Nutrient-Gene Expression

Secretion of Phospholipid Transfer Protein by Human Hepatoma Cell Line, Hep G2, Is Enhanced by Sodium Butyrate\(^1,2\)

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ABSTRACT Hep G2 cells were used to study the synthesis and secretion of phospholipid transfer protein (PLTP). Upon incubation of the cells at confluence with serum-free Dulbecco’s modified Eagle’s medium (DMEM), phosphatidylcholine (PC) transfer activity was found to accumulate in the culture media. The PC transfer activity in the media was effectively inhibited by rabbit anti-human PLTP immunoglobulin (IgG), thus indicating that the PC transfer activity was due to secreted PLTP. The molecular weight of Hep G2 PLTP was ~78 kDa by Western blot analysis, in agreement with the molecular weight obtained for purified human plasma PLTP. The PLTP secreted by Hep G2 also possessed an HDL conversion activity similar to that of human plasma PLTP. The addition of butyrate to the cell culture media resulted in a marked increase in the secretion of PLTP. After 24 h incubation with 4 mmol/L sodium butyrate, a more than twofold increase \((P < 0.01)\) of PC transfer activity in the cell-conditioned media was obtained. The dose-dependent increase in the PC transfer activity in the media upon butyrate treatment was well correlated \((r = 0.80, P < 0.01)\) with that of PLTP mass as determined by immuno-slot blot analysis of cell-conditioned media. The increased secretion of PLTP by Hep G2 treated with sodium butyrate was accompanied by a greater increase in the level of PLTP mRNA in the cells as determined by ribonuclease protection assay. In the presence of 4 mmol/L sodium butyrate, a fourfold increase \((P < 0.01)\) in mRNA level was obtained at 24 h. No stabilizing effect of butyrate on PLTP mRNA was apparent upon treatment of the cultured cells with the RNA synthesis inhibitor, actinomycin D. Thus, the up-regulatory effect of butyrate on PLTP gene expression seemed to have occurred at the transcriptional level. J. Nutr. 129: 1984–1991, 1999.

KEY WORDS: • Hep G2 • phospholipid transfer protein • sodium butyrate • messenger RNA

Butyrate is produced in the colon of mammals upon microbrial fermentation of dietary fiber, undigested starch and proteins (Morita et al. 1998, Velázquez et al. 1997, Wolin 1981). It was found that butyrate was not only metabolized by colonocytes but also represented the primary colonocyte nutrient (Roediger 1982, Schepbach et al. 1992). Butyrate inhibits cell proliferation and stimulates cell differentiation in several cell lines, including rat and human hepatoma cells (Hague et al. 1993, Saito et al. 1991). Furthermore, in an in vivo murine model of colon cancer, intravenous infusion of butyrate as well as intraluminal butyrate treatments had significant antitumor effects (Velázquez and Rombeau 1997). Thus, butyrate may have a potential role in colon cancer prevention and treatment. Expression of a number of genes in cultured mammalian cells is influenced by butyrate by either promotion or inhibition of the transcription (McKnight et al. 1980). Butyrate treatment was shown to alter nuclear protein phosphorylation and methylation as well as DNA methylation and histone acetylation (Cogrvoe and Cox 1990, Parker et al. 1986, Rigs et al. 1977). However, the exact mechanisms causing the changes in gene expression by butyrate are not known.

Phospholipid transfer protein (PLTP)\(^6\) is a multifunctional protein. PLTP was shown to facilitate the transfer of not only phospholipids (Tall et al. 1983 and 1985), but also cholesterol (Nishida and Nishida 1997) among plasma lipoproteins and lipid particles, and potentially between cells and lipoproteins.


\(^{2}\) Supported in part by USH, National Institutes of Health Grant HL 17597 and by funds from the Illinois Agriculture Experiment Station, the University of Illinois Foundation and American Heart Association, Illinois Affiliate, Student Stipend SS-03 (to Z.G.).

