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Soy extract has different effects compared with the isolated isoflavones on the proteome of homocysteine-stressed endothelial cells

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Epidemiological studies suggest that soy consumption may provide a protection in the development and progression of atherosclerosis. It is under debate, however, whether the soy isoflavones or other compounds are the "active principle". As apoptosis is a driving force in the process of atherosclerosis, we tested whether a soy extract or a combination of the two predominant isoflavones genistein and daidzein, in concentrations as found in the extract, exert similar or different effects on apoptosis in EA.hy 926 endothelial cells after exposure to the endothelial stressor homocysteine. Plasma membrane disintegration and nuclear fragmentation served as relevant apoptosis markers. To assess whether the extract and the genistein/daidzein mixture differently affect cellular target proteins changed in amount by homocysteine treatment, proteome analysis was performed by two-dimensional gel-electrophoresis and peptide mass fingerprinting of regulated protein spots. Homocysteine induced apoptosis in the cells, and both extract and genistein/daidzein inhibited apoptosis to a comparable extent. Whereas the extract prevented for 10 proteins the changes in expression levels as caused by homocysteine, the genistein/daidzein mixture reversed the homocysteine effects on the proteome for 13 proteins. The cytoskeletal protein matrin 3 and a U5 snRNP-specific 40-kDa protein were the only protein entities where both extract and genistein/daidzein reversed the homocysteine-induced changes in a common way. In conclusion, our studies provide evidence that an isoflavone containing soy extract and isolated isoflavones, despite similar effects on inhibition of homocysteine-induced apoptosis in endothelial cells, affect a quite different spectrum of cellular target proteins.

Keywords: Apoptosis / Atherosclerosis / EA.hy 926 cells / Proteome

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1 Introduction

Atherosclerosis is the primary cause of cardiovascular disease that accounts for 50% of all deaths in industrialized countries [1]. Compared to the Western countries the rates of cardiovascular disease in South East Asia are much lower. A higher consumption of soy-containing foods was suggested as an important factor in preventing atherosclerosis in Asian populations [2–4]. However, the "active principle" is still a subject of controversy as there is evidence for distinct biological activities of both the protein fraction as well as of the isoflavones in soy [5]. Several *in vitro* and *in vivo* studies have shown favorable effects of isoflavones on atherosclerosis relevant parameters assessed in a variety of models [6]. Those include the reduction of homocysteine (Hcy) levels in plasma [7], prevention of LDL oxidation [8], improvement of vascular reactivity [9], inhibition of pro-inflammatory cytokines, cell adhesion proteins [10, 11] and reactive nitrogen species [12], as well as a reduction of platelet aggregation [13]. On the other hand, it was suggested that soy ingredients such as beta-conglycinine has atheroprotective effects that greatly exceed those of isoflavones [14]. Moreover, in monkeys, it was shown that soy

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Abbreviations: CCT-delta, chaperonin containing t-complex polypeptide 1 delta subunit; **Dusp3**, dual specificity phosphoprotein phosphatase; **GenDai**, 2.5 μM genistein plus 1.0 μM daidzein; **Hcy**, homocysteine; **Hsp70**, Heat shock protein 70; **IKK**, inhibitor of nuclear factor kappa B kinases; **S40cl**, solgen 40 cleaved; **VDAC-1**, Voltage-dependent anion-selective channel protein 1.

protein with isoflavones but not isoflavone-rich supplement improves arterial low-density lipoprotein metabolism and prevents atherosclerosis development [15].

Apoptosis of endothelial cells is of central importance for the development of atherosclerosis because it leads to an increased exposure of phosphatidylserine groups on the outer leaflet of the plasma membrane that in the presence of coagulation factors V and VII, promotes thrombin generation [16]. In addition, membrane vesicles and blebs from apoptotic endothelial cells contain oxidized phospholipids that induce monocyte adhesion to the endothelium [17]. We have previously shown that the endothelial stressor Hcy induces caspase-independent apoptosis in the endothelial cell line EA.hy 926 [18]. EA.hy 926 were generated by a fusion of human umbilical vein endothelial cells with the human lung carcinoma cell line A549 [19] and represents currently the best-characterized macrovascular endothelial cell line [20]. In the present study, we tested whether the effects of an isoflavone containing soy extract on apoptosis in EA.hy 926 cells when stressed with 25 μM homocysteine differs from those of a mixture of the two most abundant soy isoflavones genistein and daidzein. The extract used here contained 2.5 μM genistein and 1.0 μM daidzein. These are concentrations commonly found in plasma of Japanese whereas on western diets plasma concentrations are usually <40 nM [21]. Hcy was used at a concentration of 25 μM, which resembles a mild hyperhomocysteinemia [22]. Proteome analysis was performed to assess the effects of the soy extract in comparison to that of the isolated isoflavones on target proteins in endothelial cells. Real-time PCR was additionally performed to determine for selected genes whether their regulation by soy extract or genistein/daidzein occurs through effects on transcription or through altered protein degradation.

2 Materials and methods

2.1 Materials

Media and supplements for cell culture were from Invitrogen (Karlsruhe, Germany). Cell culture plates were purchased from Renner (Dannstadt, Germany) and Quadriperm wells from Merck (Darmstadt, Germany). Pharmalyte and IPG strips were from Amersham Biosciences (Freiburg, Germany) and sequencing grade modified trypsin from Promega. Complete mini protease inhibitor cocktail was purchased from Roche (Mannheim, Germany), CBB G250 from Serva (Heidelberg, Germany), and the protein assay from Bio-Rad (Munich, Germany). Soy extract was obtained from Solbar Plant Extracts (Ashdod, Israel), β-glucosidase, genistein, daidzein, the Hoechst dyes 33258 and 33342 and all other materials were from Sigma (Deisenhofen, Germany).

2.2 Soy extract solgen 40 cleaved

Enzymatic treatment for releasing de-glycosylated isoflavones was carried out using two units β-glucosidase per miligram isoflavones. Enzyme and solgen 40 were mixed in 0.1 M sodium acetate/acetic buffer pH 5.0. This mixture was put in a 37°C shaking water bath at 100 rpm for 19 h. Subsequently the material was freeze-dried.

