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Peptides and hydrolysates from casein and soy protein modulate the release of vasoactive substances from human aortic endothelial cells

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Abstract

Food proteins were shown to affect atherogenic risk factors, which is supposed to be related to specific peptide sequences encrypted within their primary sequence. The aim of this study was to evaluate the effects of peptides and hydrolysates from two food proteins, casein and soy protein, on endothelial cell functions (cell proliferation and release of vasoactive substances). Cell proliferation was not influenced by dipeptides and most of the tripeptides, whereas several total hydrolysates from casein and soy protein inhibited cell proliferation at higher concentrations (>0.25 mg/mL; $P<0.05$). The release of one or more of the vasoactive substances, thromboxan B_2 (stable marker of thromboxan A_2), 6-keto-prostaglandin $F_{1\alpha}$ (stable marker of prostaglandin I_2), endothelin-1, and nitric oxide, was significantly influenced by the incubation with various peptides compared with control cells ($P<0.05$). Various hydrolysate fractions from casein and soy protein influenced the release of 6-keto-prostaglandin $F_{1\alpha}$ and nitric oxide ($P<0.05$) but did not influence the release of thromboxan B_2 and endothelin-1. In conclusion, the present study demonstrates that peptides and hydrolysate fractions from casein and soy protein influence endothelial cell function as evidenced by the modulation of endothelial cell proliferation and alterations in the release of vasoactive substances.

Keywords: Peptide; Hydrolysate; Endothelial cell; Casein; Soy protein

1. Introduction

It is well established that apart from their basic nutritional function, food proteins are capable of modulating specific biological functions. These effects are supposed to be mediated by specific peptide sequences encrypted within the primary sequence of many food proteins. For example, the soybean 7S globulin β-conglycinin was demonstrated to be biologically active influencing cholesterol homeostasis by up-regulation of liver high-affinity LDL receptors [1,2] and reduction of plasma triaclyglycerols in humans and rats [3,4]. Milk proteins were also described to contain bioactive peptides within their primary structure, e.g., angiotensin-I-converting enzyme (ACE) inhibitory peptides [5,6], which can be released during gastrointestinal digestion. Studies using milk protein-derived ACE inhibitors could demonstrate in vivo hypotensive effects in spontaneously hypertensive rats [7,8]. In addition, a recent study demonstrated that oral administration of 7S globulin and its α′ subunit to rats lowers plasma lipids and up-regulates β-VLDL receptors [9]. This suggests that biologically active peptides probably pass from the intestine to the serum in sufficient

Abbreviations: ACE, angiotensin-I-converting enzyme; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetate; C, chymotrypsin; E, elastase; ET-1, endothelin-1; HBSS, Hank's buffered salt solution; HEPES-BSS, HEPES buffered salt solution; mTOR, mammalian Target of Rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; NO, nitric oxide; P, pepsin; PGI_2, prostaglandin I_2; T, trypsin; TXA_2, thromboxan A_2; TXB_2, thromboxan B_2; TNFα, tumor necrosis factor-α; TNS, trypsin neutralizing solution; VEGF, vascular endothelial growth factor; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin$_{1\alpha}$

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amounts to exert biological effects, although peptides are probably largely degraded by gastrointestinal proteinases and peptidases and serum peptidases. However, the observed inhibition of ACE, a metallocarboxydipeptidase, which is located on the surface of vascular endothelial cells, suggests that dietary peptides probably influence endothelial function.

The endothelium is strongly involved in the atherogenic process. The formation of vasoactive substances such as nitric oxide (NO) and eicosanoids by normal endothelial cells plays an important role for the maintenance of vascular homeostasis. Alteration of normal endothelial cell function as observed during endothelial dysfunction is characterized by an altered formation of NO and eicosanoids, which promotes injury to vessel walls and the development of atherosclerosis [10,11]. Whereas the proliferation rate of normal endothelial cells is low, endothelial cells are induced to enter the cell cycle at sites of endothelial microdamage and promote reendothelialisation. However, an increased proliferation of endothelial cells probably favours plaque development as also suggested from others [12]. In addition, at sites of atherosclerotic lesions the vessel wall shows an altered response to the vasodilating effects of NO [13]. Therefore, alterations in the proliferation rate of endothelial cells and the formation of vasoactive substances are probably critical with respect to the development of atherosclerosis. However, to our knowledge, studies dealing with the effects of peptides or hydrolysates derived from food proteins on endothelial function are lacking so far. Therefore, the aim of this study was to evaluate the effects of di- and tripeptides, which are known to be transported into the enterocyte and at least partially reach the circulation, and hydrolysates from food proteins on endothelial function. Concerning that soy protein was demonstrated to have antiatherogenic properties [14], whereas casein showed proatherogenic features in vivo [15,16], peptides and hydrolysates from soy protein and casein were used. Di- and tripeptides were rather selected according to their occurrence in the casein fractions α_{s1}-casein, α_{s2}-casein and β-casein and in the soy proteins β-conglycinin and glycinin than their actual generation during pancreas digestion aiming to explore the principle question whether dietary peptides influence endothelial function or not. The hydrolysates were obtained from in vitro digestion of casein and soy protein by enzymatic hydrolysis using the gastrointestinal proteases pepsin, trypsin, chymotrypsin and elastase and simulating gastrointestinal conditions. As parameters of endothelial cell function, we determined endothelial cell proliferation and the release of vasoactive substances such as NO, eicosanoids and endothelin-1 (ET-1).

