Eur J Nutr (2005) 44 : 95–104
DOI 10.1007/s00394-004-0499-6

ORIGINAL CONTRIBUTION

Dagmar Fuchs
Sonia De Pascual-Teresa
Gerald Rimbach
Fabio Virgili
Roberto Ambra
Rufus Turner
Hannelore Daniel
Uwe Wenzel

Proteome analysis for identification of target proteins of genistein in primary human endothelial cells stressed with oxidized LDL or homocysteine

Summary *Background* Epidemiological studies suggest that soy consumption contributes to the prevention of coronary heart disease. The proposed anti-atherogenic effects of soy appear to be carried by the soy isoflavones with genistein as the most abundant compound. *Aim of the study* To identify proteins or pathways by which genistein might exert its protective activities on atherosclerosis, we analyzed the proteomic response of primary human umbilical vein endothelial cells (HUVEC) that were exposed to the pro-atherosclerotic stressors homocysteine or oxidized low-density lipoprotein (ox-LDL). *Methods* HUVEC were incubated with physiological concentrations of homocysteine or ox-LDL in the absence and presence of genistein at concentrations that can be reached in human plasma by a diet rich in soy products (2.5 μM) or by pharmacological intervention (25 μM). Proteins from HUVEC were separated by two-dimensional polyacrylamide gel electrophoresis and those that showed altered expression level upon genistein treatment were identified by peptide mass fingerprints derived from tryptic digests of the protein spots. *Results* Several proteins were found to be differentially affected by genistein. The most interesting proteins that were potently decreased by homocysteine treatment were annexin V and lamin A. Annexin V is an antithrombotic molecule and mutations in nuclear lamin A have been found to result in perturbations of plasma lipids associated with hypertension. Genistein at low and high concentrations reversed the stressor-induced decrease of these anti-atherogenic proteins. Ox-LDL treatment of HUVEC resulted in an increase in ubiquitin conjugating enzyme 12, a protein involved in foam cell formation. Treatment with genistein at both doses reversed this effect. *Conclusions* Proteome analysis allows the identification of potential interactions of dietary components in the molecular process of atherosclerosis and consequently provides a powerful tool to define biomarkers of response.

Key words human umbilical vein endothelial cells – genistein – ox-LDL – homocysteine – atherosclerosis – proteomics

Received: 30 September 2003
Accepted: 18 February 2004
Published online: 22 April 2004

D. Fuchs · Prof. Dr. H. Daniel · Dr. U. Wenzel (✉)
Dept. of Food and Nutrition
Molecular Nutrition Unit
Technical University of Munich
Hochfeldweg 2
85350 Freising, Germany
Tel.: +49-8161/71-3997
Fax: +49-8161/71-3999
E-Mail: uwenzel@wzw.tum.de

Dr. S. de Pascual-Teresa · Prof. Dr. G. Rimbach · Dr. Rufus Turner
Hugh Sinclair Unit of Human Nutrition
School of Food Biosciences
University of Reading
Reading, UK

Dr. Fabio Virgili · Dr. Roberto Ambra
National Institute for Food and Nutrition Research
Rome, Italy

Abbreviations

AIP1	apoptosis-linked gene 2 interacting protein 1
CHD	Coronary heart disease
2D-PAGE	two-dimensional polyacrylamide gel-electrophoresis
FBS	Fetal bovine serum
HEPES	4-(2-hydroxyethyl)–1-piperazine ethanesulfonic acid
HUVEC	human umbilical vein endothelial cells
IEF	isoelectric focussing
IPG	immobilized pH-gradient
MALDI-TOF-MS	Matrix-assisted laser desorption

European Journal of Nutrition (2005) Vol. 44, Number 2

	ionization time-of-flight mass spectrometry
ox-LDL	oxidized low-density lipoprotein
SDS	sodium dodecylsulfate
smc1	chromosome segregation protein

Introduction

Coronary heart disease (CHD) represents the most common cause of mortality in the industrialized world, causing over 40 % of all deaths in the United States and Western Europe [1]. Soy consumption is suggested to play a role in the prevention of CHD, primarily by lowering plasma levels of LDL and homocysteine [2, 3]. Although there is no final proof that the cardiovascular effects of soy consumption are mediated by the isoflavones fraction, many studies have shown beneficial effects of isolated isoflavones on parameters altered in the pathogenesis of atherosclerosis. It has been demonstrated that a diet supplemented with an isoflavone aglycone-rich extract containing no soy protein attenuated atherosclerosis in cholesterol fed rabbits [4], and in human subjects consumption of soy, containing naturally occurring amounts of isoflavones, reduced lipid peroxidation *in vivo* and increased the resistance of LDL to oxidation [5, 6]. Those effects were not observed with soy preparations from which the isoflavones had been extracted [6].

Since isoflavones are structurally very similar to estrogen it has been postulated that genistein may exert its anti-atherogenic effects, at least partly via interaction with estrogen-receptors [7]. However, besides its binding to estrogen-receptor β with an affinity close to that of endogenous 17β-estradiol [8], genistein possesses a variety of other biological activities including inhibition of tyrosine kinases [9, 10] and anti-inflammatory actions in different cell types [7, 11].

Methods of the large scale analysis of protein expression are becoming important tools in the field of cardiovascular research and molecular nutrition. For identification of the molecular targets of genistein action in an atherogenesis model, we used human umbilical vein endothelial cells exposed to the stressors homocysteine or ox-LDL in the absence or the presence of genistein followed by proteome analysis.

