Regulation of expression of human intestinal bile acid-binding protein in Caco-2 cells

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Molecular mechanisms of the bile acid active transport system in the ileal enterocytes remain unknown. We examined whether bile acids affect human enterocyte gene expression of intestinal bile acid-binding protein (I-BABP), a component of this transport system. Differentiated Caco-2 cells were incubated in the presence of human bile, bile acids or other lipids. The level of I-BABP expression was evaluated by Northern and Western blot analyses. A 24 h incubation of Caco-2 cells in a medium containing either bile or bile acids resulted in a remarkable 7.5-fold increase in the I-BABP mRNA level over the control level. Neither cholesterol, palmitic acid, phosphatidylcholine nor cholestyramine treated bile showed any difference in I-BABP mRNA expression from the control. Bile acid treatment increased the level of I-BABP mRNA in Caco-2 cells in a time- and dose-dependent manner. Western blot analysis showed that this induction led to increase in cytosolic I-BABP. Chenodeoxycholic acid and deoxycholic acid showed greater induction effects than other hydrophilic bile acids, including their own glycine conjugates. Pretreatment by actinomycin D or cycloheximide completely inhibited the up-regulation of I-BABP expression by bile acid. Bile acids, especially lipophilic bile acids, increase the I-BABP expression in Caco-2-cells, suggesting that luminal bile acids play an important role in regulating the I-BABP gene expression.

INTRODUCTION

Bile acids, which are synthesized from cholesterol in the liver and secreted with bile into the small bowel, where they form micelles with luminal lipids, play an important role in efficient digestion and absorption of dietary fats [1]. On reaching the terminal ileum, the luminal bile acids are actively reabsorbed by enterocytes and are returned to the liver via portal circulation. This recycling system for bile acids (enterohepatic circulation) is important for maintenance of bile acid and cholesterol homoeostases [2]. In spite of many physiological and pharmacological studies which have been conducted since the early 1960s [3–5], the molecular mechanisms of the ileal bile acid transport system remain poorly understood.

Recently, biochemical studies using photoaffinity labelling techniques have identified many putative proteins that constitute this ileal bile acid transport system [6–8]. Of these candidates for bile acid transporters, two proteins are well characterized at the molecular level. The ileal Na^+-dependent bile acid transporter (ISBT) cDNA was isolated by expression cloning strategy and was shown to encode a 348-amino acid glycoprotein having seven potential transmembrane domains, suggesting that ISBT is an integral membrane transporter for luminal bile acids [9–11]. The other candidate for bile acid transporter is a 14–15 kDa cytosolic binding protein termed intestinal bile acid-binding protein (I-BABP [12] or I-15P [13]. Sequence analysis [12–14] revealed that I-BABP belongs to a family of hydrophobic ligand-binding proteins, the fatty acid-binding proteins (FABPs). In fact, several sets of data suggest that I-BABP is an intracellular transporter for bile acids [12,15]. Additionally, I-BABP may also be associated with the bile acid transporter found in the brush border of the ileal enterocytes [8]. However, the regulatory mechanisms for I-BABP expression in the ileal enterocytes remain unknown.

More recently, we have shown that human bile dramatically induces the expression of I-BABP mRNA [16]. To determine the biliary components responsible for this induction, we examined whether bile acids or other lipids derived from human bile affect I-BABP gene expression in Caco-2 cells, which share the morphological and functional properties of the ileal enterocytes, including transcellular transport of taurocholic acid [17–21]. In this study we provide direct evidence showing that bile acids, but not other lipids in human bile, up-regulate the gene expression of I-BABP in Caco-2 cells.

EXPERIMENTAL

Chemicals

Bile acids and their glycine conjugates were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Cholesterol, palmitic acid, phosphatidylcholine, cholestyramine, actinomycin D (AMD) and cycloheximide (CHX) were purchased from Sigma (St. Louis, MO, U.S.A.) All chemicals used were analytical grade.

Bile

Drainage bile was obtained from a 66-year-old woman who underwent choledocholithotomy and T-tube drainage on the seventh day after surgery, as approved by the Institutional Review Board of Niigata University School of Medicine. Total bile acid concentration was 18.4 mM. The composition of the

Abbreviations used: AMD, actinomycin D; CA, cholic acid; CDCA, chenodeoxycholic acid; CHX, cycloheximide; CYP7, cholesterol 7-α-hydroxylase gene; DCA, deoxycholic acid; GCA, glycocholic acid; GCDC, glycochenodeoxycholic acid; I-BABP, intestinal bile acid-binding protein; ISBT, ileal Na^+-dependent bile acid transporter; L-FABP, liver fatty acid-binding protein.

