Carbohydrate Polymers 84 (2011) 153–161

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Effect of oat and barley β-glucans on inhibition of cytokine-induced adhesion molecule expression in human aortic endothelial cells: Molecular structure–function relations

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ARTICLE INFO

Article history:
Received 8 September 2010
Received in revised form 5 November 2010
Accepted 9 November 2010
Available online 16 November 2010

Keywords:
Oat and barley β-glucans
Anti-inflammatory activity
Aortic endothelial cells
Cell adhesion molecules
Molecular structure

ABSTRACT

Endothelial cell adhesion molecules have been recognized as an early step in inflammation and atherogenesis. The inhibition of TNF-α-induced expression of vascular cell adhesion molecule (VCAM-1) and intracellular cell adhesion molecule (ICAM-1) in human aortic endothelial cells (HAEC), following pretreatment with mixed-linkage (1 → 3), (1 → 4)-β-D-glucans (β-glucans) isolated from oat and barley, and the possible molecular structure–function relations, have been explored. The apparent molecular weight (Mw) of β-glucans varied in the ranges of 0.71–2.42 × 10^5 (barley) and 0.36–2.55 × 10^5 (oat); a higher ratio of 3-O-β-cellobiosyl-D-glucose to 3-O-β-cellotriosyl-D-glucose oligomers in the polymeric chains was also found for barley β-glucans. Analysis of variance showed that polysaccharide concentration, Mw and fine structure of β-glucans affected the VCAM-1 expression ($P < 0.05$); the reduction of VCAM-1 expression in stimulated HAEC was also dose-dependent (concentration range of 3.75–200 μg/ml β-glucans) and significant at polysaccharide levels > 6.25 μg/ml ($P < 0.05$). The inhibition of VCAM-1 expression was greater for barley β-glucans compared to oat, displaying a maximum anti-inflammatory activity at Mw ~1.40 × 10^5. Instead, the expression of ICAM-1 was suppressed ($P < 0.05$) only at high polysaccharide concentrations (>100 μg/ml), with maximum activity at Mw ~2.50 × 10^5. Structural differences between the oat and barley β-glucans did to seem to influence the ICAM-1 expression.

1. Introduction

Atherosclerosis, the underlying condition of coronary artery disease (CAD), is one of the major health problems, among elderly individuals, in most Western societies (Meng, 2006). Endothelial dysfunction has been shown to be associated with anatomically overt CAD, while the inflammatory process, in terms of interaction of endothelium with the immune system, plays an important role in the pathogenesis of atherosclerosis (Feletou & Vanhoutte, 2006). The cytokine-induced expression of adhesion molecules is among the numerous signalling pathways which have been implicated in the immune response of endothelium (Han, Quon, & Koh, 2007). The adhesion molecules, namely vascular cell adhesion molecule (VCAM-1) and intracellular cell adhesion molecule (ICAM-1), activated by pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), seem to participate in the initiation of this interaction.

Cell walls of cereal grains, mostly barley and oat and in lesser extent rye, sorghum and wheat, are rich in mixed-linkage (1 → 3), (1 → 4)-β-D-glucans, which are linear homopolysaccharides of D-glucose residues interlinked via β-(1 → 3) and β-(1 → 4) linkages; their structure consists of consecutively (1 → 4)-linked β-D-glucose in blocks (i.e. oligomeric cellulose segments) that are separated by single (1 → 3)-linkages (Lazaridou & Biliaderis, 2007).

Oat and barley derived β-glucans have been implicated with cardiovascular protection in humans, exerting their effects mainly by lowering the levels of serum total cholesterol and low-density-lipoprotein (LDL)-cholesterol (Ames & Rhymer, 2008; Brown,

Abbreviations: CAD, coronary artery disease; VCAM-1, vascular cell adhesion molecule; ICAM-1, intracellular cell adhesion molecule; TNF-α, necrosis factor alpha; LDL-cholesterol, low-density-lipoprotein-cholesterol; GI, gastrointestinal; IL-1β, interleukin 1β; HAEC, human aortic endothelial cells; NAGREF, National Agricultural Research Foundation; OBC, oat bran concentrate; EBM, endothelial cell basal medium; hEGF, human epidermal growth factor; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid); HBS, Hepes Buffer Saline; DMEM, Dulbecco's minimal essential medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Mw, molecular weight; HPSEC, high performance size exclusion chromatography; RI, refractive index; HPAEC, high-performance anion-exchange chromatography; PAD, pulsed amperometric detector; EGM, endothelial growth medium; PBS, phosphate buffer saline; DP, degree of polymerization; BARS, brachial artery reactivity studies.

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doi:10.1016/j.carbpol.2010.11.019

Rosner, Willett, & Sacks, 1999). As result, the US Food and Drug Administration (FDA) approved health claims for the use of oat and barley β-glucan based foods for lowering the risk of heart disease suggesting a dosage of 3 g β-glucan per day, with a recommendation of 0.75 g of β-glucan per serving (Anonymous, 1997, 2005). Recently, a similar health claim has also been approved by European Food Safety Authority (EFSA), stating that oat and barley β-glucans can contribute to maintenance of normal blood cholesterol concentrations at a dosage of at least 3 g per day in one or more servings (EFSA, 2009). Furthermore, supplementation with either oatmeal and whole-grain oat or wheat cereal products has been shown to prevent the postprandial impairment of vascular reactivity in response to a high-fat meal diet (Katz, Nawaz, Boukhalil, Giannamore, et al., 2001; Katz, Nawaz, Boukhalil, Chan, et al., 2001). Another known mechanism of soluble dietary fibers, such as cereal β-glucans, by which they can prevent endothelial dysfunction and decrease coronary heart disease risk is through their ability to attenuate postprandial elevations in insulin and glucose levels (Dikeman & Fahey, 2006; Vogel, 1999). Indeed, consumption of cereal β-glucans has been linked to lower postprandial glucose and insulin responses (Cavallero, Empilli, Brighenti, & Stanca, 2002; Wood, 2002). It is important also to mention that the hypoglycemic and hypocholesterolemic physiological actions of cereal β-glucans were found to largely depend on concentration and molecular weight of the solubilized polysaccharide in the gastro-intestinal (GI) tract (Lazaridou & Biliaderis, 2007; Wood, 2002).