2. Materials and methods

2.1. Materials

Di- and tripeptides were purchased from BACHEM (Bubendorf, Schweiz) except for H-Leu-Leu-Leu-OH (Senn Chemicals AG, Dielsdorf, Schweiz). Their occurrence in the primary sequence of caseins and the soy proteins glycinin G1 and β-conglycinin is shown in Table 1. Endothelial cell basal medium MV (ECBM) with SupplementPack, HEPES buffered salt solution (HEPES-BSS), trypsin–EDTA (0.025% trypsin and 0.01% EDTA) and trypsin neutralizing solution (TNS) containing 0.05% trypsin inhibitor and 0.1% bovine serum albumin (BSA), and human recombinant vascular endothelial growth factor (VEGF) were purchased from PromoCell (Heidelberg, Germany). Ultroser G and Hanks balanced salt solution (HBSS) were obtained from BioSepra (Cergy St. Christophe, France) and Invitrogen (Karlsruhe, Germany), respectively. Human recombinant tumor necrosis factor-α (TNFα) and BSA pepsin (from porcine stomach mucosa, 471 U/mg) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Chymotrypsin (from bovine pancreas, 60–90 U/mg) was a product of Boehringer Mannheim (Germany). Trypsin (from porcine pancreas, 50–80 U/mg) and elastase (from porcine pancreas, 17–200 U/mg) were obtained from Serva (Heidelberg, Germany).

2.2. Production of hydrolysates from casein and soy protein

Casein and soy protein derived hydrolysates were produced by in vitro digestion of casein (Fa. Meggle, Wasserburg, Germany) and soy protein isolate (Numico research, Wageningen, Netherlands), simulating gastrointestinal conditions in temperature, pH value, digestion time, as

Table 1
Occurrence of the di- and tripeptides investigated in the primary sequence of caseins and the soy proteins glycinin G1 and β-conglycinin

Peptides	Caseins	Glycinin G1	β-Conglycinin
H-Ser-Glu-OH	X	–	–
H-Val-Pro-OH	X	–	–
H-Lys-Glu-OH	X	–	–
H-Pro-Ile-OH	X	–	–
H-Pro-Leu-OH	X	–	–
H-Glu-Ser-OH	X	–	–
H-Glu-Glu-OH	–	–	X
H-Pro-Gln-OH	–	X	–
H-Gln-Gln-OH	–	X	X
H-Gln-Glu-OH	–	X	–
H-Ala-Leu-OH	–	X	–
H-Val-Ala-OH	–	X	–
H-Phe-Leu-OH	–	–	X
H-Ser-Ser-OH	–	–	X
H-Leu-Ser-OH	–	X	X
H-Ala-Pro-Phe-OH	X	–	–
H-Leu-Gly-Tyr-OH	X	–	–
H-Pro-Leu-Gly-NH2	X	–	–
H-Glu-Glu-Glu-OH	X	X	X
H-Leu-Leu-Leu-OH	X	–	X
H-Ser-Ser-Ser-OH	X	–	–
H-Val-Pro-Leu-OH	X	–	–
H-Gly-Lys-Gly-OH	–	X	–
H-Glu-(Gly-Gly-OH)-OH	–	X	–
H-Glu-γ-Glu-γ-Gln-OH	–	–	X
H-Glu-Glu-Asp-OH	–	–	X

Table 2
Casein and soy protein-derived total hydrolysates

Name	In vitro digestion[a]	Average molecular weight (Da)	Content of free amino acids (%)
$1C_1$	Casein//$_{E+T}$	1000	25.6
$1C_2$	Casein//$_{E+T+C}$	930	29.2
$1C_3$	Casein//$_{P+E+T}$	940	31.5
$1C_4$	Casein//$_{P+E+T+C}$	860	30.2
$1C_5$	Casein//$_{P/C}$	1300	27.8
$1C_6$	Casein//$_{P/E}$	1500	22.9
$1C_7$	Casein/$_{P/T}$	1300	34
$1S_1$	Soy protein//$_{P+T}$	4550	32.5
$1S_2$	Soy protein//$_{P+T,C}$	1700	42.2
$1S_3$	Soy protein//$_{P+T,C,E}$	1700	39.9
$1S_4$	Soy protein//$_{P/C}$	3600	36.2
$1S_5$	Soy protein//$_{P/E}$	2800	42.1
$1S_6$	Soy protein//$_{P/T}$	3450	35.2
$1S_7$	Soy protein//$_{P/T+C+E}$	1000	48.1

