Original Article

Sodium Butyrate Induces Apoptosis of Human Colon Cancer Cells by Modulating ERK and Sphingosine Kinase 2*

XIAO Min[1,2,+], LIU Yun Gang[3,+], ZOU Meng Chen[4], and ZOU Fei[1,#]

1. Department of Occupational Health and Occupational Medicine, School of Public Health and Tropical Medicine, Southern Medical University, Guangzhou 510515, Guangdong, China; 2. Department of Clinical Laboratory, Affiliated Hospital of Guangdong Medical College, Zhanjiang 524001, Guangdong, China; 3. Department of Toxicology, School of Public Health and Tropical Medicine, Southern Medical University, Guangzhou 510515, Guangdong, China; 4. Department of Endocrinology, Nanfang Hospital, Guangzhou 510515, Guangdong, China

Abstract

Objective To investigate the role of extracellular signal-regulated kinase (ERK) in apoptosis of human colon cancer (HCT116) cells.

Methods After the HCT116 cells were pretreated with specific ERK inhibitor (U0126) or specific siRNA and exposed to 10 mmol/L sodium butyrate (NaBT) for 24 h, their apoptosis was detected by flow cytometry, levels of SphK2 and ERK protein were measured by Western blot, and translocation of SphK2 was assayed by immunofluorescence microscopy.

Results The U0126 and siRNAs specific for SphK2 blocked the export of SphK2 from nuclei to cytoplasm and increased the apoptosis of HCT116 cells following NaBT exposure. Over-expression of PKD decreased NaBT-induced apoptosis of HCT116 cells, which was reversed by U0126. Furthermore, transfection of HCT116 cells with constitutively activated PKD plasmids recovered the U0126-blocked export of SphK2.

Conclusion ERK regulates the export of SphK2 and apoptosis of HCT116 cells by modulating PKD. Modulation of these molecules may help increase the sensitivity of colon cancer cells to the physiologic anti-colon cancer agent, NaBT.

Key words: Sodium butyrate; Apoptosis; ERK; Sphingosine kinase 2; Colon caner

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INTRODUCTION

Colorectal cancer is the third most common cancer in the world, and almost 60% of the cases have occurred in developed regions. The incidence of colon cancer varies 10-fold in each sex of population in different areas, which is highest in Australia/New Zealand and Western Europe, and lowest in Africa (except Southern Africa) and South-Central Asia[1]. These variations may be partially explained by varied cultures and life styles in different countries and regions, and lacking of

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+The authors contributed equally to this work.

#Correspondence should be addressed to Prof. ZOU Fei, Tel: 86-20-61648301; Fax: 86-20-61648324; E-mail: zoufeimed@126.com

Biographical note of the first author: XIAO Min, female, born in 1981, PhD, majoring in occupational health and environmental health.

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dietary fibers, smoking, overweight, obesity, processed consumption and excessive alcohol consumption are the potential risk factors for colon cancer[2]. Although the application of some medical procedures, such as fecal occult blood testing and endoscopy, contributes greatly to the early diagnosis and treatment of colorectal cancer, its mortality keeps increasing in some countries, such as some east Asian countries (including China)[3].

Sphingolipid metabolites have been regarded as the important regulators in many fundamental biological processes, such as cell growth and apoptosis, which include sphingosine-1-phosphate (S1P), ceramide, and sphingosine. Sphingosine kinase (SphK), which phosphorylates sphingosine to S1P, is an important mediator in these processes. Of the two isoforms of SphK (SphK1 and SphK2), SphK1 has been proved to promote colon cancer progression in mice[4]. The SphK1level is significantly higher in breast, colon, lung, ovary, stomach and kidney cancer tissues than in normal tissues of the same patient[5-6]. Many stimuli, such as epidermal growth factor (EGF), nerve growth factor (NGF), insulin growth factor (IGF), transforming growth factor beta (TGF-β), and phorbol 12-myristate 13-acetate (PMA), induce activation of SphK1 and its rapid translocation from cytoplasm to plasma membrane[7]. The activation of SphK1 is regulated by extracellular signal-regulated kinase (ERK) 1/2-mediated phosphorylation of SphK1 on Ser225[8]. ERK1/2 and protein kinase C (PKC) seem to play an important role in the up-regulation of SphK1 expression[9-10]. In contrast to SphK1, much less is known about the biological function of SphK2, especially its involvement in cancer. Mitogen-activated protein kinase (MAPK) is a family of serine/threonine protein kinases that modulate cell growth/apoptosis processes, which includes extracellular signal-regulated kinase (ERK), Jun N-terminal kinase and p38 MAPK. The latter two involved in the cellular response to environmental stress are well-known as stress-activated protein kinase[11-13]. On the other hand, ERK has been recognized as an important regulator of cell survival. It was reported that activation and translocation of SphK2 from nuclei to cytoplasm were mediated by protein kinase D (PKD), leading to sensitization of the apoptosis of human colon cancer (HCT116) cells. We are curious whether ERK is also engaged in the activation and translocation of SphK2, thus participating in the mechanism underlying the apoptosis of HCT116 cells.