There is a growing body of both *in vitro* and *in vivo* studies indicating that (1 → 3), (1 → 4)-β-D-glucans can also modulate various aspects of mammalian immune response. It has been demonstrated that mixed-linkage (1 → 3), (1 → 4)-β-D-glucans can stimulate the immune system showing anti-inflammatory activity, as well as exert antimicrobial and antitumor function under *in vitro* conditions or after oral, intraperitoneal, intragastrical or parenteral administration (Cheung & Modak, 2002; Davis et al., 2004; Delaney, Carlson, Frazer, et al., 2003; Di Renzo, Yefenof, & Klein, 1991; Estrada et al., 1997; Estrada, Van Kessel, & Laarveld, 1999; Hong et al., 2004; Porter, Morel, & Coles, 2006; Yun et al., 1998). Interestingly, the *in vitro* secretion of interleukin 1β (IL-1β) by rat monocytes stimulated by barley β-glucan was dependent on the molecular weight and dose of the polysaccharide (Porter et al., 2006). Additionally, an *in vitro* study showed that fecal water from ileostomic patients consuming oat β-glucans enhances the immune response of cytokine-stimulated enteric epithelium (Ramakers et al., 2007). In this context, previous studies have shown that the source (yeast, fungi, bacteria) and the structural and physical features of microbial (branched) β-(1 → 3), (1 → 6)-glucans, i.e. primary structure (degree of branching, polymer charge, linkage pattern and ratio), molecular weight, solubility and chain ordering, can considerably affect the physiological functionality on the immune system responses (Bohn & BeMiller, 1995; Giavasis & Biliaderis, 2007; Kulicke, Lettau, & Thielking, 1997; Zhang, Li, Xu, & Zeng, 2005).

To our knowledge, however, the effects of cereal β-glucans on the early stage of inflammatory process in endothelium leading to atherogenesis, have not yet been examined. The objective of the present study was to explore the anti-inflammatory potential of cereal β-glucans on immune stimulated endothelium *in vitro* and examine possible relationships between molecular structure of the polysaccharides and physiological function. Therefore, in this work the ability of barley and oat β-glucans, differing in molecular structure (molecular weight and distribution of cellulosic oligomers in polymeric chain), to inhibit the expression of adhesion molecules (VCAM-1 and ICAM-1) in TNF-α-activated human aorta endothelial cells (HAEC) and their effect on cell viability have been examined.

2. Materials and methods

2.1. β-Glucan sources

Eight mixed-linkage (1 → 3), (1 → 4) β-D-glucan samples varying in their molecular and structural features were used. Samples oat 255, oat 144 and oat 106 were β-glucans extracted from whole oat flours of three registered Greek oat varieties, Pallini, Kassandra and Flega, respectively; these oat cultivars, belonging to two different oat species (Pallini and Flega to *Avena sativa* L, and Kassandra to *Avena byzantina*), were obtained from the National Agricultural Research Foundation (NAGREF), Cereal Institute, Thessaloniki, Greece. Sample oat 36 was isolated from an oat bran concentrate (OBC) that was β-glucanase treated and was provided by CEBA (Lund, Sweden); the β-glucan and protein contents of the initial concentrate were 25.4% (dry basis, d.b.) and 17.7% (d.b.), respectively. The preparation bar 242 was isolated from whole barley flour of a registered Greek cultivar of normal covered barley seeds, Thessaloniki, provided by NAGREF, whereas bar 142, bar 113 and bar 71 samples were products derived from bar 242 by mild acid-hydrolysis.

2.2. Chemicals and cell culture

The HAEC culture, as well as the endothelial cell basal medium (EBM) and Single Quots Bulletin kit, containing human epidermal growth factor (hEGF), hydrocortisone, gentamycin, amphotericin B, bovine brain extract and fetal bovine serum (FBS), were purchased from Clonetics® (Cambrex Corporation, Athens Greece); all other cell culture materials, such as 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES) buffered saline solution (HBS), trypsin/EDTA solution and Dulbecco's minimal essential medium (DMEM), were obtained from Invitrogen Life Technologies (Thessaloniki, Greece). TNF-α (T0157), (±)-α-tocopherol (vitamin E, T3251), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT, M5655) and the peroxidase substrate *o*-phenylendiamine hydrochloride (FAST™OPD, P9187) were obtained from Sigma–Aldrich (Athens, Greece). VCAM-1 (BBA4) and ICAM-1 antibodies (BBA3) were obtained from R&D System (Thessaloniki, Greece) and sheep anti-mouse IgG secondary antibody (NA931) was purchased from Amersham (Athens, Greece). All other chemicals were of analytical grade and purchased from Sigma–Aldrich, Fluka and BioRad (Athens, Greece).

2.3. Isolation of cereal β-glucans

The isolation-purification protocols and the acid hydrolysis procedures followed for the production of the oat and barley β-glucan isolates are described in detail in Fig. 1. Oat and barley grains from the Greek cultivars were ground in a Camas mill to pass a 0.8 mm screen. The reflux of oat and barley flours and of OBC with 82% (v/v) aqueous ethanol at 85 °C removed most of the lipids and aimed at the inactivation of endogenous β-glucanases. The aqueous extraction of β-glucans at a temperature (52 °C) below the gelatinization temperature of starch resulted in very little starch solubilization. The purification procedure involved a dual-enzyme digestion with a heat stable α-amylase (Termamyl 120L, Novozymes A/S, Bagsvaerd, Denmark) and porcine pancreatin (Sigma–Aldrich Chemie Gmbh, Steinheim, Germany), followed by removal of starch and protein hydrolyzates by exhaustive dialysis. After the precipitation of the polysaccharide with two volumes of ethanol, the material was suspended in 2-propanol, filtered and dried to obtain soluble oat (oat 255, oat 144, oat 106 and oat 36) and barley (bar 242) β-glucan isolates. Samples of barley β-glucans

