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THE MECHANISM OF BUTYRATE-INDUCED COLLAGEN BIOSYNTHESIS IN CULTURED FIBROBLASTS

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Abstract: The data showing that butyrate may play an important role in cellular metabolism led us to study its effect on collagen biosynthesis in cultured fibroblasts. Since insulin-like growth factor-I (IGF-I) is the most potent stimulator of collagen biosynthesis in fibroblasts, the effect of butyrate on IGF-I receptor (IGF-IR) expression was evaluated. Confluent human dermal fibroblasts were treated with millimolar concentrations of sodium butyrate (NaB) for 48 hours. It was found that butyrate induced collagen biosynthesis and prolidase activity. It was found that the exposure of the cells to 4 mM butyrate contributed to a distinct increase in IGF-IR. It was accompanied by a parallel increase in the expression of SOS protein and MAP-kinases (ERK_1, ERK_2). It was found that the MEK inhibitor decreased collagen biosynthesis and expression of MAP-kinases (ERK_1, ERK_2), while NaB counteracted the process. The data suggest that butyrate-dependent stimulation of collagen biosynthesis in cultured human skin fibroblasts undergoes through IGF-IR signaling.

Keywords: butyrate; collagen biosynthesis; IGF-I receptor; prolidase

Butyrate is the microbial fermentation product generated in the human colon at millimolar concentrations (1). Butyrate, (at least in some cancer cells) was found to modulate cellular growth, differentiation and survival (2, 3), as well as some enzyme activities (4). Differentiation, growth and cellular gene expression are regulated by the interaction between cell and extracellular matrix (ECM) proteins, e.g. collagen (5, 6). An important point of collagen biosynthesis regulation is at the level of insulin-like growth factor-I receptor (IGF-IR). IGF-I is one of the potent collagen-stimulating factor in collagen-synthesizing cells (7). Stimulated IGF-I receptor induces interaction of several signaling proteins, such as Grb2, Src and Shc. This interaction allows activating further the cascade of signaling pathway through several proteins including SOS and two MAP kinases: ERK_1 and ERK_2 (8). The end point of this signaling is induction of transcription factor that up-regulates collagen gene expression. Deregulation of the signaling cascade may therefore contribute to the impairement of collagen synthesis. Another level of collagen biosynthesis regulation is at prolidase activity. Prolidase [EC 3.4.13.9] is a cytosolic enzyme that catalyses the hydrolysis of imidodipeptides with C-terminal proline or hydroxyproline (9-11). The enzyme plays an important role in the recycling of proline (from imidodipeptides derived from degradation products of collagen) for collagen re-synthesis (12) and cell growth (13). The efficiency of recycling of proline was found to be about 90% (14). It seems that the enzyme activity (despite the collagen gene expression) may be a step-limiting factor in regulation of collagen biosynthesis. This has been supported by several studies (15, 16).

In this study we examined the effect of butyrate on collagen biosynthesis, prolidase activity and expressions of IGF-IR and some signaling proteins (SOS, ERK1, ERK2) in cultured human dermal fibroblasts.

EXPERIMENTAL

Reagents

Alkaline phosphatase-labeled anti-mouse IgG, anti-rabbit IgG and anti-goat IgG antibodies, aprotinin, bacterial collagenase, Fast BCIP/NBT reagent, L-glycyl-proline, L-proline, leupeptin, monoclonal (mouse) anti-IGF-IR antibody, monoclonal (mouse) anti-phosphorylated MAPK antibody, Nonidet P-40, phenylmethylsulfonyl fluoride, sodium butyrate were

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provided by Sigma Corp., USA., as were most other chemicals and buffers used. Dulbecco's minimal essential medium (DMEM) and fetal bovine serum (FBS) used in cell culture were products of Gibco, USA. Glutamine, penicillin and streptomycin were obtained from Quality Biologicals Inc., USA. Nitrocellulose membrane (0.2 μm), sodium dodecylsulfate (SDS), polyacrylamide, molecular weight standards and Coomassie Briliant Blue R-250 were received from Bio-Rad Laboratories, USA. L-5[^{3}H] proline (28 Ci/mmol) was purchased from Amersham, UK. Polyclonal (goat) anti-β-actin antibody was the product of Santa Cruz Biotechnology Inc., USA. Monoclonal (mouse) anti-SOS antibody was obtained from Becton, Dickinson Co., USA. MEK 1/2 inhibitor was purchased from Cell Signaling, USA.

