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Protocatechualdehyde possesses anti-cancer activity through downregulating cyclin D1 and HDAC2 in human colorectal cancer cells

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ABSTRACT

Protocatechualdehyde (PCA) is a naturally occurring polyphenol found in barley, green cavendish bananas, and grapevine leaves. Although a few studies reported growth-inhibitory activity of PCA in breast and leukemia cancer cells, the underlying mechanisms are still poorly understood. Thus, we performed *in vitro* study to investigate if treatment of PCA affects cell proliferation and apoptosis in human colorectal cancer cells and define potential mechanisms by which PCA mediates growth arrest and apoptosis of cancer cells. Exposure of PCA to human colorectal cancer cells (HCT116 and SW480 cells) suppressed cell growth and induced apoptosis in dose-dependent manner. PCA decreased cyclin D1 expression in protein and mRNA level and suppressed luciferase activity of *cyclin D1* promoter, indicating transcriptional downregulation of *cyclin D1* gene by PCA. We also observed that PCA treatment attenuated enzyme activity of histone deacetylase (HDAC) and reduced expression of HDAC2, but not HDAC1. These findings suggest that cell growth inhibition and apoptosis by PCA may be a result of HDAC2-mediated cyclin D1 suppression.

1. Introduction

Colorectal cancer is the third leading cause of cancer mortality in both male and female in the United States [1]. Although the most effective treatment of CRC is surgery and adjuvant chemotherapy, chemoprevention using dietary factors has received attention as an effective approach to reduce incidence and malignancy of colorectal cancer. For last two decades, there have been numerous studies for testing anti-tumorigenic activities of natural products in several fruits, vegetables, and plants using various types of cancer [2]. Especially, polyphenols in the diet have been extensively studied due to their abilities to reduce malignancies of colorectal cancer [3].

Protocatechualdehyde (PCA) is a polyphenol (3, 4-dihydroxybenzaldehyde) found in barley [4], green cavendish bananas [5] and grapevine leaves [6]. It has been reported that PCA has antiatherosclerosis [7], anti-oxidant and anti-inflammatory effects [8]. Regarding anti-cancer activity, PCA decreased proliferation of human breast cancer [9] and induced apoptosis with inhibition of casein kinase II activity in leukemia cells [10]. However, the potential anti-cancer mechanisms of PCA have not been elucidated and the effects of PCA in human colorectal cancer have not been tested so far.

Histone acetylation and deacetylation are regulated by histone acetylase (HAT) and histone deacetylase (HDAC), respectively, and they play a significant role in regulating eukaryotic gene expression. Aberrant histone acetylation by enhanced HDAC is prevalent in many types of cancer and causes transcriptional repression of many cancer-related genes [11]. So, HDAC inhibitors have been developed clinically for the purpose of reducing malignancies [12]. Among HDAC families, HDAC2 is highly overexpressed in colorectal cancer [13] and HDAC2 expression is positively associated with β-catenin signaling [13]. In addition, HDAC2 elevates expressions of cyclin D1 and cyclin-dependent kinase (CDK)4, resulting in increase of cell proliferation [14–17]. Cyclin D1 regulates the transition from G1 to S phase by forming the complex with CDK4 and 6 [18] and overexpression of cyclin D1 protein was detected in 68.3% of CRC cases [19]. Thus, deregulation of cyclin D1 proteins correlates with colorectal tumorigenesis and improves the outcome in CRC patients [20]. HDAC inhibitors show anti-proliferative activity and induce cancer cell growth arrest and apoptosis through inhibition of transcriptional activation of cell cycle regulatory genes [21,22]. Especially, it has been reported that HDAC2 inhibition attenuates the expression of cell cycle regulatory genes such as cyclin D1 and CDK4, resulting in cell growth inhibition and apoptosis in myeloma cells [23], gastric cancer cells [24], and stromal sarcoma cells [25]. Therefore, it is accepted that the control of HDAC2-mediated cyclin D1 expression may provide promising cancer preventive and therapeutic way.

