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Fatty acids in component of milk enhance the expression of the cAMP-response-element-binding-protein-binding protein (CBP)/p300 gene in developing rats

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Fatty acids in milk are thought to play an important role in intestinal maturation and gene expression in the rat small intestine during the suckling–weaning period. In the present study, we determined the jejunal mRNA level of the cAMP-response-element-binding-protein-binding protein (CBP)/p300, which is one of the chromatin remodelling factors and regulates histone acetylation, during the postnatal period in rats. The mRNA level of CBP/p300 was high during the suckling and middle of the weaning period (day 5 to 20) and then declined sharply to a low level at the end of the weaning period and after weaning. *In situ* hybridisation also showed that CBP/p300 mRNA levels in the villus as well as the basal membrane clearly decreased after weaning. Rat pups at age 17 d, weaned to a high-fat diet, showed higher levels of CBP/p300 mRNA than those weaned to a low-fat diet. Oral administration of caprylic acid, oleic acid and linoleic acid, which are major fatty acid components in milk, induced jejunal CBP/p300 gene expression. The present results suggest that fatty acids in components of milk enhance expression of the CBP/p300 genes in the small intestine.

CBP/p300: Fatty acids: Postnatal development: Small intestine

Rat pups ingest fatty acids from milk as major nutrients during the 4-week period following birth which corresponds to the suckling–weaning period. Several studies have shown that fatty acids in milk are important for the development of the small intestine in rat pups during the suckling–weaning period[1–4]. During the transition period from suckling to weaning (13–27 d after birth), the small intestine matures dramatically to its adult form as an adaptation to altered nutrition associated with the change from a diet rich in fat (milk) to one rich in carbohydrate (solid food). The expression of many genes related to digestion or absorption, such as sucrase–isomaltase (SI)[5], GLUT5[6], cellular retinol-binding protein type II (CRBPII)[7], and liver-type and intestinal-type fatty acid-binding proteins (L-FABP and I-FABP)[8], increase during this period. Many studies, including some from our own laboratory, indicate that putative transcriptional factors for these intestinal genes are also up regulated during this period. Our previous research indicates that caudal-related homeobox 2 (Cdx-2) and hepatocyte nuclear factor (HNF)-1, which are transcriptional factors for SI gene expression, are highly expressed during the suckling–weaning transitional period[9]. Furthermore, our recent work has demonstrated that thyroid hormone receptor α-1, which is a transcriptional factor for GLUT5 gene expression, is highly expressed during this period[6]. Additionally, the gene expression of many other transcriptional factors for intestinal gene expression, such as retinoid X receptor α, retinoic acid receptor (RAR)-α and HNF-4, are highly expressed during this period[7]. Our previous research has shown that some of the nuclear receptors, the PPAR[10,11], which is activated by fatty acids, as well as its target genes, L-FABP and I-FABP[7,8,12–16], are highly expressed during this period. Additionally, several studies, including some of ours, have demonstrated that PPARα regulates L-FABP and I-FABP during the weaning period[8] as well as during adulthood[17–20]. These results suggest that fatty acids in milk may play a role in inducing small-intestinal maturation and differentiation and increasing intestinal gene expression through enhancing gene expression of transcriptional factors. Many recent studies suggest that acute induction of gene expression involved in the cell cycle and differentiation processes occurring during development are regulated not only by transcriptional factors,

Abbreviations: BW, body weight; CBP, cAMP-response-element-binding-protein-binding protein; Cdx-2, caudal-related homeobox 2; CRBPII, cellular retinol-binding protein type II; HNF, hepatocyte nuclear factor; I-FABP, intestinal-type fatty acid-binding protein; L-FABP, liver-type fatty acid-binding protein; RAR, retinoic acid receptor; RXR, retinoid X receptor; SI, sucrase–isomaltase; SSC, sodium chloride and sodium citrate; TR, thyroid hormone receptor; Tris, tri(hydroxymethyl)-aminomethane.

