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EFFECTS OF BUTYRATE HOMOLOGUES ON METALLOTHIONEIN INDUCTION IN RAT PRIMARY HEPATOCYTE CULTURES

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SUMMARY

Sodium butyrate (NaB), a 4-carbon fatty acid, has been reported to activate the metallothionein (MT) gene in certain carcinoma cell lines. Because the effects of NaB are dependent on the cell type investigated, this study was conducted to determine if NaB and its homologues induce MT in rat primary hepatocyte cultures. Hepatocytes were grown on monolayer for 12 h and subsequently treated with formate, acetate, propionate (NaP), NaB, and valeric acid for 10 to 58 h. To examine their interaction with known MT inducers, cadmium (Cd), zinc (Zn), or dexamethasone (Dex) were added to some cultures. MT protein in the cells was quantitated by the Cd-hemoglobin assay; MT-1 mRNA was analyzed by Northern blot hybridizations with oligonucleotide probes, and quantitated by slot-blot analysis. Among the 1 to 5 carbon carboxylic acids, only NaP (3 carbon) and NaB (4 carbon) induced MT. NaP and NaB alone produced a moderate increase in MT two- to fourfold over control), but when combined with Cd or Dex, an additive increase was observed. However, when combined with Zn, a synergistic increase was detected. NaB and Zn synergistically increased MT protein, but produced only an additive increase in MT mRNA, suggesting the involvement of some posttranscriptional event(s) in the NaB-Zn induction of MT. In conclusion, NaP and NaB induced MT in normal cultured rat hepatocytes, producing an additive increase in MT protein with Cd and Dex, and a synergistic increase in MT protein with Zn.

Key words: metallothionein; butyrate; hepatocyte.

INTRODUCTION

The metallothioneins (MT) are small, cysteine-rich, metal-binding proteins that play an important role in the homeostasis of essential metals (zinc and copper), and in the detoxification of heavy metals such as cadmium (10,14,32). MT can be increased by a variety of factors including metals, glucocorticoid hormones, acute stress such as inflammation and endotoxin, and some organic chemicals (3,13,14).

Sodium butyrate, a 4-carbon fatty acid, produces a wide variety of effects on cells in culture (19,23,24). These effects include the regulation of gene expression, cell growth, and differentiation, as well as the induction of proteins and enzymes. Recently it has been shown that butyrate selectively stimulates the MT gene in rat hepatoma, teratocarcinoma, and osteosarcoma cell lines (2,5,6,29). The effect was specific, as no changes in the expression of several other genes was detected (2). More interestingly, the induction of MT by known inducers, such as metals and dexamethasone, was enhanced by butyrate co-treatment (2,5,31).

The major effect of butyrate on the cells is believed to be mediated through histone hyperacetylation and changes in chromatin structure (19,24,30). Effects of butyrate homologues (2–6 carbon) on the levels of histone acetylation correlate with their biological effects on the cells (19). However, there is no information on MT induction by other monocarboxylic acids.

Most studies on the effects of butyrate have utilized malignant cell lines or embryonic cells in culture, and their response to butyrate-induced changes varies depending on the cell type investigated (19,23,24). The most consistent effect of butyrate on these transformed cells in culture is their differentiation into more mature or "normal" cells (19,24). In contrast, studies on the effects of butyrate on nontransformed normal cells is limited (16). Primary cultures of adult rat hepatocytes represent a normal cell type that resembles many in vivo metabolic and morphologic characteristics of liver parenchymal cells (7,17,22), and provide a good model for studying the cellular effects of butyrate (16,27), as well as the induction of MT (8,21).

The present study was therefore designed to answer the following: a), to determine if butyrate increases MT in normal rat primary hepatocyte cultures, b), to determine the effects of butyrate homologues (formate, acetate, propionate, and valerate) on the induction of MT, and c), to examine the interactions between butyrate, its homologues and known MT inducers (zinc, cadmium and dexamethasone) on MT gene expression and MT protein induction in rat primary hepatocyte cultures.

MATERIALS AND METHODS

Chemicals. Sodium formate (NaF), sodium acetate (NaA), sodium propionate (NaP), sodium butyrate (NaB), valeric acid (Va), and dexamethasone (Dex) were purchased from Sigma Chemical Co. (St. Louis, MO).

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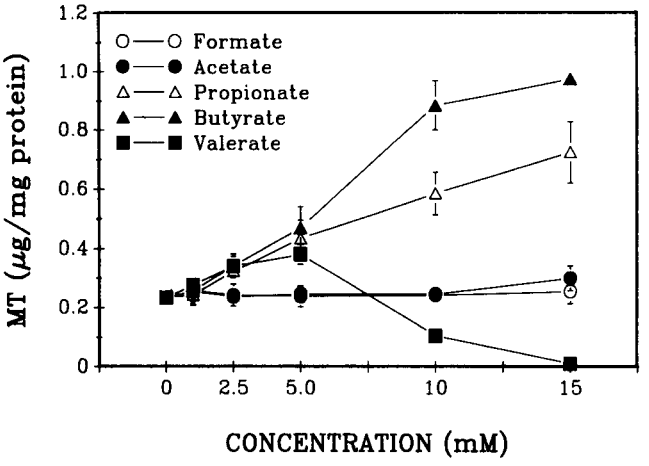

FIG. 1. Dose-dependent MT induction by butyrate and its homologues in rat cultured hepatocytes. Hepatocytes were grown on monolayer for 12 h and subsequently treated with 0 to 15 m*M* formate (NaF), acetate (NaA), propionate (NaP), butyrate (NaB), and valerate (Va) for 34 h. Results are mean ± SE of four to six rats.

