The human peripheral blood mononuclear cell proteome responds to a dietary flaxseed-intervention and proteins identified suggest a protective effect in atherosclerosis

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Flaxseed is one of the richest sources of lignans that are converted to enterolactone by the intestinal microflora. Enterolactone has been suggested to be the prime active compound mediating atherosclerosis-protective effects that were shown for flaxseed. The effects of a 1-wk intervention with 0.4 g of flaxseed/kg body weight *per* day on enterolactone plasma levels in seven healthy men revealed that all participants (PAs) responded with enhanced enterolactone plasma levels. Proteome analysis of peripheral blood mononuclear cells (PBMC) from donors before, during, and after the intervention showed that flaxseed consumption affected significantly the steady-state levels of 16 proteins of which four were altered in a similar manner when blood mononuclear cells were exposed *ex vivo* to enterolactone. Enhanced levels of peroxiredoxin and reduced levels of the long-chain fatty acid β-oxidation multienzyme complex may be taken as indicators of a reduced oxidative stress whereas reduced levels of glycoprotein IIIa/II could indicate improved protection from thrombotic and inflammatory processes. In conclusion, the blood mononuclear cell proteome responds to dietary flaxseed intake with changes in a number of atherosclerosis-relevant proteins that may be taken as biomarkers of exposure and some of these changes observed can be attributed to the action of the lignan metabolite enterolactone.

Keywords:
Atherosclerosis / Enterolactone / *In vivo* / Lignans / Peripheral blood mononuclear cells

1 Introduction

Lignans are a class of phytoestrogens to which preventive effects have been ascribed in several diseases including atherosclerosis [1, 2], chronic renal disease [3, 4], or hormone-dependent cancers such as those of the prostate [5] or mammary gland [6]. Enterolactone, produced by the colonic microflora from the precursors matairesinol, secoisolariciresinol, their glycosides, and other lignans provided by the diet, is suggested to be the prime active compound mediating these effects [7–9]. The richest known dietary source of lignans is flaxseed that provides especially the main lignan precursor secoisolariciresinol diglucoside [10]. With regard to atherosclerosis, flaxseed intake has been shown to mod-

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Abbreviations: PBMC, peripheral blood mononuclear cells; **VASP,** vasodilator-stimulated phosphoprotein

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estly improve lipid profiles and thereby contribute to reduce cardiovascular disease risks [2]. Secoisolariciresinol diglucoside was demonstrated to directly lower serum cholesterol levels [11] but has also been discussed to provide antioxidant activities [12] and to act as an antagonist of platelet-activating factor [13]. Other constituents of flaxseed, such as α-linolenic acid, also have been demonstrated to possess antioxidant, anti-inflammatory, antithrombotic, and antihypertensive activities that may contribute to the overall atherosclerosis-preventive activities reported for flaxseed or flaxseed oil [2]. Overall, those effects of flaxseed and flaxseed oil has led the American Heart Association to cite both as potentially useful foods for the prevention of cardiovascular disease [14].

In the present study, we used a proteomics approach to identify alterations in the steady-state levels of proteins in peripheral blood mononuclear cells (PBMC) of healthy males ingesting daily 0.4 g of flaxseed/kg body weight as a single bolus for a week. PBMC consist of monocytes and lymphocytes and both cell types are involved in the initiation and progression of atherosclerotic lesions [15, 16]. We have used PBMC as reporter cells *in vivo* to monitor whether flaxseed intake can affect atherosclerosis-relevant processes in humans. PBMC from the same study subjects were also exposed to enterolactone *ex vivo* to assess whether similar effects on the proteome could be observed as those caused by dietary flaxseed intake. Enterolactone plasma levels were determined in the study subjects before, during, and after the flaxseed intervention period to assure that plasma levels of this lignan metabolite increased by intake of flaxseed and to derive concentrations of enterolactone to be applied in the *ex vivo* treatment of the human PBMC.

2 Materials and methods

2.1 Study design

Seven healthy male volunteers between ages of 20 and 40 not taking any medication including antibiotics during the last 3 months were recruited. The study period consisted of a 1 wk long prephase, 1 wk of intervention, and a washout phase of 2 wk. During the 7 days of the dietary intervention, subjects consumed every morning 0.4 g/kg body weight of a commercially available shooted flaxseed (one batch). The participants (PAs) were asked to retain their usual diet but to strictly avoid consumption of flaxseed, pumpkin seed, and soy products. Fasting blood samples were collected 1 wk before the start of the intervention, on day 0 just before the intervention as well as on the first, third, and seventh day as well as 2 wk after the last flaxseed intake.

2.2 Analysis of plasma enterolactone levels

Enterolactone in plasma was analyzed by a time-resolved fluoroimmunoassay [17] using the enterolactone test (Labmaster, Turku, Finland) with minor modifications, as described earlier [7]. This assay measures total enterolactone concentrations after enzymatic hydrolysis of enterolactone glucuronides and sulfates. All analyses were carried out in duplicate. Intra- and interassay coefficients of variation ranged between 3.1–6.1 and 6.1–8.6%, respectively [18]. Coefficients of variations <10% were usually obtained with two quality control samples analyzed within each batch.

