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STIMULATION OF DNA SYNTHESIS IN PRIMARY CULTURES OF ADULT RAT HEPATOCYTES BY SODIUM BUTYRATE

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Summary: Replicative DNA synthesis in primary cultures of adult rat hepatocytes is increased by the addition of the histone deacetylase inhibitors, propionate or butyrate. DNA synthesis was increased by the addition of 0.5 or 1.0 mM butyrate; in contrast 5.0 mM butyrate inhibited replicative DNA synthesis. Replicative DNA synthesis was increased only when low levels of butyrate were added as the hepatocytes entered the S phase. The observed apparent increase in replicative DNA synthesis was real, and not owing to changes in the specific activity of the dTTP precursor pool. The effects of butyrate and propionate on DNA synthesis appear to be related to their effects on histone acetylation.

In eukaryotic cells, most chromatin is packaged into compact 30 nm fibers, which presumably must be disrupted for processes such as transcription and replication to occur (1). One possible means to relax chromatin structure is through postsynthetic modification of histones. The core histones (H2A, H2B, H3, and H4) are reversibly acetylated at sites on the basic N-terminus of the proteins (2). The partial negation of the positive charges on the N-terminus of the core histones by acetylation may relax chromatin structure, thereby contributing to the processes of transcription (3,4), cellular differentiation (4), removal of histones during spermatogenesis (5-7), and DNA repair (8-10). Dynamic histone acetylation of internal lysines of core histones has also been postulated to expose chromatin domains in a sequential manner (11).

Sodium butyrate is an agent that increases histone acetylation levels by inhibiting histone deacetylation (12-14). Increases in histone acetylation (in the presence or absence of butyrate) have been correlated with changes in either the transcriptional or the differentiation state (4). An additional effect of butyrate treatment is inhibition of replicative DNA synthesis and cell division by arrest of cells in the G_1 phase of the cell cycle (4). Evidence that the cell cycle arrest by butyrate is caused by inhibition of histone deacetylation has been provided by Chalkley and Shires, who have isolated 10 variant HTC lines that continue to divide in the presence of butyrate (15). Each of the variant HTC lines grown in the presence of butyrate has reduced

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levels of histone acetylation when compared with control cells treated with butyrate. Although histone hyperacetylation inhibits cellular DNA synthesis and division, there is relatively little evidence that histone acetylation is involved in normal replicative DNA synthesis (16-20).

Mechanisms by which histone acetylation appears to play a role in DNA repair (i.e., sequential exposure of chromatin and/or relaxation of particular chromatin domains) may also be important in replicative DNA synthesis (11). Primary culture of adult rat hepatocytes is the only cell culture system in which histone acetylation levels spontaneously decline (Staecker et al., submitted). To investigate the possibility that histone acetylation contributes to changes in chromatin structure which occur prior to or during replicative DNA synthesis, we investigated the effect of butyrate on DNA synthesis in cultured rat hepatocytes.

MATERIALS AND METHODS

Reagents: Dulbecco's modified Eagle medium (DMEM) with high glucose, Ham's F-12 medium (F-12), penicillin, and streptomycin were obtained from KC Biological Inc. (Lenexa, KS). Insulin, transferrin, selenium, and epidermal growth factor (EGF) were purchased from Collaborative Research, Inc. (Lexington, MA). Albumin (crystallized and lyophilized), collagenase (type I), and deoxynucleotide and nucleotide standards were obtained from Sigma Chemical Co. (St. Louis, MO). Dexamethasone was obtained from Organon, Inc. (W. Orange, NJ). HEPES was purchase from Research Organics, Inc. (Cleveland, OH). Collagen type I was obtained from Collagen Corporation (Palo Alto, CA), and Percoll from Pharmacia (Uppsala, Sweden). Methyl-^{3}H-thymidine (80.0 Ci/mmol) was obtained from DuPont (Wilmington, DE). All tissue culture dishes were a product of Corning (Corning, NY), except for 150-mm dishes, which were a Falcon product from Scientific Products, Inc. (McGaw Park, IL).

Isolation and Culture of Rat Hepatocytes: Hepatocytes were isolated by a collagenase perfusion technique (21) from male Harlan Sprague-Dawley rats (Madison, WI) weighing 200-240 g, and viable (based on trypan blue exclusion) hepatocytes were subsequently purified as previously described (22). These cells were suspended in a mixture of DMEM and F-12, supplemented as previously described (23). In the present study, the cell viability exceeded 95%. The cells (2×10^5 cells in 2 ml of the medium) were plated in 35-mm plastic culture dishes which had previously been coated with 20 µg collagen type I and dried at room temperature. For experiments in which the specific activity of the dTTP pool was determined, 4×10^6 cells were plated in 20 ml of medium in 150-mm plastic culture dishes coated with 300 µg of type I collagen.