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\(^{6}\) Abbreviations used: apo, apolipoprotein; CETP, cholesteryl ester transfer protein; DMEM, Dulbecco’s modified Eagle’s medium; EBP, enhancer binding protein; FBS, fetal bovine serum; GGE, gradient gel electrophoresis; Ig, immunoglobulin; PC, phosphatidylcholine; PCR, polymerase chain reaction; PLTP, phospholipid transfer protein; RPA, ribonuclease protection assay; SDGC, sucrose density gradient centrifugation.


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PLTP promotes an enlargement of HDL$_{3^r}$ to HDL$_{2}$-sized particles in vitro. This enlargement was accompanied by the release of apolipoprotein A-I (apo A-I) and the formation of smaller HDL particles (Jauhiainen et al. 1993, Tu et al. 1993). The release of apo A-I in vivo may generate nascent HDL particles, which could participate in reverse cholesterol transport. Dietary cholesterol increased plasma PLTP levels in mice (Jiang and Bruce 1995, Meijer et al. 1993). No detailed study has yet been conducted on the effects of dietary and other factors on plasma PLTP levels or phospholipid transfer activity. The regulatory mechanisms of PLTP synthesis and secretion have also not yet been clarified.

PLTP mRNA was shown to be widely distributed in many tissues (Day et al. 1994, Jiang and Bruce 1995). Although the PLTP mRNA level in liver is relatively low, liver may contribute to a major portion of plasma PLTP because of its large mass (Jiang and Bruce 1995). Hep G2, a human hepatoma-derived cell line, has been widely used to study the secretion of lipoproteins and apolipoproteins such as apo A-I and apo B-100 (Kaptein et al. 1991 and 1994). Recent study of transient expression of the luciferase gene fused with the PLTP 5’ flanking region in Hep G2, COS and CHO cells showed that Hep G2 has the highest luciferase activity. Therefore, it was speculated that Hep G2 cells may contain all transcription factors necessary for the full function of the PLTP (Tu et al. 1994 and 1997). In this study, Hep G2 was used as a model of human hepatocyte to study PLTP secretion and gene expression. Butyrate was found to greatly increase the secretion of PLTP and PLTP mRNA levels by increasing primarily the gene transcription.

**MATERIALS AND METHODS**

**Cell culture.** Hep G2 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL of penicillin, 0.1 g/L of streptomycin and 4 mmol/L glutamine, under 95% air and 5% CO$_2$ (Jiang and Bruce 1995). Hep G2, a human hepatoma-derived cell line, has been widely used to study the secretion of lipoproteins and apolipoproteins such as apo A-I and apo B-100 (Kaptein et al. 1991 and 1994). Recent study of transient expression of the luciferase gene fused with the PLTP 5’ flanking region in Hep G2, COS and CHO cells showed that Hep G2 has the highest luciferase activity. Therefore, it was speculated that Hep G2 cells may contain all transcription factors necessary for the full function of the PLTP (Tu et al. 1994 and 1997). In this study, Hep G2 was used as a model of human hepatocyte to study PLTP secretion and gene expression. Butyrate was found to greatly increase the secretion of PLTP and PLTP mRNA levels by increasing primarily the gene transcription.

**PLTP activity assay and immunoinhibition.** Phosphatidylcholine (PC) transfer activity in Hep G2 cell–conditioned media was determined with the $[^{3}H]$PC-vesicles/HDL$_{3}$ assay in a manner similar to that described previously (Tu et al. 1993). The PC transfer activity was computed by subtracting the blank value (PC transfer in Hep G2 media. The cells were lysed with 0.1 mol/L NaOH, and the concentration of the cell protein was measured byBio-Rad protein assay (Bio-Rad, Hercules, CA).

**Sucrose density gradient centrifugation (SDGC).** The PC transfer from cell vesicles to HDL$_{1}$ by Hep G2 cell–conditioned media was assessed from the labeled PC distribution profile obtained by SDGC. The cell-conditioned media (24 h), supernatant of anti-PLTP IgG or nonimmunized IgG (100 μg) were incubated with PLTP activity assay and immunoinhibition. Phosphatidylcholine (PC) transfer activity in Hep G2 cell–conditioned media was determined with the $[^{3}H]$PC-vesicles/HDL$_{3}$ assay in a manner similar to that described previously (Tu et al. 1993). The PC transfer activity was computed by subtracting the blank value (PC transfer in Hep G2 media. The cells were lysed with 0.1 mol/L NaOH, and the concentration of the cell protein was measured by Bio-Rad protein assay (Bio-Rad, Hercules, CA).