2.3 Preparation of PBMC and protein extraction

Peripheral blood from the seven volunteers was collected in CPT™ Cell Preparation Tubes (Becton Dickinson, Cowley, UK). PBMC were separated by centrifugation according to the manufacturer's instructions and washed twice with ice-cold 0.35 M sucrose containing CompleteMini protease inhibitor (Roche, Mannheim, Germany), prior to protein extraction. Protein from isolated PBMC was extracted in 200 μL of ice-cold lysis buffer (7 M urea, 2 M thiourea, 1% dithiothreitol (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 (ten strokes, low amplitude) on ice. Lysed cells were centrifuged for 30 min at 100 000 × *g* at 4°C and the supernatant was stored at 80°C. Protein concentration of samples was determined using the BioRad protein assay (BioRad, Munich, Germany). Samples of one PA were excluded from subsequent proteome analysis due to their very low protein concentrations.

2.4 Exposure of human PBMC to enterolactone *ex vivo*

PBMC of three participating persons isolated directly prior to starting the flaxseed intervention were incubated *ex vivo* for 24 h in their own plasma either in the presence or absence (control) of 50 nM enterolactone. After the incubation period, PBMC were washed twice with 0.35 M sucrose and centrifuged for 10 min at 500 × *g* and 4°C. The supernatants were discarded and the cell pellets resuspended in 200 μL of lysis buffer for protein extraction, as described above. Subsequent proteome analysis was performed as described below.

2.5 2-D PAGE

2-D PAGE was performed as described by Görg *et al.* [19] with minor modifications. Briefly, IEF was performed on 18 cm (pH 3–10) IPG strips 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 cuploading. IEF was performed under the following conditions: 500 V (10 min, gradient), 4000 V (1.5 h, gradient), 8000 V (25 000 V·h, step-n-hold). After 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. One millimeter thick 12.5% SDS-polyacrylamide gels were cast according to the method of Laemmli [20] 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 a Coomassie solution containing 10% $(NH_4)_2SO_4$, 2% phosphoric acid, 25% methanol, and 0.625% CBB G250. Gels were destained in bidistilled water until the background was completely clear.

2.6 Analysis of proteins using the ProteomWeaver software

Gels stained with Coomassie were scanned using an ImageScanner (Amersham Biosciences) and spots detected by the ProteomWeaver software (Definiens). Background subtraction and volume normalization were made automatically by the software. After spot detection, all gels were matched to each other. Two independent gels from cells derived from each PA and each time point were run. Those gels derived from the two time points of the preintervention phase and also those derived from the three time points of the intervention phase were grouped, respectively. Spots differing significantly ($p<0.05$) by at least two-fold in density and where changes in spot intensity was consistent in at least four out of six PAs were picked for MALDI-TOF MS analysis.

2.7 Enzymatic digestion of protein spots for MALDI-TOF MS

Coomassie-stained spots were picked with a 2 or 3 mm "skin picker". The destaining of spots was performed by alternate 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 (Promega, Mannheim, Germany) on ice. The trypsin supernatant was removed and proteins in the soaked gel spots were digested by overnight incubation at 37°C. Seven microliters of 1% TFA was added to each spot and peptide fragments were extracted by ultrasonication for 10 min. The supernatants derived from each spot were collected and used for MS.

2.8 MALDI-TOF MS analysis of tryptic peptides

Peptide mass analysis was performed using the Autoflex mass spectrometer of Bruker Daltonics (Bremen, Germany). Sample (2–4 μL) was spotted onto CHCA 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. Peak picking was done by FlexAnalysis 2.0 and the resulting mass list was evaluated using Bio Tools 3.0 with the search engine MASCOT (version 1.9.00, www.matrixscience.com) and the MASCOT Search database (Matrix Science, London, UK). The search parameters allowed for carboxyamidomethylation of cysteine and one missing cleavage. The criteria for positive identification of proteins were set as follows: ±50–150 ppm peptide mass tolerance, zero or one missed cleavage, carbamidomethyl modification of cysteine as global and methionine oxidation as variable modification, and charged state as MH^+. A protein was seen as validated when three samples satisfactorily showed the same results with a probability based MOWSE score being significant ($p<0.05$).

3 Results

3.1 Plasma enterolactone levels in healthy men are increased by flaxseed consumption

The seven healthy volunteers showed fasting enterolactone baseline concentrations of 4.8 ± 1.5 and 8.4 ± 3.9 nmol/L 1 wk before flaxseed intervention and on the first intervention day, respectively. Individual levels ranged from 0.3 to 28.9 nmol/L (Table 1, Fig. 1). This rather large variation at the start of the intervention remains unexplained as all volunteers reported compliance to the recommendation not to eat flaxseed or any products derived thereof during the past week. However, all subjects responded to ingestion of flaxseed with an elevation of plasma enterolactone levels with mean values of 24.9 ± 5.9 nmol/L on day 2, 30.4 ± 7.0 nmol/L on day 3, and 22.0 ± 5.2 nmol/L on day 7 of the intervention period, respectively (Table 1, Fig. 1).

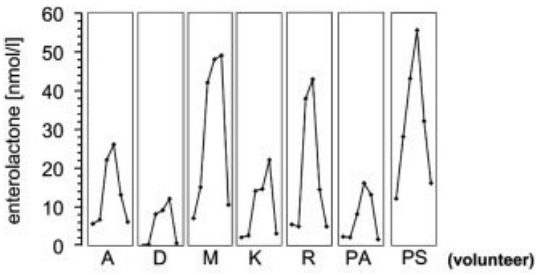

Figure 1. Enterolactone concentration profiles in fasting plasma samples (nmol/L) obtained from seven male volunteers at six time points during the flaxseed intervention study as measured by time-resolved fluoroimmunoassay. Baseline values were measured in fasting plasma 7 days before and on the day of the start of the intervention. During the intervention, enterolactone levels were determined at days 2, 3, and 7 and washout effects were determined 2 wk following the last flaxseed intake. Letters refer to the different individuals.

