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RESEARCH ARTICLE

Effect of flavonoids on basal and insulin-stimulated 2-deoxyglucose uptake in adipocytes

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Scope: The adipose tissue is a major site of insulin action and contributes substantially to energy homeostasis. Insulin increases the extraction of glucose from circulation into adipose tissue by recruiting the glucose transporter GLUT4 to the plasma membrane. It has been proposed that dietary flavonoids may interfere with glucose transport processes.
Methods and results: We have used murine 3T3-L1 adipocytes and isolated mature human adipocytes to assess the interaction of selected flavonoids with glucose uptake, both in the basal state and after insulin stimulation. Kinetic characterization of 2-deoxyglucose uptake in the basal state revealed in both cell types an apparent K_m of around 8 mM with no change in affinity but a significant increase in maximal influx in the presence of insulin. A screening of representative flavonoids of different structural classes revealed the flavanone naringenin and the isoflavone daidzein to affect glucose transport significantly with half-maximal inhibition at concentrations of around 60–80 μM for basal and 70–110 μM for insulin-stimulated glucose uptake in both 3T3-L1 adipocytes and mature human adipocytes.
Conclusion: Considering attainable plasma concentrations of flavonoids *in vivo*, we assume that even under physiological conditions naringenin and daidzein could impair glucose removal from plasma, which may pose a risk to patients with diabetes mellitus.

Keywords:
Adipocytes / Flavonoids / Glucose transporter / GLUT4 / Insulin

1 Introduction

Glucose transporters are important regulators of metabolism and glucose uptake is often rate-limiting for cellular glucose utilization. Members of the mammalian facilitative glucose transporter family GLUT differ in tissue distribution, substrate specificities, kinetic properties and in response to hormonal regulation [1]. A common feature of adipose tissue and skeletal muscle metabolism is the increase in glucose influx in response to insulin mainly mediated by translocation of GLUT4 from storage vesicles into the plasma membrane [2, 3]. Although insulin-dependent postprandial glucose disposal *in vivo* is believed to occur mainly by uptake into skeletal muscle [4], insulin-enhanced glucose uptake into adipose tissue also contributes to whole-body glucose homeostasis [5].

Flavonoids represent a large heterogeneous group of plant-derived compounds that occur both in glycosylated and non-glycosylated forms. Based on their structure, they are classified into six subgroups: flavones, flavonols, flavanones, isoflavones, catechins or flavanols, and anthocyanidins [6]. Flavonoids have been shown to mediate their biological effects by their ability to modulate cell signaling pathways based on molecular interactions with protein-tyrosine kinases [7], phosphodiesterases [8], phosphatases [9], and numerous other enzymes.

Moreover, they can also directly interfere with plasma membrane transport proteins. For several flavonoids the inhibition of glucose transport processes has been reported in erythrocytes, monocytic U937 cells, and HL-60 cells

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Abbreviations: 2-DG, 2-deoxyglucose; **EGCG,** epigallocatechin gallate; **FCS,** fetal calf serum; **GLUT,** glucose transporter; **KRH,** Krebs-Ringer-Hepes buffer; **KRP,** Krebs-Ringer-phosphate buffer; **SGLT,** sodium dependent glucose transporter

[10, 11]. Genistein, quercetin and myricetin were shown to inhibit GLUT1-mediated uptake of hexoses and dehydroascorbic acid into transfected Chinese hamster ovary (CHO) cells over-expressing GLUT1 and into human erythrocytes [12]. GLUT2 expressed in *Xenopus laevis* oocytes or in intestinal Caco-2E cells was demonstrated to be inhibited by selected flavonoids [13]. The intestinal sodium-dependent glucose transporter SGLT1 was also shown to be inhibited by different flavonoids in both glycosylated forms but also various aglycons [14].

In adipocytes, not only inhibition but also enhancement of glucose uptake by flavonoids have been reported [15–18]. These controversial findings may derive from a direct interaction of the compounds with the GLUT-proteins but also from an interference with the insulin-receptor signaling pathway by their ability to interact with kinases and phosphatases that control down-stream signaling processes and GLUT4 trafficking.

To be able to assess the direct interference of flavonoids with glucose transport on the transporter level before a significant uptake of flavonoids into cells can occur and may modify signaling processes, it is necessary to obtain initial rates of glucose influx. Therefore, we carefully characterized the kinetics of uptake of 2-deoxyglucose (2-DG) as a non-metabolized GLUT-substrate into 3T3-L1 and mature human adipocytes in basal state and after insulin stimulation. Subsequently, the effect of selected flavonoids on glucose transport kinetics was investigated.

