

## Modulation of angiogenesis-related proteins synthesis by sodium butyrate in colon cancer cell line HT29

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**Sodium butyrate (NaB), a short-chain fatty acid naturally present in the human colon, is able to induce cell cycle arrest, differentiation and apoptosis in colon cancer cells. In addition to these effects, we investigated the effect of NaB on two angiogenesis-related proteins in a colon carcinoma cell line (HT29): vascular endothelial growth factor (VEGF), the most potent angiogenic factor, and hypoxia-inducible factor (HIF)-1 $\alpha$ , the main transcription activator of the VEGF gene, which are both constitutively expressed at high levels in HT29 also in normoxic conditions. NaB treatment had a different effect on VEGF165 and HIF-1 $\alpha$  expression. In fact, it induced a dose-dependent down regulation of the VEGF165 protein level that was not paralleled by a concomitant down regulation of the corresponding mRNA, suggesting a post-translational regulation of the factor. Conversely, after 24 h of treatment all the tested NaB concentrations reduced the HIF-1 $\alpha$  protein level, whereas after a longer time of exposure HIF-1 $\alpha$  level increased in the presence of a high NaB concentration (2 mM) with a concomitant increase in HIF-1 $\alpha$  mRNA. These results indicate that NaB, besides regulating other fundamental cellular processes, is able to modulate the expression of two important angiogenesis-related molecules and suggested a further possible clinical application of this short-chain fatty acid as an anti-angiogenic compound in association with conventional chemotherapeutic agents.**

### Introduction

Solid tumors including colon carcinoma require neovascularization for their initiation, progression and metastasis. A variety of angiogenesis inducers have been described, and among these vascular endothelial growth factor (VEGF) has been identified as the most important. This factor plays an essential role in tumor development and is correlated with progression and metastatization of colon cancer (1,2). Among the VEGF isoforms produced through alternative exon splicing of the gene, VEGF165 is the predominant form related to colon cancer progression (3).

VEGF is physiologically expressed in different tissues (4) and is regulated by tissue oxygen tension. Exposure to hypoxia induces the expression of VEGF through both increased transcription and stabilization of its mRNA (5,6). The

**Abbreviations:** HIF, hypoxia-inducible factor; NaB, sodium butyrate; VEGF, vascular endothelial growth factor.

principal inducer of VEGF synthesis is hypoxia-inducible factor (HIF)-1, a heterodimeric transcription factor composed of HIF-1 $\alpha$  and HIF-1 $\beta$  subunits (7). The HIF-1 $\alpha$  protein is ubiquitinated and degraded in normoxia (8) but stabilized by hypoxia, whereas HIF-1 $\beta$  is constitutively expressed in both normoxia and hypoxia. Regions of hypoxia are very common in a tumor mass and the consequent stabilization of HIF-1 $\alpha$  protein results in a constitutive activation of specific hypoxia-induced pathways including VEGF synthesis. Several studies have documented hypoxic induction of HIF-1 $\alpha$  protein and up regulation of HIF-1 target genes in several clinical cancer histotypes and in pre-malignant lesions (9–11).

Sodium butyrate (NaB), a short-chain fatty acid naturally present in the human colon, is able to modulate a variety of fundamental cellular processes and to induce cell cycle arrest, differentiation and apoptosis in transformed cells (12–14). The observation that many transformed cells may retain their capacity to respond to a differentiation stimulus has suggested the possibility of using differentiation inducers as an alternative therapeutic approach to conventional antitumor therapy (15,16). In recent years, some differentiation-inducing agents have been proved to also affect the angiogenic activity of the tumor cells and block endothelial cells mobility (17–19).

In the present study we investigated whether NaB, in addition to the activities on proliferation, differentiation and apoptosis already reported (13), is able to modulate the levels of angiogenesis-related factors (in particular, VEGF and HIF-1 $\alpha$ ) in HT29, a human colon adenocarcinoma cell line.