Sodium butyrate (NaBT) is a sodium salt of butyric acid, a natural metabolite of dietary fibres transformed by intestinal bacteria. It has been shown that NaBT can suppress cell proliferation and induce blockage of the cell cycle in G1/S and/or G2/M in various cancer cells *in vitro*, such as colon cancer cells, neuroblastoma cells, leukemic cells, and myeloma cells[14-16]. NaBT may also induce apoptosis of some categories of cancer cells *in vitro*, such as breast carcinoma cells and colon cancer cells[17-18]. However, its precise anti-tumour mechanism remains unclear. We have recently identified that PKD and SphK2 negatively regulate NaBT-induced apoptosis of human colon cancer (HCT116) cells, and PKD regulates SphK2 in their activation and translocation.

The present study is designed to clarify whether ERK is correlated with NaBT-induced apoptosis of HCT116 cells and to investigate the mechanisms by which ERK modulates the cell apoptosis with a view to establishing some molecular checkpoints, based on which modulation of these molecules by appropriate pharmaceutical agents and sensitization of colon cancer cells to chemotherapies or natural anti-colon cancer substances (such as NaBT) could be achieved.

MATERIALS AND METHODS

Reagents

NaBT and U0126 were purchased from Sigma-Aldrich (St Louis, MO), fetal bovine serum and Dulbecco's modified Eagle medium (DMEM) were purchased from Invitrogen (Calsbad, CA), antibodies to human SphK1, total human ERK and phosphorylated human ERK were purchased from Cell Signaling (Danvers, MA), antibodies to human SphK2 were purchased from Abcam (Cambridge, UK), antibodies to phosphorylated human SphK2 were bought from Assay Biotechnology (Sunnyvale, CA), antibodies to glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were bought from Boster (Wuhan, China), and Lipofectamine 2000 was bought from Invitrogen (Calsbad, CA).

Cell Culture

HCT116 cells were cultured as previously described[19].

Cell Transfection

The sequences of siRNA against human SphK2, PKD1, PKD2, PKD3, and control siRNA were identified as previously described[19]. The siRNA was

synthesized by GenePharma Co. Ltd (Shanghai, China). Wild type PKD plasmids and constitutively activated PKD plasmids were the gifts from Professor Alex Toker (Harvard Medical School, USA). The HCT116 cells were transfected with each set of siRNA sequences or PKD-encoding plasmids as previously described[19]. The efficiency of each set of siRNA for expression interference and that of the plasmids for over-expression of PKD were detected as previously described[19].

Detection of Cell Apoptosis

Apoptosis of HCT116 cells after each treatment was detected with the Annexin V apoptosis detection kit (BD ParMingen San Diego, CA) as previously described[19].

Immunoblot Assay

Cellular lysates were prepared as previously described[19]. Proteins were separated by SDS-PAGE, with acrylamide at the concentration of 8%-10%, and transferred to PVDF membranes. The membranes were incubated with specific antibodies. Immunocomplexes were detected using appropriate secondary antibodies conjugated to horseradish-peroxidase followed by enhanced chemiluminescence (Pierce Chemical Co.).

Immunofluorescence and Confocal Microscopy

The HCT116 cells were seeded on coverslips (Macalaster Bicknell, New Haven, CT), washed with cold PBS, fixed in 4% paraformaldehyde, and permeated with 0.1% Triton X-100. Coverslips were blocked in PBS containing 5% non-fat milk, and primary antibodies were added. The cells were labeled with the appropriate secondary antibodies conjugated with FITC, incubated with DAPI and images were obtained with OLYMPUS 200M (Japan) microscope system.