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**Effect of Hep G2 PLTP on HDL conversion.** The HDL conversion assay was carried out in a manner similar to that described previously (Tu et al. 1993). Isolated human HDL$_{1}$ samples (18 μg of protein) were incubated for 12 h with aliquots of partially purified Hep G2 PLTP at 37°C (4°C for controls). For immunoinhibition of the HDL conversion, the same amount of the PLTP was preincubated with rabbit anti-PLTP IgG (100 μg) or nonimmunized IgG (100 μg) at 4°C overnight. The supernatant solutions of the IgG-treated samples were incubated with isolated HDL$_{1}$ (18 μg) at 37°C for 12 h. After incubation, gradient gel electrophoresis (GGE) of the incubated mixtures was carried out on 4–20% nondenaturing gradient gels (Bio-Rad) in 0.09 mol/L Tris-HCl/0.08 mol/L borate buffer (pH 8.35) for 2000 V 4°C. The GGE patterns were obtained by staining the gels with Sudan Black B (0.48%, Bio-Rad) in acetone/acetate/water (20:15:54, v/v/v) and subsequent destaining in the same solvent mixture. The gels were scanned using a gel scanner (ISCO). Particle sizes of HDL were determined by using high-molecular-weight standards (Pharmacia, Piscataway, NJ).

**Ribonuclease protection assay (RPA).** The total RNA extracted from Hep G2 cells was used for reverse transcription. The human plasma PLTP cDNA was amplified from total cDNA by the polymerase chain reaction (PCR) using primers based on the human plasma PLTP cDNA sequence (5’-CTGCGCATCCTCCCCCT-TCCG3’ and 5’TGAATGACAGCTGCCAGCTTG3’) (Day et al. 1994). The PCR reaction was carried out on a Hybrid Combi (TR2) Thermal Reactor (Hybaid Ltd, Teddington, Middlesex, UK). The PLTP cDNA fragment (1521 bp) was cloned into a pGEM-3Z (Promega, Madison, WI) vector at the Sma I site and subsequently transformed into Escherichia coli (JM 109). A 32P-labeled antiensense RNA probe (220 bases) was generated for the RPA by in vitro transcription of linear PLTP cDNA (1404–1602) linked to the T7 promoter of the pGEM-3Z plasmid. This linear PLTP template was generated by treatment of the PLTP cDNA plasmid with Bsu36 I. In vitro transcription was carried out by using the MAXScript T7 kit (Ambion, Austin, TX). Briefly, the reaction mixture containing the linear PLTP cDNA template, NTP, 1.9 × 10$^{-5}$ M of [$^{3}P$-UTP] (3.0
**RESULTS**

Characterization of PLTP secreted by Hep G2 cells. The time-course study suggested that PC transfer activity of Hep G2 cell–conditioned media increased linearly with incubation times up to 48 h (Fig. 1). The activity reached a maximum at ~60 h and started to decline with further incubation. Western blot analysis of partially purified Hep G2 PLTP preparations with anti-PLTP IgG exhibited a single immunoreactive band of PLTP (Fig. 1, inset). The apparent molecular weight of PLTP, 78 kDa, was identical to that of purified human plasma PLTP. SDGC was used to show actual transfer of labeled PC from vesicles to HDL₃ by the cell-conditioned media and inhibition of this transfer by anti-PLTP IgG. The changes in the labeled PC distribution in [³H]PC vesicles/HDL₃ mixtures were obtained in the presence and absence of cell-conditioned media and upon addition of anti-PLTP IgG. When the mixtures were incubated with Hep G2 cell–conditioned media, the decrease in PC radioactivity in vesicle fractions, compared with the control, was accompanied by a corresponding increase in PC radioactivity in HDL₃ density fraction (d = 1.14 kg/L) (Fig. 2A). These changes in the labeled PC distribution were nullified upon incubation with the cell-conditioned media pretreated with anti-PLTP IgG as a result of the immunoinhibition of PC transfer activity in the cell-conditioned media (Fig. 2B). When various concentrations of rabbit anti-PLTP IgG (0–200 μg) were incubated with the Hep G2 cell–conditioned media, immunoinhibition of the PC transfer activity occurred in a dose-dependent manner (Fig. 3A). The addition of nonimmunized rabbit IgG did not inhibit the PC transfer activity. The PC transfer activity of the Hep G2 cell media was heat labile at 58°C as reported previously for human plasma PLTP (Albers et al. 1984). The activity decreased with an increase in the incubation time (Fig. 3B). Less than 25% of the PC transfer activity was left in the cell media after 2 h of heat treatment.

