Effects of Butyrate on Epidermal Growth Factor Receptor Binding, Morphology, and DNA Synthesis in Cultured Rat Hepatocytes

Ivar P. Gladhaug, Magne Refsnes, Tor-Erik Sand, and Thoralf Christoffersen

Department of Pharmacology, School of Medicine, University of Oslo, N-0316 Oslo 3, Norway

ABSTRACT

We have examined the effect of butyrate on morphology, DNA synthesis, and epidermal growth factor (EGF) receptor binding in primary cultures of rat hepatocytes. Butyrate added 2 h after plating retarded the flattening and maintained the polyhedral shape of the hepatocytes in culture. Both insulin- and EGF-stimulated DNA syntheses were slightly stimulated by butyrate at 1 mM but strongly inhibited at 5 mM. EGF receptor binding was also strongly affected by butyrate treatment of the hepatocytes. The freshly isolated hepatocytes (prior to plating) and the early-stage cultures (2 h) exhibited two classes of surface EGF receptors with high and low affinity (Kd ~ 0.05 nM and ~ 0.7 nM, respectively). With increasing time in culture there was a decrease in the total EGF receptor number and a corresponding reduction in the capacity for receptor-mediated EGF internalization. The high-affinity receptor class was more strongly reduced than the low-affinity class and was almost absent after 40 h in culture. Butyrate dose-dependently counteracted the decrease in the number of surface EGF receptors during culturing and preserved the high-affinity binding component. Thus, after 40 h, the cells cultured in the presence of butyrate (5 mM) had an approximately 50% elevation in the total number of receptors and the capacity to endocytose EGF compared to control cells, whereas the binding at low ligand concentration (0.02 nM) was increased 4-fold. The results suggest that butyrate, in addition to affecting morphology and DNA synthesis, also has marked effects on the hepatocyte EGF receptor status.

INTRODUCTION

EGF is a M, 6045 polypeptide mitogen with multiple effects on a number of cells and tissues (1). Several lines of evidence have implicated EGF in growth control, differentiation, and oncogenesis (reviewed in Refs. 2–5). The cellular events are initiated by binding of EGF to specific cell surface receptors (6), which show homology with the erbB-1 oncogene product (7) and contain a tyrosine-protein kinase activity (8, 9). Upon binding, the complex of EGF and the receptor is rapidly cleared from the cell surface by receptor-mediated endocytosis (10–12). In many experimental systems, a correlation between stimulation by growth-promoting factors unrelated to EGF and a reduction of surface EGF binding capacity has been observed (13, 14). In liver, when quiescent cells are induced to divide by the flattening and maintained the polyhedral shape of the hepatocytes in culture. Both insulin- and EGF-stimulated DNA syntheses were slightly stimulated by butyrate at 1 mM but strongly inhibited at 5 mM. EGF receptor binding was also strongly affected by butyrate treatment of the hepatocytes. The freshly isolated hepatocytes (prior to plating) and the early-stage cultures (2 h) exhibited two classes of surface EGF receptors with high and low affinity (Kd ~ 0.05 nM and ~ 0.7 nM, respectively). With increasing time in culture there was a decrease in the total EGF receptor number and a corresponding reduction in the capacity for receptor-mediated EGF internalization. The high-affinity receptor class was more strongly reduced than the low-affinity class and was almost absent after 40 h in culture. Butyrate dose-dependently counteracted the decrease in the number of surface EGF receptors during culturing and preserved the high-affinity binding component. Thus, after 40 h, the cells cultured in the presence of butyrate (5 mM) had an approximately 50% elevation in the total number of receptors and the capacity to endocytose EGF compared to control cells, whereas the binding at low ligand concentration (0.02 nM) was increased 4-fold. The results suggest that butyrate, in addition to affecting morphology and DNA synthesis, also has marked effects on the hepatocyte EGF receptor status.