Table 1. Mean, SEM, median, 25. and 75. percentile (nmol/L) of enterolactone concentrations in fasting plasma samples during the three phases of the human intervention study

Phase	Day	Mean (nmol/L)	SEM	Median (nmol/L)	25. Percentile	75. Percentile
Prephase	**−7**	4.8	1.5	5.5	1.4	7.2
	0	8.4	3.9	5.4	1.4	14.2
Intervention phase	**1**	24.9	5.9	21.8	8.1	42.4
	3	30.4	7.0	25.7	14.7	48.5
	7	22.0	5.2	14.8	12.1	32.0
Washout phase	**21**	6.2	2.0	5.3	1.5	10.6

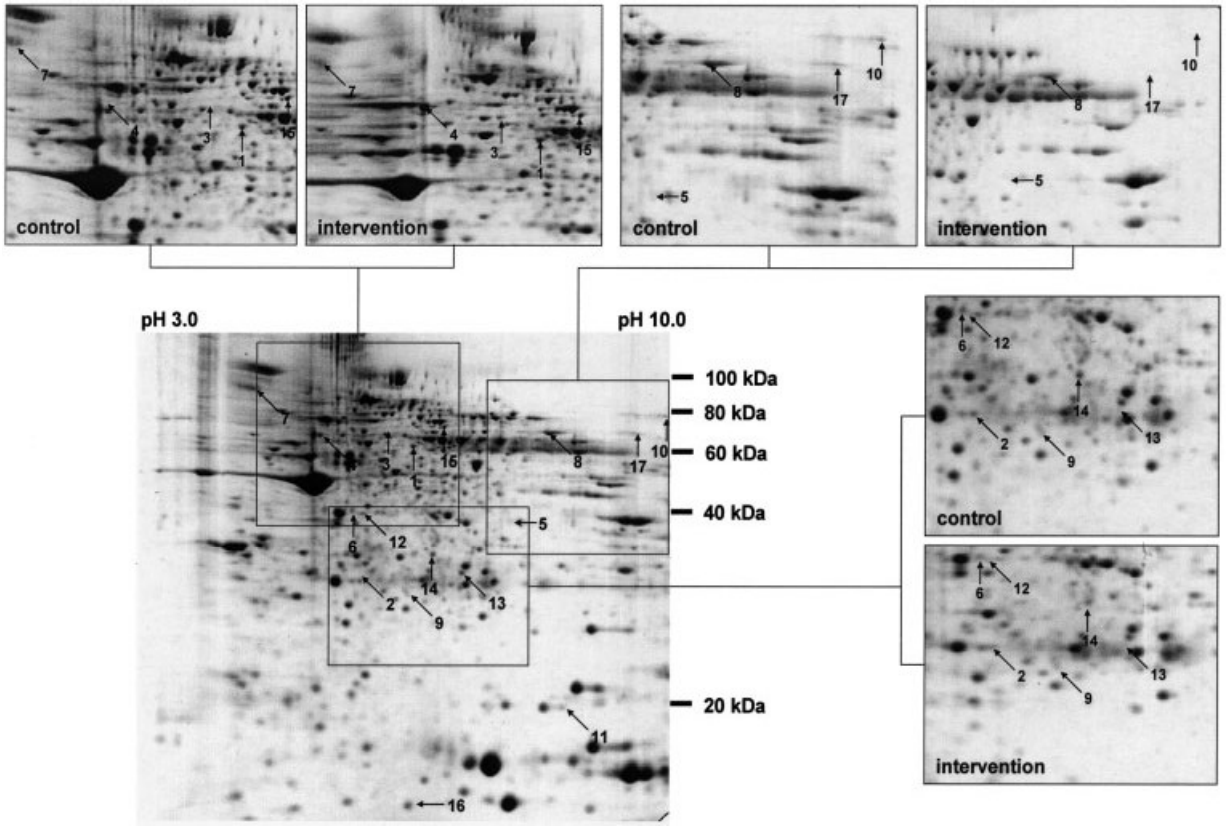

Figure 2. 2-D PAGE of proteins derived from PBMC of healthy men before and after an intervention with 0.4 g of flaxseed/kg body weight *per* day. Proteins from PBMC were separated on a pH 3–10 IPG-strip in the first dimension and on a 12.5% SDS-polyacrylamide gel in the second dimension. A typical Commassie-stained gel from PBMC isolated prior flaxseed intervention is shown in the middle section. Enlarged areas of gels derived from PBMC prior intervention or during flaxseed intervention are shown around the middle panel.

Individual response reached maximum plasma concentrations of 11.8 nmol/L (D) up to 55.8 nmol/L (PS) (Fig. 1). Interestingly, three out of seven subjects (A, R, and PS) displayed distinctly lower plasma enterolactone levels on day 7 than on day 3 of the intervention phase (Fig. 1), suggesting adaptation possibly by changes in the gut microflora. Two weeks after the last flaxseed intake, enterolactone levels in plasma had returned to baseline levels in all subjects (Table 1, Fig. 1).

