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Genistein inhibits glucose and sulphate transport in isolated rat liver lysosomes

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Genistein and daidzein are known to have both beneficial and adverse effects on human health due to their many biological actions at the cellular level. Both isoflavones have been shown to inhibit GLUT-mediated glucose transport across the plasma membrane of mammalian cells. Since lysosomal membrane transport is essential for maintaining cellular homeostasis, the present study examined the effects of genistein and daidzein on glucose and sulphate transport in isolated rat liver lysosomes. Both genistein and daidzein significantly inhibited lysosomal glucose uptake. Genistein was a more potent glucose transport inhibitor than daidzein, with a half-maximum inhibitory concentration (IC_{50}) of 45 μmol/l compared with 71 μmol/l for daidzein. Uptake kinetics of D-glucose showed a significant decrease in V_{max} (control:genistein treat = 1489 (SEM 91):507 (SEM 76) pmol/unit of β-hexosaminidase per 15 s) without a change in K_m. The presence of 50 μM-genistein in the medium also reduced glucose efflux from lysosomes preloaded with 100 mM-D-glucose. Genistein also inhibited lysosomal sulphate transport. Similar to its effects on glucose uptake kinetics, genistein treatment caused a significant decrease in sulphate uptake V_{max} (control:genistein treat = 87 (SEM 4):59 (SEM 5) pmol/unit of β-hexosaminidase per 30 s), while the K_m was not affected. The evidence provided by the present study suggests that the most likely mechanism of lysosomal glucose transport inhibition by genistein is via direct interaction between genistein and the transporter, rather than mediation by tyrosine kinase inactivation. Genistein likely has a similar mechanism of directly inhibiting sulphate transporter.

Daidzein: Genistein: Lysosome: Glucose transport: Sulphate transport

Soyabeans and soya products are major foods in Asian diet. Soya is rich in nutrients and phytochemicals, such as isoflavones, a class of Phyto-oestrogen. Evidence suggests that soya isoflavones may have protective effects against many chronic diseases including cancer[1,2], CHD[3,4] and osteoporosis[5]. Thus, soya isoflavones may be used to supplement or even substitute standard oestrogen replacement for menopausal women[6,7]. However, potential toxic effects of phyto-oestrogens in animals and in human subjects have also been described[8]. Therefore, it is important to fully understand the potential positive and negative effects that a physiological and pharmacological dose of soya isoflavones may have on cellular activity, before any adequate dietary recommendation of soya or isoflavones is made to the public.

Via their oestrogenic[9,10] and non-oestrogenic[11,12] effects, genistein and daidzein, the two major isoflavones found in soya, are known to modulate various cellular processes. Numerous data have shown that soya isoflavones can change membrane structure and properties[13], as well as regulate transport activities of various ion channels[14–16], carrier-mediated transporters[17–19] and cotransporters[15,17]. In a recent review, genistein has been shown to induce hormonal and metabolic changes in both *in vitro* and *in vivo* studies[20]. Among these changes, genistein was found to affect carbohydrate and lipid metabolism partially via alterations in glucose transport across plasma membrane. Genistein inhibits glucose uptake[19] and glucose efflux[21,22] in human erythrocytes via its effects on GLUT1. Genistein also inhibits GLUT4-mediated basal and insulin-stimulated glucose transport in rat adipocytes[23], 3T3-1L adipocytes[24] and the contraction-stimulated glucose transport in rat soleus and epitrochlearis muscles[25]. Although plasma glucose transport is important in supplying energy for the cells and in maintaining normal blood glucose levels, cellular glucose homeostasis also partially relies on lysosomal transport systems. Lysosomes are a major site for the degradation of macromolecules including complex carbohydrates, proteoglycans, protein and nucleic acids. More than twenty-five lysosomal membrane transport systems have been identified, which function to release the degradation products, such as glucose and sulphate, for the cell to reuse[26]. These systems are known to play important roles in cellular homeostasis. For example, lysosomal glucose transport is important in regulating glycogen metabolism in liver[27] and muscle[28]. The glucose released from lysosomes may be of little importance for the acute energy needs of the body during 24 h

Abbreviations: CD, circular dichroism; CFTR, cystic fibrosis transmembrane conductance regulator.

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fasting[29], but it is an important fuel source for newborns[30]. Alterations in glucose either across the plasma or the lysosomal membrane may disturb blood glucose homeostasis and lead to the development of diabetes and related diseases. Although the lysosomal glucose transport system has been well characterised[31,32], its regulation by genistein and daidzein remains to be evaluated.

Since genistein and its metabolites are anionic in nature, it is not surprising to learn that genistein acts as a competitive inhibitor of multidrug resistance-associated protein, a membrane transporter that pumps anticancer drugs out of tumour cells[18]. Genistein also stimulates Cl^- secretion via cystic fibrosis transmembrane conductance regulator (CFTR) in various tissues[15,16]. A specific pH-regulated anion transport system that accounts for recycling of sulphate, a major degradation product of glycosaminoglycans and sulpholipids, has been identified and characterised in rat liver lysosomes[33,34]. Dysfunction or alteration of lysosomal sulphate transport may also lead to metabolic derangements and certain diseases.

