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Sodium butyrate inhibits angiogenesis of human intestinal microvascular endothelial cells through COX-2 inhibition

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Abstract We examined the effect of sodium butyrate on in vitro angiogenesis and cyclooxygenase (COX) expression using primary cultures of human intestinal microvascular endothelial cells (HIMEC). Butyrate inhibited VEGF-induced cellular proliferation, transmigration and tube formation of HIMEC. Butyrate also inhibited COX-2 expression as well as prostaglandin (PG)E_2 and PGI_2 production, and administration of PGI_2 analog partially reversed the effect of butyrate on HIMEC angiogenesis. These results indicate that sodium butyrate inhibits HIMEC angiogenesis through down-regulation of COX-2 expression and PG production, and suggest that anti-angiogenic mechanisms may also be involved in the inhibitory effect of sodium butyrate on tumor growth.

Key words: Human intestinal microvascular endothelial cell; Angiogenesis; Sodium butyrate; Cyclooxygenase-2; Prostaglandin I_2

1. Introduction

Sodium butyrate, a short-chain fatty acid (SCFA) produced during colonic microbial fiber fermentation, is currently being evaluated as an anti-neoplastic therapeutic [1], and clinical trials of butyrate and its derivatives in cancer patients have already been initiated [2,3]. The preventive action of butyrate against tumor growth is believed to be mediated through a direct effect on tumor cells which results in cell cycle arrest, differentiation or apoptosis [4–7]. However, the effect of sodium butyrate on microvascular endothelial cells, which play a key role in tumor-induced angiogenesis, is presently not defined. In the present study, we examined the effect of sodium butyrate on in vitro angiogenesis using primary cultures of human intestinal microvascular endothelial cells (HIMEC). Sodium butyrate demonstrated a potent anti-angiogenic effect on HIMEC as well as inhibitory effects on cyclooxygenase-2 (COX-2) expression and prostanoid production. The COX-2-specific inhibitor NS398 impaired in vitro angiogenesis of HIMEC, suggesting that the anti-angiogenic mechanism of butyrate was related to this enzyme. Administration of exogenous prostaglandin (PG)I_2 analog reversed the inhibitory effect of sodium butyrate on HIMEC. These findings suggest that in addition to effects on epithelial cells, sodium butyrate is a potent anti-angiogenic agent.

2. Materials and methods

2.1. Western blotting for COX-1 and COX-2

HIMEC isolation was performed from normal-appearing small or large bowel mucosa as described previously [8] and pure HIMEC monolayers were cultured in media containing 5% fetal bovine serum (FBS) overnight prior to assay. Confluent HIMEC monolayers were assessed directly or following stimulation with vascular endothelial growth factor (VEGF; R&D systems, Minneapolis, MN, USA; 50 ng/ml, 4 h) with or without sodium butyrate pre-treatment (Sigma Chemical, St. Louis, MO, USA; 0.05–5 mM, 2 h). Cellular protein was extracted as described previously [8], and 10 μg of protein was applied for Western blot analysis with anti-COX-1, -2 monoclonal antibodies or anti-actin goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Following incubation with secondary horseradish peroxydase (HRP)-conjugated anti-mouse or -goat IgG (Santa Cruz Biotechnology), bands were detected using enhanced chemiluminescence (ECL) reagents (Amersham Bioscience, Piscataway, NJ, USA), according to the manufacturer's protocol.

For statistical analysis, films were scanned and the intensity of each band was determined using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). The relative ratio of COX-2/COX-1 expression was determined as [COX-2/COX-1 in each group]/[COX-2/COX-1 in HIMEC with no stimulation]. Experiments were carried out using three independent HIMEC cultures and results are expressed as mean ± S.E.M.

2.2. Determination of PGE_2 and 6-keto $PGF_{1\alpha}$ concentration in culture supernatant

Confluent HIMEC monolayers were assayed directly or following VEGF stimulation (50 ng/ml, 8 h) with sodium butyrate pre-treatment (0–5 mM, 2 h). The concentration of PGE_2 and 6-keto $PGF_{1\alpha}$ (the stable metabolite of PGI_2) in the culture supernatant was determined using a commercial enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA). In order to determine the COX isozyme which

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Abbreviations: COX, cyclooxygenase; HIMEC, human intestinal microvascular endothelial cell; PG, prostaglandin; TX, thromboxane; SCFA, short-chain fatty acid; FBS, fetal bovine serum; VEGF, vascular endothelial growth factor; HRP, horseradish peroxydase; LPS, lipopolysaccharide; NSAID, non-steroidal anti-inflammatory drugs; eNOS, endothelial nitric oxide synthase

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produces PGE_2 and PGI_2 in HIMEC, the monolayers were incubated with the COX-2-specific inhibitor NS398 for 2 h (0.05–5 μM; Cayman Chemical) and stimulated with VEGF for 8 h. The concentration of PGE_2 and 6-keto $PGF_{1\alpha}$ was determined as described above. Experiments were carried out in triplicate and results are shown as mean pg/ml ± S.D.