$CdCl_2$ and $ZnCl_2$ were obtained from Fisher Scientific Company (Fairlawn, NJ), and carrier-free radioactive $CdCl_2$ (^{109}Cd) was obtained from New England Nuclear (Boston, MA). All other chemicals were reagent grade.

Animals. Male Sprague-Dawley rats (Sasco, Omaha, NE) weighing 250 to 350 g were housed in plastic cages maintained at 70° ± 2° F with a 12-h light/dark cycle. Food (Purina laboratory rodent chow, St. Louis, MO) and tap water were provided ad libitum.

Preparation and culture of hepatocytes. Hepatocytes were prepared by a two-stage single pass perfusion method (4) as modified by Bissell and Guzelian (7). Briefly, an oxygenated Hanks' balanced salt solution (Ca^{++}, Mg^{++}-free) supplemented with Tris-HCl (25 m*M*) and EGTA (0.5 m*M*) was perfused through the liver via the portal vein for 15 min at 37° C. The liver was then perfused with 200 ml serum-free media containing 0.05% collagenase type II (Worthington Biochemical Co. Freehold, NJ) at a flow rate of 12 to 15 ml/min. After enzymatic digestion, the liver was removed, minced with scissors and filtered through four layers of sterile gauze. Hepatocytes were separated from nonparenchymal cells and debris by centrifugation (50 g, 1 min) and the cell pellet washed 3 times with culture medium, and purified by Percoll centrifugation (18). Cell viability exceeded 90% as determined by trypan blue dye exclusion.

Primary cultures were established by seeding 3×10^6 cells onto 60-mm cultures coated with collagen (Collagen Corp. Palo Alto, CA) and incubating at 37° C (5% CO_2:95% humidity) for 60 min. Medium was changed after cell attachment, and the cultures incubated in fresh medium for an additional 22 h before treatment. The medium was changed every 24 h thereafter. The culture medium was a modified Waymouth 752/1 serum-free medium as previously described (8). Insulin (1.0 μ*M*), ornithine (20 mg/liter), ascorbic acid (0.25 m*M*), methionine (0.3 m*M*), and cysteine (0.5 m*M*) were added.

Experimental treatment. Twelve hours after hepatocyte isolation, treatments were initiated by adding various concentrations of NaF, NaA, NaP, NaB, or Va for 10 to 58 h. To examine their interactions with known MT inducers, $ZnCl_2$ (20 μ*M*), $CdCl_2$ (1.0 μ*M*), and Dex (1.0 μ*M*) were added 10 h after butyrate or its homologues. All treatments were 1% volume of medium and renewed every 24 h together with medium changes.

Quantitation of MT protein. At various time points after treatment, cells were rinsed with isotonic saline 3 times, then collected in 2.0 ml Tris-HCl buffer (10 m*M*, pH 7.4) and sonicated with a W-10 cell disrupter (Ultrasonics Inc., Plainview, NY). Aliquots of sonicated cell suspensions were assayed for MT by the Cd-hemoglobin assay (15), and total cellular protein was assayed by a protein dye-binding method (9).

Hepatocellular uptake of Cd and Zn. Ten hours after butyrate (10 m*M*) treatment, medium was removed and fresh medium containing Cd (1.0 μ*M*, 0.2 μCi ^{109}Cd/dish), or Zn (20 μ*M*) was added to cultures and incubated for 1, 2, 4, 6, 8, 12, and 24 h. At the end of each incubation, medium was removed and washed 3 times with isotonic saline. For assessment of Cd uptake, cells were collected in 2 ml of 10 m*M* Tris-HCl buffer, and metal content was determined by gamma scintillation spectrometry (Packard model 5000, Downers Grove, IL). For the examination of Zn uptake, cells from four to five dishes were collected in 1.0 ml of 10 m*M* Tris-HCl buffer, followed by sonication and centrifugation (10 000 *g*, 5 min), and Zn content in the supernatant was determined by atomic absorption spectrometry (Perkin-Elmer, model 2380, Norwalk, CT).

Isolation of RNA from hepatocyte. Total RNA was isolated from cultured hepatocytes using RNAzol-B as previously described (11). Briefly, at various times after treatment, cells were washed 3 times with isotonic saline and lysed by adding 1.0 ml of RNAzol-B (CINNA/BIOTECX, Friendswood, TX) directly to the cells. RNA was solubilized by passing the lysate several times through a sterile syringe with a 18-gauge needle, extracted with chloroform, precipitated with isopropanol, washed with 75% ethanol, and dissolved in 0.5% sodium dodecyl sulfate (SDS). All solutions were treated with 0.1% diethylpyrocarbonate (DEPC) and autoclaved before use. RNA yield and purity were determined by absorbance at 260 nm and A260/A280 ratios (1.6 to 1.9).