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but also by chromatin remodelling factors such as coactivators, the TRIP/DRIP complex and the SWI/SNF complex[21]. In particular, coactivators, which have histone acetyltransferase activity are thought to play a critical role for transcription regulation, because histone acetylation by histone acetyltransferase including coactivators is the first step for recruiting transcriptional complexes[22]. Recent studies including our own have shown that one of the coactivators, cAMP-response-element-binding-protein-binding protein (CBP)/p300, is expressed in the small intestine and enables transmission of fatty acid signalling via PPAR[16,23,24]. Additionally, it has already been demonstrated that CBP/p300 is bound to other transcriptional factors for small-intestinal gene expression such as Cdx-2[25], HNF-1[26], HNF-4[27], retinoid X receptor (RXR)[28] and RAR[28] and that it activates their target genes. However, it is not clear whether small-intestinal expression of CBP/p300 occurs during the suckling–weaning period and whether its expression is regulated by fatty acids in milk.

In the present study, we focused on changes in expression of the jejunal CBP/p300 gene in rats during the suckling–weaning period. The present study indicates that CBP/p300 expression, which is associated with postnatal intestinal maturation during the suckling–weaning period, may be regulated by fatty acids in milk.

Materials and methods

Animals

Sprague–Dawley suckling rats (Japan SLC, Hamamatsu, Japan) were kept with their mothers, and both mothers and pups were given free access to a standard laboratory chow diet (MF; Oriental Yeast, Tokyo, Japan) throughout the experimental period. To examine the effect of time post-partum on gene expression, we chose the following sampling times: samples collected at 5 d post-partum represented the suckling period; samples collected at 13, 20 and 27 d marked the start, middle and end of weaning, respectively; samples collected at 42 d represented the period after weaning. Rat pups were killed by decapitation, without prior fasting, between 14.30 and 15.00 hours at the ages of 5, 13, 20, 27 and 42 d. To assess the effect of dietary fat during the postnatal stage on intestinal gene expression, in a further experiment, rat pups were removed from their mothers at age 17 d, and they then received free access to either a low-fat diet containing 2·4 % (w/w) maize oil (essentially TAG, consisting of glycerol esterified to the fatty acids, mainly oleic acid and linoleic acid in maize oil), or a high-fat diet containing 24·7 % (w/w) maize oil[29]. They were killed at the ages of 21 and 28 d without prior fasting. In a further experiment, weanling rats were orally administered with 1 ml/kg body weight (BW) of 20 % glycerol without fatty acids (control), or with 180 μmol/kg BW of various fatty acids (caprylic acid (25·96 mg/kg BW), oleic acid (50·84 mg/kg BW), linoleic acid (50·48 mg/kg BW) and arachidonic acid (54·80 mg/kg BW)) once per d for four consecutive days starting at age 27 d (between 09.00 and 09.30 hours). These rats were killed without prior fasting between 14.30 and 15.00 hours at age 30 d. The experimental procedure used in the present study met the guidelines of the Animal Usage Committee of the University of Shizuoka.

Ribonucleic acid analysis

The entire small intestine was flushed with ice-cold 0·9 % NaCl solution. The jejuno-ileum extending from the ligament of Treitz to the ileocaecal valve was divided into two equal parts along its length. A portion (100 mg) was excised from the middle of the proximal half of the jejuno-ileum (jejunum) and immediately used for RNA extraction. Total RNA was extracted by the acidified guanidinium thiocyanate method as described by Chomczynski & Sacchi[30]. Northern blot analysis of CBP/p300 and 18S rRNA was performed using ^{32}P-labelled cDNA probes as described previously[16,29]. The probe for CBP/p300 in the present study was the rat p300 probe which detects both CBP and p300 signals[16]. The specific mRNA signals from Northern blots were quantified using an image analyser (BAS 2000; Fuji Film, Tokyo, Japan) for Northern blot analysis, and the signals were standardised to the 18S rRNA signal.