2.3 Analysis of isoflavone content and composition

A representative sample (0.1 g) was taken from the freeze-dried product ($n = 3$) and the isoflavone content was determined after extraction and HPLC analysis as described [23]. Samples were extracted by stirring with 20 mL 50% ACN/50% MilliQ water for 2 h at room temperature [23]. The obtained solution was filtered (Spartan 30 0.45-μm RC filter units) and if necessary diluted with extraction medium. The sample was injected on a Waters 2690 separations module with a column oven and a Waters 960 PDA detector attached to it. Separation was achieved using a Symmetry C18 column (250 × 4.6 mm, 5 μm). The chromatographic conditions were as follows: flow 1.5 mL/min; volume of injection 10 μL; column temperature 40°C; and solvents, A, 10 mM ammonium formate in MilliQ water (pH 2.8 with formic acid) and B, gradient grade ACN. The gradient consisted of 15% of B isocratic for 5 min, 15 – 29% of B for 31 min and 29 – 35% of B in 4 min. Detection was carried out in a diode array detector. PDA data between 225 and 300 nm were collected and a signal of 260 nm was extracted for integration. Peaks were identified based on their retention time and UV spectrum against previously recorded standards. HPLC analysis revealed solgen 40 to contain 148.9 mg daidzin, 5.8 mg daidzein, 309.9 mg genistin and 2.4 mg genistein per gram extract. After enzymatic treatment, solgen 40 cleaved (S40cl) contained no detectable amounts of daidzin, 52.4 mg daidzein, 8.8 mg genistin and 139.1 mg genistein per gram extract. S40cl was dissolved in DMSO as a 100-fold stock solution and was used in the cell culture studies with a final concentration of 2.5 μM genistein and 1.0 μM daidzein.

2.4 Cell culture

EA.hy 926 cells were a generous gift from Prof. C-J. S. Edgell, University of North Carolina at Chapel Hill and were used at passage 28. Culturing of the cells was carried out in 75-cm^2 flasks with DMEM supplemented with 25 mM HEPES, 10% fetal bovine serum, 12.5 μg/mL gentamycin, 1 × modified Eagle's medium-amino acid-solution and 1 × basal medium Eagle's-vitamins. Added antibiotics were 400 U/mL penicillin and 400 μg/mL streptomycin.

The cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. Cells were passaged at preconfluent densities by the use of a solution containing 0.1% trypsin and 0.04 mM EDTA.

2.5 Apoptosis assays

Changes in membrane permeability as an early apoptosis marker were assessed after 3×10^4 EA.hy 926 cells were grown on glass slides placed into Quadriperm wells and then incubated with the test compounds for 24 h. Cells were stained with 1 μg/mL Hoechst 33342 and rate of accumulation of the dye in early apoptotic cells [24] was detected using an inverted fluorescence microscope (Leica DMIRBE, Bensheim, Germany) equipped with a bandpass excitation filter of 340–380 nm and a longpass emission filter of 425 nm.

Nuclear fragmentation as a late marker of apoptosis was determined by staining of DNA with Hoechst 33258. EA.hy 926 cells (3×10^4) were seeded on glass slides placed into Quadriperm wells and then incubated with the test compounds for 24 h. Thereafter, cells were washed with PBS, allowed to air-dry for 30 min and then fixed with 2% paraformaldehyde prior to staining with 1 μg/mL Hoechst 33258 and visualization using the inverted fluorescence microscope.

2.6 Sample preparation for 2-D-PAGE

Following a 24-h incubation period, cells were washed three times with ice-cold 350 mM sucrose, containing Complete-Mini proteinase inhibitor and then scraped off with a cell scraper. Cells of two flasks were combined in 6 mL of ice-cold sucrose solution and subsequently centrifuged for 7 min at 2500 × g. The supernatant was discarded and 200 μL of lysis buffer (7 M urea, 2 M thiourea, 2% CHAPS, 1% DTT, 0.8% Pharmalyte, CompleteMini) was added to the pellet. Homogenization of the cells was achieved by ultra sonification (10 strokes, low amplitude) on ice. Lysed cells were centrifuged for 30 min at 100 000 × g at 4°C and the supernatant containing solubilized proteins was used immediately or stored at −80°C. Protein concentration of samples was determined using the Bio-Rad protein assay.

2.7 2-D-PAGE

The 2-D-PAGE, IEF in the first dimension and SDS-PAGE in the second dimension, was performed as described by Görg *et al.* [25] with minor modifications. Briefly, IEF was performed on 18-cm IPG strips, pH 3–10 using an Amersham IPGphor unit. Each strip was rehydrated for 12 h with 340 μL of rehydratation buffer (8 M urea, 0.5% CHAPS, 15 mM DTT, 0.5% IPG buffer). Protein-suspension (500 μg) was then loaded onto the strip by cup loading. IEF was performed under the following conditions: 500 V (10 min, gradient), 4000 V (1.5 h, gradient), 8000 V (25 000 Vh, Step-n-hold). Subsequent to IEF, strips were incubated for 15 min in equilibration-buffer 1 (1.5 M Tris-HCl, pH 8,8, 6 M urea, 26% glycerol, 2% SDS, 1% DTT) and then for another 15 min in equilibration-buffer 2 (1.5 M Tris-HCl, pH 8.8, 6 M urea, 26% glycerol, 2% SDS, 4% iodoacetamide) before loading onto SDS-PAGE gels. The 1-mm-thick 12.5% SDS-polyacrylamide gels were cast according to the method of Laemmli [26] and were run using an Amersham Biosciences Ettan-Dalt II System employing the following conditions: 4 mA per gel for 1 h, then 12 mA per gel.

The proteins in the gels were fixed in 40% v/v ethanol and 10% v/v acetic acid for 5 h. Gels were then stained overnight in Coomassie-solution containing 10% w/v $(NH_4)_2SO_4$, 2% v/v phosphoric acid, 25% v/v methanol and 0.625% w/v CBB G250. Gels were destained in double distilled water until the background was completely clear.

2.8 Analysis of proteins using the ProteomWeaver Software

Gels stained with CBB were scanned using an ImageScanner (Amersham Biosciences) and spots detected by the ProteomWeaver software (Definiens, Munich, Germany). Background subtraction and volume normalization were made automatically by the software. After spot detection, all gels were matched to each other. Gels from at least three independent runs of cells derived from at least two independent cell batches after different treatments were compared to each other. Spots differing significantly ($p < 0.05$, Student's *t*-test) in density were picked for MALDI-TOF-MS analysis. Data of relative spot intensities are given as the mean ± SD.

