JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2008,** *56,* 4987–4994 **4987**

Screening for Nutritive Peptides That Modify Cholesterol 7α-Hydroxylase Expression

NORBERT NASS,*,†,‡ REGINA SCHOEPS,§ RENATE ULBRICH-HOFMANN,§ ANDREAS SIMM,† LARS HOHNDORF,‡ CHRISTIAN SCHMELZER,|| KLAUS RAITH,|| REINHARD H. H. NEUBERT,|| AND KLAUS EDER‡

Department of Cardiothoracic Surgery, Martin Luther University Halle-Wittenberg, Ernst-Grube Str. 40, D-06120 Halle/Saale, Germany, Institute of Biochemistry and Biotechnology, Martin Luther University Halle-Wittenberg, Kurt-Mothes Str. 3, D-06120 Halle/Saale, Germany, Institute of Agricultural and Nutritional Sciences, Martin Luther-University of Halle-Wittenberg, Emil-Abderhalden-Str. 26, D-06108 Halle (Saale), Germany, and Institute of Pharmacy, Martin Luther University Halle-Wittenberg, Wolfgang-Langenbeck-Str. 4, D-06120 Halle/Saale, Germany

Bioactive peptides with a variety of effects have been described from several nutritive proteins. They exhibit antimicrobial, blood-pressure lowering, antithrombotic, immunomodulatory, and cholesterol-modulating effects. In this study, we have examined whether peptides derived from food proteins might influence bile acid synthesis. A reporter gene cell line that carries a cholesterol 7α-hydroxylase promoter fragment fused to firefly luciferase (*cyp7a-luc*) was used to screen for nutritive peptides affecting *cyp7a* expression, the enzyme catalyzing the rate-limiting step in bile acid synthesis. Proteolytic hydrolysates were prepared from soy protein and bovine casein with pepsin, trypsin, chymotrypsin, and elastase and size fractionated using ultrafiltration. Several bioactive hydrolysates could be identified that inhibited luciferase expression. Also, an activation of kinase (AKT, ERK, p38-MAPK) signaling could be observed. Selected hydrolysates were further fractionated by reversed-phase HPLC. Bioactive HPLC-fractions were obtained from casein but not from soy hydrolysates; however, activity could not be recovered in single peak fractions. Peptides in such fractions were identified by mass spectrometry. Five selected peptides from α_{S1}-casein present in active fractions were synthesized, but none of these showed activity in the *cyp7a-luc* screening system. However, two of them activated MAP-kinase signaling similar to the hydrolysates, which suggests, that these peptides are involved in *cyp7a* regulation by the casein hydrolysates.

KEYWORDS: Casein; soy protein; cholesterol-7α-hydroxylase; bile acid synthesis; luciferase reporter gene

INTRODUCTION

Epidemiological studies and animal experiments have demonstrated that the source of food protein influences risk factors to develop diseases such as atherosclerosis. This is well-documented for proteins from soy (*1, 2*) and white lupin (*3*) and other legumes (*4–9*), but also effects of milk proteins are known (*10–13*). It is hypothesized that such effects depend on the release of bioactive peptides from the nutritional proteins, which are capable of influencing metabolic activities in target cells. Well-documented examples are proteolytic hydrolysates of caseins and several other nutritional proteins that contain potent inhibitors of angiotensin I cleaving enzyme (ACE) (*14–18*). In case of soy storage proteins, the α′ subunit of the 7S globulin has been identified as the source of a peptide that was able to increase low density lipoprotein (LDL) uptake in cultivated liver cells (*19*).

Although peptides are mainly transported as di- or tripeptides (*20*), there are several observations that suggest that larger peptides are absorbed as well, thus reaching peripheral tissues. For example, it was found that the oral administration of ACE-inhibiting peptides, which usually comprise three to seven amino acids (*11*), had hypotensive effects in rats (*21*). Furthermore, it has recently been shown that immunoreactive fragments of caseinomacropeptide, a 63 amino acid peptide that derives from κ-casein appeared in peripheral plasma (*22*). For insulin, a transport through intestinal cells was also observed (*23–25*).

One of the major risk factors to develop atherosclerosis is the homeostasis of cholesterol. High LDL-cholesterol and low HDL-cholesterol levels are associated with high incidence of atherosclerosis and cardiovascular events (*26*). Uptake, synthesis,

* Phone: +49 345 557 3314. Fax: +49 345 557 3317. E-mail: norbert.nass@medizin.uni-halle.de.
† Department of Cardiothoracic Surgery.
‡ Institute of Agricultural and Nutritional Sciences.
§ Department of Biochemistry and Biotechnology.
|| Institute of Pharmacy.

10.1021/jf072806p CCC: $40.75
Published on Web 06/11/2008

and degradation of cholesterol contribute to high LDL-cholesterol levels. Soy protein has already been shown to upregulate VLDL- and LDL-receptor in vivo, thus decreasing LDL-cholesterol concentration (*27*).

We were interested whether proteolytic hydrolysates derived from soy protein and casein are able to modulate cholesterol degradation. The rate-limiting step in the major neutral pathway of bile acid synthesis is catalyzed by cholesterol 7α-hydroxylase (*cyp7a*). *cyp7a* gene expression is regulated via a feedback mechanism, which involves signaling via the nuclear bile acid receptor FXR, the "small heterodimeric partner" (SHP), and liver receptor homologue-1 (LRH-1) (**Figure 7**) (*28*). *cyp7a* is also regulated via a bile acid activated growth factor signal cascade involving the fibroblast growth factor 19 (Fgf-19), its receptor FGFR4, and c-jun N-terminal kinase (JNK) (*29*). Bile acids were also found to activate the extracellular signal regulated MAPKs (ERK), p38 MAPK, and protein kinase B (AKT) (*30, 31*). Last but not least, the gene is under the control of insulin signaling and altered activity was found to be associated with diabetes (*32–34*).

A reporter gene construct has been developed by Chiang and co-workers (*35*). It is responsive to bile acid conjugates and insulin in a stably transformed human hepatoma cell line derived from HEP-G2. Thus, this cell line was proposed for screening processes for novel substances regulating bile acid synthesis. In this study, we used this cell line to evaluate whether protein hydrolysates are capable of influencing the expression of the *cyp7a* gene which would contribute to the anti- or proatherogenic potential of food proteins such as casein and soy protein.

MATERIALS AND METHODS

Materials. Commercial food protein isolates with intact subunits, almost free of impurities such as casein (Meggle, Wasserburg, Germany) and soy protein isolate (Numico Research, Wageningen, Netherlands), were used to produce physiological-like hydrolysates as described earlier (*36*). Peptides C_1 (QGLPQEVLNENLLR), C_2 (FFVAPFPEVFGK), C_3 (YKVPQLEIVPNS), C_4 (YPELFR), and C_5 (YVPLGTQYTDAPSFSDIPNPIGSENSEK) were synthesized by Panatecs (Tübingen, Germany) and further purified by reversed-phase HPLC as described for the protein hydrolysates. Pepsin derived from porcine stomach mucosa (471 U/mg) was obtained from Sigma-Aldrich (Taufkirchen, Germany), chymotrypsin (from bovine pancreas, 60−90 U/mg) was a product of Boehringer Mannheim, and trypsin (from porcine pancreas, 50−80 U/mg) and elastase (from porcine pancreas, 170−200 U/mg) were obtained from Serva (Heidelberg, Germany). Acetonitrile and trifluoroactic acid were of HPLC grade.

