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The p38 MAPK-regulated PKD1/CREB/Bcl-2 pathway contributes to selenite-induced colorectal cancer cell apoptosis *in vitro* and *in vivo*

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ABSTRACT

Supranutritional selenite has anti-cancer therapeutic effects *in vivo*; however, the detailed mechanisms underlying these effects are not clearly understood. Further studies would broaden our understanding of the anti-cancer effects of this compound and provide a theoretical basis for its clinical application. In this study, we primarily found that selenite exposure inhibited phosphorylation of cyclic adenosine monophosphate (cAMP)-response element binding protein (CREB), leading to suppression of Bcl-2 in HCT116 and SW480 colorectal cancer (CRC) cells. Moreover, the selenite-induced inhibitory effect on PKD1 activation was involved in suppression of the CREB signalling pathway. Additionally, we discovered that selenite treatment can upregulate p38 MAPK phosphorylation, which results in inhibition of the PKD1/CREB/Bcl-2 survival pathway and triggers apoptosis. Finally, we established a colorectal cancer xenograft model and found that selenite treatment markedly inhibits tumour growth through the MAPK/PKD1/CREB/Bcl-2 pathway *in vivo*. Our results demonstrated that a supranutritional dose of selenite induced CRC cell apoptosis through inhibition of the PKD1/CREB/Bcl-2 axis both *in vitro* and *in vivo*.

Introduction

The essential trace element selenium (Se) is involved in several physiological processes of the body and has been applied to cancer prevention and treatment [1,2]. Selenite compounds induce apoptosis, an important cellular event that accounts for the anti-cancer effects of selenite [3,4]. Evidence from several cancer cell lines has demonstrated that sodium selenite treatment leads to cell apoptosis [5–7].

The cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) is necessary for the proliferation, growth, survival, and differentiation of many cell types [8]. Recent research has demonstrated that CREB regulates the expression of a repertoire of genes associated with cell survival, such as Bcl-2, Bcl-xL, c-fos and tumour necrosis factor-α (TNF-α) [9–12]. Furthermore, the selenite-induced inhibition of CREB-Ser133 phosphorylation results in the suppression of anti-apoptotic genes, which induces cell death [13–15]. Numerous reports have proposed that CREB is regulated by multiple protein kinases, such as extracellular signal-regulated kinase (ERK), protein kinase D1 (PKD1) and mitogen- and stress-activated protein kinase (MSK) [16–19]. Clarifying the regulatory network of CREB would greatly contribute to the discovery of novel molecular mechanisms controlling apoptosis and would provide a broader significance for future clinical applications of selenium.

In the present study, we discovered that selenite down-regulates the PKD1/CREB/Bcl-2 axis and induces apoptosis in CRC cells. *In vivo* experiments confirmed these therapeutic effects of selenite and the alterations of these proteins that were observed *in vitro*. Together, these experiments indicate that selenite has potential for future clinical applications.

Materials and methods

Cell lines and culture

HCT116 and SW480 colorectal cancer cells were maintained in DMEM (Invitrogen, Paisley, Scotland) supplemented with 10% foetal bovine serum (FBS; Hyclone, Logan, UT, USA) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) in a humidified 5% CO_2 atmosphere at 37 °C. All of the cell cultures were discarded after 2 months, and new cell lines were propagated from frozen stocks.

Reagents and antibodies

Sodium selenite was purchased from Sigma-Aldrich (St. Louis, MO, USA) and a 10 μM working solution was used in this study. SB203580, phorbol-12-myristate-13-acetate (PMA), and CID755673 were purchased from Merck Calbiochem (San Diego, CA, USA). The CBP–CREB interaction inhibitor was purchased from MED Chemicals (San Diego, CA, USA). The pCF-CREB, p-CDNA3-FLAG-p38, and p-CDNA3-FLAG-p38DN vectors were obtained from Addgene (www.addgene.org), and the pEGFP-