2.3. Thymidine incorporation assay

Cellular DNA synthesis was assessed by [^{3}H]thymidine uptake. HIMEC were seeded onto fibronectin-coated 24-well plates (1.5×10^4 cells/cm^2) and grown for 24 h. After incubation in media containing 1% FBS overnight, cells were treated with sodium butyrate (0–5 mM) for 2 h, and stimulated with VEGF (50 ng/ml). When indicated, PGE_2 (11-deoxy Prostaglandin E2; Cayman Chemical), carbacyclin (stable analog of PGI_2; Cayman Chemical) or U46619 (thromboxane A_2 (TXA_2) receptor agonist; Cayman Chemical) were added to the media at either 0.1 or 1 μM. Following 15 h of stimulation, [^{3}H]thymidine (1 μCi/ml; Amersham Biosciences) was added to media and cells were cultured another 5 h. After washing twice with PBS, cells were fixed for 10 min on ice with 5% (v/v) trichloroacetic acid. DNA was then released from precipitated material by alkaline lysis in 0.25 N NaOH and supernatants were quantified in a gamma counter. Data from triplicate wells were expressed as a mean ± S.D.

2.4. Quantification of cell number and viability

To determine the effect of butyrate on cellular proliferation and viability, sub-confluent HIMEC monolayers in 1% FBS media were treated with butyrate (0.5 or 5 mM) for 2 h and stimulated with VEGF (50 ng/ml). After 18 h stimulation, cells were detached by trypsin–EDTA treatment, and cell number and viability were quantified under a light microscope by trypan blue staining. Data from triplicate wells were expressed as a relative ratio of cell number (% of unstimulated cells) ± S.D.

2.5. Endothelial cell chemotaxis assay

HIMEC (3×10^4 cells/cm^2) were cultured on fibronectin-coated polycarbonate filters (8 μm pore size, BD Biosciences, Bedford, MA, USA). After incubation in media containing 2% FBS overnight, HIMEC were incubated with butyrate (0–5 mM) or NS398 (0.05–0.5 μM) for 2 h, and buffers containing VEGF (50 ng/ml) were filled into the lower compartment of the 12-well plates. When indicated, PGE_2, carbacyclin or U46619 were added into both upper and lower chambers at either 0.1 or 1 μM. After overnight incubation, cell culture inserts were removed and the upper surface of the membrane was gently wiped to remove non-migrated cells. Filters were stained with DiffQuik (Baxter Scientific, McGraw, IL, USA), air-dried, and mounted onto glass slides. Migrated HIMEC adherent to the lower side of the membrane were counted (at least 15 random high-power fields (×200) per condition) and data were expressed as a mean ± S.D.

2.6. In vitro tube formation assay

Endothelial tube formation was assessed using Matrigel™ (BD Biosciences), a solubilized extracellular basement membrane matrix extracted from the Engelbreth–Holm–Swarm mouse sarcoma, as described previously [9]. Multiwell dishes (24-well) were coated with 250 μl of MCDB 131 medium containing 5 mg/ml Matrigel™ and 10% FBS. HIMEC were pre-treated with butyrate (0–5 mM) or NS398 (0.05–2 μM) for 30 min, and were seeded at a density of 5×10^4 cells/well. The concentration of butyrate or NS398 was maintained in both the cell suspension and Matrigel™ during the assay. When indicated, the medium was supplemented with PGE_2, carbacyclin or U46619. Cells were cultured on Matrigel™ for 10–16 h and endothelial tube formation was assessed by inverted phase-contrast microscopy. At least 10 high-power fields per condition were examined, and experiments were repeated in two independent HIMEC cultures. Data were expressed as a mean number of tubes per high-power field (×200) ± S.D.