Preparation of Hydrolysates. A method mimicking gastrointestinal conditions in temperature, pH value, digestion time, as well as protein and protease concentrations has been developed to yield protein hydrolysates expected to arise in human digestion of adults. Protein (2.5%) solutions (0.01 M HCl) were digested with pepsin (1 mg/mL, protein to pepsin ratio 1/25) for 3 h at pH 2 and 37 °C. After 10 min, the pH was readjusted with HCl to pH 2. For digestion with pancreatic proteases, sodium carbonate was added (85 mM final) and pH was adjusted to 8 with NaOH. Hydrolysis was performed with trypsin (0.5 mg/mL, trypsin to protein ratio 1/50) and/or chymotrypsin (0.5 mg/mL, chymotrypsin to protein ratio 1/50) and/or elastase (0.12 mg/mL, ealastase to protein ratio 1/20) for 3−4 h at 37 °C. Hydrolysis was carried out in a stirred ultrafiltration cell (Amicon, Millipore, Schwalbach, Germany) to remove low digested protein and residual protease activity immediately after digestion by use of a membrane disk filter with 5 kDa cutoff. Surprisingly, low levels of trypsin activity were frequently found in the ultrafiltrate in amounts that would compromise the cell-based assay. To inactivate this residual activity, hydrolysates were heated for 15 min in a boiling water bath. Ultrafiltration for separation of molecular weight fractions >5, 3−5, 1−3, and <1 kDa was carried out in a stirred ultrafiltration cell (Amicon, Millipore, Schwalbach, Germany) using membrane disk filters of 5, 3, and 1 kDa cutoff.

Fractionation of Hydrolysates. Reversed-phase HPLC was carried out on a Merck-Hitachi HPLC system with Eurospher-100, C_{18}, 5 μm columns (Knauer, Berlin, Germany), using a solvent system of A (5% acetonitrile, 0.05% trifluoroacetic acid) and B [50% acetonitrile, 0.05% trifluoroacetic acid with a gradient of 0−100% B (5−50 min)]. Chromatograms were recorded at 220 nm. For peptide "fingerprints", a 250 × 4 mm column was used at a flow rate of 1 mL/min. For preparative HPLC, a column with the same stationary phase of 250 × 20 mm with a flow rate of 5 mL/min was used. In total, 200 mg of hydrolysate was chromatographed in four portions, and fractions were pooled for further analysis. After lyophilization, fractions were redissolved in 5−10 mL of cell culture medium. If necessary, the pH was adjusted with sodium hydroxide (1 M) to 7.4. After filter-sterilization, the preparations were directly used for incubation experiments.

HPLC−ESI-MS and Tandem MS. The system used for HPLC−ESI-MS consisted of a Spectra System P 4000 pump, equipped with an AS 3000 autosampler and an SN 4000 controller (Thermo Fisher, San Jose, CA). The MS and tandem MS experiments were performed on a Finnigan LCQ Classic ion trap mass spectrometer (Thermo Fisher, San Jose, CA) with electrospray interface operated in the positive ion mode. Ten microliters of each sample solution was loaded onto a Nucleosil 120−5 C_{18} column (125 × 2 mm, Macherey Nagel, Düren, Germany), and peptides were eluted using a linear gradient: 5−50% acetonitrile in 0.1% formic acid over 60 min. The column was maintained at 30 °C and the flow rate was 0.2 mL/min. Electrospray conditions were as follows: capillary temperature, 200 °C; sheath gas flow rate, 1.2 L/min; ESI voltage, +4.5 kV; capillary voltage, 46 V; tube lens offset, 40 V. Full scans were performed between *m/z* 50 and 2000. Peptides were selected manually for tandem MS experiments using collision-induced dissociation (CID). The mass isolation window for CID was set to 3u, depending on experimental conditions. Fragmentation was carried out by varying the relative collision energy between 15 and 35% to achieve optimal results for $[M + H]^+$ ions. The fragment ion spectra were processed using Mascot Distiller (Matrix Science, London, UK) and analyzed by searching the sequence database MSDB with Mascot (Matrix Science) (*37*). The searches were taxonomically restricted to "other mammalia", the enzyme entry was set to "none", and phosphorylation of serine and threonine was considered (*38, 39*).

Cell Culture and Incubation Conditions. HEP-G2 and CRL-11997 cells that were derived from HEP-G2 by transformation with the *cyp7a* reporter gene were obtained from ATCC (via Promochem, Wesel, Germany), were cultured in RPMI 1640 medium (Gibco, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS, Gibco, Karlsruhe, Germany) and gentamicin (50 mg/L) at 37 °C and 5% CO_2 in 25 cm^2 flasks (Greiner, Germany). For gene expression studies, 7×10^5 cells were plated per well of a 24 well plate. For analysis of luciferase activity, cells were plated in 48-well plates and incubations started at approximately 90% confluency. Usually, cells were incubated for 16 h with protein hydrolysates in RPMI 1640 medium (Invitrogen, Gibco, Karlsruhe, Germany) supplemented with 0.5% BSA (Applichem, Darmstadt, Germany) for luciferase assays, RNA isolation, and viability tests. Experiments were performed in six replicates per plate. Cell viability was controlled by optical inspection and by using the MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] viability test (*40*). Absorption was measured using a microplate reader (Tecan Spectra Fluor Plus, Crailsheim, Germany) at 595 nm.

Luciferase Assays and Western Blot. Cells were washed once with PBS and then lysed with 25 μL passive lysis buffer (Promega, Mannheim, Germany) for 20 min at room temperature with continuous shaking. Twenty microliters was transferred to a white 96-well plate (Greiner, Frickenhausen, Germany) and luminescence determined in a Mithras multilabel reader (Bertholt, Bad Wildberg, Germany) after injection of 90 μL of luciferase assay reagent (*41*). Protein concentration was estimated from the residual cell lysate, using the BCA protein assay (Perbioscience, Bonn, Germany), calibrated with bovine serum albumin. Luciferase activity was expressed at relative light units per μg protein and normalized toward the untreated controls.

In the case of Western blot analysis, cells were lysed after 15 min incubation with hydrolysates or peptides with lysis buffer (Tris/Cl 50 mM, pH 6.8, 2% SDS, 1 mM Na_3VO_4, 0.1 μM ocadaic acid) protease

Table 1. Effect of Protein Hydrolysates on *cyp7a*-Luciferase Expression in HEP-G2 Cells[a]

hydrolysate[b]	concentration, mg/mL	relative specific luciferase activity
control	–	1.00 ± 0.07
	Casein Hydrolysates	
C//T	5.1	0.37 ± 0.07**
C//T	2.55	0.55 ± 0.04**
C//T	0.51	0.89 ± 0.06
C//P+*T* > 1 kDa	5	0.62 ± 0.11**
C//P+T 1−3 kDa	5	0.87 ± 0.06**
C//P+T,C >1 kDa	5	0.73 ± 0.06**
C//P+T,C 1−3 kDa	5	0.96 ± 0.05
C//P+T,C,E >1 kDa	5	0.46 ± 0.05**
	Soy Hydrolysates	
S//P+T > 1 kDa	5	0.35 ± 0.10*
S//P+T 1−3 kDa	5	0.50 ± 0.13*
S//P+T,C >1 kDa	5	0.59 ± 0.20*
S// P+T,C 1−3 kDa	5	0.55 ± 0.16**
S// P+ T,C,E >1 kDa	5	0.90 ± 0.17
S// P+T,C,E 1−3 kDa	5	1.04 ± 0.19