2 Materials and methods

2.1 Materials

Naringenin, daidzein, myricetin, flavanone, flavone, catechin, epigallocatechin gallate (EGCG), genistein, and 2-deoxy-D-glucose were purchased from Sigma-Aldrich, Steinheim, Germany. 2-Deoxy-D-[1-^{3}H]-glucose was purchased from GE Healthcare Europe GmbH, Freiburg, Germany. Cytochalasin B and phloretin were obtained from Alexis Biochemicals, Lörrach, Germany. All cell culture and tissue culture materials were supplied from Gibco™, Invitrogen, Karlsruhe, Germany. 3T3-L1 cells were obtained from ATCC, (LCG Standards GmbH, Wesel, Germany). Insulin, dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), pyruvate, biotin, bovine serum albumin (BSA), and PBS were purchased from Sigma-Aldrich. Gentamycin was from Roth, Karlsruhe, Germany. Collagenase was obtained from Biochrome AG, Berlin, Germany. Trypsin-EDTA was from PAA Laboratories GmbH, Pasching, Austria.

2.2 Cell culture and differentiation

3T3-L1 pre-adipocytes were maintained at 37°C in 5% CO_2 in DMEM containing 10% fetal calf serum (FCS) and 4.5 g/L glucose. At pre-confluence (90%), differentiation was induced by adding insulin (5 μg/mL), dexamethasone (250 nM) and 3-isobutyl-1-methylxanthine (0.5 mM) (day 0). After 72 h, the induction medium was replaced by a differentiation medium containing 10% FCS, 4.5 g/L glucose, and insulin (5 μg/mL). Medium was changed every other day until the experiment.

2.3 Glucose uptake assay into 3T3-L1 adipocytes

The stimulatory effect of insulin on glucose uptake was shown to be more pronounced in 3T3-L1 adipocytes on day 12 of differentiation compared to cells 8 days after the adipogenic induction (data not shown). Therefore, the uptake assay was performed only with fully differentiated 3T3-L1 adipocytes (day 12), with >90% of the cells exhibiting the adipocytic phenotype (assessed by Oil-Red-O staining). Sixteen hours before the uptake assay, differentiated 3T3-L1 adipocytes were washed twice with PBS and were then incubated in DMEM containing 5 mM glucose and 5% FCS. About 2.5 h before the experiment, cells were completely deprived of glucose and incubated at 37°C in Krebs-Ringer-Hepes buffer (KRH) containing 10 mM Hepes, 2.5 mM pyruvate, and 0.5% BSA (pH 7.38). Subsequently, 3T3-L1 adipocytes were incubated for another 30 min either in the presence or absence of insulin (1 μM). Uptake was initiated by the addition of KRH containing 0.5% BSA, 2.5 mM pyruvate, 2-DG and [^{3}H]-2-DG (concentration depending on the experiment) in the presence or absence of a specific flavonoid and dimethyl sulfoxide (DMSO), respectively. Uptake was terminated by addition of two volumes of ice-cold KRH containing 150 μM phloretin and 15 μM cytochalasin B to the incubation medium, before washing with ice-cold KRH containing 100 μM phloretin and 10 μM cytochalasin B. After stopping glucose uptake, cells were lysed in 0.1 M NaOH. Radioactivity was measured using liquid scintillation counting. Quenching of radioactivity was considered applying an external standard. 2-DG transport values were corrected for protein content determined by the bicinchoninic acid method (BCA Protein Assay Reagent, PIERCE, Rockford, IL, USA).

2.4 Patients

Adipose tissue samples were obtained from 17 subjects (12 female and 5 male) undergoing elective abdominal surgery. The mean age of the patients was 43 years (range 23–65 years) and the mean BMI was 28 kg/m^2 (range 20–36 kg/m^2). All subjects were healthy and did not suffer from acute infections or metabolic diseases. The procedure was approved by the ethical committee of the Technische Universität München, Germany.

2.5 Isolation and culture of mature adipocytes

Adipose tissue samples were immediately transported to the laboratory in DMEM containing 20 μg/mL gentamycin. Connective tissue and visible blood vessels were removed with scissors. For preparation of mature adipocytes, the tissue was minced and digested in Krebs-Ringer-phosphate buffer (KRP; 122.8 mM NaCl, 12.4 mM NaH_2PO_4, 4.9 mM KCl, 1.3 mM $MgSO_4$, 1.2 mM $CaCl_2$, and pH 7.4) containing 100 U/mL collagenase and 4% BSA for 60 min at 37°C in a shaking water bath. After this step, the undigested tissue was removed by filtrating twice through a nylon mesh with a pore size of 250 μm (VWR, Darmstadt, Germany). The floating adipocytes were washed three times with KRP containing 0.1% BSA. The first medium exchange was performed after 1 h. Cells were incubated in DMEM/Ham's F12 (1:1 v/v) with gentamycin (50 μg/mL) supplemented with 5 mM glucose for 2 days in order to allow them to recover from preparation. Cell viability was assessed using a standard lactate dehydrogenase activity assay.