Materials and methods

Materials

Endothelial growth factor and genistein were from Sigma (Poole, UK) and neutral red dye from Fisher Scientific (Leicestershire, UK). Sequencing grade modified trypsin was purchased from Promega (Mannheim, Germany), Pharmalyte and IPG strips from Amersham Biosciences (Freiburg, Germany), CompleteMini protease inhibitor cocktail from Roche (Mannheim, Germany), Bio-Rad dye reagent for protein determination was from Bio-Rad (Munich, Germany) and Coomassie brilliant blue G250 from Serva (Heidelberg, Germany).

Cell culture

Primary human endothelial cells (HUVEC) were obtained from the umbilical cord vein according to [12]. Umbilical cords were kindly provided by the nursery of "Fatebenefratelli" hospital of Rome. HUVEC were grown as described below and utilized for experiments at 90–100 % apparent confluence within the passage 3 through 6. Passages were performed according to standardized protocols [13] and by diluting the cell population 1 to 3. The identity of endothelial cells was confirmed by routine analysis for the expression of typical endothelial proteins as indicated in the original paper [12].

HUVEC were grown in Medium 199 supplemented with 20 % heat-inactivated FBS, 2 mM l-glutamine, 1 % d-glucose, 20 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin. Endothelial cell growth factor (1 x) was added to fresh culture medium after each media change.

Preparation of ox-LDL

Blood was collected in vacutainer tubes containing EDTA (1 mg/ml) from the antecubital vein of fasting healthy volunteers. The LDL fraction, corresponding to a density of 1.019–1.063 g/ml, was isolated from plasma by sequential ultracentrifugation in salt solutions, according to Havel et al. [14], using a Beckman T-100 bench-top ultracentrifuge (T-100.3 rotor). The LDL fraction was stored under nitrogen at 4 °C and used within 1 week of isolation. Lipoprotein concentration was expressed in terms of protein content. Protein was measured by the Bradford reaction using the Bio-Rad protein assay.

Prior to the experiments, LDL was dialyzed in the dark for 24 h at 4 °C against three changes of buffer (1 l each) containing 0.01 M phosphate-buffered saline, 2.7 mM KCl, and 138 mM NaCl, pH 7.4. Dialyzed LDL (200 µg of protein/ml) was oxidized with 5 µM $CuCl_2$ in phosphate-buffered saline at 37 °C. Oxidation was followed by monitoring the increased formation of conjugated dienes at 234 nm using a Beckman DU 70 spectrophotometer and was stopped by using 6 µM EDTA to chelate all the metal ions. Upon chelation, the solution then is dialyzed over 24 h using three changes of buffer. In order to standardize the level of oxidation, LDL was

administered to HUVEC when its oxidation reached the midpoint of the propagation phase, at a final concentration of 25 μg/ml. To further assess the extent of oxidation of LDL in terms of the modified surface charge on the apolipoprotein B-100, lipoprotein electrophoresis was performed using a Beckman Paragon electrophoresis system.

Determination of cell viability

The uptake of the neutral red dye was used to measure cell viability as described previously [15]. Assessment was made after HUVEC were treated with medium alone (control), 25 μM genistein, 5 μg/ml ox-LDL, or combinations of homocysteine or ox-LDL with either 2.5 μM genistein or 25 μM genistein for 24 h. For cytotoxicity measurements, the culture medium was removed and replaced with fresh medium containing 60 μg neutral red/ml for 3 h at 37 °C. Following incubation with the neutral red dye, the medium was removed and the cells extracted using a solution comprising methanol, water and glacial acetic acid (50:49:1, v/v). Absorbance was recorded at 540 nm using a microplate reader. The various treatments did not affect HUVEC cell viability.

Sample preparation for 2D-PAGE

Following 24 h incubation period, cells were washed three times with ice cold 350 mM sucrose, containing CompleteMini 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) were added to the pellet. Homogenisation 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.

2D-PAGE

2D-PAGE, IEF in the first dimension and SDS-PAGE in the second dimension, was performed as described by Görg et al. with minor modifications [16]. 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). 300 μg of protein-suspension 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 (25000 Vh, Step-n-hold). Subsequent to IEF, strips were incubated for 15 min in equilibration-buffer 1 (1.5 M TrisHCl, 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 TrisHCl, pH 8.8, 6 M urea, 26 % glycerol, 2 % SDS, 4 % iodoacetamide) before loading onto SDS-PAGE gels. 1 mm-thick 12.5 % SDS-polyacrylamide gels were cast according to the method of Laemmli [17] 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.

Staining of proteins on gels was performed by fixing in 40 % ethanol and 10 % acetic acid for 5 h. Gels were then stained overnight in Coomassie-solution containing 10 % $(NH_4)_2SO_4$, 2 % phosphoric acid, 25 % methanol and 0.625 % Coomassie brilliant blue G250. Gels were destained in bi-distilled water until the background was completely clear.

Analysis of proteins using the Proteome Weaver Software

Gels stained with Coomassie were scanned using an ImageScanner (Amersham Biosciences) and spots detected by the Proteom/Weaver software (Definiens, Munich, Germany). Background subtraction and volume normalization are 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 different treatments were compared to each other. Spots differing by at least twofold in density were picked for MALDI-TOF-MS analysis.