The present study was therefore designed to examine the regulatory roles of genistein and daidzein on lysosomal glucose and sulphate transport. The results may provide a better understanding of soya isoflavone effects on cellular metabolism and improved dietary guidelines for soya consumption and on soya isoflavone supplementation.

Materials and methods

Chemicals

All chemicals were purchased from Sigma Chemicals (St Louis, MO, USA) unless otherwise stated. Dulbecco's PBS without Ca was obtained from Irvine Scientific (Santa-Ana, CA, USA), GFC/GFF glass fibre filters from Whatman, Ready Safe (counting solution) from Backmann (Fullerton, CA, USA), D-[U-^{14}C]-glucose (310 mCi/mmol, or 11·47 GBq/mmol) from NEN Life Science Products (Boston, MA, USA), *N*-Acetyl-D-[1-^{3}H]-glucosamine (8·30 Ci/mmol or 307 GBq/mmol) from Amersham (Little Chalfont, Bucks, UK) and [^{35}S]-Na_2SO_4 (1325 Ci/mmol or 49 025 GBq/mmol) from Perkin-Elmer (Boston, MA, USA). Bio-Rad protein assay kits were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

Animals and lysosomal purification

The animal experimental protocols were approved by the Institutional Animal Care and Use Committee, Tzu-Chi University. Female Sprague–Dawley rats were obtained from BioLASCO Laboratories (Yilan, Taiwan). Rats (190–250 g) were injected with 1000 units of heparin via tail vein 15 min before sacrifice with CO_2 narcosis. Livers were removed and flushed with ice-cold saline containing 1 mM-EDTA, and homogenised in 0·25 M-sucrose with 1 mM-EDTA. Lysosomes were purified from liver homogenates by differential centrifugation in buffered solutions of iso-osmotic sucrose, pH 7·0, followed by Percoll density gradient centrifugation with 45 % (v/v) buffered Percoll as previously described[35]. The dense lysosomal fraction was removed from the gradient, washed and resuspended in 0·25 M-sucrose or suitable buffer solution at pH 7·0.

Membrane vesicle formation

The lysosomal fraction (about 1 ml) collected from the Percoll gradient was incubated at 25°C with 0·25 M-sucrose, 20 mM-HEPES, pH 7·0, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 500 μM-phenylmethylsulphonyl fluoride, 2 mM-$MgCl_2$, 2 mg/ml bovine serum albumin and 5 mM-methionine methyl ester for 15 min. Lysosomes ruptured owing to the accumulation of methionine and spontaneously resealed to form membrane vesicles. The sealed vesicles were mixed with an equal volume of ice-cold-buffered Percoll and separated by centrifugation at 20 000 ***g*** for 15 min at 4°C. The dark-banded vesicles were removed from the top of the gradient, washed and resuspended in suitable buffer solution at pH 7·0[33].

Glucose transport

Glucose transport was measured using a filtration system[33]. Briefly, glucose uptake by intact lysosomes was determined over a 15 s (or as indicated) interval at 25°C using a 50 μl assay mixture that consisted of 0·25 M-sucrose, 20 mM-HEPES, pH 7·0 (or 20 mM-4-morpholineethanesulphonic acid, pH 5·0), 10 mM-D-glucose, 74 kBq (2 μCi) [U-^{14}C]-D-glucose, treated chemicals (genistein and daidzein) and lysosomes (50–100 μg of protein). After incubation, transport was stopped by the addition of ice-cold PBS without Ca containing 100 μM-phloretin. Lysosomes were collected by filtration through GFC filters, and washed with 10 ml of ice-cold PBS without Ca. Filters were dried and subjected to scintillation counting. For determining the half-maximal inhibitory concentrations (IC_{50}) for genistein and daidzein on glucose uptake, lysosomes were incubated for 15 s in the similar assay mixture, but containing various concentrations of either genistein (0, 5, 10, 20, 30, 60, 100, 150 and 200 μmol/l) or daidzein (0, 10, 20, 30, 60, 100, 150, 200, 250, 300 and 350 μmol/l). Glucose uptake was determined as described above and expressed as percentage of control (i.e. glucose uptake in the absence of isoflavones). The IC_{50} for genistein and daidzein were plotted and estimated by using the Graphpad prism program. *N*-acetyl-D-glucosamine uptake by lysosomes was determined using the similar assay mixture, but contained 74 kBq (2 μCi) *N*-acetyl-D-[1-^{3}H]-glucosamine and 1 mM-*N*-acetyl-D-glucosamine, and the incubation time was 2 min.

For glucose efflux studies, Percoll-free lysosomes (about 1 mg protein) were incubated for 30 min at 25°C in 170 μl of 0·15 M-sucrose in 20 mM-HEPES, pH 7·0, 100 mM-D-glucose with 740 kBq (20 μCi) radiolabelled D-glucose. Lysosomes were then diluted 100-fold in 0·25 M-sucrose, 20 mM-HEPES, pH 7·0, ±50 μM-genistein at 25°C, and 0·9 ml samples were removed in 1 min interval for a total of 12 min, filtered through GFC, washed, dried and counted. Efflux was expressed as glucose remaining as percentage of value at zero time.