Nondenaturing GGE was performed to test the HDL conversion activity of the Hep G2 PLTP. When the incubations were performed in mixtures containing isolated HDL₃ and PLTP partially purified from Hep G2 cell–conditioned media, immunoinhibition of the PC transfer activity occurred in a dose-dependent manner (Fig. 3A). The addition of nonimmunized rabbit IgG did not inhibit the PC transfer activity. The PC transfer activity of the Hep G2 cell media was heat labile at 58°C as reported previously for human plasma PLTP (Albers et al. 1984). The activity decreased with an increase in the incubation time (Fig. 3B). Less than 25% of the PC transfer activity was left in the cell media after 2 h of heat treatment.

**FIGURE 1** Time course of phospholipid transfer protein (PLTP) secretion by Hep G2 cells. The cell-conditioned media were collected at indicated time periods for assay of phosphatidylcholine (PC) transfer activity. Values are means ± SEM (n = 9). The inset shows Western blot analysis of Hep G2 PLTP and human plasma PLTP. The PLTP samples were subjected to SDS-PAGE (12% gel) and subsequently blotted onto a nitrocellulose membrane. Monoclonal anti-PLTP immunoglobulin (IgG) (P2E5) was used for immunodetection. Lane 1 represents purified human plasma PLTP (500 ng); lane 2 shows PLTP isolated from the Hep G2 cell–conditioned media.

**FIGURE 2** Labeled phosphatidylcholine (PC) transfer from vesicles to HDL₃ facilitated by Hep G2 phospholipid transfer protein (PLTP) as determined by sucrose density gradient centrifugation (SDGC). The mixtures of [³H]PC vesicles and HDL₃ were incubated with cell-conditioned media or conditioned media pretreated with rabbit anti-human PLTP IgG (300 μg). Dulbecco’s modified Eagle’s medium (DMEM) was used as a control. The [³H]PC distribution profiles were obtained after SDGC of the mixtures as described in Materials and Methods. Panel A represents the profiles obtained for the mixtures containing cell-conditioned media and control DMEM. Panel B is for the mixtures containing cell-conditioned media pretreated with rabbit anti-human PLTP IgG and control DMEM.
from Hep G2 cell–conditioned media was carried out at 37°C for 12 h, isolated human HDL3 (8.3 nm) was enlarged to particles with a Stokes’ diameter of 9.0 nm (Fig. 4, pattern 3). This enlarging effect of Hep G2 PLTP was prevented by pretreatment of the partially purified Hep G2 PLTP with rabbit anti-PLTP IgG (pattern 4). The incubation mixtures kept at 4°C also showed no enlargement (pattern 5). The Stokes’ diameters of particles obtained by treatment with rabbit anti-PLTP IgG and the control sample kept at 4°C were 8.4 and 8.5 nm, respectively. Nonimmunized IgG showed no inhibitory effect on HDL3 conversion (pattern 6).

**Effect of butyrate on PLTP secretion.** The treatment of Hep G2 cells with sodium butyrate profoundly enhanced the secretion of PLTP in the cell-conditioned media. The PC transfer activity increased with increasing concentrations of sodium butyrate (0–4 mmol/L). A twofold (300% of control, P < 0.01) increase of PC transfer activity was observed at the concentration of 4 mmol/L (Fig. 5, panel A). The Hep G2 PLTP mass in the cell-conditioned media was determined with immuno-slot blot analysis. Figure 5 (panel B) shows that PLTP mass also increased upon treatment of butyrate in a dose-dependent manner (p < 0.01) and was well correlated with the increase in PLTP activity (r = 0.80, P < 0.01).