Measurement of DNA Synthesis: EGF treatment of the cultures was begun after the 2nd h of culture. Since previous results have shown that DNA synthesis peaked near the 44th h of culture (23 and Sawada et al., submitted), DNA synthesis was measured by the incorporation of [^{3}H]-thymidine from the 44th to 46th h. Assays for replicative DNA synthesis were performed according to Nakamura et al. (24) as previously described (23).

Determination of dTTP Specific Activity: Two 150-mm collagen-coated dishes were used for each determination. The cells were rinsed twice with ice-cold PBS prior to the addition of 1.5 ml of 10% TCA (trichloroacetic acid) to each dish. The cells were scraped off the dishes, which were then rinsed with an additional 1.5 ml of 10% TCA. The material removed from the dishes was

centrifuged (1000 X g for 5 min). The pellet was used to determine the amount of [^{3}H]-thymidine incorporated into acid-insoluble material, and the nucleotides and deoxynucleotides in the supernatant were extracted and prepared for HPLC as described by Riss et al. (25). The lyophilized samples were dissolved in 100 µl water, and 50 µl of the resulting solution was used for HPLC analysis. HPLC analyses were performed by use of Water 510 pumps with a Beckman 160 UV detector (A_{254}) and a Kipp and Zonen BD41 strip chart recorder. Chromatography on a Whatman 10-SAX (4.6 X 250 mm) was carried out as described (Sawada et al., submitted). The KH_2PO_4 used for HPLC chromatography was purified to remove UV-absorbing polyphosphates (26).

RESULTS

DNA synthesis in cultured hepatocytes was measured in a system in which a majority of cultured hepatocytes are capable of replicative DNA synthesis. Since butyrate is rapidly metabolized by cultured hepatocytes (results not shown; also 27), it is possible to treat cultured hepatocytes with a pulse of butyrate. In addition, the rapid metabolism of butyrate by cultured hepatocytes enabled us to determine the effects of butyrate on DNA synthesis at various intervals of the cell cycle. In the system used in these studies, hepatocytes begin to enter S phase at 24 h; a peak of replicative DNA synthesis occurs at approximately 46 h of culture (23,28 Sawada et al., submitted). DNA synthesis was inhibited when 5 mM butyrate was added prior to the S phase, but the inhibition of DNA synthesis by 5 mM butyrate declined as more cells entered the S phase (Fig 1a); this is in agreement with previous results obtained with other types of cultured cells (4). Whereas 5 mM butyrate inhibited DNA synthesis when added prior to the S phase, 0.5 or 1.0 mM butyrate increased replicative DNA synthesis when added during the 24th h of culture, a time corresponding to the time cells are entering the S phase (Fig. 1a). Butyrate (0.5 or 1.0 mM) had little effect on DNA synthesis when added at times other than 24 h (Fig. 1B).

Experiment-to-experiment variability was greatly reduced when viable hepatocytes were purified prior to being plated (22), but there was still appreciable variability between experiments when DNA synthesis was measured (Fig. 1b). The cause of this variability is not known but may be due to small changes in hepatocyte density that gave rise to relatively large changes in DNA synthesis (unpublished data). In both control and butyrate-treated hepatocytes DNA synthesis varied between experiments; however, DNA synthesis in butyrate-treated hepatocyte cultures was invariably higher than that observed in control cultures (Fig. 1b).

In previous studies with this system to study DNA synthesis in cultured hepatocytes, we found that the incorporation of [^{3}H]-thymidine accurately represents replicative DNA synthesis under a wide range of experimental conditions (23,28,29, Sawada et al., submitted). However, it has been reported that under certain conditions butyrate treatment may increase the specific activity of the dTTP pool when [^{3}H]-thymidine incorporation is used to measure