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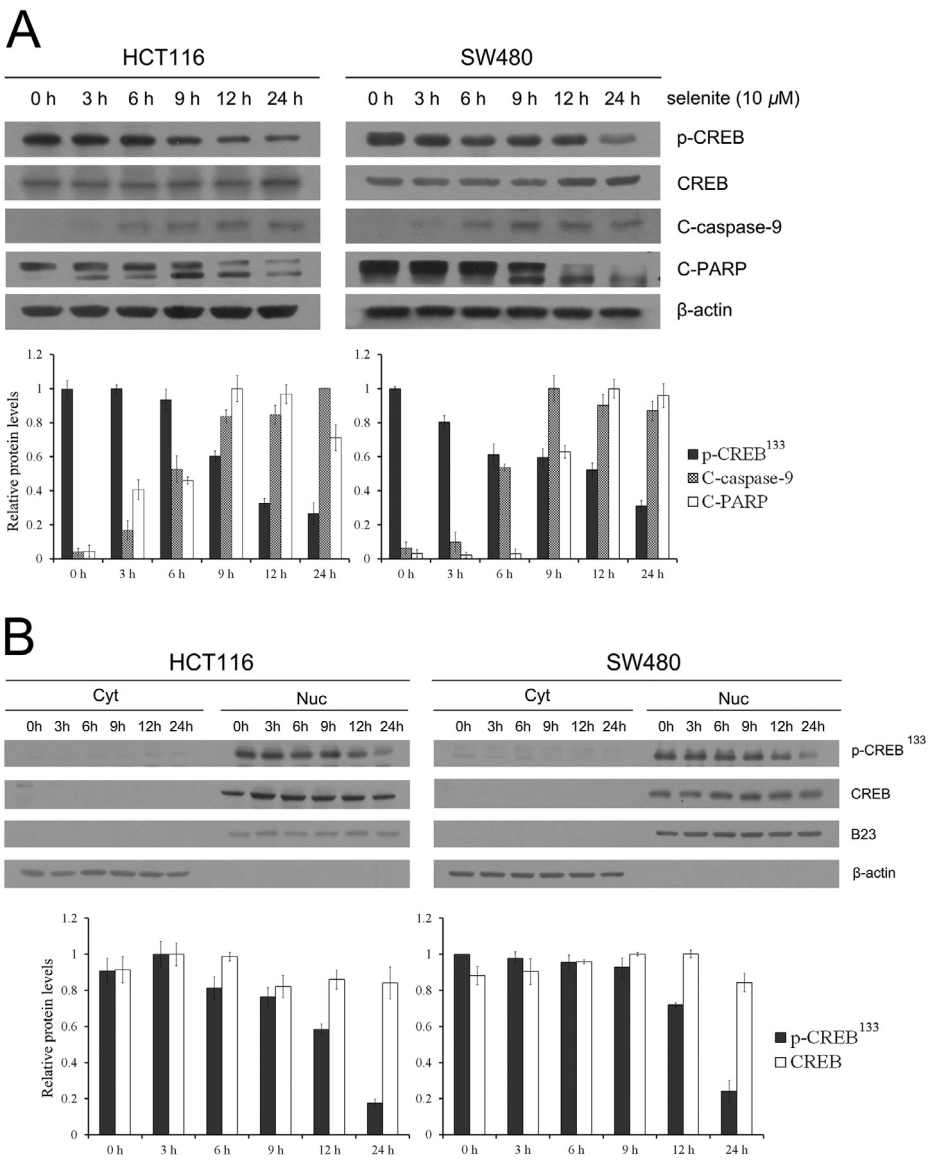
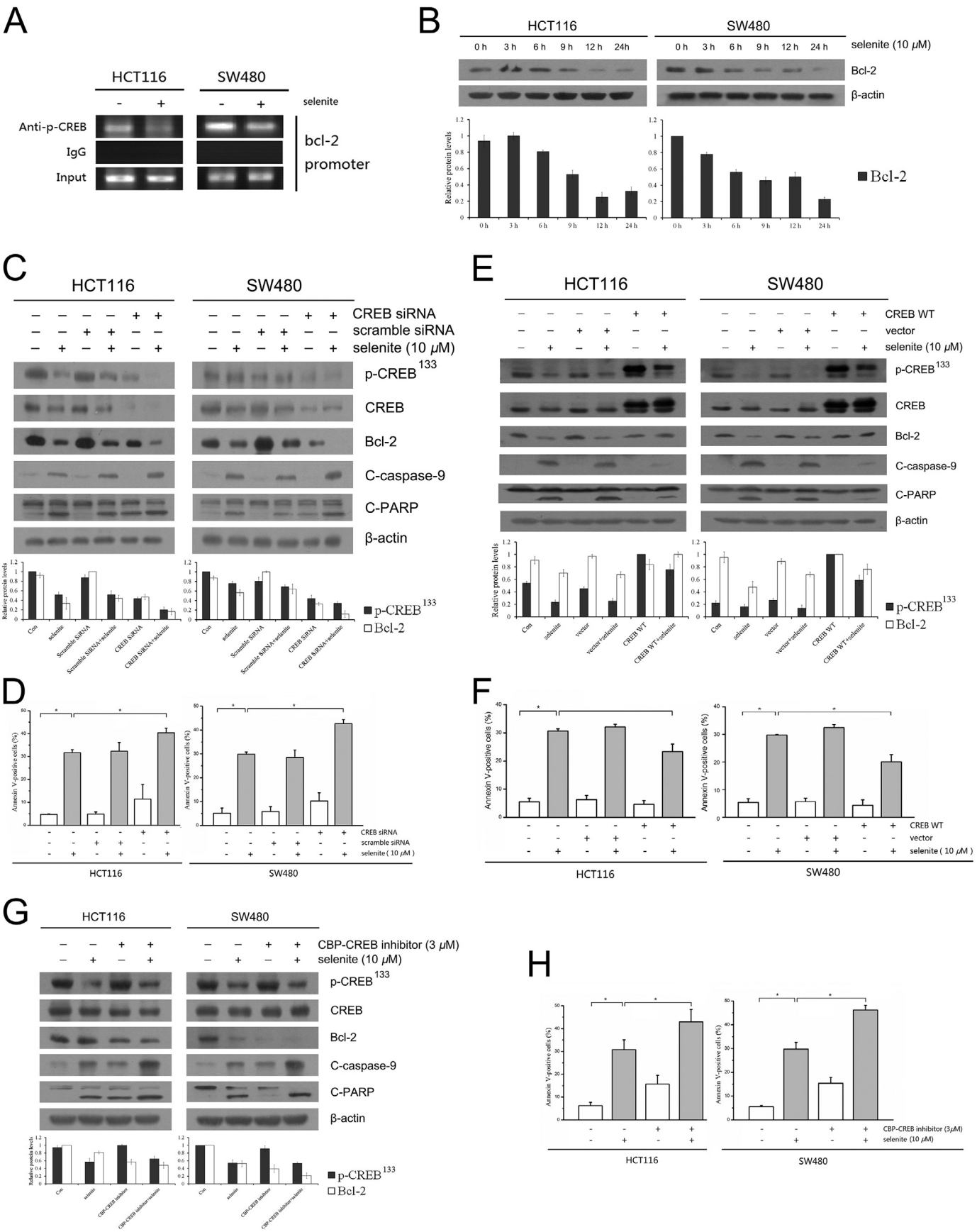


Fig. 1. Sodium selenite inhibits CREB signalling in CRC cells. (A) Sodium selenite inhibited CREB phosphorylation and induced apoptosis in CRC cells. HCT116 and SW480 cells were treated with selenite (10 μM) for the time points indicated. CREB, p-CREB and apoptosis markers were detected by western blot and quantified by pixel densitometry. (B) Selenite treatment specifically decreased p-CREB in the nucleus of CRC cells. The nuclear and cytoplasmic fractions were prepared from selenite-treated and control cells, and p-CREB was detected by western blot and quantified by pixel densitometry. β-actin and B23 were used as loading controls for the cytoplasmic and nuclear fractions, respectively.