[a] Experiments were performed at least twice with four replicates each. Standard deviation (SD) is given, and statistical significance was calculated according to Student's *t* test. *, $p < 0.05$; **, $p < 0.01$. [b] S, soy protein; C, casein; /T, digested by trypsin; /P+T,C,E, first digested by pepsin and afterward simultaneously by trypsin, chymotrypsin, and elastase, respectively.

inhibitor cocktail (Sigma, Deisenhofen, Germany). Mercaptoethanol (5%) and glycerol (10%) were added for denaturation (80 °C, 20 min, continuous shaking). After denaturing SDS–polyacrylamide electrophoresis (10%) proteins were transferred to supported nitrocellulose membranes (0.45 μm, Whatman, Dassel, Germany) using a semidry blotting apparatus (BioRad, Munich, Germany) in transfer buffer (50 mM CAPS, 1 mM 3-mercaptopropionic acid, 10% methanol, pH 10). Membranes were stained with ponceau red for control and blocked with TBS (Tris/Cl, 50 mM, pH 7.5, NaCl 150 mM, 2% BSA, 0.2% NP-40, 0.03% NaN_3). For detection, membranes were incubated with phospho-modification specific monoclonal rabbit antibodies (Cell Signalling NEB, Frankfurt, Germany) overnight at 4 °C, and after three washes in TBS-NP40, bound antibodies were detected with secondary peroxidase conjugated antibodies (Dianova, Hamburg, Germany) and chemiluminescence-luminol reagent (Tris/Cl 50 mM, pH 8.5, 2.5 mM luminol, 400 μM *p*-coumaric acid) using a Fuji LAS 3000 imager. Data were quantified using AIDA 3.5 software.

RNA Isolation and cDNA Synthesis. Culture medium was removed and 250 μL of Trizol Reagent (Invitrogen, Karlsruhe, Germany) was added per well for RNA extraction according to the manufacturer's protocol. For reverse transcription, 1.2 μg of RNA was incubated with 0.125 pmol of T_{18} primer (Operon, Cologne, Germany), 0.4 mM dNTPs (GeneCraft, Münster, Germany), and 60 units Revert Aid reverse transcriptase (MBI Fermentas, St. Leon-Roth, Germany). Reverse transcription was performed at 42 °C for 1 h, followed by 10 min at 70 °C in a thermocycler (MWG-Biotech, Ebersberg, Germany).

RESULTS

Protein hydrolysates were prepared from bovine casein and soy protein preparations by limited digestion, using pepsin, trypsin, chymotrypsin, and elastase in various combinations. For all protein sources, hydrolysates could be obtained that lowered the activity of the *cyp7a-luc* reporter gene (**Table 1**) at a fixed concentration of 5 mg/mL. However, activity depended on protease combinations used for digestion and molecular mass range of the product. It was also critical to inactivate residual trypsin activity by heating, as low amounts of active trypsin caused a strong reduction of luciferase activity (data not shown). This effect became evident after 4−6 h and remained stable for up to 24 h (data not shown). Cell vitality was usually unaffected under assay conditions, as judged from the MTT

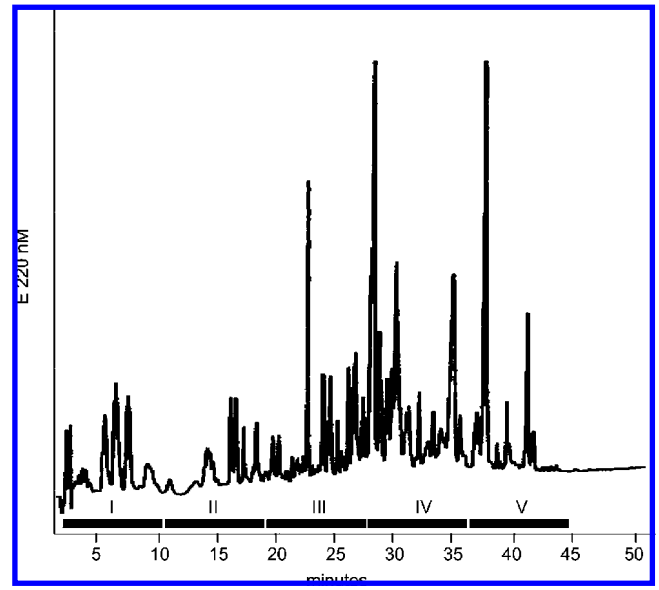

Figure 1. HPLC fingerprint of casein hydrolysate C//P+T (<1 kDa). Hydrolysates were subjected to reversed-phase HPLC as described in Materials and Methods. Fractions I−V, corresponding to **Figure 2**, are indicated.

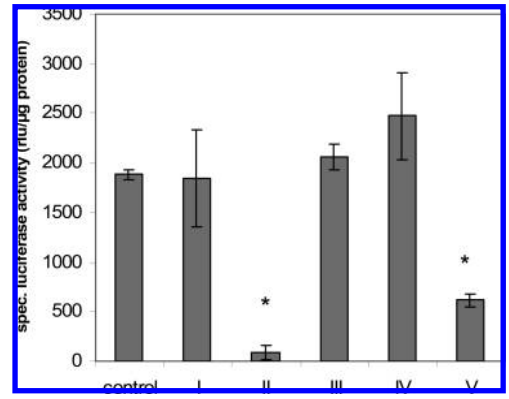

Figure 2. Effect of fractions I−V of C//P+T (<1 kDa) on the activity on the *cyp7a-luc* reporter gene. CRL-11997 cells, harboring the *cyp7a-luc* reporter gene, were incubated with the fractions for 16 h and luciferase activity and protein content were determined as described in Materials and Methods. Error bars represent the standard deviation of four replicates each. Significance was calculated using Student's *t* test. *, $p < 0.05$.

assay (data not shown) and optical inspection. In control experiments with selected casein hydrolysates and insulin, the mRNA-amount of *cyp7a* correlated well with reporter gene activity (data not shown).

Active hydrolysates were further fractionated by reversed-phase HPLC. In a first step, five fractions were collected based on hydrophobicity as shown in **Figure 1** for a casein hydrolysate. From several casein hydrolysates two active subfractions could be obtained (**Figure 2**). When these fractions were further separated into single peaks, the activity was lost. In the case of soy hydrolysates, no active fractions could be identified after chromatographic separation.

We then analyzed early signal-tranduction events that are associated with *cyp7a* regulation, such as MAP-kinase signaling. Interestingly, a strong phosphorylation of protein kinase b (PKB, AKT) was observed for several hydrolysates, 15 min after addition. Stress associated protein kinase (SAPK), cJUN-kinases (JNK), p38 MAPK, and extracellular signal regulated kinase (p42/44 ERK) were also affected (**Figure 3**).