3. Results

3.1. Sodium butyrate inhibits COX-2 expression and prostanoid production in HIMEC

HIMEC cultured in 5% FBS-containing media demonstrated detectable COX-2 protein expression, which was significantly increased by VEGF stimulation for 4 h (Fig. 1A,B). Pre-treatment with sodium butyrate inhibited COX-2 expression in a dose-dependent manner. In contrast, COX-1 expression was not affected by VEGF or sodium butyrate. Corresponding with the effect on COX-2 expression, sodium butyrate inhibited both PGE_2 and PGI_2 (determined as 6-keto $PGF_{1\alpha}$) production induced by VEGF (Fig. 1C). At greater than 2 mM butyrate, both PG levels were less than the basal production without VEGF stimulation. Both PGE_2 and PGI_2 production are strongly inhibited by pre-treatment with NS398 as low as 0.05 μM, indicating that production of these prostanoids is largely dependent on COX-2 activity in HIMEC.

3.2. Sodium butyrate inhibits HIMEC proliferation, migration and tube formation, which are partially reversed by PGI_2 analog administration

Cellular proliferation was determined by measuring both [^{3}H]thymidine uptake and cell number. [^{3}H]thymidine uptake was significantly increased after VEGF stimulation, which was inhibited by sodium butyrate pre-treatment in a dose-dependent manner (Fig. 2A). VEGF stimulation for 18 h increased the number of cells, although it was not statistically significant. Pre-treatment with 5 mM butyrate reduced cell number significantly in VEGF-stimulated HIMEC (Fig. 2B). The cellular viability remained over 98% in all groups (data not shown). The inhibitory effect of sodium butyrate on HIMEC [^{3}H]thymidine uptake was partially reversed by administration of the PGI_2 analog carbacyclin, but not by 11-deoxy PGE_2 or U46619 (stable PGE_2 or TXA_2 analog, respectively) (Fig. 2C).

HIMEC transmigration was also increased by VEGF stimulation and was significantly inhibited by pre-treatment with sodium butyrate (either 0.5 or 5 mM; Fig. 3A). Similar to the results of the [^{3}H]thymidine uptake assay, carbacyclin partially reversed the inhibitory effect of sodium butyrate on HIMEC transmigration, but PGE_2 or TXA_2 analogs did not exert an effect (Fig. 3B).

The endothelial in vitro tube formation assay using Matrigel™ was performed in the absence of VEGF, because VEGF stimulation did not significantly affect microvascular tube formation in our preliminary experiments (data not shown). Similar to the results from the cell proliferation and transmigration assays described previously, the number of endothelial tubes which formed in Matrigel™ was significantly inhibited by sodium butyrate (2.5 mM). The inhibitory effect of sodium butyrate on endothelial tube formation was also partially reversed by the administration of carbacyclin, but not by 11-deoxy PGE_2 or U46619 (Fig. 4).

3.3. The COX-2-specific inhibitor NS398 inhibits HIMEC transmigration and tube formation

In order to determine the causal relationship between inhibition of COX-2 and inhibition of in vitro angiogenesis of HIMEC, transmigration and tube formation assays were performed using the COX-2 specific inhibitor NS398. NS398 exerts a potent effect on HIMEC, inhibiting prostanoid production as demonstrated above. As shown in Fig. 5, both transmigration and tube formation were significantly inhibited by NS398 in concentrations as low as 0.05 μM. These results indicate that inhibition of COX-2 activity is linked to impaired HIMEC angiogenesis in vitro.