3.2 Flaxseed consumption alters the steady-state levels of atherosclerosis-relevant proteins in PBMC *in vivo*

More than 700 different protein spots could be resolved by 2-D PAGE of which 17 were altered significantly in quantity and identified by MALDI-TOF MS (Table 2, Fig. 2). Two of them corresponded to the α- and β-subunits of TCP-1, thus representing the same protein (Table 2). For many of the

Table 2. PBMC proteins affected in their SSLR (steady-state level ratio) by the ingestion of flaxseed in healthy men

Spot no.	Protein description	Theoretical M_r/p*I*	Measured M_r/p*I*	Protein amount		Residues of identified peptides	% Sequence coverage	Accession no.
				Intervention phase/ prephase	Washout phase/ prephase			
	Chaperons							
1	Chaperonin-containing TCP-1 β-subunit homolog	96/6.4	58/6.0	2.05	0.86	26–40, 90–111, 121–131, 190–203, 205–222, 285–293, 323–342, 389–402, 406–427, 445–466, 467–481, 502–516	36	AAC98906
2	Peroxiredoxin 4	38/5.3	31/5.9	3.70	1.05	1–18, 46–66, 48–66, 67–78, 81–99, 140–164, 174–186, 187–200, 201–208, 213–223, 231–240, 241–263	64	Q13162
3	T-complex protein 1 subunit-α (TCP-1-α)	121/5.8	61/5.8	2.01	0.95	74–84, 131–145, 190–199, 234–243, 299–309, 356–365, 379–390, 391–400, 391–401, 434–443, 469–480, 469–481, 500–510, 516–526, 539–556, 542–556	30	P17987
4	60 kDa heat shock protein, mitochondrial precursor (hsp60)	210/4.6	61/5.7	Only in intervention	n.d.	61–72, 97–121, 158–180, 206–218, 222–233, 237–249, 250–268, 269–290, 370–387, 371–389, 421–429, 430–446, 447–462, 462–493, 494–516	41	P10809
	Cytoskeletal proteins							
5	LIM protein	48/7.7	38/7.6	2.88	1.55	85–96, 97–111, 138–150, 151–166, 177–187, 188–197, 211–221, 222–230, 236–257	37	JC2324
6	β-5 Tubulin	51/5.4	38/5.4	0.50	0.77	3–19, 47–58, 63–77, 78–103, 104–121, 155–162, 163–174, 217–241, 242–252, 253–262, 263–276, 283–297, 310–318, 325–336, 337–350	49	AAH20946
7	Platelet glycoprotein IIIa/II	220/4.5	86/5.0	0.45	0.52	46–61, 125–136, 181–190, 216–234, 239–252, 352–359, 390–401, 412–421, 422–446, 598–514, 519–529, 580–599	23	B36268
	Metabolism							
8	Pyruvate kinase isozymes M1/M2	134/8.7	58/8.0	2.04	1.84	32–42, 43–55, 73–88, 151–161, 173–185, 188–205, 206–223, 247–254, 270–277, 278–293, 279–293, 294–304, 319–335, 342–366, 367–375, 383–398, 383–399, 400–421, 422–432, 467–474, 475–488, 489–497	51	P14618
9	Protein-L-isoaspartate (D-aspartate) *O*-methyltransferase (EC 2.1.1.77) splice form I	38/6.6	25/6.8	0.24	0.60	4–17, 27–36, 27–41, 81–97, 113–123, 124–134, 135–143, 178–196, 204–218	49	P22061
10	Long-chain fatty acid β-oxidation multienzyme complex α chain precursor, mitochondrial	160/10.4	83/10.4	Only in prephase	0.99	1–12, 39–46, 67–86, 112–125, 167–187, 214–235, 268–279, 310–326, 327–334, 338–350, 391–399, 423–440, 494–505, 541–549, 550–560, 611–625, 661–676, 665–676, 720–728, 735–742, 743–759, 743–760	35	543064
11	Cyclophilin A	21/8.7	18/7.7	Only in prephase	0.76	2–19, 20–28, 20–31, 56–69, 77–91, 83–91, 132–144, 134–144, 155–165	50	P62937

Table 2. Continued

Spot no.	Protein description	Theoretical M_r/p*I*	Measured M_r/p*I*	Protein amount		Residues of identified peptides	% Sequence coverage	Accession no.
				Intervention phase/ prephase	Washout phase/ prephase			
12	TALDO 1 protein	54/5.4	37/5.8	Only in intervention	n.d.	101–111, 114–120, 133–144, 183–194, 195–205, 221–229, 236–248, 286–297	26	AAH18847
13	Phosphoglycerate mutase 1 (phosphoglycerate mutase isozyme B) (PGAM-B) (BPG-dependent PGAM 1)	39/6.9	29/6.8	2.86	1.65	10–20, 21–38, 21–39, 46–60, 90–99, 116–137, 117–137, 140–156, 141–156, 141–161, 162–175, 180–190, 222–239	56	P18669
	Gene regulation							
14	Purine-nucleoside phosphorylase (EC 2.4.2.1)	47/6.9	32/6.5	2.06	1.89	12–22, 23–41, 42–58, 68–76, 102–123, 124–133, 134–148, 155–168, 186–207, 212–229, 230–244, 235–244, 245–254	62	P00491
15	Stress-induced phosphoprotein 1	154/6.8	63/6.4	2.01	Only in prephase	14–32, 33–44, 64–73, 79–87, 94–109, 110–118, 145–153, 154–160, 208–223, 253–272, 306–315, 316–325, 318–325, 352–364, 416–429, 454–462, 463–470, 463–478, 489–505	44	P31948
	Other proteins							
16	Chain B, crystal structure of desoxy-human hemoglobin β6 Glu->Trp	13/6.5	16/7.3	0.30	0.54	1–17, 9–17, 18–30, 31–40, 41–59, 66–82, 67–82, 83–95, 96–104	67	6HBWB
17	Gelsolin precursor	103/9.6	86/5.9	Only in prephase	1.42	148–162, 178–188, 328–338, 369–390, 398–419, 420–447, 458–481, 585–597, 598–615, 627–348	23	P06396