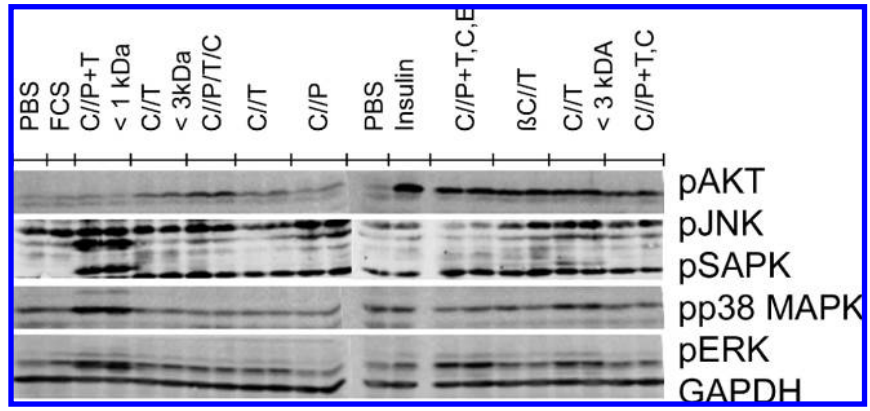

Figure 3. MAP-kinase signaling is activated by casein hydrolysates. HEP-G2 cells were stimulated for 15 min with the hydrolysate indicated (5 mg/mL) and phosphorylated kinases were visualized by Western blotting. For hydrolysate designations, refer to **Table 1**. Experiments were performed in duplicate, and both results of a representative experiment are shown. PBS represents the solvent control, and FCS and insulin served as positive controls. For loading control, GAPDH was included in Western blot experiments.

Table 2. Peptides Identified in Active Fractions of Casein Hydrolysates by Mass Spectrometry[a]

sequence	origin
DMPIQAF	β-casein
DMPIQAFL	β-casein
FLL	β-casein
FPPQSVL	β-casein
GPFPII	β-casein
GPFPIIV	β-casein
LGPVR	β-casein
LLYQEPVLGPVR	β-casein
QPEVLGVSKVKEAMA	β-casein
H**QGLPQEVLNENLLR C_1**	α_{S1}-casein
GLPQEV**LNENLLR C_1**	α_{S1}-casein
LNENLLR C_1	α_{S1}-casein
NENLLR C_1	α_{S1}-casein
PQEVLNENLLR C_1	α_{S1}-casein
VAPFPEVFGK C_2	α_{S1}-casein
FVAPFPEVFGK C_2	α_{S1}-casein
PFPEVFGK C_2	α_{S1}-casein
VAPFPEVF C_2	α_{S1}-casein
YKVPQLEIVPN C_3	α_{S1}-casein
YPELFR C_4	α_{S1}-casein
AYF**YPEL C_4**	α_{S1}-casein
YF**YPEL C_4**	α_{S1}-casein
YVPLGTQYTDAPSFSDIPNPIGSENSEK C_5	α_{S1}-casein
YLGYLEQLLR	α_{S1}-casein
YQLDAYPSGAW	α_{S1}-casein
YLGYL	α_{S1}-casein
PEL	α_{S1}-casein
EPMIGVNQELAY	α_{S1}-casein
TTMPLW	α_{S1}-casein
FAL	α_{S2}-casein
FALPQY	α_{S2}-casein
FALPQYL	α_{S2}-casein
FALPQYLK	α_{S2}-casein
FPQYLQY	α_{S2}-casein
FPQYLQYL	α_{S2}-casein
WQVL	κ-casein
YIPIQY	κ-casein

[a] Peptides C_1−C_5 from α_{S1}-casein and peptides containing partial sequences are indicated in bold.

Although single peak fractions were not active in the *cyp7a* reporter gene assay, we were interested in effects of single peptides that were present in the active fractions as it could be that such peptides were lost or diluted during the purification of single peaks. Furthermore, because hydrolysates derived from α-casein were as active as hydrolysates of total bovine casein, we decided to focus on peptides derived from α_{S1}-casein for further studies. On the basis of the mass spectrometric determination of peptide sequences, five peptides were synthesized that were present in most of the active fractions (**Table 2**, **Figure 4**), and their ability to alter *cyp7a* expression and to activate signal-transduction pathways was tested. None of these peptides affected *cyp7a-luc* expression after 6−16 h exposure, but two peptides had effects on early events, i.e. MAP-kinase phosphorylation. Peptide C_1 was able to activate AKT, SAPK, JNK, and ERK signaling at concentrations of about 1 mg/mL (0.6 mM), whereas peptide C_2 repressed AKT-phosphorylation at 1 mg/mL (0.7 mM) but showed weak activation of SAPK/JNK and strong activation of ERK at this concentration. Both peptides were also able to activate p38-MAPK; again peptide C_1 was more effective in this manner (**Figures 5** and **6**). Phosphorylation of p38 MAPK was enhanced by C_2 at concentrations down to 0.0016 mg/mL (ca. 10^{-6} M).

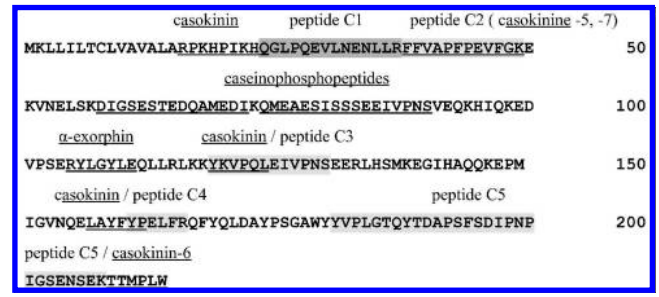

Figure 4. Sequence of bovine α_{S1}-casein. Previously described bioactive sequences are underlined and the five peptides that were selected as putative bioactive compounds are indicated.

DISCUSSION

In recent years, a multitude of bioactive peptides has been isolated from milk proteins (*11, 42*). These peptides exhibit a variety of effects in vitro and also in vivo. They either stimulate or antagonize opioid receptors, inhibit angiotensin converting enzyme (ACE), support immune responses by stimulating phagocytosis or proliferation of lymphocytes, carry calcium ions, and have antimicrobial effects. Many of these peptides derive from α_{S1}-casein. These include α-casomorphins (exorphins), α-casokinins, and caseinophosphopeptides (**Figure 4**).

Peptides from soy protein were described as antihyperlipidemic (*43–45*) if compared to a casein-based diet. Cholesterol homeostasis is determined by endogenous synthesis, intestinal uptake, degradation, and excretion (*46*). The accumulation of LDL-bound cholesterol in blood plasma is considered as a major

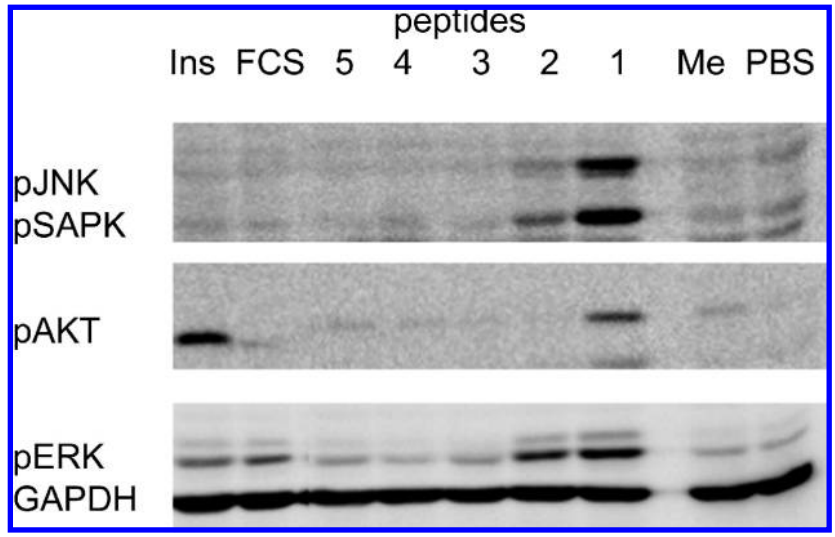

Figure 5. Effect of five selected peptides (1 mg/mL; corresponding to 0.6 mM C_1, 0.7 mM C_2 and C_3, 1.2 mM C_4, and 0.3 mM C_5) from α_{S1}-casein on the phosphorylation of MAP-kinases after 15 min incubation. Me, growth medium control; PBS, solvent control. Insulin and FCS (10%) served as positive controls. GAPDH was included as loading control.

