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 hormon-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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estly improve lipid profiles and thereby contribute to reduce cardiovascular disease risks [2]. Secoisolariciresinol digluco-
side 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 have 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 vitro to monitor whether flax-
seed 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 showered 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
fluorimmunoassay [17] using the enterolactone test (Lab-
master, Turku, Finland) with minor modifications, as de-
scribed earlier [7]. This assay measures total enterolactone
concentrations after enzymatic hydrolysis of enterolactone
gluconorides 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]dimethyl-
ammonio]-1-propanesulfonate (CHAPS), 0.8% Pharmalyte
(pH 3–10), and protease inhibitor cocktail) by homogeniza-
tion of the cells through ultrasonication (ten strokes, low
amplitude) on ice. Lysed cells were centrifuged for 30 min at
100 000 x 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

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 peri-
од, PBMC were washed twice with 0.35 M sucrose and
centrifuged for 10 min at 500 x 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. Sub-
sequent 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 rehy-
dration 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% (NH₄)₂SO₄, 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 ProteomeWeaver software

Gels stained with Coomassie were scanned using an ImageScanner (Amersham Biosciences) and spots detected by the ProteomeWeaver 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 NH₄HCO₃ and ACN/pure 50 mM NH₄HCO₃ 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 carbamoylomethylation 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, carbamomethylation 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](image_url)  

**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 fluorimunnoassay. 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

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

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 Coomassie-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>
<thead>
<tr>
<th>Spot no.</th>
<th>Protein description</th>
<th>Theoretical M/Mr</th>
<th>Measured M/Mr</th>
<th>Protein amount</th>
<th>Residues of identified peptides</th>
<th>% Sequence coverage</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intervention phase/prephase</td>
<td>Washout phase/prephase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Protein-γ-isoaspartate (γ-aspartate) O-methyltransferase (EC 2.1.1.77) splice form I</td>
<td>38/6.6</td>
<td>25/6.8</td>
<td>0.24</td>
<td>0.60</td>
<td>4–17, 27–36, 27–41, 81–97, 113–123, 124–134, 135–143, 178–196, 204–218</td>
<td>49</td>
</tr>
<tr>
<td>Spot no.</td>
<td>Protein description</td>
<td>Theoretical M_/p/</td>
<td>Measured M_/p/</td>
<td>Protein amount</td>
<td>Residues of identified peptides</td>
<td>% Sequence coverage</td>
<td>Accession no.</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Intervention phase/ prephase</td>
<td>Washout phase/ prephase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Chain B, crystal structure of deoxy-human hemoglobin (β8 Glu–Trp)</td>
<td>13/6.5</td>
<td>16/7.3</td>
<td>0.30</td>
<td>0.54</td>
<td>1–17, 9–17, 18–30, 31–40, 41–59, 66–82, 67–82, 83–95, 96–104</td>
<td>67</td>
</tr>
</tbody>
</table>

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.

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 isoelectric 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 IIa/IIb, peroxiredoxin 4 and the long-chain fatty acid β-oxidation multienzyme complex (Fig. 4).
<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Protein description</th>
<th>Theoretical $M_r/p$</th>
<th>Measured $M_r/p$</th>
<th>Protein amount 50 nM enterolactone/ control</th>
<th>Residues of indent peptides</th>
<th>% Sequence coverage</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chaperons</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Cytoskeletal proteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>γ-Actin</td>
<td>26/5.6</td>
<td>27/5.8</td>
<td>0.42</td>
<td>54–63, 71–95, 73–85, 96–111, 149–169, 217–229</td>
<td>38</td>
<td>AAA51580</td>
</tr>
<tr>
<td></td>
<td>glycoprotein Iib</td>
<td></td>
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<tr>
<td>8</td>
<td>Metabolism</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>enzyme complex α-chain precursor, mitechnodrial</td>
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<td></td>
<td>enzyme complex α-chain precursor, mitechnodrial</td>
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<td>12</td>
<td>Gene regulation</td>
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<td></td>
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</tr>
<tr>
<td>13</td>
<td>Other proteins</td>
<td></td>
<td></td>
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<td>14</td>
<td>Chain B, crystal structure of desoxy-human hemoglobin β6 Glu-&gt;Trp</td>
<td>16/7.3</td>
<td>15/7.4</td>
<td>0.45</td>
<td>18–30, 31–40, 41–59, 66–82, 83–95, 96–104, 105–120, 121–132, 133–144</td>
<td>82</td>
<td>6HBWB</td>
</tr>
</tbody>
</table>

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 pI (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 \textit{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 \textit{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 entero lactone 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 sulfation of enterolactone for increasing body clearance by excretion [26, 27]. Despite these differences in the responses in enterolactone 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 \textit{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 IkB 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/IIb, that was observed in PBMC after flaxseed intervention \textit{ex vivo} and after enterolactone exposure \textit{ex vivo}, can also be connected to inhibition of thrombosis. Due to its critical role in mediating platelet aggregation, glycoprotein IIIa/IIb has become a primary target in the development of antiplatelet 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/IIb is based on lowered levels in platelets. However, glycoprotein IIIa/IIb 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 \textit{vivo} and \textit{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 \textit{in vivo} but not after \textit{ex vivo} exposure of PBMC to enterolactone. This may suggest that those changes found \textit{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 status. 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-isoaspartate (0-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 enteralactone, we identified for example vasodilator-stimulated phosphoprotein (VASP), fibrinogen and myeloperoxidase. The increase in myeloperoxidase levels points also to some adverse effects of enteralactone 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 atheros [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 enteralactone 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 enteralactone 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 enteralactone.

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 enteralactone, suggesting that this enteralignane 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