2.6 Glucose uptake assay in mature human adipocytes

About 2.5 h before the uptake assay, adipocytes were placed in a glucose-free medium. Cells were washed twice with pre-warmed KRP containing 2.5 mM pyruvate. Two hundred microliters of packed cells and 490 μL of pre-warmed KRH buffer containing 2.5 mM pyruvate as an energy substrate were incubated without shaking for 2 h at 37°C. Adipocytes were either exposed to insulin (1 μM) or were kept without insulin for 30 min at 37°C. Uptake was initiated by the addition of adequate concentrations of 2-DG and [^{3}H]-2-DG. Solutions were mixed by carefully flicking the tubes. Further mechanical agitation was avoided as far as possible during the transport assay. Flavonoids were dissolved in DMSO and added at a final DMSO concentration of 0.01% that was tested not to affect transport (data not shown). Termination of the uptake was achieved by covering the cell suspension with a layer of 500 μL of dinonyl phthalate (Alexis Biochemicals) and immediate centrifugation for 30 s at 100 × *g*. Tubes were then rapidly frozen at −20°C. For analysis, the frozen adipocyte layer was cut out using a lathe (Wabeco D 6000 E, Remscheid, Germany) and a scintillation cocktail (Rotiszint, Roth) was added. Radioactivity was measured with quench-correction based on external standard for each of the samples.

2.7 Statistical analysis

Data are expressed as means ± SD. The significance of differences between means was assessed by one-way ANOVA and Tukey post hoc testing using the Graph Prism program version 5 (GraphPad software, San Diego, CA, USA).

3 Results

3.1 Basal and insulin-stimulated 2-DG uptake into adipocytes

For assessing the selective effect of flavonoids on the GLUT membrane transporters, it was necessary to thoroughly characterize the kinetics of basal and insulin-stimulated 2-DG uptake into adipocytes under zero-trans conditions.

3.2 Kinetics of basal and insulin-stimulated 2-DG uptake in differentiated 3T3-L1 adipocytes

The time course of 2-DG uptake (50 μM) under basal conditions and after insulin stimulation in 3T3-L1 adipocytes is shown in Fig. 1 (A–C). The influx rate of 2-DG increased six-fold with insulin and uptake was completely blocked by cytochalasin B and phloretin that were previously shown to inhibit facilitative glucose transport in a competitive manner [19]. Unidirectional uptake of 20 mM 2-DG was found to occur linear to time for at least 2 min (data not shown).

Kinetics of 2-DG influx as a function of substrate concentration assessed at 2 min revealed a Michaelis–Menten function (Fig. 1B) including a linear component with a slope of $\gamma_b = 20.91x$ for basal and $\gamma_i = 18.56x$ for insulin-stimulated conditions, respectively. This linear component representing either non-specific binding or transport was experimentally confirmed by assessing the transport rate of 20 mM 2-DG in the presence of 100 μM phloretin and 10 μM cytochalasin B (Fig. 1B). The residual flux rate accounted for the predicted non-carrier-mediated component. Apparent K_m and V_{max} parameters were determined by non-linear regression analysis of data corrected for the linear component (Fig. 1C). The Eadie–Hofstee transformation of insulin-stimulated 2-DG influx revealed two transport components (data not shown). For basal uptake in 3T3-L1 adipocytes, the apparent K_m and V_{max} values were 9.1 mM and $508\,\text{pmol} \times \mu\text{g}^{-1} \times 120\,\text{s}^{-1}$, respectively. Accordingly, insulin-stimulated 2-DG uptake revealed a K_m of 7.5 mM with a doubled V_{max} ($973.5\,\text{pmol} \times \mu\text{g}^{-1} \times 120\,\text{s}^{-1}$) and the calculated difference between insulin-stimulated and basal uptake, when fitted to the Michaelis–Menten equation, yielded an apparent K_m value of 6.2 mM and a V_{max} of $470.9\,\text{pmol} \times \mu\text{g}^{-1} \times 120\,\text{s}^{-1}$ for the insulin-responsive component (data not shown). Transformation of insulin-responsive data according to Eadie–Hofstee confirmed that a single transporter isoform was examined which is assumed to be GLUT4 (data not shown).

3.3 Kinetics of basal and insulin-stimulated 2-DG uptake into mature human adipocytes

Kinetics of basal and insulin-stimulated 2-DG uptake into mature human adipocytes was determined as described for