Northern blot analysis. Denaturation of RNA, agarose gel electrophoresis, and Northern transfer were done essentially as described by Lehrach et al. (20). RNA (8 μg) was denatured in a solution containing 50% formamide, 2.2 *M* formaldehyde, 20 m*M* 3-(N-morpholino) propanesulphonic acid, 5 m*M* sodium acetate, 1 m*M* Na_2EDTA, pH 7.0, by heating at 65° C for 15 min. RNA was then cooled on ice before loading a 1.2% (5 × 7.5 cm) agarose gel. After 3 h electrophoresis, the gel was washed in deionized water before capillary transfer of RNA in 10× sodium chloride-sodium citrate (SSC) to a Zeta-probe membrane (Bio-Rad, Richmond, CA). Gel loading, transfer efficiency, and RNA integrity were monitored by methylene blue staining of 28S and 18S ribosomal RNA (25).

Slot-blot analysis. Slot-blot analysis of RNA was done using a Bio-Rad slot-blot vacuum apparatus (Richmond, CA). RNA (8 μg) was denatured with 0.5 ml of 10 m*M* NaOH containing 1 m*M* EDTA just before loading on the Zeta-probe membrane.

Labeling MT-I probe. Oligonucleotide MT-I probe (20 mers) was prepared using known rat MT cDNA sequences (1) from a nontranslated region to obtain probe specific for MT-I ('5-GAGGGCAGCAGCACTGTTCG-3'). The probe was 3'-end labeled (12) with deoxyadenosine [α-^{32}P]triphosphate (6000 Ci/mmol) using a DNA tailing kit (Boehringer Mannheim, Inc.). The tailing reaction (15 μl) contained 3.0 μl tailing buffer, 4.5 μl $CoCl_2$, 3.4 pmol of probe, 45 pmol deoxyadenosine triphosphate (270 μCi on reference date), and 55 U terminal deoxytransferase. The reaction was initiated with addition of enzyme and allowed to incubate for 25 min at 37° C. The reaction was stopped by adding 10 μl of ice-cold 0.5 *M* EDTA. Unincorporated label was removed using G-25 Sephadex quick-spin col-

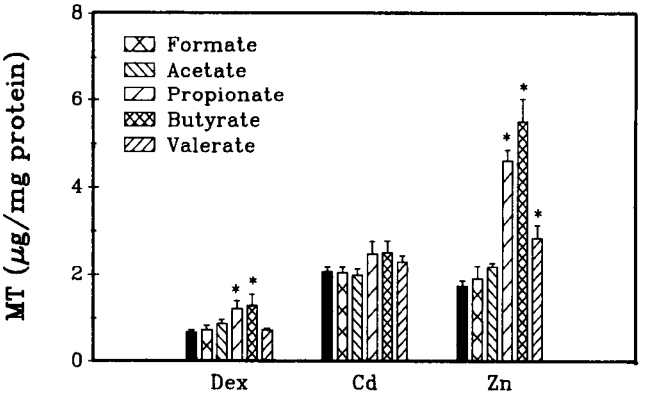

FIG. 2. Interaction of butyrate and its homologues with Dex, Cd, and Zn. Hepatocytes were pretreated with butyrate (NaB), propionate (NaP), acetate (NaA), formate (NaF) at 10 m*M* and valerate at 5 m*M* for 10 h, followed by adding Dex (1 μ*M*), Cd (1 μ*M*), or Zn (20 μ*M*) for an additional 24 h. Results are mean ± SE of four rats. *Asterisks* indicate significantly different from inducer-controls ($P < 0.05$).

