Proteomic biomarkers of peripheral blood mononuclear cells obtained from postmenopausal women undergoing an intervention with soy isoflavones[1–3]

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ABSTRACT

Background: The incidence of cardiovascular diseases increases after menopause, and soy consumption is suggested to inhibit disease development.

Objective: The objective was to identify biomarkers of response to a dietary supplementation with an isoflavone extract in postmenopausal women by proteome analysis of peripheral blood mononuclear cells.

Design: The study with healthy postmenopausal woman was performed in a placebo-controlled sequential design. Peripheral mononuclear blood cells were collected from 10 volunteers after 8 wk of receiving daily 2 placebo cereal bars and after a subsequent 8 wk of intervention with 2 cereal bars each providing 25 mg of isoflavones. The proteome of the cells was visualized after 2-dimensional gel electrophoresis, and peptide mass fingerprinting served to identify proteins that by the intervention displayed altered protein concentrations.

Results: Twenty-nine proteins were identified that showed significantly altered expression in the mononuclear blood cells under the soy-isoflavone intervention, including a variety of proteins involved in an antiinflammatory response. Heat shock protein 70 or a lymphocyte-specific protein phosphatase and proteins that promote increased fibrinolysis, such as α-enolase, were found at increased intensities, whereas those that mediate adhesion, migration, and proliferation of vascular smooth muscle cells, such as galectin-1, were found at reduced intensities after soy extract consumption.

Conclusion: Proteome analysis identified in vivo markers that respond to a dietary intervention with isoflavone-enriched soy extract in postmenopausal women. The nature of the proteins identified suggests that soy isoflavones may increase the antiinflammatory response in blood mononuclear cells that might contribute to the atherosclerosis-preventive activities of a soy-rich diet. *Am J Clin Nutr* 2007;86:1369–75.

KEY WORDS Soy, isoflavones, cardiovascular disease, postmenopausal women, peripheral blood mononuclear cells, proteome, heat shock protein 70

INTRODUCTION

Coronary heart disease represents the leading cause of death in Western societies (1). Although its incidence is quite low in premenopausal women, cardiovascular disease risk increases strongly after menopause because of diminished estrogen production (2). Supply of exogenous estrogen as part of a hormone replacement therapy was shown to reduce plasma concentrations of LDL- and increase HDL-cholesterol fractions (3). However, an increased risk of thrombosis (4) and inflammation (5) and a greater risk of developing hormone-dependent cancers (6) questioned the benefits of hormone replacement therapy and addressed the need for alternative concepts in the prevention of atherosclerosis. Soy-based diets were shown frequently to promote a cardioprotective effect in postmenopausal women (7), and the effect is commonly attributed to soy isoflavones, a class of phytoestrogens, with genistein and daidzein representing the major compounds. Protein components of soybeans appear also to have beneficial effects on cardiovascular health, and the combination of soy protein and isoflavones probably conveys more benefit than does either component alone (8–10). Although the overall evidence for soy products affecting cardiovascular health is still weak, the American Heart Association concluded that foods rich in soy protein may indirectly reduce cardiovascular risk if they replace animal-based products in the diet (11).

A variety of markers related to atherosclerosis have been measured in healthy postmenopausal women undergoing different types of intervention and supplementation trials, either with soy protein extracts depleted of isoflavones or with isoflavone-enriched extracts (8–15). The present study was part of a European multicenter intervention program in postmenopausal women on the effects of cereal bars that contained an isoflavone-rich soy extract and provided 50 mg isoflavones/d with a

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genistein-to-daidzein ratio of 2:1 (12–15). As previously reported, the intervention did not cause significant alterations in plasma homocysteine concentrations (12) or clinically relevant markers of inflammation, apart from significantly decreased odds for achieving a C-reactive protein value > 1 mg/L after isoflavones (13). In addition, no effects were observed on lipid metabolism or plasma concentrations of glucose or insulin (14). However, some beneficial effects could be shown when the study population was grouped by genotype, and this included a decrease of plasma vascular cell adhesion molecule-1 in subjects carrying the estrogen receptor β (*Alu*I) genotype AA (13) and an elevation of HDL cholesterol in subjects with estrogen receptor β*(cx) Tsp*509I genotype *AA* (14). Moreover, endothelium-independent vasodilation was improved in a subset of these women (15).