Cell culture

All studies were performed on normal human skin fibroblasts (CRL-1474), that were purchased from American Type Culture Collection, Manassas, VA, USA. The cells were maintained in growth medium (DMEM supplemented with 5% fetal bovine serum (FBS), 2 mmol/L glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin) at 37°C in a 5% CO_2 incubator. Cells were counted in hemocytometer and cultured at 1×10^5 cells per well in 2 mL of growth medium in 6 well plates (Costar). Cells reached confluence at day 6 and in most cases such cells were used for assays. Cells were used in the 8th to 14th passages. Confluent cells were treated with sodium butyrate in growth medium.

Methods

Determination of prolidase activity. The activity of prolidase was determined according to the method of Myara (17). Protein concentration was measured by the method of Lowry (18). Enzyme activity was reported as nanomoles of proline released from synthetic substrate, during one minute per milligram of supernatant protein of cell homogenate.

Collagen production. Incorporation of radioactive precursor into proteins was measured after labeling of confluent cells in growth medium with butyrate for the last 24 h with 5[^{3}H] proline (5 μCi/mL, 28 Ci/mM) as described previously (19). Incorporation of tracer into collagen was determined by digesting proteins with purified *Clostridium histolyticum* collagenase, according to the method of Peterkofsky (20). The results are shown as combined values for cell plus medium fractions.

SDS-PAGE. Slab SDS/PAGE was used, according to the method of Laemmli (21), using 10% SDS-polyacrylamide gel.

Western Immunoblot Analysis. After SDS-PAGE, the gels were allowed to equilibrate for 5 min in 25 mmol/L Tris, 0.2 mol/L glycine in 20% (v/v) methanol. The protein was transfered to 0.2 μm pore-sized nitrocellulose at 100 mA for 1 hour using an LKB 2117 Multiphor II electrophoresis unit. The nitrocellulose was incubated with: polyclonal antibody against β-actin at concentration 1:3,000; monoclonal anti-SOS antibody at concentration 1:5,000; monoclonal antibodies against phosphorylated-MAPK protein at concentration 1:5,000; monoclonal anti-IGF-IR antibody at concentration 1:1,000 in 5% dried milk in TBS-T (20 mmol/L Tris-HCl buffer, pH 7.4, containing 150 mmol/L NaCl and 0.05% Tween 20) for 1 hour. In order to analyze IGF-IR, SOS protein and phosphorylated MAP kinases second antibody-alkaline phosphatase conjugated, anti-mouse IgG (whole molecule) was added at a concentration 1:7,500 in TBS-T; in order to analyze β-actin second antibody-alkaline phosphatase conjugated, anti-goat IgG (whole molecule) was added at a concentration 1:5,000 in TBS-T and incubated for 30 min with slow shaking. Then nitrocellulose was washed with TBS-T (5 × 5 min) and submitted to Sigma-Fast BCIP/NBT reagent. The intensity of the bands was quantified by densitometric analysis using apparatus for gel documentation Syngen UVI-KS400 I (CO, USA) with digital densitometry (in arbitral units).

Cell viability assay. The assay was performed according to the method of Carmichael (22) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The cells were cultured for 48 h with various concentrations of NaB in six-well plates, washed three times with PBS and then incubated for 4 h in 1 mL of MTT solution (0.5 mg/mL of PBS) at 37°C. The medium was removed and 1 mL of 0.1 mol/l HCl in absolute isopropanol was added to attached cells. The absorbance of converted dye in living cells was measured at a wavelength of 570 nm. Cell viability in the presence of NaB was calculated as a percent of control cells.

Statistical analysis. In all experiments, the mean values for three independent experiments done in duplicates ± standard deviation (S.D.) were calculated, unless otherwise indicated. The results were submitted to statistical analysis using the Student's t-test, accepting $p < 0.05$ as significant.