In situ *hybridisation*

The cRNA probe of rat CBP/p300 for *in situ* hybridisation was prepared using rat p300 cDNA[16] as a template. Antisense and sense riboprobes were labelled with digoxigenin using a digoxigenin RNA labelling kit (Roche Molecular Biochemicals, Tokyo, Japan) according to the manufacturer's instructions. Tissue sections were deparaffinised in xylene and ethanol and treated with 0·2 M-HCl for 8 min, 0·1 M-triethanolamine-HCl (pH 8·0) for 1 min, and 0·26 % (v/v) acetic anhydride in 0·1 M-triethanolamine (pH 8·0) for 15 min. After rinsing for 10 min with 2 × sodium chloride and sodium citrate (SSC) buffer (0·03 M-sodium citrate, 0·3 M-NaCl, pH 7·0), the sections were incubated with a hybridisation solution which contained digoxigenin-labelled probe (6 ng/μl) in hybridisation buffer (50 % (v/v) formamide, 2 × SSC, 10 % (w/v) dextran sulfate, yeast t-RNA (1 μg/μl), sonicated salmon sperm DNA (1 μg/μl) and bovine serum albumin (1 μg/μl)) at 50°C for 16 h. After hybridisation, the slides were sequentially washed with 5 × SSC (0·075 M-sodium citrate, 0·75 M-NaCl, pH 7·0) solution at 60°C for 10 min, 50 % (v/v) formamide and 2 × SSC at 60°C for 30 min, and TNE buffer (0·5 M-NaCl, 10 mM-tri(hydroxymethyl)-aminomethane (Tris)-HCl, pH 7·6) at 37°C for 10 min. The sections were treated with RNase A (2 μg/ml) in TNE buffer at 37°C for 30 min, followed by washing with TNE buffer at 37°C for 30 min, with 2 × SSC at 48°C (30 min, twice) and 0·2 × SSC at 48°C (30 min, twice). After washing with 0·1 M-Tris-HCl (pH 7·5) at room temperature for 10 min, the slides were incubated with a blocking buffer containing 1 % (w/v) blocking reagent (Roche Molecular Biochemicals, Tokyo, Japan) in 0·1 M-Tris-HCl (pH 7·5) at room temperature for 60 min. The slides were incubated for 18 h at 4°C with a blocking buffer containing anti-digoxigenin-alkaline phosphatase and antigen-binding fragments (Fab; Roche Molecular Biochemicals, Tokyo, Japan). These slides were washed four times with 0·1 M-Tris-HCl (pH 7·5) for 10 min and soaked in buffer containing 0·1 M-Tris, 0·1 M-NaCl, 0·2 M-$MgCl_2$ (pH 9·5) at room temperature for 5 min. Finally, immunoreactivity of sections was visualised by incubating for 18 h at 4°C with 450 μg NBT (nitroblue tetrazolium chloride) and 175 μg BCIP (5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt)

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solution in 1 ml of buffer containing 0·1 M-Tris, 0·1 M-NaCl and 0·2 M-$MgCl_2$ (pH 9·5).

Statistical analysis

Results are expressed as mean values with their standard errors. The significance of differences was determined by ANOVA followed by Tukey's multiple-range test to compare among each stage of postnatal development (Fig. 1), or Student's *t* test to compare between two groups (Fig. 2) or Dunnett's multiple-range test to compare between CBP/p300 mRNA treated with glycerol without any fatty acids (control) and those with fatty acids (Fig. 3) as appropriate.

Results

Postnatal changes in gene expression of CBP/p300 in rat jejunum

In the first experiment, the rat pups were kept with their mothers until weaning was completed. In the present study, we focused on jejunal expression of CBP/p300 because the bulk of digestion and absorption of nutrients in the small intestine occurs in the jejunum. At the ages of 5, 13, 20, 27 and 42 d, the jejunal total RNA was extracted and subjected to Northern blot hybridisation for CBP/p300 mRNA. CBP/p300 mRNA levels were high during the suckling and middle of the weaning period (5–20 d after birth), and reached maximal levels at 20 d after birth. At their peak CBP/p300 mRNA levels were 1·5, 2·4, and 4·0 times the level seen in 5 (suckling period), 27 (end of weaning period) and 42 d old rats (period after weaning), respectively. The CBP/p300 mRNA level then declined to a low basal level at the end of the weaning period (at age 27 d). This low level of expression was also observed at age 42 d (Figs. 1 (A) and (B)). *In situ* hybridisation analysis showed that CBP/p300 mRNA was expressed in the entire villus and basal membrane at age 5 and 20 d and its expression had clearly disappeared by age 42 d (Fig. 1 (C)).

Dietary fat-induced CBP/p300 gene expression in the postnatal small intestine

Suckling rats receive milk which is rich in fatty acids from their mother. To investigate whether the expression of the CBP/p300 gene observed in the suckling–weaning period (Fig. 1) was associated with the fat intake from milk, we performed Northern blot analyses for CBP/p300 mRNA in the rat pups that were weaned to a low- or a high-fat diet at age 17 d. CBP/p300 mRNA levels at ages 21 and 28 d in the rats fed a high-fat diet were 1·5-fold ($P<0·05$) and 1·6-fold ($P<0·05$) higher, respectively, than the corresponding mRNA levels in the rats fed a low-fat diet (Fig. 2).