The spot numbers are identical to those given in Fig. 2. Protein levels altered by the intervention with flaxseed by at least two-fold and significantly were identified by MALDI-TOF-MS as described. n.d., no spot detected.

proteins altered in level upon flaxseed ingestion, a distinct role in atherosclerotic processes has been suggested such as for peroxiredoxin that displayed increased levels, or cyclophilin A and the long-chain fatty acid β-oxidation multienzyme complex with reduced protein spot intensity. Likewise the platelet membrane glycoprotein IIb, that is part of the platelet glycoprotein IIIa/II complex, a membrane receptor for fibrinogen and von Willebrand factor [21], showed reduced levels (Table 2, Fig. 2). Most of the alterations observed under flaxseed ingestion were reversible and 2 wk after the end of the intervention the levels of chaperonin-containing TCP-1, peroxiredoxin, TCP-1-α, hsp60, long-chain fatty acid β-oxidation multienzyme complex, and gelsolin precursor had returned to baseline values (Table 2). Stress-induced phosphoprotein 1 was no longer detectable after the washout phase although it was detectable prior to the intervention and did increase upon flaxseed intake (Table 2). There were also proteins, however, that remained unaltered in their steady-state levels from the intervention throughout the washout phase such as β-5 tubulin, pyruvate kinase isozymes M1/M2, or platelet glycoprotein IIIa/II protein levels (Table 2).

3.3 Effects of enterolactone on the proteome of human PBMC *ex vivo*

Exposure of isolated PBMC to 50 nM enterolactone altered the spot intensities of 15 proteins that could be identified by MALDI-TOF MS (Table 3, Fig. 3). These correspond to 13 protein entities since two spots differing in isolectric point were identified as β-actin and two other spots differing primarily in molecular weight were identified as long-chain fatty acid β-oxidation multienzyme complex (Table 3). Whereas the two β-actin spots could be due to a varying degree of phosphorylation the different long-chain fatty acid β-oxidation multienzyme complex spots appear to be caused by different multimeric states. Four of the proteins that displayed significantly altered spot intensities in PBMC by enterolactone exposure were also identified as regulated in an almost identical manner *in vivo* by flaxseed intake (Table 3, Fig. 3) including the atherosclerosis-relevant proteins platelet glycoprotein IIIa/II, peroxiredoxin-4 and the long-chain fatty acid β-oxidation multienzyme complex (Fig. 4).

Table 3. SSLR of proteins from PBMC that were incubated in the absence (control) or presence of 50 nM enterolactone *ex vivo*

Spot no.	Protein description	Theoretical M_r/p*I*	Measured M_r/p*I*	Protein amount 50 nM enterolactone/ control	Residues of indent peptides	% Sequence coverage	Accession no.
	Chaperons						
1	Peroxiredoxin-4	31/5.9	28/6.0	2.81	46–66, 48–66, 81–99, 140–164, 174–186, 187–200, 213–223, 231–240, 241–263	50	Q13162
	Cytoskeletal proteins						
2	γ-Actin	26/5.6	27/5.8	0.42	54–63, 71–95, 73–95, 96–111, 149–169, 217–229	36	AAA51580
3	Platelet membrane-glycoprotein IIb	114/5.2	95/4.5	0.31	73–90, 91–104, 156–170, 185–195, 185–196, 313–334, 335–348, 359–366, 367–386, 387–399, 400–417, 418–431, 432–453, 487–502, 511–532, 533–543, 551–571, 693–702, 766–774, 783–799	28	P08514
4	Actin, β	41/5.6	50/8.1	0.40	12–21, 22–32, 33–43, 89–106, 190–199, 190–203, 207–231, 209–231, 232–247, 278–284, 284–305, 285–305, 329–352, 353–365	46	AAH08633
5	Actin, β	41/5.6	41/5.5	2.09	12–21, 44–54, 78–88, 89–106, 190–199, 207–231, 209–231, 232–247, 285–305, 329–352	39	AAH08633
6	Talin	272/5.8	49/9.7	2.97	16–30, 34–57, 36–57, 36–58, 67–74, 75–82, 107–118, 119–131, 165–178, 182–194, 196–207, 208–220, 221–234, 235–254, 307–316, 346–357	7	AAF27330
7	VASP	40/9.1	46/10.2	7.95	10–21, 22–34, 35–47, 52–67, 53–67, 71–82, 86–95, 257–272, 321–345, 361–374	34	P50552
	Metabolism						
8	Long-chain fatty acid β-oxidation multi-enzyme complex α-chain precursor, mitochondrial	84/9.2	83/10.5	0.35	67–86, 112–125, 133–159, 167–187, 191–205, 215–235, 236–249, 310–326, 338–350, 363–383, 423–440, 441–455, 494–505, 541–549, 570–597, 635–644, 647–660, 665–676, 720–728, 743–759	42	543064
9	Myeloperoxidase precursor	94/9.3	35/9.1	2.05	597–609, 676–688, 678–689, 714–737, 715–738, 763–786, 811–827	11	P05164
10	Long-chain fatty acid β-oxidation multi-enzyme complex α-chain precursor, mitochondrial	84/9.2	80/10.2	0.37	67–86, 112–125, 133–159, 167–187, 191–205, 215–235, 310–334, 338–350, 363–383, 423–440, 441–455, 494–505, 541–549, 570–597, 647–660, 743–759, 743–760	38	543064
11	Proteasome β-3-subunit	23/6.1	27/6.8	0.5	28–41, 49–66, 100–115, 178–192, 178–194	31	AAH13008
	Gene regulation						
12	Heterogenous ribonucleo-protein A1 (helix-destabilizing protein) (single-stranded binding protein) (hnRNP core protein A1)	39/9.3	36/10.3	0.23	14–30, 15–30, 31–46, 92–104, 122–129, 122–139, 130–139, 145–160, 146–160, 166–177	24	P09651
	Other proteins						
13	Chain B, crystal structure of fibrinogen fragment D	36/7.7	44/7.6	0.36	2–19, 35–47, 71–90, 91–108, 109–117, 137–151, 177–190, 219–233, 234–244, 250–259, 282–294, 307–313	52	1FZAB
14	Chain B, crystal structure of desoxy-human hemoglobin β6 Glu->Trp	16/7.3	15/7.4	0.45	18–30, 31–40, 41–59, 66–82, 83–95, 96–104, 105–120, 121–132, 133–144	82	6HBWB
15	Igκ light chain VLJ region	30/5.8	28/6.3	3.5	74–82, 136–154, 137–155, 155–170, 217–235, 219–235, 246–272	32	BAC01700