### Materials and methods

#### Cell culture and treatments

HT29 colon cancer cells were grown as a monolayer in RPMI 1640 medium (Sigma Chemical, St Louis, MO) supplemented with 10% fetal calf serum (vol/vol) and 1% glutamine (Sigma), cultured in T-75 cm<sup>2</sup> plastic flasks (Corning Industries, Corning, NY), maintained at 37°C in 5% CO<sub>2</sub> humidified atmosphere, and passaged weekly. At the beginning of the experiments, mycoplasma-free cells in the exponential growth phase were removed from the flasks with a 0.05% trypsin–0.02% EDTA solution and seeded in T-75 cm<sup>2</sup> flasks (20 000 cells/cm<sup>2</sup>) in RPMI 1640 medium supplemented with 10% fetal calf serum. The cells were allowed to adhere for 24 h; the seeding medium was removed and replaced with experimental medium. Early modulatory effects of NaB, without any concomitant anti-proliferative activity, and medium-term effects were investigated keeping the cells for 24 or 72 h in the medium supplemented with increasing concentrations (0.1, 0.5, 1 and 2 mM) of NaB (ICN Biomedicals, South Chillicothe, OH). To avoid the activation of apoptosis we excluded NaB concentrations >2 mM (13). Moreover, to investigate the effect of NaB on HIF-1 $\alpha$  and VEGF expression under hypoxic conditions, 24 h experiments were carried out in the absence and in the presence of 100  $\mu$ M cobalt chloride (CoCl<sub>2</sub>).

#### VEGF determination by quantitative immunoassay

Alternative exon splicing of the VEGF gene results in different VEGF isoforms containing 121, 165, 189 or 206 amino acid residues; VEGF165 is the most frequently occurring VEGF isoform its concentration was determined using a commercial quantitative immunoassay kit for human VEGF165 (Quantikine, human VEGF; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Concentrations were expressed as picograms of VEGF protein per milligram of total protein.

Western blotting

Total extracts were obtained from cells treated with increasing concentrations of NaB for 24 or 72 h. The cells were rinsed twice with PBS at 4°C, centrifuged at 800 g for 10 min and lyzed in 0.1 ml buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 10 mM EDTA, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 1 mM phenylmethanesulfonyl fluoride and 1% Triton X-100) and incubated for 1 h on ice. A 5 µl aliquot was used to determine the protein concentration with the Bradford microassay kit (Life Science Research, Bio-Rad Laboratories, Segrate, Italy). Samples were then adjusted with an appropriate volume of buffer and 50 µg of total proteins per lane were subjected to electrophoresis on discontinuous mini gel with a 9% sodium dodecyl sulfate acrylamide-bisacrylamide running gel at 100 V for 2.5 h. The gel was equilibrated in transfer buffer (25 mM base, 192 mM glycine and 10% methanol) and then transferred to a nitrocellulose membrane (Hybond-ECL, Amersham, Life Science, Little Chalfont, Buckinghamshire, UK) at 4°C overnight. The membrane was equilibrated in transfer buffer and blocked for 1 h with 5% milk in PBS, 0.1% Tween-20. HIF-1α levels were determined by immunoblotting with the commercially available monoclonal antibody (H1α67, Abcam, Cambridge, UK). To be sure that there is no cross-reactivity with other VEGF isoforms, VEGF165 expression was also investigated by western blot analysis using an anti-VEGF monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) which recognizes all but 206 amino acid splice variants. Actin was used as an internal control and was detected with an anti-actin mouse monoclonal antibody (Sigma). Alkaline phosphatase (Chemicon International, Temecula, CA) was used as a marker of differentiation in colonic epithelial cells induced by sodium butyrate (20). The membrane was subsequently washed with PBS 0.1% and Tween-20 and incubated with a secondary antibody conjugated with horseradish peroxidase. Immune complexes were detected using the ECL chemoluminescent system (Amersham), which impressed the Hyperfilm-ECL (Amersham). Bands were detected by a ScanJet IIIcx/T (Hewlett Packard Co., Greley, CO), quantified by TotalLab Software (Nonlinear Dynamics, Durham, NC), normalized according to the internal control and expressed as normalized densitometric units

(NDU). Before using actin bands to normalize the results the presence of a linear range of protein concentrations (i.e. a proportional increase in the actin signal by increasing the amount of starting protein concentration) was verified (rs = 0.95, P = 0.001). The results represent the mean of three independent experiments.