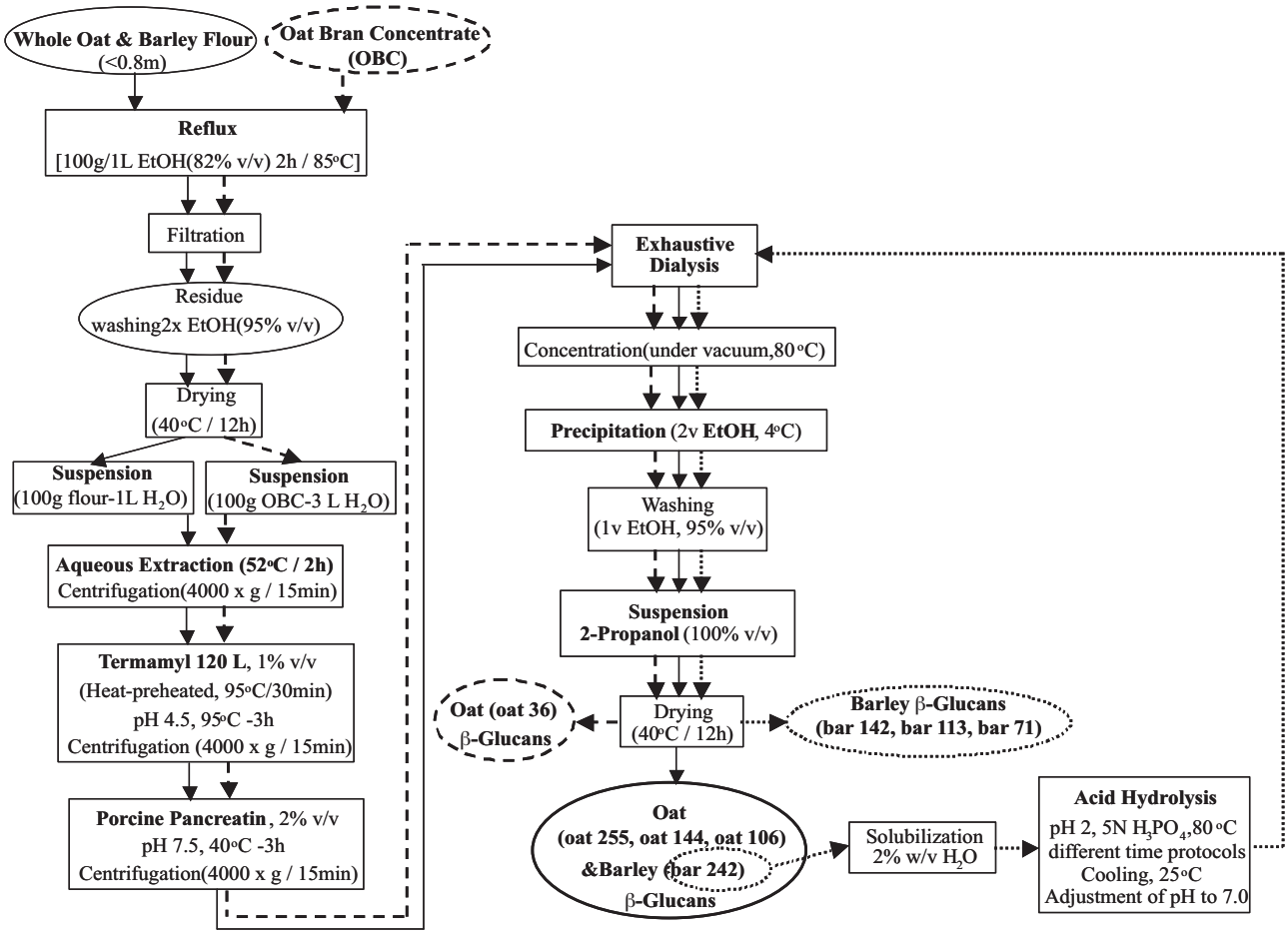

Fig. 1. Extraction – purification scheme of oat and barley β-glucans from whole oat and barley flours and an oat bran concentrate; the arrows indicate the steps followed for preparation of oat 255, oat 144, oat 106, bar 242 (solid arrows), oat 36 (dashed arrows) and bar 142, bar 113, bar 71 (dotted arrows).

with different molecular size, bar 142, bar 113 and bar 71, were also obtained following a stepwise acid hydrolysis of 2% (w/v) barley β-glucan solutions obtained from the bar 242 isolate with H_3PO_4 (pH 2.0) at 80 °C for 20, 30 and 40 min, respectively. After neutralization, a dialysis step was necessary for the removal of salt and low molecular weight carbohydrates released during acid hydrolysis.

2.4. Compositional and molecular characterization of cereal β-glucans

The protein content of oat and barley β-glucan preparations was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin (Merck KGaA, Damstadt, Germany) solutions as standards. The β-glucan content was determined by the method of McCleary and Glennie-Holmes (1985) using the mixed linkage β-glucan assay kit, purchased from Megazyme International Ltd. (Bray, Ireland).

The apparent peak molecular weight (Mw) of the β-glucan samples was obtained with a high performance size exclusion chromatography (HPSEC) system, which consisted of a single pump (Marathon IV, Rigas Labs, Thessaloniki, Greece), a guard TSK PWH column, a 7.8 mm × 600 mm TSK G5000 PW – SEC column, and a refractive index detector (RI, ERC-7515A, ERC-Inc. Nishiaoki, Kawaguchi-City, Japan). Calculation of the Mw for the eluted polysaccharides was based on calibration with β-glucan standards (Mw of 0.40×10^5, 1.23×10^5, 1.83×10^5, 2.45×10^5, as characterized by a light scattering technique) obtained from Megazyme International Ltd. (Bray, Ireland); eluting volumes of peak fractions for both standards and unknown samples were used in this calculation. Description of the sample preparation and running conditions of this HPLC method as well as of the determination of limiting viscosities [η] and second critical concentration (c^{**}) of the β-glucan samples using Ubbelodhe capillary viscometers are described in detail by Lazaridou, Biliaderis, and Izydorczyk (2003).

The distribution of cellulosic oligomers in the chain of β-glucans was determined by treatment with lichenase [(1 → 3), (1 → 4)-β-glucan-4-glucanohydrolase, EC 3.2.1.73] and chromatography. β-Glucan samples were dissolved in phosphate buffer (2 mg/ml, 0.01 M, pH 6.5) and incubated with lichenase (4 U/ml) from Megazyme for 20 h at 40 °C. Oligosaccharides released from barley and oat β-glucan preparations by lichenase action were analyzed by high performance anion exchange chromatography (HPAEC). A Dionex system, equipped with a Waters pump, a 715 WISP sample injector (Waters Associates, Milford MA), a Danaus CarboPac PA1 column (4 mm × 250 mm) with a PA1 guard column (4 mm × 50 mm), and a pulsed amperometric detector (PAD-2; Danaus Canada Ltd., Etobicoke, ON), was employed for chromatography; the operating conditions of Wood, Erfle, Teather, Eisz, and Miller (1994) were adopted.