We present data on changes in the proteome of peripheral blood mononuclear cells (PBMCs) isolated from postmenopausal women consuming cereal bars as placebo for 8 wk and from the same volunteers after a subsequent 8-wk intervention with the bars providing the isoflavone-enriched soy extract. Proteome analysis was performed to identify markers of response to a dietary intervention with isoflavone-enriched soy extract. The study finally aims at identifying sensitive markers for in vivo effects of soy isoflavones and to further explain through which molecular mechanism such compounds exert their atherosclerosis-preventing or cardioprotective effects.

SUBJECTS AND METHODS

Subjects and study design

Selection of human volunteers and study design was described previously (11–14). A total of 133 healthy postmenopausal women between 45 and 70 y of age were recruited from 4 European countries to take part in the ISOHEART intervention study. Of them, 34 healthy postmenopausal women were recruited from the surrounding areas of the University of Reading, United Kingdom. Of the 34 recruited women at the University of Reading, 27 subjects completed the study. All subjects had a body mass index (in kg/m^2) between 20 and 32. Confounding factors were eliminated as far as possible (12–15).

Participants were requested to incorporate 2 cereal bars/d into their normal diet during the trial, one with breakfast and one in the late afternoon or evening. During the test period, subjects consumed 2 identical cereal bars/d as during the placebo phase except that the product contained an isoflavone-enriched soy extract that provided 50 mg isoflavones/d. The extract used (Solgen 40; Solbar Plant Extracts Ltd, Ashdod, Israel) had a genistein-to-daidzein ratio of 2:1. The cereal bars (40 g), manufactured by a commercial company in the United Kingdom (Efamol, Ltd, Manchester, United Kingdom), had an average nutrient content of energy (652 kJ), protein (2.6 g), carbohydrate (17.3 g), fat (8.5 g), fiber (1.8 g), and sodium (0.012 g). The isoflavone content of cereal bars was measured at Wageningen UR, the Netherlands, by HPLC as described elsewhere (15). The isoflavone extract contained 148.9 mg daidzin/g (8.0 mg/bar), 5.8 mg daidzein/g (0.3 mg/bar), 309.9 mg genistin/g (16.6 mg/bar), and 2.4 mg genistein/g (0.1 m/bar).

Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of all Reading volunteers after 8 wk of placebo or soy-isoflavone consumption. Samples from 10 women, whose first intervention phase was the placebo and underwent the isoflavone treatment after the washout period, were collected after each phase for proteome analysis. Blood samples were collected into an 8-mL CPT Cell Preparation Tube (Becton Dickinson, Cowley, United Kingdom). PBMCs were separated by centrifugation (1500 × *g*, 20 min, room temperature) according to the manufacturer's instructions and washed twice with ice-cold 0.35 mol/L sucrose containing CompleteMini proteinase inhibitor (Roche, Mannheim, Germany) before protein extraction.

Protein extraction from PBMCs

Protein from isolated PBMCs was extracted in 200 μL ice-cold lysis buffer [7 mol/L urea, 2 mol/L thiourea, 1% ditheiothreitol (DTT), 2% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.8% Pharmalyte (pH 3–10)] and protease inhibitor cocktail by homogenization of the cells through ultrasonication (10 strokes, low amplitude) on ice. Lysed cells were centrifuged for 30 min at 100 000 × *g* at 4 °C, and the supernatant fluid was stored at −80 °C. Protein concentration of samples was measured with the use of the Bio-Rad protein assay (Bio-Rad, Munich, Germany).