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bile acids, analysed by HPLC, was as follows: glycocholic acid (GCA), 11.5 mM; glycochenodeoxycholic acid (GCDC), 4.5 mM; taurocholic acid, 1.7 mM; taurochenodeoxycholic acid, 0.6 mM; and cholic acid (CA), 0.1 mM. Other bile acids and their conjugates were not detected. After addition 2.0 ml of the bile to 150 mg of cholestyramine, the mixture was agitated overnight at 4 °C. After centrifugation of the mixture, the supernatant was used as a bile acid-depleted bile. The total bile acid concentration was estimated at 0.5 mM. Bile samples were filtered through 0.2 μm cellulose acetate filters (Gelman Sciences, Ann Arbor, MI, U.S.A.) before use.

Cell culture

Caco-2 cells (passage number 40) obtained from the Riken Cell Bank (Saitama, Japan) were derived from the European Collection of Animal Cell Cultures (Wiltshire, U.K.: ECA86010202). Cells were maintained at 37 °C in 5 % CO_2 in Dulbecco's modified Eagle's medium (4.5 g glucose/l; Bio Whittaker, Wakersville, MD, U.S.A.) supplemented with 20 % (v/v) fetal calf serum (JRH Bioscience, Lenexa, KS, U.S.A.), 4 mM glutamine, 1 % non-essential amino acids and 60 μg/ml kanamycin. Media were changed every 2 days. The cells were plated onto 6.0-cm polystyrene culture dishes (Corning Glass Works, Corning, NY, U.S.A.) at approx. 1.4×10^4 cells/cm^2 and grown to total confluence with high differentiation (dome formation). All the cells used were from passage numbers 42–46.

Northern blot analysis

At the fourth day post-confluence, media were changed. The cells were incubated for an appropriate period in the medium containing bile, bile acids or other lipids. Total RNA was isolated following the method of Chromczynski and Sacchi [22] using an RNA preparation kit (Isogen; Nippon Gene Corp., Tokyo, Japan).

The RNA (20 μg) was fractionated by electrophoresis in a 1 % agarose gel containing formaldehyde and transferred to a Nytran nylon membrane (Schleicher and Schuell, Dassel, Germany). The membrane was hybridized with radioactive cDNA probes and the radioactive signals on the membrane were quantified by densitometric scanning with an image-analysing system (BAS 1000; Fuji Film Inc., Tokyo, Japan). The hybridization conditions have been described previously [23].

Molecular probes

As previously described [16], we used the full-length human I-BABP cDNA as a probe for Northern blot analysis. A human liver FABP (L-FABP) cDNA probe was synthesized by PCR from a human ileal cDNA library [15] using the primers corresponding to the C- and N-terminal regions based on the human L-FABP cDNA sequence [24]. A β-actin probe was prepared as previously described [25]. These probes were labelled with [α-^{32}P]dCTP (3000 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.) using a random-primed DNA labelling kit (Megaprime; Amersham, Buckinghamshire, U.K.).

Western blot analysis

Cells were harvested after 24 h incubation with chenodeoxycholic acid (CDCA) (250 μM). Homogenate was prepared in ice-cold 154 mM KCl/10 mM sodium phosphate (pH 7.4) containing 1 mM PMSF and then centrifuged for 10 min at 18000 ***g***. The resulting supernatant was centrifuged for 1 h at 105000 ***g***. Cytosolic proteins (30 μg) were fractionated by SDS/12 %-PAGE and transferred on to a PVDF membrane (New England). After blocking with 2 % BSA, the membrane was probed either with anti-human I-BABP antiserum (1:1000) or anti-rat L-FABP antiserum (1:1000). The sensitivities and specificities of these antisera have been described elsewhere [26,27]. Antigen–antibody complexes were revealed with enhanced chemiluminescence detection (New England Nuclear).

Inhibitor assay

Differentiated Caco-2 cells were preincubated for 30 min with AMD (4 μg/ml) or vehicle alone (0.1 % dimethylsulphoxide). This was followed by incubation with or without CDCA (250 μM) for an additional 12 h. After the treatments, total RNA was extracted and the transcripts were analysed by Northern hybridization. In experiments with CHX, cells were preincubated for 30 min with CHX (10 μM) or ethanol vehicle alone and then incubated with or without CDCA (250 μM) for an additional 24 h. Procedures for RNA extraction and Northern hybridization were similar to those used for the AMD experiments.