RNA preparation and RT-PCR

Cells were kept for 24 or 72 h in the medium supplemented with increasing concentrations (0.1, 0.5, 1 and 2 mM) of NaB. After harvesting of the cells, total RNA was extracted by means of Trizol (Life Technologies, Frederick, MD) according to the manufacturer's instructions. The one-step reverse transcription-polymerase chain reaction (RT-PCR) amplification of a specific target RNA was carried out from 0.5 µg of total RNA using the GeneAmp Gold RNA PCR reagent kit (Perkin-Elmer Biosystems, Foster City, CA). Samples were processed in a Perkin-Elmer 9600 GeneAmp thermocycling system under the following conditions: 12 min at 45°C for the reverse transcriptase reaction; 10 min at 95°C for AmpliTaq Gold activation followed by 27 amplification cycles (1 min at 94°C for denaturation, 1 min at 60°C for primer annealing and 45 s at 72°C for primer extension), and final extension at 72°C for 10 min. The optimal conditions for RT-PCR experiments were verified in terms of linearity of the reaction as a function of the number of amplification cycles (Figure 1) and of the amount of starting RNA in the range of 0.05–0.9 µg when the target (VEGF or HIF-1α) and the reference (β-actin) genes were co-amplified in the same tube (Figure 2A and B).

Amplification of HIF-1α (460 bp) was obtained by use of published primer sequences: sense strand 5'-CTC AAA GTC GGA CAG CCT CA-3' and antisense strand 5'-CCC TGC AGT AGG TTT CTG CT-3' (21) at the concentration of 50 pmol. Likewise, amplification of VEGF isoforms (320 bp for 206, 300 bp for 189, 230 bp for 165, 100 bp for 121) was obtained by use of published primer sequences: sense strand 5'-CAC ATA GGA GAG ATG AGC TTC-3' and antisense strand 5'-CCG CCT CGG CTT GTC ACA T-3' (22) at the concentration of 50 pmol. RT-PCR with primers encoding for β-actin (700 bp; sense strand 5'-ACA CTG TGC CCA TCT ACG AGG-