Two-dimensional gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was performed as described by Görg et al (16) with minor modifications. Briefly, isoelectric focusing (IEF) was performed on 18-cm pH 3–10 immobilized pH-gradient (IPG) strips with the use of an Amersham IPGphor unit (Amersham Biosciences, Freiburg, Germany). Each strip was rehydrated for 12 h with 340 μL of rehydration buffer (8 mol/L urea, 0.5% CHAPS, 15 mmol/L 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). After IEF, strips were incubated for 15 min in equilibration buffer 1 [1.5 mol/L Tris HCl, pH 8.8, 6 mol/L urea, 26% glycerol, 2% sodium dodecyl sulfate (SDS), 1% DTT] and then for another 15 min in equilibration buffer 2 (1.5 mol/L Tris HCl, pH 8.8, 6 mol/L urea, 26% glycerol, 2% SDS, 4% iodoacetamide) before submitting to SDS-PAGE. The 1-mm thick 12.5% SDS-polyacrylamide gels were cast according to the method of Laemmli (17) and were run with the use of an Amersham Biosciences Ettan-Dalt II System with the following conditions: 4 mA/gel for 1 h, then 12 mA/gel. In each analysis (gel chamber load), 5 gels with samples from the placebo group and 5 from the isoflavone group were separated with ≥2 gels for each sample.

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 a Coomassie solution containing 10% $(NH_4)_2SO_4$, 2% phosphoric acid, 25% methanol, and 0.625% Coomassie brilliant blue G250. Gels were destained in bidistilled water until the background was completely clear.

Analysis of proteins with the use of PROTEOMWEAVER software

Gels stained with Coomassie were scanned with the use of an ImageScanner (Amersham Biosciences), and spots were detected by the PROTEOMWEAVER software (version 3.1;