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In light of the therapeutic potential of PCA in colorectal cancer, this study was performed to elucidate the biological mechanism by which PCA induces the inhibition of cell growth and apoptosis in human colorectal cancer cells. Here, for the first time, we report that PCA leads to transcriptional downregulation of *cyclin D1* gene which may be associated with the decrease of HDAC2 expression and enzyme activity in human colorectal cancer cells.

2. Materials and methods

2.1. Materials

Cell culture media was purchased from Thermo Scientific (Rockford, IL), and protocatechualdehyde (PCA) was purchased from Sigma Aldrich (St. Louis, MO). Antibodies against cyclin D1, CDK4, CDK6, HDAC1 and 2, PARP, and β-actin were purchased from Cell Signaling (Danvers, MA). The luciferase construct containing −1745 to +134 of human *cyclin D1* promoter was kindly provided by Dr. Pestell (Thomas Jefferson University). All chemicals were purchased from Fisher Scientific, unless otherwise specified.

2.2. Cell culture and treatment

Human colorectal cancer cells, HCT116 and SW480 were purchased from American Type Culture Collection (Manassas, VA) and grown in DMEM/F-12 1:1 Modified medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were incubated at 37 °C under a humidified atmosphere of 5% CO_2. PCA was dissolved in dimethyl sulfoxide (DMSO) and treated to cells. The final DMSO concentration was not exceeded 0.1% (v/v).

2.3. Cell proliferation assay

Cell growth was measured using the Cell Proliferation Assay system (Promega, Madison, WI). Briefly, HCT116 (1000 cells/well) and SW480 (3000 cells/well) cells were seeded in 96-well plates and incubated overnight. The cells were treated with 0, 50, 100 and 200 μM of PCA in media containing 1% FBS for 0, 24 and 48 h. Then, the cells were incubated with 20 μL of Cell Titer96 Aqueous One solution for 3 h at 37 °C under 5% CO_2, and absorbance was recorded at 490 nm in an enzyme-linked immunosorbent assay plate reader (Bio-Tek Instruments Inc, Winooski, VT).

2.4. Cyclin D1 promoter activity

Transient transfection for *cyclin D1* promoter was performed using PolyJet DNA transfection reagent (SignaGen Laboratories, Ijamsville, MD) according to the manufacturer's instruction. Briefly, HCT116 and SW480 cells (2×10^5 cells/well) were seeded in 12-well plate. After 24 h, plasmid mixture containing 1 μg of *cyclin D1* promoter containing −1745 to +134 of human *cyclin D1* promoter region and 0.1 μg of *pRL-null* vector was transfected for 24 h. After transfection, the cells were treated with 0, 100 and 200 μM of PCA for 48 h and then harvested in 1× luciferase lysis buffer. Then the luciferase activity was normalized to the *pRL-null* luciferase activity using a dual-luciferase assay kit (Promega, Madison, WI).

2.5. HDAC activity assay

HDAC activity was performed using EpiQuik™ HDAC activity/Inhibition Assay Kit (Epigentek, Farmingdale, NY) according to the manufacturer's instruction. Briefly, HCT116 and SW480 cells were seeded in 6-well plate. After 24 h, cells were treated with 0, 100 and 200 μM of PCA for 48 h. After PCA treatment, the nucleus was prepared using Nuclear Extract Kit (Active Motif, Carlsbad, CA). Briefly, the cells were washed with ice-cold PBS containing phosphatase inhibitors, re-suspended with hypotonic buffer and detergent, and then centrifuged to obtain cytosolic fraction. Nuclear fractions were collected by suspending nuclear pellet with lysis buffer and centrifugation. Biotinylated HDAC substrate was added to 8-well strip plate for 45 min. After washing, the mixtures containing nuclear extracts and HDAC assay buffer were added to the plate. After 1 h, the plate was washed and the capture antibody was added for 1 h. After adding detection antibody and developing solution, the absorbance was recorded at 450 nm in an enzyme-linked immunosorbent assay plate reader (Bio-Tek Instruments Inc.) after adding stop solution.