Adult rat hepatocytes kept in vitro as primary cultures retain the normal in vivo characteristics and express certain properties during the culture period. Partial hepatectomy, both the number of EGF receptors and DNA synthesis, and epidermal growth factor (EGF) receptor binding in primary cultures of rat hepatocytes. Butyrate added 2 h after plating retarded the flattening and maintained the polyhedral shape of the hepatocytes in culture. Both insulin- and EGF-stimulated DNA syntheses were slightly stimulated by butyrate at 1 mM but strongly inhibited at 5 mM. EGF receptor binding was also strongly affected by butyrate treatment of the hepatocytes. The freshly isolated hepatocytes (prior to plating) and the early-stage cultures (2 h) exhibited two classes of surface EGF receptors with high and low affinity (Kd ~ 0.05 nM and ~ 0.7 nM, respectively). With increasing time in culture there was a decrease in the total EGF receptor number and a corresponding reduction in the capacity for receptor-mediated EGF internalization. The high-affinity receptor class was more strongly reduced than the low-affinity class and was almost absent after 40 h in culture. Butyrate dose-dependently counteracted the decrease in the number of surface EGF receptors during culturing and preserved the high-affinity binding component. Thus, after 40 h, the cells cultured in the presence of butyrate (5 mM) had an approximately 50% elevation in the total number of receptors and the capacity to endocytose EGF compared to control cells, whereas the binding at low ligand concentration (0.02 nM) was increased 4-fold. The results suggest that butyrate, in addition to affecting morphology and DNA synthesis, also has marked effects on the hepatocyte EGF receptor status.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium with low glucose (5.6 mM) and Waymouth's medium MAB 87/3 modified as described (32) were from Gibco (Grand Island, NY). Collagenase (type I), n-butyrate (sodium salt), dexamethasone, bovine serum albumin (fraction V), and collagen (rat tail, type VII) were from Sigma Chemical Co. (St. Louis, MO). Insulin was from Novo (Copenhagen, Denmark). 125I-EGF (specific activity, 561–683 Ci/mmol) and [6-3H]Thymidine (specific activity, 22 Ci/mmol) were from Amersham International (Buckinghamshire, United Kingdom). Unlabeled EGF (receptor grade) was from Collaborative Research, Inc. (Bedford, MA).

Isolation and Culture of Hepatocytes. Male Wistar rats (180–220 g) were fed ad libitum. Hepatocytes were isolated by in vitro collagenase perfusion and low speed centrifugation (33) with modifications previously described (34). The hepatocytes were seeded into Costar plastic culture wells with collagen in a 1:100 dilution (35), at cell densities of 25,000 cells/cm² for binding studies (12-well dishes) and 16,000 cells/cm² for estimation of DNA synthesis (6-well dishes). The cells were cultured without serum in a 1:1 combination of Dulbecco's modified Eagle's medium and Waymouth's medium (0.25 ml/cm²) as described (24) with dexamethasone (0.25 µM), insulin (0.4 µM), penicillin (67 µg/ml), and streptomycin (100 µg/ml). The culture dishes were maintained at 37°C in a 95% air/5% CO2 atmosphere. The medium was not changed during the culture period. Sodium butyrate at the indicated concentrations was added 2 h after plating.

125I-EGF Binding Assays. The monolayers were washed twice with ice-cold Krebs HCO3- (2-hydroxyethyl)-1-piperazineethanesulfonic acid: Ringer buffer with 1% bovine albumin, pH 7.4 (binding buffer). For estimation of surface binding the cells were chilled on ice for 30 min and incubated with 125I-EGF in a total volume of 600 µl/well at 0°C for 20 h. Under these conditions equilibrium was reached (data not shown). The surface binding capacity was linear at cell densities of 25,000–30,000 cells/cm² (data not shown). For Scatchard analysis labeled EGF was diluted with unlabeled EGF in a fixed ratio of 1:9 as described (24) with dexamethasone (0.25 µM), insulin (0.4 µM), penicillin (67 µg/ml), and streptomycin (100 µg/ml). The culture dishes were maintained at 37°C in a 95% air/5% CO2 atmosphere. The medium was not changed during the culture period. Sodium butyrate at the indicated concentrations was added 2 h after plating.