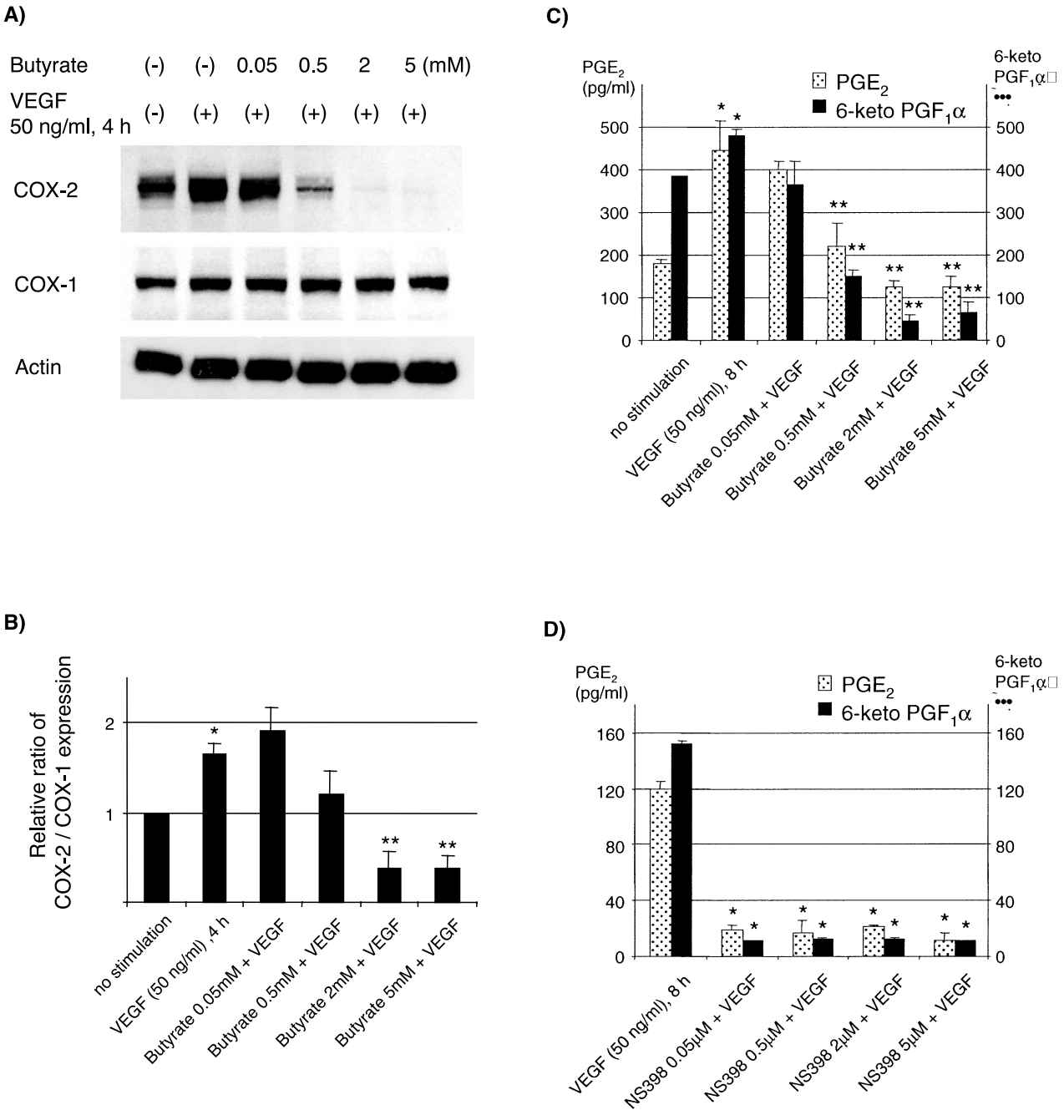


Fig. 1. Effect of sodium butyrate on COX protein expression and prostanoid production in HIMEC. A: The results of Western blotting for COX-1 and COX-2. VEGF stimulation increased COX-2 expression, which was significantly inhibited by sodium butyrate. COX-1 expression was not affected by VEGF or sodium butyrate. Actin served as an internal control. Representative result from a total of three independent experiments. B: The result of the statistical analysis for the relative ratio of COX-2/COX-1 expression. The intensity of each band was determined using image analysis software and the relative ratio of COX-2/COX-1 expression was determined as [COX-2/COX-1 in each group]/[COX-2/COX-1 in HIMEC with no stimulation]. Experiments were carried out using three independent HIMEC cultures and results are expressed as mean ± S.E.M. * = $P < 0.05$ compared to no stimulation; ** = $P < 0.05$ compared to VEGF stimulation by analysis of variance (ANOVA). C: The results of enzyme immunoassay for PGE_2 and 6-keto $PGF_{1\alpha}$ in HIMEC culture supernatants. VEGF stimulation significantly increased both PGE_2 and 6-keto $PGF_{1\alpha}$ production in HIMEC. Sodium butyrate inhibited production of both prostanoids in a dose-dependent manner. Representative result from a total of three independent experiments. Data were expressed as pg/ml PG production ± S.D. * = $P < 0.05$ compared to no stimulation; ** = $P < 0.05$ compared to VEGF stimulation by ANOVA. D: The results of enzyme immunoassay for PGE_2 and 6-keto $PGF_{1\alpha}$ with NS398 treatment. NS398 inhibited production of both prostanoids as low as 0.05 μM in HIMEC. * = $P < 0.05$ compared to VEGF stimulation by ANOVA.