2.6. Cell death assay

Cell death was performed using Cell Death Detection ELISA^{PLUS} Kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instruction. Briefly, HCT116 and SW480 cells were seeded in 6-well plate. After 24 h, cells were treated with 0, 100 and 200 μM of PCA for 48 h. After the PCA treatment, the cytosol was prepared using Nuclear Extract Kit (Active Motif). Equal amounts of cytosolic extracts, immunoreagent containing anti-histone-biotin, and anti-DNA-POD were added to microplate well and incubated for 2 h under shaking. After washing, the ABTS solution was added to each well for 20 min and then the ABTS stop solution was added. The absorbance was recorded at 405 nm and 490 nm in an enzyme-linked immunosorbent assay plate reader (Bio-Tek Instruments Inc.).

2.7. Western blot

Cells were washed with 1× phosphate-buffered saline (PBS), and lyzed in radio immunoprecipitation assay (RIPA) buffer supplemented with mixture of protease and phosphatase inhibitors (Sigma Aldrich) and centrifuged at 12,000×*g* for 10 min at 4 °C. The protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) using a bovine serum albumin (BSA) as the standard. The proteins were separated on SDS–PAGE and transferred to the nitrocellulose membranes (Osmonics, Minnetonka, MN). The membranes were blocked for non-specific binding with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1 h at room temperature and then exposed to specific primary antibodies in 5% nonfat dry milk at 4 °C overnight. After washing with TBS-T, the blots were incubated with horse radish peroxidase (HRP)-conjugated immunoglobulin G (IgG) for 1 h at room temperature, and a chemiluminescence was detected with Pierce ECL Western blotting substrate (Thermo Scientific) and visualized by ChemiDoc MP Imaging system (Bio-Rad, Hercules, CA).

2.8. Isolation and analysis of RNA

The total RNA was extracted from cells using an RNeasy Mini Kit (Qiagen, Valencia, CA) and the total RNA (1 μg) was reverse-transcribed using Verso cDNA kit (Thermo Scientific, Pittsburgh, PA) according to the manufacturer's instruction for complementary DNA (cDNA) synthesis. Polymerase chain reaction (PCR) was performed for 28 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min using PCR Master Mix kit (Promega) with human primers for cyclin D1 and GAPDH as followed: cyclin D1: forward 5′-aactacctggaccgcttcct-3′ and reverse 5′-ccacttgagcttgttcacca-3′, GAPDH: forward 5′-acccagaagactgtggatgg-3′ and reverse 5′-ttctagacggcaggtcaggt-3′.

2.9. Statistical analysis

A statistical analysis was performed with the Student's unpaired t-test, with statistical significance set at $^{*}P < 0.05$.

3. Results

3.1. Inhibitory effect of PCA on the growth of HCT116 and SW480 cells

To investigate whether PCA affect proliferation of human colorectal cancer cells, HCT116 (APC wild type) and SW480 (APC mutant) were treated with PCA for 0, 24 and 48 h and the cell proliferation was measured. As shown in Fig. 1A, HCT116 cells treated with 50, 100, and 200 μM of PCA reduced the cell growth by 21%, 30% and 41% in 24 h and 31%, 67% and 71% in 48 h, respectively. And SW480 cells treated with 50, 100, and 200 μM of PCA reduced the cell growth by 21%, 36% and 43% in 24 h and 24%, 44% and 58% in 48 h, respectively (Fig. 1B). The results indicate that PCA treatment suppressed cell growth of human colorectal cancer cells in dose- and time-dependent manner and anti-proliferative activity of PCA is APC-independent. Because the prominent inhibition of cell growth was observed at 48 h after PCA treatment, further study was performed by incubating the cells with PCA for 48 h.