[a] (//…)=digested with…; (+…)=further digestion of the whole pepsin digest, (/…)=further digestion of the insoluble residue of pepsin digestion in a successive (+…+) or simultaneous (+…,…) manner; P=pepsin; T=trypsin; C=chymotrypsin; E=elastase.

well as protein and protease concentrations. The commercial food proteins were checked for purity and the presence of intact subunits (glycinin, β-conglycinin in case of soy protein) or complete components (α_{S1}-, α_{S2}-, β- and κ-casein in case of casein) by reversed-phase HPLC and SDS-electrophoresis in comparison to the literature [17]. Casein or soy protein isolate was suspended in water (25 mg/mL), adjusted to pH 2 by adding 1 N HCl and digested with pepsin (1 mg/mL) at 37 °C for 3 h. Either the whole pepsin digest or the insoluble, partly digested components only were neutralised by adding $NaHCO_3$, adjusted to pH 8 and digested by adding pancreas proteases (trypsin and chymotrypsin: 0.5 mg/mL; elastase: 0.12 mg/mL), stepwise or simultaneously, at 37 °C. In addition, samples of water suspended protein, without proceeding pepsin digestion, were treated with pancreas proteases in the same way. Resulting total hydrolysates were boiled for 15 min and centrifuged for removing residual protease activity and insoluble material. In case of fractionation of selected hydrolysates, protease activity and partly digested components were removed by ultrafiltration, using 5-kDa membrane disc filters (Omega-Series, PALL, Ann Arbor, USA), over 3–4 h, while digestion proceeded. For further fractionation, peptide fractions of 1–3 kDa and smaller than 1-kDa membrane disc filters with cutoff sizes of 3 and 1 kDa were used, respectively. Total hydrolysates and peptide fractions were generally lyophylized. The parameters for the definition of the hydrolysates are shown in Tables 2 and 3. Average molecular weight and content of free amino acids were calculated according to Ref. [18] by spectrophotometrical determination of free amino residues with ninhydrin-reagent (Sigma-Aldrich) in relation to protein quantity, and by the copper-complex-method [19]. Residual active trypsin was determined spectrophotometrically after incubation of 20 mg/mL lyophilisate with the chromogenic trypsin reagent *N*α-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (Sigma-Aldrich) at 37 °C for 20 min. The peptide content of salt containing low molecular fractions was determined using the BCA protein assay kit from Pierce (Perbio Science Deutschland GmbH, Bonn) with a salt-free hydrolysate as standard. In addition, the hydrolysates were characterized by reversed-phase HPLC fingerprint and, for selected single hydrolysate fractions, by mass spectrometrical sequencing of the contained peptides (data not shown).

2.3. Cell culture

Human aortic endothelial cells (HaoEC), obtained from a 28-year-old female donor, were purchased from PromoCell. The cells were isolated from the ascending aorta by enzymatic digestion. PromoCell characterized the cells by Factor VIII-related antigen expression. The cells were cultured as previously described [20]. Ultroser G, containing the trypsin-inhibitor aprotinin, was used instead of fetal calf serum in order to protect peptides and hydrolysates from hydrolysis during incubation. Cells were passaged after reaching confluence by using trypsin/EDTA. After trypsinization, TNS was added to prevent enzymatic damage to the cells. Only cells from passages 3–5 were used for this study.

2.4. Cell count, cell viability and protein determination

The cell count was determined with a Neubauer chamber after cells were harvested by trypsinization and pelleted by centrifugation (170×*g* for 5 min). Cell viability was examined by the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazoleum bromide (MTT) assay [21]. After the cells were incubated for 24 h at 37 °C with fresh medium alone

Table 3
Casein and soy protein-derived hydrolysate fractions

Name	In vitro digestion[a]	Peptide fraction (kDa)	Residual trypsin (%)[b]	Peptide content (%)[c]
$2C_1$	Casein//$_{P/T}$	<1	<0.005[d]	61
$2C_2$	Casein//$_{P/T}$	1–3	0.9	89
$2C_3$	Casein//$_{P+T,C}$	<1	<0.005[d]	66
$2C_4$	Casein//$_{P+T,C}$	1–3	<0.005[d]	82
$2C_5$	Casein//$_{P+T,C,E}$	<1	<0.005[d]	54
$2C_6$	Casein//$_{P+T,C,E}$	1–3	0.34	82
$2S_1$	Soy protein//$_{P+T}$	<1	<0.005[d]	57
$2S_2$	Soy protein//$_{P+T}$	1–3	0.09	100
$2S_3$	Soy protein//$_{P+T,C}$	<1	<0.005[d]	37
$2S_4$	Soy protein//$_{P+T,C}$	1–3	0.08	85
$2S_5$	Soy protein//$_{P+T,C,E}$	<1	<0.005[d]	57
$2S_6$	Soy protein//$_{P+T,C,E}$	1–3	0.02	100

[a] (//P)=digested with pepsin; (+…,…)=simultaneous further digestion of the whole pepsin digest; (//P/T)=further digestion of the insoluble residue of pepsin digestion with trypsin; P=pepsin; T=trypsin; C=chymotrypsin; E=elastase.
[b] Active trypsin in lyophilized fractions.
[c] Of salt containing fractions.
[d] Limit of detection.