Expression of jejunal CBP/p300 gene in weanling rats supplemented with fatty acids

To examine which particular fatty acids in components of milk up-regulat gene expression of CBP/p300 in the rat postnatal small intestine, we orally administered caprylic acid, oleic acid, linoleic acid and arachidonic acid at a dose rate of 180 μmol/kg BW to weanling rats. The CBP/p300 mRNA level was induced 3·0-fold by caprylic acid ($P<0·01$), 3·6-fold by oleic acid ($P<0·01$) and 2·8-fold by linoleic acid ($P<0·05$). Arachidonic acid tended to enhance the CBP/p300 mRNA level (Fig. 3) but not significantly.

Discussion

Rat pups ingest fatty acids as major nutrients from milk during the suckling–weaning period, which normally lasts until 27 d after birth in rats[2,3]. During this period, expression of many genes related to digestion and absorption increases markedly.

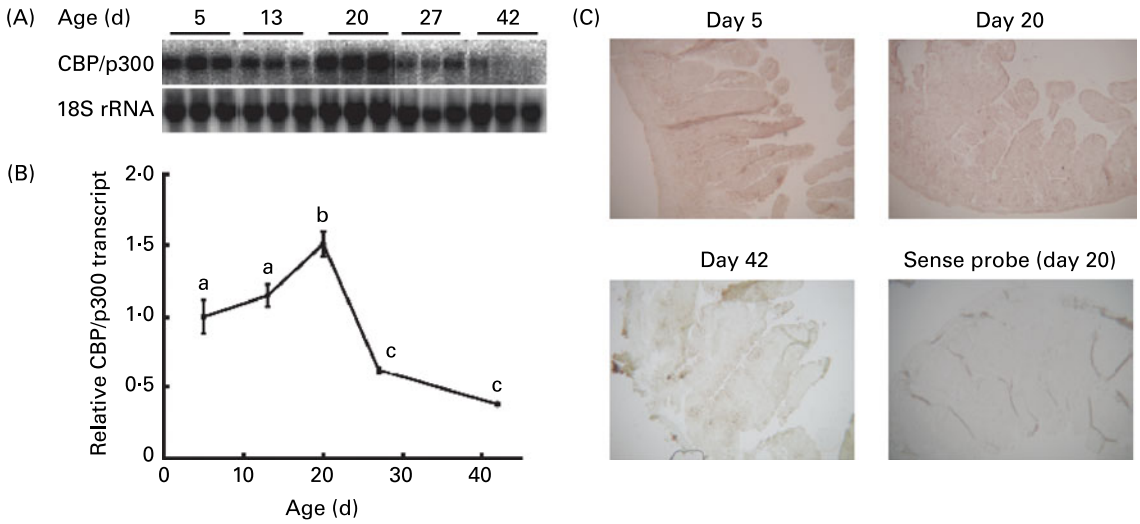

Fig. 1. Postnatal changes of cAMP-response-element-binding-protein-binding protein (CBP)/p300 mRNA level in rat jejunum. (A) Northern blotting. Total RNA (10 μg) was analysed for CBP/p300 mRNA by Northern blot hybridisation. (B) mRNA levels normalised to 18S rRNA abundance. Values are means for four animals, with their standard errors represented by vertical bars. [a,b,c] Values with unlike letters are significantly different from one another by Tukey's multiple range test ($P<0·05$). (C) *In situ* hybridisation for CBP/p300 mRNA using digoxigenin-labelled antisense cRNA in rat jejunum at 5, 20 and 42 d after birth. Sense cRNA (lower right) was used to show that unrelated cRNA did not hybridise in tissue. All pictures were taken at 100-fold magnitude.