**Effect of butyrate on PLTP gene expression.** The effect of butyrate on Hep G2 PLTP gene expression was determined by RPA. It was shown that the increase in sodium butyrate concentration from 0 to 4 mmol/L progressively increased the PLTP mRNA level (Fig. 6); a fourfold increase in mRNA level was observed at 4 mmol/L sodium butyrate compared with control (P < 0.01). The time course of butyrate effect on the level of PLTP mRNA in confluent Hep G2 cells showed a notable increase of PLTP mRNA level as early as 6 h of incubation with 4 mmol/L sodium butyrate. Further incubation caused a nearly linear increase in the mRNA level, giving...
a fourfold increase above the level in control cells (Fig. 7). When butyrate was removed from the culture media after 12 h of exposure to 4 mmol/L sodium butyrate, PLTP mRNA levels decreased to the control levels in 24 h (data not shown). These results indicated that sodium butyrate increased PLTP mRNA levels in both a dose- and time-dependent manner.

Effect of butyrate on PLTP mRNA stability. To ascertain whether the enhancing effect of butyrate on PLTP gene expression was due to promotion of transcription or also involved an increase in mRNA stability, the rate of decay of Hep G2 PLTP mRNA abundance was determined. Hep G2 cells were incubated with serum-free DMEM in the presence and absence of 4 mmol/L sodium butyrate for 12 h. RNA synthesis inhibitor, actinomycin D (5 mg/L), was then added into the cell culture media followed by further incubation. Hep G2 PLTP mRNA concentrations were measured by RPA after incubation of the cells with actinomycin D for 4, 8 and 12 h.

The rates of decrease of PLTP mRNA level upon addition of actinomycin D were not different in the presence and absence of butyrate (Fig. 8). After 12 h of incubation, ~50% decreases were observed in both cases. It appeared that sodium butyrate did not have a significant stabilizing effect on Hep G2 PLTP mRNA.

DISCUSSION

In this study, phospholipid transfer activity secreted from Hep G2 was found to accumulate in the cell-conditioned media. The secreted PC transfer activity was inhibited by anti-human plasma PLTP IgG in a manner similar to that observed for purified human plasma PLTP or PLTP present in plasma (Tu et al. 1993). The secreted PLTP possessed an apparent molecular weight of 78 kDa and a thermosensitive property similar to that of human plasma PLTP (Albers et al. 1984, Tu et al. 1993). We demonstrated that the PLTP se-
sodium butyrate. Hep G2 cells were incubated with 4 mmol/L sodium butyrate for indicated time periods and the cellular RNA were extracted as described in Materials and Methods. Total RNA (50 μg) was subjected to ribonuclease protection assay (RPA) as given for Figure 6. The control represents the cells incubated with serum-free Dulbecco’s modified Eagle’s medium (DMEM) without sodium butyrate. The results are expressed as the percentage of the time-matched control value. Values are means ± SEM (n = 6).

Our results showed that the secretion of PLTP from Hep G2 cells as well as PLTP gene expression in the cells was greatly enhanced by inclusion of sodium butyrate in the cell media. The butyrate effect on the PLTP mRNA level was more pronounced than its effect on the PLTP secretion, as shown by both PC transfer activity and mass determinations. Although modest increases in PLTP secretion were observed at butyrate concentrations <2 mmol/L, a more than twofold increase in the secretion occurred at 4 mmol/L. In contrast, the increase in mRNA levels upon increase in butyrate concentrations from 1 to 4 mmol/L gave a more linear response. It is yet to be determined whether PLTP synthesis reaches a threshold before secretion. Further study of the butyrate effects is necessary to clarify the interrelationship of PLTP gene transcription, translation and secretion.