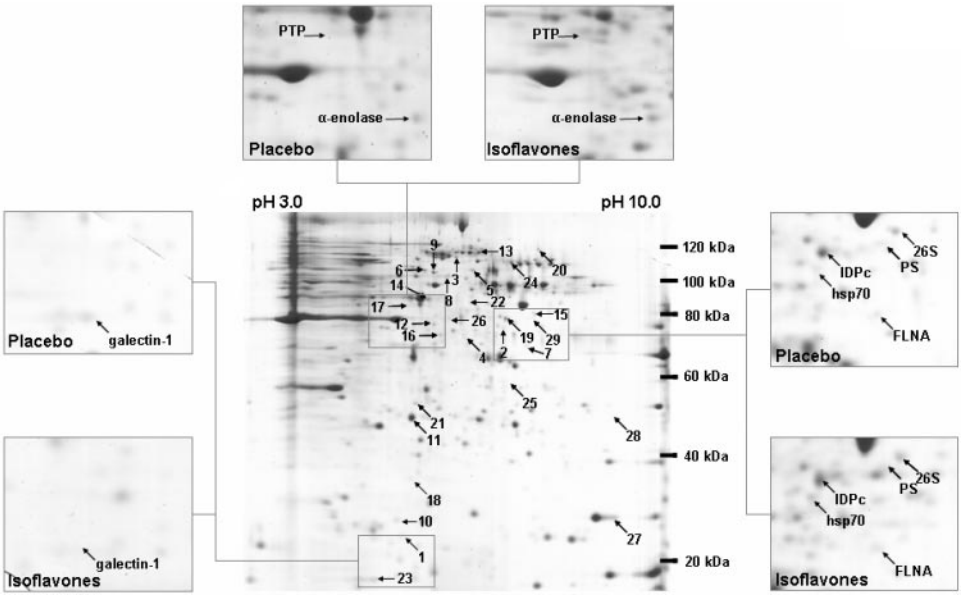

FIGURE 1. Two-dimensional polyacrylamide gel electrophoresis of proteins from peripheral blood mononuclear cells (PBMCs) of postmenopausal women ($n = 10$) as influenced by the consumption of isoflavone-enriched cereal bars. Proteins were separated on a pH 3–10 immobilized pH-gradient strip in the first dimension and on a 12.5% sodium dodecyl sulfate–polyacrylamide gel in the second dimension. The middle section shows a representative Coomassie-stained gel derived from PBMCs of control donors. Protein spots that responded significantly and by >2-fold to the isoflavone intervention are marked by arrows. Protein identifications are given in Table 1. Around the typical control gel-enlarged areas of gels derived from PBMCs of either the placebo group or the isoflavone-supplemented group ($n = 10$) are shown with only those spots marked that were discussed. PTP, protein tyrosine phosphatase; 26S, 26S protease regulatory subunit 8; PS, pleckstrin; IDPc, cytosolic $NADP^+$-dependent isocitrate dehydrogenase; hsp70, heat shock protein 70; FLNA, filamin A.

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 runs of cells derived from 10 probands from each treatment period were compared with each other. Spots differing ≥2-fold and significantly ($P < 0.05$, Mann-Whitney test) in density were picked for matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS).

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 50 mmol/L NH_4HCO_3 and acetonitrile/pure 50 mmol/L NH_4HCO_3 1:1. After the blue color was fully removed, a last washing step with pure acetonitrile followed, and the spots were dried in a SpeedVac (Fouan, Fernwald, Germany). The dry spots were rehydrated for 1 h at 4 °C with 5 μL of 0.02 μg/μL sequencing grade-modified trypsin (Promega, Mannheim, Germany) on ice. The trypsin-supernatant fluid was removed, and proteins in the soaked gel spots were digested by overnight incubation at 37 °C. Seven microliters of 1% trifluoracetic acid were added to each spot, and peptide fragments were extracted by ultrasonication for 10 min. The supernatant fluids derived from each spot were collected and used for MS.

MALDI-TOF MS analysis of tryptic peptides

Peptide mass analysis was performed with the use of the Autoflex mass spectrometer from Bruker Daltonics (Bremen, Germany). Portions (2–4 μL) of the sample were spotted onto α-cyano-4-hydroxycinnamic acid AnchorChip targets with the use of 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 limits allowed for carboxyamidomethylation of cysteine and one missing cleavage. The criteria for positive identification of proteins were set as follows: *1*) a minimum score of 63, *2*) a mass accuracy of ±0.01%, *3*) ≥2-fold analysis from 2 independent gels, and *4*) that the protein exhibits a significant difference in the number of matched peptides to the next potential hit.

RESULTS

On average, >700 different protein spots from the PBMC protein extract could be resolved by 2D-PAGE (**Figure 1**). Of those, 41 showed significant changes in protein density with ≥2-fold difference between the samples after ingestion of isoflavones or the placebo, and 29 proteins could be identified by MALDI-TOF MS (**Table 1**).

The intervention with isoflavone-enriched bars caused the decline of 4 proteins in PBMCs (Figure 1; Table 1) with a 3-fold reduction of galectin 1 concentrations, whereas spots of pleckstrin, aconitase 2 precursor, and RNA polymerase III transcription initiation factor B were no longer detectable.

All remaining PBMC proteins identified as regulated showed an increase in steady state values in the intervention period (Figure 1; Table 1). Most of those were cytoskeleton associated. The second most prominent protein class identified could be grouped to metabolism. A variety of proteins affected in values by isoflavone-extract consumption have been described in context with atherosclerotic processes such

TABLE 1
Steady state ratios of proteins identified by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) analysis of the proteome of peripheral blood mononuclear cells (PBMCs) from postmenopausal women after supplementation with an isoflavone-containing cereal bar compared with consumption of a placebo bar[1]