Enzymatic digestion of protein spots for MALDI-TOF-MS

Coomassie-stained spots were picked with a 2 mm or 3 mm "SKIN-Picker". The destaining of spots occurred with alternating washing procedures in pure acetonitrile and 50 mM NH_4HCO_3 until the blue colour was fully removed. After drying in a SpeedVac, the spots were incubated 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 and gel spots washed twice with bi-distilled water. Digestion of the protein was performed by incubating spots in 5 μl of 50 mM NH_4HCO_3 for 20 h at 37 °C. Peptide fragments were extracted twice by incubating each spot in 7 μl of 40 % trifluoracetic acid/60 % acetonitrile (v/v). The supernatants derived from each spot were collected and dried in a SpeedVac.

European Journal of Nutrition (2005) Vol. 44, Number 2

MALDI-TOF-MS analysis of tryptic peptides

Peptide mass analysis was performed using the Autoflex mass spectrometer of Bruker Daltonics (Leipzig, Germany). The dried protein sample was resuspended in 3 µl of 1 % TFA and 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 accurancy of ± 0.1 %; 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.

Results

The steady-state level of a spectrum of proteins changed after exposure of cells to homocysteine or ox-LDL in the absence or presence of low or high concentrations of genistein. More than 700 protein spots were resolved per 2D-gel from the total cell lysate (Figs. 1 and 2, mid panels). Of the proteins with changed expression level by the pro-atherosclerotic stressors homocysteine or ox-LDL, ten of those altered by homocysteine (Table 1, Fig. 1) and nine of those altered by ox-LDL (Table 2, Fig. 2) were found to respond also to genistein treatment and could be identified by MALDI-TOF analysis.

The desmoplakin I level was found to be reduced in HUVEC treated with a combination of genistein and homocysteine compared to cells treated with homocysteine alone (Table 1). Desmoplakin I, however, was not found to be altered by homocysteine exposure of cells (Table 1), suggesting that genistein does not block homocysteine-mediated effects by altering the amount of desmoplakin I. Similarly to desmoplakin I, fascin, a protein involved in cell motility, was also not affected by homocysteine alone, whereas genistein caused an up-regulation of fascin levels in cells treated with homocysteine (Table 1). The homer-2 protein was also potently up-regulated by genistein, but here an up-regulation was also found in HUVEC exposed to homocysteine alone (Table 1), so that genistein and homocysteine changed protein levels in the same direction. Eight proteins could be identified by MALDI-TOF-MS analysis that were regulated by genistein at low and/or high concentrations in an opposite direction to their change after homocysteine treatment (Table 1). Amongst those proteins we identified annexin V, an anti-thrombotic protein. Annexin V was not detectable after homocysteine treatment (Table 1). Annexin V levels were up-regulated 3-fold by 2.5 µM genistein treatment and 4-fold by 25 µM genistein (Table 1, Fig. 1, spot 4). Similarly, the expression of lamin A, a protein with a proposed central role in prevention of hypertension, was decreased significantly after homocysteine exposure of cells (Table 1), whereas genistein, especially at high concentrations, reversed this effect (Table 1, Fig. 1, spot 9). The effect of homocysteine treatment on ubiquitin carboxyterminal hydrolase L1 levels was also reversed when the cells were treated additionally with genistein (Table 1) and the reduction of apoptosis-linked gene 2 interacting protein 1 by homocysteine could be counteracted by genistein even at low concentrations.

Of nine proteins with altered expression level upon genistein exposure of ox-LDL treated cells, seven proteins appeared not to respond specifically, as they were either not affected by ox-LDL alone or they were regulated by genistein in the same direction as by ox-LDL (Table 2).

The ubiquitin conjugating enzyme 12, known to be involved in foam cell formation, was increased more than two-fold by ox-LDL exposure and genistein treatment, even with low concentrations (Table 2, Fig. 2, spot 2) reduced the response and lowered protein levels again. Similarly, aldehyde dehydrogenase was increased by ox-LDL, although less than two-fold, but was significantly reduced by 2.5 µM and 25 µM genistein, respectively (Table 2, Fig. 2, spot 4).

Discussion

Homocysteine and ox-LDL are well recognized as independent risk factors with an involvement in the development of vascular diseases [18–21]. Normal plasma homocysteine concentrations range from 1 µM to 12 µM [22], whereas 25 µM, as used in this study, corresponds to mild hyperhomocysteinemia [22]. Ox-LDL was used at a concentration of 5 µg/ml as in previous studies in HUVEC [23]. Subjects with an increased cardiovascular risk exhibit ox-LDL levels of up to 30 µg/ml [24]. However, such concentrations are cytotoxic in HUVEC (data not shown). Genistein was supplemented at a concentration of 2.5 µM, a level within the range found in human plasma after intake of a single, high dose of isoflavones [25]. A concentration of 25 µM genistein, as used in a different set of experiments, may only be achieved by pharmacological intervention.

One target protein that was highly upregulated by genistein treatment of HUVEC was annexin V. Whereas homocysteine caused the corresponding protein spot to decline below the level of detection, genistein reversed this effect. Since annexin V was shown to efficiently inhibit endothelial-cell-mediated thrombin formation *in*