For kinetic studies, the glucose concentration in each sample varied from 1·25 to 60 mmol/l, and the initial rate of glucose uptake was examined over a 15 s interval at 25°C.

Glucose uptake was expressed as pmol glucose/unit of β-hexosaminidase per incubation time.

Sulphate transport

Lysosomal sulphate transport was assayed as described for glucose transport with some modifications. The standard assay mixture contained 0·25 M-sucrose buffered with 20 mM-4-morpholineethanesulphonic acid, pH 5·0 (or 20 mM-HEPES, pH 7·0), 100 μM-anhydrous Na_2SO_4, 74 kBq (2 μCi) [^{35}S]Na_2SO_4 ± 50 or 100 μM-genistein or daidzein and 15 μl lysosomes in a final volume of 50 μl. The assay mixture was incubated for 30 s at 25°C.

For kinetic studies, the Na_2SO_4 concentration in each sample varied from 25 to 1000 μmol/l.

For sulphate countertransport, vesicles were formed in the presence of 10 mM-Na_2SO_4. Loaded vesicles (approximately 10 mg protein/tube) were incubated at 25°C in 50 μl of 0·25 M-sucrose, 20 mM-HEPES, pH 7·0, with 100 μM-Na_2SO_4, 74 kBq (2 μCi) [^{35}S]Na_2SO_4 ± 50 or 100 μM-genistein or daidzein. At the end of incubation, the vesicles were collected by filtration through GFF filters. The filters were washed, dried and counted. Sulphate uptake was expressed as pmol sulphate/unit of β-hexosaminidase per incubation time or nmol sulphate/mg protein per incubation time[34].

Circular dichroism spectroscopy

Isolated rat lysosomal vesicles resuspended in 0·25 M-sucrose, 20 mM-HEPES, pH 7·0 solution (approximately 30–45 μg protein per tube) were incubated with or without 50 μM-genistein at 25°C for 15 s, and then subjected to circular dichroism (CD) spectra analysis. CD spectra were recorded using a Jasco 715 spectropolarimeter (Jasco, Tokyo, Japan) with a thermal circular accessory. All measurements were performed in quartz cells with path length of 0·1 cm. Data were collected by scan rate of 100 nm/min from 190 to 300 nm wavelength in 0·2 nm increments. Every CD spectrum reported is the average obtained from at least three individual samples. The reported CD spectra were corrected for baseline using the solution containing 0·25 M-sucrose, 20 mM-HEPES, pH 7·0. All measurements were carried out at 25 ± 0·2°C. Secondary structure analysis was performed in online web server:Dichroweb[36,37]. CDSSTR program[38] was used to estimate the related secondary structure.

Assays

Activities of lysosomal β-hexosaminidase were measured fluorometrically[39] and defined as 1 unit equals 1 μmol of product formed per min at 37°C. Protein content was measured colorimetrically using Coomassie Brilliant Blue[40].

Statistical analysis

Data were analysed using MINITAB release 13.1 for Windows (Minitab, State College, PA, USA). Results were expressed as means with their standard errors. Differences between individual means were compared using Student's *t* test. The level of statistical significance was set at $P<0·05$.

Results

Using isolated rat liver lysosomes, series of *in vitro* studies were conducted to examine the effects of soya isoflavones (genistein and daidzein) on lysosomal membrane transport systems.

Glucose transport

Time-course studies were performed to examine the effects of isoflavones on lysosomal glucose uptake. Incubation of lysosomes with 10 mM-D-glucose and 74 kBq (2 μCi) D-[^{14}C]-glucose resulted in a rapid uptake of radiolabel. Addition of 100 μM-daidzein significantly decreased the rate of glucose uptake (Fig. 1). Addition of genistein also caused an inhibitory effect on glucose transport as daidzein did (data not shown). Both genistein and daidzein inhibited glucose uptake in a concentration-dependent manner. The half-maximum inhibitory concentrations (IC_{50}) of genistein and daidzein were determined to be 45 and 71 μmol/l, respectively (Fig. 2(a) and (b)). Uptake kinetics of D-glucose showed a concentration-dependent saturable process with a K_m of 77 (SEM 9) mmol/l and V_{max} of 1489 (SEM 91) pmol/unit of β-hexosaminidase per 15 s. The addition of 50 μM-genistein caused a significant reduction in V_{max} (507 (SEM 76) pmol/unit of β-hexosaminidase per 15 s), but no change in K_m (64 (SEM 13) mmol/l; Fig. 3(a) and (b)), suggesting a non-competitive inhibition of genistein on glucose uptake. Efflux of D-glucose from lysosomes loaded with 100 mM-glucose by equilibration was time dependent, and was also significantly inhibited by the presence of 50 μM-genistein in the medium (Fig. 4).

