. Flavonoids inhibit glucose uptake at the μM ranges, in a competitive manner. The transports of glucose were reported to occur in sodium-independent and sodium-dependent manner (13). But, this inhibition by flavonoids was unknown about the dependence of sodium. Experiments were performed using the buffer with and without sodium, to determine what mechanism of the glucose transport was inhibited by flavonoids. The previous study indicated that the majority of glucose uptake in U937 was inherently independent of sodium in the buffer (unpublished data). Therefore, it looks like that sodium independent glucose transporters play a major role in glucose uptake in U937, and the inhibition of glucose uptake by flavonoids is not altered in sodium and sodium-free buffer. As expected, the tested flavonoids (quercetin, fisetin, and genistein) showed 50% inhibition of glucose uptake at the concentrations of 10–20 μM, in regardless of sodium in the buffer. In Fig. 4, only the inhibition of glucose uptake in sodium-free buffer was shown. In U937, the majority of the inhibitory effect of flavonoids on glucose uptake seems to be due to the sodium-independent glucose transporters, and the competitive inhibition of glucose uptake by flavonoids is independent of sodium in the buffer.

Determination of a moiety of flavonoids accountable for the inhibition of glucose uptake. As indicated above, this sodium-independent and competitive inhibition of glucose uptake seems to be related with a structure of flavonoids. Therefore, the following studies were performed to determine the moiety of the structure of flavonoids with the blocking activity. For the studies, a basic structure of flavonoids was dissected into benzopyran-4-one and phenyl group (see Fig. 1). Since the catechol (1,2-benzenediol) structure is found in the B ring of many flavonoids including quercetin and fisetin, it was investigated to determine whether an inhibitory effect comes from B ring structure alone. The data in Table 1 clearly indicate that the catechol structure of B ring alone is not responsible for the blocking effect on glucose uptake. Next, I investigated benzopyran group for its inhibitory activity. I used chromone (benzopyran-4-one), coumarin (1,2-benzopyrone), and 4-chromanone to investigate the blocking effect of glucose uptake, since they have the same or similar chemical sturcture as benzypyran-4-one (A and C ring of flavonoids). Chromone and 4-chromanone did not inhibit glucose uptake in U937 cells (Table 1). Also, coumarin that contains a ketone group at the 2 position and a saturated C ring structure, showed no inhibitory effect on glucose uptake (Table 1). These experiments

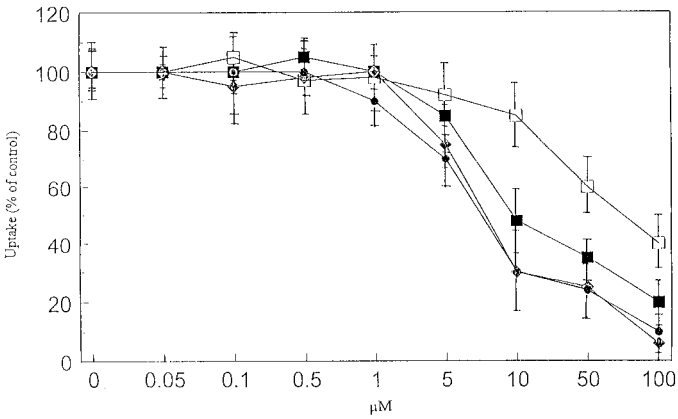

FIG. 4. Effect of quercetin, fisetin, naringenin, and genistein on glucose uptake in sodium free buffer. The diamonds, circle, and open and closed squares represent quercetin, fisetin, naringenin and genistein, respectively.

indicated that the inhibition of glucose uptake by flavonoids might attribute to the flavone structure (an united form of benzopyran and phenyl groups). Therefore, I examined several flavones (flavone, 3-hydroxyflavone, 5-hydroxyflavone, 7-hydroxyflavone, and 5,7-dihydroxyflavone), in order to determine whether the inhibition is truly originated from the flavone structure of flavonoids (Fig. 5). Surprisingly, flavone, a backbone structure of flavonoid, could not inhibit glucose uptake even at the concentration of 100 μM. Since fisetin and myricetin contain 3-hydroxyl group at their flavone structure, 3-hydroxyflavone was tested for its inhibitory activity, but it did not show any inhibition of glucose uptake at the concentrations of 50 and 100 μM. However, 5-hydroxyflavone, 7-hydroxyflavone, and 5.7-hydroxyflavone inhibited glucose uptake in U937 cells (Table 1).

