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Original Article

Sodium butyrate activates Notch1 signaling, reduces tumor markers, and induces cell cycle arrest and apoptosis in pheochromocytoma

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Abstract: *Background:* Pheochromocytoma is a neuroendocrine (NE) tumor of the adrenal medulla for which surgical resection is the only therapy. However, 10-46% of tumors are metastatic or have malignant features, and are often inoperable. Our lab has demonstrated the importance of the Notch1 signaling pathway in NE neoplasia, indicating that this pathway could be a target for emergent treatments in pheochromocytoma. It has recently become clear that histone deacetylase (HDAC) inhibitors influence Notch1 signaling. We hypothesized that the HDAC inhibitor Sodium Butyrate (NaB) might activate Notch1 in pheochromocytoma resulting in altered tumor cell proliferation. *Methods:* Pheochromocytoma (PC-12) cells were treated with increasing concentrations of NaB. MTT cellular proliferation assay was used to determine the effect of NaB on PC-12 cell growth. Expression of Notch1, NE markers, and cell cycle proteins was studied using Western analysis. *Results:* Untreated PC-12 cells lack Notch1 activity. Treatment with NaB led to a dose-dependent induction of Notch1 signaling, reduction of NE markers ASCL1 and CgA, and a significant reduction in cellular proliferation. Levels of expression of cyclin D1, p21, cleaved PARP, and cleaved caspase 3 proteins indicated the presence of cell cycle arrest and apoptosis following NaB treatment. *Conclusion:* NaB activated Notch1 signaling, inhibited cellular proliferation, reduced NE markers, and induced cell cycle arrest and apoptosis in pheochromocytoma cells. This data indicates that activation of Notch1 signaling is a promising potential therapy or palliative measure for pheochromocytoma that warrants further investigation.

Key Words: Butyrate, pheochromocytoma, PC-12, Notch1, neuroendocrine, HDAC inhibitor.

Introduction

Pheochromocytoma is a rare catecholamine-secreting neuroendocrine tumor of the chromaffin cells of the adrenal medulla and less commonly of extra-adrenal tissues such as the sympathetic ganglia [1]. Management of the pheochromocytoma patient remains a challenge because, in addition to possessing an incidence of only 2-8 cases per million per year, it causes a debilitating and variable paraneoplastic syndrome secondary to excess catecholamine production that may feature anxiety, tremulousness, pallor, chest pain, visual disturbances, nausea and vomiting, polyuria, polydypsia, heat intolerance, orthostatic hypotension, palpitations, headaches, diaphoresis, uncontrollable diarrhea, skin rashes, and hypertension [2]. Hypertension occurs in 61-100% (0.1 to 1% of all hypertensives) of pheochromocytoma patients and presents as paroxysmal, potentially fatal attacks which are resistant to standard treatments [1, 3-5]. Surgical resection represents the only potential cure [6]. Precluding this approach, the tumors are metastatic or have malignant features in 10-46% of cases [7-9]. Accordingly, the development of novel treatments, palliative or curative, for non-resectible pheochromocytoma would represent a significant advancement for this subset of patients.

Notch1 is a transmembrane receptor protein that functions in a variety of contexts including development, differentiation, proliferation, and survival [10]. Notch1 is activated by cleavage following Delta-like ligand binding whereupon the active Notch1 intracellular domain (NICD) translocates to the nucleus where it functions as a transcription factor in conjunction with the C promoter-binding factor 1 (CBF-1) and other DNA binding complexes [10, 11]. In cancer, its function as an oncogene or tumor suppressor is also context-dependent [10]. The Notch1 signaling pathway is highly conserved, but inactive in NE malignancies including medullary thyroid cancer, carcinoid, and small cell lung cancer. Our lab has shown that over-expressing active Notch1 protein inhibits growth and hormone secretion in medullary thyroid and carcinoid NE tumors [10, 12-16]. This suggests that activating Notch1 signaling is a promising strategy for developing targeted therapies for NE tumors such as pheochromocytoma.

Very few small molecules have been identified as Notch1 activators. Histone deacetylase (HDAC) inhibitor compounds are well understood in their ability to alter chromatin structure by causing hyper-acetylation of the nucleosomal histone cores around which DNA molecules are coiled following inhibition of HDAC enzymes. The conformational change allows increased access to the DNA molecules by various transcription factors [17]. We have previously found that HDAC inhibitors activate Notch1 signaling in NE tumors [14-16].

