Biosci. Biotechnol. Biochem., **71** (3), 821–825, 2007

Note

Effects of Dipeptides Having a C-Terminal Lysine on the Cholesterol 7α-Hydroxylase mRNA Level in HepG2 Cells

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Received November 14, 2006; Accepted December 27, 2006; Online Publication, March 7, 2007
[doi:10.1271/bbb.60639]

Inducing expression of the cholesterol-catabolizing enzyme cholesterol 7α-hydroxylase (CYP7A1) in the liver can be an effective strategy in preventing hypercholesterolemia and atherosclerosis. We used HepG2 cells to investigate the effects of 1 mM dipeptides having a C-terminal lysine group on the CYP7A1 mRNA level. We found that the dipeptides Asp-Lys, Glu-Lys, and Trp-Lys significantly increased the CYP7A1 mRNA level.

Key words: dipeptide; cholesterol 7α-hydroxylase; apolipoprotein B; HepG2 cells; lysine

Hypercholesterolemia is a serious risk factor for cardiovascular disease, one of the leading causes of death worldwide.[1,2] Although the suppression of hypercholesterolemia and the suppression of atherosclerosis are linked, current methods of treatment are incapable of decisively preventing or improving either condition. Researchers are working to solve the problem by developing good reformative theories and technologies to improve cholesterol metabolism. Part of their work focuses on dietary control, an important strategy in preventing and improving hypercholesterolemia.[3] Dietary protein, for example, can be used effectively to ameliorate hypercholesterolemia and atherosclerosis in animals and humans.[4,5] Yet the hypocholesterolemic action induced by dietary peptide remains something of a mystery.

In our work, we have identified the serum cholesterol-lowering peptide lactostatin (Ile-Ile-Ala-Glu-Lys: IIAEK), which comes from β-lactoglobulin in bovine milk.[6] Animal tests have shown that lactostatin provides a more potent serum cholesterol-lowering action than the drug β-sitosterol.[6] We have also reported that lactostatin induced changes in CYP7A1 mRNA in mice accompanying hypocholesterolemia.[7] However, the precise mechanism behind the hypocholesterolemic action of lactostatin remains unclear.

Cholesterol levels in the blood are determined by a number of factors, including cholesterol absorption, synthesis, storage, degradation, and elimination.[8] The liver-specific enzyme cholesterol 7α-hydroxylase (CYP7A1, EC 1.14.13.17) serves as a rate-limiting enzyme in the classical (neutral) pathway of bile acid conversion into cholesterol.[9] This enzyme, which is specifically expressed in the liver, provides a major route for the removal of excess cholesterol from the body. Overexpression of CYP7A1 has been reported to improve hypercholesterolemia and atherosclerosis in mice, suggesting that agents that activate CYP7A1, including natural substances, can play a role in improving the outcome in hypercholesterolemia and atherosclerosis.[10,11] The lipophilic liver X receptor (LXR) ligands are the only known agents capable of activating CYP7A1 gene transcription.[12,13] But LXR ligands are inappropriate for medical application due to their adverse effects.[14,15] Unfortunately, only limited information is available about the activator of human CYP7A1 gene transcription. Hence, studies of the activator of human CYP7A1 and the hepatic regulatory pathway for human CYP7A1 gene transactivation are crucial to the prevention and improvement of atherosclerosis. Recently we discovered that lactostatin in human hepatic HepG2 cells increases the mRNA level for CYP7A1, the rate-limiting enzyme in the synthesis of bile acids from cholesterol.[16] We also found that lactostatin treatment at concentrations between 10 μM and 1 mM increased the CYP7A1 mRNA level in a concentration-dependent manner, up to about double the pretreatment level.[16] Moreover, we found that the C-terminal lysine residue in the amino acid sequence configuring lactostatin plays an important role in the induction of CYP7A1 mRNA, and that dipeptide EK is the smallest unit in the lactostatin induction of CYP7A1 mRNA.[16] However, only limited information is available about the effects of dipeptides on cholesterol metabolism.