2.9 Enzymatic digestion of protein spots for MALDI-TOF-MS

CBB-stained spots were picked with a 2-mm or 3-mm "skin-picker". The destaining of spots occurred with alternating washing procedures in pure 50 mM NH_4HCO_3 and ACN/pure 50 mM NH_4HCO_3 1/1. After the blue color was fully removed, a last washing step with pure ACN followed and the spots were dried in a SpeedVac. The dry spots were rehydrated for 1 h at 4°C with 5 μL of 0.02 μg/μL sequencing grade modified trypsin for 60 min on ice. The trypsin-supernatant was removed to perform the digestion of the proteins by incubating overnight at 37°C. 7 μL of 1% TFA

was added to each spot and peptide fragments were extracted by ultra sonification for 10 min. The supernatants derived from each spot were collected and used for MS.

2.10 MALDI-TOF-MS analysis of tryptic peptides

Peptide mass analysis was performed using the Autoflex mass spectrometer of Bruker Daltonics (Leipzig, Germany). 2–4 μL protein sample were spotted onto HCCA AnchorChip™ targets using the double-layer method from Bruker Daltonics. Detection was performed in the positive ion reflector mode and a peptide calibration standard (Bruker Daltonics) was used for external calibration. Proteins were identified by the use of the MASCOT Server 1.9 (Bruker Daltonics) based on mass searches within human sequences only. The search parameters allowed for carboxyamidomethylation of cysteine and one missing cleavage. The criteria for positive identification of proteins were set as follows: (i) a minimum score of 63; (ii) a mass accuracy of ±0.01%; (iii) at least a twofold analysis from two independent gels, and (iv) that the protein exhibits a significant difference in the number of matched peptides to the next potential hit.

2.11 LightCycler real-time RT-PCR

Quantitative RT-PCR was performed as described by Pfaffl *et al*. [27]. Briefly, 1 μg of total RNA from EA.hy 926 cells was reversely transcribed to generate cDNA pools, and 3.3 ng reversely transcribed total RNA was added to each PCR reaction. The relative amount of target mRNA normalized to the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT) was calculated according to the method described [28]. HPRT was chosen as housekeeping gene since other housekeeping genes like glyceraldehyde dehydrogenase, 18S or β-actin have been described to be affected by Hcy at the mRNA-level in endothelial cells [29]. Primer design was done using the LightCycler Probe design software. Blast search in the published sequence database GenBank (http://www.ncbi.nlm.nih.gov/BLAST) revealed that primers were gene specific. If possible, primers that spanned at least one intron were chosen. Based on these criteria the following primers were used: Annexin II (forward primer 3–22 (position in the open reading frame); reverse primer 305–290); dual specificity phosphatase 3 (Dusp3) (168–183, 358–342); Capping protein alpha subunit isoform 1 (18–34, 244–227); heat shock 70 kDa protein 9B (1415–1430, 1623–1607); Matrin 3 (939–955, 1158–1143); and HPRT (191–207, 456–441).

For each variable at least three independent experiments were carried out. Variance analysis between groups was performed by one-way ANOVA and significance of differences between groups was determined by a Tukey's Multiple Comparison test (GraphPadPrism, San Diego, CA, USA). Data are given as the mean ± SD.

3 Results

3.1 S40cl and genistein/daidzein both inhibit Hcy-induced apoptosis in EA.hy 926 cells

Apoptosis as an atherosclerosis-promoting factor was assessed in EA.hy 926 endothelial cells in response to the endothelial stressor Hcy. As reported previously [18], Hcy induced plasma membrane disintegration (Fig. 1A) as an early marker of apoptosis and nuclear fragmentation and chromatin condensation (Fig. 1B) that are markers of late stages in the apoptotic process. Both the soy extract S40cl containing 2.5 μM genistein and 1.0 μM daidzein and the mixture of isolated genistein and daidzein (GenDai) at the same concentrations inhibited Hcy-induced membrane disintegration and nuclear fragmentation completely (Fig. 1).

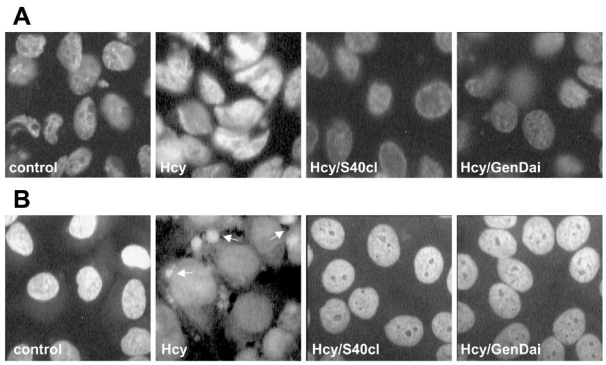

Figure 1. Hcy-induced apoptosis in EA.hy 926 cells is inhibited by S40cl and GenDai. (A) Detection of plasma membrane disintegration after 24 h incubation was assessed based on the uptake of Hoechst 33342 in control cells, cells exposed to 25 μM Hcy or the combination of 25 μM Hcy with either S40cl (final concentration of genistein 2.5 μM and of daidzein 1.0 μM) or with 2.5 μM genistein/1.0 μM daidzein (GenDai). (B) Detection of nuclear fragmentation with Hoechst 33258 was achieved in cells treated as described in (A) after fixation. Arrows indicate nuclear fragmentation and chromatin condensation. Representative photographs from three independent cell batches are shown.

3.2 S40cl and GenDai affect Hcy-induced changes in the endothelial cell proteome differently

2-D-PAGE separated more than 700 different protein spots of which 51 proteins were significantly affected in steady state level by Hcy (data not shown). For 13 of these 51 proteins the Hcy-induced changes were reversed by the co-administration of extract S40cl and 10 of these 13 proteins

Table 1. Steady state level ratios of those proteins for which changes upon Hcy treatment could be altered by the addition of soy extract S40cl in EA.hy 926 cells