(control) or with medium supplemented with peptides at final concentrations ranging from 1 nM to 10 mM or various hydrolysates at concentrations from 0.025 to 5 mg/mL, cell medium was removed and the cells were incubated with medium containing 0.5 mg/mL MTT for 2 h at 37 °C and 5% CO_2 atmosphere. Subsequently, medium was aspirated and 2-propanol (Roth, Karlsruhe, Germany) was added to dissolve the formazan. The supernatants were transferred to a 96-well microtitre plate and the absorbance was read at 595 nm in a microtitre plate spectrophotometer (Tecan, Crailsheim, Germany). Protein concentration was measured by the method of Bradford [22] using Bio-Rad protein assay (München, Germany) with BSA as standard.

2.5. Cell proliferation assay

The effect of various peptides on endothelial cell proliferation was assessed using the Biotrak cell proliferation ELISA system (Amersham, Buckinghamshire, UK), which is based on the measurement of 5-bromo-2′-deoxyuridine (BrdU) during DNA synthesis in proliferating cells. The cell proliferation ELISA was performed according to the manufacturer's protocol (Amersham). In brief, cells were seeded in 96-well microtitre plates at a density of 2500 cells/well. After reaching 70–80% confluence, cells were incubated for 24 h with labelling medium (100 μM BrdU) alone (control) or with labelling medium (100 μM BrdU) and various peptides at final concentrations of 1 nM to 1 mM or various hydrolysates at concentrations from 0.025 to 2.5 mg/mL. As further controls, the cell proliferation assay was also performed with VEGF and TNFα at final concentrations of 10 and 0.1 ng/mL, respectively. At the end of the labelling period, cells were fixed and DNA denatured using a fixative, which was followed by a 30-min blocking step. After removing the blocking buffer, cells were subsequently incubated with peroxidase-labelled anti-BrdU for 90 min at room temperature. The immune complex was detected by the subsequent reaction with TMB and reading the resultant colour at 450 nm in a microtitre plate spectrophotometer (Tecan).

2.6. Release of vasoactive substances

For determination of vasoactive substances, cells were seeded in six-well plates. After reaching 70–80% confluence, growth medium was removed and cells were incubated for 24 h with medium alone (control) or with medium supplemented with various peptides at final concentrations of 100 nM and 50 μM or various hydrolysates at concentrations of 0.05 to 0.5 mg/mL. At the end of the incubation period, the incubation medium was assayed for the concentrations of endothelin-1 (ET-1), and nitric oxide (NO). As an index of the NO concentration, nitrate and nitrite concentrations were determined by a Nitrate/Nitrite Colorimetric Assay kit (no. 765001, Cayman Chemical, Ann Arbor MI). ET-1 was measured using Endothelin ELISA (no. 59231, Cayman Chemical). For the measurement of eicosanoids (6-keto-$PGF_{1\alpha}$ and TXB_2), the 24-h incubation period was followed by an additional 1-h incubation period in HBSS. 6-Keto-$PGF_{1\alpha}$ and TXB_2 were determined in the HBSS supernatant as measures of the unstable PGI_2 and TXA_2 using EIA-kits (nos. 515211, 519031, Cayman Chemical).

2.7. Statistics

Means were compared by Student's *t*-test. Means were considered significantly different for $P<0.05$.

3. Results

3.1. Cell viability

Cell viability was not affected by incubation with di- and tripeptides at concentrations ranging from 1 nM to 1 mM but was reduced at 10 mM compared to untreated cells (data not shown). The viability of cells treated with casein and soy protein derived hydrolysates was not affected at concentrations ranging from 0.025 to 2.5 mg/mL but was reduced at 5 mg/mL compared to untreated cells (data not shown).