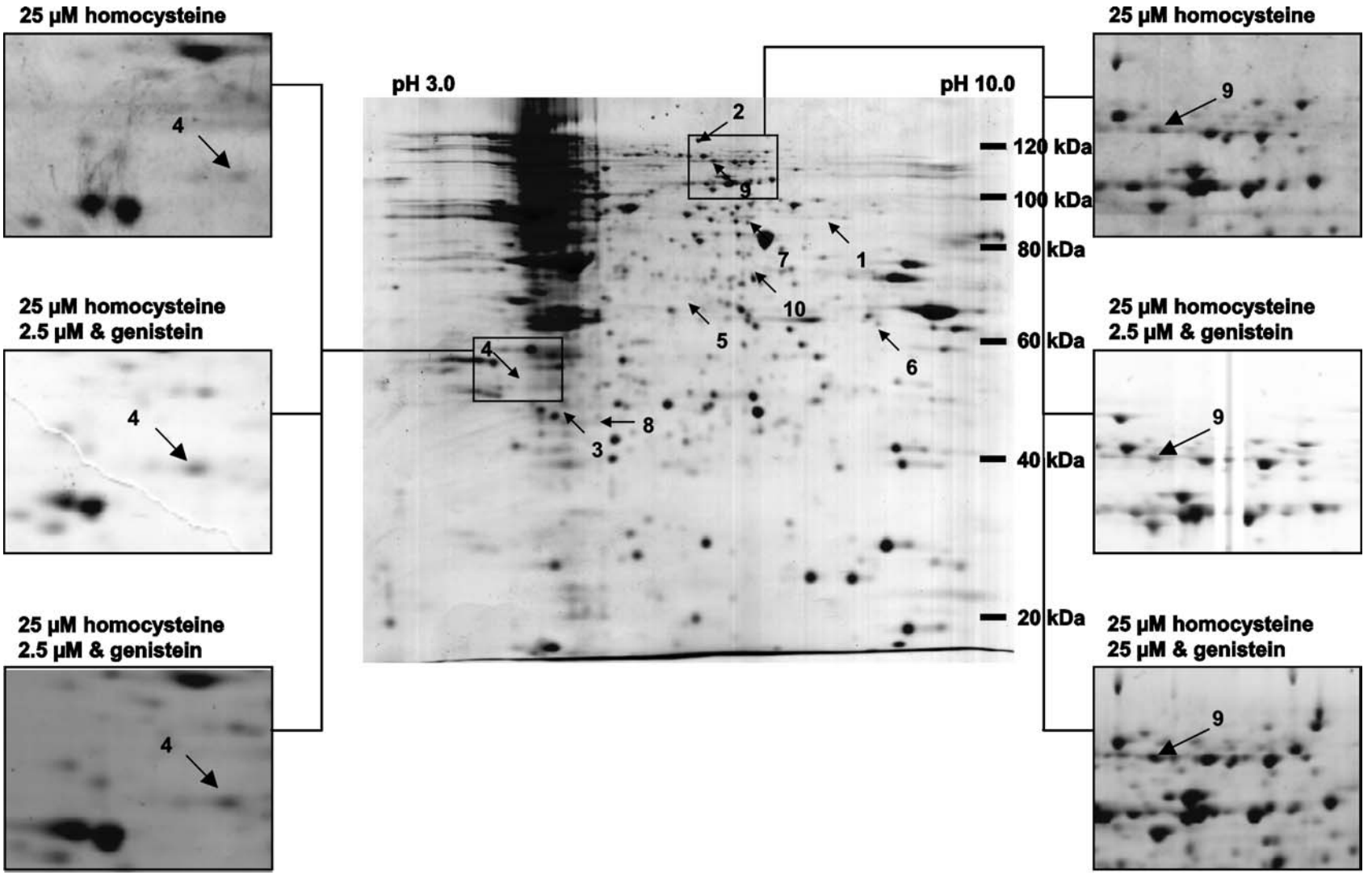

Fig. 1 2D-PAGE of proteins from HUVEC grown for 24 h in the presence of 25 µM homocysteine alone or in the presence of 25 µM homocysteine plus 2.5 µM or 25 µM genistein. Proteins were separated on a pH 3–10 IPG-strip in the first dimension and on a 12.5 % SDS-polyacrylamide gel in the second dimension. A typical Coomassie-stained gel derived from HUVEC control cells is shown in the middle section. Enlarged areas of gels derived from differently treated HUVEC are shown around the representative control gel