HA-PKD1 WT and pEGFP-HA-PKD1 KD vectors were a kind gift from Dr. Angelika Hausser. Antibodies recognising cleaved PARP, cleaved caspase-9, p-PKD1 (Ser744), p-PKD1 (Ser916), PKD1, p-p38 MAPK, HA-tag and FLAG-tag were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies recognising p-CREB (Sre133) and CREB were obtained from ExCell Biology (Shanghai, China). Antibodies against B23, Bcl-2 and p38 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For immunohistochemical staining, an anti-p-PKD1 (Ser916) antibody was purchased from Abcam (Cambridge, UK), and the antibody against PKD1 was obtained from Santa Cruz Biotechnology. The antibody against p-PKD1 (Ser916) used for immunofluorescence was purchased from GeneTex (GeneTex, CA, USA).

Fig. 2. Selenite suppresses CREB phosphorylation and subsequently reduces Bcl-2 expression. (A) Selenite promotes p-CREB binding to the Bcl-2 promoter. CRC cells were treated with or without selenite (10 μM) for 24 h, and CREB-DNA binding was then analysed with a ChIP assay. The transcriptional level of β-actin was detected as input. (B) Selenite decreased Bcl-2 expression in both HCT116 and SW480 CRC cells. The western blot assay showed that selenite decreased Bcl-2 expression in both HCT116 and SW480 CRC cells. (C) CREB knockdown decreased the Bcl-2 levels. The cells were transfected with a siRNA targeting CREB and then treated with 10 μM selenite for 24 h. Next, the Bcl-2, CREB and apoptosis markers levels were detected by western blot and quantified by pixel densitometry. (D) CREB knockdown enhanced the degree of apoptosis induced by selenite. The cells were treated as described in (C), and the number of apoptotic cells was then determined by flow cytometry. The data are represented as the means ± SD (n = 3). $^{*}P < 0.05$. (E) The overexpression of constitutively active CREB reversed the selenite-induced apoptosis and decrease in Bcl-2 expression. After transfection with constitutive actively CREB plasmids, the cells were cultured for 24 h and then treated with 10 μM selenite for 24 h. The Bcl-2, CREB and apoptotic markers levels were detected by western blot and quantified by pixel densitometry. (F) The overexpression of constitutively active CREB reduced the selenite-induced apoptosis. The cells were treated as described in (E), and the apoptotic cells were then detected by flow cytometry. (G) Inhibition of CREB transcriptional activity further decreased the levels of Bcl-2 and enhanced the extent of selenite-induced apoptosis. The cells were pretreated with 3 mM CBP–CREB interaction inhibitor for 2 h and then treated with 10 μM selenite for 24 h. The Bcl-2, CREB and apoptosis marker levels were detected by western blot and quantified by pixel densitometry. (H) CREB inhibition further enhanced the degree of apoptosis induced by selenite. The cells were treated as described in (G), and the apoptotic cells were then detected by flow cytometry. The data are represented as the means ± SD (n = 3). $^{*}P < 0.05$.


A
HCT116
SW480
selenite
Anti-p-CREB
IgG
Input
bcl-2
promoter
B
HCT116
SW480
selenite (10 μM)
Bcl-2
β-actin
Relative protein levels
C
CREB siRNA
scramble siRNA
selenite (10 μM)
p-CREB133
CREB
Bcl-2
C-caspase-9
C-PARP
β-actin
E
CREB WT
vector
D
F
G
CBP-CREB inhibitor (3 μM)
H
Annexin V-positive cells (%)

Western blot assay

The total proteins were extracted and separated as described previously and then quantified by pixel densitometry using the ImageJ® software and expressed as means ± SD (n = 3) [7]. Specifically, the cytoplasmic and nuclear fractions were prepared according to the manufacturer's instructions for the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL, USA).

Transient transfection analysis

Approximately 4×10^5 cells were plated into six-well plate 1 day before the transfection experiments to allow for cell attachment and growth. The cells were transfected with the indicated plasmids using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, approximately 4 μg of each plasmid was transfected into cells together with 5 μl of Lipofectamine 2000 reagent per well, and the cells were treated as indicated 24 h later.

Small interfering RNAs

The p38 MAPK siRNA (5′-GGAAUUCAAUGAUGUGUA-3′) was chemically synthesised by GenePharm (Shanghai, China). The CREB siRNA (h) and PKD1 siRNA (h) were purchased from Santa Cruz Biotechnology. The cells were cultured in six-well plates before transfection with 100 pM siRNA using Lipofectamine 2000 according to the guidelines described earlier. The cells were then subjected to further treatment as indicated.

Co-immunoprecipitation

The cells were harvested and washed twice with ice-cold PBS and then lysed in RIPA buffer for 30 min. Then 200-μg aliquots of the lysates were incubated with the appropriate antibodies to immunoprecipitate the proteins of interest. The proteins bound to the antibodies were then captured with 25 μl of protein A + G agarose beads (Santa Cruz Biotechnology). After washing and elution, the immunoprecipitates were resuspended in western blot sample buffer and boiled for 10 min. The samples were then used for the western blot assays.

Immunofluorescence

The cells were grown on coverslips for 24 h before treatment with 10 μM selenite for 24 h. The cells were then fixed in Immunol staining fix solution (Beyotime, Haimen, Jiangsu, China) for 10 min. After washing with PBS three times for 5 min each, samples were blocked with Immunol staining blocking buffer (Beyotime) for 30 min at room temperature. Subsequently, the slides were incubated with the indicated primary antibodies overnight at 4 °C. The cells were then incubated with the appropriate secondary antibodies for 1 h at room temperature, followed by counterstaining with DAPI. Images were acquired on an Olympus laser scanning confocal FV1000 microscope (Olympus, Tokyo, Japan) and analysed using the Olympus Fluo View software.