Statistical analysis

Values are expressed as means $\pm$ S.E.M. and were considered significant when P was < 0.05 using Student's t test for unpaired values.

RESULTS

Since our previous study showed that human bile is a potent inducer of I-BABP gene expression *in vivo* and *in vitro* [16], we first attempted to identify biliary factors responsible for this regulation using Caco-2 cells. We hypothesized that the major components of bile, i.e. bile acids, are physiological regulators of I-BABP expression [16]. To test this hypothesis, well-differentiated Caco-2 cells were incubated for 24 h in a medium containing human bile or a bile acid, then the levels of I-BABP mRNA were assessed by Northern blot analysis. As shown in Figure 1, the level of I-BABP mRNA in the control was very low.

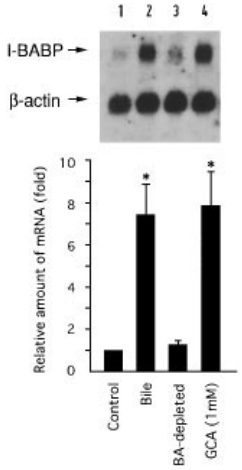

Figure 1 Effects of bile and bile acids on the expression of I-BABP mRNA in Caco-2 cells

The expression of I-BABP mRNA in Caco-2 cells was assessed by Northern blot analysis using human I-BABP cDNA as a probe. Representative results are shown in the upper panel. Lanes: 1, cells in control medium; 2, cells in the medium containing 5 % (v/v) of human bile; 3, cells in the medium containing cholestyramine-treated 5 % (v/v) human bile; 4, cells in the medium containing glycocholic acid (GCA) (1 mM). The level of I-BABP mRNA was quantified in the lower panel from three independent experiments. * $P < 0.02$ compared with control.

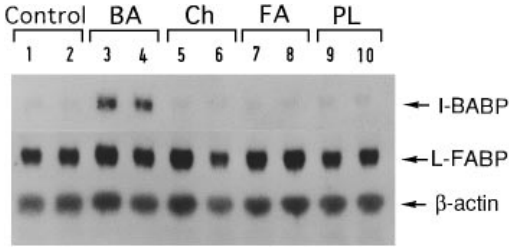

Figure 2 Specific regulation by bile acids of I-BABP gene expression

Caco-2 cells were incubated for 24 h in the medium containing a bile acid (BA; glycocholic acid, 1 mM), cholesterol (Ch; cholesterol, 100 μM), long-chain fatty acid, (FA; palmitic acid, 100 μM), or phospholipid (PL; phosphatidylcholine, 100 μM). Each experiment was done in duplicate. The blot was reprobed with human L-FABP and β-actin probes.

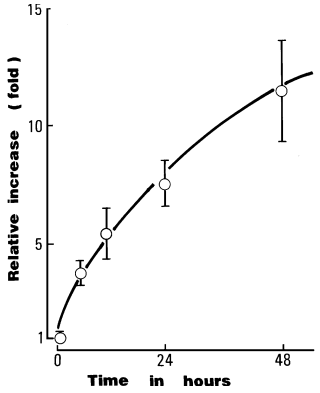

Figure 3 Time course of the expression of I-BABP mRNA in bile acid-treated Caco-2 cells

On the fourth day of post-confluence, the cells were incubated in medium containing 1 mM GCA. The level of I-BABP mRNA was evaluated by Northern blot analysis using a human I-BABP cDNA probe. Each point represents the mean $\pm$ S.E.M. ($n = 3$).

This level increased 7.46 $\pm$ 1.40-fold in Caco-2 cells incubated in a medium containing 5% (v/v) human bile. In contrast, the bile treated with the bile acid-sequestrant cholestyramine had little effect on the level of I-BABP mRNA (1.30 $\pm$ 0.18, $P = 0.275$). This suggests that bile acids are strong candidates as physiological regulators. Consistent with this finding, GCA, a major component of bile acids in human bile, increased the level of I-BABP mRNA in much the same way as when bile was added to the medium (7.87 $\pm$ 1.60). The level of β-actin mRNA was not significantly changed (control, 1.0 $\pm$ 0.09; bile, 0.98 $\pm$ 0.14; bile acid-depleted bile, 0.97 $\pm$ 0.03; GCA, 0.92 $\pm$ 0.04). No significant difference in cell morphology or viability between the control and treated cells was evidenced by microscopic observation or Trypan Blue exclusion tests, suggesting that this effect of GCA is not due to differences in cell growth and/or differentiation between the two experiments.