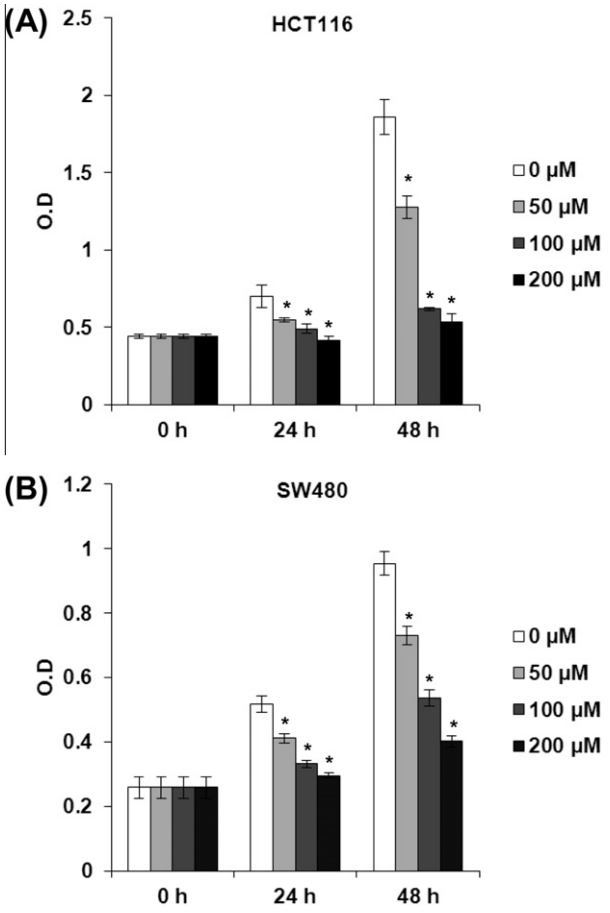

Fig. 1. The inhibitory effect of PCA on cell growth in human colorectal cancer cells. HCT116 (A) and SW480 (B) were treated with 0, 50,100 and 200 μM of PCA in 1% FBS containing media for 0, 24 and 48 h. Cell growth was measured using CellTiter96 Aqueous One Solution Cell Proliferation Assay Kit and expressed as absorbance (A_{490}). $^{*}P < 0.05$ compared to DMSO-treated cells.

3.2. Inhibitory effect of PCA on cyclin D1 expression

Cell growth is regulated by cell cycle regulatory proteins, cyclins and CDKs. Cyclin D1, one of the G1 cyclins, forms a complex with CDK4 and CDK6, resulting in phosphorylation of retinoblastoma (Rb) for G1/S transition [26]. To investigate whether PCA modulates cyclin D1 protein level, Western blot was performed. As shown in Fig. 2A and B, PCA treatment attenuated cyclin D1 levels in a dose-dependent manner in HCT116 and SW480 cells treated with 100 and 200 μM for 48 h. To test if a decrease of cyclin D1 expression is associated with transcriptional downregulation of *cyclin D1* gene, we examined mRNA level of cyclin D1 using RT-PCR after treatment with PCA for 48 h. As shown in Fig. 2C and D, mRNA level of cyclin D1 was decreased in HCT116 and SW480 cells treated with PCA. Then, the *cyclin D1* promoter linked to the luciferase reporter was transfected into HCT116 and SW480 cells, and then the cells were treated with PCA for 48 h and luciferase activity was measured. As a result, cyclin D1 promoter activity was significantly inhibited by PCA treatment in dose-dependent manner in both cells (Fig. 2E and F). These results indicate that PCA-induced decrease of cyclin D1 expression is associated with transcriptional downregulation of *cyclin D1* gene in human colorectal cancer cells. In addition, PCA inhibited CDK4 expression in both HCT116 and SW480 cells, while CDK6 expression was inhibited by the PCA in HCT116 cells alone (Fig. 2G and H).

3.3. Effect of PCA on histone deacetylase (HDAC) protein level and HDAC activity

There is growing evidences that HDAC inhibition can reduce the cyclin D1 expression through the suppression of cyclin D1 transcriptional activity [27]. To investigate whether the PCA-mediated inhibition of cyclin D1 expression is associated with HDAC inhibition, Western blot for HDAC and analysis of enzyme activity for HDAC were performed. As shown in Fig. 3A and B, the PCA significantly decreased HDAC2 protein levels, but not HDAC1 in the HCT116 and SW480 cells. We also confirmed that the PCA affects the enzyme activity of HDAC and observed that PCA treatment resulted in inhibition of HDAC activity in dose-dependent manner (Fig. 3C and D). These results indicate that PCA may play a role as the potential novel HDAC inhibitor, and the inhibition of cyclin D1 expression by PCA may be mediated by the decrease of HDAC2 protein level and HDAC activity in APC-independent manner in human colorectal cancer cells.