3.2. Cell proliferation

Treatment of cells with 10 ng/mL of VEGF stimulated endothelial cell proliferation, whereas treatment with 0.1 ng/mL of TNFα inhibited cell proliferation compared to untreated controls (VEGF 110±5%, TNFα 79±6%, control 100±8%; $n=3$; $P<0.05$). No effect on endothelial cell proliferation was observed for the dipeptides H-Glu-Glu-OH, H-Gln-Glu-OH, H-Val-Pro-OH, H-Ser-Glu-OH, H-Glu-Ser-OH, H-Lys-Glu-OH, H-Pro-Ile-OH, H-Pro-Leu-OH, H-Phe-Leu-OH, H-Ser-Ser-OH, H-Leu-Ser-OH, H-Ala-Leu-OH, H-Gln-Gln-OH, H-Val-Ala-OH and H-Pro-Gln-OH in the concentration range investigated (1 nM–1 mM, data not shown). From the tripeptides investigated, only H-Glu-Glu-Glu-OH and H-Val-Pro-Leu-OH influenced endothelial cell proliferation at concentrations ≥1 nM (Fig. 1); incubation with H-Glu-Glu-Glu-OH caused a stimulation of endothelial cell proliferation at concentrations from 1 nM to 100 μM, whereas H-Val-Pro-Leu-OH caused an inhibition of endothelial cell proliferation at 1 nM but stimulation at 1 μM ($P>0.05$). Endothelial cell proliferation was not influenced by the tripeptides H-Glu-Glu-Glu-OH, H-Pro-Leu-Gly-NH_2, Glu-γ-Glu-γ-Gln-OH, H-Glu-Glu-Asp-OH, H-Glu-(Gly-Gly-OH)-OH, H-Ala-Pro-Leu-OH, H-Ala-Pro-Phe-OH, H-Leu-Gly-Tyr-OH, H-Leu-Leu-Leu-OH and H-Gly-Lys-Gly-OH in the concentration range investigated (1 nM–1 mM, data not shown). Hydrolysates from casein inhibited endothelial cell proliferation at concentrations ≥1 mg/mL ($1C_1$, $1C_2$) and ≥2.5 mg/mL ($1C_3$,

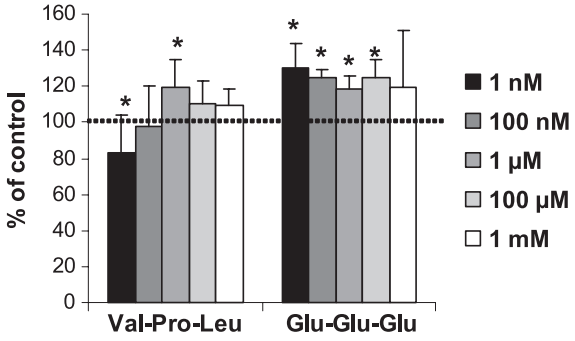

Fig. 1. The effect of H-Val-Pro-Leu-OH and H-Glu-Glu-Glu-OH on the proliferation of human aortic endothelial cells compared to untreated cells (control). Bars representing means ± S.D. for $n=3$ show relative cell proliferation compared to control (% of control = 100 ± 8) at the concentrations 1 nM, 100 nM, 1 µM, 100 µM and 1 mM. Control is indicated by the broken line. *Different from untreated cells; $P<0.05$.

$1C_5$) compared to control treatment ($P<0.05$, Fig. 2A). The casein hydrolysates $1C_4$, $1C_6$, $1C_7$, $2C_1$, $2C_3$ and $2C_5$ had no effect on endothelial cell proliferation in the concentration range investigated (0.05–2.5 mg/mL, data not shown). Hydrolysates from soy protein inhibited endothelial cell proliferation at concentrations ≥0.25 mg/mL ($1S_3$, $1S_4$) and ≥0.5 mg/mL ($1S_2$) and at 2.5 mg/mL ($1S_1$, $1S_5$) ($P<0.05$, Fig. 2B). Treatment of cells with $1S_6$, $1S_7$, $2S_1$, $2S_3$ and $2S_5$ had no effect on endothelial cell proliferation compared to incubation of cells with medium alone (data not shown).

3.3. Release of vasoactive substances

The release of vasoactive substances was significantly influenced by the incubation with various di- and tripeptides (Table 4). Cells treated with 50 µM of H-Leu-Leu-Leu-OH released more TXB_2 into the incubation medium than control cells ($P<0.05$). Treatment of cells with 100 nM of either H-Val-Pro-Leu-OH, H-Glu-Glu-Glu-OH or H-Ser-Ser-Ser-OH and 50 µM and 1 mM of H-Ser-Glu-OH increased concentrations of 6-keto-$PGF_{1\alpha}$ in the incubation medium compared to control treatment ($P<0.05$). Cells treated with 100 nM and 50 µM of either H-Val-Pro-Leu-OH or H-Ser-Ser-Ser-OH released more ET-1 into the incubation medium than control cells ($P<0.05$). Incubation of cells with 50 µM of H-Glu-Glu-OH reduced concentrations of ET-1 in the incubation medium compared with control cells ($P<0.05$). Endothelial cells treated with 100 nM and 50 µM of H-Val-Pro-Leu-OH released more NO during incubation than control cells ($P<0.05$).