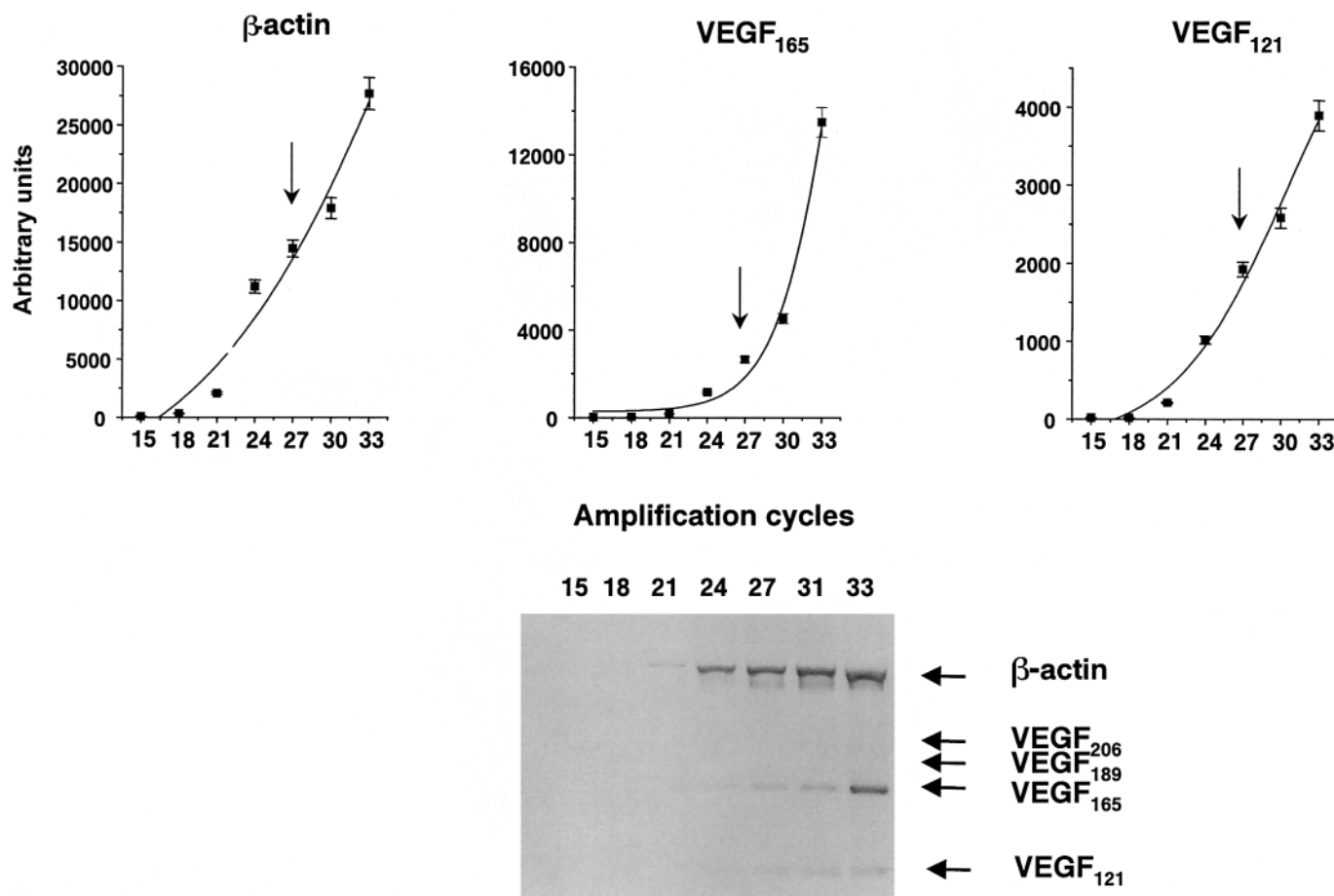


Fig. 1. Set-up of the optimal cycles number for RT-PCR experiments using a starting RNA concentration of 0.5 µg. For both the target (VEGF) and reference (β-actin) gene a relation between increasing number of cycles and cDNA amplification was observed. For RT-PCR experiments 27 amplification cycles were adopted. Data represent mean ± SD obtained from three replicates.

3' and antisense strand 5'-AGG GGC CGG ACT CGT CAT ACT-3' (23) was used as internal control at the concentration of 25 pmol. Primers were purchased from MWG-Biotech AG (Ebersberg, Germany). PCR products were analyzed on 3% agarose gels. Bands were recorded impressing Polaroid 665 PN black and white instant pack film (Polaroid, Cambridge, MA), detected by a ScanJet IIIcx/T (Hewlett Packard), quantified by TotalLab Software, normalized according to the internal control and expressed as normalized densitometric units (NDU). The results represent the mean of three independent experiments.

#### Statistical analysis

The Spearman rank correlation coefficient analysis was applied to assess the linearity between amounts of loaded protein concentrations and actin signal and Student's *t*-test was used to assess the difference between independent groups.

## Results

As shown in Figure 3, HT29 cells constitutively expressed a high level of HIF-1 $\alpha$  protein, which diminished already after a 24 h treatment.

After 24 h of treatment, concentrations of NaB  $\geq 0.5$  mM significantly reduced protein expression by  $\sim 30\%$  with respect to control ( $P = 0.05$ ) with a further decrease to 50% after a 72 h treatment ( $P = 0.01$ ). In contrast, in the presence of a NaB concentration of 2 mM a dramatically increased HIF-1 $\alpha$  level was observed (+60% with respect to the control). As expected, alkaline phosphatase, used as a marker of differentiation in colonic epithelial cells following NaB treat-