2.5. Culture of endothelial cells

HAEC were cultured at 37 °C in a humidified 95% air–5% CO_2 atmosphere in EBM supplemented with hEGF (10 ng/ml), hydrocortisone (1 mg/ml), gentamycin (50 mg/ml), amphotericin B (50 ng/ml), bovine brain extract (12 μg/ml) and fetal bovine serum (2%) (called thereafter endothelial growth medium or EGM), as previously described (Papoutsi et al., 2008). At 70–80% con-

fluence, cells were washed twice with HBS solution pH 7.2–7.5, harvested with 0.025% trypsin–0.01% EDTA and plated at a density 2500 cell/cm^2. All experiments used HAEC of passage four to eight. The cells were grown to confluence in 96-well plated using EGM. Cells were washed once with HBS pH 7.2–7.5 and then fresh EGM, containing β-glucans at final concentrations 3.75–200 μg/ml, was added and an 18 h incubation period followed. Subsequently, TNF-α (1 ng/ml) was added and the cells were incubated for an additional 6 h period. Cells treated with TNF-α (1 ng/ml) alone and cells treated with TNF-α (1 ng/ml) plus α-tocopherol (20 μM) were also included. Cells (HAEC) were incubated in EGM alone (without TNF-α or compounds) and used as blank. Subsequently, cells were used for measurement of protein levels of the cell adhesion molecules (VCAM-1 and ICAM-1) by cell ELISA and for assessment of cell viability by MTT assay.

The preparation of α-tocopherol solution was as follows: a stock solution of α-tocopherol at 10 mg/ml was prepared in ethanol and stored at −70 °C. To prepare the α-tocopherol solution for cell culture, a stock solution was first mixed with FBS at a ratio of 1:20, then incubated at 37 °C for 15 min during which time brief vortexing was conducted every 5 min. The FBS-α-tocopherol solution was then diluted by EGM medium to make the final concentration of α-tocopherol for supplementing HAEC (Wu, Koga, Martin, & Meydani, 1999).

2.6. Measurement of cell adhesion molecules by cell ELISA

Surface expression of VCAM-1 and ICAM-1 was quantified by cell ELISA performed on HAEC monolayer in flat-bottom 96-well plates as described previously (Zhang & Frei, 2002; Zhang, Stocker, McCall, Forte, & Frei, 2002). Briefly, following incubation, the cells were fixed with 0.1% glutaraldehyde in phosphate buffer saline (PBS) for 30 min at 4 °C. Plates were blocked at 37 °C for 1 h with 5% skimmed milk powder in PBS and incubation at 4 °C overnight with a primary monoclonal mouse antibody anti-human ICAM-1 or VCAM-1, at final concentration of 2 μg/ml in 5% skimmed milk PBS, followed. The plates were then washed three times with 0.1% Tween-20 in PBS and incubated with a horseradish peroxidase-conjugated sheep anti-mouse secondary antibody at a dilution 1:5000 at room temperature for 1.5 h. Subsequently, the plates were washed three times with 0.1% Tween-20 in PBS and finally the expression of cell adhesion molecules was quantified by the addition of the peroxidase substrate *o*-phenylendiamine hydrochloride. The absorption of each well was measured at 492 nm using a microplate spectrophotometer.

Data reported are means of three independent experiments (each experiment was also conducted in triplicate). The data are expressed as percentage of TNF-α response, which was calculated as follows [(value for cells pre-treated with compounds) − (value for blank cells)/(value for cells treated with TNF-α) − (value for blank cells)] × 100.

2.7. Cell viability

Cell viability was assessed by morphological observations under a phase contrast microscope and by reduction of tetrazolium salt MTT by mitochondrial dehydrogenases as described elsewhere (Denizot & Lang, 1986; Papoutsi et al., 2008). Briefly, after incubation of cells under the experimental conditions employed, the HAEC culture was washed twice with PBS and the medium was replaced with MTT dissolved at a final concentration of 1 mg/ml in DMEM serum-free, phenol red free, and a further 4 h incubation followed. Then, the MTT-formazan was solubilized in isopropanol and the optical density was measured at a wavelength of 550 nm and a reference wavelength of 690 nm.

2.8. Statistical analysis

The experiment was conducted to evaluate the influence of source, molecular weight and concentration of β-glucans on VCAM-1 and ICAM-1 expression in TNF-α-activated HAEC. The differences in β-glucan source (oat and barley) reflect differences in the primary structure of polysaccharide chain between oat and barley β-glucans. A three factor (structure × Mw × concentration) factorial experiment was employed for this study in a completely random design. Each of the combined treatments of either VCAM-1 or ICAM-1 expression (2 structures × 4 Mw's × 7 concentrations) had three replicates. The data were analyzed by a three-way ANOVA according to a linear model and differences between means were compared using the least square difference (LSD) test at $\alpha = 0.05$ significance level. All statistical analyses were performed by SPSS statistical software (version 16.0 SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Molecular characteristics of barley and oat β-glucans

The compositional, molecular and structural features of barley and oat β-glucan samples used are given in Table 1. The isolation/purification protocol employed (Fig. 1) seemed to provide β-glucan isolates of high purity, with β-glucan content greater than 89% (d.b.) (Table 1). Apparently, β-glucans from cereals, such as barley and oat, have a similar generalized chemical structure. They are linear homopolysaccharides of D-glucopyranosyl residues (Glc*p*) linked via β-(1 → 3) and β-(1 → 4) linkages, with blocks of consecutive (1 → 4)-linked residues (i.e. oligomeric cellulose segments) separated by discontinuous (1 → 3)-linkages (Fig. 2). Although most of the cellulose segments are trimers and tetramers, longer cellodextrin segments are also present in the polymeric chains. Lichenase, specifically cleaves the (1 → 4)-glycosidic bond of the 3-substituted glucose residues in β-glucans yielding oligomers with different degrees of polymerization (DP); as a result, the major products for cereal β-glucan lichenase digests are 3-O-β-cellobiosyl-D-glucose (DP3) and 3-O-β-cellotriosyl-D-glucose (DP4), as well as cellodextrin-like oligomers (DP ≥ 5) which are produced from the polymer regions containing more than three consecutive C4-linked glucose residues.

Among the β-glucan isolates there was a diversity in molecular size and structural features. Apparent Mw values, estimated from the peak fraction of the HPLC polysaccharide elution profiles (Fig. 3), varied in the ranges of 0.71–2.42 × 10^5 and 0.36–2.55 × 10^5 for barley and oat β-glucans, respectively (Table 1). Also, as it was expected, with increasing molecular size the limiting viscosity ([η]) values increased from 0.8 to 3.7 dl/g, whereas the critical concentration (c^{**}) values decreased from 2.2 to 0.8 g/dl. Moreover, the estimated [η] values were similar for oat and barley β-glucan samples having equivalent molecular size. Differences in the molecular weight of cereal β-glucans may reflect variations due to genetic and environmental (growth) factors as well as due to different methods followed for extraction – isolation and Mw determination of these polysaccharides (Lazaridou & Biliaderis, 2007). For instance, the apparent peak Mw of water-extractable β-glucans derived from 18 varieties of normal covered barley seeds and four oat varieties, grown in the same year and location in Greece, varied between 1.26–2.39 × 10^5 and 0.44–1.10 × 10^5, respectively (Papageorgiou, Lakhdara, Lazaridou, Biliaderis, & Izydorczyk, 2005).