Protein description	Theoretical	Measured	Protein amount[2]	*P*	Sequence coverage	Accession no.
	M_r/*pI*	M_r/*pI*			%	
Annexins						
Calelectrin (spot no. 1)	76/5.5	18/5.5	2.63 ± 0.60[3]	0.0001	10	AAA35656
Chaperons						
Chain A, heat shock 70-kDa protein, 42-kDa ATPase *N*-terminal domain (spot no. 2)	42/6.7	41/7.1	2.33 ± 1.39	0.047	25	1HJOA
Cytoskeletal proteins						
Talin (spot no. 3)	272/5.8	76/6.4	2.15 ± 0.89	0.004	10	AAF27330
Myosin heavy chain, nonmuscle type A (spot no. 4)	228/5.5	41/6.7	3.40 ± 2.04	0.0001	7	MYH9_HUMAN
Talin (spot no. 5)	272/5.8	76/6.4	3.62 ± 1.73	0.002	8	AAF27330
Vinculin (spot no. 6)	117/5.8	64/5.9	2.76 ± 0.88	0.003	16	AAH39174
FLNA protein (spot no. 7)	89/5.9	38/7.5	2.37 ± 0.97	0.043	22	AAH14654
Talin (spot no. 8)	272/5.8	76/6.4	2.70 ± 1.80	0.041	8	AAF27330
Vinculin (spot no. 9)	117/5.8	64/5.9	3.93 ± 2.31	0.006	18	AAH39174
ACTG1 protein (spot no. 10)	19/5.2	19/5.4	4.18 ± 1.50	0.0001	30	AAH10417
γ-Actin (spot no. 11)	26/5.7	28/5.7	2.60 ± 0.75	0.0001	40	AAA51580
Actin β (spot no. 12)	41/5.6	41/6.0	5.79 ± 2.14	0.0001	33	AAH08633
Talin (spot no. 13)	272/5.8	76/6.4	3.66 ± 1.64	0.0001	12	AAF27330
Myosin heavy chain, nonmuscle type A (spot no. 14)	228/5.5	41/6.7	2.06 ± 0.76	0.006	6	MYH9_HUMAN
Metabolism						
26S protease regulatory subunit 8 (spot no. 15)	31/7.0	43/7.6	2.24 ± 1.34	0.011	36	O43208
α-Enolase (spot no. 16)	47/7.0	40/6.1	3.96 ± 1.38	0.0001	35	ENOA_HUMAN
Protein tyrosine phosphatase (spot no. 17)	50/5.6	51/5.6	2.33 ± 0.81	0.014	41	AAA59531
YWHAZ protein (spot no. 18)	33/4.9	22/5.7	3.13 ± 1.18	0.003	37	AAH68456
$NADP^+$-dependent isocitrate dehydrogenase IDPc (spot no. 19)	47/6.5	42/7.1	3.39 ± 1.69	0.028	25	AAD29284
Aconitase 2, precursor (spot no. 20)	86/7.6	82/7.7	only in control	NA	44	AAH26196
Gene regulation						
Prohibitin (spot no. 21)	30/5.6	29/5.7	2.39 ± 1.03	0.008	55	I52690
RNA polymerase III transcription initiation factor B (spot no. 22)	16/5.0	53/6.5	only in control	NA	10	AAG30220
Other proteins						
Galectin 1 (spot no. 23)	15/5.3	14/5.1	0.31 ± 0.09	0.0001	45	LNHUGB
Fibrinogen alpha chain precursor, extended splice form (spot no. 24)	96/5.7	26/5.7	3.37 ± 1.55	0.0001	15	D44234
Thrombospondin (spot no. 25)	42/6.6	34/7.3	4.92 ± 2.60	0.033	38	AAA61237
Cytosolic thyroid hormone-binding protein (spot no. 26)	59/8.0	43/6.3	2.13 ± 1.37	0.044	32	AAA36672
Chain A, human platelet profiling complexed with the L-Pro10 peptide (spot no. 27)	19/8.2	20/8.8	2.76 ± 1.83	0.0001	57	1AWIA
Electron transfer flavoprotein β chain (spot no. 28)	28/8.6	28/8.8	2.03 ± 0.58	0.0001	38	S32482
Pleckstrin (spot no. 29)	40/8.3	42/7.5	only in control	NA	45	S00755