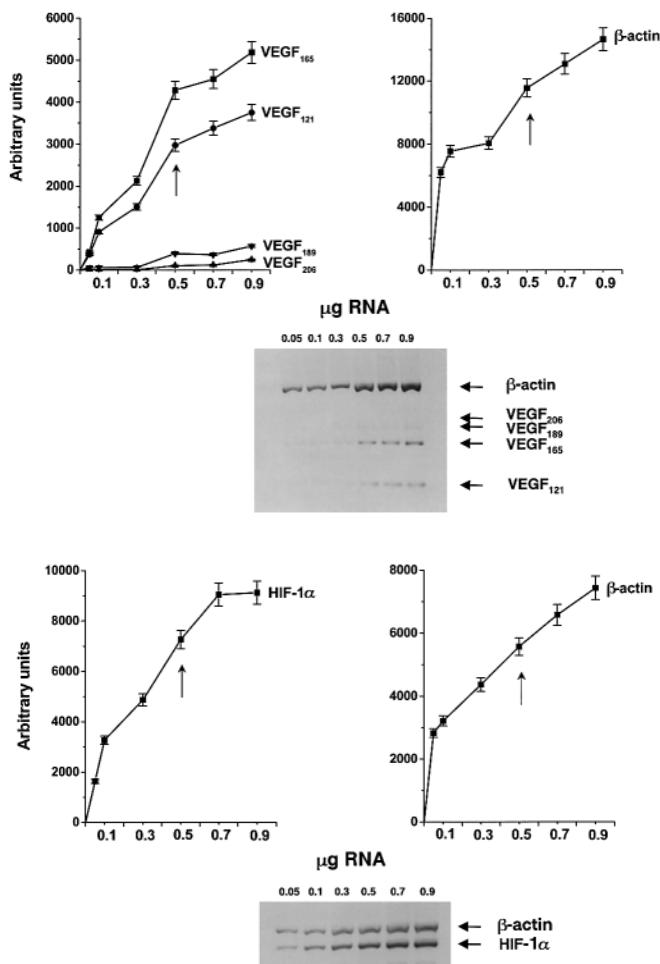
ment, was induced in a dose-dependent manner already after 24 h ( $P = 0.01$ ).

The inhibitory effect of NaB on VEGF165 levels is shown in Figure 4.

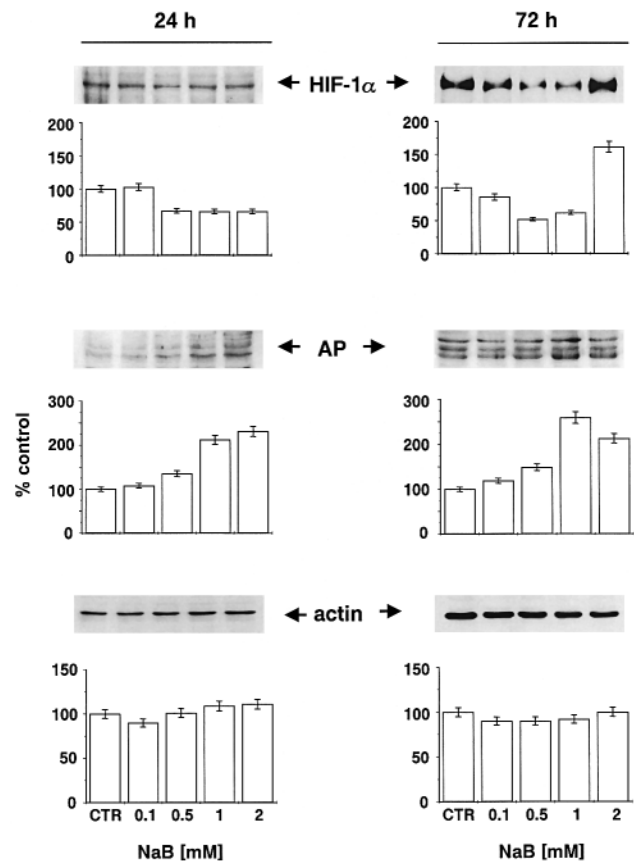
NaB induced a dose-dependent decrease in the VEGF165 protein, which is constitutively present at very high levels (125 and 221 pg/mg protein, respectively, at 24 and 72 h) in the HT29 cell line. Western blot analysis (Figure 5) showed that after 72 h of treatment all VEGF isoforms but VEGF189 were down regulated by increasing concentrations of NaB and that VEGF165 isoform decreased in a similar manner as observed by immunoassay, with a significant association between the two approaches.

The decrease of the VEGF165 protein was not paralleled by a concomitant decrease in the corresponding mRNA, which remained the most frequent isoform expressed in HT29 cells even under NaB treatment. In fact, RT-PCR analysis (Figure 6) showed that only the VEGF121 isoform was affected by treatment with NaB: a clear, dose-dependent up regulation of this isoform was observed already after a 24 h treatment, but was not paralleled by a concomitant increase in VEGF121 protein.

Conversely, VEGF165 isoform was almost unaffected by NaB treatment. Also HIF-1 $\alpha$  mRNA, constitutively over-expressed in this colon cancer cell line, was affected by a NaB treatment. A dose-dependent increase of HIF-1 $\alpha$  mRNA expression, with respect to control, was observed already after a 24 h treatment (Figure 7).