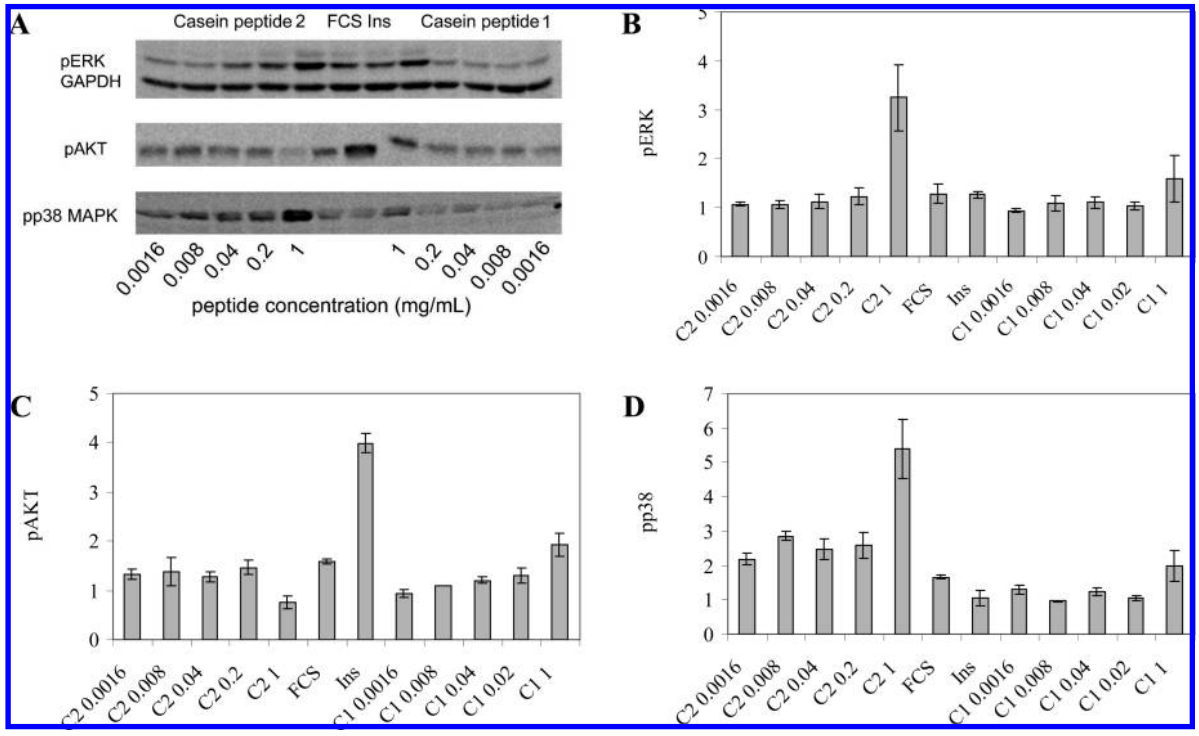

Figure 6. Dose response of MAPK activation by casein peptides C_1 and C_2 after 15 min incubation time. (**A**) Western blots of one representative experiment are shown. Concentrations are given in mg/mL. Fetal calf serum (FCS, 10%) and insulin were included as positive controls. (**B**, **C**, **D**) Quantification of MAPK-phosphorylation. Signals are normalized to GAPDH and control experiments are set to 1. Standard error (SEM) is given.

risk factor for atherosclerosis. Cholesterol synthesis, uptake of LDL-cholesterol by the LDL-receptor, and cholesterol degradation are regulated in a highly concerted manner, mainly by end product feedback regulation (**Figure 7**). Intracellular cholesterol levels are sensed by the SREBP system, whereas oxidized sterols are detected by the nuclear liver-X-receptor and bile acids by the nuclear farnesyl-X-receptor (*46*). If cellular cholesterol concentrations are high, proteolytic SREBP activation is inhibited; thus, expression of lipogenic enzymes and LDL-receptor is repressed (*47*). Furthermore, the amount of oxidized sterols increase with cholesterol concentration, which results in LXR activation, which then activates bile acid synthesis by increasing *cyp7a* transcription. If subsequently bile acid concentration rises, FXR is activated, which results in *cyp7a* repression. Both processes are also under hormonal control. Insulin downregulates *cyp7a* expression while upregulating LDL-receptor transcription via SREBPs. In this paper, we present evidence that bioactive peptides released from food protein hydrolysates can interfere with this regulation of bile acid synthesis and may result in decreased degradation of cholesterol to bile acids.

Hydrolysates were obtained by a method that mimicked gastrointestinal conditions. We therefore assume that these hydrolysates are similar to the products of human digestion in adults. On the basis of HPLC fingerprints, the method produced a magnitude of peptides in a reproducible manner.

For the screening approach, such hydrolysates were directly analyzed for their ability to influence the activity of a *cyp7a-luc* reporter gene, which was stably introduced into the human