Furthermore, between the oat and barley β-glucan isolates differences were evident in the distribution pattern of cellulosic oligomers of their polymeric chains. From the HPAEC chromatograms the weight percent values of the oligosaccharides in the lichenase digests are summarized in Table 1. The weight per-

Table 1
Compositional, molecular and structural features of oat and barley β-glucan isolates.

Samples	β-Glucans (% d.b.)[a]	Protein (% d.b.)[a]	Mw[b]	[η] (dl/g) (20 °C)	c** (g/dl)	DP3[c]	DP4[c]	DP(5–14)[c]	Molar ratio DP3/DP4
Barley β-glucans									
bar 242	94.8	1.3	2.42×10^5	3.1	0.9	62.0	29.2	8.8	2.8
bar 142	91.4	0.8	1.42×10^5	2.1	1.1	62.1	29.0	8.9	2.8
bar 113	89.1	3.2	1.13×10^5	1.8	1.2	61.9	29.3	8.8	2.8
bar 71	90.5	1.6	0.71×10^5	1.2	1.8	62.3	29.2	8.5	2.8
Oat β-glucans									
oat 255	97.2	0.5	2.55×10^5	3.7	0.8	54.2	36.6	9.2	2.0
oat 144	94.4	3.1	1.44×10^5	2.4	1.1	55.3	35.5	9.1	2.1
oat 106	93.5	3.3	1.06×10^5	1.9	1.2	56.2	35.3	8.5	2.1
oat 36	93.0	3.6	0.36×10^5	0.8	2.2	60.6	35.5	3.9	2.3

[a] Percentage on dry basis.
[b] From the peak fraction of main eluting peak of the HPLC chromatograms.
[c] Weight percent from the chromatograms of the lichenase digests.

cent of trimers was higher for barley β-glucan samples, in the range 61.9–62.3%, compared to the values of oat β-glucans, which ranged between 54.2% and 60.6%, whereas the reverse order was observed for the weight percent of tetramers, being lower for β-glucans derived from barley (29.0–29.3%) than those from oat (35.3–36.6%). As a result, the molar ratio of DP3/DP4 was higher for barley β-glucans (2.8) compared to β-glucans isolated from oat (2.0–2.3). These values are in close agreement with available literature data (Lazaridou & Biliaderis, 2007); apparently, the ratio of DP3/DP4 has been widely used as a structural fingerprint of cereal β-glucans, with oat β-glucans exhibiting the lower values of this ratio (1.5–2.3), compared to barley (1.8–3.5). For all β-glucan isolates, the total weight percent of long cellulose-like oligomers with DP from 5 to 14 (DP5–14) varied between 3.9% and 9.2%, without observing differences between oat and barley β-glucans (Table 1).

3.2. Inhibitory effect of barley and oat β-glucans on TNF-α-induced VCAM-1 and ICAM-1 expression in HAEC

The binding and recruitment of circulating monocytes to vascular endothelial cells are early steps in the development of inflammation and atherosclerosis, mediated through cell adhesion molecules that are expressed on the surface of endothelial cells. The importance of adhesion molecules in atherogenesis was evidenced by their presence in atherosclerotic plaque (Cybulsky et al., 2001) and the association of their increased plasma levels in apparently healthy individuals with risks of future coronary events (Ridker, Hennekens, Roitman-Johnson, Stampfer, & Allen, 1998). In this context, the potential of β-glucans from oat and barley to influence the expression of VCAM-1 and ICAM-1 in cytokine-activated HAEC, and therefore to control the initiating steps of atherosclerosis, was explored. Assessment of cell viability revealed that neither the morphology nor the reduction of MTT salt in HAEC cells was affected by any of the compounds (β-glucans, α-tocopherol or TNF-α) incorporated at any of the concentration level or experimental conditions used (data not shown).

Table 2
Analysis of variance, *F*-values and significance of model effects, for the VCAM-1 and ICAM-1 expression in TNF-α-activated HAEC, following pretreatment with oat and barley β-glucans of different Mw's and concentrations.

Factors	VCAM-1 expression[a]		ICAM-1 expression[a]	
	F-value	*P*-value	*F*-value	*P*-value
Structure (Str)	128.901	<0.001	0.134	0.715
Molecular weight (Mw)	29.322	<0.001	4.121	0.008
Concentration (Con)	71.688	<0.001	59.093	<0.001
Str × Mw	29.623	<0.001	5.430	0.002
Str × Con	5.558	<0.001	5.901	<0.001
Mw × Con	3.117	<0.001	2.011	0.014
Str × Mw × Con	2.038	0.013	1.723	0.045

[a] Estimated as percentage of α-TNF response.

The TNF-α induced expression of VCAM-1 (Fig. 4) and ICAM-1 (Fig. 5) in β-glucan pre-treated endothelial cells, which was assayed by ELISA, indicated that cereal β-glucans can selectively inhibit the cytokine-stimulated HAEC. As a positive control, vitamin E (α-tocopherol) was used, an antioxidant known for its inhibitory effect on cytokine-stimulated expression of adhesion molecules in HAEC (Wu et al., 1999).

The effects of β-glucan structure, Mw and concentration as well as their interactions on TNF-α-induced VCAM-1 protein expression in HAEC were significant as showed by analysis of variance (Table 2). Between samples from the two β-glucan sources used, barley β-glucans displayed a stronger inhibitory effect on TNF-α-induced VCAM-1 expression compared to preparations obtained from oat; i.e. lower mean values of VCAM-1 expression (~57% of TNF α-response) for barley β-glucan pre-treated HAEC vs. oat (~80%) (Table 3). Among the β-glucan isolates varying in molecular weight, the samples with apparent peak Mw about 1.40×10^5 showed the maximum inhibition of VCAM-1 expression. Gener-