[1] Protein levels that changed ≥2-fold after 8 wk of isoflavone consumption were identified by MALDI-TOF MS as described. Spot numbers are identical to those given in Figure 1. M_r, relative molecular weight; p*I*, isoelectic point; NA, not available, ie, no statistical analysis could be performed for spots present only in isoflavone- or placebo-exposed PBMCs; FLNA, filamin A; ACTG1, actin γ 1; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, ζ polypeptide; IDPc, cytosolic NAD P^+-dependent isocitrate dehydrogenase.

[2] Spot intensities obtained from isoflavone-exposed PBMCs compared with those obtained from placebo-exposed PBMCs.

[3] $\bar{x}$ ± SD (all such values).

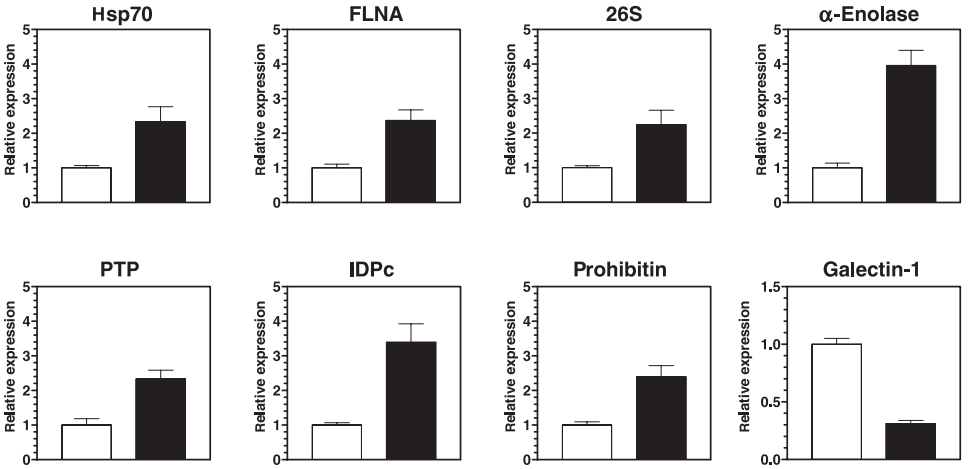

FIGURE 2. Relative expression of proteins from peripheral blood mononuclear cells after the soy-isoflavone intervention. Given are the median protein concentrations and their SDs after the soy-isoflavone intervention (■) in relation to their concentrations after consumption of placebo (□) bars. Shown is the relative expression of proteins from Figure 1 with documented relevance for atherosclerosis, except pleckstrin (spot 29) that completely vanished on isoflavone consumption. Moreover, regulation of prohibitin (spot 21), that was not shown in Figure 1, is given here. Hsp70, heat shock protein 70; FLNA, filamin A; 26S, 26S protease regulatory subunit 8; PTP, protein tyrosine phosphatase; IDPc, cytosolic $NADP^+$-dependent isocitrate dehydrogenase.

as the heat shock protein 70 (hsp70), filamin A protein, 26S protease subunit, α-enolase, protein tyrosine phosphatase, cytosolic $NADP^+$-dependent isocitrate dehydrogenase (IDPc), prohibitin, and galectin-1 (**Figure 2**).