3.4. Induction of apoptosis by PCA

It has been reported that HDAC2 attenuates apoptosis in pancreatic cancer cells [28], and HDAC2 knockdown enhances the apoptosis in several types of cancer including cervical cancer, breast cancer and colorectal cancer cells [29]. To determine whether the decrease of HDAC2 by PCA affects apoptosis in HCT116 and SW480 cells, cells were treated with 0, 100 and 200 μM of PCA for 48 h, and apoptosis-related cleaved poly (ADP-ribose) polymerase (PARP) and the cell death assay were performed by Western blot and ELISA-based cell death assay, respectively. As shown in Fig. 4A and B, the PARP cleavage was dramatically increased in HCT116 and SW480 cells treated with 100 and 200 μM of PCA. In addition, significant increases of cell death were observed in the cells treated with PCA (Fig. 4C and D) in a dose-dependent manner. These results indicate that PCA may induce apoptosis through the decrease of HDAC2 protein level in human colorectal cancer cells.

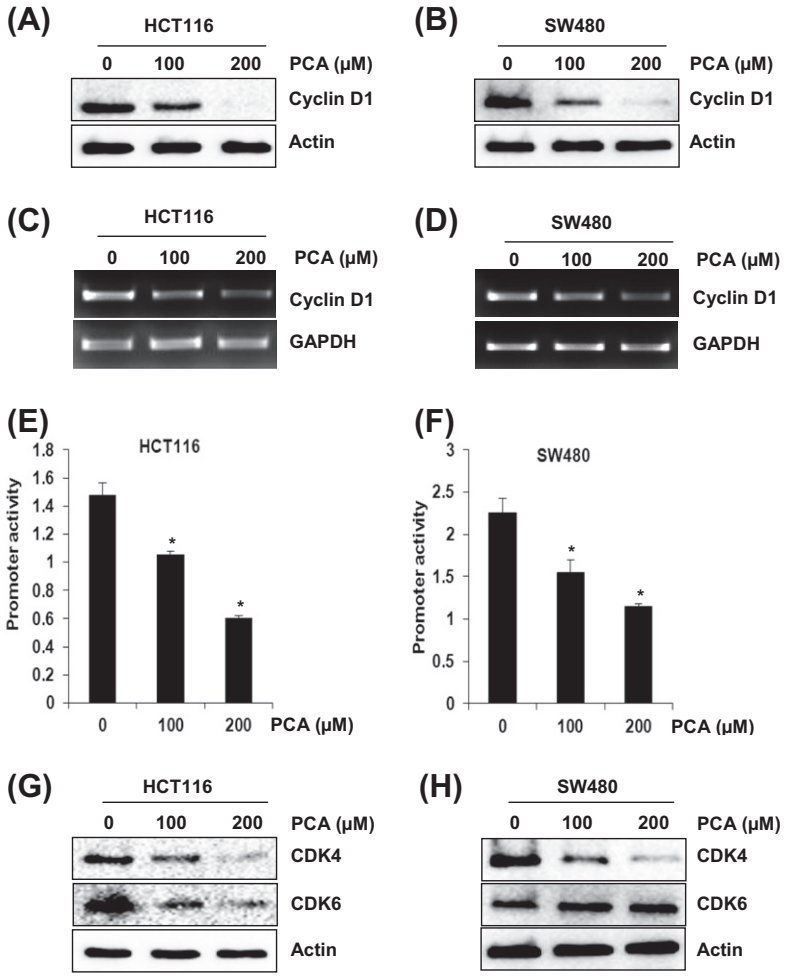

Fig. 2. The inhibitory effect of PCA on expression of cyclin D1 and CDK4 in human colorectal cancer cells. For Western blot (A, B, G, H), HCT116 and SW480 cells were treated with 0, 100 or 200 μM of PCA for 48 h. Cell lysates were subjected to SDS–PAGE and the Western blot was performed using antibodies against cyclin D1, CDK4, CDK6 and actin. For RT-PCR analysis of cyclin D1 gene expression (C, D), total RNA was prepared after PCA treatment for 48 h. Actin and GAPDH were used as internal control for Western blot and RT-PCR, respectively. For *cyclin D1* promoter activity (E, F), luciferase construct containing −1745 to +134 of human *cyclin D1* promoter region was co-transfected with *pRL-null* vector and the cells were treated with PCA for 48 h and luciferase activity was measured. $^{*}P < 0.05$ compared to cells without PCA treatment.