To further examine the specificity of isoflavones on lysosomal glucose transport, two other distinct transport systems, *N*-acetyl-*d*-glucosamine and sulphate transport, were studied. The addition of genistein or daidzein in the incubation medium also caused a significant decrease in the

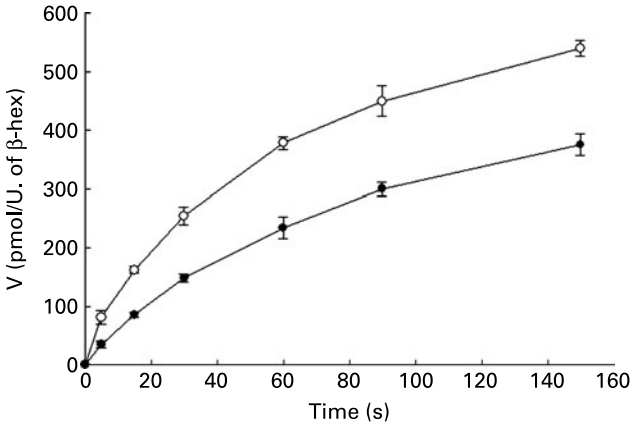

Fig. 1. Glucose uptake *v.* time. Intact lysosomes were incubated at 25°C in sucrose/HEPES buffer, pH 7·0, containing 10 mM-D-glucose, 74 kBq (2 μCi) [U-^{14}C]-D-glucose and in the presence (●, daidzein) and absence (○, control) of 100 μM-daidzein for various time intervals as indicated. At the completion of the incubation period, lysosomes were collected by filtration, washed, dried and counted. Glucose uptake was expressed as pmol/unit of β-hexosaminidase (U. of β-hex). Values are means with their standard errors, *n* 3. Means between groups were significantly different at all time points ($P<0·05$).

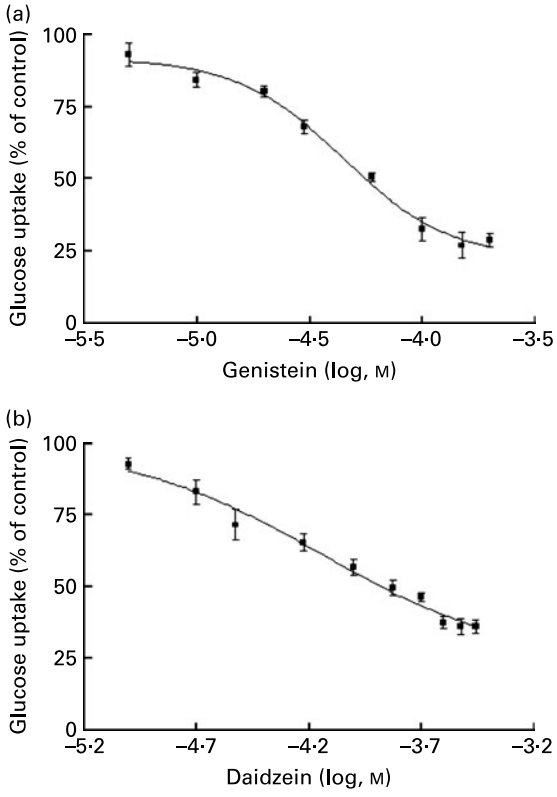

Fig. 2. Dose-dependent effects of isoflavones on D-glucose uptake. Glucose uptake was measured in isolated lysosomes incubated for 15 s in the similar assay mixture as described in Fig. 1, but containing various concentrations of either (a) genistein (0–200 μmol/l) or (b) daidzein (0–350 μmol/l). Glucose uptake was expressed as percentage of control (i.e. glucose uptake in the absence of isoflavones). Values are means with their standard errors, n 3–6 (a), n 4 (b). The half-maximal inhibitory concentrations (IC_{50}) for genistein and daidzein were estimated to be 45 μmol/l (a) and 71 μmol/l (b), respectively.