Next, to determine the inducing specificity of bile acids for the expression of I-BABP mRNA, we examined whether other lipid components in human bile could affect the expression of I-BABP mRNA. Caco-2 cells were incubated in a medium containing GCA (1 mM), cholesterol (100 μM), palmitic acid (100 μM) or phosphatidylcholine (100 μM); lipids other than GCA were used at a lower concentration due to their insolubility and/or cytotoxicity. As shown in Figure 2, the level of the I-BABP mRNA was increased markedly only with GCA treatment. No significant increase in the I-BABP mRNA level occurred in 24 h incubation with fatty acids, cholesterol or phospholipids. Furthermore, reprobing with a cDNA for L-FABP, a member of the FABP family, showed no significant changes in mRNA level after incubation with GCA or other lipids, indicating that bile acids in human bile specifically induce I-BABP gene expression.

To examine the time course of I-BABP mRNA induction by bile acid, Caco-2 cells were exposed to 1 mM GCA for 6–48 h. I-BABP mRNA expression was induced within 6 h, and the level was increased 7.5-fold after a 24 h incubation with GCA. A marked increase in the I-BABP mRNA level persisted throughout the experiment (to 48 h). Thus, treatment with bile acid (GCA) produced a time-dependent increase in the level of I-BABP mRNA (0 h, 1.0 $\pm$ 0.19; 6 h, 3.85 $\pm$ 0.33; 12 h, 5.41 $\pm$ 0.89; 24 h, 7.64 $\pm$ 0.78; 48 h, 11.4 $\pm$ 1.78) (Figure 3). Similar results were obtained by experiments using other bile acids (results not shown).

To compare the potency of bile acid derivatives in inducing the expression of I-BABP mRNA, Caco-2 cells were incubated with various bile acids or their glycine conjugates at a final concentration of 250 μM for 24 h. When the amount of I-BABP mRNA from cells treated with CA was defined as 1.0, results from treatment with other bile acids were as follows; CDCA, 7.09 $\pm$ 0.25; deoxycholic acid (DCA), 2.74 $\pm$ 0.42; lithocholic acid, 1.60 $\pm$ 0.19; GCA, 0.87 $\pm$ 0.09; GCDC, 1.26 $\pm$ 0.29; glycodeoxycholic acid, 0.77 $\pm$ 0.21; glycolithocholic acid, 0.98 $\pm$ 0.20 (Table 1). Thus, the inducing potencies of CDCA ($P < 0.01$) and DCA ($P < 0.02$) were significantly higher than those of other bile acids.

Table 1 Comparison of the potency of bile acids to induce the expression of I-BABP mRNA

Caco-2 cells were incubated for 24 h in a medium containing 250 μM of natural bile acid or the corresponding glycine conjugate. The relative amounts of I-BABP mRNA in Caco-2 cells are given as means $\pm$ S.E.M. for five independent experiments. The amount of I-BABP mRNA from the cells treated with CA is defined as 1.00. ** $P < 0.01$; * $P < 0.02$; no asterisk, not significant.

Condition	I-BABP mRNA
Cholic acid	1.00 $\pm$ 0.16
Chenodeoxycholic acid	7.09 $\pm$ 0.25**
Deoxycholic acid	2.74 $\pm$ 0.42*
Lithocholic acid	1.60 $\pm$ 0.19
Glycocholic acid	0.87 $\pm$ 0.09
Glycochenodeoxycholic acid	1.26 $\pm$ 0.29
Glycodeoxycholic acid	0.77 $\pm$ 0.21
Glycolithocholic acid	0.98 $\pm$ 0.20

Table 2 Effects of bile acid concentration on the expression of I-BABP mRNA

Caco-2 cells were incubated for 24 h in a medium containing CDCA at concentrations of 10, 50, or 250 μM. The level of I-BABP mRNA was evaluated by Northern blot analysis using a human I-BABP cDNA probe. The relative amounts of I-BABP mRNA are given in the form of means $\pm$ S.E.M. for three independent experiments. * $P < 0.05$.