Spot Id[a)]	Protein description	Theoretical M_r/p*I*	Measured M_r/p*I*	Protein amount		% Sequence coverage	Accession no.
				Hcy/control	Hcy & S40cl/ Hcy		
Annexins							
1	Annexin A2	39/7.6	39/4.8	3.343	0.186	24	ANX2_HUMAN
Chaperones							
2	Heat shock protein gp precursor	90/4.7	43/3.8	0.3234	1.9570	11	AAK74072
3	dnaK-type molecular chaperone precursor, mitochondrial	74/5.9	66/4.7	0.416	8.754	30	B48127
Cytoskeletal proteins							
4	Capping protein alpha	33/5.5	39/4.5	2.668	0.297	41	G02639
5	Matrin 3	95/5.9	63/7.4	0.323	2.512	23	AAH15031
Detoxification							
6	Probable thioredoxin peroxidase (EC 1.11.1.-) PAGA	22/8.3	24/8.9	0.497	3.320	59	A46711
Kinases/Phosphatases							
7	Dual specificity phosphoprotein phosphatase (EC 3.1.3.-) DUSP3	21/7.7	22/7.1	0.444	2.048	60	A47196
8	IKK interacting protein, isoform 1	43/4.9	53/3.4	Only in control	Only in Hcy & S40cl	26	AAH58933
Gene regulation							
9	Splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated)	76/9.5	103/9.5	Only in Hcy	Only in Hcy	21	AAH51192
10	U5 snRNP-specific 40 kDa protein	40/8.3	39/8.5	Only in control	Only in Hcy & S40cl	28	AAC69625

a) The spot numbers are identical to those given in Fig. 2.

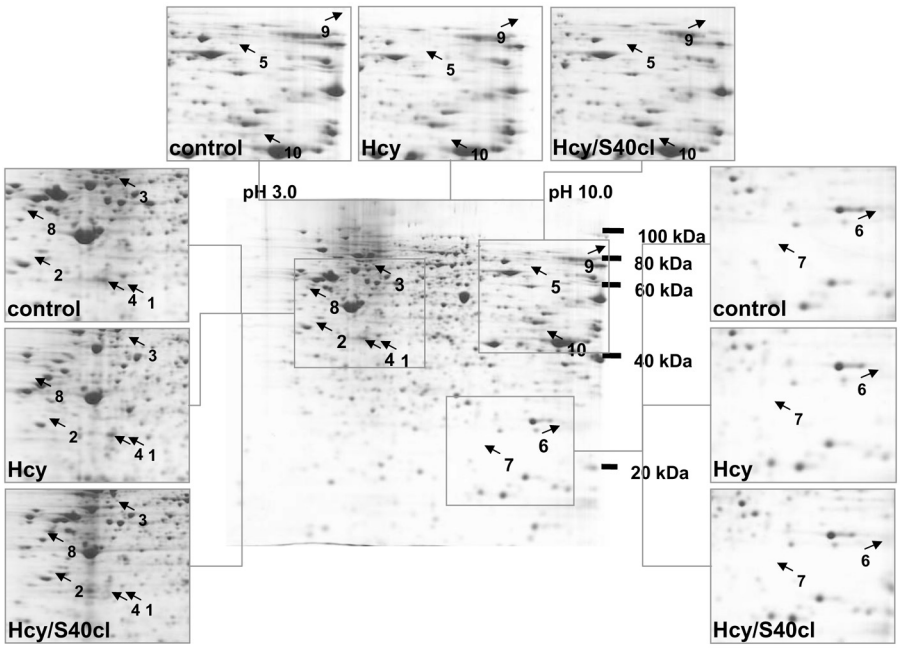

Figure 2. 2D-PAGE of proteins from EA.hy 926 cells exposed for 24 h to 25 μM Hcy in the absence or presence of the cereal bar extract S40cl. Proteins were separated on pH 3-10 IPG-strips in the first dimension and on 12.5% SDS-polyacrylamide gels in the second dimension. The middle section shows a representative Coomassie-stained gel derived from EA.hy 926 control cells surrounded by enlarged gel areas showing the comparative analysis of protein spots from control cells, cells exposed to Hcy, and cells treated with Hcy and S40cl.

could be identified by MALDI-TOF-MS (Table 1, Fig. 2). For example, the mitochondrial dnaK-type molecular chaperone precursor was decreased in expression level more than twofold by Hcy treatment whereas in the simultaneous presence of S40cl protein levels increased almost fourfold over that in untreated control cells (Table 1, Fig. 2). Similarly, the dual specificity phosphoprotein phosphatase Dusp3 showed a reduced spot intensity after treatment with Hcy which was reversed by the addition of S40cl (Table 1, Fig. 2).

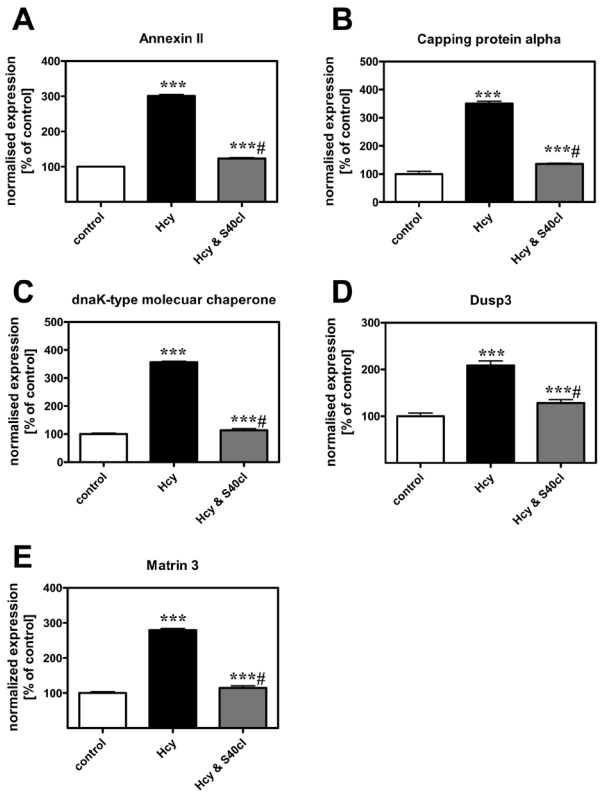

Figure 3. Relative mRNA levels of selected genes in control EA.hy 926 cells or EA.hy 926 cells treated with 25 μM Hcy in the absence or presence of S40cl. Transcript levels of five genes were determined by real-time PCR in comparison to the housekeeping gene HPRT. Relative expression of untreated control cells were set as 100%.$^{***}p < 0.001$ versus control cells; $^{***\#}p < 0.001$ versus HG_1-treated cells

Interestingly, both genes were regulated at the mRNA levels in the opposite direction, *i.e.*, the up-regulation as caused by Hcy was reversed by the co-administration of S40cl (Fig. 3). This suggests that dnaK-type molecular chaperone precursor and Dusp3 protein levels are mainly regulated via protein degradation or are extensively modified to yield forms that escaped detection. However, that this is not a general theme is seen in case of annexin A2 and capping protein alpha that were increased by Hcy-treatment and decreased by S40cl in Hcy-exposed cells and that changed at both the mRNA and protein levels (Table 1, Fig. 3).