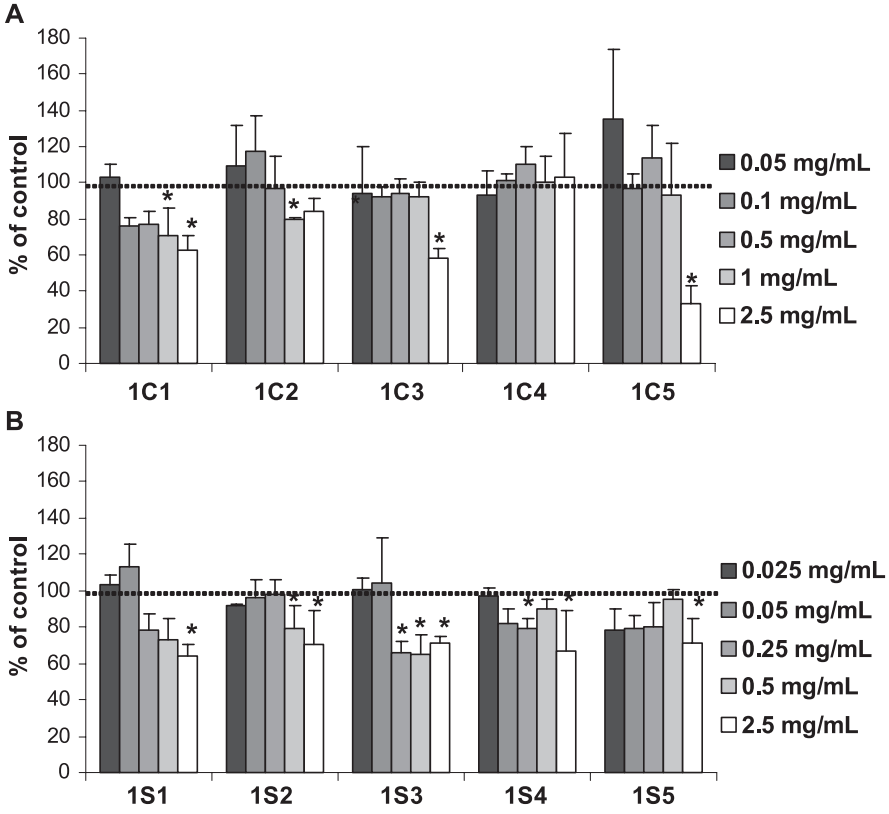

Fig. 2. The effect of hydrolysates derived vom casein (A) and soy protein (B) on the proliferation of human aortic endothelial cells compared to untreated cells (control). (A) Bars representing means ± S.D. for $n=3$ show relative cell proliferation compared to control (% of control = 100 ± 8) at the concentrations 0.025, 0.05, 0.25, 0.5 and 2.5 mg/mL. Control is indicated by the broken line. *Different from untreated cells; $P<0.05$. (B) Bars representing means ± S.D. for $n=3$ show relative cell proliferation compared to control (% of control = 100 ± 8) at the concentrations 0.05, 0.1, 0.5, 1.0 and 2.5 mg/mL. *Different from untreated cells; $P<0.05$.

Table 4
Relative concentrations of vasoactive substances per milligram of cell protein in cell medium of human aortic endothelial cells treated with various di- and tripeptides compared to untreated cells[a]

Peptides/concentration	Relative concentration of vasoactive substances (% of control)			
	TXB_2 (control=100±11)	6-keto-$PGF_{1\alpha}$ (control=100±15)	ET-1 (control=100±19)	NO (control=100±29)
H-Val-Pro-Leu-OH				
100 nM	101±30	124±10*	184±21*	195±38*
50 μM	108±49	135±42	160±46*	198±49*
H-Glu-Glu-Glu-OH				
100 nM	121±37	183±41*	101±17	94±8
50 μM	105±43	122±32	97±14	110±37
H-Ser-Ser-Ser-OH				
100 nM	100±10	146±20*	113±2*	108±25
50 μM	106±13	121±47	118±2*	105±7
H-Leu-Leu-Leu-OH				
100 nM	97±33	106±56	94±3	108±8
50 μM	160±42*	158±60	94±7	96±10
H-Val-Pro-OH				
50 μM	102±14	83±24	88±12	77±38
H-Glu-Glu-OH				
50 μM	108±23	121±22	78±16*	85±39
H-Ser-Glu-OH				
50 μM	–[b]	192±47*	96±37	116±48

[a] Results are means±SD for n=6.
[b] Not determined.
* Different from untreated cells; $P<0.05$.

Incubation of endothelial cells with various hydrolysates from casein and soy protein influenced the release of 6-keto-$PGF_{1\alpha}$ and NO but did not influence the release of TXB_2 and ET-1 (Table 5); endothelial cells treated with 0.5 mg/mL of either $2C_1$, $2C_3$, $2S_1$ or $2S_3$ released more NO into the medium than control cells ($P<0.05$). Treatment of cells with 0.05 mg/mL of $2C_3$ reduced concentrations of 6-keto-$PGF_{1\alpha}$ in the incubation medium compared with control treatment ($P<0.05$).