**Fig. 2.** Densitometric quantification of PCR products for target [VEGF isoforms (A); HIF-1 $\alpha$  (B)] and reference ( $\beta$ -actin) genes as a function of the amount of starting RNA. Data represent mean  $\pm$  SD obtained from three replicates.



**Fig. 3.** Modulatory effect of increasing concentrations (0.1–2 mM) of NaB on the level of HIF-1 $\alpha$  and alkaline phosphatase (AP), after 24 or 72 h of treatment, analyzed by western blotting. The graphs show the corresponding quantification by densitometric analysis normalized by the internal control (actin).

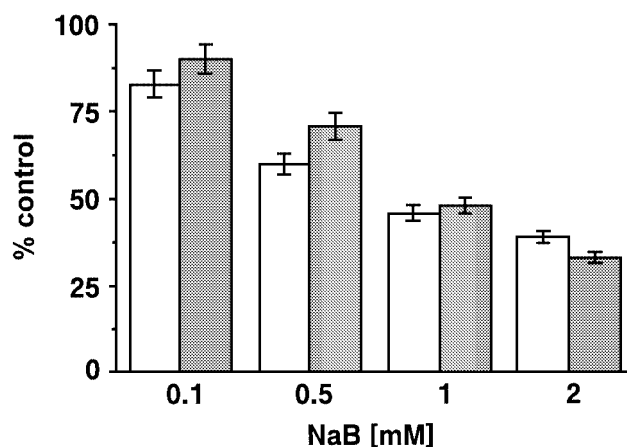


Fig. 4. Modulatory effect of increasing concentrations (0.1–2 mM) of NaB on the VEGF165 concentration quantified by an enzyme immunoassay kit after 24 (white) or 72 h (gray) of treatment.

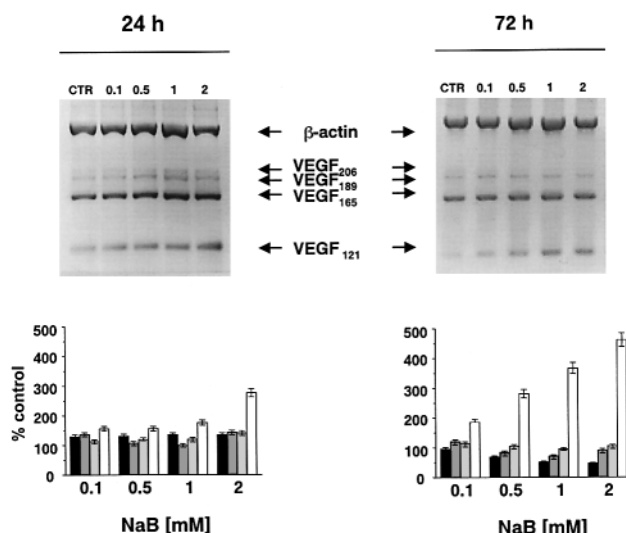


Fig. 6. Modulatory effect of increasing concentrations (0.1–2 mM) of NaB on the expression of VEGF mRNA, after 24 or 72 h of treatment, analyzed by RT-PCR. The graphs show the corresponding quantification by densitometric analysis normalized by the internal control ( $\beta$ -actin). VEGF206 isoform (black), VEGF189 isoform (dark gray), VEGF165 isoform (light gray) and VEGF121 isoform (white).