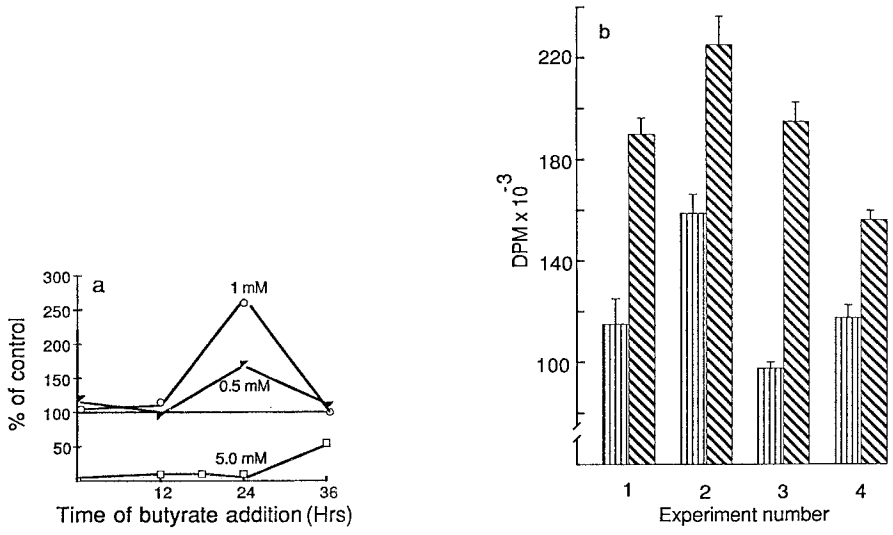

Figure 1. (a) The effect of butyrate on DNA synthesis. DNA synthesis was measured as described in Materials and Methods. Butyrate was added at the indicated time of culture: 5.0 mM butyrate □-□, 1.0 mM butyrate ○-○, or 0.5 mM butyrate ▼-▼. Each point represents the average of four determinations and is expressed as percentage of control (untreated) cultures. (b) The effect of 1.0 mM butyrate added at 24 h of culture on DNA synthesis (±2 SEM) from four separate experiments (i.e., cells from perfusions performed on four different days). Control cultures ▥, butyrate-treated (1.0 mM) cultures ▨. DNA synthesis is expressed as the incorporation of ^{3}H-thymidine into acid-soluble material per plate.

DNA repair synthesis (29). To insure that the increase in [^{3}H]-thymidine incorporation observed in hepatocytes treated with low levels of butyrate reflects a real increase in DNA synthesis, and not just an increase in the specific activity of the dTTP pool, we measured the effect of butyrate on DNA synthesis while simultaneously measuring the specific activity of the dTTP pool. We found that butyrate has little effect on the specific activity of the dTTP pool and that 1.0 mM butyrate treatment results in a real increase in replicative DNA synthesis (Table 1).

To control for some of the effects of butyrate unrelated to histone deacetylation, we also examined the effect of sodium propionate on DNA synthesis in cultured hepatocytes. Propionate is nearly as effective as butyrate in inhibiting histone deacetylation (31), but being a fatty acid with an odd number of carbons, propionate is presumably metabolized in a manner different from the four carbon fatty acid butyrate. In addition, propionate treatment of primary cultures of adult rat hepatocytes has the opposite effects of butyrate on glucose production, the glycolytic rate, and pH changes of the culture medium (32). We determined that the addition of propionate (1 mM) at 24 h of culture resulted in higher levels of DNA synthesis, similar to the observations when butyrate (1 mM) is added at 24 h of culture (Table II).

Table I
Effect of 1 mM butyrate on ^3H TDR incorporation into the dTTP pool and into DNA in cultured hepatocytes[a]

Additions to medium	H^3 in dTTP	nMol dTTP	Specific Activity dTTP[b]	^3H in DNA[c] (Acid ppt.)	nMol TTP[d]	% change
EGF[e] (2 h)	25,650	171.6	149.5	97,200	650	
EGF[e] (2 h) Plus Butyrate (24 h)	22,660	178	127.4	196,000	1540	+236

a - dTTP was separated from other acid soluble material by HPLC, the dTTP peak was collected in 0.5-min intervals, and the [^3H]-content was measured by liquid scintillation counting. The maximum amount of radioactivity always occurred in the 18.0- to 19.0-min fractions (corresponding to the dTTP peak), although radioactivity was present in the fractions from 17.5 to 19.5 min, and the DPMs from these fractions were included in calculations for determining dTTP activity.
b - Specific activity is expressed as DPM/nmol dTTP.
c - The amount of ^3H-thymidine incorporated into acid-insoluble material.
d - The amount of dTTP incorporated into acid-insoluble material was calculated by dividing the ^3H-DPM in the acid-insoluble material by the specific activity of the dTTP pool.
e - Epidermal growth factor (EGF) was added after the 2nd h of culture at a concentration of 20 ng/ml.