Similarly to the number of proteins affected by S40cl in Hcy-treated cells, GenDai reversed the effects of Hcy on the steady state levels of 18 proteins of which 13 were clearly identified by peptide mass fingerprinting (Table 2, Fig. 4). Matrin 3 and U5 snRNP-specific 40-kDa protein turned out to be the only proteins that changed similarly in level by S40cl and GenDai treatment of Hcy-exposed cells (Tables 1 and 2, Figs. 2 and 4). In contrast to S40cl, GenDai prevented in Hcy-treated cells the decrease of lamin A and of the lysosomal aspartyl protease cathepsin D (Table 2, Fig. 4). A protein where GenDai caused a decrease of the Hcy-induced increase in steady state level was the voltage-dependent anion-selective channel protein 1 (VDAC-1) (Table 2, Fig. 4).

3.3 Effects of S40cl and of GenDai on the proteome of EA.hy 926 cells in the absence of Hcy

The effects of S40cl and GenDai on the endothelial cells' proteome were also assessed without treating cells with the stressor Hcy. S40cl altered the expression levels of 54 proteins of which 34 distinct protein entities were identified by MALDI-TOF-MS (Table 3, Fig. 5A) and GenDai changed levels of 30 proteins of which 14 could be identified (Table 4, Fig. 5B). Of the S40cl regulated proteins 5 were also identified as changed in Hcy-treated cells when S40cl was co-administered. Whereas the putative thioredoxin peroxidase was regulated by S40cl in the same direction in control and in Hcy-exposed cells, this was not the case for the dnaK-type molecular chaperone precursor, annexin A2, inhibitor of nuclear factor kappa B kinase (IKK) interacting protein and the U5 snRNP-specific 40-kDa protein (Tables 1 and 3). These results demonstrate that the effects caused by S40cl are largely dependent on the presence or absence of Hcy. In GenDai-treated cells, also 5 proteins were identified as regulated by the isoflavones in the presence and absence of Hcy (Table 4, Fig. 5 B). Here, cathepsin D, 6-phosphogluconolactonase, the U5 snRNP-specific 40-kDa protein and the KH-type splicing regulatory protein were regulated in opposite directions by GenDai, depending on whether Hcy was present or not (Tables 2 and 4). Only the heterogeneous nuclear ribonucleoprotein H was reduced in its expression levels in the presence and the absence of Hcy (Tables 2 and 4).

4 Discussion

Soy consumption has been consistently demonstrated to provide preventive effects on the development and progression of coronary heart disease [3, 4]. Although soy contains a vast number of different phytochemicals, the beneficial effects of soy were mainly ascribed to the isoflavones due to their ability to bind to estrogen receptors and to behave as weak agonists/antagonists in both animals and humans [30]. To decrease cardiovascular risk a minimum intake of 40 – 60 mg isoflavones/day, together with 25 g of soy protein is recommended [30]. The isoflavones daidzein and genistein occur naturally in most soy foods mainly as glucose-conjugates. To reach circulation and for entering cells, hydrolysis with the release of the sugar moiety by intestinal or bacterial beta-glucosidases is needed [31, 32]. Plasma

Table 2. Steady state level ratios of those proteins for which changes upon Hcy treatment were significantly affected by 2.5 μM genistein plus 1.0 μM daidzein (GenDai) exposure in EA.hy 926 cells

Spot Id[a)]	Protein description	Theoretical M_r/pI	Measured M_r/pI	Protein amount		% Sequence coverage	Accession no.
				Hcy/control	Hcy & GenDai/Hcy		
Chaperones							
1	Chaperonin containing t-complex polypeptide 1, delta subunit; CCT-delta	58/8.0	58/7.8	0.4313	2.0647	29	AAC96010
Cytoskeletal proteins							
2	Lamin A	74/6.6	72/5.8	0.4124	2.705	33	VEHULA
3	Matrin 3	95/5.9	64/8.1	0.229	2.5266	23	AAH15031
4	VDAC-1 (hVDAC1) (Outer mitochondrial membrane protein porin 1) (Plasmalemmal porin) (Porin 31HL) (Porin 31HM)	31/8.6	34/7.3	2.0566	0.4998	43	POR1_HUMAN
Metabolism							
5	Cathepsin D Chain B, Cathepsin D At Ph 7.5	26/5.3	32/4.5	0.1027	6.2268	47	1LYWB
6	6-Phosphogluconolactonase	28/5.7	29/5.3	2.6064	0.4965	44	CAB57866
Gene regulation							
7	Heterogeneous nuclear ribonucleoprotein H	49/5.9	27/5.9	2.2014	0.4987	28	I39358
8	Heterogeneous nuclear ribonucleoprotein H	49/5.9	34/4.6	2.1582	0.4896	28	I39358
9	54-kDa nuclear RNA- and DNA-binding protein (p54(nrb)) (p54nrb) (55 kDa nuclear protein) (NMT55) (Non-POU domain-containing octamer-binding protein) (DNA-binding P52/P100 complex, 52 kDa subunit)	54/9.0	60/8.9	0.4322	2.492	30	NR54_HUMAN
10	Putative spliceosome associated protein (breast carcinoma amplified sequence 2)	26/5.5	29/4.6	2.6051	0.3403	51	AAC64059
11	Translation elongation factor EF-Tu precursor	50/7.7	35/5.5	2.2021	0.498	44	S62767
12	U5 snRNP-specific 40 kDa protein	40/8.3	41/8.0	0.3455	3.5	41	AAC69625
Other proteins							
13	KH-type splicing regulatory protein	73/6.8	74/6.5	0.4987	1.8209	27	AAB53222

a) The spot numbers are identical to those given in Fig. 4.

concentrations of genistein and daidzein in the low micromolar range are generally observed in Asian populations with an adequate dietary intake of soy products [21]. Such plasma concentrations can also be reached after a single high dose of isoflavones [32].