European Journal of Nutrition (2005) Vol. 44, Number 2

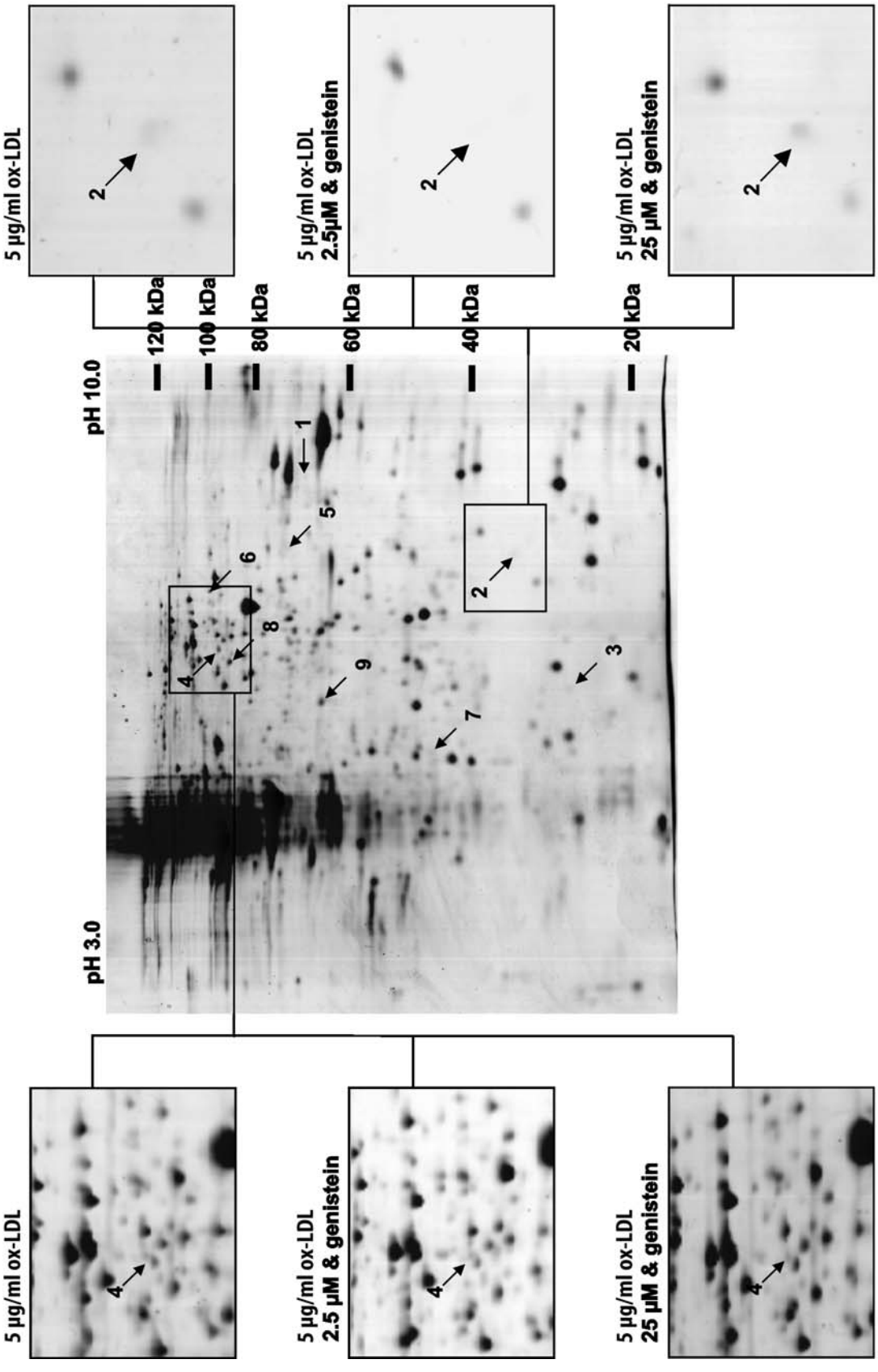

Fig. 2 2D-PAGE of proteins from HUVEC incubated for 24 h with 5 µg/ml ox-LDL or in addition with 2.5 µM or 25 µM genistein. Enlargements around the control gel in the center part show identical sections of gels from cells treated with ox-LDL alone or in addition with 2.5 µM or 25 µM genistein

Table 1 Effects of 2.5 μM or 25 μM genistein on the steady-state levels of proteins in HUVEC exposed for 24 h to 25 μM homocysteine

Spot-ID	Protein description	Protein amount			Residues of identified peptides	Accession No.
		homocysteine/ control	homocyst. & 2.5 μM genistein/homocyst.	homocyst. & 25 μM genistein/homocyst.		
1	Desmoplakin I	0.90	0.40	0.85	240–249, 285–291, 292–298, 366–373, 460–469, 470–480, 470–482, 483–491, 484–491, 541–558, 622–628, 606–675, 676–683, 684–689, 684–692, 699–706, 723–731, 756–765, 837–848, 840–848, 917–928	A38194
2	Apoptosis-linked gene 2 interacting protein 1	0.40	2.05	4.33	24–41, 52–70, 61–70, 152–164, 216–229, 358–373, 439–446, 584–606, 676–686, 700–707	AAF08220
3	Keratin, type 2 cytoskeletal 1	0.54	2.23	1.35	185–198, 267–275, 343–363, 395–406, 417–431, 443–454	K2C1_HUMAN
4	Annexin V	0.11	2.90	4.27	3–14, 26–41, 42–54, 76–85, 86–97, 105–113, 123–147, 148–157, 183–197, 190–197, 209–223, 242–267, 257–267, 273–281, 273–282, 287–297, 298–305, 306–312	1HVE
5	Chromosome segregation protein smc1	0.32	1.92	2.31	97–110, 238–245, 256–264, 273–282, 318–327, 364–373, 386–395, 402–412, 414–420, 415–422, 447–453, 469–475, 541–551, 700–713, 769–787, 800–806, 862–880, 888–896, 898–910	I54383
6	Heterogenous nuclear ribonucleoprotein (hnRNP) A2	0.42	1.43	2.61	10–26, 102–117, 109–125, 118–135, 141–156, 142–161, 202–216	AAB60650
7	Fascin	1.41	1.97	2.62	68–81, 90–99, 100–108, 109–117, 118–130, 151–166.185–193, 185–196, 201–216, 313–329, 330–340, 379–388, 389–397, 398–407, 408–425	FSC1_HUMAN
8	Ubiquitin carboxyterminal hydrolase L1	0.32	1.04	3.04	69–90, 91–100, 117–138, 121–138, 164–180, 164–184, 188–198	AAD09172
9	Lamin A	0.46	1.64	3.95	9–25, 29–41, 51–62, 79–89, 100–108, 102–108, 134–144, 136–144, 145–156, 156–166, 157–166, 182–190, 241–249, 241–260, 281–296, 297–311, 299–311, 367–377, 367–378, 379–386, 428–435, 428–439, 440–453, 585–597, 598–624, 628–644	VEHULA
10	Homer-2 protein, Homer-2B splicing form	only in homocysteine	5.08	5.60	1–10, 23–29, 47–54, 103–111, 121–133, 169–181, 171–182, 214–219, 229–241, 264–271, 273–296, 291–304, 297–304, 321–327, 324–336	CAB75538