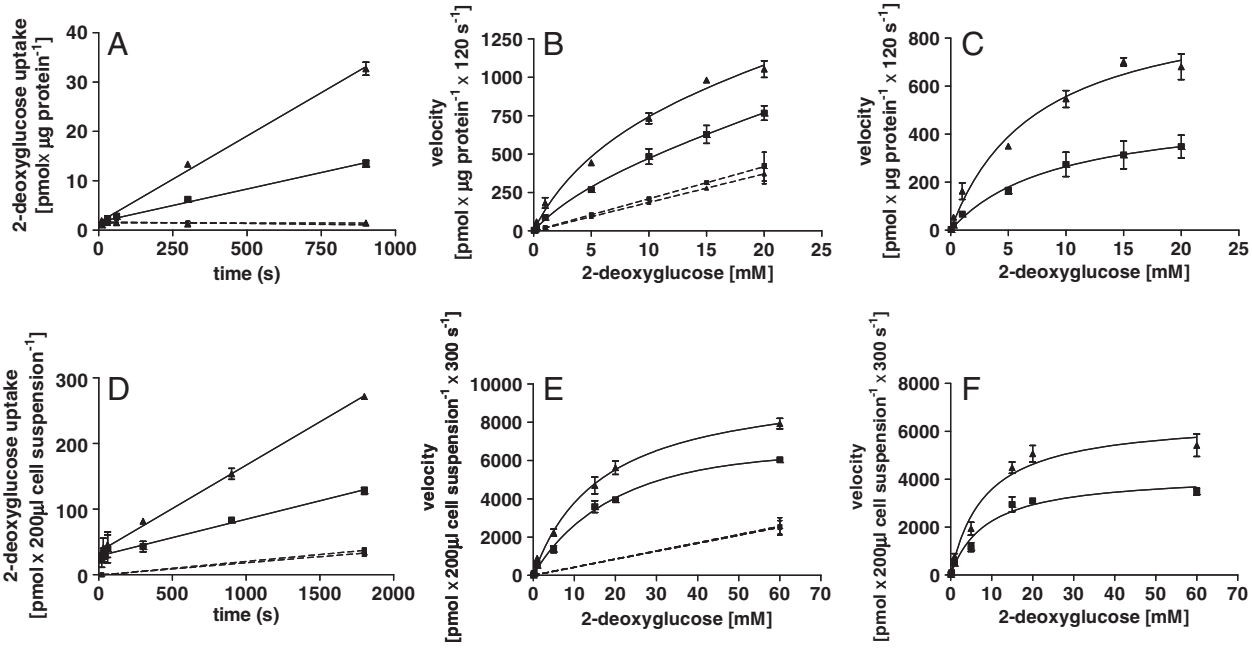

Figure 1. Analysis of basal (squares) and insulin-stimulated (triangles) 2-DG uptake in different adipocyte models. For glucose uptake 3T3-L1 (A–C) and human adipocytes (D–F) were incubated at 37°C in KRH containing [^{3}H]-2-DG and various concentrations of 2-DG in the presence (dotted line) or absence (solid line) of 100 µM phloretin and 10 µM cytochalasin B for the times indicated. Time course experiments (A and D) were performed using 50 µM 2-DG and 2 µCi/mL [^{3}H]-2-DG. Cells were harvested at the times indicated. For the concentration dependency (B and E) adipose cells were incubated at 37°C for 120 s (B) or 300 s (E) with KRH and various concentrations of 2-DG and a constant amount of [^{3}H]-2-DG glucose [3.2 µCi/mL]. Panels (C) and (F) display concentration-dependent data corrected for non-carrier-mediated transport fitted by non-linear regression. Symbols represent mean ± SD of three independent experiments performed in duplicate (A and D) or mean ± SEM of two experiments performed in duplicate (B, C, E, F).

the 3T3-L1 adipocytes. Figure 1 (D–F) depicts the time course of 2-DG uptake in basal and insulin-stimulated mature human adipocytes. Uptake was linear for at least 300 s in the presence of 50 µM and 60 mM 2-DG (data not shown). When mature human adipocytes were treated with 1 µM insulin, the transport rate increased by 2.1-fold as compared to the basal state. Initial flux rates as a function of substrate concentration are provided in Fig. 1E. As an estimate of passive diffusion, uptake rates of 60 mM 2-DG were determined in the presence of 10 µM cytochalasin B and 100 µM phloretin and yielded $y_b = 42.59x$ for the basal and $y_i = 41.72x$ for the insulin-stimulated component. After subtraction, the saturation kinetics (Fig. 1F) allowed apparent K_m and V_{max} parameters to be determined by non-linear regression analysis with V_{max} values accounting to 4215 and 6551 pmol × 200 µL^{-1} × 300 s^{-1} for basal and insulin-stimulated uptake, respectively. K_m values were calculated to be 8.8 and 8.3 mM. The Eadie–Hofstee plot of insulin-stimulated 2-DG uptake data indicated two participating transport systems (data not shown). To derive the insulin-responsive part, the difference was calculated and revealed a K_m of 7.6 mM and a V_{max} of 2337 pmol × 200 µL^{-1} × 300 s^{-1}. The Eadie–Hofstee plot of the data confirmed linearity as evidence for a single transport system (data not shown).

In summary, in both adipocyte cell models the kinetics of 2-DG uptake under zero-trans conditions revealed transport components that increase in V_{max} by insulin exposure of cells whereas the apparent K_m values remain essentially unaffected. The findings are in agreement with the insulin action to increase the number of GLUT4-transporters in the cell membrane without affecting substrate affinity.

3.4 Effect of flavonoids on basal and insulin-stimulated 2-DG uptake in adipocytes

Several flavonoids were screened for their effect on 2-DG uptake into differentiated 3T3-L1 adipocytes. We pre-selected representative compounds of each of the five subgroups of flavones, flavonols, flavanones, isoflavones, and catechins (or flavanols). The pre-selection also took into account the concentrations of the compounds as found in food items and mean intake rates *via* diet as well as the attainable plasma concentrations as described in the literature. These flavonoids were screened for their effects on basal and insulin-stimulated influx of 2-DG into adipocytes by using a concentration of 10 µM. As shown in Fig. 2A, the flavanols catechin and EGCG and the flavonol myricetin failed to cause transport inhibition. In contrast, flavone was found to