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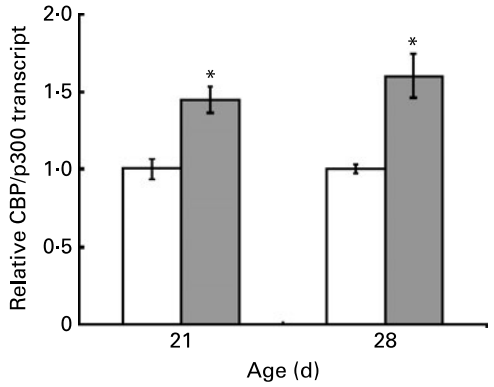

Fig. 2. Effects of dietary fat on the expression of jejunal cAMP-response-element-binding-protein-binding protein (CBP)/p300 genes during the weaning period. Rat pups (17 d old) were removed from their mothers and they received a high-fat (■) or a low-fat (□) diet for 4 and 11 d. Each mRNA level was normalised to 18S rRNA abundance. Values are means for four animals, with their standard errors represented by vertical bars. *Mean value is significantly different from that for the low-fat-fed control rats ($P<0·05$).

This is especially so for genes associated with fatty acid absorption and induced in the small intestine. During this period intestinal morphology also changes dramatically[5,7,8,13,14,31]. It is thought that fatty acids in milk play an important role in stimulating gene expression as well as intestinal maturation and differentiation during this period. Recent studies indicate that chromatin remodelling factors such as coactivators which have histone acetyltransferase activity regulate expression of genes involved with the cell cycle and differentiation[22]. In particular, CBP/p300, which is a major coactivator, is expressed in the small intestine and transmits fatty acid signalling through PPARα[16,23]. CBP/p300 has multiple functions in the cell cycle, in differentiation, in cancer and in transcription. One major role for CBP/p300 involves activation of transcription by acetylating histones and binding to transcriptional factors including PPAR on the target genes[32]. CBP/p300 also transmits signals of many transcriptional factors for small-intestinal gene expression such as Cdx-2[25], HNF-1[26], HNF-4[27], RXR[28] and RAR[28]. It is probable that CBP/p300 regulates intestinal gene expression as well as differentiation stimulated by fatty acids in milk during the suckling–weaning period.

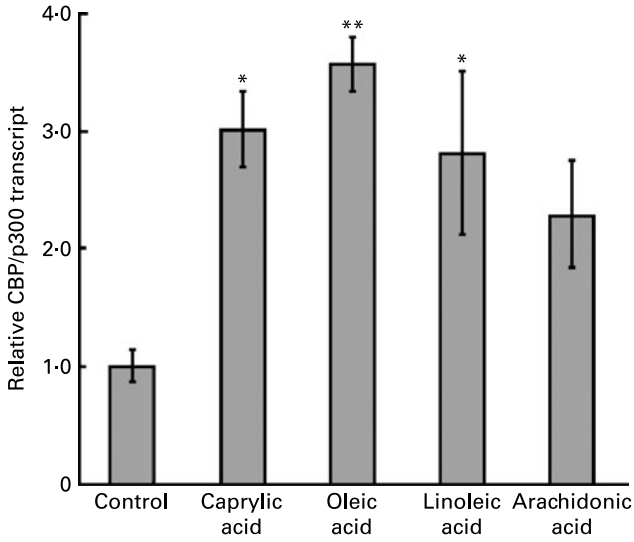

Fig. 3. Effects of fatty acids in milk on mRNA level of cAMP-response-element-binding-protein-binding protein (CBP)/p300 in the jejunum of weanling rats. Total RNA (10 μg) was analysed for CBP/p300 by Northern blot hybridisation. Northern blots of RNA were derived from the jejunum of 30 d old rats that had been orally administered with caprylic acid, oleic acid, linoleic acid or arachidonic acid in 20 % glycerol or vehicle (control) at ages 27, 28, 29 and 30 d. Each mRNA level was normalised to 18S rRNA abundance. Values are means for four animals, with their standard errors represented by vertical bars. Mean value is significantly different from that for the control rats: * $P<0·05$, ** $P<0·01$.

