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SELECTIVE INHIBITION BY SODIUM BUTYRATE OF THE GLUCOCORTICOID INDUCTION OF GLYCEROL PHOSPHATE DEHYDROGENASE IN GLIAL CULTURES

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SUMMARY: We examined the effect of millimolar concentrations of the short chain fatty acid, n-butyrate, on the induction of two cytoplasmic enzymes in the C6 rat glioma cell line. Hydrocortisone addition to the culture medium produced a 2- to 3.5-fold induction of glycerol phosphate dehydrogenase after 24 h, and norepinephrine produced a 2-fold increase in lactate dehydrogenase activity in this time period. Five mM sodium butyrate completely abolished the glucocorticoid-mediated response, while the catecholamine-mediated response was unaffected. This selective inhibition was rapid and reversible, and did not affect the level of cytoplasmic steroid binding. Furthermore, high doses of hydrocortisone did not overcome the inhibition. Finally, this phenomenon was also seen in primary cultures of pure oligodendrocytes.

INTRODUCTION

In a variety of vertebrate cell lines, millimolar concentrations of sodium butyrate (NaB) exert diverse effects upon cellular morphology, metabolism, differentiation, and gene expression (1-5). The only specific biochemical effect of NaB shared by all vertebrate cell types reported to date is the hyperacetylation of nucleosomal histone proteins (6) most probably due to inhibition of deacetylase activity (7). No conclusive proof exists yet that the NaB-induced histone hyperacetylation underlies any of the wide number of observed cellular changes. The suggestion has been made that an important role for histone acetylation is in regulating chromatin transcriptional activity (8,9). Recent work on NaB-induced hyperacetylated chromatin (10) shows that the nucleosomal structure has been altered by the criterion of

Abbreviations: NaB, sodium butyrate; GPDH, glycerol phosphate dehydrogenase; HC, hydrocortisone.

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DNAse I cleavage, in which susceptibility of DNA in chromatin to DNAse I attack is generally believed to be correlated with increased transcriptional activity (11). By this test NaB is thought to increase transcription by the hyperacetylation mechanism.

These observations led us to investigate whether NaB could alter the rate of synthesis of specific proteins known to be regulated by hormonal agents. Steroid hormones alter the rate of synthesis, both in vivo and in vitro, of a number of proteins in different cell types. The great body of evidence supports the view that this class of hormones, via the interaction of their cytoplasmic receptors with specific nuclear 'acceptor' sites, increases the rate of transcription of these proteins' respective mRNAs (12). While the chemical nature of the nuclear 'acceptor' site is still a matter of controversy, it appears that DNA is the most promising candidate (13), with chromosomal proteins playing an important, accessory role (e.g., directing specificity of and accessibility to the critical DNA binding sites) (14). The nature of this accessory function has yet to be precisely elucidated. The knowledge that the specificity of protein-DNA interactions is altered by NaB (10) further increased our interest in the possibility of their modifying the steroid receptor's interaction with nuclear sites. A second class of hormones, the catecholamines, act through a different series of events. They bind to membrane receptors and appear to regulate transcriptional processes via a cyclic AMP-regulated rise in the intracellular level of cyclic AMP which causes increased protein phosphorylation (see 15 for references).

Specifically, in our laboratory the cytoplasmic enzyme, glycerol phosphate dehydrogenase (glycerol-3-phosphate: NAD^+2-oxidoreductase, EC 1.1.1.8; GPDH), in the rat C6 glial tumor cell line is inducible by hydrocortisone (HC) and related glucocorticoids (16). Furthermore, this induction involves de novo RNA and protein synthesis, the continual presence of the steroid (16,17), and a classical steroid receptor-mediated mechanism (18). In the presence of 5 mM NaB, the increase in GPDH level induced by hydrocortisone

was completely abolished. In contrast, the induction of lactate dehydrogenase (EC 1.1.1.27) by catecholamines (16) was not affected by this concentration of NaB.