In the present study, we tested whether a combination of the soy isoflavones genistein and daidzein presented as a mixture or in a soy extract affect apoptosis in endothelial cells combined with the identification of cellular target proteins. The soy extract was enzymatically treated to release the isoflavone aglycones from the glycosides and to be able to compare their actions on Hcy-stressed cells. Apoptosis is of crucial importance as a stress-response in pro-atherosclerotic lesions [16, 17] and Hcy was shown to induce apoptosis in EA.hy 926 endothelial cells that was associated with the regulation of a number of apoptosis-relevant proteins [18].

Both S40cl and GenDai inhibited Hcy-induced apoptosis as shown by a reduced uptake of Hoechst 33342 that was used as an indicator of early apoptosis by membrane permeability impairments. Similarly, late apoptosis events that are indicated by DNA-fragmentation assessed by staining with Hoechst 33258 were also diminished in Hcy-treated cells by the extract and the mixture. Irrespective of these similar anti-apoptotic effects of S40cl and GenDai, their actions on the proteome of Hcy-exposed cells were quite different. Matrin 3 and the U5 snRNP-specific 40-kDa protein were the only proteins where both S40cl and GenDai prevented the changes in the steady state levels caused by Hcy. Functionally, the Hcy-dependent reduction of the cytoskeletal protein matrin 3 may be best explained by its specific cleavage by proteases activated during apoptosis induction [33]. Although the function of matrin 3 and U5 snRNP-specific 40-kDa protein in atherosclerosis is not clear yet, it

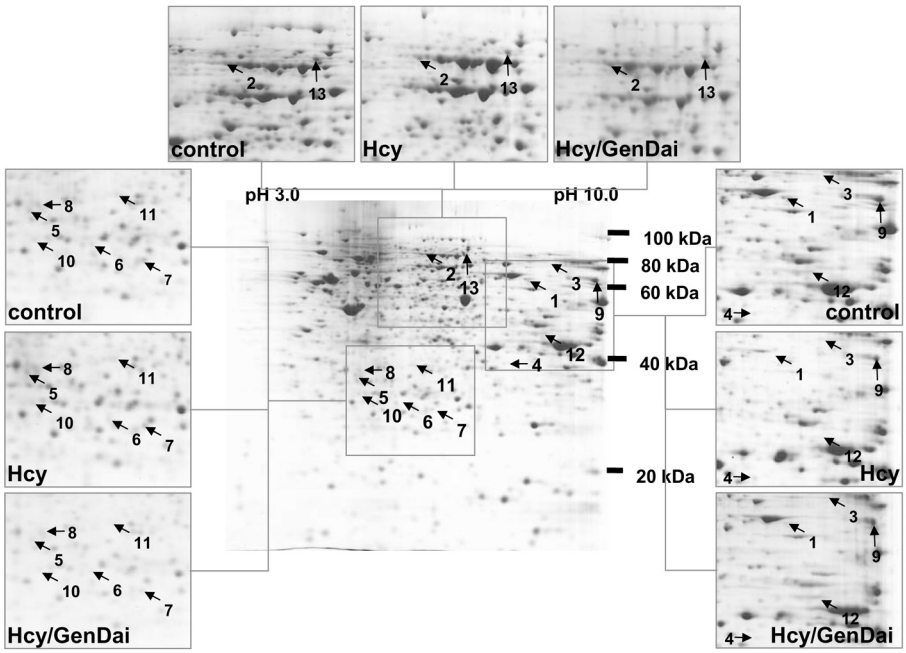

Figure 4. 2D-PAGE of proteins from EA.hy 926 cells incubated for 24 h with medium alone (control) or with 25 μM Hcy either in the absence or in the presence of the 2.5 μM genistein and 1.0 μM daidzein (GenDai). The middle section shows a representative Coomassie-stained gel derived from EA.hy 926 control cells surrounded by enlarged gel areas derived from protein separations of control cells, cells exposed to Hcy alone or in combination with GenDai.

cannot be excluded that their regulation contributes to the proposed anti-apoptotic/anti-atherosclerotic actions of both GenDai and S40cl. Other specified proteins with relevance to apoptosis, however, were only identified in cells exposed to S40cl or GenDai. This was the case, *e.g.*, for VDAC-1 where only GenDai reversed its Hcy-induced increase. VDAC-1 is a receptor for plasminogen kringle 5, which was shown to induce apoptosis in human endothelial cells [34, 35]. The reduction in the levels of dual specificity (threonine/tyrosine) phosphoprotein phosphatase DUSP3 caused by Hcy, on the other hand, were only prevented by S40cl but not by GenDai. This effect might support the anti-apoptotic actions of S40cl based on an inactivation of stress-induced mitogen-activated protein kinase signaling pathways by DUSP family members [36, 37]. Moreover, the increased expression of the identified thioredoxin peroxidase by S40cl in Hcy-treated cells could augment the anti-apoptotic actions of the extract since thioredoxin peroxidase acts as a cellular antioxidant system in protecting endothelial cells against oxidative damage and apoptosis [38].

Besides proteins with an obvious role in the process of apoptosis, a number of other proteins with relevance in atherogenesis were identified in response to either S40cl or GenDai in EA.hy 926 cells exposed to Hcy. The dnaK-type molecular chaperone that is also known as mortalin-2 or heat shock protein 70 (Hsp70) was found to be reduced in Hcy-treated cells and S40cl reversed this effect. Hsp70 has been demonstrated to suppress angiotensin II – induced hypertension that seems to be mediated via an interaction of Hsp70 with the NF-κB pathway [39, 40]. Hsp70 was demonstrated in this context to deplete IKK-α and to suppress the depletion of IκB-α [38], thus inhibiting NF-κB mediated signal transduction. Whereas we did not find altered steady state levels of IKK-α or IκB-α in Hcy-treated cells we observed a reduction in the level of an IKK-interacting protein isoform 1 by Hcy that was blocked by S40cl, suggesting that this protein could inhibit NF-κB activity. GenDai exposure resulted in Hcy-exposed cells in the prevention of a reduction in cathepsin D, a protein that is important for the degradation of atherogenic low-density lipoproteins [41]. Similarly, GenDai reversed the Hcy-induced decline in the levels of chaperonin containing t-complex polypeptide 1 delta subunit (CCT-delta) and nuclear lamin A. CCT-delta is essential for the biogenesis of tubulin [42], and tubulin levels in normal aortic intima were found to be generally higher than in aortic intima with fibro-fatty lesions [43]. Moreover, a functional loss of lamin A is associated with lipodystrophy, hyperinsulinemia, dyslipidemia, diabetes and hypertension [44]. Thus, reported anti-atherogenic activities of GenDai may be mediated by the effects on CCT-delta and lamin A. It has to be stressed here that there seem to be different types of lamins with different properties due to posttranslational modifications by glycosylation, phosphorylation and methylation [45]. Hcy reduced mainly the levels of the smaller sized lamins and GenDai reversed this effect. In accordance with these data, we observed an Hcy-induced decline of the low molecular mass lamin A in EA.hy 926 cells also previously and this decrease was blocked by genistein [18]. In the present study, S40cl reduced the amount of the higher molecular weight lamin A forms too.