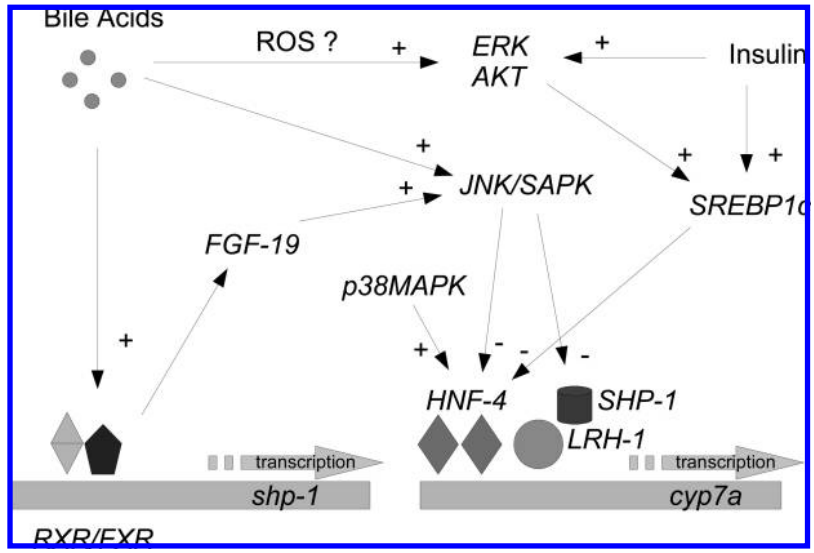

Figure 7. Summary of signal transduction pathways regulating *cyp7a* expression. In the presence of bile acids, feedback inhibition of *cyp7a* transcription is achieved by binding to the farnisoyl-X-receptor (FXR), which usually forms a heterodimer with the retinoid X-receptor (RXR) and then binds to the bile acid response elements in several genes (*50*). It thereby activates synthesis of the small heterodimer partner (SHP-1) and fibroblast growth factor-19 (FGF-19). SHP-1 binds to liver receptor homologue 1 (LRH-1) and inactivates the hepatic nuclear factor 4 (HNF-4) and LRH-1 dependent transcription of *cyp7a* (*51*). FGF-19 activates JNK via its receptor FGFR4 (*52*). Activation of JNK results in increased phosphorylation of SHP-1 and HNF-4, which further increases *cyp7a* repression (*53, 54*). Additionally, bile acids were found to activate the kinases ERK and AKT (*55*), which are also parts of the insulin signaling cascade. Long-term exposure (several hours) to insulin results in repression of the *cyp7a* gene. Sterol regulatory element binding protein 1c (SREBP1c) is activated by insulin and also phosphorylated by ERK (*56*), which results in inhibition of HNF-4. HNF-4 is phosphorylated at many residues by several kinases, such as AMPK, PKC and p38MAPK. As a result of these phosphorylations, DNA-binding activity and biological half-life is modulated, which results in regulation of the *cyp7a* gene (*30*).

hepatoma cell line HEP-G2, an established model for the analysis of cholesterol and lipid homeostasis (*35*). Activity of the hydrolysates was analyzed under serum free conditions, where repression of the reporter gene by insulin and bile acid conjugates can be observed. Bioactive hydrolysates were identified that decreased luciferase expression at concentrations above 2 mg/mL, but no obvious correlation with the particular nutritive protein and combination of proteases could be observed.

In order to isolate active peptides, the hydrolysates were fractionated by reversed-phase HPLC. First, five fractions based on hydrophobicity were isolated and analyzed for activity. Interestingly, for soy protein, we could not obtain active HPLC fractions with the *cyp7a-luc* assay, whereas from most active casein hydrolysates two active fractions were isolated. An example is shown in **Figure 2**. When these active casein hydrolysate fractions were further purified into single peak fractions, the activity in the bioassay could not be further resolved. We have no convincing explanation for the observation that the activity of soy protein hydrolysates could not at all be enriched by RP-HPLC. Nevertheless, a repression of *cyp7a-luc* expression might rely on unspecific effects of the hydrolysates, or the active compounds are simply lost during chromatography. It should also be noted that soy protein was described as hypolipidemic, and a repression of bile acid synthesis would counteract these effect.

For the *cyp7a-luc* bioassay, hydrolysates and peptides were incubated with the cells for at least 6 h, which might not be suitable for the analysis of low amounts of pure peptide, because the stability of isolated peptides under cell culture conditions is limited. The half-life of peptides in HEP-G2 cell culture supernatants depends on the peptide sequence and ranges from 2 to 6 h (F. Hirche, University of Halle, Germany, unpublished). However, it is also possible that active compounds were lost during chromatography and drying or that active peptide sequences were present in a variety of peptides with diverse hydrophobicity and could therefore not be separated by RP-HPLC into single peaks. Indeed, shortened versions of C_1 and C_2 were identified in the bioactive fractions (**Table 2**). Additionally, the failure of chromatographic separation could also be due to necessary, synergistic effects of individual compounds.

To further simplify the system, we focused on the peptides derived from bovine α_{S1}-casein, as we could show that active hydrolysates could also be obtained from α-casein (data not shown). We were able to identify five peptides that were apparently present in most of the active α-casein hydrolysate fractions. These peptides were synthesized and analyzed with the *cyp7a-luc* reporter assay, but disappointingly, no effect could be observed. Consistent with our theory that isolated peptides are not stable enough to influence the *cyp7a* expression, we attempted to analyze early signaling events that are involved in bile acid synthesis or lipid metabolism in general. An early event in insulin signal transduction is the phosphorylation of AKT but also ERK. The activation of the JNK is also implicated in the regulation of *cyp7a*. Indeed, an influence of the hydrolysates upon phosphorylation of these kinases could be observed, which suggests that their activation is linked with *cyp7a* repression, as it is in case of insulin action. With two of the selected peptides derived from α_{S1}-casein, these early effects could be observed. However, the peptides exhibited differential activation of AKT, and peptide C_1 resulted in a stronger activation of the kinases. Interestingly, peptide 2 contained two peptide sequences that were described as casokinin-5 and -7. As ACE inhibition cannot be the reason for the effects of the peptides, another independent mechanism must be responsible for the effects. One possible mechanism is receptor-mediated activation of signaling mechanisms. Another hypothesis might be the uptake of the peptides and intracellular interference with signaling events, but as kinase signaling was evident already after 15 min of incubation, we think that such mechanisms are unlikely. It has been proposed by Wissler et al. (*48*) that casein hydrolysates contain peptides

that exhibit insulin-like activity, and such compounds were called casoinsulins or exinsulins. Also, a patent has been filed by Wissler et al. describing peptide sequences from caseins that have insulin-like structure (*49*). Although none of the peptides identified in our study shares the described active sequences, the effects of casein peptide 2, especially the activation of AKT, is consistent with an insulin-like action on the HEP-G2 cells. However, further experiments are needed to confirm that the peptides indeed interact with the insulin or insulin-like growth factor receptor to facilitate AKT phosphorylation. Also, the link between the responses to the peptides and the primary effect on *cyp7a* regulation, observed with crude and purified hydrolysates, should be further investigated. Last but not least, the results from the HEP-G2 cell culture model must be tested in feeding experiments to establish a link between casein and casein peptide consumption and *cyp7a* downregulation and, furthermore, possible proatherogenic effects. Nevertheless, this study provides evidence for the first time that casein peptides might be able to influence bile acid synthesis mediated by MAP-kinase signaling.

ACKNOWLEDGMENT

Sandra Barth and Dieter Peschel are acknowledged for excellent technical assistance.

LITERATURE CITED

(1) Torres, N.; Torre-Villalvazo, I.; Tovar, A. R. Regulation of lipid metabolism by soy protein and its implication in diseases mediated by lipid disorders. *J. Nutr. Biochem.* **2006**, *17*, 365–373.

(2) Sirtori, C. R.; Even, R.; Lovati, M. R. Soybean protein diet and plasma cholesterol: From therapy to molecular mechanisms. *Ann. N.Y. Acad. Sci.* **1993**, *676*, 188–201.

(3) Sirtori, C. R.; Lovati, M. R.; Manzoni, C.; Castiglioni, S.; Duranti, M.; Magni, C.; Morandi, S.; D'Agostina, A.; Arnoldi, A. Proteins of white lupin seed, a naturally isoflavone-poor legume, reduce cholesterolemia in rats and increase LDL receptor activity in HepG cells. *J. Nutr.* **2004**, *134*, 18–23.

(4) Alper, C. M.; Mattes, R. D. Peanut consumption improves indices of cardiovascular disease risk in healthy adults. *J. Am. Coll. Nutr.* **2003**, *22*, 133–141.