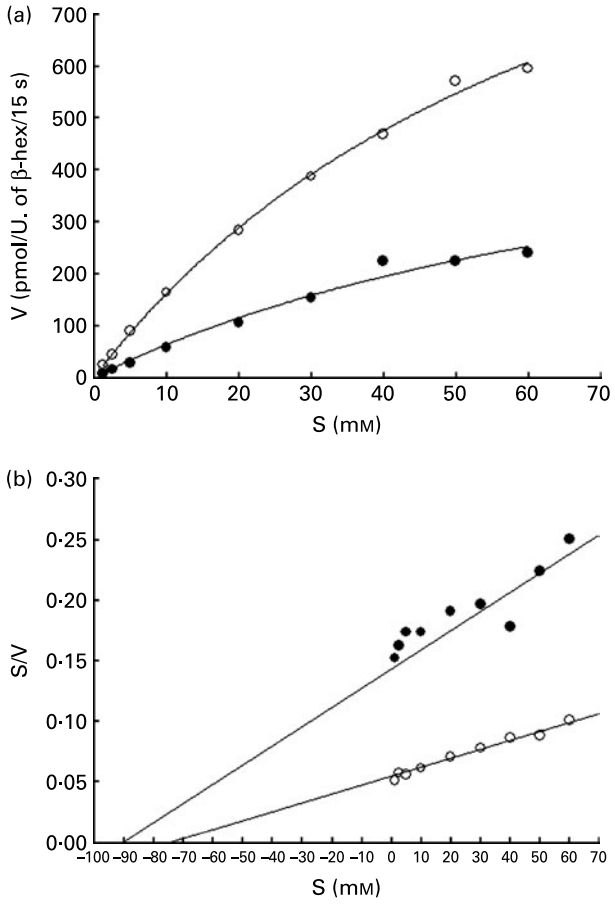

Fig. 3. Effects of genistein on the kinetics of D-glucose transport. Lysosomes were incubated for 15 s at pH 7·0 in sucrose/HEPES buffer containing various concentrations of glucose as indicated, and in the presence (●, genistein) and absence (○, control) of 50 μM-genistein. Michaelis–Menten plot of the initial rate of glucose uptake as a function of the glucose concentration (a); Hanes plot of the same data (b). Results of one representative experiment are displayed. Overall, mean values with their standard errors calculated from three separate experiments are control: $V_{max} = 1489$ (SEM 91) pmol/unit of β-hexosaminidase (U. of β-Hex)/15 s, $K_m = 77$ (SEM 9) mmol/l; and genistein treated: $V_{max} = 507$ (SEM 76) pmol/unit of β-hexosaminidase per 15 s, $K_m = 64$ (SEM 13) mmol/l. V_{max} for glucose uptake was significantly decreased in genistein-treated lysosomes as compared with that of controls ($P < 0{\cdot}005$).

rate of lysosomal *N*-acetyl-D-glucosamine uptake. Both genistein and daidzein showed greater inhibitory effects on glucose uptake than on *N*-acetyl-D-glucosamine uptake, but daidzein was less effective than genistein. Furthermore, the simultaneous presence of 50 μM-genistein and daidzein caused a 60 % reduction in glucose uptake, which is less than the sum total reduction (80 %) resulted from the separate effects of genistein and daidzein (Table 1).

Sulphate transport

The effects of isoflavones on sulphate transport were also examined. As shown in Table 2, the amount of lysosomal sulphate uptake was pH dependent. Appreciable amounts of sulphate uptake were demonstrated at low pH (pH 5·0), but only slight uptake was observed at neutral pH. At pH 5·0, genistein at 50 and 100 μmol/l caused a dose-dependent decrease in sulphate uptake, but the effects were less potent than that on glucose and *N*-acetyl-D-glucosamine transport. Daidzein, on the other hand, had a very small inhibitory effect on sulphate uptake at 100 μmol/l. Kinetic analysis of sulphate transport revealed a significantly lower V_{max} in the presence of 100 μM-genistein compared with that of the controls (control:genistein treated = 87 (SEM 4):59 (SEM 5) pmol/unit of β-hexosaminidase per 30 s). K_m was unaffected by the addition of genistein (control:genistein treated = 216 (SEM 25):260 (SEM 42) μmol/l; Fig. 5(a) and (b)). The data again suggested a non-competitive inhibition of genistein on sulphate uptake.

Sulphate countertransport was demonstrated at external buffer pH 7·0, internal buffer pH 7·0, when lysosomal vesicles were preloaded with 10 mM-Na_2SO_4. This marked stimulation of sulphate uptake as a consequence of sulphate preloading was only moderately decreased by the presence of 100 μM-genistein in the external medium. Daidzein, on the other hand, failed to affect sulphate countertransport (Table 3).

Far-UV circular dichroism

To examine whether genistein has direct effects on the conformation of membrane proteins, lysosomal membrane vesicles

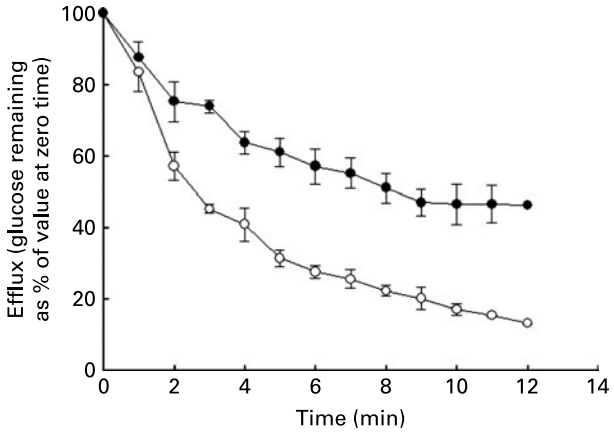

Fig. 4. Effects of genistein on glucose efflux. Isolated lysosomes were equilibrated with 100 mM-D-glucose and 740 kBq (20 μCi) radiolabelled D-glucose for 30 min. Lysosomes were then diluted 100-fold with sucrose/HEPES buffer, and in the presence (●, genistein) and absence (○, control) of 50 μM-genistein. Samples were removed in 1 min intervals and lysosomes were collected, washed, dried and counted. Glucose efflux was expressed as glucose remaining as percentage of values at zero time. Values are means with their standard errors (*n* 3, except the last two points, *n* 2). Differences between groups were significant ($P<0{\cdot}05$) at all time points and past 3 min of incubation.

were incubated in sucrose/HEPES buffer with and without 50 μM-genistein for 15 s. The far-UV CD spectra were recorded and the secondary structures of the membrane proteins were analysed. The results are summarised in Table 4. CD spectra indicated that membrane proteins from both control and genistein-treated lysosomal membrane vesicles contained a high percentage of α-helix. The presence of genistein, however, caused a small transition from α-helix to β-sheet structure.