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2 Research Fellow of the Norwegian Cancer Society. To whom requests for reprints should be addressed, at the Department of Pharmacology, University of Oslo, P. O. Box 1057, Blindern, N-0316 Oslo 3, Norway.
3 The abbreviation used is: EGF, epidermal growth factor.
EGF. Specific EGF binding activity was expressed as fmol EGF/mg protein. For internalization studies, the acid/salt elution method (36) was used to distinguish between surface-bound and intracellular EGF. After free ligand had been removed by rinsing the monolayers, the surface-bound ligand was eluted with a solution of acetic acid (0.2 M) and sodium chloride (0.5 M), pH 2.5, for 10 min. The radioactivity resistant to the acid/salt treatment was interpreted to represent intracellular ligand. Internalization in freshly isolated hepatocytes in suspension was measured as described previously (12). Protein was determined according to the method of Lowry et al. (37).

Estimation of DNA Synthesis. [3H]Thymidine incorporation into DNA was measured biochemically after a 60-min pulse of [3H]thymidine (2.5 μCi/ml, 0.23 nM) to cells cultured for 68 h. The cells were precipitated with 5% trichloroacetic acid, and the DNA was hydrolyzed as described previously (38, 39). To minimize the effects of variation in cellular uptake of thymidine, the results were expressed as the ratio of cpm in DNA by cpm in the acid-soluble fraction (38).

RESULTS

Characterization of Surface EGF Receptor Populations during Short Time Primary Culture of Hepatocytes. We have previously reported that freshly isolated rat hepatocytes exhibit two affinity classes of surface EGF receptors (12). Fig. IA shows Scatchard analysis of binding in cells plated in culture for 2 h compared to later stages during culture (24 h, 40 h). Cells plated in culture for only 2 h displayed curvilinear binding plots. Upon further culturing the EGF binding capacity was significantly reduced, and linear binding plots emerged. Thus, with increasing time in culture the heterogeneous receptor population was converted to a homogeneous population. Data from numerous experiments indicated that at the early stage of culture (2 h) there was a high-affinity population with $K_d$ of about 0.05 nM and a low-affinity population with $K_d$ of about 0.7 nM. This is in close agreement with the binding characteristics of freshly isolated rat hepatocytes (12). The decline in total binding sites was somewhat variable, but based on a number of experiments the overall decrease in binding was 50–75%. The gradual loss of EGF binding capacity which occurred during continued culture consisted of a reduction in the number of low-affinity sites and a virtually complete loss of the high-affinity receptor population.

The loss of surface binding sites was accompanied by a proportional reduction in endocytosis of EGF (Fig. 1B). During continuous endocytosis in freshly isolated hepatocytes, the initial increase in intracellular EGF is followed by a rapid loss of cell-associated ligand, consistent with cellular degradation of the internalized ligand (12). This subsequent reduction in cell-associated EGF was greatly diminished in the cultured hepatocytes, suggesting a difference in the overall processing of EGF between freshly isolated cells and cultured cells.

Effects of Butyrate on Hepatocyte Morphology and DNA Synthesis. Butyrate blocks entry into S phase (27–29) and induces differentiated characteristics in many cells (26). Since transgression of a critical point in G1 might be associated with an alteration in the EGF receptor expression, we treated the cells with butyrate and assessed the DNA synthesis and the EGF binding as well as the morphology.

Butyrate (5 mM) had a profound effect on the morphology of the cultured hepatocytes (Fig. 2). When freshly isolated hepatocytes are placed in culture in the presence of dexamethasone and insulin, they first acquire a polyhedral shape and form trabecular chord-like structures, but subsequently the cells gradually flatten out and form irregular stellate structures (34, 40). Butyrate treatment retarded the characteristic flattening of the hepatocytes, and the cells more or less maintained the uniform polyhedral shape and the tendency to form trabeculae (Fig. 2).

The morphological effects were accompanied by a complete, dose-dependent inhibition of DNA synthesis, in both the absence and presence of EGF (Fig. 3). At the lowest butyrate concentration tested (1 mM), there was a slight stimulation of DNA synthesis. This is consistent with data in a recent report where low levels of butyrate added at the time of cell entry into S phase increased hepatocyte DNA synthesis (41).