Apoptosis assay

The percentage of cells undergoing apoptosis was determined with an Annexin V/PI double staining kit from Merck Calbiochem according to the manufacturer's instructions. The fluorescence was detected with an Accuri C6 flow cytometer (Accuri Cytometers Inc., Ann Arbor, MI, USA). We calculated the apoptotic ratio by calculating the sum of the Annexin V+/PI– cells ratios and the Annexin V+/PI+ cells' ratio. All of the experiments were repeated thrice independently, and the data are represented as the means ± SD (n = 3). $^*P < 0.05$.

ChIP assay

ChIP assays were performed using the Simple ChIP Enzymatic Chromatin IP Kit purchased from Cell Signaling Technology. Chromatin extracts were immunoprecipitated using an antibody against p-CREB and analysed by subsequent PCR with the following primers for β-actin and Bcl-2: β-actin forward, 5′-CATGTTTGAGACCTTCAACACCCC-3′; β-actin reverse, 5′-GCCATCTCTTGCTCGAAGTCTAG-3′; Bcl-2 forward, 5′-GCCCGACTTTGCAGAGATGTCCAG-3′; and Bcl-2 reverse, 5′-GCCATGCCGGTTCAGGTACTCAG-3′.

Xenograft tumour model

BALB/c (nu/nu) nude mice were individually caged in standard laboratory conditions and provided food and water *ad libitum*. Four-week-old female nude mice were injected with HCT116 and SW480 cells. After tumours were detectable, the mice were randomly divided into three groups (each group contained seven mice) and treated with PBS or selenite every 2 days (2 mg/kg/day or 1 mg/kg/day). After 3 weeks of this treatment, the mice were killed for analysis. The animals were maintained and tested according to the Guidelines for the Declaration of Helsinki and/or the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health.

Immunohistochemical staining

Portions of the tissue samples of the xenograft tumour model were homogenised and subjected to western blotting, and the remaining tissues were embedded for immunohistochemical analysis as described previously [20].

Statistical analysis

Each experiment was repeated at least thrice. For all of the quantitative analyses represented in the histograms, the values are expressed as the mean values ± SD. Two-tailed Student's t-tests were performed to compare two groups. $P < 0.05$ was considered significant.

Results

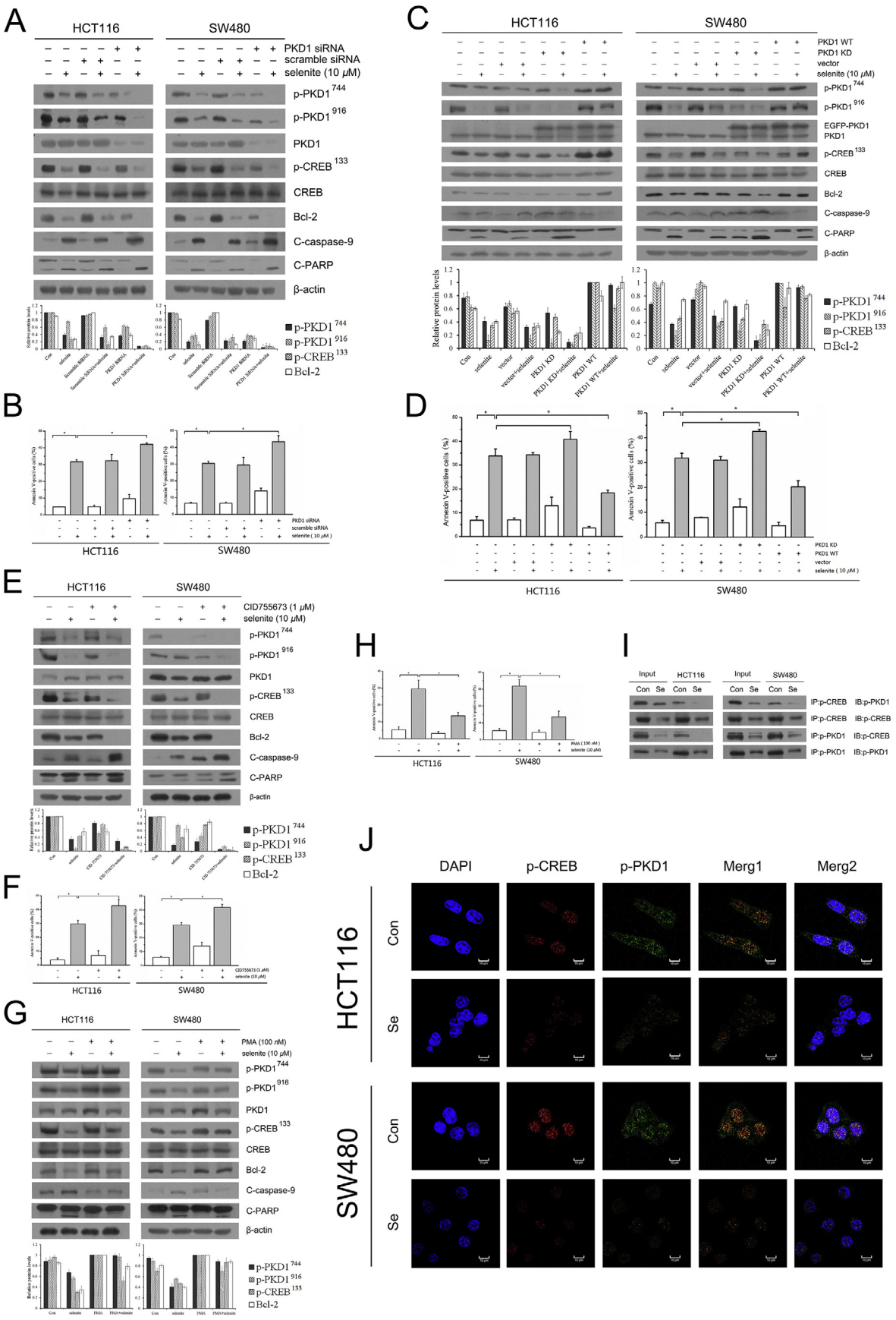
Selenite suppresses CREB phosphorylation and induces apoptosis in CRC cells