DISCUSSION

Rearrangement of nucleosomes occurs during UV-induced DNA repair synthesis (33), and butyrate treatment has been shown to result in increased DNA repair synthesis of DNA damaged by UV treatment (8). Acetylation of acid-soluble nuclear proteins increases in UV-irradiated human fibroblasts (9), and butyrate treatment of a human adenocarcinoma cell line has been shown to increase chromatin accessibility to DNA repair enzymes (10). Smith has proposed (10) that the histone acetylation-based surveillance system, as proposed by Perry and Chalkley (11), allows access to damaged DNA by sequentially relaxing DNA domains.

If one assumes that relaxation of chromatin structure is a necessary prerequisite of replication, it is reasonable to postulate that histone acetylation may play a role in relaxing chromatin structure prior to, or during,

Table II
The effects of propionate and butyrate on DNA synthesis when added at 24 hours

Addition to Medium	Exp. 1	Exp. 2
Control	159,260 ± 3112	118,056 ± 3593
butyrate (1 mM)	225,688 ± 5201	155,208 ± 4356
propionate (1 mM)	180,698 ± 5618	152,011 ± 3372

Data are expressed as the incorporation (DPM) of [^3H]-thymidine/dish ± 2 SEM and represent the averages of four (experiment 1) or three (experiment 2) separate determinations.

replicative DNA synthesis. Increased histone acetylation is associated with increased DNA synthesis during EGF-induced DNA synthesis in Chang liver cells (16), after a partial hepatectomy (17), and during sea urchin embryonic development (20). Newly synthesized histone H4 is rapidly and extensively acetylated prior to deposition onto DNA (20,34,35), and it has been suggested that acetylation of histones H3 and H4 is necessary for deposition of histones onto newly replicated DNA (18,19). The demonstration that histone hyperacetylation appears to be the cause of the arrest of butyrate-treated cells in G_1 (15) indicates that histone acetylation levels are important in replication and suggests to us that there may be an optimal histone acetylation state for dividing cells. There is evidence that histone acetylation plays a role in relaxing chromatin structure during DNA repair synthesis (see above). A similar process could play a role in increasing chromatin accessibility prior to or during replicative DNA synthesis. In addition, presumably histones must be temporarily displaced to other domains, or removed from DNA, during replication (36). Investigators have already demonstrated that histone acetylation plays a role in a similar process in which hyperacetylated histones are displaced by protamines during spermatogenesis (5-7).

We have shown in this paper that replicative DNA synthesis in primary cultures of adult rat hepatocytes can be stimulated by the addition of low levels of butyrate or propionate. We believe that the mechanism of DNA synthesis stimulation may be due to increased histone acetylation in response to the addition of butyrate and propionate for the following reasons: 1) While butyrate and propionate have many dissimilar effects on cultured hepatocytes (see results), both effectively inhibit histone deacetylation; 2) cultured hepatocytes have deficient histone acetylation levels, i.e., acetylation levels decline during culture from levels observed in freshly isolated hepatocytes or *in vivo* (Staecker et. al., submitted); and 3) the stimulation of replicative DNA synthesis by butyrate is restricted to a relatively short period when the majority of cells are entering S phase, corresponding to the point (G_1) at which butyrate-induced histone hyperacetylation blocks progression through the cell cycle.

The use of primary culture of adult rat hepatocytes provides an unique system for studying the role histone acetylation plays in various cellular processes since histone acetylation levels spontaneously decline when hepatocytes are cultured as described. In addition, acetylation levels can be increased, as in other systems, by the addition of propionate or butyrate to cultured cells (Staecker et at., submitted). The rapid metabolism of butyrate by cultured hepatocytes allows one to determine the effects of butyrate on hepatocytes during particular points in the cell cycle. Indeed, our results suggest that increasing histone acetylation levels as cultured hepatocytes enter the S phase of the cell cycle increases replicative DNA synthesis. Since

a butyrate concentration of 1 mM does not completely inhibit histone deacetylation (37), it appears that histone aceylation levels intermediate between the high levels observed with a replication-inhibiting concentration of 5 mM butyrate and the low levels observed in untreated hepatocyte cultures are optimal for replicative DNA synthesis. In the future, we wish to determine the effects of 1 mM butyrate on histone acetylation that is rapidly (vs slowly) turning over. It has been shown previously that rapidly turning over histone acetylation is the kinetic class of acetylation that appears to be responsible for arresting cells in the G_1 phase of the cell cycle with high (5 mM) concentrations of butyrate (34). We have not yet been able to look specifically at rapid acetylation because of the low levels of [^{3}H]-acetate incorporated into histones observed in cultured hepatocytes (Staecker et al., submitted).

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