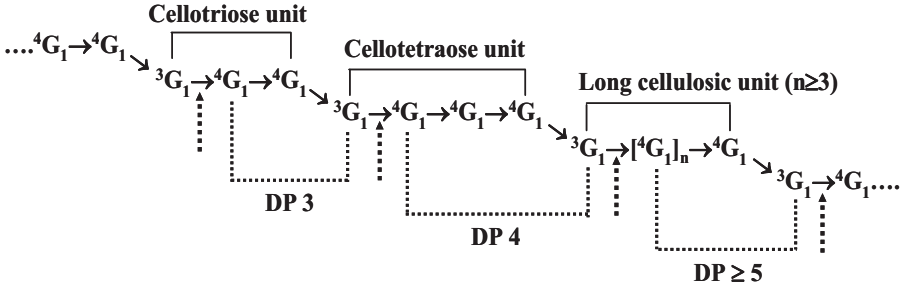

Fig. 2. Generalized structure of linear mixed linkage (1 → 3, 1 → 4) cereal β-D-glucans; dotted arrows indicate hydrolysis sites on the polysaccharide chain by lichenase. G: β-D-glucopyranosyl unit; DP3: 3-O-β-cellobiosyl-D-glucose; DP4: 3-O-β-cellotriosyl-D-glucose; DP ≥ 5: cellodextrin-like oligosaccharides containing more than three consecutive 4-O-linked glucose residues.

Table 3
Effect of structure, Mw and concentration of β-glucans on VCAM-1 and ICAM-1 expression in TNF-α-activated HAEC, following pretreatment with cereal β-glucans.

Factors	Levels	VCAM-1 expression[a]		ICAM-1 expression[a]	
Structure	1 (barley β-glucans)	$n = 84$	57.40[b]	$n = 84$	92.56
	2 (oat β-glucans)		80.25		92.00
		$LDS_{0.05} = 3.99$		$LDS_{0.05} = 3.04$	
Mw	1 ($2.42–2.55 \times 10^5$)	$n = 42$	70.42	$n = 42$	88.11
	2 ($1.42–1.44 \times 10^5$)		53.02		92.61
	3 ($1.06–1.13 \times 10^5$)		74.65		92.73
	4 ($0.36–0.71 \times 10^5$)		77.21		95.67
		$LDS_{0.05} = 5.64$		$LDS_{0.05} = 4.30$	
Concentration	1 (200 μg/ml)	$n = 24$	30.91	$n = 24$	57.99
	2 (100 μg/ml)		51.71		90.05
	3 (50 μg/ml)		59.67		96.40
	4 (25 μg/ml)		77.03		99.11
	5 (12.5 μg/ml)		79.79		101.89
	6 (6.25 μg/ml)		87.13		100.19
	7 (3.75 μg/ml)		95.54		100.33
		$LDS_{0.05} = 7.46$		$LDS_{0.05} = 5.69$	

[a] Estimated as percentage of α-TNF response.
[b] Values are the means of n determinations.

ally, the reduction of VCAM-1 expression was dose-dependent in the tested concentration range of 3.75–200 μg/ml β-glucans with significant responses above 6.25 μg/ml, as evidenced by analysis of variance (Table 3). The inhibitory effect of cereal β-glucans on TNF-α-induced VCAM-1 expression was significantly affected by the

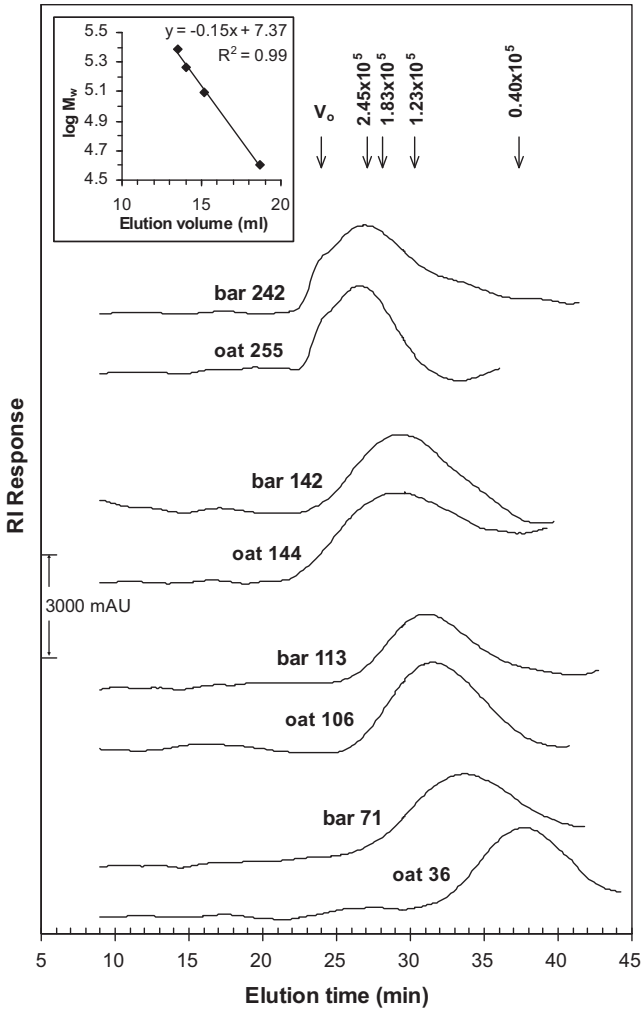

Fig. 3. HPSEC elution profiles of barley and oat β-glucan samples detected by RI; the arrows indicate the elution time of the peak fraction of four (1 → 3, 1 → 4) β-glucan standards used for plotting of the standard curve (inset).

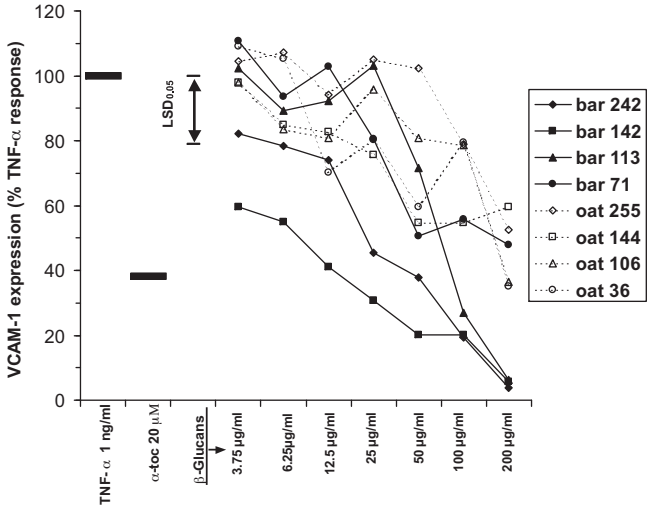

Fig. 4. Effect of barley and oat β-glucans on TNF-α induced VCAM-1 protein expression in HAEC. HAEC were pre-treated with α-tocopherol (α-toc) at 20 μM, or with different concentrations of β-glucans (200, 100, 50, 25, 12.5, 6.25 and 3.75 μg/ml) for 18 h, followed by stimulation with TNF-α (1 ng/ml) for additional 6 h. Adhesion molecules were measured by cell ELISA; HAEC pre-treated with α-toc used as positive control. Data are expressed as percentage of the α-TNF response (TNF-α-stimulated HAEC without any pretreatment with compounds) and shown as means of three independent experiments (each conducted in triplicate); differences between means were compared by LSD test at $\alpha = 0.05$.