Sodium Butyrate is a naturally occurring short-chain fatty acid that is among the longest acknowledged HDAC inhibitors [18]. Its activity in pheochromocytoma as a promoter of cellular differentiation and inhibitor of cell division has been described previously [19]. However, previous studies do not examine the molecular mechanisms involved in the observed phenomena or the quantitative effect of varying doses of NaB on pheochromocytoma cell proliferation. Knowing that some HDAC inhibitors activate Notch1 signaling in NE tumors, we hypothesized that NaB may activate this pathway, affect expression of NE markers, and affect cell proliferation in pheochromocytoma cells. In the present study we report that NaB activates Notch1 signaling, suppresses cellular proliferation via apoptosis and cell-cycle arrest, and reduces NE tumor markers in pheochromocytoma cells *in vitro*.

Materials and methods

Cell culture

PC-12 rat pheochromocytoma cells purchased from the American Type Culture Collection company (ATCC, Manassas, VA) were maintained in the following medium: Ham's F12K (ATCC) medium supplemented with 15% horse serum (Sigma, St. Louis, MO), 2.5% fetal bovine serum (Sigma), 100 IU/ml penicillin (Invitrogen, San Diego, CA), and 100 µg/ml streptomycin (Invitrogen) [12, 20].

Sodium butyrate (NaB) treatment

PC-12 cells were plated in circular 100-mm dishes and allowed to adhere overnight. The cells were treated with increasing concentrations of NaB (0-50 mM, Sigma) dissolved in a combination of dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO) and deionized water in a 1:3 ratio, controlling for identical concentrations of DMSO among the treatment groups. Cells were harvested and lysed and whole cell protein extracts prepared and quantified after 2 days of treatment as previously described [12, 20].

Western blot analysis

Whole cell protein extracts were denatured and resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

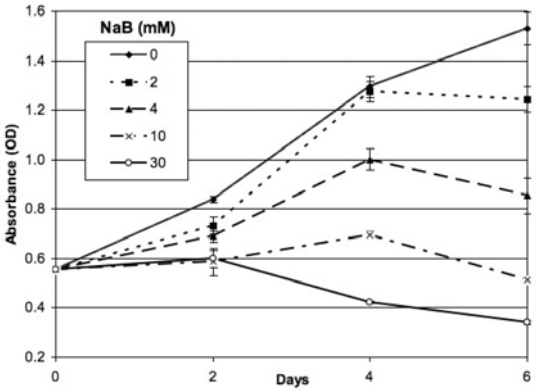

Figure 1. Two-day treatment with NaB (0-30 mM) inhibited proliferation of PC-12 cells as measured by MTT assay. Proliferation was significantly inhibited at day 4 using concentrations >2 mM ($p < 0.05$).

10% precast gels (Invitrogen), transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH), blocked for 1 hour in milk (1x phosphate-buffered saline, 5% nonfat dry milk, 0.05% Tween-20), and subjected to antibody hybridization incubation as previously described, overnight at 4˚C [20]. The following primary antibody dilutions were used: 1:500 for ASCL1 (anti-MASH1, BD Pharmingen, San Diego, CA) and chromogranin A (Invitrogen), 1:1000 for Notch1 (Santa Cruz Biotechnology, Santa Cruz, CA), p21, cyclin D1, cleaved caspase-3, and PARP (Cell Signaling), and 1:10,000 for G3PDH (Trevigen, Gaithersburg, MA). Following overnight incubation, membranes were washed 3 x 5 minutes with wash buffer (1x phosphate-buffered saline, 0.05% Tween-20). Horseradish-peroxidase (HRP)-conjugated goat antimouse or goat antirabbit secondary antibodies (Cell Signaling) were applied as dictated by the source of the primary antibody in a dilution of 1:2000 in milk for 1 hour at room temperature. Membranes were then washed 3 x 10 minutes with wash buffer. Protein signal was visualized using either Immunstar (Bio-Rad Laboratories, CgA, G3PDH, cyclin D1) or Super Signal West Femto (Pierce Biotechnology, ASCL1, PARP, cleaved caspase-3, Notch1, p21) developing kit according to the manufacturer's instructions and used to expose x-ray films (ISC BioExpress, Kaysville, UT) [12, 20].

Cell proliferation assay

We performed and repeated the MTT (methylthiazolyldiphenyl-tetrazolium bromide) assay (Sigma) to measure cellular proliferation following NaB treatment at various concentrations. Cells were seeded in quadruplicate on 24-well plates and allowed to adhere overnight. NaB treatment was carried out for 6 days, with cell viability being assayed after 2, 4, or 6 days using a spectrophotometer (μQuant by Bio-Tek Instruments, Winooski, VT) as previously described [15, 16, 20].

