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EFFECT OF *n*-BUTYRATE ON THE SYNTHESIS OF SULFATED GLYCOSAMINOGLYCANS AND HYALURONATE BY RAT LIVER FAT-STORING CELLS (ITO CELLS)

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Abstract—The effect of naturally occurring aliphatic carboxylic acid *n*-butyrate on total and type-specific synthesis of sulfated proteoglycans and hyaluronic acid by rat liver fat-storing cells (Ito cells, vitamin A-storing cells), the main connective tissue producing cell type in liver, was studied. Concentrations of *n*-butyrate equal to and higher than 5 mM inhibited significantly in a dose-dependent manner the incorporation of [^{35}S]sulfate and [^{3}H]glucosamine into the carbohydrate chains of sulfated proteoglycans. Maximum inhibition of 70% was reached at 15 mM. Similarly, the formation of hyaluronic acid was impaired by *n*-butyrate. The synthesis profile of specific sulfated proteoglycans was not affected by the compound. β-Xyloside, an artificial carbohydrate chain initiator, reversed only partially the inhibitory effect of *n*-butyrate. Thus, the mechanism of *n*-butyrate inhibition may involve an impairment of both proteoglycan core protein formation and glycosaminoglycan chain elongation.

Fat-storing cells also known as perisinusoidal lipocytes, vitamin-A-storing cells, and Ito cells are a highly specialized non-parenchymal type of liver cells located in the space of Disse in recesses of adjacent hepatocytes [1–3] and devoted primarily to the storage of vitamin A [4–8]. It was shown by immunofluorescence, light- and electron-microscopic autoradiography and cell culture studies that fat storing cells are able to synthesize significant amounts of several types of collagens [9–12] and structural glycoproteins like fibronectin and laminin [11, 13]. Recently, we have shown *in vitro* their high capacity to synthesize a wide spectrum of sulfated proteoglycans [14] and hyaluronic acid [15], of which about 80% are secreted into the medium. It is suggested by these studies that fat-storing cells are the principal cell type for the production of extracellular matrix proteoglycans and hyaluronic acid, respectively, in liver. In chronically injured and inflamed liver tissue fat-storing cells proliferate and transform into (myo-)fibroblasts [16–20], a mechanism which is thought to be of crucial importance for the development of liver fibrosis, i.e. the exaggerated production and extracellular deposition of collagens and proteoglycans [21]. The factors controlling proteoglycan and hyaluronic acid synthesis/secretion by fat-storing cells in liver are poorly understood. n-Butyric acid, a short chain, naturally occurring aliphatic carboxylic acid has been found capable of a diversity of effects on cells in culture including modulation of specific gene expression [22], hyperacetylation of histone [23–25], inhibition of replicative DNA synthesis and cell division by arrest of cells in G_1 phase [26–29], and stimulation of certain enzyme activities [30–32]. So far, the effect of this compound on the production of connective tissue molecules in hepatic cells, in particular on that of glycosaminoglycans is unknown. Previous studies on the influence of *n*-butyrate on glycosaminoglycan synthesis in a variety of tumor cells and nonmalignant cells have provided diverse results. In mastocytoma cells [33, 34] butyrate increases severalfold cellular heparin and heparan sulfate and the formation of heparin molecules with high affinity for antithrombin [34]. In human kidney tumor cells *n*-butyrate decreases the incorporation of [^{35}S] sulfate into heparin/heparan sulfate whereas the synthesis of hyaluronic acid and of chondroitin sulfate/dermatan sulfate measured by incorporation of [^{3}H]glucosamine was found to be stimulated [35]. A recent study with human skin fibroblasts reports on an inhibition of the synthesis of hyaluronic acid by butyrate leaving that of sulfated proteoglycans unaffected [36]. Thus, the response of glycosaminoglycan synthesis to butyrate obviously depends on the type of cell under investigation. We studied *in vitro* the effect of *n*-butyrate on the formation of these complex carbohydrates by the principal connective tissue producing cell type in liver.