Table 3. Steady state level ratios of proteins identified by MALDI-TOF MS analysis of the proteome of EA.hy 926 cells exposed to S40cl

Spot Id[a)]	Protein description	Theoretical M_r/p*I*	Measured M_r/p*I*	Protein amount S40cl/control	Seq. cov. in %	Accession no.
Annexins						
1	Annexin A2	39/7.6	34/8.3	Only in control	19	ANX2_HUMAN
2	Annexin A2	39/7.6	39/4.8	5.378	24	ANX2_HUMAN
Chaperones						
3	dnaK-type molecular chaperone precursor, mitochondrial	74/5.9	66/4.7	Only in control	30	B48127
4	DnaJ protein SB73	36/5.8	36/4.9	Only in control	32	AAL56008
5	Endoplasmin precursor	93/4.8	100/3.3	0.3581	28	A35954
Cytoskeletal proteins						
6	NAG22 protein	56/5.7	83/5.9	0.421	28	AAF69498
7	Lamin A	74/6.6	80/5.8	0.497	33	VEHULA
8	Motor protein	80/5.7	90/5.3	0.489	28	BAA04654
9	Oxygen-regulated protein 150K precursor	111/5.2	55/8.9	0.498	17	JC5278
10	VPS29-like phosphoesterase-related protein	21/6.3	23/6.2	0.455	36	JC7515
11	Endoplasmic-reticulum-lumenal protein 28	29/6.8	28/5.2	Only in control	42	T09549
12	Lamin C	65/6.4	72/6.1	2.1642	39	VEHULC
Metabolism						
13	TKT protein	59/6.5	70/7.2	0.478	36	AAH24026
14	Coiled-coil-helix-coiled-coil-helix domain containing 3	26/8.5	8/8.7	0.385	28	AAH11596
15	26S protease regulatory subunit 6A (TAT-binding protein 1) (TBP-1) (Proteasome subunit P50)	49/5.1	53/4.0	2.065	25	PRSA_HUMAN
16	Protein disulfide-isomerase (EC 5.3.4.1) precursor	57/4.8	42/4.0	2.474	24	ISHUSS
17	3-Methylcrotonyl-CoA carboxylase subunit MCCB	62/7.6	59/6.4	2.438	32	AAK49409
18	Triosephosphate isomerase 1	27/6.5	29/6.3	0.469	46	AAH17917
19	Mitochondrial processing peptidase beta-subunit	55/6.2	47/5.5	Only in control	29	AAC39915
Detoxification						
20	Aldehyde dehydrogenase family 7 member A1 (Antiquitin 1)	56/6.2	55/6.2	0.426	33	D7A1_HUMAN
21	Probable thiol-specific reductase (EC 1.-.-.-) AOE37-2	31/5.9	29/4.6	2.145	36	G01790
22	Probable thioredoxin peroxidase (EC 1.11.1.-) PAGA	22/8.3	24/8.9	2.004	59	A46711
Kinases/Phosphatases						
23	Phosphoprotein phosphatase (EC 3.1.3.16) 1-alpha catalytic chain, splice form 2 [validated]	39/6.2	40/5.4	0.381	54	A46240
24	IKK interacting protein, isoform 1	43/4.9	53/3.4	0.3387	26	AAH58933
Gene regulation						
25	Heterogeneous nuclear ribonucleoprotein H	49/5.9	57/5.5	0.395	48	I39358
26	DEAD-box protein 3 (Helicase-like protein 2) (HLP2) (DEAD-box, X isoform)	73/6.7	80/6.5	0.433	31	DDX3_HUMAN
27	hnRNP 2H9B	35/6.4	40/6.2	0.497	62	AAF68844
28	mRNA export protein	42/8.0	43/7.8	0.482	40	AAC28126
29	hnRNP protein A2	36/8.7	36/8.1	2.813	34	AAB60650
30	HXC-26 protein	39/6.2	41/6.0	Only in control	36	JC5276
31	Splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated)	76/9.5	103/9.5	Only in S40cl	21	AAH51192
32	u5 snrnp-specific protein, putative	40/8.3	40/8.4	0.214	39	O95320
33	U5 snRNP-specific 40 kDa protein	40/8.3	39/8.5	Only in control	28	AAC69625
Other proteins						
34	Alpha-complex protein 1	38/6.7	43/5.9	0.408	57	S58529

a) The spot numbers are identical to those given in Fig. 5A.

Most of the proteins altered in steady state level by S40cl or GenDai in Hcy-treated cells were not influenced in the absence of Hcy. This finding suggests that the extract and the isoflavone mixture act mainly by blocking Hcy-effects rather than inducing a Hcy-independent expression of mRNA/protein. However, a variety of proteins that were