Results

NaB inhibits PC-12 cell proliferation

We examined the effect of NaB treatment on pheochromocytoma cell proliferation using the MTT assay. PC-12 cells treated with 2 mM NaB display suppressed growth and proliferation rates compared to untreated cells (**Figure 1**). Further, the MTT results suggest that the extent of growth suppression varies in a dose-dependent manner with the concentration of NaB applied to cells. Complete growth inhibition was achieved using NaB concentrations >10 mM. After 6 days of treatment, all concentrations used had effected a significant growth inhibition ($p < 0.05$).

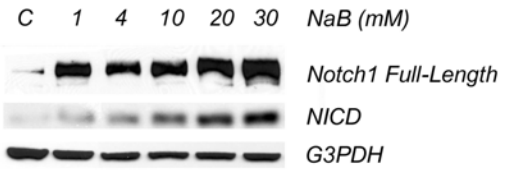

Figure 2. Two-day treatment of PC-12 cells with NaB (0-30 mM) induced both full-length and cleaved, activated (NICD) Notch1 protein expression in a dose-dependent manner as measured by western blot.

NaB Activates Notch1 Expression in PC-12 Cells in vitro

Having confirmed that NaB inhibited growth of PC-12 cells, we tested our hypothesis that NaB activated Notch1 signaling concurrently. PC-12 cells had minimal Notch1 protein expression at baseline. Two-day treatment with NaB resulted in a dose-dependent increase in expression of both the full-length Notch1 transmembrane receptor protein and the cleaved, activated Notch1 intracellular domain (NICD) (**Figure 2**).

NaB Reduces NE Markers

Our laboratory has previously shown that differentiation away from the malignant NE tumor cell phenotype, inhibition of cell proliferation, and reduction of hormone production is consistently associated with reduced expression of the helix-loop-helix transcription factor achaete-scute complex-like 1 (ASCL1) and the acidic glycoprotein chromogranin A (CgA) [10, 12-16, 21]. Moreover, activation of Notch1 signaling has been shown to silence *ASCL1* transcription as well as reduce CgA expression in other NE tumors [12, 15, 16, 22]. With this in mind, we sought to determine whether the Notch1 activation and growth inhibition exhibited by PC-12 cells following NaB treatment was

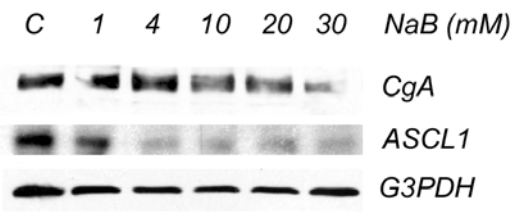

Figure 3. Two-day treatment of PC-12 cells with NaB (0-30 mM) suppressed expression of the NE marker chromogranin A (CgA) as well as the NE marker and transcription factor ASCL1 dose-dependently as measured by western blot.

associated with a reduction in these ubiquitously expressed NE tumor markers. Western analysis revealed that NaB in concentrations >4 mM abolished ASCL1 expression, and that NaB caused conservative but evidently dose-dependent reduction in CgA levels in PC-12 cells (**Figure 3**).

The Mechanism of Growth Inhibition of PC-12 Cells by NaB is a Combination of Cell-Cycle Arrest and Apoptosis

We studied the mechanism by which NaB inhibited PC-12 cell proliferation by performing western analysis to determine whether cells were undergoing cell cycle arrest, apoptosis, or both. Expression of the universal cyclin-dependent kinase inhibitor p21 causes degradation of cyclin D1, and these two events are associated with a G1-phase cell cycle arrest [12]. NaB treatment led to increased expression of p21 at the lowest dose used (1 mM), and cyclin D1 was reduced dose-dependently and abolished at concentrations >10 mM (**Figure 4**). These results suggest that the growth inhibition observed in PC-12 cells following NaB treatment is at least partially due to cell cycle arrest.

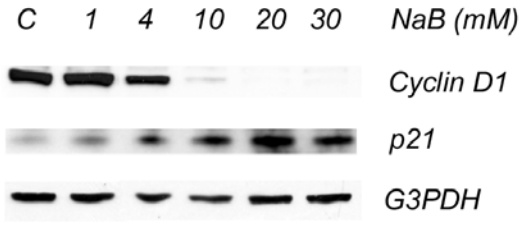

Figure 4. Two-day treatment of PC-12 cells with NaB (0-30 mM) led to a dose-dependent increase in the expression of the CDK inhibitor p21 concurrent with a dose-dependent abolition of cyclin D1 as measured by western blot.