MATERIALS AND METHODS

Isolation and culture of fat-storing cells. The cells were prepared from 1-year-old male Sprague–Dawley rats (body weight 500–700 g, Lippische Versuchstierzucht Extertal, F.R.G.), which had free access to a standard laboratory chow diet (type Han

* Abbreviations used: NEM, *N*-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate].

MR5 containing 15000 U vitamin A/kg) and tap water. Nonparenchymal liver cells were isolated by the pronase-collagenase method [9] in a sequence of non- and recirculating perfusions incorporating some minor modifications described elsewhere [14]. Fat-storing cells were purified from the nonparenchymal liver cell suspension by single-step density gradient centrifugation with Nycodenz® (analytical grade, Nyegaard and Co. AS, Oslo, Norway), which has been described in detail previously [14]. The mean purity of freshly isolated cells was at least 85%, cell viability as checked by trypan blue exclusion was more than 80%, and the yield ranged from $5 \cdot 10^6$ to $80 \cdot 10^6$ cells/liver. After the first change of medium most of the contaminating cells were removed, and the monolayers were essentially free of impurities. Fat-storing cells were identified by their typical light microscopic appearance, transmission electron microscopy [14], positive immunofluorescence staining for desmin [37], and vitamin A-specific autofluorescence at an excitation wavelength of 328 nm [14]. The cells were seeded with an initial density of $0.4 \cdot 10^6/2\ cm^2$ and maintained as monolayers in 24-well culture plates (Falcon®, Becton & Dickinson, Oxnard, U.S.A.) in 1 ml/2 cm^2 well of Dulbecco's modification of Eagles medium (Flow Laboratories GmbH, Bonn, F.R.G.) containing 4 mmol/l L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), 0.1% fungizone, and 10% (v/v) fetal calf serum (Boehringer GmbH, Mannheim, F.R.G.) in a humidified atmosphere of 5% CO_2 and 95% air. The first change of medium was made about 20 hr after seeding, the following changes were made daily. Sodium butyrate (Sigma Chem. Co., Munich, F.R.G.) was added to the cultures at the first day of culture for 24 hr, then medium was changed and butyrate was removed. The following isotope incorporation was performed always in absence of butyrate. At the time of labeling cells had reached preconfluency.

Determination of the synthesis of total and specific types of proteoglycans. The synthesis of sulfated proteoglycans was determined by the incorporation of either [^{35}S]sulfate (18.5–22.2 GBq/mmol; 370 kBq/ml medium; New England Nuclear Corp., Boston, U.S.A.) or D-[6-^{3}H] glucosamine hydrochloride (1.48 TBq/mmol; 370 kBq/ml medium, Amersham Buchler GmbH, Braunschweig, F.R.G.) during a labeling period of 24 hr. Labeled carbohydrates were measured in the medium of the cultures and isolated by two different procedures. [^{3}H]glucosamine labeled glycosaminoglycans were isolated from the cell-free medium (after centrifugation for 6 min, 1000 *g*, 4°) after exhaustive proteolysis in 0.1 M sodium acetate, pH 6.2, with papain (EC 3.4.22.2, Boehringer GmbH, Mannheim, F.R.G.) for 38 hr at 60° [38] and batchwise chromatography on DEAE-Sephacel (Pharmacia Fine Chem., Uppsala, Sweden) equilibrated with 0.1 M sodium acetate, pH 6.2 as described [14, 39]. The efficiency of proteolytic glycosaminoglycan extraction was more than 90%. An aliquot of total labeled glycosaminoglycans eluted from the resin with 2.2 M NaCl was counted for radioactivity, which was expressed in relation to the DNA content of the culture plate. The latter was determined fluorometrically [40] using calf thymus DNA (type I, Sigma Chem. Co., Munich, F.R.G.) as a standard. [^{35}S]Sulfate-labeled proteoglycans were isolated from the medium without proteolytic extraction. An aliquot of the medium (700 μl) was mixed with 3 ml buffer A (0.13 M Tris–HCl, pH 7.5, containing 7 M urea, 0.1% CHAPS, 1 mM EDTA, 1 mM PMSF and 10 mM NEM). The mixture was incubated for 1 hr at room temperature with DEAE-sephacel equilibrated in buffer A, centrifuged, and the resin was extensively washed with buffer A supplemented with 0.1 M NaCl. Total proteoglycans were eluted with 0.8 M NaCl in buffer A.