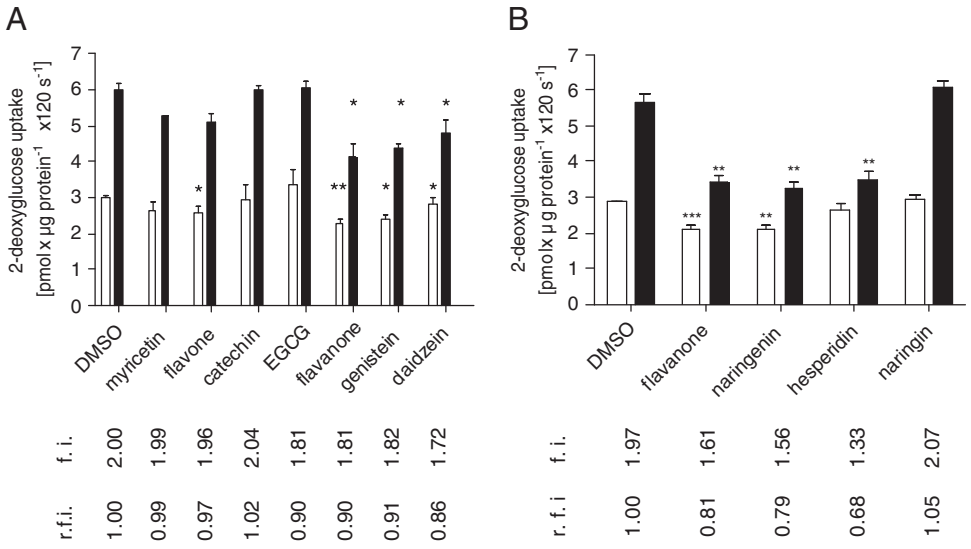

Figure 2. Effect of different flavonoids on basal (white columns) and insulin-stimulated (black columns) 2-DG uptake in differentiated 3T3-L1 adipocytes. (A) Effect of representative compounds from different subgroups of flavonoids. Cells were incubated at 37°C in KRH containing [^{3}H]-2-DG [2 µCi/mL], 50 µM 2-DG and 10 µM of myricetin, flavone, catechin, EGCG, flavanone, genistein, or daidzein for 120 s. Corresponding volumes of DMSO were used as a control. (B) Effect of selected flavanones. Cells were incubated at 37°C in KRH containing [^{3}H]-2-DG [2 µCi/mL], 50 µM 2-DG and 20 µM of flavanone, naringenin, hesperidin, or naringin or corresponding volumes of DMSO (control) for 120 s. Columns represent mean ± SD of three experiments performed in duplicate. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. f. i. ('fold-induction') is the ratio of glucose uptake under insulin-stimulated over basal conditions. r. f. i. ('relative fold-induction') is fold-induction in the presence of the respective flavonoid over control as a measure for inhibition of insulin-stimulated glucose uptake.

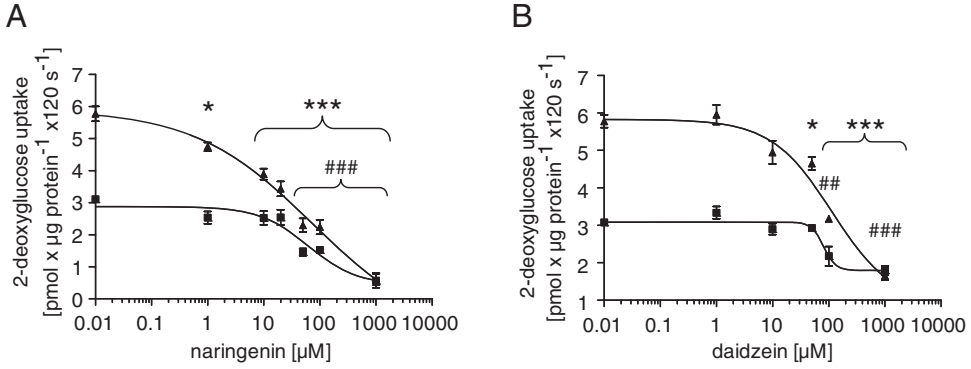

Figure 3. Effect of naringenin (A) and daidzein (B) on basal (squares) and insulin-stimulated (triangles) 2-DG uptake in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated at 37°C for 120 s with KRH buffer containing 50 µM 2-DG and [^{3}H]-2-DG [2 µCi/mL] and indicated concentrations of naringenin (A) or daidzein (B), respectively, or corresponding volumes of DMSO (control). Symbols represent mean ± SD of three independent experiments performed in duplicate. * indicates significantly different from control in insulin-stimulated conditions; # indicates significantly different from control in basal conditions. $^*p<0.05$, $\#\ \#p<0.01$, ***and $\#\#\#p<0.001$.

significantly reduce basal 2-DG influx, whereas flavanone and the isoflavonoids genistein and daidzein significantly inhibited both basal and insulin-stimulated 2-DG uptake.

To further explore the inhibitory effect of selected flavanones, naringenin, its glycoside naringin and the glycoside hesperidin were tested also at a concentration of 20 µM. Except for naringin, all other compounds significantly inhibited basal as well as insulin-stimulated 2-DG transport (Fig. 2B).