MATERIALS AND METHODS

Cells and Cell Culture: The C6 glioma cell line was originally established from a chemically induced tumor in rat brain (19). The 2BD subclone was used in these experiments. All cultures were grown at 37°C without antibiotics in a humidified atmosphere of 95% air/5% CO_2. Culture medium was Ham's F-10 containing 10% fetal calf serum (Irvine Scientific Co., Irvine, CA). Medium was changed 1 d prior to any experimental treatment. All drugs were first added to the medium, the pH adjusted to 7.4, and the medium then filter-sterilized prior to addition to the cultures. All experiments were carried out on confluent cultures (10-12 d). Primary cultures of pure oligodendrocytes from fetal rat brains were prepared as described (20) and maintained in basal medium Eagle's (with Hank's salts) in the same atmosphere as C6 cells.

Enzyme Assays: GPDH and lactate dehydrogenase activities were determined at 30°C (17,21). One unit of enzyme is defined as that amount which causes the oxidation of 1 nmol of NADH/min at 30°C. Specific activity is expressed as units of enzyme activity/mg of total cell protein. Protein determinations (22) and enzyme assays were done in triplicate and expressed as an average ± standard deviation.

Cytoplasmic Receptor Assay: A high speed cytosol preparation (105,000 x g) was made from C6 cells treated with or without 5 mM NaB. The amount of specific binding of [^{3}H]dexamethasone, corrected for protein taken from cell homogenates, was determined as previously reported (23), except that the charcoal used to separate free from bound hormone was coated with albumin to prevent adsorption of cytosol proteins on the charcoal.

Incorporation of [^{3}H]leucine into Total Protein: C6 cells were incubated for 30 min at 37°C with 2-4 µCi/ml [^{3}H]leucine in leucine-free Ham's F-10 medium supplemented with 10% dialyzed fetal calf serum and 0.26 µg/ml leucine. Cells were processed as previously described (17).

Whole Cell Uptake of [^{3}H]hydrocortisone: C6 cells were incubated with 10 µM [^{3}H]hydrocortisone in complete medium for 1 h at 37°C either in the presence or absence of 5 mM NaB. Cells were then washed 4 times with ice-cold phosphate-buffered saline, harvested in phosphate-buffered saline, and then sonicated for 20 s. Aliquots of this suspension were taken for liquid scintillation counting and protein determination.

RESULTS

Effect of Sodium Butyrate on GPDH Induction: Hydrocortisone treatment of C6 cells increased GPDH activity nearly 4-fold over a 24 h period. Five mM NaB in the medium resulted in a complete block of the induction while the GPDH basal level remained unaffected. Pretreatment of the cells for 24 h with 5 mM NaB followed by a rapid medium wash did not inhibit subsequent GPDH