RESULTS

As can be seen in Table 1, 48 h incubation of fibroblasts with NaB contributed to an increase in collagen biosynthesis. At 4 mM of NaB, about 30%

Table 1: Viability, prolidase activity and collagen biosynthesis measured as 5[^{3}H]-proline incorporation into proteins susceptible to the action of bacterial collagenase in confluent human skin fibroblasts incubated for 48 h with different concentrations of sodium butyrate (NaB).

Concentration (μM of NaB)	Viability of cells (% of control)	5-[^{3}H] proline incorporation (dpm × 10^3/mg protein)	Prolidase activity nmoles/min/mg protein
0	100	185 ± 5	159 ± 5
1	100	185 ± 5	160 ± 8
2	99 ± 1	210 ± 10	170 ± 9
3	99 ± 1	213 ± 7	175 ± 8
4	98 ± 1	250 ± 10*	238 ± 10*
5	98 ± 1	251 ± 6 *	238 ± 8 *
6	98 ± 1	250 ± 8 *	238 ± 7 *

Mean values ± SD from three independent experiments done in duplicates are presented.
* $p < 0.05$

increase in collagen biosynthesis was observed. Parallel increase in prolidase activity was found in cells treated with NaB, particularly at 4 mM (149% of control values). No significant effect of 4 mM NaB on cell viability was found as demonstrated by MTT assay (Table 1). The data suggest that the NaB-dependent increase in collagen biosynthesis in skin fibroblasts may result from stimulation of prolidase activity.

Since collagen biosynthesis is regulated due to the signal induced by activated insulin-like growth factor-I (IGF-I) receptor (7) the expression of this receptor in cells treated for 48 h with NaB was measured. As shown in Figure 1, in NaB-treated cells a distinct increase in the IGF-I receptor expression (to about 160% of control), compared to control cells was found.

The expression of some signaling proteins activated by IGF-I receptor in NaB-treated cells was measured by Western immunoblot analysis. NaB affected the expression of SOS protein and MAP kinases (ERK1, ERK2). We found that NaB increased the expression of SOS protein by about 3-fold (Figure 2 A) and phosphorylated ERK1 and ERK2 by about 200% (Figure 2 B), as determined by densitometric analysis. Since phosphorylation of MAP kinases ERK1 and ERK2 is the end point of IGF-I receptor signaling (23) and collagen biosynthesis is regulated due to the signal induced by activated insulin-like growth factor-I (IGF-I) receptor

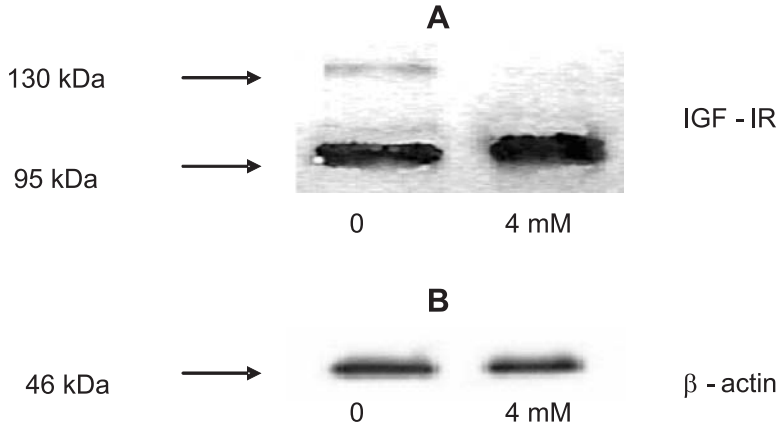

Figure 1. Western immunoblot analysis of IGF receptor (A) in control human skin fibroblasts (lane 1) and the cells cultured for 48 h with 4 mM of NaB (lane 2). The intensity of the bands was quantified by densitometric analysis. The arrows indicate the molecular mass of standards. The same amount of supernatant protein (20 μg) was run in each lane. The expression of β-actin served as a control for protein loading (B).