CREB is essential for the proliferation, growth and survival of various types of cancer cells. Phosphorylation of the Ser133 residue in CREB is necessary for the transcriptional activation of anti-apoptotic gene expression [10,15,21]. To elucidate the detailed molecular mechanism of selenite-induced apoptosis in CRC cells, we first investigated the effect of selenite on CREB in CRC cells. Our previous work indicated that a supranutritional dose of sodium selenite (10 μM) induces apoptosis in CRC cells. As shown in Fig. 1A, in the presence of 10 μM selenite, CREB phosphorylation at Ser133 decreased over time in both CRC cell lines tested, whereas PARP and caspase-9 cleavage increased. It is widely recognised that CREB is located in the nucleus as a transcription factor [14,16,22]. We extracted the nuclear fraction of selenite-treated CRC cells and discovered that 10 μM selenite treatment decreased CREB-Ser133 phosphorylation in the nucleus at 24 h without altering the total CREB levels (Fig. 1B). Taken together, these results demonstrate that selenite inhibits CREB phosphorylation in the nucleus.

Selenite suppresses CREB phosphorylation and subsequently reduces Bcl-2 expression

In the nucleus, activated CREB can bind to promoters containing a consensus sequence to enhance the transcription of various survival proteins including Bcl-2 [8,16,18,22]. Bcl-2 family proteins are essential regulators of selenite-induced apoptosis [23–25].

Fig. 3. Selenite-treatment suppressed CREB/Bcl-2 signalling by inhibiting PKD1 activity. (A, C and E) PKD1 inhibition decreased the levels of CREB phosphorylation and Bcl-2 expression and increased selenite-induced apoptosis. The cells were transfected with siRNA targeting PKD1 or a PKD1-overexpressing KD plasmid or pre-treated with 1 μM CID 755673 for 2 h. The cells were then treated with selenite (10 μM) for 24 h, and the PKD1, CREB, Bcl-2 and apoptosis marker levels were detected by western blot and quantified by pixel densitometry. (B, D and F) PKD1 inhibition further enhanced the degree of apoptosis induced by selenite. The cells were treated as described in (A, C and E), and the apoptotic cells were measured by flow cytometry. The data are represented as the means ± SD (n = 3). $^*P < 0.05$. (C and G) PKD1 activation increased CREB phosphorylation and Bcl-2 expression. The cells were transfected with PKD1 WT or pretreated with 100 nM PMA for 2 h. After the cells were treated with 10 μM selenite for 24 h, the PKD1, CREB, Bcl-2 and apoptosis marker levels were detected by western blot and quantified by pixel densitometry. (D and F) PKD1 activation reversed the selenite-induced apoptosis. The cells were treated as described in (C and G), and the apoptotic cells were measured by flow cytometry. The data are represented as the means ± SD (n = 3). $^*P < 0.05$. (I) PKD1 interacts with CREB. The cells were exposed to 10 μM selenite for 24 h, and co-immunoprecipitation was used to detect the interactions between PKD1 and CREB. (J) The co-localisation of PKD1 and CREB was reduced after selenite exposure. The cells were treated with 10 μM selenite for 24 h, and PKD1 and CREB were indirectly labelled with primary antibodies. The images were generated with a confocal microscope. Bar: 10 μm.


A
HCT116
SW480
PKD1 siRNA
scramble siRNA
selenite (10 μM)
p-PKD1 744
p-PKD1 916
PKD1
p-CREB 133
CREB
Bcl-2
C-caspase-9
C-PARP
β-actin
p-PKD1 744
p-PKD1 916
p-CREB 133
Bcl-2
B
HCT116
SW480
C
HCT116
SW480
PKD1 WT
PKD1 KD
vector
selenite (10 μM)
p-PKD1 744
p-PKD1 916
EGFP-PKD1
PKD1
p-CREB 133
CREB
Bcl-2
C-caspase-9
C-PARP
β-actin
Relative protein levels
p-PKD1 744
p-PKD1 916
p-CREB 133
Bcl-2
D
HCT116
SW480
E
HCT116
SW480
CID755673 (1 μM)
selenite (10 μM)
p-PKD1 744
p-PKD1 916
PKD1
p-CREB 133
CREB
Bcl-2
C-caspase-9
C-PARP
β-actin
p-PKD1 744
p-PKD1 916
p-CREB 133
Bcl-2
F
HCT116
SW480
G
HCT116
SW480
PMA (100 nM)
selenite (10 μM)
p-PKD1 744
p-PKD1 916
PKD1
p-CREB 133
CREB
Bcl-2
C-caspase-9
C-PARP
β-actin
p-PKD1 744
p-PKD1 916
p-CREB 133
Bcl-2
H
HCT116
SW480
I
Input
HCT116
Input
SW480
Con Se Con Se
Con Se Con Se
IP:p-CREB IB:p-PKD1
IP:p-CREB IB:p-CREB
IP:p-PKD1 IB:p-CREB
IP:p-PKD1 IB:p-PKD1
J
DAPI
p-CREB
p-PKD1
Merg1
Merg2
HCT116
Con
Se
SW480
Con
Se

Thus, we performed chromatin immunoprecipitation (ChIP) experiments to examine whether selenite treatment influences p-CREB binding to the Bcl-2 promoter and subsequently regulates Bcl-2 transcription. Indeed, as shown in Fig. 2A, selenite treatment in HCT116 and SW480 CRC cells reduced CREB binding to the Bcl-2 promoter, which may result from the decreased CREB phosphorylation levels observed previously. Accordingly, our western blot results indicated that selenite treatment suppressed Bcl-2 expression (Fig. 2B).