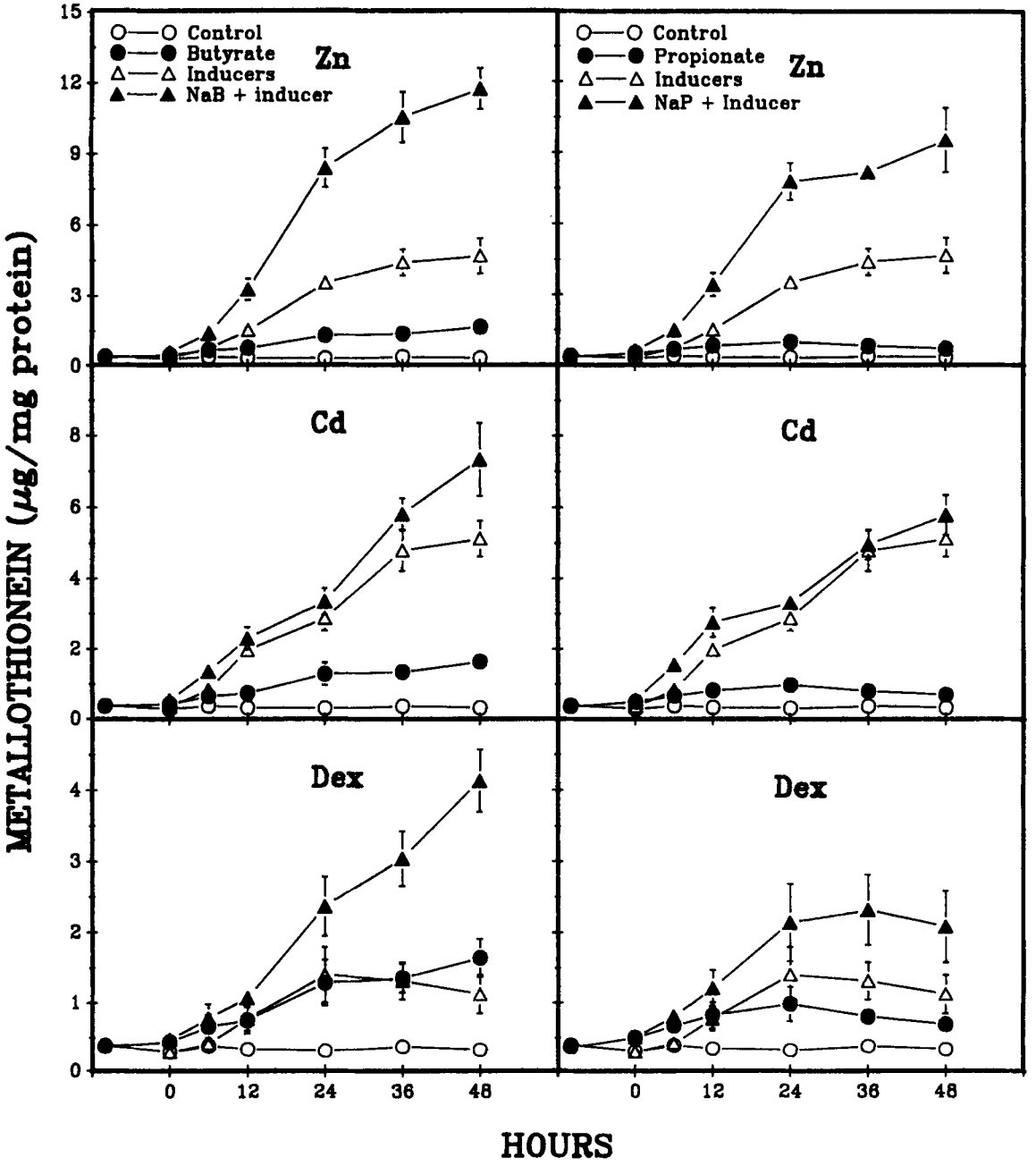

FIG. 3. Time-course of MT induction by butyrate and propionate in combination with MT inducers. Hepatocytes were pretreated with 10 m*M* butyrate or propionate for 10 h, and subsequently exposed to Zn (20 μ*M*), Cd (1 μ*M*), or Dex (1 μ*M*) for an additional 6 to 48 h. Results are mean ± SE of four rats.

umns (Boehringer Mannheim, Inc.). This procedure yielded a tail length of approximately five bases with a specific activity of 8.0×10^9 dpm/μg DNA.

Hybridization of labeled probes. Prehybridization and hybridization reactions were identical for Northern and slot-blots. Prehybridization (20 m*M* $NaPO_4$, pH 7.0, 7% SDS, 20% formamide, 5× SSC, 5× Denhart's solution, 100 μg/ml salmon sperm DNA, 250 μg/ml d[pA]$_5$) was at 47° C for 8 to 12 h. Hybridization incubations were similar, except that the Denhart's solution was 1×, and the incubation was 18 to 20 h. The membranes were washed at 47° C in 200 ml of 3× SSC and 2% SDS for 30 min, and then in four subsequent washes with decreasing salt and increasing temperature to final conditions of 0.5× SSC, 2% SDS at 55° C. Autoradiographs were developed by exposing film in conjunction with a high-plus intensifying screen at −80° C for an appropriate time. Semiquantitative comparisons were made by cutting slots from the membrane to quantitate ^{32}P in each hybrid by liquid scintillation spectrometry.

Statistical analysis. The 0.05 level of probability was used as the criteria for significance. Comparisons between control and treatment were made by a one-way analysis of variance (ANOVA) followed by Duncan's test for multiple unplanned comparisons (28).

RESULTS

The dose-response for MT induction by NaB and its homologues is shown in Fig. 1. Twenty-four hours after the incubation with 2.5

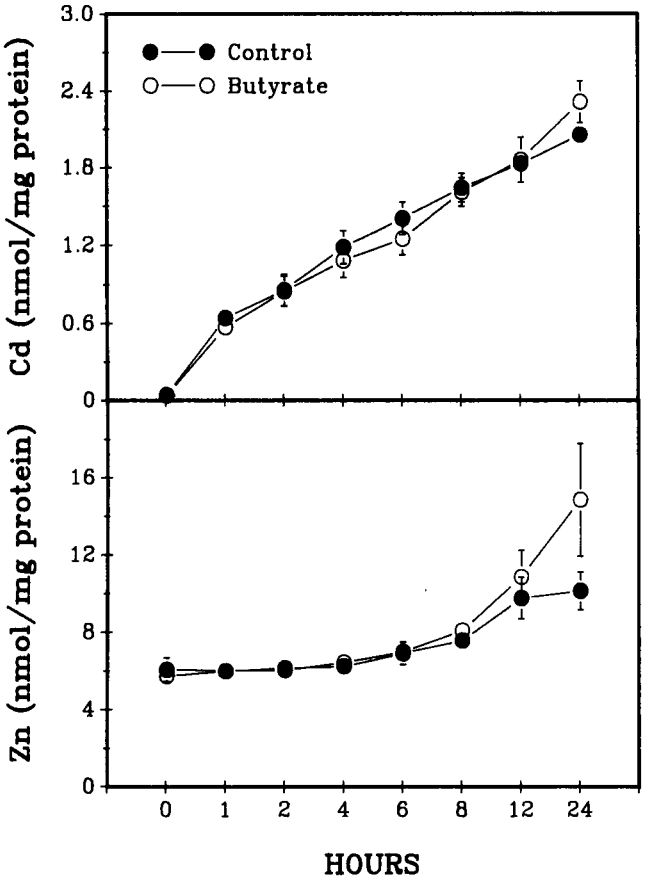

FIG. 4. Effect of butyrate on the uptake of Cd and Zn into cultured hepatocytes. Hepatocytes were pretreated with butyrate (10 m*M*) for 10 h, and subsequently exposed to Cd (1 μM, 0.2 μCi/dish) or Zn (20 μM, four to five dishes pooled for atomic absorption spectrometry detection) for an additional 1 to 24 h. Results are mean ± SE of three to six rats.