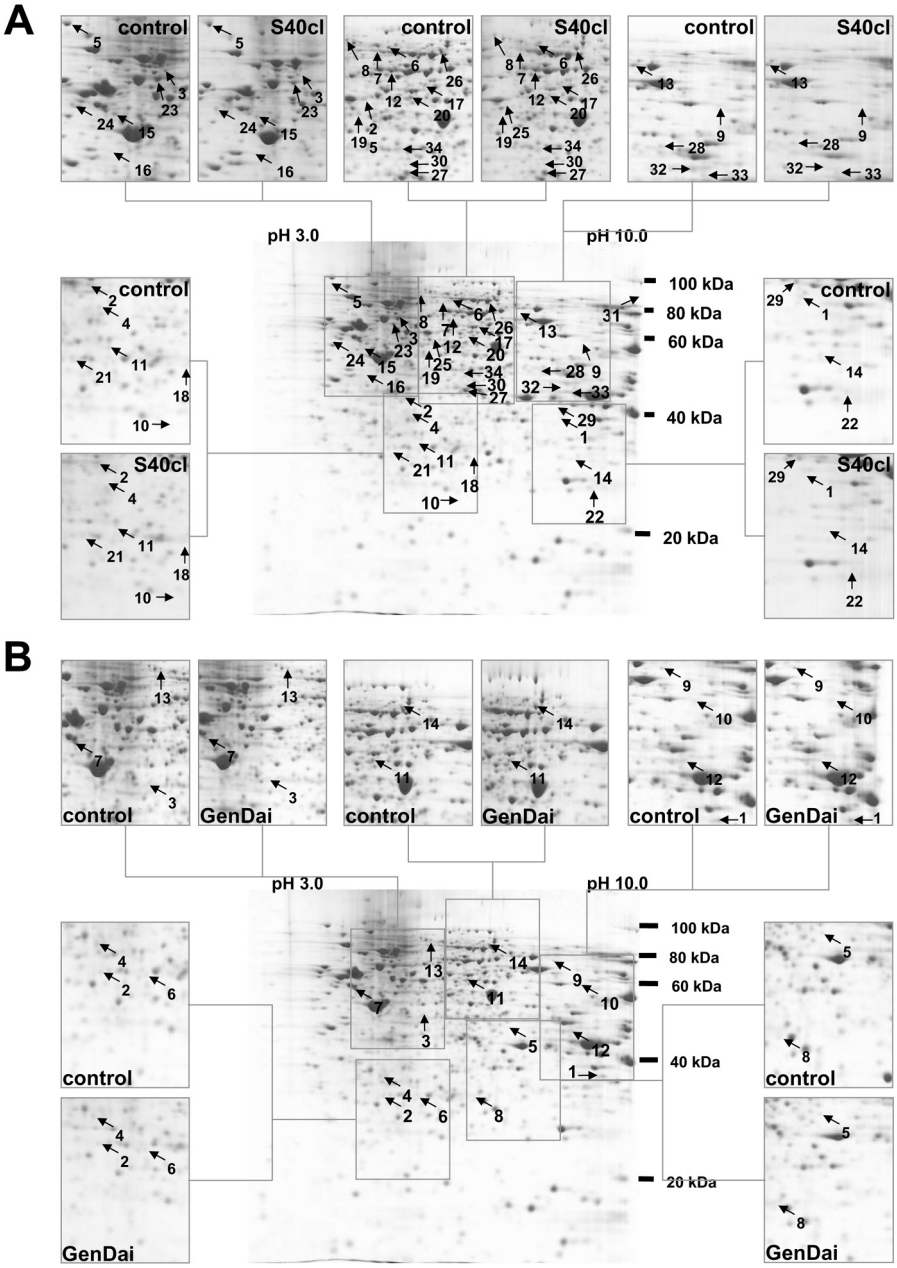

Figure 5. 2D-PAGE of proteins from EA.hy 926 exposed for 24 h to S40cl (A) or to GenDai (B). Enlarged gel areas around the central control gel display protein spots with altered densities in cells exposed to S40cl (A) or GenDai (B) versus control cells.

altered by S40cl or GenDai in the absence of Hcy can also be linked to atherosclerosis. Lamin C expression, for example was increased by S40cl and as it is the case for lamin A impaired functions of lamin C, as a result of common polymorphisms of the LMNA gene encoding nuclear lamin A/C, have been associated with traits related to atherosclerosis [46]. Another anti-atherosclerotic protein increased in expression level by GenDai, was ATP sulfurylase/adenosyl-5′-phosphosulfat kinase 2. This enzyme catalyzes the synthesis of 3′-phosphoadenosyl-5′-phosphosulfate and plays a role in the sulfation of many target molecules such as proteoglycans that are commonly decreased in atherosclerotic vessels [47]. The anaplastic lymphoma kinase fusion protein moesin, which promotes the attachment of monocytes to the plasma membrane of endothelial cells and thereby impacts their adhesion and migration [48], was found to be decreased in expression level by GenDai treatment of the cells.

In conclusion, our studies show that both an isoflavone containing soy extract and a mixture of the isoflavones genistein and daidzein inhibit apoptosis in EA.hy 926 human endothelial cells and are thereby able to interfere with a key process in atherosclerosis development. The soy extract and the isoflavone mixture, however, exert their effects on cell function and apoptosis by altering the expression levels of

Table 4. Steady state level ratios of proteins identified by MALDI-TOF MS analysis of the proteome of EA.hy 926 cells exposed to GenDai

Spot Id[a)]	Protein description	Theoretical M_r/p*I*	Measured M_r/p*I*	Protein amount GenDai/control	Seq. cov. in %	Accession no.
Cytoskeletal proteins						
1	MSN protein (moesin)	39/9.4	32/8.6	0.4801	33	AAH01112
Metabolism						
2	Putative spliceosome associated protein (breast carcinoma amplified sequence 2)	26/5.5	29/4.6	2.4356	51	AAC64059
3	Acyl-CoA dehydrogenase (EC 1.3.99.-) short/ branched chain specific precursor	48/6.5	42/5.3	2.2395	38	A55680
4	Cathepsin D Chain B, Cathepsin D At Ph 7.5	26/53	32/4.5	0.2735	47	1LYWB
5	Methionine adenosyltransferase regulatory beta subunit	38/6.9	41/6.9	2.1412	47	AAF28477
6	6-Phosphogluconolactonase *(Hydrolase)*	28/5.7	29/5.3	2.388	44	CAB57866
7	P5 protein precursor	48/4.9	52/4.0	Only in control	30	JC4369
8	Triosephosphate isomerase 1	27/6.4	29/6.1	2.0017	35	AAH17917
Kinases/Phosphatases						
9	ATP sulfurylase/APS kinase 2	70/7.9	64/7.9	2.4837	23	AAC64583
10	Oxygen-regulated protein 150K precursor	111/5.1	53/8.3	0.5057	16	JC5278
Gene regulation						
11	Heterogeneous nuclear ribonucleoprotein H	49/5.9	55/6.1	0.2734	36	I39358
12	U5 snRNP-specific 40 kDa protein	40/8.3	41/8.0	0.1674	41	AAC69625
Other proteins						
13	Hypothetical protein	83/6.1	77/5.5	0.4902	29	CAD97612
14	KH-type splicing regulatory protein	73/6.8	74/6.5	0.4128	27	AAB53222

a) The spot numbers are identical to those given in Fig. 5B.

completely different sets of target proteins. Our observations provide another line of evidence for the notion that the ability of soy foods to prevent atherosclerosis cannot be attributed solely to the isoflavones.

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