As the inhibitory effect of naringenin was most pronounced, its interaction with the GLUTs was further studied by assessing the concentration-dependent inhibition of 2-DG uptake in 3T3-L1 adipocytes. This resulted in IC_{50} values of 61 and 71 µM for basal and insulin-stimulated 2-DG transport, respectively (Fig. 3A).

To assess whether naringenin not only directly inhibits GLUT4 but can also alter GLUT4 vesicle recruitment to the plasma membrane, cells were either pre-treated with naringenin for 60 min together with insulin before assaying 2-DG uptake in the presence of naringenin or naringenin was only added together with 2-DG during the 120 s uptake assay. As shown in Fig. 4, insulin-stimulated uptake

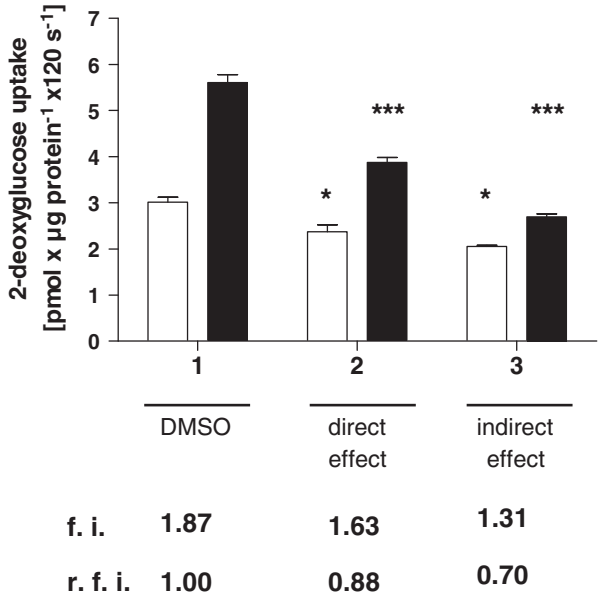

Figure 4. Direct and indirect effect of naringenin on basal (white columns) and insulin-stimulated (black columns) 2-DG uptake in differentiated 3T3-L1 adipocytes. Cells were incubated at 37°C for 120 s with 2-DG uptake solution containing 2 μCi/mL [^{3}H]-2-DG either in the absence of naringenin (DMSO), in the presence of 10 μM naringenin (direct effect at the transporter level) or in the presence of 10 μM naringenin after 60 min pre-incubation with 10 μM naringenin (indirect effect at a cellular level). Columns represent mean ± SD of five (DMSO, direct) and two (indirect) experiments performed in duplicate, respectively. $^*p<0.05$, $^{***}p<0.001$. f. i. and r. f. i. as in Fig. 2

was inhibited in both cases by naringenin with more pronounced effects when cells were pre-treated with the compound (52 *versus* 31% inhibition), whereas basal transport was inhibited in a similar manner during both treatments.

For the isoflavonoid subgroup, the apparent inhibitory affinity of daidzein was assessed in 3T3-L1 adipocytes (Fig. 3B) and revealed IC_{50} values of 81 μM for basal and 114 μM for insulin-stimulated uptake conditions, respectively.

In the mature human adipocytes, naringenin and daidzein at concentrations of 20 μM significantly inhibited the basal as well as insulin-stimulated 2-DG uptake (Fig. 5A and C). At concentrations of 1 μM, naringenin also decreased transport rates significantly (Fig. 5B), whereas daidzein failed to cause any transport inhibition (Fig. 5D).

4 Discussion

Dietary flavonoids are reported to possess a large variety of metabolic effects. They also have been shown to alter membrane protein functions and we therefore investigated whether selected flavonoids can interfere with glucose transport into adipocytes. Uptake of glucose occurs *via* GLUT-proteins and is increased by insulin action *via* the recruitment of GLUT 4-transporters from vesicles to the cell membrane, leading to increased transporter density and enhanced glucose influx. We first kinetically characterized substrate influx in two different cell models of adipose tissue: differentiated 3T3-L1 adipocytes of murine origin and freshly isolated human adipocytes. All studies were carried out with 2-DG as a non-metabolizable GLUT substrate. As metabolism acts as a sink for unidirectional transport processes, it is important for any kinetic analysis that uptake is assessed under zero-trans conditions. Facilitative GLUTs can be inhibited by cytochalasin B and phloretin, and we demonstrate that both compounds completely blocked carrier-mediated uptake, resulting in a dose-linear diffusion component. Kinetic analysis of transporter-mediated 2-DG uptake in differentiated 3T3-L1 adipocytes revealed apparent K_m values of 7.5–9 mM for basal and insulin-stimulated uptake, respectively. Reported K_m values for glucose transport with model substrates such 3-*O*-methylglucose in 3T3-L1 adipocytes, range between 11 and 20 mM for basal and insulin-stimulated uptake [20, 21]. Since human GLUT1- and GLUT4-transporters expressed in oocytes show a higher affinity for 2-DG than for 3-*O*-methylglucose [22], the different substrate may explain why the affinity we determined is slightly higher. In isolated mature human adipocytes, however, we found similar K_m values for 2-DG influx as those obtained in differentiated 3T3-L1 adipocytes, providing a cross-validation of the two models.