Statistical Analysis

All data were obtained from at least 3 separate experiments and analyzed by one-way analysis of variance. $P<0.05$ was considered significantly different.

RESULTS

SphK2 and ERK Regulate Apoptosis on the Same Signal Pathway

The effect of NaBT on ERK activity in HCT116 cells was detected. As shown in Figure 1A, the phosphorylation of ERK increased after NaBT treatment, reached its peak at 20 min and then started to decrease at 60 min. Western blot analysis demonstrated that the total level of ERK was not affected. To further investigate whether ERK plays a role in the translocation of SphK2 and apoptosis of HCT116 cells in response to NaBT, a specific ERK inhibitor U0126[20-22] was used to inhibit the ERK. U0126 blocked the export of SphK2 from nuclei to cytoplasm (Figure 1C) and sensitized the apoptosis of HCT116 cells treated with NaBT (Figure 1D). To identify whether ERK and SphK2 stay in the same signaling pathway for regulating apoptosis of HCT116 cells, the effect of down-regulated SphK2 and U0126 on the export of SphK2 and apoptosis of HCT116 cells was investigated. Western blot analysis showed that the SphK2 level in HCT116 cells transfected with the specific siRNA was only 8%-12% of that detected in HCT116 cells transfected with non-silencing RNA, with the SphK1 level unchanged (Figure 1B). This down-regulation of SphK2 suppressed the translocation of SphK2 and sensitized the apoptosis of HCT116 cells, very similar to that observed using U0126 (Figures 1C, 1D). Furthermore, joint application of SphK2-siRNA and U0126 increased the apoptosis of HCT116 cells at the same level as observed after either siRNA or U0126 treatment (Figure 1D), suggesting that SphK2 and ERK might modulate the apoptosis of HCT116 cells in the same signal pathway.

ERK Stays Upstream to SphK2 and PKD for Regulating Apoptosis

To verify that ERK stays upstream to SphK2 in the signal pathway regulating cell apoptosis, the effect of SphK2-siRNA on ERK activity and that of ERK on SphK2 activation in HCT116 cells were detected. Down-regulation of SphK2 did not change the ERK activity (Figure 2A), and U0126-inhibited ERK blocked the phosphorylation of NaBT-induced SphK2 (Figure 2B), demonstrating that the activity of SphK2 was positively regulated by ERK.

To answer the question whether ERK stays between PKD and SphK2 or just upstream to PKD in the signal pathway for regulating SphK2 translocation and cell apoptosis, the wild-type PKD was expressed in the cells, leading to a 1.9-time increase in the expression of PKD (Figure 3A). The effect of PKD over-expression, PKD-siRNA and U0126 on cell apoptosis was investigated following NaBT treatment. As shown in Figure 3B, treatment of the cells with U0126, PKD-siRNA, U0126 and PKD-siRNA in combination, the vector of wild-type PKD, or PKD