Next, following pre-treatment with a siRNA targeting CREB, the cells were exposed to selenite for 24 h. The western blot results indicated that the knockdown of CREB further reduced Bcl-2 expression while simultaneously increasing PARP and caspase-9 cleavage (Fig. 2C). An analysis of the apoptotic rate of these cells demonstrated that CREB suppression enhanced apoptosis in selenite-treated CRC cells (Fig. 2D, Supplementary Fig. 2D). On the contrary, when we over-expressed a constitutively active CREB, we observed that the selenite-induced Bcl-2 down-regulation was partially reversed, and the selenite-induced activation of PARP and caspase-9 was suppressed (Fig. 2E). The Annexin V/PI double staining results demonstrate that the selenite-induced apoptosis was suppressed after the overexpression of CREB (Fig. 2F, Supplementary Fig. 2F).

Finally, to fully determine the function of CREB in this system, we treated CRC cells with CREB binding protein (CBP)–CREB interaction inhibitor, and then exposed these cells to selenite for 24 h. The western blot results indicated that the inhibition of CREB transcriptional activity further resulted in enhanced selenite-induced inhibition of Bcl-2 expression and increased PARP and caspase-9 cleavage (Fig. 2G). The apoptotic rate of these cells, as detected with the Annexin V/PI double staining assay, demonstrated that CREB inhibition enhanced the amount of apoptosis of selenite-treated CRC cells (Fig. 2H, Supplementary Fig. 2H).

Selenite-induced inhibition of PKD1 activation is required for CREB signalling pathway suppression

A large body of evidence has indicated that activated PKD1 phosphorylates CREB at Ser133, which enhances its transcriptional activity [21,26,27]. To determine whether selenite regulates PKD1 activation and whether this effect could exert any impact on CREB signalling in CRC cells, we performed a series of experiments. First, we found that the knockdown of PKD1 by siRNA transfection, the over-expression of a dominant negative PKD1 (PKD1 kinase dead (KD)) or the administration of CID 755673 (a PKD1 inhibitor) inhibited CREB phosphorylation, which decreased p-CREB-dependent Bcl-2 expression and PARP and caspase-9 cleavage (Fig. 3A, C, E). The analysis of the apoptotic rate with the Annexin V/PI double staining assay demonstrated that PKD1 inhibition increased the apoptosis rate of selenite-treated CRC cells (Fig. 3B, D, F and Supplementary Fig. 3B, D, F).

In another set of experiments, we over-expressed wild-type PKD1 (PKD1WT) or treated cells with the PKD1 activator PMA. The western blot results demonstrated that both PKD1 WT over-expression and PMA treatment sharply increased the p-CREB and Bcl-2 levels even in the presence of selenite treatment, compared with selenite treatment alone (Fig. 3C, G). The analysis of the apoptotic rate with the Annexin V/PI double staining assay demonstrated that PKD1 activation or over-expression could partially rescue selenite-induced apoptosis (Fig. 3D, H and Supplementary Fig. 3D, H).

Next, to verify whether CREB is a PKD1 substrate, we performed a co-immunoprecipitation experiment. As shown in Fig. 3I, selenite treatment greatly decreased the association between p-PKD1 and p-CREB in CRC cells. This result suggested that the reduction in CREB phosphorylation may be partly attributable to the selenite-induced disruption of the association between PKD1 and CREB. Furthermore, our immunofluorescence results (Fig. 3J) support the conclusion earlier that selenite reduced CREB phosphorylation in the nucleus. Taken together, these results indicate that selenite inhibited CREB/Bcl-2 survival signalling via PKD1 activity in CRC cells.

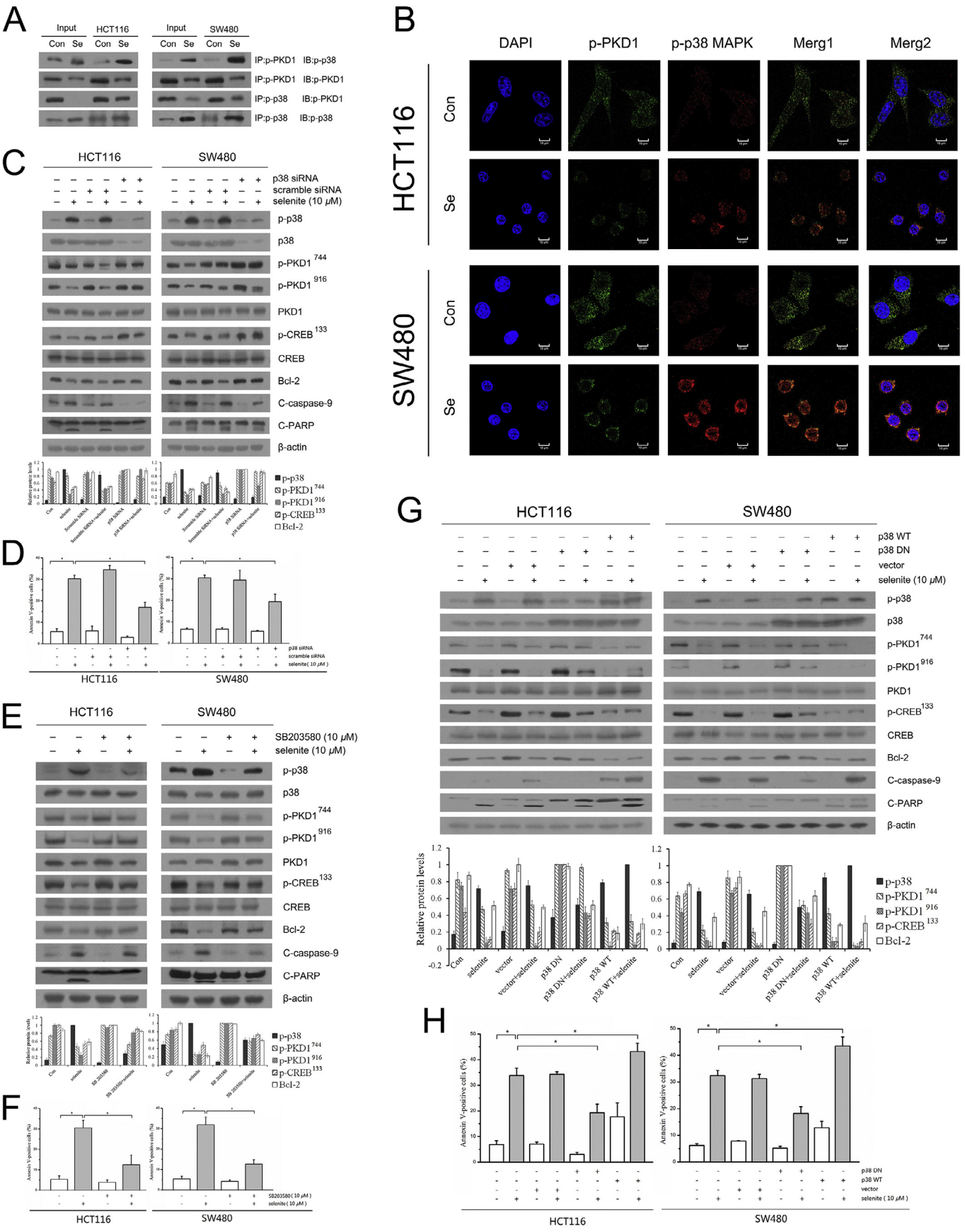
p38 MAPK inhibits the PKD1/CREB/Bcl-2 survival signalling pathway in selenite-treated CRC cells