Concentration (μM)	I-BABP mRNA
Control	1.00 $\pm$ 0.05
10	1.49 $\pm$ 0.28
50	2.34 $\pm$ 0.25*
250	15.4 $\pm$ 2.3*

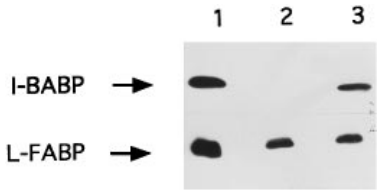

Figure 4 Effect of bile acid on the cytosolic I-BABP and L-FABP levels in Caco-2 cells

Cells were incubated for 24 h in the presence of 250 μM CDCA. Control Caco-2 cells were cultured without bile acid. The cytosolic level of I-BABP and L-FABP were evaluated by Western blot. Lane 1, purified I-BABP or L-FABP (100 ng); lane 2, culture without bile acid (control); lane 3, culture with CDCA. Each experiment was done in duplicate.

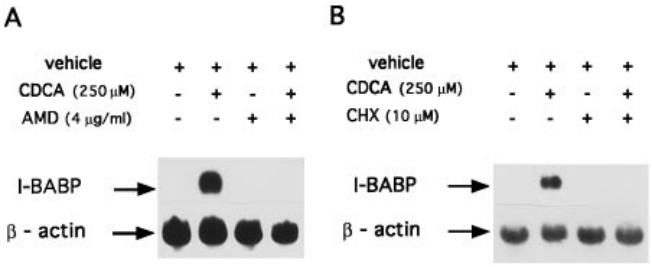

Figure 5 Effect of AMD or CHX on the up-regulation of I-BABP mRNA level by CDCA in Caco-2 cells

(**A**) Cells were preincubated for 30 min with AMD (4 μg/ml) or vehicle alone (0.1% DMSO) and then incubated for an additional 12 h with 250 μM CDCA or vehicle alone. The Figure represents Northern blot analysis of three independent experiments. (**B**) Cells were preincubated for 30 min with CHX (10 μM) or vehicle alone (0.1% absolute ethanol) and then incubated for an additional 24 h with 250 μM CDCA or vehicle alone. The Figure is representative of four independent experiments.

Additionally, we examined the effect of bile acid (CDCA) concentrations on the expression of I-BABP mRNA. Caco-2 cells were incubated for 24 h in a medium containing the indicated concentrations of CDCA. As shown in Table 2, CDCA triggered a dose-dependent increase in the I-BABP mRNA level (control, 1.0 ± 0.05; 10 μM, 1.49 ± 0.28; 50 μM, 2.34 ± 0.25, 250 μM, 15.4 ± 2.3).

To determine whether this up-regulation led to an increase in the cytosolic I-BABP content, Western blot analysis was carried out. In contrast to L-FABP, I-BABP protein level was undetectable in Caco-2 cells cultured in standard medium (Figure 4, lane 2). However, 24 h after addition of 250 μM CDCA, a strong signal was found in cytosol while the FABP level remained unchanged (Figure 4, lane 3).

Finally, in order to explore the mechanism by which bile acids might induce I-BABP expression, Caco-2 cells were cultured with AMD or CHX, which are respectively known to be strong inhibitors of gene transcription and protein synthesis. Both AMD and CHX totally suppressed induction of the expression of I-BABP mRNA by CDCA (Figure 5). These inhibitions were gene-specific and were not due to toxic effects of these drugs since β-actin mRNA level was unchanged.

DISCUSSION

The present study using Caco-2 cells demonstrates three major points with regard to regulation of I-BABP gene expression.

First, bile acids in human bile specifically induce the expression of the I-BABP gene in a time- and dose-dependent manner. Although bile is of complex composition, containing bioactive peptides such as epidermal growth factor [28], the pretreatment of bile with heat or protease did not eliminate its inducing activity (results not shown). In contrast, the inducing action of I-BABP was completely eliminated in cholestyramine-treated bile. GCA, one of the main bile acids in human bile, caused an increase in the I-BABP mRNA level compatible with that caused by bile. Taken together, these findings strongly suggest that bile acid is the biliary factor responsible for the induction of I-BABP expression by bile. Fatty acids, cholesterol or phospholipids, minor components of the biliary lipids, showed no significant effect on I-BABP expression, supporting the specific induction by bile acids. In the initial series of experiments (Figures 1–3), 1 mM GCA provided gene induction similar to that found with 5% of bile. Based on HPLC analysis of human bile (see the Experimental procedures section), we chose GCA (1 mM) as a representative bile acid. The concentration of conjugated bile acids in the human terminal ileum has been reported to be 1–2 mM [29], close to the concentration at which substantial induction of I-BABP expression was observed in the present study. However, since bile acids in the terminal ileum bind to other intestinal components, the free bile acid concentration must be lower than 1–2 mM. The free bile acid concentration in the present study using Caco-2 cells could also be expected to be lower than the added concentrations, because bile acids will be bound to serum albumin in culture medium [15]. Importantly, the I-BABP mRNA level in Caco-2 cells, as mentioned below, was significantly increased by treatment with a much lower concentration (0.05 mM) of CDCA (Table 2). Thus, I-BABP gene expression might be physiologically up-regulated by bile acids at micromolar doses.