Butyrate, a naturally occurring 4-carbon fatty acid, is produced by the microbial fermentation of undigested material reaching the colon. Both dietary fiber and undigested starch and protein constitute the principal substrates for the fermentation of short-chain fatty acids in humans (Velázquez et al. 1997). It has been demonstrated that total short-chain fatty acid concentrations are high in the colon, ranging from 80 to 131 mmol/kg intestinal contents, with butyrate comprising >20% of the total concentrations (Cummings 1994). A major proportion (72%) of butyrate is very rapidly metabolized in the colonic epithelial cells. The concentration of butyrate (4 mmol/L) that gave very pronounced effects on PLTP secretion and mRNA expression could be within physiologic concentration ranges for colon cells. However, it is a pharmacologic concentration for other cells such as hepatocytes and peripheral cells. Butyrate concentrations in plasma and portal vein remain low in general (Cummings 1994, Roediger 1994). Other short-chain fatty acids such as acetate and propionate also gave significant increases in the PLTP secretion (data not shown), although the extent of the enhancements was considerably less than that observed for butyrate.

Sodium butyrate is likely to regulate PLTP gene expression by promoting transcriptional activation. Our results showed that the reduction in PLTP mRNA concentrations by addition of actinomycin D was of the same magnitude in the presence and absence of sodium butyrate. Apparently, butyrate did not stabilize PLTP mRNA. Previous studies revealed that the effects of butyrate on the expression of various genes could take place either transcriptionally or post-transcriptionally (Fan et al. 1991, Saini et al. 1990). In most cases, butyrate seemed to act via a transcriptional mechanism (Kruh et al. 1994). It is interesting to note that low levels of cholesteryl ester transfer protein (CETP) gene transcription in Hep G2 cells was due to low levels of C/enhancer binding protein (EBP) (Agellon et al. 1992). The enhancing effect of butyrate on CETP mRNA levels in Hep G2 cells was attributed to up-regulation of the expression of C/EBP (Sperker et al. 1993), which activates the CETP gene promoter (Agellon et al. 1992). Although consensus sequences for potential binding of C/EBP are present in the 5′-flanking region of the PLTP gene, these sequences are not located in the functional promoter.
region, −230 to −72 (Tu et al. 1995). Whether C/EBPα is involved either directly or indirectly in enhanced transcription of PLTP gene by sodium butyrate treatment requires clarification. Although butyrate treatment of Hep G2 cells gave degrees of increase in CETP mRNA levels comparable to those observed with PLTP mRNA levels, its enhancing effect on CETP secretion was less pronounced than on PLTP secretion (Sperker et al. 1993). Furthermore, in contrast to PLTP secretion, very low or undetectable levels of CETP are secreted in the Hep G2 media in the absence of butyrate (Agellon et al. 1992, Clark et al. 1995, Richardson et al. 1996). Transcription as well as post-transcriptional factors that cause these differences in the expression of CETP and PLTP are yet to be investigated. Both CETP and PLTP belong to the lipid transfer/lipopolysaccharide-binding protein family (Lagrost et al. 1998).

The importance of PLTP in lipid and lipoprotein metabolism has recently been well recognized. PLTP was shown to promote the transfer and exchange of not only phospholipids (Tall et al. 1983 and 1985) but also cholesterol (Nishida and Nishida 1997). PLTP may enhance reverse cholesterol transport. In transgenic mice, PLTP expression appears to be positively related to HDL cholesterol level (Albers et al. 1996, Jiang et al. 1996). PLTP could release apoA-I during the conversion of HDL₃ to HDL₂-sized particles (Pussinen et al. 1995, Tu et al. 1993) and transform discoidal HDL into vesicular structures (Nishida et al. 1997). The apoA-I released could promote cholesterol efflux from plasma membranes of various cells (Fielding and Fielding 1995, Oram and Yokoyama 1996). The very pronounced effects of butyrate on both PLTP gene expression and PLTP secretion observed in this study suggest that sodium butyrate may have a role as a useful agent with which to investigate the mechanisms of transcriptional regulation of PLTP gene expression.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the excellent technical assistance of Daniel Klock and Lena Powers.

LITERATURE CITED


plasma cholesteryl-ester-transfer protein in Hep G2 cells is induced by sodium butyrate quantification of low mRNA levels by polymerase chain reaction. Eur. J. Biochem. 218: 945–950.