interactions among the three factors ($P < 0.05$) (Table 2 and Fig. 4). In particular, pre-treatment of activated HAEC with barley β-glucans resulted in a greater reduction of the VCAM-1 expression and over a wider polysaccharide concentration range compared to oat β-glucans (Fig. 4); these effects were also depended on the Mw of barley β-glucans. Thus, bar 142 showed maximum inhibitory effect at a concentration range of 3.75–200 μg/ml, followed by bar 242 at 6.25–200 μg/ml, while the two relatively low Mw samples, bar 113 and bar 71, displayed significant inhibitory activity at concentration above 50 μg/ml. In some cases, the inhibitory effect of β-glucans on VCAM-1 expression of activated endothelium was comparable to that provoked by pretreatment with α-tocopherol at 20 μM.

In contrast with the cytokine-induced VCAM-1 expression in HAEC, the inhibition of ICAM-1 expression was significant only at high polysaccharide levels (>100 μg/ml) (Table 3) and was influenced by Mw and concentration of the polysaccharide and not by the structural features/differences between barley and oat β-glucans (Table 2). Moreover, the inhibitory effects of β-glucans

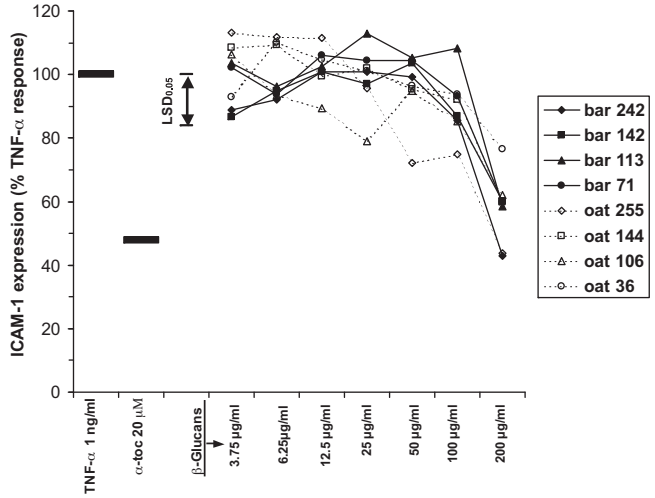

Fig. 5. Effect of barley and oat β-glucans on TNF-α induced ICAM-1 protein expression in HAEC. HAEC were pre-treated with α-tocopherol (α-toc) at 20 μM, or with different concentrations of β-glucans (200, 100, 50, 25, 12.5, 6.25 and 3.75 μg/ml) for 18 h, followed by stimulation with TNF-α (1 ng/ml) for additional 6 h. Adhesion molecules were measured by cell ELISA; HAEC pre-treated with α-toc used as positive control. Data are expressed as percentage of the α-TNF response (TNF-α-stimulated HAEC without any pretreatment with compounds) and shown as means of three independent experiments (each conducted in triplicate); differences between means were compared by LSD test at $\alpha = 0.05$.

on expression of ICAM-1 molecules were less pronounced that those of VCAM-1. Among the β-glucans differing in molecular weight, isolates with an apparent Mw $\sim 2.50 \times 10^5$ showed the maximum inhibition of ICAM-1 expression (Table 3). Differences in ICAM-1 endothelial expression were mostly attributed to β-glucan concentration ($P < 0.001$) and partly to the molecular size of the isolate ($P = 0.008$) (Table 2). Additionally, the inhibitory effect of cereal β-glucans on TNF-α-induced ICAM-1 expression was significantly affected by the interactions among the three tested factors ($P < 0.05$). The majority of β-glucan isolates showed a significant reduction of the TNF-α-induced ICAM-1 expression only at the highest polysaccharide concentration (200 μg/ml) (Fig. 5); the inhibitory effect for the two high Mw isolates (oat 255 and bar 242) seemed to be comparable to that found for the positive control of α-tocopherol.

In agreement with the present findings, a recent study showed that the oral administration of a crude polysaccharide preparation from *Lentinus edodes* mushroom can significantly inhibit the increased expression levels of VCAM-1 mRNA in thoracic aorta endothelial cells of rats fed with a high-fat diet (Xu, HaiYan, JianHong, & Jing, 2008). Similarly, a marked decrease in ICAM-1 expression of lung tissue has been found for lung-injured rats after their treatment with a β-(1 → 3)-D-glucan (Bedirli et al., 2007). In contrast, incubation of various enterocytes from small intestine and colon with fecal water of ileostomic patients consuming 5 g of oat β-glucans increased ICAM-1 expression in T84 and Caco-2 cells, compared to placebo fecal water (Ramakers et al., 2007); however, no significant changes in the inflammatory expression profile were found for another two cell lines (HT29 and INT407). Although it has been suggested that β-glucans can modulate immune activity *in vivo*, very few studies have evaluated the inflammatory responses following oral intake. Apparently, Delaney, Carlson, Frazer, et al. (2003), Delaney, Carlson, Zheng, et al. (2003) showed that a 28-day oral consumption of a barley β-glucan concentrate did not cause treatment-related inflammatory or other hematological or toxicological adverse effects in mice or rats when blended into feed at concentrations of 0.7, 3.5, and 7.0% β-glucan. Moreover, a human study showed that consumption of 4.8 g oat β-glucan for 4 weeks had no impact on inflammatory markers in hypercholesterolemic subjects (Theuwissen, Plat, & Mensink, 2009).