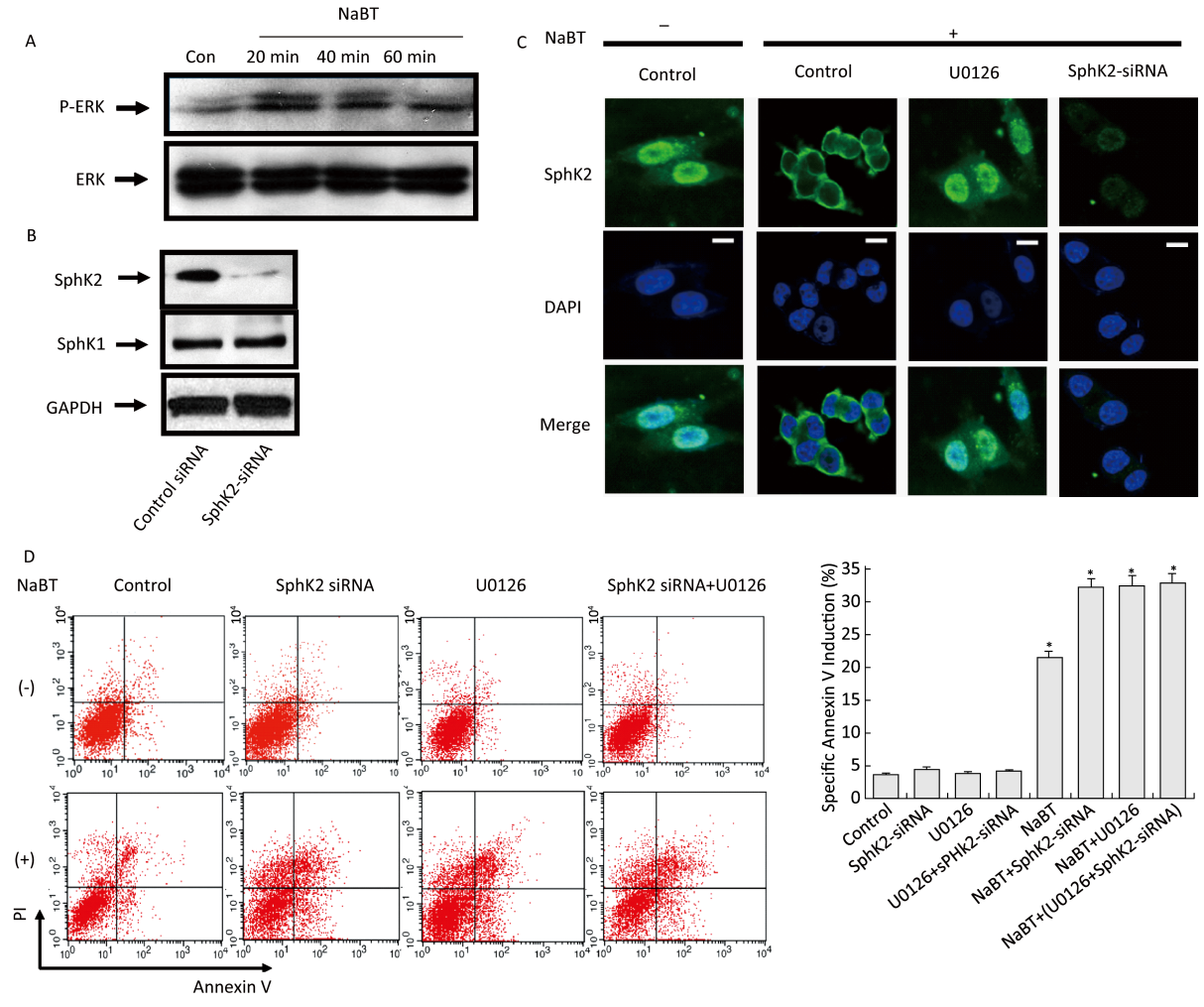

Figure 1. Phosphorylation of ERK (A) after treatment of HCT116 cells with NaBT and effect of SphK2-siRNA and U0126 on NaBT-induced export of SphK2 from nuclei to cytoplasm (B, C) and cell apoptosis (D). Cells were exposed to NaBT (10 mmol/L) for the indicated time span, and the protein fraction was isolated from the cell extract. Western blot was performed with anti-ERK or anti-phosphorylated ERK antibodies, with the total ERK serving as the loading control (A). SphK2 was down-regulated by specific siRNA, with SphK1 serving as a parallel control, and GAPDH serving as the loading control (B). After treatment with SphK2-siRNA and U0126 (20 μmol/L), the cells were exposed to NaBT and the distribution of SphK2 was detected by confocal microscopy with immunofluorescence staining (C). *Bars*, 10 μm. Data are typical representatives of 3 independent experiments. Cell apoptosis was detected by flow cytometry (D). The bottom part of panel D indicates the percentages of cells positive for Annexin V. Data are expressed as mean±SD of 3 independent experiments. $^{*}P<0.05$ *vs.* control.

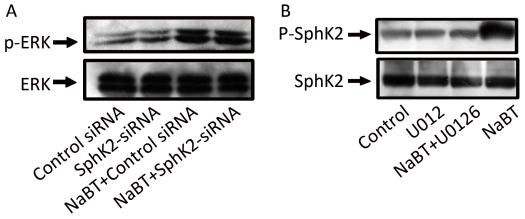

Figure 2. Effect of U0126 and SphK2-siRNA on phosphorylation of SphK2 (A) and ERK (B). Following treatment with either SphK2-siRNA or U0126 (20 μmol/L), HCT116 cells were exposed to NaBT (10 mmol/L), Western blot showed the phosphorylation of ERK and SphK2[19] (Figure 1).