Discussion

The present report provides evidence for the first time that soya isoflavones, including genistein and daidzein, inhibit glucose and sulphate transport in isolated rat liver lysosomes. Isoflavones are known potent inhibitors of GLUT-mediated glucose transport across plasma membranes of mammalian cells[21–25]. The present findings show similar, but not identical, inhibition profiles of glucose transport across lysosomal membranes. For instance, the sensitivity of lysosomal glucose transporters to genistein inhibition appears less than that of plasma membrane glucose transporters. For liver lysosomal glucose transport, 50 % inhibition is achieved at a genistein concentration of 45 μmol/l, compared with 15[20] and 20 μmol/l[24] for GLUT1 and GLUT4, respectively. This difference in response may be related to the structural features of the transporter proteins. Although the plasma membrane and lysosomal membrane glucose transporter systems have both been characterised and compared[31,32,41,42], without gene and protein identification, it is unclear whether the latter belongs to the GLUT family. There is some evidence suggesting that GLUT8 is the lysosomal glucose transporter. In Chinese hamster ovary cells, GLUT8 is located with late endosome and lysosome and is associated with a lysosomal marker, lysosomal-associated membrane protein 1[43]. However, other studies have found GLUT8 to be expressed mainly in the testis and brain, and to a much lesser extent in the liver[44]. Additionally, GLUT8 has a much higher affinity for glucose ($K_m = 2{\cdot}4$ mmol/l)[44] than lysosomal glucose carriers ($K_m = 22–77$ mmol/l)[31,32]. While GLUT8 and the rat liver lysosomal glucose transporter we have studied have similar properties, it remains to be determined whether they are indeed the same protein, or two different proteins that colocalise on the same membrane. Further studies will also be required to elucidate whether and how GLUT8 is regulated by isoflavones, including genistein.

Lysosomal glucose transport is inhibited by genistein, as noted above. Interestingly, the addition of genistein (50 μmol/l) outside of the lysosome resulted in an inhibition of both glucose uptake (Table 1) and glucose efflux (Fig. 4) with similar potency. One explanation is that genistein is permeable and can cross the lysosomal membrane, blocking transport from both sides, although actual binding sites of genistein on the transporter are unknown.

It has been shown that genistein inhibition of plasma membrane glucose transport may be dependent on pH[22], but the present study has not found this to be true for lysosomal glucose transport. In human red cell membranes, Martin *et al.* [22] reported an inverse relationship between genistein inhibition and pH (6·5–8·5), with a decrease in inhibition at higher pH.

Table 1. D-Glucose and *N*-acetyl-D-glucosamine (GlcNAc) uptakes by rat liver lysosomes treated with genistein and/or daidzein*

(Mean values with their standard errors)

	Uptake (% of control)†					
	D-Glucose (*n* 4)			GlcNAc (*n* 2)		
Treatment	Mean	SEM	*P*	Mean	SEM	*P*
None (control)	100	0·0		100	0·0	
Genistein (50 μmol/l)	50·5	5·5	0·003	61·9	0·8	0·013
Genistein (100 μmol/l)	32·0	4·3	0·001	40·2	1·8	0·019
Daidzein (50 μmol/l)	67·8	7·7	0·041	77·0	0·4	0·012
Daidzein (100 μmol/l)	52·0	3·1	0·005	63·0	0·9	0·015
Genistein (50 μmol/l)+daidzein (50 μmol/l)	40·2	4·0	0·002	–		–

* Student's *t* test was used to compare the means between control and treatment groups for each sugar uptake.
† Actual uptake rates for controls were 299·4 (SEM 27) pmol of glucose/unit of β-hexosaminidase per 30 s and 46·8 (SEM 4·6) pmol of GlcNAc/unit of β-hexosaminidase per 2 min.

Table 2. Sulphate uptake by rat liver lysosomes treated with genistein and/or daidzein at pH 5·0 or pH 7·0*

(Mean values with their standard errors)

	Sulphate uptake (pmol/U. of β-hex per 30 s)					
	pH 5·0 (*n* 4)			pH 7·0 (*n* 3)		
Treatment	Mean	SEM	*P*	Mean	SEM	*P*
None (control)	18·10	2·3		0·630	0·015	
Genistein (50 μmol/l)	13·34	1·9	0·001	0·583	0·007	0·046
Genistein (100 μmol/l)	8·79	1·4	0·013	0·510	0·045	0·065
Daidzein (50 μmol/l)	15·98	2·9	0·580	0·572	0·024	0·110
Daidzein (100 μmol/l)	13·19	2·4	0·190	0·613	0·067	0·820
Genistein (50 μmol/l)+daidzein (50 μmol/l)	10·42	2·0	0·046	0·535	0·030	0·048

β-hex, β-hexosaminidase.

* Student's *t* test was used to compare the means between control and treatment groups at different pH.