The spot numbers are identical to those given in Fig. 3.

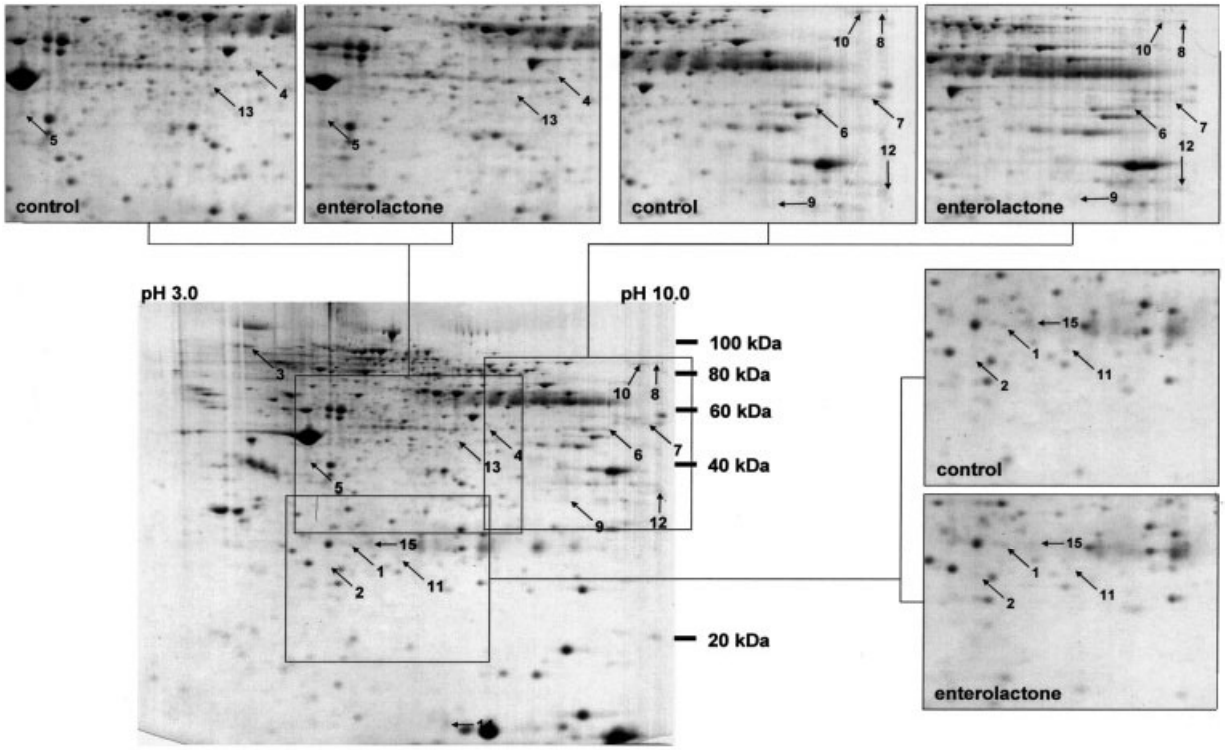

Figure 3. 2-D PAGE of proteins from PBMC that were isolated in the preintervention phase and exposed thereafter for 24 h to plasma with 50 nM enterolactone added or not (control). Enlargements around the control gel in the middle section show identical areas of the gels from PBMC grown in plasma alone or in addition with 50 nM enterolactone.