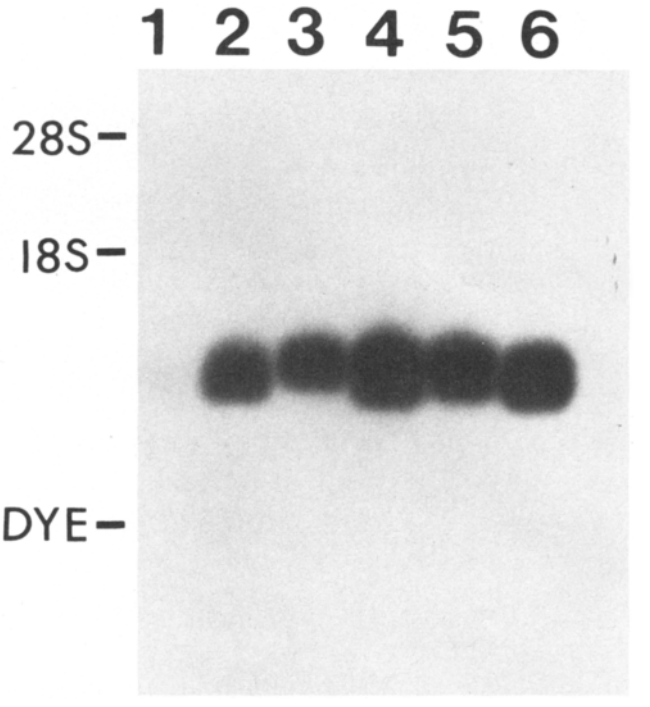

FIG. 5. Northern-blot analysis of MT-I mRNA . *Lane 1*, control; *lane 2*, NaB (10 m*M*, 16-h treatment); *lane 3*, Zn (20 μM, 6-h treatment); *lane 4*, NaB + Zn; *lane 5*, Cd (1 μM, 6-h treatment); *lane 6*, NaB + Cd.

to 15 m*M* NaB and 5.0 to 15 m*M* NaP there was a dose-related MT induction, but the increase was small (two- to fourfold). A maximum effect was achieved with a 15-m*M* concentration of NaB; higher concentrations produced cytotoxicity in the cells (data not shown). NaA and NaF were ineffective at these concentrations. Va had a slight effect at low concentrations (2.5 to 5.0 m*M*); however, higher concentrations were toxic to cells.

Based on the data of this dose-response study, we chose 10 m*M* NaF, NaA, NaB, NaP, and 5 m*M* Va to examine their interactions with known MT inducers, such as Dex (1.0 μM), cadmium (Cd, 1.0 μM), and zinc (Zn, 20 μM). NaB and NaP pretreatment (10 h) followed by the addition of Dex seemed to produce an additive increase in MT protein, whereas a synergistic effect was observed with Zn. Va (5 m*M*) also produced an additive increase in MT protein with Zn, whereas NaF and NaA had no interactions with MT inducers (Fig. 2).

To obtain more information about the interactions between NaB or NaP and MT inducers, a time course of MT induction by NaB or NaP in combination with Zn, Cd, or Dex was examined (Fig. 3). NaB (10 m*M*), or NaP (10 m*M*) alone produced mild increases in MT, with peak concentrations observed around 24 h (plus 10 h pretreatment). Pretreatment of cells with NaB or NaP (10 h) enhanced the MT increases produced by Zn, Cd, and Dex, with a peak concentration of MT observed 48 h after the addition of MT inducers. Both NaB and NaP produced synergistic effects with Zn, whereas additive effects were observed with Cd and Dex.

From the above studies, it seems that NaP had similar effects on MT as did NaB, but to a lesser degree. Therefore, we chose NaB for additional studies that were aimed at describing the possible mecha-

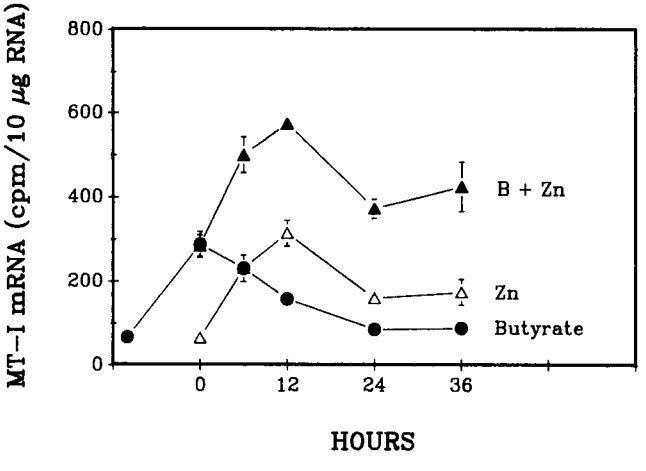

FIG. 6. Time course of MT-I mRNA induction. Hepatocytes were grown on monolayer for 12 h and subsequently treated with butyrate (10 m*M*, -10 to 36 h), Zn (20 μM, 0 to 36 h), or butyrate plus Zn. Results are mean ± SE of three rats.