For analysis of specific types of glycosaminoglycans or proteoglycans, the material was subjected to consecutive degradations with nitrous acid to yield the incorporation of label into heparan sulfate [41] and to enzymatic digestion with chondroitin AC- (EC 4.2.2.5) and -ABC-lyases (EC 4.2.2.4) (Seikagaku Fine Chemicals, Tokyo, Japan) to obtain the fractions of chondroitin 4,6-sulfate and dermatan sulfate [42], respectively. Analytical details of the procedure are reported elsewhere [38].

Determination of hyaluronic acid synthesis. Total [^{3}H]glycosamine-labeled glycosaminoglycans were subjected to enzymatic digestion with hyaluronate lyase (EC 4.2.2.1, from *Streptomyces hyalurolyticus*, Seikagaku Co., Tokyo, Japan) by incubation for 3 hr at 60° in 33 mM sodium acetate, pH 5.0 with 5 TRU hyaluronate lyase (dissolved in 0.1 M NaCl, 12.5 TRU/ml) in a total reaction volume of 1 ml [15]. Control incubations were done with a similar volume of 0.1 M NaCl instead of enzyme. After termination of the reaction by cooling in ice those GAG not degraded by hyaluronate lyase were precipitated for 16 hr at room temperature with 4 vol. of sodium acetate saturated ethanol and centrifuged (4500 *g*, 10 min). The sediment was dried, dissolved in water and counted for radioactivity. The amount of labeled hyaluronic acid was calculated from the difference between control and enzyme incubation. Specificity and efficiency of degradation of hyaluronic acid were higher than 90%. In previous experiments hyaluronic acid in the medium was isolated and characterized by chromatography on DE-52 cellulose (Whatman Chem. Sep., U.K.) and sepharose Cl-6B, respectively [15].

Ion exchange chromatography of glycosaminoglycans. [^{35}S]sulfate labeled glycosaminoglycans isolated from the medium after proteolysis as described above were dissolved in 0.15 M NaCl and subjected to anion exchange chromatography on a column (0.5 × 3 cm) of Dowex 1 × 2 (Serva Biochemicals, Heidelberg, F.R.G.). The material was eluted with a stepwise gradient of increasing molarity of NaCl. The fractions were extensively dialysed against water and counted for radioactivity.

Statistical analysis. Differences of $n \geq 4$ independent determinations were tested with the Scheffé multiple range test for pairwise comparisons of means; $P < 0.05$ was accepted to be statistically significant [43].

RESULTS

The typical light and electron microscopic morphology of fat-storing cells was not affected by the