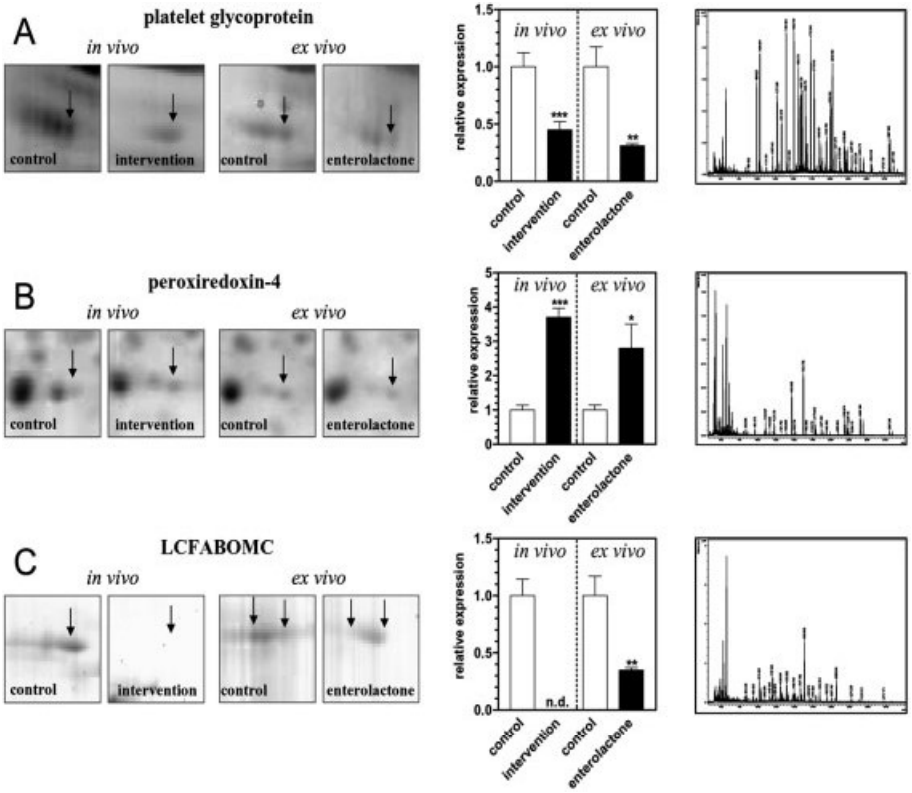

Figure 4. Expression levels of atherosclerosis-relevant proteins platelet glycoprotein (A), peroxiredoxin-4 (B), and long-chain fatty acid β-oxidation multienzyme complex (LCFABOMC) (C) in PBMC in response to a flaxseed intervention *in vivo* or an enterolactone exposure *ex vivo*. The left panels show representative protein spots from gels that were derived from the respective treatments and the mid panels show the relative expression *vs.* the control, with * $p<0.05$, ** $p<0.01$, and *** $p<0.001$; n.d., no spot detectable. In PBMC exposed *ex vivo* to enterolactone two isoforms of LCFABOMC were identified that both responded with reduced expression to the treatment (left panels in C). *Ex vivo* expression in the mid panel is given for LCFABOMC with higher p*I* (right-handed arrows). In the right panels, a representative annotated spectrum as obtained by MALDI-TOF MS from those spots is given.

4 Discussion

We have employed a proteomics approach to address the question of whether marker proteins in PBMC can be identified that respond with robust changes to a dietary intervention with flaxseed, which is suggested to exhibit atherosclerosis-preventing and cardioprotective effects [22–24], and whether enterolactone as the prominent lignan derivative found in plasma can affect by itself protein expression levels in PBMC upon exposure *ex vivo*.

PBMC were chosen as easily obtainable reporter cells that encompass cell populations such as monocytes and lymphocytes known to play an important role in atherosclerotic processes [15, 16]. Enterolactone plasma concentrations were determined in the volunteers throughout the trial (prephase, intervention phase, and washout phase) to assess compliance and for deriving relevant concentrations for the *ex vivo* study in isolated PBMC exposed to enterolactone. All volunteers responded to flaxseed intake with markedly increased plasma enterolactone levels although both baseline levels and maximal plasma concentrations varied considerably between subjects. In general we observed that subjects with higher baseline levels also showed higher peak plasma levels during the intervention indicating that enterolactone production in the large intestine is dependent on the individual microflora [25]. Moreover, in three out of seven study subjects the plasma enterolactone values at day 7 of flaxseed intake were much lower than at day 3, which suggests that an effective adaptation occurred based on either a reduced production of enterolactone in the colonic flora and/or by enhanced glucuronidation and/or sulfatation of enterolactone for increasing body clearance by excretion [26, 27]. Despite these differences in the responses in enterlactone plasma levels, PBMC protein changes showed similar and fairly robust changes in all study subjects.