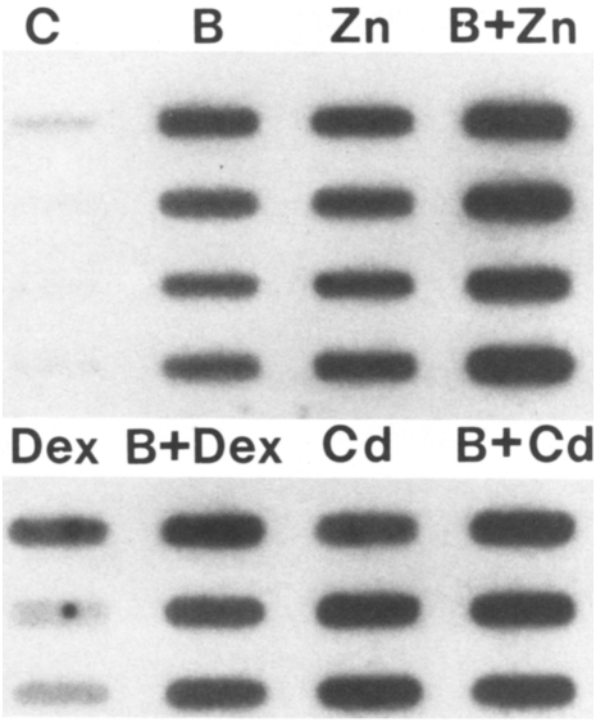

FIG. 7. Slot-blot analysis of MT-I mRNA induction by butyrate, Zn, Cd, and Dex. Hepatocytes were pretreated with butyrate (10 m*M*) for 10 h and subsequently exposed to Zn (20 μM), Cd (1 μM), or Dex (1 μM) for an additional 6 h. Results are from three to four rats.

nism(s) of this interaction. First, the ability of NaB to enhance the uptake of Cd and Zn into cultured hepatocyte were examined (Fig. 4). NaB (10 m*M*) pretreatment (10 h) had no appreciable effect on the uptake of Cd or Zn, especially at the early incubation times (1 to 8 h, i.e. before MT was induced). At 24 h of incubation, both Cd and Zn accumulation in the NaB pretreated cells were higher than controls; however, this might be due to the increase in MT concentration observed at this time point. The increase in MT could bind metals in the cytosol, thus more metals would accumulate in the cells to associate with MT.

It was important to determine if increases in MT following NaB and MT inducers could be due to an increase in MT mRNA. Northern hybridizations were done to determine probe specificity, mRNA size, and appropriate stringency conditions. The data in Fig. 5 clearly shows that MT-I mRNA is significantly increased over controls after all the treatments, and that hybridization procedures were satisfactory. To quantitate MT-I mRNA expression, slot-blot analysis was used. The time course of MT-I mRNA induction is shown in Fig. 6. The maximum MT-I mRNA induction seems to be obtained after 10 h treatment with butyrate, 6 to 12 h treatment with Zn or Zn plus butyrate pretreatment.

Based on the data in Fig. 6, we used 6-h exposure time (after the addition of MT inducers) to quantitate MT-I mRNA in control cells and cells treated with NaB, Zn, Cd, or the combinations. The slot-blot is shown on Fig. 7. Butyrate alone produced a mild increase in MT mRNA; however, NaB pretreatment (10 m*M*, 10 h), followed by the addition of known MT inducers, seemed to produce an additive increase in MT-I mRNA. The inductive and enhanced effects of NaB on MT-I mRNA and protein are summarized in Table 1. NaB produced approximately a fourfold increase in both MT-I mRNA and protein; however, when combined with Dex, NaB produced approximately an additive increase in both MT protein and MT-I mRNA. Although butyrate produced a synergistic effect with Zn in MT protein (from 11-fold to 27-fold), it only produced an additive increase on MT-I mRNA (from 5-fold to 8-fold).

DISCUSSION

The present studies demonstrate that butyrate can induce MT in rat primary hepatocyte cultures. In this nontransformed cell type, butyrate only produced a mild increase in MT (2- to 4-fold) as compared to marked increase in MT (up to 20 fold) reported in some carcinoma cell lines (2,5). Butyrate treatment of cultured cells leads to multiple changes in chromatin. Some of these changes such as hyperacetylation of histone, inhibition of histone deacetylase, and changes in MT gene regulatory factors (2,5,19) may be responsible for the observed effects of butyrate on MT gene expression. However, some effects such as changes in nuclease sensitivity of chromatin (6) are not correlated with enhanced transcription of the MT gene.