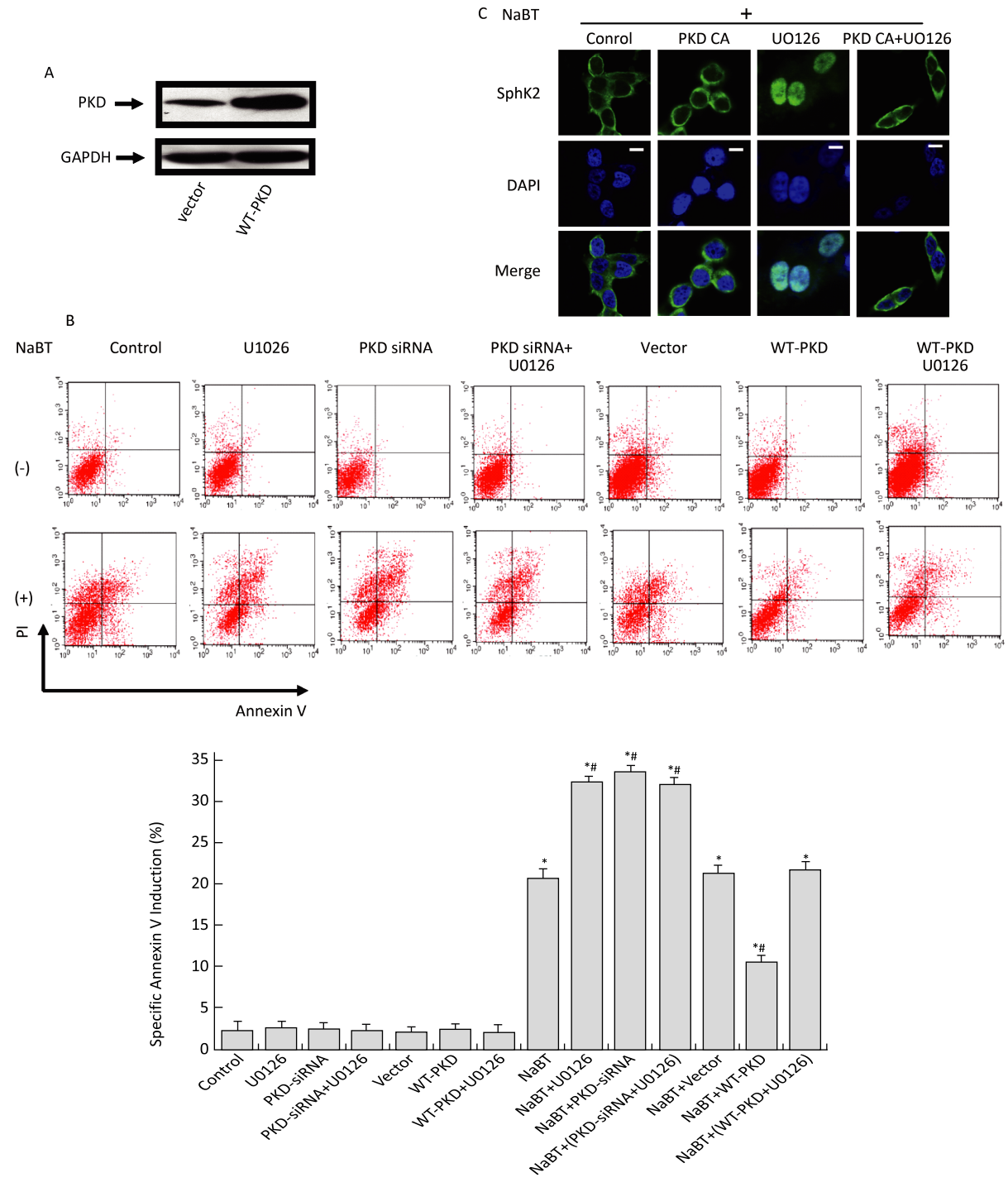

Figure 3. Effect of PKD over-expression, PKD-siRNA, and U0126 on apoptosis of HCT116 cells exposed to NaBT. Western blot analysis showed the efficiency of wild-type PKD expression in the cells (A). The cells were treated with U0126, PKD-siRNA, and wild-type PKD transfection separately or two in combination. Twenty-four hours after the cells were exposed to NaBT (10 mmol/L), their apoptosis was analyzed and detected by flow cytometry (B). The bottom part of panel B indicates the percentages of cells positive for Annexin V. Data are expressed as mean±SD of 3 independent experiments. $^{*}P<0.05$ *vs.* the control, $^{\#}P<0.05$ *vs.* the group with only NaBT. After treatment with constitutively activated PKD plasmids (PKD CA) and U0126 (20 μmol/L), the cells were exposed to NaBT and the distribution of SphK2 was detected with immunofluoreseence staining by confocal microscopy (C). *Bars*, 10 μm. Data are the typical representatives of 3 independent experiments.