These data imply that flavone structure and some types of hydroxylation (especially, the 5, and 7 position) are required for exhibiting the blocking effect on glucose uptake. Since 3-hydroxylation did not have an influence on the inhibition, the structural difference between flavones and flavonols seems to have no impact on their inhibition of glucose uptake, if they are hydroxylated at 5 and/or 7 positions. Based on these assumptions, the structures of apigenin, quercetin, fisetin, and myricetin are consistent with the chemical structure proposed as being responsible for the inhibition of glucose uptake; indeed, those flavonoids exhibited the highest blocking effect. However, the flavonoid glycones such as rutin and naringin surprisingly did not inhibit glucose uptake in U937 cells.

DISCUSSION

In plants, flavonoids were proposed to be antioxidants, enzyme inhibitors, pigments, phytohormones and to block UV light (14–16). Flavonoids have been also reported to contain anti-inflammatory, antiviral, antiproliferative, and estrogen-like activities in mammalian systems (17, 18). Our results demonstrate that flavonoids have the potential to inhibit the glucose uptake in a myeloid cell, U937. Previously, genistein, an isoflavonoid, was reported to have an inhibitory effect on the glucose uptake in HL-60 cells (8). In the same paper, it was also reported that daidzein, another isoflavonoid, was incapable of inhibiting glucose uptake (8). In contrast, our data demonstrate that daidzein also has the potential to inhibit glucose uptake, but at relatively higher concentrations than genistein. The discrepancy between our data and the previously reported data might be attributed to use of different cell lines (U937 versus HL-60). We also found that the solubility of genistein and daidzein differ depending on whether the solvent used is acetone, methanol, ethanol, or dimethyl sulfoxide (DMSO). The decreased solubility of daidzein may also have contributed to the underestimation of the inhibitory effect on glucose uptake.

Flavonoids occur most often as glycosylated forms (glycones) in plants, but non-glycosylated forms of flavonoids (aglycones) exist in small amounts. Recently, it was reported that glycones might be inhibitors of glucose transport and also transport into cells via the glucose transporter (9). However, this proposition could not be confirmed in our study, since rutin (a glycone form of quercetin) and naringin (a glycone form of naringenin) did not inhibit glucose uptake in U937 cells. Most flavonoids tested in this study are aglycone forms of flavonoids, and are an effective blocker of glucose uptake. Furthermore, these flavonoids inhibit glucose uptake competitively with inhibition constant of $K_i = 10 \pm 2$ μM. Even if the data indicated that

TABLE 1

The Comparison of the Inhibitory Activities of Flavonoid Analogues on Glucose Uptake in U937

	Concentrations	
Flavonoid analogues	40 μM	100 μM
Fisetin	≥30%	≥20%
Quercetin	≥30%	≥20%
Catechol	100%	100%
Chromone	100%	100%
4-Chromanone	100%	100%
Coumarin	100%	100%
Flavone	100%	95%
3-Hydroxyflavone	100%	100%
5-Hydroxyflavone	≥40%	≥30%
7-Hydroxyflavone	≥50%	≥35%
5,7-Dihydroxyflavone	≥40%	≥30%

Note. The remaining activities of glucose uptake in U937 were represented as percentage, at two concentrations of flavonoid analogues.