4. Discussion

It has been reported that naturally occurring polyphenols such as epicatechins, quercetin, and resveratrol have chemopreventive activities in humancolorectal cancer cells [3]. Like other polyphenols, protocatechualdehyde (PCA) showed anti-cancer activity [9,10]. However, the biological mechanism by which PCA exerts anti-cancer effect has not been elucidated. In this study, we, for the first time, report that PCA leads to the inhibition of cell growth and the promotion of apoptosis through HDAC2-mediated cyclin D1 suppression in human colorectal cancer cells.

Cyclin D1 is a protein regulating G1- to S-phase progression in cell cycle by forming active complex with cyclin-dependent kinase (CDK)4 and CDK6 [18]. Cyclin D1 is commonly overexpressed in human colorectal cancers and the cyclin D1 overexpression is associated with a prolonged survival in human colorectal cancer cells [30]. Therefore, it is estimated that cyclin D1 may be an important target of colorectal cancer prevention. In this study, PCA inhibited the cell growth and the cyclin D1 expression through transcriptional downregulation of *cyclin D1* gene in HCT116 and SW480 cells. It also suppressed the expression of CDK4. These results indicate that PCA-mediated inhibition of the cell growth might result from cell cycle arrest from G1 to S phase.

On the other hand, it has been reported that the expression of cyclin D1 and CDK4 is regulated by HDAC2 [14]. The HATs and HDACs dynamically regulate many genes associated with cellular proliferation and differentiation. Histone acetylation by HATs induces gene activation, while deacetylation by HDACs mediates transcriptional repression [31]. Therefore, it is regarded that the HDAC inhibition is a candidate target for cancer therapy by re-expressing genes. Colorectal cancer is associated with aberrant Wnt pathway due to the loss of the tumor suppressor adenomatosis polyposis coli (APC) and/or mutations in the β-catenin gene. In fact, loss of APC function induces HDAC2 expression and elevated HDAC2 levels are associated with poor prognosis and advanced stage [13]. Our data indicate that PCA attenuated HDAC2 protein levels and HDAC activities in HCT116 and SW480 cells in APC-independent manner. These results support that PCA may play a role as the potential novel HDAC inhibitor and the PCA-induced downregulation of cyclin D1 is mediated from modulation of HDAC2 level and HDAC activity.

In addition, it was reported that HDAC2 overexpression induces the inhibition of apoptosis in various cancer cells such as pancreatic cancer, cervical cancer, breast cancer, and colorectal cancer cells [28,29]. Especially, Schuler et al. [28] reported that knockdown of HDAC2 gene using siRNA enhanced apoptosis. In our