DISCUSSION

Arterial function diminishes at a significant rate in postmenopausal women as a result of low estrogen concentrations with a number of alterations in markers linked to coronary risk such as increased serum concentrations of triacylglycerols and cholesterol, a reduced fibrinolytic activity, or increased concentrations of the plasminogen activator inhibitor type 1 (18). Moreover, the decline in ovarian function leads to an increase of proinflammatory cytokines, in particular interleukin-1, interleukin-6, and tumor necrosis factor-α (TNF-α) (19). A high dietary intake of soy is generally found to be associated with lower risk of cardiovascular diseases (20), which has led to an approved health claim by the Food and Drug Administration that "25 g of soy protein a day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease" (11, 21). Soy consumption has been shown to lower the plasma concentrations of the endothelial stressors oxidized LDL or homocysteine (22), and these effects have been attributed to the isoflavone fraction of soy (23, 24). However, isolated isoflavones quite frequently failed in intervention studies to show atherosclerosis-preventive effects, and it was suggested that other compounds in soy than the isoflavones might be responsible for the discrepancies in clinical outcome with the use of soy or the isolated isoflavones (20).

Previously, in a randomized placebo-controlled dietary intervention trial, bars containing an isoflavone-enriched soy extract were used as a dietary supplement, and effects on clinically relevant markers that indicated an atherosclerosis risk in postmenopausal volunteers were small and limited to particular genotypes (25). It was therefore concluded from the study that isoflavones have beneficial effects only on C-reactive protein, but not on other cardiovascular risk markers, and that the differences in response of HDL cholesterol and vascular cell adhesion molecule 1 to isoflavones were evident only in subgroups with specific estrogen receptor-β genotypes (25).

Here, a more comprehensive proteome profiling approach to PBMCs of the postmenopausal women was applied to identify sensitive markers of response to the intervention with the soy extract, and a variety of changes in ≈40 proteins were observed. PBMCs can easily be obtained and may be considered to serve as reporter cells (26, 27) because they reach various body compartments and encompass subpopulations of cells such as monocytes and lymphocytes (28, 29) that participate in a variety of processes underlying the genesis and progression of atherosclerotic lesions. The study was conducted in a sequential design; therefore, temporal influences cannot be excluded.

Hsp70 was one of the proteins with increased concentrations in PBMCs of the postmenopausal women after intake of the isoflavone-enriched soy extract. This response could be taken as an indicator of an antiinflammatory activity of isoflavones because hsp70 has been shown to block TNF-α production in monocytes or macrophages in vitro after bacterial lipopolysaccharide stimulation and in vivo after an injurious insult (30). TNF-α is mainly secreted by macrophages after the stimulation with various atherogenic factors (31) and is involved in different stages of atherogenesis, eg, by promoting the synthesis of adhesion molecules in endothelial cells (32) or by inducing vascular calcification (31). The importance of an enhanced hsp70 concentration for the inhibition of atherosclerotic processes is further stressed by findings showing that hsp70 protects cellular elements of the arterial wall from injury by reducing oxidation, inflammation, and apoptosis and by promoting the refolding of damaged proteins (33). Moreover, it was shown that circulating hsp70 concentrations are predictors of the development of atherosclerosis in subjects with established hypertension (34) and that high serum concentrations of human hsp70 are associated with a low risk of coronary artery disease, probably through its multiple protective effects on a cell's response to stress (35). Because the heat shock proteins are also secretory products (36), their increased concentrations in PBMCs could contribute as well to increased serum concentrations.

Similar to hsp70, the filamin A protein displayed increased concentrations in PBMCs in the soy-extract intervention period. This response could augment the actions of hsp70 on the suppression of TNF-α activity by blocking TNF-receptor–associated factor 2 protein (37). This intracellular protein is involved in signal transduction from TNF receptor I and II, which is required for TNF-activated apoptosis-inducing stress-response signaling pathways that also activate the proinflammatory transcription factor-κB (NF-κB) (37). NF-κB activity could also be reduced through the observed enhanced concentrations of the 26S protease subunit 8 that has been shown to inhibit NF-κB activation in vitro and in vivo (38). Another line of antiinflammatory response in PBMCs to the soy extract comes from the observation of enhanced concentrations of a lymphocyte-specific protein-tyrosine-phosphatase because this enzyme was shown to be central to maintain or restore a "resting lymphocyte phenotype" (39). Increased concentrations of prohibitin in PBMCs caused by isoflavone consumption could also be taken as an indicator of an antiinflammatory activity. This is based on recent findings in which prohibitin was identified as a novel vitamin D target, mediating antiproliferative actions of vitamin D (40). Similar antiproliferative actions in PBMCs could then be amplified by the observed reduction in galectin-1 concentrations, a β-galactoside-binding lectin involved in cell-cycle progression (41). Although galectin-1 is devoid of a signal peptide, it is secreted from cells by mechanisms independent of normal secretory functions (41). Galectin-1 was shown to be involved in adhesion, migration, and proliferation of vascular smooth muscle cells (42) as prime steps in the development of atherosclerotic lesions and restenosis, and its reduced expression in PBMCs might therefore also contribute to an atherosclerosis preventive effect within the arterial wall.