The spot numbers are identical to those given in Fig.1. Proteins altered in level at least twofold by treatment of cells with 2.5 or 25 μM genistein in the presence of 25 μM homocysteine versus proteins altered by treatment of HUVEC with 25 μM homocysteine alone were identified by MALDI-TOF-MS. The identified peptide fragments are shown in relation to the amino acid sequences of the identified protein

vivo [26], it may represent a selective target protein through which genistein antagonizes the homocysteine-activated pro-atherosclerotic processes. It is interesting to note that a common polymorphism in the annexin V gene increases translation efficiency and plasma levels of annexin V, associated with a decreased risk of myocardial infarction [27]. A second protein of interest, which was also significantly down-regulated by homocysteine, was lamin A. However, the ability of low genistein concentrations to reverse this effect was not as pronounced as those found for annexin V. Lamin A is a nuclear lamin which participates in DNA replication, chromatin organization, nuclear pore arrangement, nuclear growth, and nuclear membranes anchorage [28]. It

Table 2 Effects of 2.5 μM or 25 μM genistein on the steady state levels of proteins in HUVEC exposed for 24 h to 5 μg/ml ox-LDL

Spot No.	Protein description	Protein amount			Residues of identified peptides	Accession No.
		ox-LDL/control	ox-LDL & 2.5 μM genistein/ox-LDL	ox-LDL & 25 μM genistein/ox-LDL		
1	KIAA 1505 Protein (fragment)	1.30	0.20	1.49	29–37, 46–57, 100–113, 130–135, 136–142, 137–149, 150–157, 185–198, 242–247, 255–263, 286–295, 312–319, 371–385, 396–403, 430–436, 468–477, 498–504, 498–505, 513–521, 530–542, 535–543, 551–557, 569–577, 583–592	BAA96029
2	Ubiquitin conjugating enzyme 12	2.42	0.26	0.58	35–45, 62–72, 158–166, 159–166, 170–180	AAC26141
3	KRAB zinc finger protein HFB101S	1.16	0.29	0.54		BAA88522
4	Aldehyde dehydrogenase 1A1 (cytosolic)	1.63	0.35	0.11	57–64, 68–77, 78–84, 113–127, 143–155, 308–320, 308–321, 353–361, 378–394, 419–434, 420–434, 490–498	P00352
5	Phosphoserine aminotransferase	0.62	0.47	0.66	6–16, 17–27, 34–45, 98–110, 118–127, 201–213, 214–222, 324–333, 324–336, 343–356	AAN71736
6	Uridine diphosphoglucose dehydrogenase	1.47	1.22	2.25	59–67, 208–220, 221–230, 299–311, 331–340, 374–393, 432–442, 471–481	JE0353
7	26S proteasome subunit p 28	only in ox-LDL	1.35	2.27	19–30, 70–85, 117–135, 136–145, 163–184, 185–203, 190–206, 207–221, 214–222	BAA33215
8	Nuclear matrix protein NMP 238	0.81	1.10	2.37	47–57, 65–76, 77–90, 109–117, 172–182, 185–201, 185–202, 269–276, 275–281, 318–333, 340–357, 379–400, 405–418, 419–427, 428–441	JE0334
9	p167	only in ox-LDL	0.75	3.01	592–597, 682–687, 695–700, 712–718, 811–817, 837–842, 862–868, 912–919, 971–979, 981–990, 991–999, 1011–1020, 1020–1026, 1102–1110	AAB41584

The spot numbers are identical to those given in Fig.2. Protein levels altered at 24 h at least twofold by the treatment of cells with 2.5 μM or 25 μM genistein plus 5 μg/ml ox-LDL versus proteins altered by exposure of HUVEC to 25 μM ox-LDL alone were identified by MALDI-TOF-MS as described

has been shown to be associated with lipodystrophy, hyperinsulinemia, dyslipidemia, diabetes, and hypertension in patients bearing a missense mutation in the lamin A gene [29, 30].

The level of the apoptosis-linked gene 2 interacting protein was found to be reduced by homocysteine which could cause the induction of apoptosis in HUVEC. Overexpression of its C-terminal half has been proven to inhibit programmed cell death induced by several stimuli [31]. Further evidence for a pro-apoptotic action of homocysteine comes from the reduction of cytoskeletal keratin type 2 protein level. Keratins are caspase substrates that are cleaved during apoptosis [32]. One of the consequences of increased rates of apoptosis by homocysteine is that the endothelial cells increase their content of phosphatidylserine on the outer cell membrane. This leads to thrombin activation, thus increasing the probability of arterial thromboses [33]. The homocysteine induced decrease of both apoptosis-linked gene 2 interacting protein and cytoskeletal keratin type 2 levels could be reversed by low and high concentrations of genistein, suggesting an anti-apoptotic activity of the isoflavone. Induction of apoptosis has also been shown to be associated with increased ubiquitin hydrolase gene expression and both were found to occur in the early phase of acute myocardial infarction due to human serum factors [34]. It is important to note that genistein, at high concentrations, reversed the effects of homocysteine on the expression of ubiquitin carboxyterminal hydrolase L1. This suggests that homocysteine may be involved also in anti-atherosclerotic mechanisms and similarly suggests that genistein, at high concentrations, may possess pro-atherogenic activities.