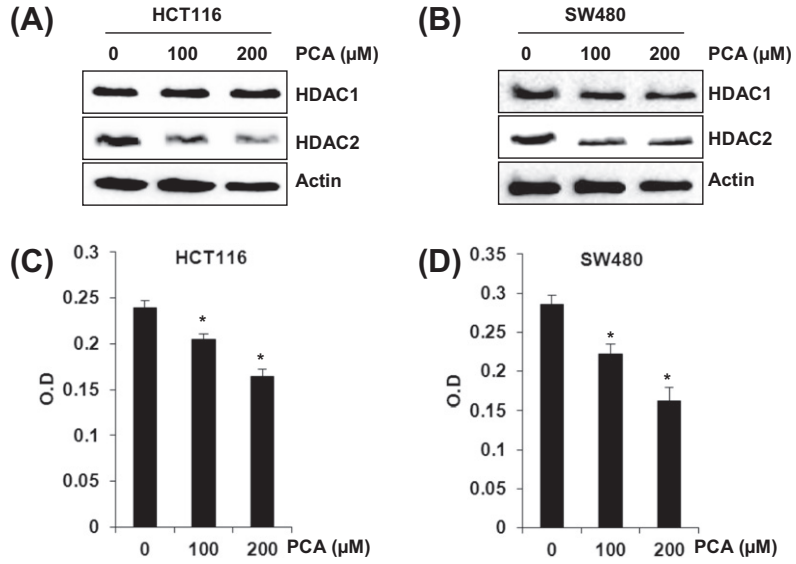

Fig. 3. The inhibitory effect of PCA on expression of HDAC2 (A, B) and enzyme activity of HDAC (C, D) in human colorectal cancer cells. HCT116 and SW480 cells were treated with 0, 100 or 200 μM of PCA for 48 h. Cell lysates were subjected to SDS–PAGE. Western blot was performed using antibodies against HDAC1, HDAC2, and actin. For HDAC activity, the nuclear fraction was extracted from the PCA-treated cells. The HDAC activity was performed using EpiQuik™ HDAC activity/Inhibition Assay Kit and expressed as absorbance (A_{450}). $^{*}P < 0.05$ compared to cells without PCA treatment.

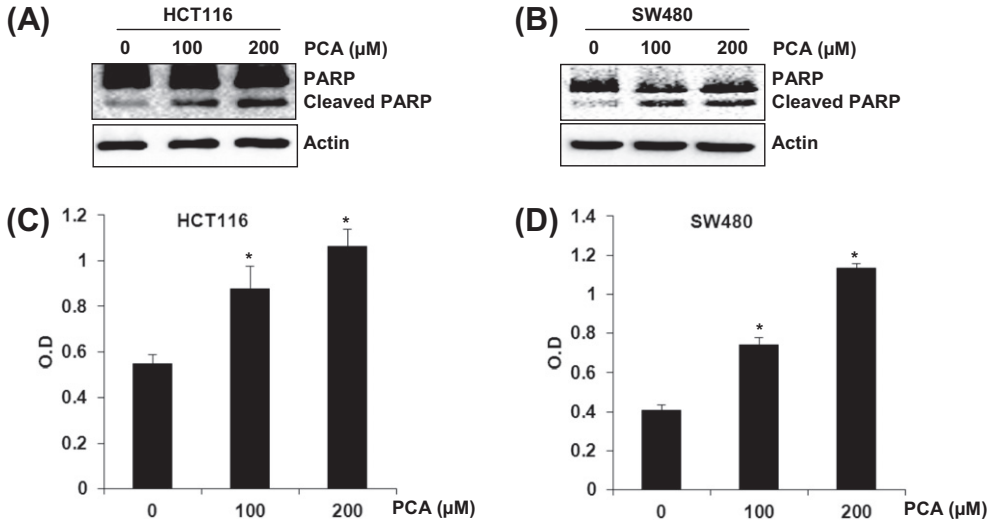

Fig. 4. The induction of apoptosis by PCA in human colorectal cancer cells. Apoptosis was determined using Western blot for Poly(ADP-ribose) Polymerase (PARP) (A, B) and ELISA was used for the cell death (C, D). HCT116 and SW480 cells were treated with 0, 100 or 200 μM of PCA for 48 h. Cell lysates were subjected to SDS–PAGE. The Western blot was performed using antibodies against PARP and actin. The proteins were then visualized using ECL detection. For the ELISA analysis of cell death, the cytosol fraction was extracted from PCA-treated cells, and the cell death was measured using the Cell Death Detection ELISA^{PLUS} Kit, and expressed as absorbance (A_{405}–A_{490}). $^{*}P < 0.05$ compared to cells without PCA treatment.

study, the PCA increased the PARP cleavage and induced the cell death in a dose-dependent manner in HCT116 and SW480 cells, indicating that increased apoptosis may be also mediated by suppression of HDAC2 in PCA-treated cells.

We do not exclude the possibility that decreased expression of cyclin D1 by PCA could be at least a partial consequence of proteasome degradation because the decrease of cyclin D1 protein is much more prominent than the decrease of mRNA and *cyclin D1* promoter activity (Fig. 2). In fact, cyclin D1 is a target of proteasomal degradation by various compounds including 6-gingerol [32] and curcumin [33].

In conclusion, PCA, a potential novel HDAC inhibitor may exert anti-cancer activity by downregulating cyclin D1 expression through modulation of HDAC2 and HDAC activity in human colorectal cancer cells.

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