Effects of Butyrate on the EGF Receptor Binding. Fig. 4 shows Scatchard plots from cultures treated with butyrate (5 mM) for 27 or 45 h. This treatment resulted in a higher total EGF receptor binding compared to controls and yielded curvilinear binding plots resembling those of freshly isolated hepatocytes and early-stage culture. To obtain a qualitative assessment of the high- and low-affinity binding, hepatocyte cultures were treated with butyrate for 42 h, and the subsequent binding of EGF was determined at both low (with 0.02 nM labeled EGF) and high (with 5.0 nM labeled EGF) degree of occupancy of the total EGF receptor population. Fig. 5 shows that exposure of the cultures to increasing concentrations of butyrate (1.0–5.0 mM) resulted in dose-dependent, distinct effects on the two receptor populations. In addition to the increase (40–50%) in total binding and low-affinity binding, the high-affinity binding component was preserved (Figs. 4 and 5). At the maximal butyrate concentration tested (5 mM), binding of a low ligand concentration (0.02 nM) was approximately 4-fold increased compared to controls (Fig. 5).

The higher EGF binding in the butyrate-treated cultures...
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Fig. 2. Morphology of hepatocytes cultured for 45 h in the absence (a) or presence (b) of 5 mM sodium butyrate. The medium of all cultures were supplemented with dexamethasone (0.25 μM) and insulin (0.4 μM) as described in "Materials and Methods." Phase contrast photomicrographs, x 125.

Fig. 3. Effect of butyrate on hepatocyte DNA synthesis. The hepatocytes were cultured for 68 h in the presence of varying concentrations of butyrate. The monolayers were maintained in medium with insulin (ins, 0.4 μM) and dexamethasone (dxm, 0.25 μM), without EGF or with 5 nM EGF added at 40 h after plating. DNA synthesis was assessed at 68 h by measuring the incorporation of [3H]thymidine into trichloroacetic acid-precipitable material during a 60-min pulse as described in "Materials and Methods." Points, means of triplicate cultures from one experiment. Bars, SE. The experiment was repeated three times under slightly different conditions, with essentially similar results.

Fig. 4. Scatchard analysis of the effect of butyrate on EGF receptor binding. Rat hepatocytes were cultured in the absence or presence of butyrate (5 mM) for 27 or 45 h and then incubated with labeled EGF as described in Fig. 1. Points, means of duplicate cultures. The experiment was repeated four times.

Fig. 5. Dose-dependent effects of butyrate on low-affinity (A) and high-affinity (B) EGF receptor binding. Cultured hepatocytes were treated with butyrate for 42 h, and the EGF binding was determined at 0.02 and 5.0 nM labeled EGF, to give predominantly high-affinity and low-affinity sites, respectively. Points, means of triplicate cultures from one experiment. Bars, SE. The experiment was repeated twice.

Fig. 6. Effect of butyrate on the kinetics of internalization of EGF. The hepatocytes were cultured in the absence (○, □) or presence (●, ■) of butyrate (5 mM) for 42 h and then incubated with 0.02 nM labeled EGF (A) or 5 nM labeled EGF (B) at 37°C. Internalized (○, ●) and surface-bound (□, ■) radioactivity was determined by the acid/salt elution method (36) as described in "Materials and Methods." Points, means of four cultures from one experiment. Bars, SE. Two identical experiments.

DISCUSSION

In this work we have studied the effect of butyrate on hepatocyte EGF receptors during short-time primary culture. A loss appeared to represent functional receptors in terms of the capacity for endocytic uptake of EGF, since the butyrate-treated cells also showed a greater ability to internalize EGF at both low (0.02 nM) and high (5 nM) concentration of the ligand (Fig. 6).
of hepatocyte EGF binding during 48 h in primary culture has been noted previously (23, 42), but it has also been reported that the number of hepatocyte EGF receptors remained unchanged after a period of 3 weeks in culture (43). We have characterized a differential reduction of high- and low-affinity EGF receptors during short-time primary culture of rat hepatocytes. Furthermore, the results demonstrate that exposure to butyrate counteracts the time-dependent reduction in surface EGF receptors and preserves the high-affinity binding component concomitant with a strong inhibition of DNA synthesis.