Because genistein has a large number of hydroxyl (OH) groups, at alkaline pH, these groups become deprotonated, producing an inactive form of genistein. However, previous data[31] and the present study did not reveal a statistically significant difference in either the control or the genistein-treated lysosomal glucose uptake at pH 5 *v.* 7. A pH greater than 7·4 is not physiologically applicable and was not tested.

Glucose uptake into lysosomes occurs through glucose transporters. Kinetic analysis revealed a non-competitive inhibition of genistein on lysosomal glucose entry (Fig. 3). At a genistein concentration of 50 μmol/l, the maximal transport rate (V_{max}) was significantly reduced; however, there was no change in glucose affinity to the transporter (K_m). This inhibition was rapid after only 15 s of incubation. For this reason, it is likely that the decrease in V_{max} is due to a direct alteration of the transporters by genistein, rather than an overall decrease in the number of available transporters. Another explanation is that genistein inhibits glucose transport by altering lysosomal membrane structure and properties, and thus affecting the transporters.

To determine whether the inhibitory effects of isoflavones on lysosomal glucose transport are due to specific effects on the transporters or general effects on membrane structure and property, we examined two other distinct lysosomal transport systems, *N*-acetyl-D-glucosamine and sulphate anion transport. In the presence of genistein, both transport systems were inhibited, although sulphate transport was inhibited to a lesser degree than both glucose and *N*-acetyl-D-glucosamine transport. Daidzein, another soya isoflavone, also inhibited glucose and *N*-acetyl-D-glucosamine uptake, but with less of an inhibitory effect than genistein. This is likely due to a greater number of hydroxyl (OH) groups in genistein, as suggested by Martin *et al.*[22]. Daidzein, as opposed to genistein, failed to affect sulphate transport. Our data clearly indicate that various lysosomal transport systems respond to genistein and daidzein differently and likely involve different mechanisms.

Different mechanisms of the inhibitory action of genistein on plasma membrane glucose transport have been proposed. One study reported that genistein inhibits plasma membrane glucose transport via inactivating protein tyrosine kinases[45]. However, numerous data have suggested that the inhibitory effect of genistein on plasma membrane glucose transport is

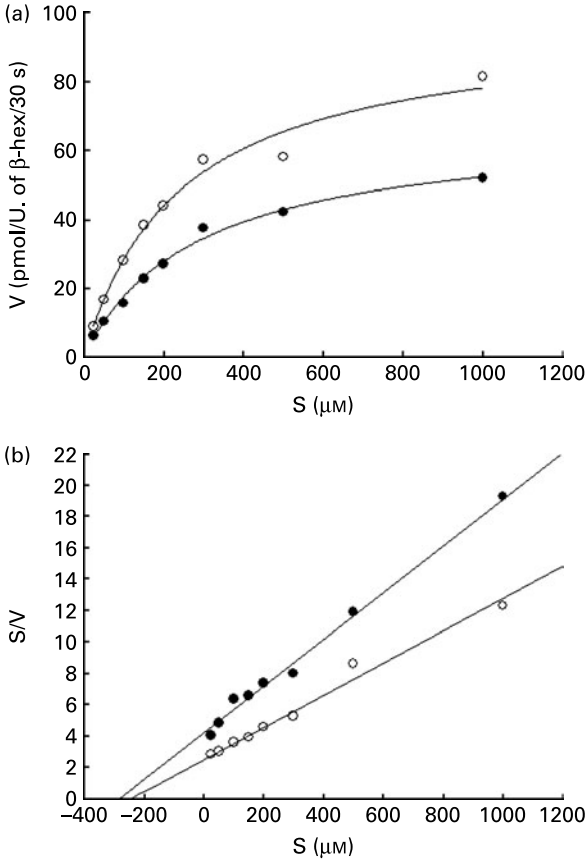

Fig. 5. Effects of genistein on the kinetics of lysosomal sulphate transport. Lysosomes were incubated for 30 s in sucrose/4-morpholineethanesulphonic acid buffer, pH 5·0, containing various concentrations of sulphate as indicated, and in the presence (●, genistein) and absence (○, contol) of 100 μM-genistein. Michaelis–Menten plot of the initial rate of sulphate uptake as a function of sulphate concentration (a); Hanes plot of the same data (b). Results of one representative experiment are displayed. Overall, mean values with their standard errors calculated from six separate experiments are control: V_{max} = 87 (SEM 4) pmol/unit of β-hexosaminidase (U. of β-hex)/30 s, K_m = 216 (SEM 25) μmol/l; and genistein treated: V_{max} = 59 (SEM 5) pmol/unit of β-hexosaminidase per 30 s, K_m = 260 (SEM 42) μmol/l. V_{max} for sulphate uptake is significantly decreased in genistein-treated lysosomes as compared with that of controls (P<0·001).