(5) Bazzano, L. A.; He, J.; Ogden, L. G.; Loria, C.; Vupputuri, S.; Myers, L.; Whelton, P. K. Legume consumption and risk of coronary heart disease in US men and women: NHANES I epidemiologic follow-up study. *Arch. Intern. Med.* **2001**, *161*, 2573–2578.

(6) Deshpande, S. S. Food legumes in human nutrition: A personal perspective. *Crit. Rev. Food Sci. Nutr.* **1992**, *32*, 333–363.

(7) Duane, W. C. Effects of legume consumption on serum cholesterol, biliary lipids, and sterol metabolism in humans. *J. Lipid Res.* **1997**, *38*, 1120–1128.

(8) Kingman, S. M.; Walker, A. F.; Low, A. G.; Sambrook, I. E.; Owen, R. W.; Cole, T. J. Comparative effects of four legume species on plasma lipids and faecal steroid excretion in hypercholesterolaemic pigs. *Br. J. Nutr.* **1993**, *69*, 409–421.

(9) Martins, J. M.; Riottot, M.; de Abreu, M. C.; Lanca, M. J.; Viegas-Crespo, A. M.; Almeida, J. A.; Freire, J. B.; Bento, O. P. Dietary raw peas (*Pisum sativum* L.) reduce plasma total and LDL cholesterol and hepatic esterified cholesterol in intact and ileorectal anastomosed pigs fed cholesterol-rich diets. *J. Nutr.* **2004**, *134*, 3305–3312.

(10) Korhonen, H.; Pihlanto, A. Food-derived bioactive peptides—Opportunities for designing future foods. *Curr. Pharm. Des.* **2003**, *9*, 1297–1308.

(11) Meisel, H. Biochemical properties of regulatory peptides derived from milk proteins. *Biopolymers* **1997**, *43*, 119–128.

(12) Meisel, H.; Bockelmann, W. Bioactive peptides encrypted in milk proteins: Proteolytic activation and thropho-functional properties. *Antonie Van Leeuwenhoek* **1999**, *76*, 207–215.

(13) Meisel, H.; Frister, H.; Schlimme, E. Biologically active peptides in milk proteins. *Z. Ernahrungswiss* **1989**, *28*, 267–278.

(14) Jiang, J.; Chen, S.; Ren, F.; Luo, Z.; Zeng, S. S. Yak milk casein as a functional ingredient: Preparation and identification of angiotensin-I-converting enzyme inhibitory peptides. *J. Dairy Res.* **2007**, *74*, 18–25.

(15) Tauzin, J.; Miclo, L.; Gaillard, J. L. Angiotensin-I-converting enzyme inhibitory peptides from tryptic hydrolysate of bovine alphaS2-casein. *FEBS Lett.* **2002**, *531*, 369–374.

(16) He, G. Q.; Xuan, G. D.; Ruan, H.; Chen, Q. H.; Xu, Y. Optimization of angiotensin I-converting enzyme (ACE) inhibition by rice dregs hydrolysates using response surface methodology. *J. Zhejiang Univ. Sci. B* **2005**, *6*, 508–513.

(17) Je, J. Y.; Park, P. J.; Kwon, J. Y.; Kim, S. K. A novel angiotensin I converting enzyme inhibitory peptide from Alaska pollack (Theragra chalcogramma) frame protein hydrolysate. *J. Agric. Food Chem.* **2004**, *52*, 7842–7845.

(18) Mallikarjun Gouda, K. G.; Gowda, L. R.; Rao, A. G.; Prakash, V. Angiotensin I-converting enzyme inhibitory peptide derived from glycinin, the 11S globulin of soybean (*Glycine max*). *J. Agric. Food Chem.* **2006**, *54*, 4568–4573.

(19) Lovati, M. R.; Manzoni, C.; Gianazza, E.; Arnoldi, A.; Kurowska, E.; Carroll, K. K.; Sirtori, C. R. Soy protein peptides regulate cholesterol homeostasis in Hep G2 cells. *J. Nutr.* **2000**, *130*, 2543–2549.

(20) Leibach, F. H.; Ganapathy, V. Peptide transporters in the intestine and the kidney. *Annu. Rev. Nutr.* **1996**, *16*, 99–119.

(21) Wu, J.; Ding, X. Hypotensive and physiological effect of angiotensin converting enzyme inhibitory peptides derived from soy protein on spontaneously hypertensive rats. *J. Agric. Food Chem.* **2001**, *49*, 501–506.

(22) Fosset, S.; Fromentin, G.; Gietzen, D. W.; Dubarry, M.; Huneau, J. F.; Antoine, J. M.; Lang, V.; Mathieu-Casseron, F.; Tomé, D. Peptide fragments released from Phe-caseinomacropeptide in vivo in the rat. *Peptides* **2002**, *23*, 1773–1781.

(23) Ziv, E.; Bendayan, M. Intestinal absorption of peptides through the enterocytes. *Microsc. Res. Tech.* **2000**, *49*, 346–352.

(24) Bendayan, M.; Ziv, E.; Gingras, D.; Ben-Sasson, R.; Bar-On, H.; Kidron, M. Biochemical and morpho-cytochemical evidence for the intestinal absorption of insulin in control and diabetic rats. Comparison between the effectiveness of duodenal and colon mucosa. *Diabetologia* **1994**, *37*, 119–126.

(25) Bendayan, M.; Ziv, E.; Ben-Sasson, R.; Bar-On, H.; Kidron, M. Morpho-cytochemical and biochemical evidence for insulin absorption by the rat ileal epithelium. *Diabetologia* **1990**, *33*, 197–204.

(26) Assmann, G. Assessment of cardiovascular risk-PROCAM and new algorithms. *Biomed. Tech. (Berlin)* **2005**, *50*, 227–232.

(27) Jenkins, D. J.; Kendall, C. W.; Connelly, P. W.; Jackson, C. J.; Parker, T.; Faulkner, D.; Vidgen, E. Effects of high- and low-isoflavone (phytoestrogen) soy foods on inflammatory biomarkers and proinflammatory cytokines in middle-aged men and women. *Metabolism* **2002**, *51*, 919–924.

(28) Davis, R. A.; Miyake, J. H.; Hui, T. Y.; Spann, N. J. Regulation of cholesterol-7alpha-hydroxylase: BAREly missing a SHP. *J. Lipid Res.* **2002**, *43*, 533–543.

(29) Holt, J. A.; Luo, G.; Billin, A. N.; Bisi, J.; McNeill, Y. Y.; Kozarsky, K. F.; Donahee, M.; Wang, D. Y.; Mansfield, T. A.; Kliewer, S. A.; Goodwin, B.; Jones, S. A. Definition of a novel growth factor-dependent signal cascade for the suppression of bile acid biosynthesis. *Genes Dev.* **2003**, *17*, 1581–1591.

(30) Stroup, D. Kinase/phosphatase regulation of CYP7A1. *Front Biosci.* **2005**, *10*, 1678–1692.

(31) Xu, Z.; Tavares-Sanchez, O. L.; Li, Q.; Fernando, J.; Rodriguez, C. M.; Studer, E. J.; Pandak, W. M.; Hylemon, P. B.; Gil, G. Activation of bile acid biosynthesis by the p38 mitogen-activated protein kinase (MAPK): Hepatocyte nuclear factor-4alpha phosphorylation by the p38 MAPK is required for cholesterol 7alpha-hydroxylase expression. *J. Biol. Chem.* **2007**, *282*, 24607–24614.