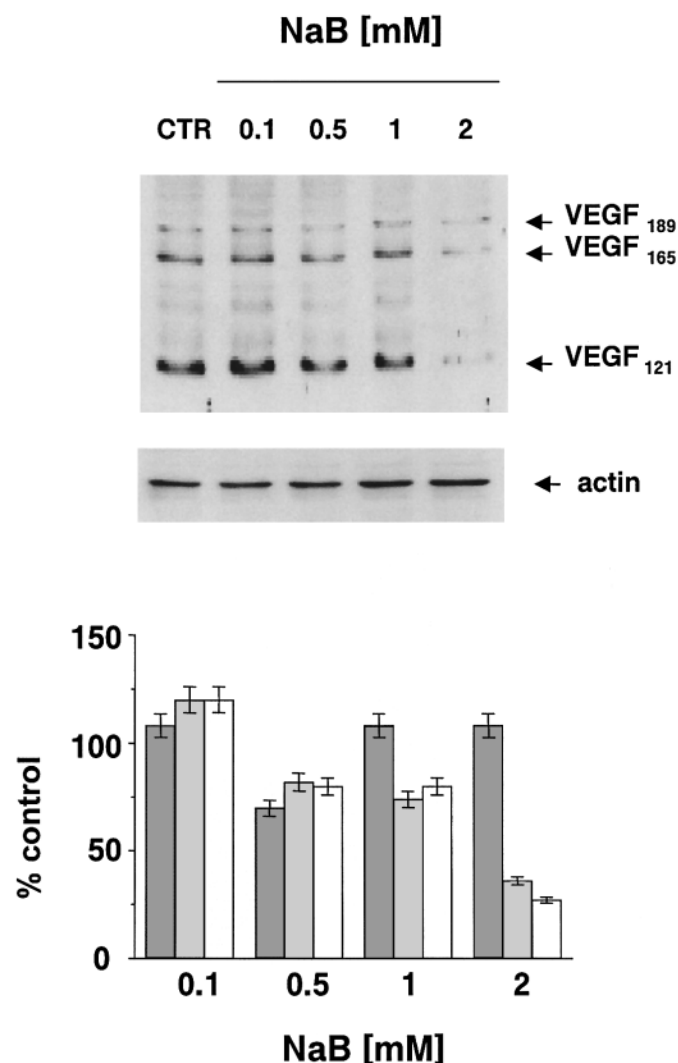


Fig. 5. Modulatory effect of increasing concentrations (0.1–2 mM) of NaB on VEGF189 (dark gray), VEGF165 (light gray) and VEGF121 (white) isoforms analyzed by western blotting after 72 h of treatment. The graph shows the corresponding quantification by densitometric analysis normalized by the internal control (actin).

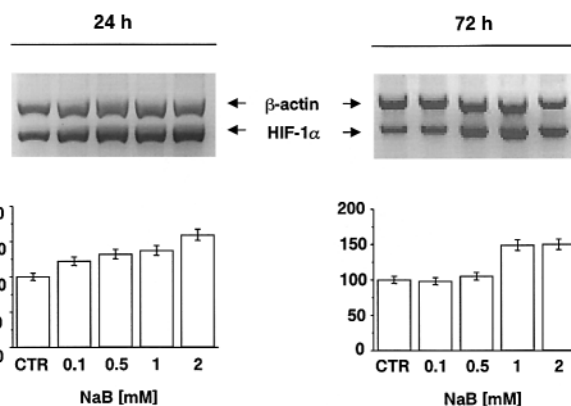


Fig. 7. Modulatory effect of increasing concentrations (0.1–2 mM) of NaB on the expression of HIF-1 $\alpha$  mRNA, after 24 or 72 h of treatment, analyzed by RT-PCR. The graphs show the corresponding quantification by densitometric analysis normalized by the internal control ( $\beta$ -actin).

Table I. Effect of increasing concentration of NaB in the absence or in presence of  $\text{CoCl}_2$  (100  $\mu\text{M}$ ) on HIF-1 $\alpha$  and VEGF protein levels

	Without $\text{CoCl}_2$		With $\text{CoCl}_2$	
	HIF-1 $\alpha$	GF <sup>a</sup>	HIF-1 $\alpha$	VEGF
CTR	0.67	125	0.55	237
NaB (mM)				
0.1	0.69	104	0.56	235
0.5	0.44	75	0.65	223
1	0.44	57	0.72	189
2	0.45	49	0.70	172

<sup>a</sup>Expressed as normalized densitometric unit.

<sup>b</sup>Expressed as pg/mg of cytosolic protein.

The effect of a 24 h NaB treatment, in the presence of CoCl<sub>2</sub>, on VEGF165 and HIF-1 $\alpha$  protein levels is reported in Table I.

NaB was able to counteract only in part the remarkable increase (+90%) in VEGF165 protein induced by CoCl<sub>2</sub>. HIF-1 $\alpha$  protein, which was surprisingly not affected by CoCl<sub>2</sub> treatment, was slightly increased by NaB co-treatment.