Heterogeneity in EGF binding sites has been described in a number of cells (44–46). Recent evidence has indicated a structural basis for the presence of distinct EGF receptor classes, suggesting that the low- and high-affinity types represent receptor monomers and dimers, respectively (47–49). The possible separate biological functions of the two receptor classes has not been resolved (46). When the hepatocyte EGF receptors were monitored 2 h after plating in primary culture, the surface binding isotherms were typically curvilinear, similar to those obtained in freshly isolated cells in suspension (12), suggesting two distinct affinity states. Upon further culturing there was a nearly complete loss of the high-affinity receptor population and a substantial decrease in low-affinity receptors with no significant change in the affinity for EGF. The linear Scatchard plots obtained in cells that had been kept in culture for 40 h are consistent with previous observations (23, 50, 51).

The explanation for the conversion from a heterogeneous to a homogeneous receptor population during hepatocyte culture is not known. Both humoral and mechanical factors might alter the EGF receptor status in the plated hepatocytes. The loss of the high-affinity receptors during primary culture resembles the reduction of high-affinity binding which has been reported to occur in certain cells as a result of treatment with phorbol esters (52, 53), platelet-derived growth factor (54, 55), vasopressin (56), bombesin (13, 57), and transforming growth factor β (58, 59). These substances modulate the EGF receptors through binding to their own receptors (transmodulation). It is conceivable that the hepatocytes, when placed in primary culture, are induced to synthesize and release peptides that attenuate the high-affinity binding through discrete receptors. Alternatively, released EGF-like peptides (transforming growth factor α) may down-regulate the EGF receptors in an autocrine fashion. Transformation of cells with the ras (60) and src (61) oncogenes results in a loss of EGF receptors, although only the ras-transformed cells secreted transforming growth factor α (60). The EGF receptor level is also significantly reduced during experimental hepatocarcinogenesis (62, 63) and in human hepatoma (64).

In a previous report we demonstrated an increased sensitivity for EGF-stimulated DNA synthesis when the hepatocytes had been cultured in the presence of insulin for 20–40 h (24). Thus, the reduction of EGF receptors shown here may be an integrated part of a complex set of cellular modifications preceding DNA synthesis and mitosis in hepatocytes. To examine this further, we treated the cells with butyrate, which has been shown to block various cells in G0 (26–29) and alter the regulation of a number of gene products with induction of differentiated cellular traits (26). The mechanism of action of butyrate is incompletely understood but has been linked to histone hyperacetylation (29, 30). Butyrate inhibited the time-dependent conversion of EGF receptor populations during short-time culture. We do not know the mechanism responsible for the butyrate-induced maintenance of the high-affinity receptor population. Recent data in this laboratory suggest that butyrate and dexamethasone interact synergistically in their effects on hepatocyte EGF receptors.4

The profound effects of butyrate on hepatocyte morphology suggest that gross effects on cytoarchitecture and cytoskeletal elements may be involved. In hepatoma cells butyrate-induced reversal of certain cytoarchitectural abnormalities correlates with an increase in cytoskeletal-associated actin (65). It should be noted that the high-affinity class of EGF receptors has been reported to be associated with the cytoskeleton (66).

Several reports from different experimental systems suggest that cells induced to divide exhibit reduced EGF receptor binding. AKR-2B cells and C3H/10T1/2 cells arrested in G0 by nutrient deficiency have a lower EGF receptor number than cells arrested in G0 by serum starvation (67). When BALB/c-3T3 cells were exposed to tetradecanoylphorbol acetate under conditions that allowed the cells to enter the cell cycle a persistent decrease in EGF receptors was detected (68). Recently it was also shown that norepinephrine, through an α-adrenoceptor-mediated effect, reduced the surface EGF receptor level and increased EGF-stimulated DNA synthesis in cultured hepatocytes (51). The present observation that butyrate blocks DNA synthesis and maintains the EGF receptor status in cultured hepatocytes is compatible with the hypothesis that there is a relationship between a low cellular EGF receptor number and a high sensitivity to the mitogenic effect of EGF.

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