We discovered that PKD1 plays an important role in regulating the CREB/Bcl-2 axis. We then determined which upstream signal causes PKDI inhibition. After screening several proteins, we found that p38 MAPK inhibits PKD1 activation in selenite-treated CRC cells. First, we validated the interaction between p38 MAPK and PKD1 by co-immunoprecipitation and found that this binding was enhanced after selenite exposure (Fig. 4A). In addition, the co-localisation of p38 MAPK and PKD1 was observed by immunofluorescence staining, which confirms the immunoprecipitation results (Fig. 4B).

To elucidate the role of p38 MAPK in the selenite-induced apoptosis in CRC cells, we pretreated CRC cells with siRNA targeting p38 MAPK or the p38 MAPK inhibitor SB203580 before exposing the cells to selenite for 24 h. Our western blot results showed that either the knockdown or inhibition of p38 MAPK reversed the selenite-mediated inhibition of the PKD1/CREB/Bcl-2 signalling pathway and resulted in decreased cleavage of PARP and caspase-9 (Fig. 4C, E). The analysis of the apoptotic rate using the Annexin V/PI double staining assay demonstrated that the knockdown or inhibition of p38 MAPK reversed the selenite-induced apoptosis in CRC cells (Fig. 4D, F and Supplementary Fig. 4D, F).

Finally, to fully demonstrate the function of p38 MAPK in this system, we over-expressed p38 MAPK WT or p38 MAPK domain negative (p38 MAPK DN) in CRC cells. We observed that p38 MAPK WT overexpression sharply enhanced the selenite-induced PKD1/CREB/Bcl-2 inhibition compared with selenite treatment alone. Simultaneously, p38 MAPK DN over-expression reversed the selenite-induced PKD1/CREB/Bcl-2 inhibition in CRC cells (Fig. 4G). We found that the selenite-induced apoptosis was suppressed upon p38 MAPK DN over-expression, whereas the selenite-induced apoptosis was further enhanced after p38 MAPK WT over-expression (Fig. 4H and Supplementary Fig. 4H). All of these data strongly suggest that p38 MAPK inhibits the PKD1/CREB/Bcl-2 survival signalling pathway in selenite-treated CRC cells.

Fig. 4. p38 MAPK induces apoptosis by inhibiting the PKD1/CREB/Bcl-2 survival signalling pathway in selenite-treated CRC cells. (A) p38 MAPK interacted with PKD1. The cells were exposed to 10 μM selenite for 24 h, and co-immunoprecipitation was used to detect the interactions between p38 MAPK and PKD1. (B) The co-localisation of p38 MAPK and PKD1. The cells were treated with 10 μM selenite for 24 h. p38 MAPK and PKD1 were indirectly labelled with primary antibodies. The images were generated with a confocal microscope. Bar: 10 μm. (C and E) Inhibition of p38 MAPK induced further inhibition of the PKD1/CREB/Bcl-2 axis inhibition. The cells were transfected with siRNA targeting p38 MAPK or pretreated with 10 μM SB203580 for 2 h. After the cells were treated with 10 μM selenite for 24 h, the p38 MAPK, PKD1, CREB, Bcl-2 and apoptosis marker levels were detected by western blot and quantified by pixel densitometry. (D and F) Inhibition of p38 MAPK reverses the selenite-induced apoptosis in CRC cells. The cells were treated as described in (C and E), and the apoptotic cells were measured by flow cytometry. The data are represented as the means ± SD (n = 3). $^{*}P < 0.05$. (G) p38 MAPK inhibits the PKD1/CREB/Bcl-2 signalling pathway. The cells were transfected with p38 MAPK WT or DN plasmids. After the cells were treated with 10 μM selenite for 24 h, the p38 MAPK, PKD1, CREB, Bcl-2 and apoptosis marker levels were detected by western blot and quantified by pixel densitometry. (H) p38 MAPK enhances the selenite-induced apoptosis in CRC cells. The cells were treated as described in (G), and the apoptotic cells were measured by flow cytometry. The data are represented as the means ± S.D (n = 3). $^{*}P < 0.05$.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Input
HCT116
Con Se Con Se
Input
SW480
Con Se Con Se
IP:p-PKD1 IB:p-p38
IP:p-PKD1 IB:p-PKD1
IP:p-p38 IB:p-PKD1
IP:p-p38 IB:p-p38
B
DAPI
p-PKD1
p-p38 MAPK
Merg1
Merg2
HCT116
SW480
Con
Se
C
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SW480
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scramble siRNA
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p38
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vector
selenite (10 μM)
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p-PKD1 916
PKD1
p-CREB 133
CREB
Bcl-2
C-caspase-9
C-PARP
β-actin
Relative protein levels
Con
selenite
vector
vector+selenite
p38 DN
p38 DN+selenite
p38 WT
p38 WT+selenite
p-p38
p-PKD1 744
p-PKD1 916
p-CREB 133
Bcl-2
H
HCT116
SW480
p38 DN
p38 WT
vector
selenite (10 μM)