Caspase-3 is an effector in the caspase family of proteases, extensively characterized in its ability induce apoptosis [23, 24]. Poly-(ADP-ribose) polymerase (PARP) is a DNA repair enzyme that is cleaved and inactivated by the active caspase cascade. This cleavage is widely accepted as a marker of apoptosis [15]. Our results indicate increased expression of caspase-3 protein in conjunction with PARP cleavage and inactivation, both present following treatment concentrations of >1mM NaB (**Figure 5**). This finding suggests that the growth inhibition observed in PC-12 cells following NaB treatment is at least in part due to cell apoptosis.

Taken together, our western data suggests that the total growth reduction observed in the MTT assay is due to a combination of cell apoptosis and cell cycle arrest mechanisms.

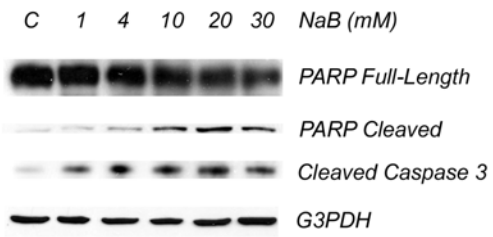

Figure 5. Western blot analysis following 2-day NaB treatment (0-30 mM) of PC-12 cells revealed a dose-dependent increase in the expression of the pro-apoptotic cleaved caspase 3 protein concurrent with the dose-dependent cleavage and inactivation of the DNA repair enzyme PARP.

Discussion

As surgery remains the only effective treatment for pheochromocytoma, patients with inoperable or metastatic tumors stand to gain tremendously from the development of novel treatments and palliative measures for the disease.

The highly conserved Notch1 signaling pathway is inactive in many NE tumor diseases including medullary thyroid cancer, carcinoid, and small cell lung cancer, and its activation is tumor-suppressive in these diseases [10, 12-16, 25]. It has previously been shown that over-expressing active Notch1 protein inhibits

growth and hormone secretion in medullary thyroid and carcinoid NE tumors [12, 13]. Furthermore, Notch1 signaling has been linked directly to the down-regulation of ASCL1, the transcription factor most tightly linked to the NE phenotype [21, 22]. These findings suggest that activating Notch1 signaling is a promising strategy for developing targeted therapies for NE tumors such as pheochromocytoma. Until recently, few small molecules had been reported as being Notch1 activators. Our lab has previously shown that HDAC inhibitors can activate Notch1 signaling in NE tumor diseases [14-16, 25].

It has been observed that NaB reduces cell division in pheochromocytoma cells *in vitro*, however, the dose-dependency and molecular mechanism involved in this phenomenon remain unclear [19]. We hypothesized that altered Notch1 signaling might be involved. Knowing that certain HDAC inhibitors activate Notch1 signaling in selected NE tumors, we aimed to determine whether NaB activated Notch1 signaling, dose-dependently inhibited cell proliferation, reduced expression of NE markers, and invoked or altered cell cycle and apoptotic pathways in pheochromocytoma cells.

In this study we report that NaB dose-dependently inhibited PC-12 cell growth as measured by MTT assay (**Figure 1**). Growth inhibition was significant after 4 days at doses >2 mM and after 6 days at all doses used.

Having established the growth inhibitory effect of NaB in PC-12, we used western analysis to determine the effect of NaB on Notch1 signaling, NE marker expression, and the mechanism underlying the observed growth inhibition. NaB led to dose-dependent increases in both the full-length transmembrane Notch1 protein and the cleaved, activated Notch1 intracellular domain (NICD). The same treatments led to dose-dependent reductions in the levels of the important NE markers ASCL1 and CgA. Observed growth reduction appears to be due to a combination of cell apoptosis and cell cycle arrest based on increased expression of the CDK inhibitor p21, abolition of the cell cycle promoter cyclin D1, increased expression of the pro-apoptotic protease caspase-3, and increased levels of the cleaved and inactivated DNA repair enzyme PARP.

NaB activated Notch1 signaling, reduced NE tumor marker expression, and induced a cell cycle arrest- and apoptosis-mediated dose-dependent growth inhibition in the NE pheochromocytoma cells, suggesting that Notch1 activation in pheochromocytoma is a promising potential targeted therapy for this disease. However, NaB may not be an ideal compound for this purpose because of its toxicity and non-specificity. Identifying suitable compounds specifically activating Notch 1 signal to treat pheochromocytoma warrants further investigation.

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