4. Discussion

In the present study, our data demonstrate that (i) sodium butyrate inhibited VEGF-induced proliferation, transmigration and tube formation in HIMEC, an in vitro strategy for modeling angiogenesis, (ii) sodium butyrate blocked VEGF-induced COX-2 expression as well as prostanoid production, (iii) the administration of PGI_2 analog carbacyclin partially

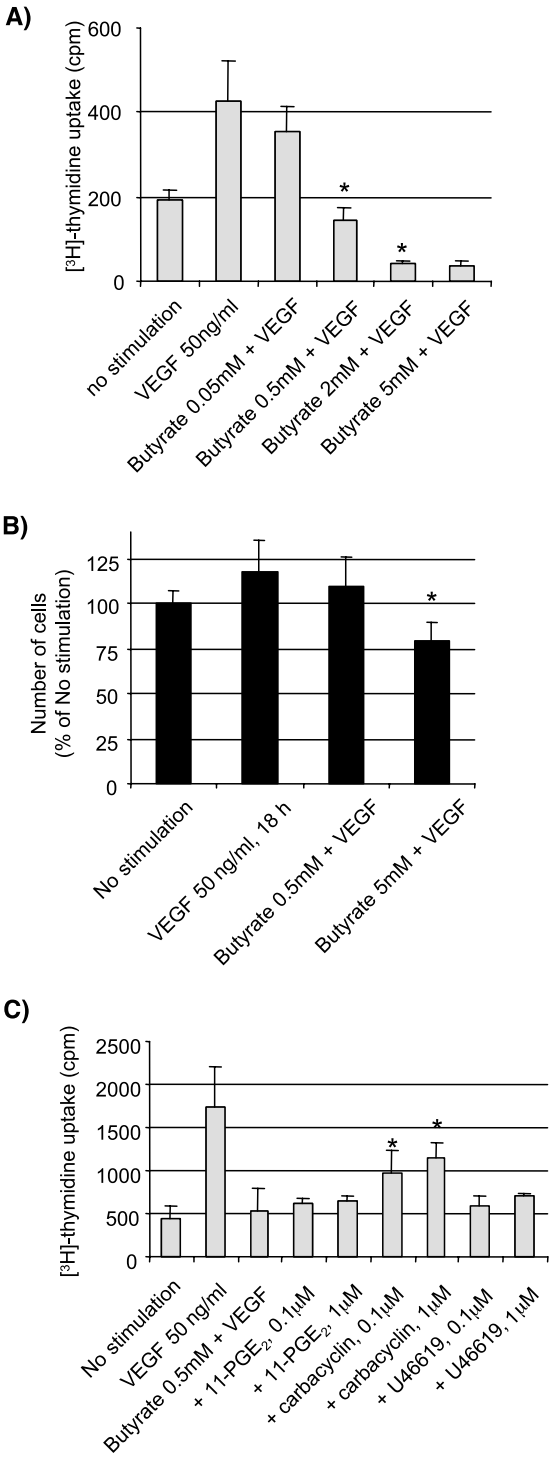


Fig. 2. Effect of sodium butyrate on HIMEC proliferation. A: Cellular DNA synthesis was assessed by measuring [^{3}H]thymidine uptake. [^{3}H]thymidine uptake was significantly increased after VEGF stimulation for 15 h and was inhibited by sodium butyrate pre-treatment for 2 h in a dose-dependent manner. Assays were done in triplicate and the data are shown as mean cpm ± S.D. Representative results from a total of three independent experiments are shown. * = $P < 0.05$ compared to VEGF-stimulated HIMEC cultures by ANOVA. B: Number of HIMEC was counted after VEGF stimulation with or without butyrate treatment. VEGF stimulation appeared to increase the number of HIMEC after 18 h, but failed to reach significance ($P > 0.05$). Sodium butyrate at 5 mM significantly decreased the number of cells compared to VEGF stimulation alone. Data from triplicate wells were expressed as a relative ratio of cell number (% of unstimulated cells) ± S.D. Representative results from a total of two independent experiments are shown. * = $P < 0.05$ compared to VEGF-stimulated HIMEC cultures by ANOVA. C: The inhibitory effect of sodium butyrate on [^{3}H]thymidine uptake was partially reversed by exogenous carbacyclin (PGI_2 analog) administration, but not by 11-deoxy PGE_2 or U46619 (PGE_2 or TXA_2 analog, respectively). * = $P < 0.05$ compared to sodium butyrate-treated HIMEC without exogenous PG administration (lane 3) by ANOVA.
←

reversed the inhibitory effect of sodium butyrate on thymidine uptake, transmigration and tube formation, and (iv) the COX-2-specific inhibitor NS398 also inhibits HIMEC transmigration and tube formation. These results suggest that sodium butyrate will inhibit microvascular endothelial growth and proliferation and suppress angiogenesis by inhibiting COX-2 expression and PG production. The inhibitory effect of sodium butyrate on [^{3}H]thymidine uptake (Fig. 2A), transmigration (Fig. 3A), tube formation (Fig. 4D) as well as prostanoid production (Fig. 1C) appears to be greater than an effect attributed to the reduction in endothelial cell numbers caused by this SCFA (Fig. 2B). Thus these data suggest that the inhibition of in vitro angiogenesis or reduced prostanoid production is the result of specific effects of butyrate on VEGF-induced activation in HIMEC.