Therefore, in the present research, we investigated the

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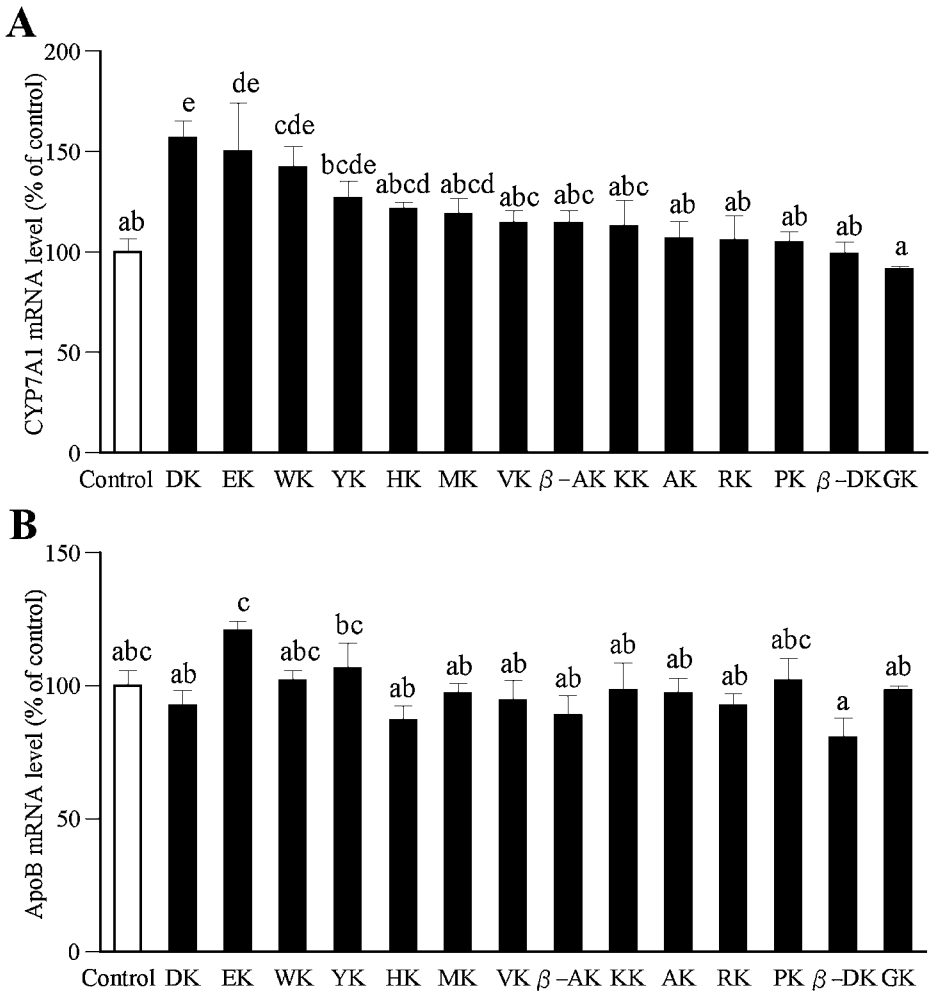

Fig. 1. Effects of Dipeptides Having a C-Terminal Lysine (K) on the Expression of CYP7A1 mRNA and ApoB mRNA in HepG2 Cells.

HepG2 cells were treated with 1 mM of dipeptide (DK, EK, WK, YK, HK, MK, VK, β-AK, KK, AK, RK, PK, β-DK, or GK) for 24 h. After treatment, total RNA was prepared. No dipeptide was added to the control. A, CYP7A1 mRNA; B, ApoB mRNA and β-actin mRNA were measured by real-time RT-PCR assay. The CYP7A1 mRNA levels and ApoB mRNA levels were expressed as percent of control calculated by CYP7A1 mRNA/β-actin mRNA or ApoB mRNA/β-actin mRNA. Values (% of control) were expressed as mean ± SEM (n = 3). Different superscript letters are significantly different ($p < 0.05$) by Duncan's multiple range test.

effects of 14 types of dipeptides (1 mM) with a C terminal lysine (Lys) on CYP7A1 mRNA levels in HepG2 cells. Additionally, apolipoprotein B (ApoB) is a major protein constituent of LDL (low-density lipoprotein). In humans there is a known association between ApoB elevation and hypercholesterolemia with elevated LDL cholesterol, suggesting the possibility that inhibition of ApoB production might be associated with inhibition of the development of hypercholesterolemia.[17] Hence we initiated an investigation into the effects of dipeptides on ApoB mRNA levels.

The 14 kinds of dipeptides with a C-terminal lysine ((Ala-Lys: AK, Val-Lys: VK, Pro-Lys: PK, Trp-Lys: WK, and β-Asp-Lys: β-DK (purity, > 95%, Bachem, Bubendorf, Switzerland); Asp-Lys: DK, Glu-Lys: EK, Gly-Lys: GK, His-Lys: HK, Lys-Lys: KK, Met-Lys: MK, Arg-Lys: RK, Tyr-Lys: YK, and β-Ala-Lys: β-AK (purity, > 95%, Kokusan Chemical, Tokyo, Japan)) and IIAEK (purity, > 95%, Peptide Institute, Osaka, Japan) were used in the present study. Cells of the human hepatoblastoma cell line, HepG2, were routinely grown in minimum essential medium (MEM) supplemented with heat-inactivated 10% (v/v) fetal bovine serum (FBS), then incubated in serum-free MEM during the dipeptide treatment periods, as shown in Fig. 1 and Fig. 2. After treatment, total RNA was isolated. Total RNA was isolated from HepG2 cells using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and treated with DNase I using an RNase-Free DNase Set (Qiagen). Total RNA was converted to cDNA using a High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was run on an ABI PRISM 7000 using a TaqMan® Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. TaqMan® Ribosomal RNA Control Reagents (Applied Biosystems) were used as primers, and the TaqMan® probe was used for 18S ribosomal RNA. The primers and TaqMan® probes for