In the absence of insulin more than 95% of GLUT4-proteins in muscle and adipocytes are sequestered intracellularly, but translocate rapidly to the cell surface following insulin stimulation [23]. Although GLUT1 can also undergo an insulin-dependent trafficking to the cell surface, the magnitude of this effect is much smaller than that of GLUT4, with a 2-fold compared to a 20-fold increase [24]. Correspondingly, the increase in transport activity after insulin stimulation is usually almost completely assigned to GLUT4. Although we could not determine to which extent GLUT1 and GLUT4-transporters contribute to overall transport, the kinetic analysis provides evidence that a single transporter phenotype is responsible for the insulin-responsive transport component. To resolve the kinetic properties of the glucose transporter GLUT1 and GLUT4 in insulin-stimulated differentiated 3T3-L1 adipocytes, Palfreyman et al. applied bis-mannose-photolabel displacement studies that revealed an apparent K_m value of 7 mM for GLUT4 [21]. Studies on mouse and human GLUT4 expressed in *X. laevis* oocytes revealed K_m values for 2-DG that almost perfectly match with the 6.2 mM derived here for adipocytes [25, 26]. In this respect, our cell models proved valid to assess the effects of flavonoids on GLUT4-mediated 2-DG transport.

Flavonoids are recognized as dietary constituents with a variety of biological effects and proposed health-promoting properties such as preventing atherosclerosis [27]

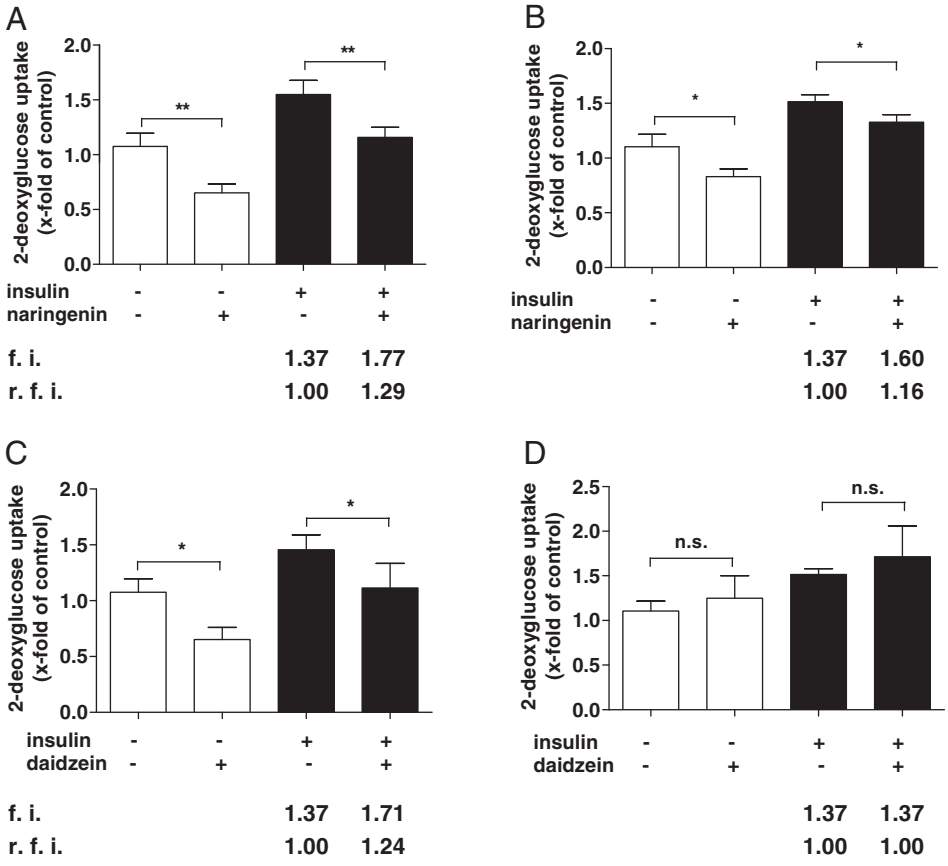

Figure 5. Effect of naringenin and daidzein on basal and insulin-stimulated 2-DG uptake in mature human adipocytes. Two hundred microliters of the cell suspension was incubated at 37°C for 300 s in KRH containing 50 μM 2-DG, [^{3}H]-2-DG [2 μCi/mL], and either 20 μM (A and C) or 1 μM (B and D) of naringenin (A and B) or daidzein (C and D). Columns represent mean ± SD of five independent experiments performed in duplicate. $^*p<0.05$; $^{**}p<0.01$. f. i. and r. f. i. as in Fig. 2

and cancer [28], and improving bone density [29]. Flavonoids were also reported to interfere with glucose transport systems in a variety of cells that express GLUT-proteins or SGLT1-proteins [12–14]. However, since flavonoids are also known to interact with numerous intracellular signaling kinases and phosphatases including those involved in insulin action, the causal effects of transport inhibition are difficult to assess. We therefore defined conditions that allowed to separate direct transporter effects from those that may occur subsequent to the uptake of flavonoids into the cell.