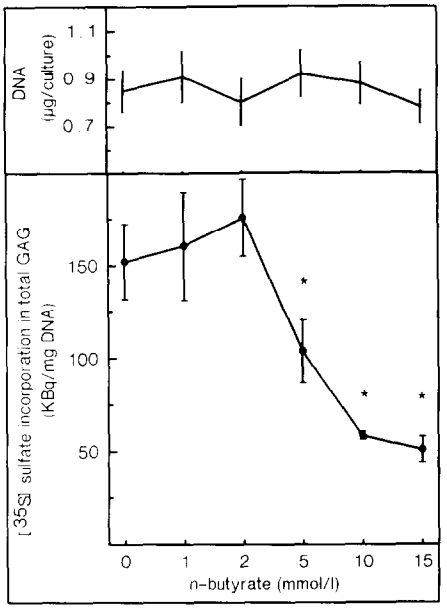

Fig. 1. Effect of various concentrations of *n*-butyrate on the DNA content and sulfated medium proteoglycan synthesis of fat-storing cell cultures. Cells at the first culture day were exposed for 24 hr with *n*-butyrate, after which time medium was renewed and butyrate was removed. The cells were labeled for the following 24 hr with [^{35}S]sulfate (370 kBq/ml medium) and the incorporation of the label into medium proteoglycans was measured. Mean values ± SD of 3 to 4 experiments are shown, asterisks mark statistically significant changes in comparison with controls. GAG = glycosaminoglycans.

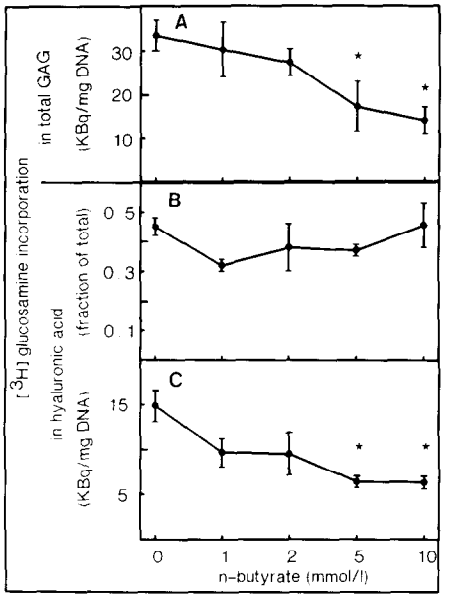

Fig. 2. Effect of various concentrations of *n*-butyrate on the incorporation of [^{3}H]glucosamine (370 kBq/ml medium) into total glycosaminoglycans (A) and hyaluronic acid (C) of the medium of fat-storing cells. The amount of hyaluronic acid expressed as fraction of total labeled glycosaminoglycans is shown in (B). The conditions are similar to those described in Fig. 1. Mean values ± SD of 4 experiments are given, significant changes are indicated by asterisks. GAG = glycosaminoglycans.

exposure with *n*-butyrate (not shown). Neither the viability of the cells (about 90%) nor the DNA content of the culture plates (Fig. 1) was reduced in the presence of up to 15 mM *n*-buyrate for 24 hr. The synthesis of sulfated glycosaminoglycans in the medium of fat-storing cells was significantly diminished at concentrations of *n*-butyrate equal to and higher than 5 mM (Fig. 1). Maximum inhibition of about 70% was reached at 15 mM *n*-butyrate. Similar results were obtained applying the rate of incorporation of [^{3}H]glucosamine as a measure of medium glycosaminoglycan synthesis (Fig. 2A).

The carboxylic acid did not interfere with the pattern of newly formed sulfated glycosaminoglycans. Both in control and *n*-butyrate-treated cultures the main fraction was dermatan sulfate comprising 70–80% of total medium glycosaminoglycans (Table 1), which is consistent with previous studies. An almost identical distribution profile was obtained with [^{3}H]glucosamine-labeled glycosaminoglycans. The formation of the unsulfated glycosaminoglycan hyaluronic acid was inhibited to a similar extent as the synthesis of total glycosaminoglycans. Significant inhibitions of hyaluronate synthesis were reached at *n*-butyrate concentrations of 5 mM and higher (Fig. 2C); its fractional synthesis rate remained constant during exposure with the agent (Fig. 2B). No changes of the ion exchange

Table 1. Effect of *n*-butyrate on the profile of [^{35}S]sulfate-labeled specific types of glycosaminoglycans in the medium of fat-storing cells

Culture	Type of glycosaminoglycans (%) Chondroitin sulfate	Dermatan sulfate	Heparan sulfate
Control	17.0 ± 4.5 n.s.	72.7 ± 3.9 n.s.	20.6 ± 7.8 n.s.
n-Butyrate	11.4 ± 9.4 n.s.	80.4 ± 8.6 n.s.	26.8 ± 3.8 n.s.