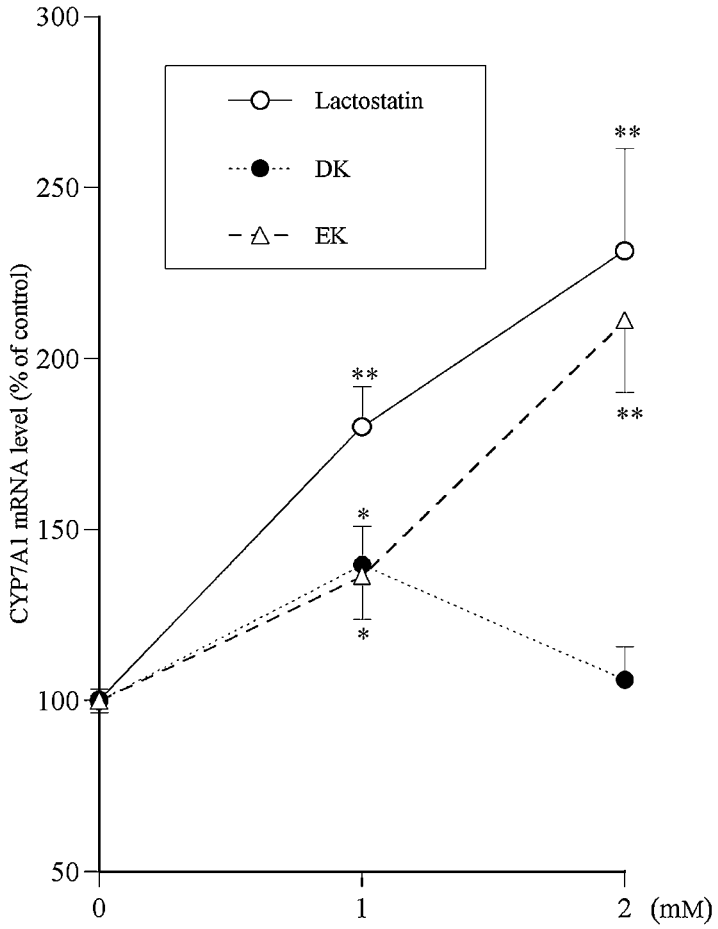

Fig. 2. The Concentration Dependent Effects of Lactostatin, DK, and EK on the Expression of CYP7A1 mRNA in HepG2 Cells.

HepG2 cells were treated with the indicated concentrations of lactostatin or dipeptide (DK or EK) for 24 h. After treatment, total RNA was prepared. No peptide was added to the control. CYP7A1 mRNA and 18S rRNA were measured by real-time RT-PCR assay. The CYP7A1 mRNA levels were expressed as percent of control calculated by CYP7A1 mRNA/18S rRNA. Values (% of control) were expressed as mean ± SEM (n = 3). Statistical significance as compared with control by Student's *t* test ($^{*}p < 0.05$, $^{**}p < 0.01$).

human CYP7A1 (Hs00167982 m1), human ApoB (Hs00181142 m1), and human β-actin (Hs99999903 ml) were purchased from Applied Biosystems as the TaqMan® Gene Expression Assay. The CYP7A1 and ApoB mRNA levels were expressed as the percent of control calculated by CYP7A1 mRNA/β-actin mRNA, ApoB mRNA/β-actin mRNA, or CYP7A1 mRNA/18S rRNA. Results were expressed as averages ± SEM. The statistical significance of differences was determined by Student's *t* test[18] and Duncan's multiple range test.[19]

In HepG2 cells, we found that dipeptides having a C-terminal lysine (K) affected the CYP7A1 mRNA level. We added 1 mM of each dipeptide to HepG2 cells, and cultured the samples for 24 h at 37 °C. The results of real-time RT (reverse transcription) PCR assay showed that the greatest rate of elevation in the CYP7A1 mRNA level was associated with the addition of DK (157%), followed by EK (150%), and then WK (142%) in comparison to the control (no added dipeptide, 100%). All of these increases were significant ($p < 0.05$) (Fig. 1A). No significant changes in comparison to the control (no added dipeptide) were noted with any of the other 11 types of dipeptide tested (Fig. 1A). Figure 1B shows the effect of dipeptides on ApoB mRNA levels, investigated in HepG2 cells as described in Fig. 1A. No significant changes in comparison to the control (no added dipeptide) were noted with any dipeptides tested (Fig. 1B).