In the present study, we focused on changes in expression of the CBP/p300 gene during the postnatal period. By measuring mRNA levels at the ages of 5, 13, 20, 27 and 42 d, we have shown in the present study that the CBP/p300 mRNA in the small intestine is highly expressed in the jejunum during the period from suckling to the middle of weaning (days 5–20) compared with levels of expression at the end of weaning (day 27) and after weaning (day 42) (Figs. 1 (A) and (B)). It should be noted that CBP/p300 protein levels in the jejunum during the suckling–weaning period were not measured in the present study. Further work is needed to investigate the jejunal CBP/p300 protein level during the suckling–weaning period. *In situ* hybridisation showed that CBP/p300 mRNA level was high at suckling (age 5 d) and in the middle of the weaning period (age 20 d), and its expression was undetectable after weaning (age 42 d) (Fig. 1 (C)). Considering that CBP/p300 mRNA in adult rat jejunum is relatively rich compared with other tissues such as liver, heart, skeletal muscle and colon[16], the mRNA levels in the jejunum during the suckling–weaning period are speculated to be physiologically abundant. These results indicate that expression of the CBP/p300 gene during the postnatal period is associated with dietary fat intake from milk.

To investigate whether dietary fatty acids are able to modulate CBP/p300 gene expression during this period, we removed rat pups from their mothers at age 17 d to wean them to either a low- or a high-fat diet. In the rats fed the high-fat diet, CBP/p300 mRNA level was enhanced (Fig. 2). The milk produced by rats is rich in fatty acids, especially the medium-chain fatty acids, *n*-6 fatty acids and *n*-9 fatty acids[33]. Several studies have suggested that medium-chain fatty acids and unsaturated fatty acids are important for development of the infant[34,35]. Thus, we focused in the present study on these fatty acids, although other fatty acids such as long-chain SFA are also present in milk. As shown in Fig. 3, we examined expression of CBP/p300 genes following oral administration of a range of fatty acids, namely the medium-chain fatty acid caprylic acid, the *n*-9 fatty acid oleic acid, and the *n*-6 PUFA linoleic acid and arachidonic acid, all of which are components of the milk of weaning rats. In the present study, we chose a dietary fat inclusion level of 24·7 % (w/w) in the diet described in Fig. 2, and fatty acid concentrations in a 20 % glycerol solution of 180 μmol/kg BW, which corresponded to concentrations in solution in the range of 2·5–6 % (w/w) fatty acids (Fig. 3). Considering that the percentage milk fat (w/w) in rodents is about 20 %[36], these concentrations of dietary fat and fatty acids are physiologically realistic. Interestingly, CBP/p300 was significantly induced by caprylic acid, oleic acid and linoleic acid

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(Fig. 3). Additionally, arachidonic acid tended to induce CBP/p300 expression. These results suggest that fatty acids in milk may be one of the primary nutritional factors that maintain an enhanced level of expression of the CBP/p300 gene during the suckling–weaning period, although other factors such as thyroid hormone and glucocorticoid hormone, which are known to increase in serum during this period[1–3], may also contribute to regulation of CBP/p300 expression.

The molecular mechanism of CBP/p300 function in the small intestine during the suckling–weaning period is unknown. CBP/p300 strongly regulates PPARα signalling in the small intestine[16]. In the postnatal small intestine, PPARα is highly expressed and mediates the expression of small-intestinal genes such as L-FABP and I-FABP, which are known to be target genes of PPARα[8]. Moreover, both L-FABP and the CRBP II gene, which is a putative target gene of PPARα, were induced by caprylic acid and oleic acid in weanling rats[38]. Thus, the expression change of CBP/p300 gene during postnatal development might regulate the transactivity of PPARα. CBP/p300 also mediates the signalling of other intestinal transcriptional factors such as Cdx-2[37], HNF-1[26], thyroid hormone receptor (TR) α-1[39], retinoid X receptor[28] and RAR[28]. Gene expression of SI a putative target gene for Cdx-2 and HNF-1, also increases during the suckling–weaning transitional period, when Cdx-2 and HNF-1 expressions are high[6,9]. The present results also indicate that TR α-1 is not only highly expressed during the suckling–weaning transitional period, but also regulates GLUT5 gene expression during this period[6]. It remains to be resolved whether CBP/p300 regulates intestinal gene expression by transmitting the signalling of PPAR as well as these intestinal transcriptional factors during postnatal development.

In conclusion, the results of the present study suggest that expression of small-intestinal CBP/p300 during postnatal development is regulated by fatty acids derived from milk. The results of the present study are the first evidence that dietary fatty acids enhance expression of the CBP/p300 gene. Further work should investigate whether intestinal expression of genes during the suckling–weaning period is regulated by CBP/p300 as well as considering the differences in expression and function between CBP and p300 in the postnatal small intestine.

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