(32) Li, T.; Kong, X.; Owsley, E.; Ellis, E.; Strom, S.; Chiang, J. Y. Insulin regulation of cholesterol 7alpha-hydroxylase expression in human hepatocytes: Roles of forkhead box O1 and sterol regulatory element-binding protein 1c. *J. Biol. Chem.* **2006**, *281*, 28745–28754.
(33) Twisk, J.; Hoekman, M. F.; Lehmann, E. M.; Meijer, P.; Mager, W. H.; Princen, H. M. Insulin suppresses bile acid synthesis in cultured rat hepatocytes by down-regulation of cholesterol 7 alpha-hydroxylase and sterol 27-hydroxylase gene transcription. *Hepatology* **1995**, *21*, 501–510.
(34) Subbiah, M. T.; Yunker, R. L. Cholesterol 7 alpha-hydroxylase of rat liver: An insulin sensitive enzyme. *Biochem. Biophys. Res. Commun.* **1984**, *124*, 896–902.
(35) Wang, D. P.; Stroup, D.; Marrapodi, M.; Crestani, M.; Galli, G.; Chiang, J. Y. Transcriptional regulation of the human cholesterol 7 alpha-hydroxylase gene (CYP7A) in HepG2 cells. *J. Lipid Res.* **1996**, *37*, 1831–1841.
(36) Schoeps, R.; Koerting, R.; Nass, N.; Schmelzer, C. E. H. Physiological-like food protein hydrolysates: A strategy to identify peptides modulating atherosclerosis-relevant factors. *NAFAS* **2005**, *3*, 59–64.
(37) Perkins, D. N.; Pappin, D. J.; Creasy, D. M.; Cottrell, J. S. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **1999**, *20*, 3551–3567.
(38) Schmelzer, C. E.; Schops, R.; Reynell, L.; Ulbrich-Hofmann, R.; Neubert, R. H.; Raith, K. Peptic digestion of beta-casein time course and fate of possible bioactive peptides. *J. Chromatogr. A* **2007**, *1166*, 108–115.
(39) Schmelzer, C. E.; Schops, R.; Ulbrich-Hofmann, R.; Neubert, R. H.; Raith, K. Mass spectrometric characterization of peptides derived by peptic cleavage of bovine beta-casein. *J. Chromatogr. A* **2004**, *1055*, 87–92.
(40) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55–63.
(41) Dyer, B. W.; Ferrer, F. A.; Klinedinst, D. K.; Rodriguez, R. A noncommercial dual luciferase enzyme assay system for reporter gene analysis. *Anal. Biochem.* **2000**, *282*, 158–161.
(42) FitzGerald, R. J.; Murray, B. A.; Walsh, D. J. Hypotensive peptides from milk proteins. *J. Nutr.* **2004**, *134*, 980S–988S.
(43) Sirtori, C. R.; Lovati, M. R. Soy proteins and cardiovascular disease. *Curr. Atheroscler. Rep.* **2001**, *3*, 47–53.
(44) Tovar, A. R.; Murguia, F.; Cruz, C.; Hernandez-Pando, R.; Aguilar-Salinas, C. A.; Pedraza-Chaverri, J.; Correa-Rotter, R.; Torres, N. A soy protein diet alters hepatic lipid metabolism gene expression and reduces serum lipids and renal fibrogenic cytokines in rats with chronic nephrotic syndrome. *J. Nutr.* **2002**, *132*, 2562–2569.
(45) Shukla, A.; Brandsch, C.; Bettzieche, A.; Hirche, F.; Stangl, G. I.; Eder, K. Isoflavone-poor soy protein alters the lipid metabolism of rats by SREBP-mediated down-regulation of hepatic genes. *J. Nutr. Biochem.* **2007**, *18*, 313–321.
(46) Repa, J. J.; Mangelsdorf, D. J. The role of orphan nuclear receptors in the regulation of cholesterol homeostasis. *Annu. Rev. Cell Dev. Biol.* **2000**, *16*, 459–481.
(47) Osborne, T. F.; LaMorte, V. J. Molecular aspects in feedback regulation of gene expression by cholesterol in mammalian cells. *Methods* **1998**, *16*, 42–48.
(48) Wissler, J. H.; Georgi, G.; Luy, B.; Jennissen, H. P.; Boehm, G. Exsulins (casoinsulins): Nutrition-derived cryptic metalloregulated peptides with insulin mimicry and amyloid propensity. *Mol. Biol. Cell* **2002**, *Suppl. 13*, 392a.
(49) Wissler, J. H.; Georgi, G.; Boehm, G. Insulin Mimetic Amino Acid Sequences. European Patent EP1434797, 2004.
(50) Claudel, T.; Staels, B.; Kuipers, F. The farnesoid X receptor: A molecular link between bile acid and lipid and glucose metabolism. *Arterioscler. Thromb. Vasc. Biol.* **2005**, *25*, 2020–2030.
(51) Repa, J. J.; Mangelsdorf, D. J. Nuclear receptor regulation of cholesterol and bile acid metabolism. *Curr. Opin. Biotechnol.* **1999**, *10*, 557–563.
(52) Yu, C.; Wang, F.; Jin, C.; Huang, X.; McKeehan, W. L. Independent repression of bile acid synthesis and activation of c-Jun N-terminal kinase (JNK) by activated hepatocyte fibroblast growth factor receptor 4 (FGFR4) and bile acids. *J. Biol. Chem.* **2005**, *280*, 17707–17714.
(53) Gupta, S.; Natarajan, R.; Payne, S. G.; Studer, E. J.; Spiegel, S.; Dent, P.; Hylemon, P. B. Deoxycholic acid activates the c-Jun N-terminal kinase pathway via FAS receptor activation in primary hepatocytes. Role of acidic sphingomyelinase-mediated ceramide generation in FAS receptor activation. *J. Biol. Chem.* **2004**, *279*, 5821–5828.
(54) Gupta, S.; Stravitz, R. T.; Dent, P.; Hylemon, P. B. Down-regulation of cholesterol 7alpha-hydroxylase (CYP7A1) gene expression by bile acids in primary rat hepatocytes is mediated by the c-Jun N-terminal kinase pathway. *J. Biol. Chem.* **2001**, *276*, 15816–15822.
(55) Fang, Y.; Han, S. I.; Mitchell, C.; Gupta, S.; Studer, E.; Grant, S.; Hylemon, P. B.; Dent, P. Bile acids induce mitochondrial ROS, which promote activation of receptor tyrosine kinases and signaling pathways in rat hepatocytes. *Hepatology* **2004**, *40*, 961–971.
(56) Kotzka, J.; Lehr, S.; Roth, G.; Avci, H.; Knebel, B.; Muller-Wieland, D. Insulin-activated Erk-mitogen-activated protein kinases phosphorylate sterol regulatory element-binding Protein-2 at serine residues 432 and 455 in vivo. *J. Biol. Chem.* **2004**, *279*, 22404–22411.

Received for review September 21, 2007. Revised manuscript received March 6, 2008. Accepted March 17, 2008. This work was supported by the German Ministry of Education and Science (BMBF) as part of the research network Molecular Nutrition. Further support was granted by the Deutsche Forschungsgemeinschaft (DFG) to N.N. and A.S. (Si-1317/1-1).

JF072806P