Proteins of cell cycle control are crucial for reinitiating cell division and endothelial cell proliferation in disorders such as atherosclerosis and restenosis [35]. In

this context, homocysteine was shown to reduce the regeneration rate of damaged endothelial cells via inhibiting endothelial cell growth [36]. It is interesting to note that two proteins necessary for proliferation of cells, the chromosome segregation protein smc1 [37] and the heterogenous nuclear ribonucleoprotein A2 [38], were reduced by homocysteine treatment, whereas genistein at low and especially at high concentrations counteracted the effects of homocysteine.

Only two proteins could be identified that were regulated in an opposite direction by ox-LDL plus genistein when compared to ox-LDL alone. Whereas the effects of genistein on the ox-LDL-mediated increases in cytosolic aldehyde dehydrogenase 1A1 can not be related to any process of atherosclerosis, the antagonizing effects on the increased levels of ubiquitin conjugating enzyme 12 may be part of an anti-atherosclerotic mechanism. The rate of ubiquitination of cellular proteins plays a crucial role in ox-LDL induced apoptosis [39]. Within this process, the ubiquitinated proteins are finally submitted to degradation by the 26S proteasome [39]. The 26S proteasome was found in increased levels in cells exposed to ox-LDL but strikingly high concentrations of genistein even further increased its density. This provides additional evidence that genistein at high concentrations may have unwanted side effects.

Uridine diphosphoglucose dehydrogenase is another protein that is found in increased levels when the cells are treated with high concentrations of genistein. This enzyme is involved in the synthesis of structural components of proteoglycans [40] and has been found with reduced levels in vessels with atherosclerotic lesions [41]. In this case, genistein at high concentrations could have beneficial effects.

Proteome analysis technologies allowed to identify proteins that are differentially expressed in primary human endothelial cells challenged with homocysteine or ox-LDL in the absence or the presence of the soy isoflavone genistein. Various of the molecular targets of genistein could be linked to processes impaired in atherosclerosis, supporting the anti-atherogenic activities of soy isoflavones in endothelial cells. Importantly, those biomarkers were found to be affected by genistein at concentrations that can be achieved *in vivo* by dietary intervention but further studies are required to determine whether the effects of genistein observed *in vitro* here are relevant to an *in vivo* situation.

Acknowledgements This work was supported by the EU-grant Isoheart QLK1-CT-2001-00221

References

1. Yutani C, Imakita M, Ishibashi-Ueda H, Tsukamoto Y, Nishida N, Ikeda Y (1999) Coronary atherosclerosis and interventions: pathological sequences and restenosis. Pathol Int 49:273–290
2. Wagner JD, Zhang L, Greaves KA, Shadoan MK, Schwenke DC (2000) Soy protein reduces the arterial low-density lipoprotein (LDL) concentration and delivery of LDL cholesterol to the arteries of diabetic and nondiabetic male cynomolgus monkeys. Metabolism 49:1188–1196
3. Tonstad S, Smerud K, Hoie L (2002) A comparison of the effects of 2 doses of soy protein or casein on serum lipids, serum lipoproteins, and plasma total homocysteine in hypercholesterolemic subjects. Am J Clin Nutr 76:78–84
4. Yamakoshi J, Piskula MK, Izumi T, Tobe K, Saito M, Kataoka S, Obata A, Kikuchi M (2000) Isoflavone aglycone-rich extract without soy protein attenuates atherosclerosis development in cholesterol-fed rabbits. J Nutr 130:1887–1893
5. Tikkanen MJ, Wahala K, Ojala S, Vihma V, Adlercreutz H (1998) Effect of soybean phytoestrogen intake on low density lipoprotein oxidation resistance. Proc Natl Acad Sci 95:3106–3110
6. Wiseman H, O'Reilly JD, Adlercreutz H, Mallet AI, Bowey EA, Rowland IR, Sanders TA (2000) Isoflavone phytoestrogens consumed in soy decrease F(2)-isoprostane concentrations and increase resistance of low-density lipoprotein to oxidation in humans. Am J Clin Nutr 72:395–400
7. Barnes S, Boersma B, Patel R, Kirk M, Darley-Usmar VM, Kim H, Xu J (2000) Isoflavonoids and chronic disease: mechanisms of action. Biofactors 12: 209–215
8. Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JA (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139:4252–4263
9. Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M, Fukami Y (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinases. J Biol Chem 262:5592–5595
10. Shimokado K, Yokota T, Umezawa K, Sasaguri T, Ogata J (1994) Protein tyrosine kinase inhibitors inhibit chemotaxis of vascular smooth muscle cells. Arterioscler Thromb 14:973–981
11. Gottstein N, Ewins BA, Eccleston C, Hubbard GP, Kavanagh IC, Minihane AM, Weinberg PD, Rimbach G (2003) Effect of genistein and daidzein on platelet aggregation and monocyte and endothelial function. Br J Nutr 89: 607–616
12. Jaffe EA, Nachman RL, Becker CG, Minick CR (2001) Culture of human endothelial cells derived from umbilical veins. J Clin Invest 52:2745–2756
13. Rimbach G, Saliou C, Canali R, Virgili F (2001) Interaction between cultured endothelial cells and macrophages: in vitro model for studying flavonoids in redox-dependent gene expression. Meth Enzymol 335:238–242
14. Havel JR, Eder HA, Bragdon JH (1955) The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J Clin Invest 34:1345–1353
15. Valacchi G, Rimbach G, Saliou C, Weber SU, Packer L (2001) Effect of benzoyl peroxide on antioxidant status, NF-kappaB activity and interleukin-1alpha gene expression in human keratinocytes. Toxicology 165:225–234