Our initial screening of representative flavonoids from the various subgroups revealed that naringenin and daidzein can significantly inhibit glucose uptake in adipocytes. For naringenin in 3T3-L1 adipocytes this has been shown before, and the inhibition was postulated to occur *via* impaired phosphatidylinositol 3-kinase (PI3K) activity and a decreased translocation of GLUT4 to the plasma membrane [16, 30]. Our studies, however, revealed a dual mode of action of naringenin. A comparison of its effects on insulin-stimulated glucose uptake rates either after direct addition or after pre-incubation of cells with naringenin suggests that about half of its effects can be attributed to a direct interaction with the transporter while the remaining effect may be ascribed to processes involved in GLUT4 trafficking. Since naringenin was shown not to affect insulin-receptor auto-phosphorylation or the tyrosine phosphorylation of IRS-1 and IRS-2 [16], these processes could impair GLUT4 recruitment. In addition to naringenin, we also found that hesperidin and flavanone can inhibit basal and insulin-stimulated 2-DG uptake into 3T3-L1 adipocytes whereas the glycone naringin lacked any effect. This is in accordance with previous data reporting that neither rutin as the glycoside of quercetin nor naringin was able to inhibit facilitative GLUTs in U937 cells [10].

The inhibition caused by naringenin was slightly more pronounced at identical concentrations than that caused by daidzein. This could originate from structural differences, mainly the bond structure between C2 and C3 in the C ring, and the number of hydroxyl groups. For the C2═C3 double bond configuration it was reported to reduce the inhibitory potency of compounds when 2-DG uptake was assessed in U937 cells [10]. For other flavonoids and GLUT1-mediated glucose flux in human erythrocytes, it was shown that an increasing inhibitory potency was associated with the number of hydroxyl groups of the test compounds [31]. It is proposed that the OH-groups of the glucose molecule *via* hydrogen bonds interact with the side chains of polar amino acids (so called QLS sites) lining the wall of the aqueous channel of the facilitative GLUTs, and that the flavonoids with OH-groups may mimic the glucose features [32]. Quercetin, myricetin and catechin-gallate have previously

been proposed to interfere with GLUT4 through a direct interaction with the QLS sites based on computer simulation data [17]. The fact that we observed a high affinity for transport inhibition in case of naringenin might therefore be attributed to the additional hydroxyl group in the A ring. The ketone at position 4 in the C ring appears also to be important for the interaction of flavonoids with GLUT-proteins. Neither catechin nor EGCG inhibited 2-DG uptake in adipocytes in our study and this is in accordance with findings from Strobel *et al.* reporting that catechin could not inhibit 3-*O*-methylglucose transport following insulin stimulation in isolated rat adipocytes [17]. Numerous other studies have shown effects of compounds such as genistein, kaempferitrin, myricetin, catechin-gallate, and quercetin on insulin-stimulated glucose uptake in 3T3-L1 adipocytes [33, 34] or in rat adipocytes [17]; but whether this occurs *via* direct inhibition of GLUT-proteins or indirectly by effects on trafficking needs more detailed analysis. In addition, it needs to be addressed whether the flavonoid concentrations used in these studies for inhibition of glucose transport are relevant under physiological conditions.

Given the enormous variety of flavonoids and the huge spectrum of dietary sources, a reliable quantification of the daily intake of individual flavonoids or subgroups is not trivial [35]. Moreover, studies on the bioavailability of the compounds have shown quite impressive differences in oral availability that are structure-specific. Yet, the concentrations of intact flavonoids in human plasma have been shown to rarely exceed 1 μM when quantities of 10–100 mg of individual compounds were administered [36].

When the flavonoids assessed in the present study were employed at a concentration of 1 μM, they either failed to show inhibition of glucose uptake as in case of daidzein or possessed small but significant effects like naringenin. Moreover, consumption of around 20 oz of grapefruit or orange juice in volunteers was shown to result in peak naringenin plasma concentrations ranging from 0.7 to 14.8 μM [37]. In addition, naringenin was shown to have plasma half-lives of 1–2 h [37]. When using 10 μM of the compound to simulate a concentration as after juice consumption, significant inhibitory effects were observed suggesting that glucose uptake into adipose tissue at those plasma naringenin concentrations could also inhibit transport *in vivo*. In view of the central role of GLUT-4 in glucose uptake into muscle and adipose tissue, our findings implicate that a high dietary intake of flavonoids such as naringenin could potentially impair glucose homeostasis in humans.

In conclusion, we have shown that flavonoids belonging to the flavanone and isoflavonoid subclasses can alter glucose uptake into adipocytes in the basal state and after insulin stimulation by direct interaction with GLUT-transporters. Considering the plasma concentrations that can be achieved in humans by dietary intake either from foods or supplements, we expect that particularly naringenin could impair glucose disposal *in vivo*. However, further studies are required to substantiate this assumption.

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