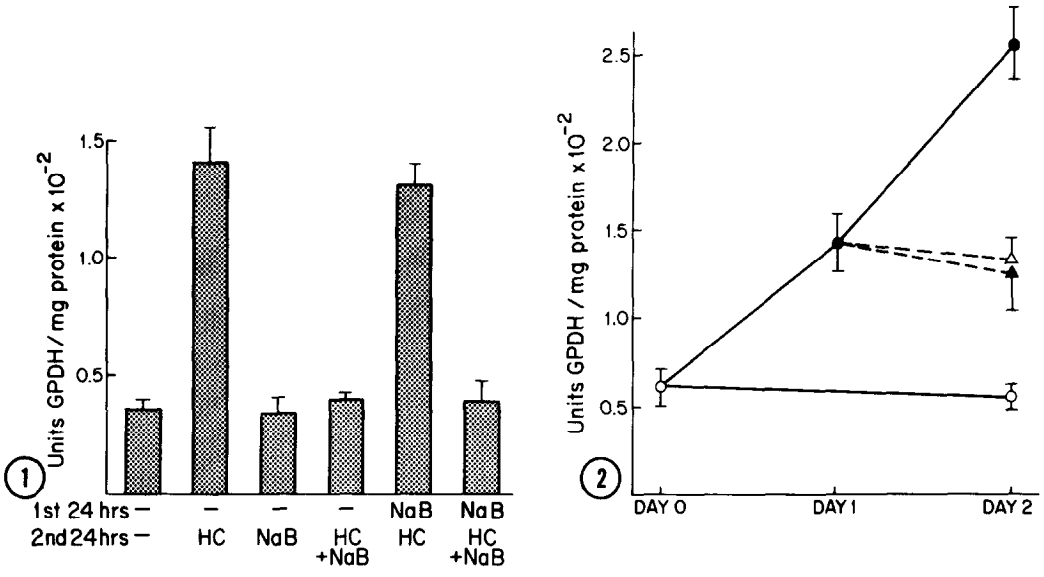

Figure 1. Effect of 5 mM NaB on the hydrocortisone (1.38 μM) induction of GPDH in C6 cells. In two experimental conditions (5th and 6th vertical bars), cells are pretreated for 24 h with NaB. The solid dash (—) simply represents changing the medium. The first vertical bar represents basal GPDH levels while the 2nd vertical bar shows 24 h hydrocortisone-induced GPDH levels. The 3rd vertical bar shows the effect of NaB on basal GPDH levels. The 4th vertical bar shows GPDH levels 24 h after the concurrent administration of NaB and hydrocortisone.

Figure 2. Basal GPDH levels at Day 0 (start of experiment) and Day 2 (48 h later) in confluent C6 cells exposed to medium alone are shown (o). Hydrocortisone (1.38 μM)-induced GPDH levels on Day 1 (24 h induction) and Day 2 (48 h induction) are shown (●). The GPDH levels of cells first exposed to hydrocortisone for 24 h, then washed with medium, and finally kept in medium for 24 additional h are shown (Δ). The GPDH levels of cells first exposed to hydrocortisone for 24 h, then washed with medium, and finally maintained in medium containing 5 mM NaB are shown (▲).

induction by hydrocortisone in the following 24 h period (Fig. 1). Thus, the effect of NaB appears to be fully reversible. Furthermore, induced levels of the enzyme were unaffected by a subsequent 24 h challenge with NaB (Fig. 2). This indicates that NaB does not preferentially inactivate newly made GPDH enzyme molecules nor cause them to leak into the extracellular media.

Dose-response analysis reveals that a concentration of NaB as low as 0.5 mM caused a significant inhibition of enzyme induction (Fig. 3), while 0.1 mM was ineffective. To rule out an effect of NaB on hydrocortisone metabolism that would result in only a partial saturation of glucocorticoid receptors, the concentration of hydrocortisone in the medium was raised to 50 μM; this had no effect upon the blockade of GPDH induction (data not shown).

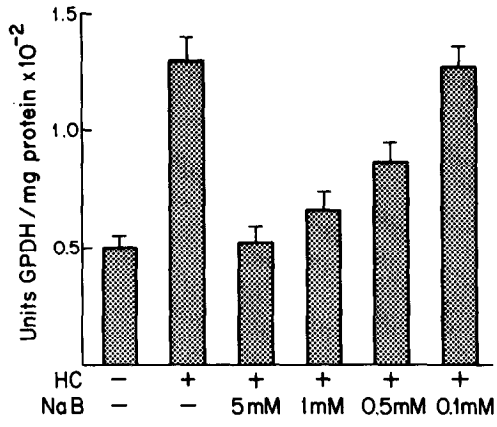

Figure 3. Effect of NaB concentration on the hydrocortisone induction of GPDH in C6 cells. Cultures were treated with 1.38 μM HC for 24 h. NaB was added to the cultures simultaneously with HC.

The butyrate inhibition of the induction appears to occur rapidly. When 5 mM NaB and hydrocortisone were added simultaneously to a culture 24 h after an initial challenge with hydrocortisone, the GPDH level assayed 24 h later was not different from a culture in which the initial hydrocortisone challenge was simply replaced with hydrocortisone-free media for 24 h (Fig. 2). Combined with the knowledge of complete inhibition of induced GPDH levels with the 5 mM NaB, this notion of the rapidity of the inhibition is further strengthened.