Recent evidence demonstrates that VEGF induces COX-2 expression in endothelial cells and COX-derived prostanoids promote angiogenesis in response to VEGF stimulation [10,11]. Our present data also demonstrate that VEGF enhanced COX-2 protein expression in HIMEC. Furthermore, we first demonstrated that COX-2 expression in HIMEC was inhibited by sodium butyrate in a dose-dependent manner. Sodium butyrate also blocked serum- or lipopolysaccharide (LPS)-induced COX-2 expression in HIMEC (data not shown), indicating that sodium butyrate is a potent inhibitor of COX-2 expression as well as prostanoid production in endothelial cells regardless of the type of stimuli. The suppressed COX-2 expression is not likely to be the result of generalized cellular unresponsiveness or damage, because sodium butyrate at 0.5–5 mM did not affect LPS-induced E-selectin expression and in fact resulted in increased ICAM-1 expression [12]. Therefore, butyrate appears to alter HIMEC gene/protein expression in a gene-specific manner. In addition, the inhibitory effect of butyrate on COX-2 expression seems to be specific to endothelial cells, because it did not affect COX-2 expression in smooth muscle cells isolated from intestinal mucosa (data not shown), and a recent report has shown that butyrate increased COX-2 expression in epithelial cells [13].

COX-2 and prostanoid have been implicated in mediating angiogenesis during tumor growth. COX-2 is up-regulated in the microvasculature surrounding colon tumors [14], and inhibition of COX activity by non-steroidal anti-inflammatory drugs (NSAIDs) reduces angiogenesis and tumor growth both in vivo and in vitro [15,16]. Recent reports also indicate that COX-2-derived PGE_2, TXA_2 or PGI_2 promote angiogenesis [10,15,17], although the predominant role of endothelial COX-2 vs. COX-1 in angiogenesis remains controversial [18]. In the present study, we demonstrate that the COX-2 inhibitor NS398 impaired in vitro angiogenesis of HIMEC, supporting the idea that COX-2-derived prostanoids play a

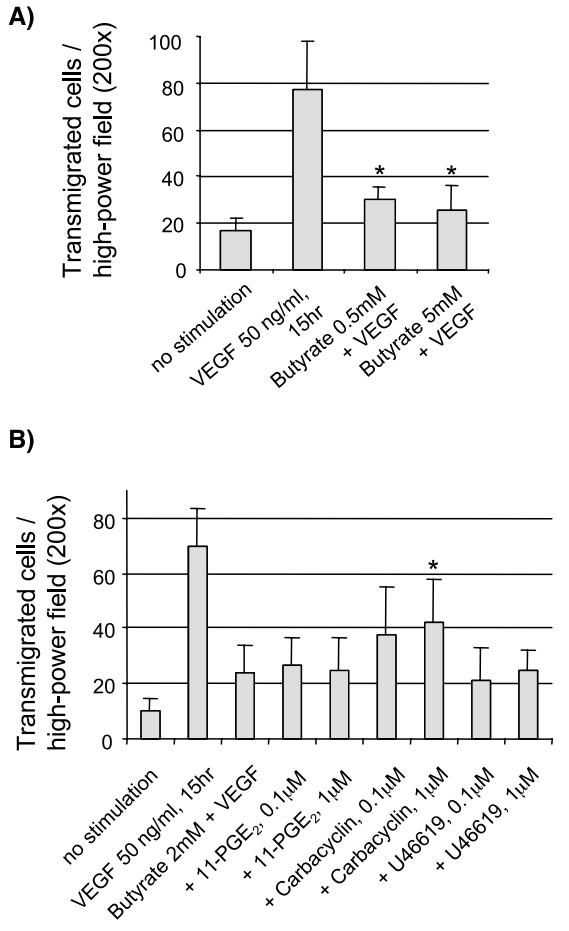
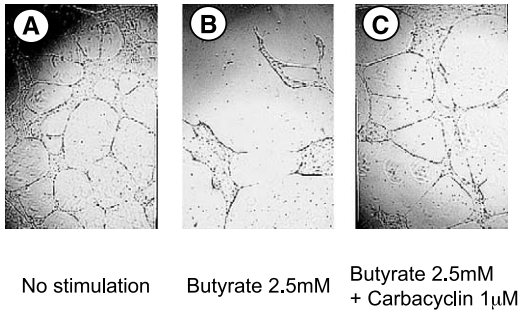