Next we added 1 mM or 2 mM of lactostatin, DK, or EK to HepG2 cells, and cultured them for 24 h at 37 °C. The treatment by lactostatin and that by EK increased the CYP7A1 mRNA level in a concentration-dependent manner (Fig. 2). As shown in Fig. 1A, the CYP7A1 mRNA level was significantly increased by the treatment by 1 mM DK and that by 1 mM EK (Fig. 2). CYP7A1 mRNA tended to increase by the treatment by 2 mM DK.

In this research using HepG2 cells, we compared findings[16] for 1 mM lactostatin with C-terminal EK and for the other dipeptides (1 mM). The results showed that dipeptide DK (1 mM) provided more potent CYP7A1 mRNA-inducing activity than was observed with 1 mM EK. For culturing conditions, we used a peptide concentration of 1 mM, which elicits the maximum CYP7A1

mRNA-inducing activity by lactostatin in HepG2 cells.[16] Samples were cultured for 24 h. The differences in increase in CYP7A1 mRNA level that were seen with the addition of these dipeptides indicated the specificity of peptides with regard to CYP7A1 mRNA induction (in other words, cholesterol-catabolizing activity). In particular, the pronounced difference between DK and β-DK with regard to CYP7A1 mRNA induction suggests that there may exist a correlation between the activity of CYP7A1 mRNA induction and the peptide structure. The newly discovered 1 mM DK and 1 mM WK also act to increase CYP7A1 mRNA levels. This suggests a principle regarding the structure and activity of previously unobtainable peptides, and indicates the possibility that in the future, based on this principle, CYP7A1 genetic expression can be used as an indicator in technology for the identification of active peptides that will improve cholesterol metabolism, and for the creation of active peptides. Detailed studies on the concentration-dependent effects on CYP7A1 mRNA of dipeptides is necessary to determine the active amino acid sequence.

In our previous study, lactostatin exhibited hypocholesterolemic action in rats *in vivo* under oral administration of 200 mg/kg/d for 3 d.[6] If the absorption efficiency of lactostatin is 100%, this dose (200 mg/kg/d) is equivalent to about 0.35 mM of the *in vivo* concentration in the rat. Thus, this calculated *in vivo* concentration of 0.35 mM is sufficient for HepG2 study using 1 mM lactostatin. Moreover, if EK or DK is administered orally to a rat at the dose of 200 mg/kg/d, the calculated *in vivo* concentration of EK or that of DK is equivalent to about 0.73 mM or 0.77 mM respectively. Thus, these calculated concentrations of 0.73 mM and 0.77 mM are also sufficient for HepG2 study using 1 mM EK or 1 mM DK. However, lactostatin is superior to dipeptides EK or DK with regard to increasing the CYP7A1 mRNA level, as shown in Fig. 2. In fact, oral administration of EK produced a decrease in serum cholesterol (but no significant change) at a dose of 524 mg/kg/d for 4 d in mice (K. Morikawa, and S. Nagaoka, unpublished results). Further studies are needed to evaluate the hypocholesterolemic action induced by dipeptides *in vivo*.

In vivo degradation of cholesterol depends solely on the pathway for which hepatic CYP7A1 is the rate-limiting enzyme.[9] CYP7A1 activators, substances such as the transcription factor LXR ligand 22-hydroxycholesterol, are associated with the adverse drug reaction of triglyceride elevation, and thus cannot be used.[13,14] Little is known about useful activators of CYP7A1 gene expression. Taurine, which increases CYP7A1 mRNA in HepG2 cells, must be present at a relatively high concentration (20 mM) before providing this effect.[20] In our experimental system using HepG2 cells, we found that relatively low levels of dipeptide (1 mM) were sufficient to produce significant elevation, and that dipeptides DK, EK, and WK provided much greater effects on CYP7A1 than did taurine. There is a positive correlation between high blood levels of LDL and hypercholesterolemia.[17] In the present research, we found that DK, EK, and WK produced no change in ApoB mRNA level. We thus consider the DK, the EK, or the WK sequence to be an important key to future investigation and evaluation of new cholesterol metabolism-improving peptides. Research on the physiological functions of dipeptides, such as hypocholesterolemic action, should contribute to peptide utilization and to amino acid utilization for health promotion and disease prevention.

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