over-expression combined with U0126 did not change the spontaneous cell apoptosis level. U0126 significantly increased the level of NaBT-induced apoptosis of the cells, and down-regulation of PKD sensitized the cell apoptosis at a similar level, which was not further increased by combined U0126 and PKD siRNA. Over-expression of PKD even decreased the NaBT-induced apoptosis of HCT116 cells, which was reversed by U0126, indicating that PKD and ERK stayed in the same signal pathway to regulate NaBT-induced apoptosis of HCT116 cells. Furthermore, the cells were transfected with constitutively activated PKD plasmids (PKD CA) for 24 h and exposed to U0126 for 48 h, showing that continuous activation of PKD could reverse the blockage effect of U0126 on export of SphK2 from nuclei to cytoplasm in HCT116 cells (Figure 3C) and that ERK stayed upstream to PKD in this regulating pathway.

DISCUSSION

In the present study, ERK was involved in NaBT-induced apoptosis of HCT116 cells and ERK was situated in the same signal pathway as SphK2 in regulating apoptosis, as indicated by the activation (phosphorylation) of ERK shortly after NaBT treatment, and the blocked translocation of SphK2 and U0126-sensitized apoptosis of HCT116 cells in response to NaBT (Figure 1). Furthermore, ERK stayed upstream to SphK2 in regulating NaBT-induced apoptosis of the cells (Figure 2). We, therefore, propose for the first time that ERK negatively regulates NaBT-induced apoptosis of HCT116 cells. Our results are inconsistent with the previous findings[23-24] and consistent with the protection against apoptosis of human hepatoma (HepG2) cells[25] and leukemia cells[26], suggesting that the role of ERK in regulating apoptosis depends on the involved cell type or other conditions, such as inducer of apoptosis. ERK is an endogenous protein that negatively regulates the apoptosis of HCT116 cells exposed to NaBT.

It has been recently reported that both SphK2 and PKD down-regulate the reactivity of HCT116 cells to NaBT-induced apoptosis and PKD stays upstream to SphK2 for activating and mediating translocation of SphK2[19]. In the present study, the same intensity of potentiated apoptosis was achieved using either U0126, siRNA of PKD or their combination following NaBT treatment. In addition, U0126 could reverse the suppressed apoptosis of HCT116 cells due to over-expressed PKD (Figure 3B), indicating that ERK and PKD also stayed in the same signal pathway in regulating NaBT-induced apoptosis of the cells. As evidenced by the reversal of U0126-blocked export of SphK2 by continuous activation of PKD in HCT116 cells (Figure 3C), ERK may stay upstream to PKD in regulating cell apoptosis, suggesting that the signalling sequence subsequent to NaBT exposure may start from the activation of ERK, followed by activation of PKD, and finally SphK2 is activated and thus exported from the nuclei to the cytoplasm, leading to suppression of apoptosis (Figure 4).

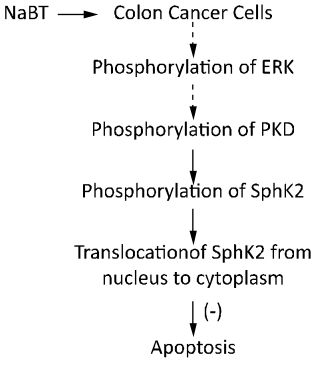

Figure 4. Proposed signal transduction pathway for negatively regulating NaBT-induced apoptosis of HCT116 cells.

Since ERK, PKD, and SphK2 are endogenous proteins that negatively regulate the apoptosis of colon cancer cells in response to NaBT, they can be considered as the potential therapeutic drug-acting targets for sensitizing the cancer cells to NaBT. It is, therefore, very important to further investigate their expression levels in colon cancer cells and the relation with their sensitivity to physiologic anti-cancer agents and chemotherapies. It is also interesting to understand whether the role of the above proteins is specific to some anti-cancer agents. Further study is needed to clarify these questions, which may potentially lead to new medications for sensitizing colon cancer cells to NaBT and other heat therapies for colon cancer.

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