One of the PBMC proteins with increased levels after intervention and after *ex vivo* treatment of PBMC was peroxiredoxin 4. Overexpression of peroxiredoxin 4 has been demonstrated to result in the inhibition of NF-κB-dependent gene expression initiated by the proinflammatory cytokine tumor necrosis factor-α [28]. Among several cellular antioxidant defense systems, the thioredoxin pathway appears to have the greatest selectivity for NF-κB-mediated responses through inhibition of IκB phosphorylation that in turn provides a potent blockade of inflammatory processes that are crucial in atherosclerosis initiation and progression [29]. NF-κB not only plays a role in inflammation but also in thrombosis which is also loosely linked to atherosclerosis [29]. Next to the increased peroxiredoxin levels, the reduced expression of platelet glycoprotein IIIa/II, that was observed in PBMC after flaxseed intervention *in vivo* and after enterolactone exposure *ex vivo*, can also be connected to inhibition of thrombosis. Due to its critical role in mediating platelet aggregation, glycoprotein IIIa/II has become a primary target in the development of antithrombotic drugs [30]. Since according to the density gradients formed within the CPT Cell Preparation Tubes platelets are cosegregated with PBMC from other blood cells, it is plausible that the diminished expression of glycoprotein IIIa/II is based on lowered levels in platelets. However, glycoprotein IIIa/II appears also to be found on the surface of monocytes/macrophages in which it could contribute to the formation of platelet–monocyte complexes and fibrin polymerization explaining also the function of monocytes in thrombotic complications of atherosclerosis [31]. Monocytes within platelet–monocyte complexes appear to have a higher state of activation and therefore could possess an increased proatherogenic capacity [32]. A third protein regulated in PBMC by enterolactone *in vivo* and *ex vivo* was the long-chain fatty acid β-oxidation multienzyme complex. Its lower levels suggest a reduced generation of ROS in the cells, according to diminished delivery of reduction equivalents to the respiratory chain [33], which might contribute to the proposed reduction of a proinflammatory state.

A number of other atherosclerosis-relevant proteins were also found altered in steady-state level in PBMC *in vivo* but not after *ex vivo* exposure of PBMC to enterolactone. This may suggest that those changes found *in vivo* are mediated by other flaxseed compounds than the lignans. Among the proteins, cyclophilin A was found at reduced levels. It is a secretory protein that has been demonstrated to be induced by cellular oxidative stress [34] and was proven to play an important role in inflammatory diseases such as atherosclerosis [35]. Its reduction in PBMC may therefore be taken as a measure of an anti-inflammatory action of flaxseed intake. Other proteins identified could have an impact on the numbers of immunocompetent cells through affecting their apoptosis rate or maturation state. For instance, transaldolase (TALDO) 1, which was found at increased level after flaxseed intervention was demonstrated to increase apoptosis in Jurkat T-cells by reducing the levels of glutathione [36] and gelsolin blocks in the same cells apoptosis initiated by different stimuli [37]. Gelsolin was found at reduced levels in PBMC upon flaxseed intake which could result from cleavage during apoptotic processes in lymphocytes [38]. However, the increased expression of chaperonin-containing TCP-1 in PBMC might indicate that programmed cell death in monocytes is reduced as TCP-1 appears to protect cells from apoptosis by unfolded protein response [39].

Although the majority of proteins with changed levels suggest that flaxseed intake could have a beneficial overall function in the prevention of atherosclerosis some alterations found also point to a stress response. Especially the increased levels of hsp60 are indicative for this. Hsp60, like other heat shock proteins, is released from PBMC as part of a "danger signaling cascade" in response for example to a bacterial infection [40]. Hsp60 has been shown to be present in human atheroma tissue and was demonstrated to activate human PBMC in atherosclerosis [41]. Moreover, immune reactions against bacterial hsp60 can lead to crossreactivity with human hsp60 expressed on endothelial cells [42] which might be augmented by enhanced hsp60 expression and

secretion through PBMC. Such autoimmune reactions could be fostered by the observed increased levels of protein-L-isoaspartate (D-aspartate) *O*-methyltransferase since this protein was proven to stimulate T-cells and to play a role in autoimmune pathology [43]. Moreover, the increased levels of the stress-induced phosphoprotein 1 under flaxseed-intake suggest a stress induction with activation of PBMC [44] since this protein affects the functions of the macrophage scavenger receptor such as internalization, cell-surface expression, and signal transduction [45]. Interestingly, phosphoprotein 1 was no longer detectable in PBMC at the end of the washout phase of our intervention trial which indicates that phosphoprotein-1 could serve as a marker for an adaptive stress response in PBMC.

Among the proteins affected in level only after *ex vivo* exposure of PBMC to enterolactone, we identified for example vasodilator-stimulated phosphoprotein (VASP), fibrinogen and myeloperoxidase. The increase in myeloperoxidase levels points also to some adverse effects of enterolactone since this protein is involved in the generation of ROS and diffusible radical species. Moreover, it contributes to the internalization of cholesterol in macrophages and colocalizes with macrophages in atheromas [46]. Diminished levels of fibrinogen, on the other hand could be taken as a measure of a reduction of thrombosis and of platelet–monocyte complexes [32]. The protein with the greatest response to enterolactone was VASP, showing eight-fold increased levels. Phosphorylation of VASP by cGMP-dependent protein kinase I is an established biochemical endpoint of NO/cGMP signalling and clearly provides a protective function to the endothelium [47]. Since these proteins were identified only when isolated cells were treated with enterolactone but did not change in volunteers taking flaxseed, one has to assume that other compounds in flaxseed can abrogate *in vivo* some of the effects of enterolactone.

In conclusion, proteome analysis revealed that the consumption of flaxseed in male human volunteers affects the steady-state levels of several proteins in PBMC that are all known to be associated with atherosclerotic processes. The regulations found suggest an overall anti-inflammatory and antithrombotic response of the cells to flaxseed intake. Some of the key proteins in this regard were also regulated in a similar direction and magnitude when PBMC were exposed *ex vivo* to enterolactone, suggesting that this enterolignane plays a causal role for the observed effects of flaxseed administration. Since some of the proteins responding to treatment are indicative of a stress response in the PBMC cell population and may represent markers of possible adverse effects of flaxseed, further studies are needed in the risk–benefit analysis before a dietary supplementation with flaxseed can be recommended.

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5 References

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