Besides inflammation as a key process, the fibrinolytic system also is commonly involved in atherogenesis, with fibrin appearing as the most prominent hemostatic risk factor for cardiovascular disease (43). α-Enolase, which showed increased concentrations in PBMCs in the isoflavone period of the trial, was proven to be responsible for most effects that promote plasminogen activation on the surface of leukocytic cells through tissue-type and urokinase-type plasminogen activators (44). Moreover, it protects cell-bound plasmin from its inhibitor α_2-antiplasmin and aggravates the proteolytic activity of cell-bound plasmin, leading in total to enhanced fibrinolysis (44). In this respect, higher α-enolase concentrations in PBMCs might also contribute to reduced fibrin formation.

Increased concentrations of IDPc were observed in PBMCs after consumption of isoflavones, a particularly interesting finding if it is assumed that other cells will respond to soy isoflavones in the same way. IDPc, an enzyme, controls cytosolic redox balance and the cellular defense against damage to reactive oxygen species (ROS) by supplying NADPH for the antioxidant enzyme systems (45). IDPc could thereby be able to reduce the ROS burden after the oxidative burst when macrophages are activated (46). It could also protect the endothelium against ROS-induced lipid peroxidation and apoptosis, if response to isoflavones in endothelial cells is similar to that observed in PBMCs. In a similar manner, reduced concentrations of pleckstrin as seen in the PBMCs could, if also reduced in endothelial cells, be taken as an indicator of an atherosclerosis-preventive action of soy isoflavones. The fact that homocysteine, an endothelial stressor, can induce expression of a member of the pleckstrin homology-related domain family (a protein that can induce anoikis, ie, detachment-mediated apoptosis) (47), is particularly pertinent to our findings in PBMCs. Expression of this pleckstrin member was also found enhanced and to correlate with apoptosis in the atherosclerotic lesions from apoE$^{-/-}$ mice fed diets that increased homocysteine concentrations (47).

In conclusion, proteome analysis applied to PBMCs of postmenopausal women undergoing a dietary intervention for 8 wk with a daily intake of 50 mg isoflavones showed a selected set of proteins responding to treatment that could be closely linked to the genesis and progression of atherosclerotic processes. The observed changes of the marker proteins suggest the soy extract to provide an antiinflammatory activity and may also protect the fibrinolytic system. Because all volunteers represented healthy women, no overall antiinflammatory activity of the soy intervention was observed when clinically relevant inflammation markers were measured in plasma (25). The identified proteins in PBMCs undergoing regulation however may be seen as more sensitive markers that also represent topical effects on inhibition of inflammatory processes and that may also respond earlier than those plasma markers classically used. Further human intervention trials are needed to validate the changes in the PBMC proteome for assessing the in vivo effects of isoflavones or soy products.

The author's responsibilities were as follows—DF, KV, WLH, HD, CMV, JHS, and UW: contributed to the design of the study and the manuscript preparation: KV, WLH, and CMV: collected blood samples; JHS: measured the isoflavone content of the soy-isoflavone-enriched cereal bars; DF: performed the proteome analysis of PBMCs. None of the authors had any financial or personal conflict of interest.

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