Toxicity of Sodium Butyrate: Exposure to 5 mM NaB for 1, 6 or 24 h had absolutely no effect upon [^{3}H]leucine incorporation into proteins (data not shown). This suggests that overall protein synthesis is unaffected at the highest NaB dose used in these experiments, while the rapid reversibility of the effect is also not consistent with an argument for toxicity. In addition, the absence of inhibition of the lactate dehydrogenase induction (Table 1) further suggests that the inhibition of the steroid response is selective and not the result of nonspecific NaB-induced toxicity.

Receptor Studies: The *in vitro* specific binding of [^{3}H]dexamethasone to the cytosol receptor in C6 cells was completely unaffected by treatment at either 1, 6, or 24 h with 5 mM NaB in the medium (data not shown).

Table 1. Differential effect of sodium butyrate on the induction of lactate dehydrogenase and glycerol phosphate dehydrogenase in C6 cells

	Control[b]	Induced[c]	1 mM NaB[d]	5 mM NaB[e]
Lactate Dehydrogenase[a]	990 ± 20	1880 ± 100	2106 ± 105	1848 ± 50
Glycerol Phosphate Dehydrogenase[a]	38 ± 3	80 ± 11	46 ± 2	34 ± 2

[a]Expressed as units/mg whole cell protein.

[b]Cultures receive only a medium change.

[c]Cultures receive 3 μM norepinephrine and 1.38 μM hydrocortisone for 24 h.

[d]Cultures receive 3 μM norepinephrine and 1.38 μM hydrocortisone for 24 h in the presence of 1 mM sodium butyrate.

[e]Cultures receive 3 μM norepinephrine and 1.38 μM hydrocortisone for 24 h in the presence of 5 mM sodium butyrate.

[f]All values are expressed as an average of 3 cultures ± S.D.

Whole Cell Uptake of [^{3}H]steroid: NaB has no effect on the whole cell uptake of the radiolabeled steroid (data not shown).

Effect of Sodium Butyrate on GPDH Induction in Primary Cultures of Oligodendrocytes: In the central nervous system, GPDH is exclusively localized in oligodendrocytes (24,25). The glucocorticoid induction of GPDH is expressed in various systems of primary cultures of nervous tissue (21,25), including pure cultures of oligodendrocytes (20). Hydrocortisone treatment of oligodendroglial cultures for 24 h increased the specific activity of GPDH from 40 to 200 units. The simultaneous presence in the medium of 5 mM NaB totally inhibited the 5-fold induction of GPDH.

DISSCUSSION

The hydrocortisone induction of GPDH in C6 glioma cells and primary cultures of oligodendrocytes is specifically blocked by NaB. The reversibility of this inhibition subsequent to removing NaB from the medium is consistent with the observed rapid reversibility of most of the other recorded cellular changes in other cell types (3). The fact that this inhibition

occurs in normal cells as well indicates that it is not merely a peculiarity of malignant transformation.

This phenomenon cannot simply be accounted for by arguments for NaB-induced nonspecific toxicity, blockade of steroid diffusion into cells, destruction (inactivation) of either basal or induced levels of the enzyme, leakage of the enzyme extracellularly, or inadequate cytoplasmic steroid binding.

We have yet to determine the underlying mechanism, most notably whether histone hyperacetylation plays a critical role. If the defect is indeed in the nucleus as a result of alteration of nuclear DNA-protein associations (10), then NaB can be used as a new tool to investigate the steps in the glucocorticoid induction process distal to the cytoplasmic binding of the steroid. Studies are currently under way in our laboratory to directly deal with this issue. The fact that complete blockade exists despite massive steroid doses (50 μM) already suggests the possibility of the total unavailability of critical nuclear acceptor sites for the steroid receptor-hormone complex. Finally, the resistance of the catecholamine induction of LDH to NaB shows that the effect of NaB on the regulation of gene expression is selective. Previously, we reported that the LDH induction is associated with increased phosphorylation of a nuclear protein (26); this study suggests that protein acetylation does not play a role in the phenomenon.

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