Fig. 3. Effect of sodium butyrate on HIMEC transmigration. Endothelial transmigration assay was performed using polycarbonate filters (8 μm pore size). HIMEC which transmigrated through the filters to the lower side of the membrane were counted (at least 15 random high-power fields (×200) per condition) and data were expressed as a mean ± S.D. A: The number of transmigrated HIMEC was increased by VEGF stimulation, which was significantly inhibited by pre-treatment with sodium butyrate. Representative result from a total of four independent experiments. * = $P < 0.05$ compared to VEGF-stimulated HIMEC by ANOVA. B: The inhibitory effect of sodium butyrate on HIMEC transmigration was significantly reversed by carbacyclin administration at 1 μM. Representative result from a total of three independent experiments. * = $P < 0.05$ compared to VEGF-stimulated HIMEC with butyrate pre-treatment (lane 3) by ANOVA.

central role in angiogenesis. The administration of PGI_2 analog carbacyclin reversed the inhibition of thymidine uptake, transmigration and tube formation by sodium butyrate. Thus, the effect of sodium butyrate could be explained in part by suppression of PGI_2 formation through inhibition of COX-2 expression. No reversal of inhibition was observed by administration of PGE_2 and TXA_2 analogs, suggesting that among the major COX products, loss of PGI_2 underlies the effect of sodium butyrate on HIMEC angiogenesis. These results are consistent with a recent report demonstrating that administration of PGI_2 analog, but not PGE_2 or TXA_2 analogs, reversed the effect of COX-2 inhibitor on blocking endothelial cellular proliferation [11]. However, it should be noted that PGI_2 analog did not overcome the anti-angiogenic effect of sodium butyrate completely, and it appears that butyrate inhibits HIMEC angiogenesis more strongly than NS398. Conversely, when we consider that NS398 inhibits prostanoid production more effectively than butyrate, the anti-angiogenic effect of this SCFA will involve mechanisms in addition to its inhibitory effect on COX-2 expression in HIMEC. In addition to the effect on COX-2 expression, sodium butyrate also affects expression of several genes associated with cell growth and/or apoptosis (e.g. Bcl2 etc.; unpublished data), and a recent report demonstrates that sodium butyrate inhibits endothelial nitric oxide synthase (eNOS) expression in endothelial cells which may contribute to impaired endothelial tube formation [19]. Sodium butyrate's effects on gene expression are believed to involve inhibition of histone deacetylase (HDAC), which may result in either the expression or silencing of specific genes. Recent reports using human and animal large-vessel endothelial cells have suggested that HDAC inhibitors are potent anti-angiogenic agents in vitro and in vivo, through



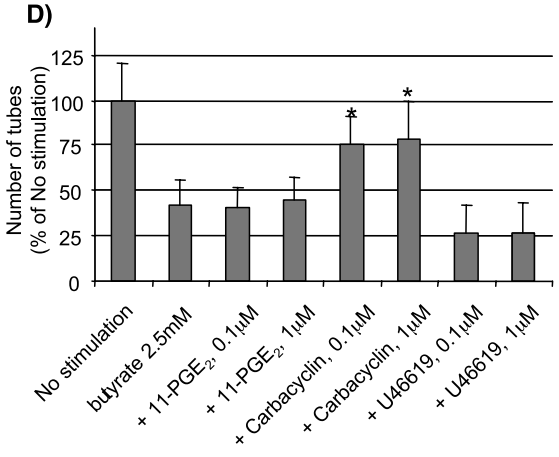


Fig. 4. Effect of sodium butyrate on in vitro tube formation of HIMEC. A: HIMEC cultured overnight on 5 mg/ml Matrigel™ form microvascular tubes. Phase-contrast photomicrograph demonstrates fine capillary-like structures which form a delicate lattice work over the tissue culture dish (×40 magnification). B: Treatment of the HIMEC with 2.5 mM sodium butyrate inhibits formation of microvascular tubes following overnight incubation (×40 magnification). C: PGI_2 analog carbacyclin partially restores endothelial tube formation in the sodium butyrate-treated HIMEC (×40 magnification). D: Quantification of Matrigel™ in vitro tube formation assay using overnight HIMEC culture. At least 10 high-power fields per condition were examined and experiments were repeated in three independent HIMEC cultures. Data were expressed as a mean number of tubes per high-power field (×200) ± S.D. Tube formation was inhibited by sodium butyrate and was significantly reversed by exogenous carbacyclin administration. * = $P < 0.05$ compared to HIMEC treated with sodium butyrate without PG administration (lane 2) by ANOVA.