Among the short-chain fatty acids there is a good correlation between their ability to inhibit histone deacetylase and their biological effects (19,24). The 3 and 5 carbon fatty acids are less effective than butyrate, whereas the others had little effect (19). In the rat primary hepatocyte cultures, both propionate (3 carbon) and butyrate produce similar effects by increasing levels of histone acetylation (26,27). In the present study, the induction of MT by butyrate and its homologues correlated well with earlier studies showing their ability to produce histone hyperacetylation (19,24,26,27). The relative abilities of butyrate and its homologues to induce MT were NaB > NaP > Va, and NaF and NaA had no effect. These data support the hypothesis that the production of more "open" chromatin by histone hyperacetylation may be responsible for the MT gene activation. Although propionate had not been shown to increase MT

TABLE 1

EFFECT OF BUTYRATE ON MT PROTEIN AND MT-I mRNA IN COMBINATION WITH Zn, Dex, OR Cd

	MT, μg/mg protein[a]	MT-I mRNA, cpm[b]
Control	0.31 ± 0.03	87 ± 13
NaB (10 m*M*)	1.29 ± 0.33 (4.20)[c]	340 ± 31 (3.9)
Zn (20 μM)	3.52 ± 0.37 (11.4)	429 ± 18 (5.0)
NaB + Zn	8.37 ± 0.83 (27.0)	719 ± 59 (8.3)
Cd (1 μM)	2.87 ± 0.36 (9.30)	550 ± 35 (6.0)
NaB + Cd	3.35 ± 0.38 (10.8)	583 ± 35 (6.7)
Dex (1 μM)	1.40 ± 0.40 (4.50)	260 ± 76 (3.0)
NaB + Dex	2.37 ± 0.42 (7.60)	481 ± 70 (5.5)

[a] Values represent mean ± SE of four rats at 24 h exposure.

[b] Values of cpm are mean ± SE of three to four rats from slot-blot data (Fig. 7).

[c] Values in parentheses indicate fold increase over controls.

gene expression in tetracarcinoma cell lines (2), it did increase MT proteins in rat primary hepatocyte cultures in the present study.

Increases in histone acetylation have been correlated with changes in transcription and differentiation, such as replicative DNA synthesis (19). In rat hepatocyte cultures, both propionate and butyrate stimulate DNA synthesis at low concentrations (0.5 to 1.0 m*M*); however, at high concentrations (> 5.0 m*M*), DNA synthesis was inhibited (27). In fact, both butyrate and hydroxyurea (a known inhibitor of DNA synthesis) are of similar potency in the inhibition of DNA synthesis. For example, at 5.0 m*M*, each agent reduces DNA synthesis to about 5% of that of controls; however, only butyrate seemed to induce MT (29). In the present study, both propionate and butyrate produced a dose-related (2.5 to 15.0 m*M*) induction of MT, but this effect was probably unrelated to DNA synthesis.

Butyrate and propionate alone only produced a mild induction of MT in rat cultured hepatocytes; however, when combined with metal ions, they produced additive, and even synergistic increases in MT. One possible explanation for this observation is an increased metal ion transport into the cells following butyrate pretreatment, and the metal ions then stimulate the MT gene. In the present studies, however, butyrate had no effect on either Zn or Cd uptake during early exposure times (before MT is induced), suggesting that the enhanced induction was not due to an increased metal uptake into cells. This conclusion is also in agreement with previous observations (5,29,31). However, we do not rule out the possibility that butyrate may alter the intracellular distribution of metals.

To better understand the enhanced inductive effects of butyrate with the known MT inducers, we examined the interaction of butyrate with Dex, Cd, or Zn. This approach differs from previous studies that used a mixture of metals (2). Both MT-I mRNA and total MT protein were measured and compared. The time-course of MT mRNA expression after the various inducers is comparable with previous observations (2,5). As expected, butyrate stimulated MT-I mRNA, with increases comparable to increases in MT protein (Table 1). Butyrate produced additive effects in both MT protein and MT-I mRNA when combined with Dex or Cd, suggesting that butyrate may regulate the expression of MT by mechanism(s) other than those for Dex or Cd. Although butyrate produced synergistic increases in MT protein with Zn, an additive increase in MT-I mRNA was observed. These data suggest that some posttranscriptional events may be involved in the butyrate-Zn interaction for the induction of MT. The processes involved in the posttranscriptional regulation of MT genes require further investigation.

In summary, butyrate induces MT in normal rat primary hepatocyte cultures. Propionate produced similar effects to those seen with butyrate. Valerate had a mild induction effect, whereas formate and acetate were ineffective. Butyrate in combination with Zn produced a synergistic induction of MT protein but an additive increase in MT mRNA. In comparison, when butyrate was combined with Cd or Dex, the induction was additive. The mechanism(s) by which butyrate and propionate induce MT and potentiate other MT inducers may be related to histone acetylation, as well as the regulatory effects on the translation of MT mRNA.