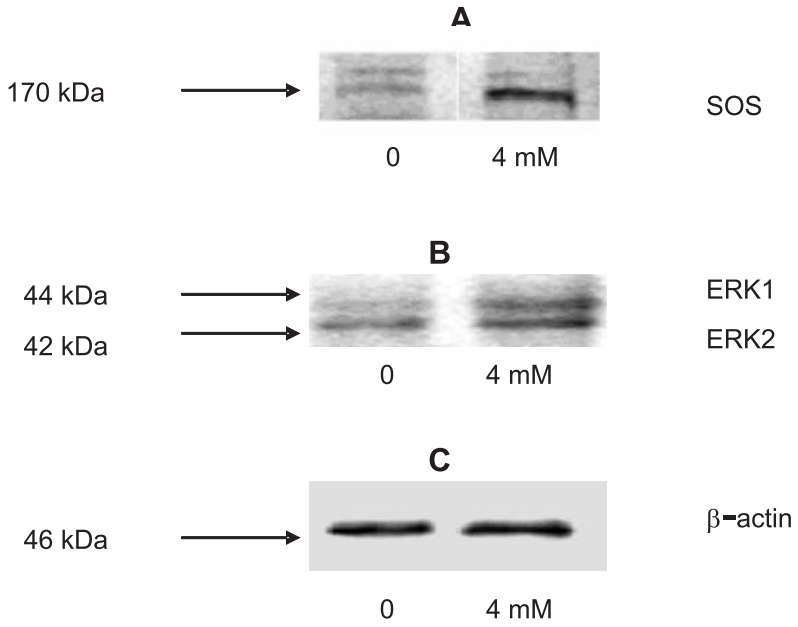

Figure 2. Western immunoblot analysis of SOS (A) and MAP kinases ERK1 and ERK2 (B), in control human skin fibroblasts (lane 1) and the cells cultured for 48 h with 4 mM of NaB (lane 2). The intensity of the bands was quantified by densitometric analysis. The arrows indicate the molecular mass of standards. The same amount of supernatant protein (20 μg) was run in each lane. The expression of β-actin served as a control for protein loading (C).