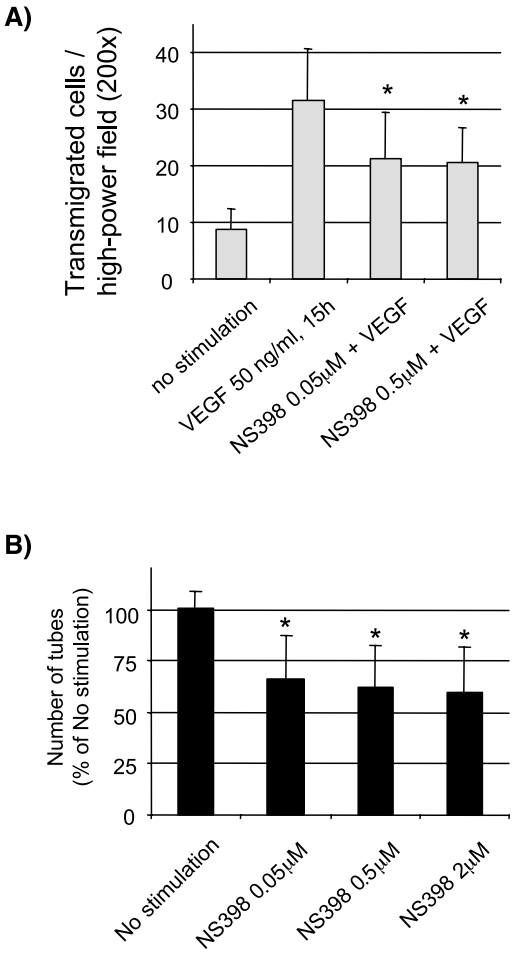


Fig. 5. Effect of sodium butyrate on transmigration and in vitro tube formation of HIMEC. A: Endothelial transmigration assay was performed with the COX-2-specific inhibitor NS398. The number of transmigrated HIMEC increased by VEGF stimulation was significantly inhibited by NS398. Representative result from a total of two independent experiments. * = $P < 0.05$ compared to VEGF-stimulated HIMEC by ANOVA. B: Quantification of Matrigel™ in vitro tube formation assay using overnight HIMEC culture with NS398 treatment. Representative result from a total of two independent experiments. Data were expressed as a mean number of tubes per high-power field (×200) ± S.D. Sodium butyrate at 0.05–2 μM inhibits formation of microvascular tubes following overnight incubation. * = $P < 0.05$ compared to HIMEC with no stimulation by ANOVA.

modulation of pathways including tumor suppressor genes and altering VEGF receptor expression [20,21]. Our experiments in HIMEC have confirmed that sodium butyrate's modulatory effect on gene expression is similar to the effect of the HDAC inhibitor tricostatin A [12]. Taken together, these results suggest that the anti-angiogenic activity of sodium butyrate involves modulation of multiple pathways in endothelial cells.

The importance of enteric bacteria in the development of a mature intestinal microcirculation was recently identified by Stappenbeck et al. [22]. These investigators demonstrated that intestinal angiogenesis during normal growth was intimately related to the presence of indigenous enteric microbes, whose proximity resulted in the production of Paneth cell-derived antimicrobial peptides which mediated postnatal vascular development. Our data demonstrate that the inter-relationship between enteric flora and the vasculature is even more complex, as the presence of bacterial fermentation products will exert potent anti-angiogenic effects on local endothelial populations. Although the local concentration of sodium butyrate within colonic mucosa is unclear, concentrations of butyrate in the human portal vein reach 0.03 mM after cecal instillation of lactulose [23] and a recent report showed that approximately half of absorbed butyrate is found in mesenteric blood as non-metabolized substrate in rats [24]. These reports suggest that mucosal microvascular endothelial cells are exposed to sodium butyrate in vivo. In addition, plasma levels of butyrate are reported to reach 0.1–9 mM in rodents by oral administration of tributyrin or sodium butyrate [25], which is similar to the butyrate doses used in the present study.

In summary, our present study indicates that sodium butyrate is a potent inhibitor of angiogenesis of HIMEC in vitro. The mechanisms involve inhibition of COX-2 expression and prostanoid production. Given the importance of angiogenesis and tumor neovascularization in cancer progression, our data suggest that the anti-cancer effects of sodium butyrate may also involve direct effects on local microvascular populations.

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