Recently, there is some evidence that small amounts of orally administrated water-soluble branched β-(1 → 3), (1 → 6)-glucans are absorbed directly into the lymphatic and vascular systems, where they can interact with circulating immune cells (Rice et al., 2005; Sandvick et al., 2007). Indeed, β-glucans have been detected in plasma of patients following fungal infection as well as of healthy individuals (Digby et al., 2003; Gonzalez et al., 2004); such findings support the notion for the transport of β-glucans from the GI tract into the bloodstream. In animal studies with rats, following oral administration, β-(1 → 3), (1 → 6)-glucans were shown to be bound and internalized by intestinal epithelial cells and gut-associated lymphoid tissue and thus, these polysaccharides can be translocated from the GI tract into the systemic circulation (Rice et al., 2005). Pharmacokinetic data allowed the estimation of β-glucan absorption within 24 h after ingestion and showed that their bioavailability was in the range of 4.0–4.9%. Interestingly, it was demonstrated that among these polysaccharides, the β-glucan with the highest Mw ($10.2 \times 10^5 - [\eta]$: 1.08 dl/g) studied, was absorbed from the intestinal lumen to the circulation system more rapidly than that a lower Mw ($0.077 \times 10^5 - [\eta]$: 0.07 dl/g) sample, reflecting a preferential absorption early in the intestinal transit, and implicating that differences in uptake from the intestinal lumen may play a key role in their final biological activity and exerted systemic effects.

The potency of cereal β-glucans to inhibit the TNF-α induced endothelial activation and expression of VCAM-1 and ICAM-1 adhesion molecules, as illustrated in Figs. 4 and 5, suggests that these polysaccharides might exert anti-inflammatory and anti-atherogenic activity in activated epithelium. β-Glucans exhibited stronger and over a wider concentration range inhibitory effects on VCAM-1 expression compared to ICAM-1 adhesion molecules. Animal studies have shown that although expression of both VCAM-1 and ICAM-1 is upregulated in antherosclerotic lesions, VCAM-1 is playing a dominant role in the initiation of atherosclerosis, while ICAM-1 seems to be involved in lesion progression (Cybulsky et al., 2001).

Generally, the inhibition of the TNF-α-induced VCAM-1 expression was more pronounced for barley β-glucans than those from oat. This response could be related to the fine structural differences between barley and oat β-glucans; barley β-glucans chains exhibit higher levels of 3-O-β-cellobiosyl-D-glucose segments (i.e. a higher proportion of β-(1 → 3) glycosyl linkages) than those from oat. However, the precise mechanism by which cereal β-glucans inhibit the adhesion molecule expression in endothelial cells, stimulated in response to cytokine signals, remains unknown; this mechanism could provide further insights about the structure–function relations shown in the present study. Nevertheless, the present findings provide some evidence for a possible relationship between physiological activity and the primary molecular structure of the mixed linkage (1 → 3), (1 → 4) cereal β-glucans.

Another molecular feature, related to the inhibitory action of β-glucans for both VCAM-1 and ICAM-1 expression in stimulated endothelial cells, was polysaccharide molecular size. Among the tested preparations, β-glucans from barley with Mw about 1.40×10^5 exhibited the strongest inhibitory effect on VCAM-1 expression. In general, the maximum inhibition of VCAM-1 and ICAM-1 expression in activated HAEC for β-glucans was shown for samples with Mw around 1.40×10^5 and 2.50×10^5, respectively. This observation seems to be in agreement with previous reports on microbial β-glucans which showed maximum biological activity at certain Mw of the polysaccharides (Kulicke et al., 1997; Zhang et al., 2005). In another study, the *in vitro* secretion of IL-1β from rat monocytes stimulated by two barley β-glucan

preparations differing in Mw was investigated (Porter et al., 2006); the amount of IL-1β released by the high Mw β-glucan was higher than that derived by the action of the low Mw preparation. However, the effect of molecular weight on the biological functionality of microbial β-glucans is surrounded by some controversy, despite of numerous studies in the literature on this issue (Bohn & BeMiller, 1995; Giavasis & Biliaderis, 2007); the latter could be attributed to the fact that Mw largely affects both chain conformation and solubility of the polysaccharide. It is worth noting here that the concentration range exhibiting the anti-inflammatory activity of both barley and oat β-glucans in stimulated HAEC was much lower than the c^{**} (i.e. critical concentration marking the transition from a semi-diluted to a concentrated solution of these biopolymers in solution, Table 1), implying a disordered state of the polysaccharide chains at these concentration levels for all β-glucan samples examined.

The cardiovascular protective effect of fibers has been generally linked to their hypocholesterolemic activity, with the soluble cereal β-glucans being considered as quite effective bioactives for cholesterol reduction (Ames & Rhymer, 2008; Brown et al., 1999). However, Delaney, Nicolosi, Wilson, et al. (2003) reported not only a significant decrease of plasma total cholesterol and LDL-cholesterol in hypercholesterolemic hamsters fed with 8% oat and barley β-glucans for 9 weeks, but also a significant lowering in the total cholesterol and cholesterol ester concentrations of the aorta. These researchers also indicated that only 50% of the reduction in aortic total cholesterol and cholesterol esters could be explained by the effects β-glucans have on plasma LDL-cholesterol reduction, suggesting the existence of additional antiatherogenic mechanisms of action of cereal β-glucans occurring outside the gut; these mechanisms may include anti-inflammatory activity of the polysaccharide in the blood vessel wall within the aorta. In a similar line, previous studies have revealed that endothelial dysfunction induced by acute fat ingestion in healthy adults, as measured by brachial artery reactivity studies (BARS), may be prevented by a month-long, daily supplementation with 5 g of β-glucans from oat grains (Katz, Nawaz, Boukhalil, Chan, et al., 2001).

4. Conclusions

There have been a limited number of studies that report on a possible association of beneficial effects of cereal β-glucans directly to the endothelium function. In the present work, it was demonstrated, that both barley and oat derived β-glucans have the ability to inhibit cytokine-induced protein expression of adhesion molecules in endothelial cells in a concentration- and molecular structure-dependent manner. The experimental findings further suggest that soluble cereal β-glucans could act beneficially in inhibiting the early stages of atherosclerosis. Significant molecular structure–function relationships have been identified, with the barley β-glucans exhibiting a higher physiological potency than oat β-glucans. Moreover, maximum inhibitory effect was displayed by β-glucans at a certain molecular weight range. However, further studies are required to explore the *in vivo* efficacy of cereal β-glucans as a potential anti-atherogenic agent and unravel additional biological activities of these polysaccharides in relation with their molecular features and the dose of consumption.

Acknowledgments

This work has been carried out with the financial support from the Greek Ministry of Development, General Secretariat for Research & Technology, International Cooperation in Industrial Research and Development Program DSEBPRO-2005, # 05 DSEBPRO-100, 2006–2008. The authors wish to express their gratitude to Dr. Marta Izydorczyk, Grain Research Laboratory, Agriculture Canada, Winnipeg, Manitoba, Canada, for conducting the lichenase – HPAEC experiments.

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