16. Görg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W (2000) The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 21: 1037–1053
17. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
18. Hajjar KA (2001) Homocysteine: a sulph'rous fire. J Clin Invest 107: 663–664
19. Haynes WG (2002) Hyperhomocysteinemia, vascular function and atherosclerosis: effects of vitamins. Cardiovasc Drugs Ther 16:391–399
20. Berliner JA, Heinecke JW (1996) The role of oxidized lipoproteins in atherogenesis. Free Radic Biol Med 20: 707–727
21. Ursini F, Davies KJ, Maiorino M, Parasassi T, Sevanian A (2002) Atherosclerosis: another protein misfolding disease? Trends Mol Med 8:370–374
22. Jacobsen DW (1998) Homocysteine and vitamins in cardiovascular disease. Clin Chem 44:1833–1843
23. Virgili F, Ambra R, Muratori F, Natella F, Majewicz J, Minihane AM, Rimbach G (2003) Effect of oxidized low-density lipoprotein on differential gene expression in primary human endothelial cells. Antiox Redox Signal 5:237–247
24. Holvoet P, Mertens A, Verhamme P, Bogaerts K, Beyens G, Verhaeghe R, Collen D, Muls E, Van de Werf F (2001) Circulating oxidized LDL is a useful marker for identifying patients with coronary artery disease. Arterioscler Thromb Vasc Biol 21:844–848
25. Izumi T, Piskula MK, Osawa S, Obata A, Tobe K, Saito M, Kataoka S, Kubota Y, Kikuchi M (2000) Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans. J Nutr 130:1695–1699
26. van Heerde WL, Poort S, van 't Veer C, Reutelingsperger CP, de Groot PG (1994) Binding of recombinant annexin V to endothelial cells: effect of annexin V binding on endothelial-cell-mediated thrombin formation. Biochem J 302: 305–312
27. Gonzalez-Conejero R, Corral J, Roldan V, Martinez C, Marin F, Rivera J, Iniesta JA, Lozano ML, Marco P, Vicente V (2002) A common polymorphism in the annexin V Kozak sequence (–1C > T) increases translation efficiency and plasma levels of annexin V, and decreases the risk of myocardial infarction in young patients. Blood 100: 2081–2086
28. Moir RD, Spann TP (2001) The structure and function of nuclear lamins: implications for disease. Cell Mol Life Sci 58:1748–1757
29. Hegele RA, Anderson CM, Wang J, Jones DC, Cao H (2000) Association between nuclear lamin A/C R482Q mutation and partial lipodystrophy with hyperinsulinemia, dyslipidemia, hypertension, and diabetes. Genome Res 10:652–658
30. Haque WA, Vuitch F, Garg A (2002) Postmortem findings in familial partial lipodystrophy, Dunnigan variety. Diabet Med 19:1022–1025
31. Chatellard-Causse C, Blot B, Cristina N, Torch S, Missotten M, Sadoul R (2002) Alix (ALG-2-interacting protein X), a protein involved in apoptosis, binds to endophilins and induces cytoplasmic vacuolization. J Biol Chem 277: 29108–29115
32. Badock V, Steinhusen U, Bommert K, Wittmann-Liebold B, Otto A (2001) Apoptosis-induced cleavage of keratin 15 and keratin 17 in a human breast epithelial cell line. Cell Death Differ 8: 308–315
33. Lesauskaite V, Ivanoviene L, Valanciute A (2003) Programmed cellular death and atherogenesis: from molecular mechanisms to clinical aspects. Medicina 39:529–534
34. Hasegawa R, Kita K, Hasegawa R, Fusejima K, Fukuzawa S, Wano C, Watanabe S, Saisho H, Masuda Y, Nomura F, Suzuki N (2003) Induction of apoptosis and ubiquitin hydrolase gene expression by human serum factors in the early phase of acute myocardial infarction. J Lab Clin Med 141:168–178
35. Bicknell KA, Surry EL, Brooks G (2003) Targeting the cell cycle machinery for the treatment of cardiovascular disease. J Pharm Pharmacol 55:571–591
36. Wang J, Dudman NP, Wilcken DE, Lynch JF (1992) Homocysteine catabolism: levels of 3 enzymes in cultured human vascular endothelium and their relevance to vascular disease. Atherosclerosis 97:97–106
37. Rocques PJ, Clark J, Ball S, Crew J, Gill S, Christodoulou Z, Borts RH, Louis EJ, Davies KE, Cooper CS (1995) The human SB1.8 gene (DXS423E) encodes a putative chromosome segregation protein conserved in lower eukaryotes and prokaryotes. Hum Mol Genet 4:243–249
38. Pino I, Pio R, Toledo G, Zabalegui N, Vicent S, Rey N, Lozano MD, Torre W, Garcia-Foncillas J, Montuenga LM (2003) Altered patterns of expression of members of the heterogeneous nuclear ribonucleoprotein (hnRNP) family in lung cancer. Lung Cancer 41:131–143
39. Vieira O, Escargueil-Blanc I, Jurgens G, Borner C, Almeida L, Salvayre R, Negre-Salvayre A (2000) Oxidized LDLs alter the activity of the ubiquitin-proteasome pathway: potential role in oxidized LDL-induced apoptosis. FASEB J 14:532–542
40. Wegrowski Y, Perreau C, Bontemps Y, Maquart FX (1998) Uridine diphosphoglucose dehydrogenase regulates proteoglycan expression: cDNA cloning and antisense study. Biochem Biophys Res Commun 250:206–211
41. Pillarisetti S (2000) Lipoprotein modulation of subendothelial heparan sulfate proteoglycans (perlecan) and atherogenicity. Trends Cardiovasc Med 10: 60–65