4. Discussion

To test whether casein and soy protein-derived peptides and hydrolysates influence endothelial cell function, several di- and tripeptides and hydrolysates were screened for their potential to modulate cell proliferation. This study shows that endothelial cell proliferation was not influenced by dipeptides and most of the tripeptides, whereas several total hydrolysates from casein and soy protein inhibited cell

Table 5
Relative concentrations of vasoactive substances per milligram of cell protein in cell medium of human aortic endothelial cells treated with casein and soy protein-derived hydrolysates compared to untreated cells[a]

Hydrolysates/concentration	Relative concentration of vasoactive substances (% of control)			
	TXB_2 (control=100±5)	6-Keto-$PGF_{1\alpha}$ (control=100±9)	ET-1 (control=100±16)	NO (control=100±19)
$2C_1$				
0.05 mg/mL	110±9	59±11*	131±22	118±39
0.5 mg/mL	100±8	56±9*	100±15	135±32*
$2C_3$				
0.05 mg/mL	100±11	43±7*	129±37	124±31
0.5 mg/mL	109±10	66±11	111±14	122±30*
$2C_5$				
0.05 mg/mL	111±45	53±16*	99±8	130±61
0.5 mg/mL	90±12	48±27*	94±23	148±52
$2S_1$				
0.05 mg/mL	99±22	113±13	112±5	140±33
0.5 mg/mL	91±15	89±33	93±7	154±19*
$2S_3$				
0.05 mg/mL	115±19	61±22	97±10	136±32
0.5 mg/mL	110±25	118±97	93±23	171±44*

[a] Results are means±SD for n=6.
* Different from untreated cells; $P<0.05$.

proliferation at higher concentrations. Until now, no data are available from the literature regarding the effects of dietary peptides or hydrolysates on endothelial cell proliferation. The observation that VEGF and TNFα caused a significant stimulation and inhibition of endothelial cell proliferation, respectively, is in accordance with findings from the literature [23,24]. It was shown that mitogens such as VEGF or PDGF modulate endothelial cell proliferation not only via specific receptors (flt-1 and KDR) and subsequent tyrosine autophosphorylation and tyrosine phosphorylation of known signal transduction proteins [25,26], but also via mTOR (mammalian Target of Rapamycin)-signaling via the PI3K/protein kinase B-pathway [27]. Function of mTOR, playing a key role in the regulation of cell proliferation, was demonstrated to be regulated by the availability of nutrients, e.g., amino acids, and ATP [28,29] and, therefore, might be, albeit highly speculative, also regulated by di- or tripeptides. However, unspecific inhibition of cell surface peptidases (e.g., angiotensin-I-converting enzyme (ACE) or neutral endopeptidase) could also account for the modulation, particularly the inhibition, of endothelial cell proliferation by the total hydrolysates investigated due to the strong antiproliferative and antimitogenic effect of ACE inhibition [30,31]. Natural inhibitors of ACE have been identified within the primary sequence of a range of food proteins such as casein [32] and were probably also generated during the in vitro digestion step. Regarding that the concentrations of the peptides evaluated in the present study are in the range of the ACE IC_{50} values (ranging from 2 μM for the potent ACE inhibitors Val-Ala-Pro and Ile-Pro-Pro to 1 mM for less potent ACE inhibitors), which are reported for different peptides from caseins or other milk proteins [32], the effect on cell surface peptidases should also be taken into consideration in future studies. Whether the concentrations evaluated in the present study are of physiological relevance is difficult to answer concerning the lack of information in the literature about peptide concentrations in the plasma. The highest concentrations reported for single peptides in the plasma are about 40 μM for Cys-Gly [33], the degradation product of glutathione, however, concentrations of peptides derived from digestion of food proteins are expected to be much lower. The biological importance of our observations is less clear, because the proliferation rate of endothelial cells is low in healthy vessels and the significance of endothelial cell proliferation for the development of atherosclerosis is still discussed controversially. Whereas an increased proliferation rate could be interpreted beneficial in view of reendothelialisation after endothelial microdamage [34], it could be shown that antiproliferative effects are beneficial in view of prevention from plaque development [35].

Concerning this controversial debate, we further investigated the potential of several peptides and hydrolysate fractions to modulate the release of vasoactive substances concerning that alterations of normal endothelial cell function are accompanied by an altered release of NO, eicosanoids and ET-1 [10,11]. Our study shows that several tripeptides and hydrolysate fractions from casein and soy protein modulated the release of vasoactive substances. The observation that the tripeptides H-Val-Pro-Leu-OH and H-Ser-Ser-Ser-OH, H-Glu-Glu-Glu-OH, which are found in casein and/or soy protein, increased the release of 6-keto-$PGF_{1\alpha}$, a stable marker of PGI_2, might be considered beneficial in view of prevention from atherosclerosis. Reduced concentrations of PGI_2 are established risk factors for ischaemic heart diseases and the concentrations of PGI_2 are strongly reduced during acute myocardial infarction [36,37]. PGI_2, through activation of the IP receptor present on smooth muscle cells and platelets, causes vasodilatation and inhibits platelet aggregation [38]. From the peptides investigated, only H-Val-Pro-Leu-OH markedly increased the release of NO. In addition, several hydrolysate fractions from casein and soy protein could be shown to increase the release of NO ($2C_1$, $2C_3$, $2S_1$, $2S_3$). These findings might be also beneficial concerning that NO acts anti-atherogenic due to its vasodilatating effect and the observation that NO concentrations are reduced during endothelial dysfunction [10,11]. The tripeptide H-Leu-Leu-Leu-OH, which is found in casein and soy protein, was shown to increase the endothelial release of TXB_2, a stable marker of TXA_2. This effect might be considered critically due to the strong vasoconstrictory potential and the promoting effect of TXA_2 on platelet aggregation [39]. However, the physiological relevance of this finding is presumably less important concerning that TXA_2 is synthesized to a lower extent by endothelial cells, whereas it is primarily formed by platelets, which possess a high activity of TX synthase [40,41].