## Discussion

In previous papers we have demonstrated that millimolar concentrations of NaB arrest cell proliferation, induce apoptosis and modulate the expression of cell cycle-related proteins (cyclin D1, p53 and cyclin-dependent kinases) in a wide range of tumor cell lines representing the most common solid tumors including breast, lung and colon cancer (12–14). The present results indicate that in the HT29 colon adenocarcinoma cell line, in addition to the above-mentioned activities, NaB also modulates the expression of two angiogenesis-related proteins, VEGF and HIF-1 $\alpha$ , particularly in normoxic conditions. This finding is in agreement with a recent paper (18) reporting the *in vitro* and *in vivo* angiogenesis inhibition by phenylbutyrate (another differentiation inducer), in association with retinoic acid, on human and rodent prostate cancer cell lines. In fact, under normoxic conditions, NaB was able, already after 24 h of treatment, to down regulate in a dose-dependent fashion the expression of VEGF, the most important angiogenic factor, and in particular the expression of VEGF165, which is the predominant isoform related to tumor progression. As the decrease in VEGF165 protein was not paralleled by a concomitant decrease in VEGF165 mRNA, a post-translational regulation with increased protein degradation should be hypothesized. Experiments carried out in the presence of CoCl<sub>2</sub> (used to mimic hypoxic induction) showed that HT29 cells, which constitutively express very high levels of HIF-1 $\alpha$  and VEGF protein, responded to NaB and CoCl<sub>2</sub> co-treatment in an unexpected manner. In fact, CoCl<sub>2</sub>, which further increase the high basal VEGF level, failed to increase HIF-1 $\alpha$  protein level and was only in part counteracted by NaB co-treatment. Thus, in HT29 cells, CoCl<sub>2</sub> might be unable to further increase a high basal expression of HIF-1 $\alpha$ , and induced VEGF synthesis through a partially HIF-1-independent mechanism, which cannot be counteracted by a 24 h treatment with NaB.

Among the VEGF mRNA isoforms only VEGF121 proved to be up regulated by NaB in a dose-dependent fashion already after a 24 h treatment even though without a concomitant up regulation in the corresponding protein. Observations related to a series of clinical colorectal carcinomas and corresponding normal colon mucosa (data not shown) indicated that VEGF121 mRNA was the predominant isoform in normal tissue but not in colorectal carcinoma and that this isoform was specific for normal mucosa. Therefore, of particular interest is the evidence that, in agreement with its differentiation activity, the only VEGF mRNA isoform affected by NaB is VEGF121 mRNA. This finding would suggest this isoform to be differentiation-related but also raises the question about the putative mechanism for this mRNA isoform selectivity not paralleled by the up regulation of the corresponding protein.

The observation that NaB up regulated HIF-1 $\alpha$  mRNA is quite surprising, considering the role of HIF-1 in carcinogenesis and progression (9,10) and is in disagreement with a recent paper (24) in which a down regulation of HIF-1 has been reported. However, HIF-1 $\alpha$  expression has been recently shown

(25) to counteract the *in vivo* growth of a breast cancer cell line, presumably through hypoxia-induced apoptosis, whereas low HIF-1 $\alpha$  levels in the presence of a high basal VEGF content were associated with a more aggressive phenotype. The overexpression of HIF-1 $\alpha$  mRNA induced by NaB treatment and paralleled by an increase in HIF-1 $\alpha$  protein could represent an early step in apoptosis induction, which was mainly evident at higher NaB concentrations or with prolonged NaB exposure (13). In addition, it has been reported (26) that NaB is able to induce some heat shock genes, such as HSP70 and HSP28, as expression of differentiation induction and that this up regulation is barely detectable at 24 h, but considerably at 72 h. In this context, the up regulation of HIF-1 $\alpha$  and the concomitant down regulation of VEGF protein could be an aspect of the reversion induced by NaB towards a more differentiated phenotype, as indicated by the overexpression of alkaline phosphatase.

In summary, our results indicate that NaB, besides regulating cell proliferation and differentiation, is able to modulate the expression of two important angiogenesis-related molecules, and suggest a further possible clinical application of this short-chain fatty acid as an anti-angiogenic agent. As the main disadvantages (the short lifetime and the high concentration required to maintain effective plasma levels) which limit the clinical use of NaB may be easily overcome by a new generation of pro-drugs, such as hyaluronic ester compounds (27), pre-clinical studies should address the anti-angiogenic effects of these new drugs in comparison to NaB and evaluate their possible clinical application.

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