Cells of the first culture day were treated for 24 hr with 10 mM *n*-butyrate, thereafter medium was changed and butyrate was removed. Control incubations were not exposed to butyrate. [^{35}S]Sulfate (370 kBq/ml) was added to the cultures for the folowing 24 hr and its incorporation into specific glycosaminoglycans was determined. Mean values of ±SD of 4 experiments are shown. n.s. = not significant.

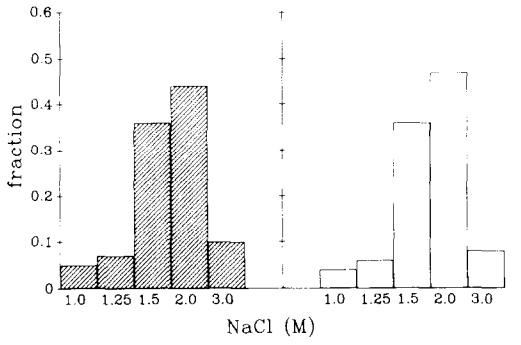

Fig. 3. Anion exchange chromatography (Dowex 1 × 2) of [^{35}S]sulfate-labeled glycosaminoglycans secreted into the medium by fat storing cells. The cells were treated for 24 hr with 10 mM *n*-butyrate prior to labeling as described in Fig. 1 (hatched bars). Control incubations were kept in the absence of butyrate (open bars).

elution profiles of [^{35}S]sulfate-labeled glycosaminoglycans were observed under the influence of *n*-butyrate (10 mM). Both in control and *n*-butyrate-treated cultures the major fractions were eluted with 1.5 M and 2.0 M NaCl, respectively (Fig. 3).

Experiments were undertaken to define the step of *n*-butyrate induced inhibition of proteoglycan synthesis either at the level of core protein synthesis or carbohydrate chain elongation. The addition of β-D-xylopyranoside, which acts as an artifical initiator of polysaccharide chain elongation, to the cultures stimulated significantly the synthesis of [^{35}S]sulfate-labeled medium glycosaminoglycans (Table 2). The inhibition induced by *n*-butyrate could be reversed only partially (by about 75% in comparison with xyloside treatment alone) in presence of the chain initiatory molecule (Table 2). Compared with untreated fat-storing cell cultures β-xyloside abolished completely the inhibitory effect of *n*-butyrate.

Table 2. Effect of β-D-xylopyranoside on the *n*-butyrate-induced inhibition of glycosaminoglycan synthesis by fat storing cells

Additions to the cell incubation	DNA (μg/culture)	Incorporation of [^{35}S]sulfate into glycosaminoglycans (kBq/mg DNA)
None	1.2 ± 0.18	260 ± 34
Xylopyranoside	1.2 ± 0.10	374 ± 33* ($P < 0.05$)
n-Butyrate	1.4 ± 0.11	135 ± 17* ($P < 0.05$)
n-Butyrate and xylopyranoside	1.2 + 0.18	285 ± 41 n.s.

Fat storing cells of the first culture day were incubated for 24 hr with *n*-butyrate (10 mM). Medium was changed and β-xylopyranoside (0.5 mM) was added for 24 hr. Thereafter medium was renewed again and supplemented with [^{35}S]sulfate for 24 hr. Control cultures received no β-xyloside and/or butyrate. The radioactivity incorporated into glycosaminoglycans of the medium was measured. Mean values ± SD of 4 experiments are shown. n.s. = not significant in comparison with untreated cultures.