To our surprise, incubation of H-Val-Pro-Leu-OH not only increased the release of NO and PGI_2, but also of the vasoconstrictory ET-1. The physiological effects of ET-1 are mediated via specific G-protein coupled endothelin receptors, ET_A and ET_B [42,43]. Stimulation of ET_A present on smooth muscle cells causes vasoconstriction and mediates smooth muscle cell proliferation. In contrast, stimulation of ET_B, which is present preferentially on endothelial cells, is linked to the formation of NO and PGI_2. It has been suggested that this process serves to restore normal vascular tone and probably explains the fact that in vivo infusions of ET-1 at low concentrations exert vasodilatory effects [44]. Therefore, the increased release of ET-1 by H-Val-Pro-Leu-OH might be interpreted as a compensatory means for the vasodilatory actions of NO and PGI_2. Until now, there is no indication from the literature explaining the altered formation of NO and eicosanoids by dietary peptides or hydrolysates. However, the effects observed on the release of vasoactive mediators might be also explained by unspecific inhibition of ACE by peptides or hydrolysates. ACE, which is located on the endothelial cell membrane, is responsible for the conversion of angiotensin I into angiotensin II, as well as for the breakdown of bradykinin. Therefore, ACE inhibition, which inhibits the breakdown of the vasodilator bradykinin, a direct stimulant of NO release

and PGI_2 production from the intact endothelial cell [31,45], increases the production of NO, PGI_2 as well as tissue plasminogen activator and reduces plasminogen activator inhibitor-1 activity [45] and, therefore, might explain the increase in mediator release by some of the hydrolysate fractions investigated. It is known that alterations in the production of vasoactive substances such as NO or PGI_2 are observed during endothelial cell proliferation. Therefore, there might be also a causal link between the alterations in endothelial cell proliferation and the formation of vasoactive substances. For instance, it has been shown that endothelial NO synthase (eNOS) expression and hsp90 interactions with eNOS are increased during proliferation, which increases the production of NO [46,47]. Studies using inhibitors of eNOS could demonstrate that mitogens exert their proliferative effects on endothelial cells by an NO-dependent mechanism [46,47]. Based on the primarily descriptive data of the present study, the actual mechanisms underlying the effects of peptides and hydrolysates can be hardly explained. Therefore, further studies are required to elucidate the role of dietary peptides and protein hydrolysates in the modulation of NO and eicosanoid release from endothelial cells. A reasonable approach to study a possible linkage between mediator release and endothelial cell proliferation should include the use of pharmacological inhibitors of NO or prostaglandin synthesizing enzymes. Possible structure–activity relationships should be also elucidated in future studies considering that this could account for the differences between the active peptides H-Val-Pro-Leu-OH, H-Glu-Glu-Glu-OH or H-Ser-Ser-Ser-OH on proliferation and mediator release in the present study. For instance, binding of natural ACE inhibitors is reported to be strongly influenced by the C-terminal tripeptide sequence, e.g., many substrates and competitive inhibitors of ACE contain hydrophobic amino acids and food protein-derived ACE inhibitors contain Pro, Lys or Arg at the C-terminal residue [32].

From the present study, no indications could be found resolving the question whether peptides or hydrolysates from soy protein are superior to those from casein with respect to antiatherogenic properties because peptides, e.g., H-Glu-Glu-Glu-OH, and hydrolysates from both proteins were shown to increase the release of vasodilatory substances such as PGI_2 and NO. The alterations in endothelial cell function by the hydrolysates and hydrolysate fractions indicate that these hydrolysates probably contain bioactive sequences mediating the observed effects. To identify the bioactive components in the total hydrolysates and hydrolysate fractions, future studies using suitable separation techniques such as RP-HPLC and analytical means such as peptide mass fingerprinting are required.

In conclusion, the present study demonstrates that peptides such as H-Val-Pro-Leu-OH, H-Glu-Glu-Glu-OH and H-Ser-Ser-Ser-OH, even at low concentrations, and hydrolysate fractions from casein and soy protein influence endothelial cell function as evidenced by the modulation of endothelial cell proliferation and alterations in the release of vasoactive substances. This indicates that dietary proteins contain specific sequences possibly mediating biological effects on the endothelium in vivo. Whether the observed effects are mediated by peptide sequences with well-established biological effects, e.g., ACE-inhibitory peptides, or by sequences with unknown effects has to be elucidated in future studies. In addition, further studies are required to clarify the physiological relevance of the findings of the present study concerning that no data are available with respect to the concentrations of various di- and tripeptides in the serum.

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