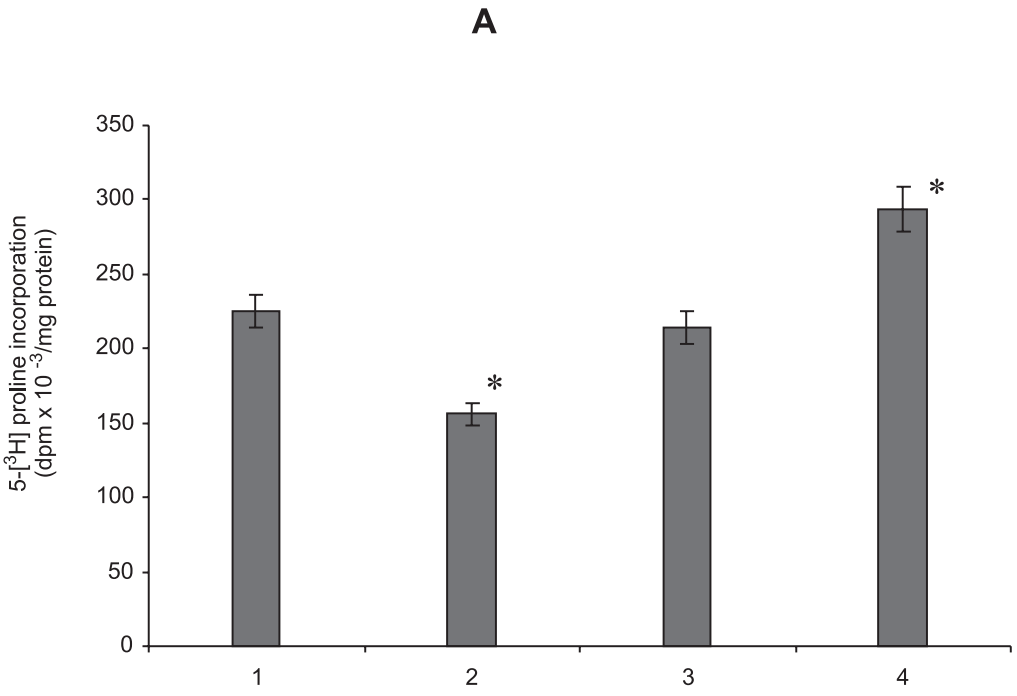

Figure 3. Collagen biosynthesis (A) measured as 5[^{3}H]-proline incorporation into proteins susceptible to the action of bacterial collagenase and western immunoblot analysis of MAP kinases ERK1 and ERK2 (B) in control, confluent human skin fibroblasts (lane 1) and the cells cultured for 48 h with 20 mM of MEK1/2 inhibitor (lane 2), plus 2 mM of NaB (lane 3), or 4 mM of NaB (lane 4). * $p < 0.05$.
The intensity of the bands was quantified by densitometric analysis. The arrows indicate the molecular mass of standards. The same amount of supernatant protein (20 μg) was run in each lane. The expression of β-actin served as a control for protein loading (C).

(7), biosynthesis of this protein was measured in confluent human dermal fibroblasts that has been treated with 20 mM of inhibitor of MEK 1/2 and different concentrations of NaB for 48 h. At 20 mM of MEK inhibitor, about 30% decrease in collagen biosynthesis was observed (Fig. 3 A, lane 2). An addition of 2 mM NaB restored this process to 95% of control value (Fig. 3 A, lanes 3, 1), while 4 mM sodium butyrate increased the process to 137% of control value (Fig. 3 A, lanes 4, 1). A similar direction of changes was observed in the MAP kinases expression. MEK kinase inhibitor blocked expression of ERK 1/2 (Fig. 3 B, lane 2), whereas an addition of NaB up-regulated the expression of these kinases (Fig. 3 B, lanes 3, 4). It suggests that the ability of NaB to induce an increase of collagen biosynthesis may involve stimulation of IGF-I receptor expression.

DISCUSSION

In the present study we provide evidence that NaB induces collagen production and prolidase activity, and countracts the inhibitory action of MEK 1/2 inhibitor on collagen biosynthesis and MAP-kinases expression.

Since collagen is regulated by insulin-like growth factor I receptor (IGF-IR) (7) and butyrate was shown to affect IGF-I activity (28), we postulated that the effects of NaB on collagen production and prolidase activity may be related to alterations in intracellular signaling pathway generated by IGF-IR. In fact, the data presented here show that NaB-induced collagen biosynthesis and prolidase activity are accompanied by the increased expression of IGF-IR. Previously, it has been shown that prolidase is stimulated by IGF-I (29). Prolidase catalyzes the final step in collagen degradation which completes the recycling of proline (12, 30). The best and most abundant substrate for prolidase is glycyl-proline (Gly-Pro). Collagen represents polypeptide containing the highest amount of imido-bonds compared to all known proteins. In a1 chains of type I collagen, Gly-Pro occurs 25 times (14). The observation that IGF-II stimulates fibroblast chemotaxis toward PDGF with concomitant increase in fibroblasts prolidase activity (31) provide an example of functional link between IGFs and prolidase. The functional link between collagen and prolidase activity has been also found in cultured human skin fibroblasts treated with anti-inflammatory drugs (32), pyrroline-5-carboxylate-P5C (16), during experimental aging of these cells (33) and fibroblasts chemotaxis (31).

The mechanism of butyrate-dependent stimulation of prolidase activity may undergo indirectly and may involve IGF-IR. An increase in the expression of IGF-IR contributed to the increase in the expression of SOS-protein and activation of MAP-kinases (ERK_1 and ERK_2). It is interesting that the effects of butyrate action are observed after 48 h of treatment that does not affect cell viability. Such an observation is supported by studies of other authors (3, 34). Considering the important role of prolidase in regulation of collagen biosynthesis it seems that the complex of processes induced by butyrate may affect the enzyme activity. It was previously found that phosphorylation of prolidase increases the activity of this enzyme in cultured human skin fibroblasts (35). This phenomenon may represent an important mechanism for up-regulation of prolidase activity and subsequently of collagen production due to IGF-I dependent signaling.

CONCLUSION

The results of present study suggest that NaB counteracted the collagen biosynthesis and expression of MAP-kinases (ERK_1, ERK_2) decreased by MEK inhibitor. NaB may exert its effect on collagen biosynthesis through modulation of prolidase activity through the expressions of IGF-IR, SOS-protein and phosphorylated MAP-kinases.

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