Acknowledgements

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References

1. Andersen, R. D.; Birren, B. W.; Taplitz, S. J., et al. Rat metallothionein-1 structural gene and three pseudogenes, one of which contain 5′-regulatory sequence. Mol. Cell. Biol. 6:302–314; 1986.
2. Andrews, G. K.; Adamson, E. D. Butyrate selectively activates the metallothionein gene in teratocarcinoma cells and induces hypersensitivity to metal induction. Nucleic Acids Res. 15:5461–5475; 1987.
3. Andrews, G. K. Regulation of metallothionein gene expression. Recent Prog. Food Nutr. Sci. 14:193–258; 1990.
4. Berry, M. N.; Friend, D. S. High-yield preparation of isolated rat liver parenchymal cells. J. Cell Biol. 43:506–520; 1969.
5. Birren, B. W.; Herschman, H. R. Regulation of the rat metallothionein-I gene by sodium butyrate. Nucleic Acids Res. 14:853–867; 1986.
6. Birren, B. W.; Taplitz, S. J.; Herschman, H. R. Butyrate-induced changes in nuclease sensitivity of chromatin cannot be correlated with transcriptional activation. Mol. Cell. Biol. 7:3863–3870; 1987.
7. Bissell, D. M.; Guzelian, P. S. Phenotypic stability of adult rat hepatocytes in primary monolayer culture. Ann. NY Acad. Sci. 349:85–98; 1980.
8. Bracken, W. M.; Klaassen, C. D. Induction of metallothionein in rat primary hepatocyte cultures: evidence for direct and indirect induction. J. Toxicol. Environ. Health 22:163–174; 1987.
9. Bradford , M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254; 1976.
10. Cherian, M. G., Nordberg, M. Cellular adaption in metal toxicology and metallothionein. Toxicology 28:1–15; 1983.
11. Chomczynski, P.; Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156–159; 1987.
12. Collins, M. L.; Hunsaker, W. R. Improved hybridization assays employing tailed oligonucleotide probes: a direct comparison with 5′-end-labeled oligonucleotide probes and nick-translated plasmid probes. Anal. Biochem. 151:211–224; 1985.
13. Cousins, R. J. Absorption, transport, and hepatic metabolism of copper and zinc: special reference to metallothionein and ceruloplasmin. Physiol. Rev. 65:238–309; 1985.
14. Dunn, M. A.; Blalock, T. L.; Cousins, R. J. Metallothionein. Proc. Soc. Exp. Biol. Med. 185:107–119; 1987.
15. Eaton, D. L.; Toal, B. F. Evaluation of the Cd/hemoglobin affinity assay for the rapid determination of metallothionein in biological tissues. Toxicol. Appl. Pharmacol. 66:134–142; 1982.
16. Engelmann, G. L.; Staecker, J. L.; Richardson, A. G. Effect of sodium butyrate on primary cultures of adult rat hepatocytes. In Vitro Cell. Dev. Biol. 23:86–92; 1987.
17. Guguen-Guillouzo, C.; Guillouzo, A. Modulation of functional activities in cultured rat hepatocytes. Mol. Cell. Biochem. 53/54:35–56; 1983.
18. Kreamer, B. L.; Staecker, J. L.; Sawada, N., et al. Use of a low-speed, iso-density percoll centrifugation method to increase the viability of isolated rat hepatocyte preparations. In Vitro Cell. Dev. Biol. 22:201–211; 1986.
19. Kruh, J. Effects of sodium butyrate, a new pharmacological agent, on cells in culture. Mol. Cell. Biochem. 42:65–82; 1982.
20. Lehrach, H.; Diamond, D.; Wozney, J. M., et al. RNA molecular weight determination by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry 16:4743–4751; 1977.
21. Liu, J.; Kershaw, W. C.; Klaassen, C. D. Rat primary hepatocyte cultures are a good model for examining metallothionein-induced tolerance to cadmium toxicity. In Vitro Cell. Dev. Biol. 26:75–79; 1990.
22. Nakamura, T.; Ichihara, A. Control of growth and expression of differentiated functions of mature hepatocytes in primary culture. Cell Struct. Funct. 10:1–16; 1985.

23. Prasad, K. N.; Sinha, P. K. Effect of sodium butyrate on mammalian cells in culture: a review. In Vitro 12:125–132; 1976.
24. Prasad, K. N. Butyric acid: a small fatty acid with diverse biological functions. Life Sci. 27:1351–1358; 1980.
25. Sambrook, J.; Fritsch, E. F.; Maniatis, T., editors. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989:7.3–7.87.
26. Staecker, J. L.; Sawada, N.; Pitot, H. C. Stimulation of DNA synthesis in primary cultures of adult rat hepatocytes by sodium butyrate. Biochem. Biophys. Res. Commun. 147:78–85; 1987.
27. Staecker, J. L.; Sattler, C. A.; Pitot, H. C. Sodium butyrate preserves aspects of the differentiated phenotype of normal adult rat hepatocytes in culture. J. Cell. Physiol. 135:356–376; 1988.
28. Steele, R. G. D.; Torrie, J. H. Principles and procedures of statistics. New York: McGraw-Hill Book Co.; 1960:99–160.
29. Thomas, D. J.; Angle, C. R.; Swanson, S. A., et al. Effect of sodium butyrate on metallothionein induction and cadmium cytotoxicity in ROS 17/2.8 cells. Toxicology 66:35–46; 1991.
30. Thorne, A. W.; Kmiciek, D.; Mitchelson, K., et al. Patterns of histone acetylation. Eur. J. Biochem. 193:701–713; 1990.
31. Waalkes, M. P.; Wilson, M. J. Enhancement of cadmium-induced metallothionein synthesis in cultured TRL 1215 cells by butyric acid pretreatment. Toxicol. Lett. 32:289–294; 1986.
32. Webb, M. Role of metallothionein in cadmium metabolism. In: Foulkes, E. C., ed. Cadmium (Handbook of experimental pharmacology, vol. 80). Berlin, Heidelberg: Springer-Verlag Press; 1986:281–328.