Table 3. Sulphate countertransport by the isolated lysosomal vesicles treated with genistein and/or daidzein*

(Mean values with their standard errors)

	Sulpate uptake (*n* 3, nmol/mg protein per 30 s)		
Treatment	Mean	SEM	*P*
None (control)	0·902	0·051	
Genistein (50 μmol/l)	0·797	0·032	0·160
Genistein (100 μmol/l)	0·708	0·022	0·025
Daidzein (50 μmol/l)	0·788	0·057	0·210
Daidzein (100 μmol/l)	0·752	0·038	0·078
Genistein (50 μmol/l) + daidzein (50 μmol/l)	0·688	0·048	0·038

* Student's *t* test was used to compare the means between control and treatment groups.

not triggered by tyrosine kinase inhibition; rather it is resulted from direct binding of genistein to the ATP-binding domain of glucose transporters, altering the conformation of the carrier and, subsequently, reducing the rate of glucose fluxes across the membrane[19,21–25,46]. The present study provides evidence that genistein's effect on lysosomal glucose transport is not mediated by tyrosine kinase inactivation, but more likely by a direct interaction with the lysosomal membrane or membrane transporter. For instance, the inhibitory effect was rapidly demonstrated, after only 15–30 s of exposure to genistein, without the long preincubation period required for tyrosine inactivation[19]. Also, the effects of genistein and daidzein on the glucose transporter were not additive (Table 1), suggesting that both inhibit lysosomal glucose uptake by the same mechanism. Since it is known that daidzein is not a tyrosine kinase inhibitor[11], the mechanism of lysosomal glucose transport inhibition by genistein is not likely via tyrosine kinase inactivation. However, unlike plasma membrane glucose transporters, which are regulated by ATP binding and ATP concentration[47], lysosomal glucose transport was not affected by the presence of ATP[48]. It is currently unknown whether or not lysosomal glucose transporter contains a nucleotide binding site. The present results including the kinetic data suggest that genistein does not interact with either the substrate binding site or the nucleotide-binding domain (if indeed present) of the lysosomal glucose transporter.

Another possible mechanism by which genistein and daidzein may inhibit lysosomal glucose transport is their general effects on membrane structure and property.

Table 4. Secondary structure analysis of lysosomal membrane proteins incubated with or without genistein, and determined by far-ultraviolet circular dichroism spectroscopy*

(Mean values with their standard errors)

	Control (%)		Genistein (%)		
Protein structure	Mean	SEM	Mean	SEM	*P*
α-helix	55·5	3·0	46·0	1·5	0·028
β-sheet	18·3	2·1	23·8	1·5	0·076
Random coil	26·3	1·5	29·3	1·6	0·220

* Student's *t* test was used to compare the means between control and treatment groups (*n* 4).

Yu *et al.* [13] reported a change in the plasma membrane protein conformation (increase β-sheet, decrease random coil and no change in α-helix content) of human colon tumour cells incubated with either genistein or daidzein. Our CD spectroscopy data revealed a significant decrease in α-helix, and a small increase in β-sheet and random coil content of lysosomal membrane proteins after the membranes were incubated with genistein. These data suggest an alteration in the membrane proteins; however, it is unknown whether the glucose and sulphate transporters are specifically affected.

Similar to its effects on glucose transport, genistein also inhibits lysosomal anion sulphate transport, although with less potency. Daidzein, as noted above, does not seem to have a significant effect on sulphate transport. A direct interaction between genistein and the transporter or membrane is once again supported by the rapid inhibition of sulphate transport. Other studies have also shown that genistein can alter the activity of a different anion transport system, the CFTR-mediated chloride channel[49]. It has been shown that both ATP binding and ATP hydrolysis are essential for opening the CFTR chloride channel[16], and genistein alters this activity by directly binding to ATP-binding domains on CFTR[49]. Genistein does not appear to act on CFTR chloride channel via cAMP, tyrosine kinase or protein phosphatase-dependent mechanisms[49]. It has been shown previously[33] and in the present study that at neutral pH, sulphate uptake by lysosomes is minimal. However, in *N*-ethylmaleimide (H^+ATPase inhibitor) -treated lysosomes at neutral pH, sulphate uptake can be stimulated by ATP, likely due to ATP binding and/or ATP hydrolysis[48]. For this reason, we think that genistein may interact with the ATP-binding domain of lysosomal sulphate transporters. Also, similar to the effects on glucose transport kinetics, genistein decreases the V_{max} of sulphate transport, without any change in K_m, indicating that genistein interacts with the transporter, but does not compete for the binding to the substrate-binding site. The present experiment using lysosomal vesicles preloaded with 10 mM-Na_2SO_4 showed marked stimulation of sulphate uptake, suggesting active countertransport at pH 7·0. With the addition of 100 μM-genistein to the medium, sulphate uptake was moderately decreased, which leads us to suspect whether genistein is a true competitor, although unlikely, for sulphate transport. However, this is an issue that will require more additional study.

In conclusion, the present studies have shown for the first time that genistein is a potent inhibitor of lysosomal glucose and sulphate transport. While further genetic and proteomic studies are necessary to understand the actual mechanism of inhibition, our data suggest that inhibition is likely due to direct interactions between genistein and the transporters. Our data have significant implications for the increasingly popular use of genistein as a dietary supplement. Although genistein has numerous health benefits, we have shown that it is important to establish guidelines and set a maximum daily intake level for genistein because excessive intake may negatively alter glucose and sulphate metabolism.

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