DISCUSSION

Sodium butyrate, when added to the medium of a variety of cultured cells, exerts numerous biological effects including the inhibition of cell division [26–29], the stimulation of some enzyme activities [30–32], and the modulation of hormone receptor density [29, 44, 45]. The effect of the compound on the formation of hyaluronic acid and sulfated proteoglycans are obviously dependent on the cell type as mentioned above [33, 35]. Recently, it was reported that *n*-butyrate inhibits specifically the synthesis of hyaluronic acid by human skin and colon fibroblasts [36]. In that study the formation of chondroitin sulfate and dermatan sulfate was not affected. We demonstrate for cultured rat liver fat-storing cells an inhibitory effect of *n*-butyrate on the synthesis of nonsulfated glycosaminoglycans and sulfated proteoglycans, respectively. The conditions used make unlikely toxic effects, morphologic changes, and a reduction of cell density by *n*-butyrate. Our finding of an unselective and quantitatively similar inhibitory action of butyrate on various types of sulfated proteoglycans and hyaluronic acid, which is contradictory to the reported specific inhibition of hyaluronate formation in skin fibroblasts [36] might have a number of reasons. The most likely one will be the diversity of the cell types studied. Liver fat-storing cells are normally engaged in the uptake, storage, and metabolism of retinoids [8]. Although these cells produce also a number of different types of collagens [9–12], laminin [11], fibronectin [13], proteoglycans [14], and hyaluronate [15] fat-storing cells are clearly distinct from fibroblasts [2]. They might be regarded as resting prefibroblasts because in injured liver tissue fat-storing cells transform and proliferate via transitional cells to myofibroblast-like cells [17, 18, 20]. It will be of interest to study the effect of *n*-butyrate on proteoglycan synthesis of transformed fat-storing cells and on the process of cellular transformation. The mechanism of inhibition of sulfated proteoglycan synthesis cannot be ascribed to a mere impairmant of protein core synthesis because the addition of β-D-xyloside could not fully reverse the effect of *n*-butyrate. Xylosides act as artifical initiators of glycosaminoglycan chain elongation in competition with the endogenous, xylosylated core protein [46] and, thus, glycosaminoglycan chain synthesis in presence of these compounds by-passes the formation of the protein core of proteoglycans [47]. Furthermore, the formation of hyaluronic acid, which is not dependent on the synthesis of a protein core, was inhibited similarly to that of proteoglycans. The results suggest that *n*-butyrate interferes, beside an effect on core protein formation, also with the carbohydrate chain elongation reaction by some as yet unknown mechanism. Since carboxylic acids can modify gene expression through acetylation [23–25], DNA methylation [48], chromatin phosphorylation [49], and ADP-ribosylation [50] it is conceivable that the syntheses of short living enzymes essential for carbohydrate chain synthesis such as UDP-glucuronyltransferase and/or -hexosaminyltransferases are diminished. It seems not likely that *n*-butyrate inhibits preferentially the sulfation of the

glycosaminoglycans since quantitatively similar results were obtained with [^{3}H]glucosamine as labeled precursor and the ion exchange elution profile of glycosaminoglycans was not altered by *n*-butyrate. Possible changes of the specific activity of the immediate precursor pool, i.e. UDP-*N*-acetylglucosamine and 3′-phosphoadenosine-5′-phosphosulfate (PSPS) [38, 51, 52] have not been measured in this study and, thus, cannot be strictly ruled out. However, it is considered unlikely that the specific activities of both precursor pools are reduced to the same extent by *n*-butyrate. The functional implications of *n*-butyrate mediated inhibition of proteoglycan- and hyaluronate synthesis must remain speculative at present. Deduced from the supposed fundamental roles of these complex carbohydrates in modulation of tissue and cell differentiation, in the regulation of cell migration and proliferation and in cell-matrix and cell-cell adhesion [53–56] it will be important to consider in further studies on the cellular response to *n*-butyrate its profound and cell-type specific action on the metabolism of proteoglycans and hyaluronic acid.

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