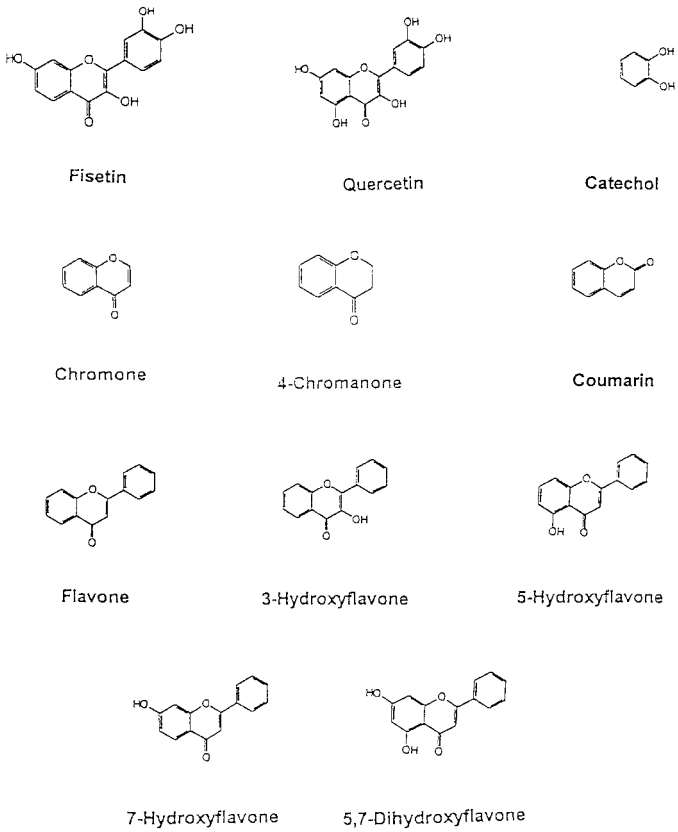

FIG. 5. Structures of flavonoid analogues. Fisetin, quercetin, catechol, chromone, 4-chromanone, coumarin, flavone, 3-hydroxyflavone, 5-hydroxyflavone, 7-hydroxyflavone, and 5,7-dihydroxyflavone.

flavonoids inhibit glucose uptake in a competitive manner in U937, the important question remains unsolved; whether flavonoids simply compete with glucose for binding to the transporters or flavonoids are competitively transported via the transporters. To answer the question is a key to find out a true mechanism of transport of flavonoids into cells. Therefore, further studies must to be performed to resolve this question.

The transport of glucose is carried out into cells by several glucose transporters in sodium-dependent and -independent manners. The glucose transporters (Glu 1–5) are sodium-independent, but the glucose transporter SGLT is sodium-dependent (19, 20). In U937, the inhibitory effect of flavonoids was not alternated when sodium-free buffer was used instead of the buffer containing sodium. These data imply that most of the inhibition of glucose uptake in U937 occurred via the sodium-independent glucose transporters. Incomplete inhibition (less than 10%) was sometimes observed and could not be eliminated at high concentrations of flavonoids (greater than 500 μM). A plausible reason for the incomplete inhibition at high concentrations might be that the sodium-dependent transporter exists in U937 cells, but at a lower level than the sodium-independent transporters.

Data from the present study demonstrate that flavonoids, especially aglycones, inhibit glucose uptake in U937 cells. A similar inhibitory effect was also observed in the Jurkat cell line. Using the analogues of flavonoids, the minimal structure of flavonoids to confer the inhibition was determined as 5 and/or 7-hydroxyflavones. The data showed that some flavonoids containing the structure, truly block the glucose uptake in the cells and the flavonoids could be used as a blocker of the glucose uptake, *in vitro*. The beneficial effect of natural flavonoids in a diet has not been fully studied, with respect to *in vivo* glucose absorption, even though many *in vitro* data for flavonoids have been published (21–25). It was currently reported that the average human intake of flavonoids has been estimated to be approximately 23 mg/day (26–28). But, the physiological concentrations of individual flavonoids in human intestine have not been conclusively established. With the incomplete information, the true effects of flavonoids on the absorption of glucose cannot be fully established *in vivo*. Therefore, the future studies of flavonoids must be performed to validate the *in vitro* effects, and evaluate the *in vivo* effects in humans.

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