Secondly, the potency of gene-inducing actions varies among the bile acids. Significantly, of the endogenous bile acids, the unconjugated dihydroxy bile acids (CDCA and DCA) proved to be particularly potent activators for inducing the expression of the I-BABP gene. The lipophilic properties of bile acids might be related to the intensity of the regulatory action which they provide. Since lipophilic unconjugated bile acids exhibit rapid transbilayer movement across artificial membranes [30], these substances may be present at higher cytosolic levels than hydrophilic bile acids in this simplified model. However, bile acid uptake by cells is more complex. Because bile-acid transport across the Caco-2 plasma membrane can be driven by an active Na^+-dependent process [19–21], the potency of bile acids to activate I-BABP gene expression is unlikely to depend only on the hydrophobic properties of these bile acids. Instead, the potency of individual bile acids to induce I-BABP gene expression may be affected by the affinity of each bile acid for putative nuclear receptors involved in this gene expression. In fact, as mentioned below, a putative nuclear receptor for bile acids could mediate regulation of the gene expression for proteins responsible for bile acid biosynthesis [31].

Thirdly, the present study shows that this up-regulation by bile acids, requiring *de novo* protein synthesis, is exerted predominantly at the transcriptional level. Bile acids showed the ability to increase markedly the expression of a very low level of I-BABP mRNA present in Caco-2 cells. Moreover, AMD completely suppressed the CDCA induction of I-BABP mRNA. Taken together, these results suggest that I-BABP gene expression may be up-regulated by bile acids at the transcriptional level.

In recent years, the nuclear receptor supergene family has been studied intensively. These nuclear receptors, functioning as ligand-dependent transcription factors, mediate the diverse

actions of vitamins or steroid/thyroid hormones in the target cells. They include nuclear factors regulating the expression of genes involved in lipid metabolism. For example, fatty acid-activated receptor has recently been shown to mediate the transcriptional effect of fatty acids on the expression of adipocyte lipid-binding protein gene, which belongs to the hydrophobic ligand-binding protein family, as well as I-BABP [32]. Thus, a novel nuclear receptor-mediated mechanism by which expression of the I-BABP gene is regulated by its ligands, bile acids, can be hypothesized. This putative transacting factor, preferentially activated by bile acids, remains to be identified.

Interestingly, the present findings form a striking contrast to earlier results which showed that expression of the cholesterol 7-α-hydroxylase gene (*CYP7*), the hepatic rate-limiting enzyme in the conversion of cholesterol into bile acids, is repressed by bile acids in HepG2 cells [33,34]. Although the mechanism of this suppression by bile acids remains unclear, the negative feedback regulation of *CYP7* gene expression may be mediated by a putative nuclear receptor which is activated by bile acids [31]. It would be interesting to compare regulatory mechanisms of gene expression between cholesterol 7-α-hydroxylase and I-BABP.

Faecal loss of bile acid is a major pathway for the elimination of cholesterol. This provides a new therapeutic approach for the treatment of hypercholesterolaemia, i.e. pharmacological blockage by specific inhibitors of the bile acid transporters existing in ileal enterocytes. Therefore, elucidating the molecular mechanisms underlying the regulation of I-BABP gene expression might lead to the discovery of a novel drug able to modulate the enterohepatic circulation of bile acids.

In conclusion, we have identified the biliary factor responsible for the expression of the I-BABP gene. Bile acids specifically up-regulate the expression of the I-BABP gene. This up-regulation is exerted predominantly at the transcriptional level, requiring protein neosynthesis. Further study to elucidate the molecular mechanisms underlying this transcriptional regulation of the I-BABP gene is in progress.

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