The p38 MAPK/PKD1/CREB signalling pathway is regulated by selenite in vivo

After exploring the important role of the p38 MAPK/PKD1/CREB axis following selenite treatment *in vitro*, further experiments were conducted to investigate the effects of selenite *in vivo*. Four-week-old female nude mice were injected with HCT116 and SW480 cells. Once tumours were detectable, the mice were randomly divided into three groups and administered PBS or selenite (2 mg/kg/day or 1 mg/kg/day) through an intraperitoneal injection every 2 days. After treatment with selenite for 3 weeks, mice were killed for analysis. We discovered that selenite treatment markedly attenuated tumour growth compared with the control group without any adverse effects on body weight and activity (Fig. 5A). Tissues from the group treated with 2 mg/kg/day sodium selenite were processed for western blot and immunohistochemistry experiments.

To verify the *in vitro* results, we first performed an analysis of the tissues from both control and selenite-treated samples by western blot. The results revealed that selenite activated p38 MAPK and inhibited the PKD1/CREB/Bcl-2 survival signalling pathway, thereby inhibiting tumour growth (Fig. 5B and Supplementary Fig. 5B). Additionally, through a series of immunohistochemistry experiments, we examined the localisation patterns of critical molecules in this signalling pathway. We observed that p38 MAPK phosphorylation was increased and that PKD1/CREB phosphorylation and Bcl-2 expression were decreased in tissues from the group treated with a supranutritional dose of sodium selenite. In contrast, p38, PKD1 and CREB did not show any obvious changes (Fig. 5C).

Discussion

This study presents evidence that the p38 MAPK/PKD1/CREB/Bcl-2 signalling pathway is closely associated with selenite-induced apoptosis in CRC cells and xenograft tumours. A model depicting our findings is presented in Fig. 6. Briefly, selenite activates p38 MAPK, which further inhibits PKD1. As a result, CREB, the downstream mediator of PKD1, becomes inactivated and then inhibits the expression of anti-apoptotic Bcl-2. This discovery elucidates a mechanism through which selenite imparts anti-tumour activity.

Selenite induces apoptosis by regulating the ATF/CREB family of transcription factors [28]. CREB, a member of the ATF/CREB family, plays an important role in cancer cell survival, and the phosphorylation of residue Ser133 is involved in its transcriptional activity [19,29]. Our data demonstrated that CREB phosphorylation was suppressed in selenite-treated CRC cells, which indicated that selenite may induce apoptosis by inhibiting the transcriptional activity of CREB. By performing a ChIP assay, we found that p-CREB interacted with the Bcl-2 promoter in CRC cells and that selenite treatment disrupted this binding. Bcl-2 is widely known for its anti-apoptotic function in various cancer cell lines, and the inhibition of Bcl-2 expression is an effective method for cancer therapy [30]. Selenite induces apoptosis by inhibiting the expression of Bcl-2, although the mechanism is unclear [6,25,31]. Notably, our overexpression and knockdown results verified that CREB is involved in selenite-induced apoptosis through its regulation of Bcl-2 expression. CREB-binding protein (CBP) is an important co-activator of CREB and plays a role in the transcription of CREB-dependent genes [32,33]. To confirm the mechanism through which CREB regulates Bcl-2 expression, we pretreated CRC cells with CBP–CREB interaction inhibitor and then treated the cells with selenite. This experiment indicated that Bcl-2 expression required the transcriptional activity of CREB. Overall, selenite decreases the Bcl-2 expression by inhibiting the transcriptional activity of CREB in CRC cells. It has been reported that CREB regulates Bcl-2 expression in other cancer cells, such as non-small cell lung cancer (NSCLC) cell lines, MCF7 breast cancer cells and HeLa cell lines, which is consistent with our conclusion [8,13,34].

An increasing body of evidence suggests a role for PKD1 in proliferation and survival by activating CREB in various cancer cell lines [21,26]. However, whether PKD1 is involved in the regulation of CREB in selenite-treated CRC cells was unknown. In this study, we found that PKD1 reversed the selenite-induced reduction of p-CREB. Moreover, studies with CRC cells treated with selenite combined with CID 755673 (PKD1 inhibitor) or PMA (PKD1 activator) indicated that the PKD1-mediated phosphorylation of p-CREB required the kinase activity of PKD1. However, a previous report indicated that the H_2O_2-induced inhibition of CREB via a PKD1-dependent mechanism did not require Ser133 phosphorylation [35]. To determine whether PKD1 phosphorylates CREB directly or indirectly in CRC cells, we performed co-immunoprecipitation and immunofluorescence experiments. The results from these experiments showed that PKD1 directly phosphorylated CREB and that the selenite-induced de-phosphorylation of PKD1 led to the inhibition of CREB phosphorylation in CRC cells. Moreover, studies conducted by Nguyen et al. [36] and Huang et al. [37] concluded that CREB can promote cancer cell survival via PI3K/AKT-dependent activation. Our previous study also provided evidence that selenite can induce CRC cells apoptosis by inhibiting the PI3K/AKT survival pathway [7]. Thus, further studies need to be performed to explore the crosstalk between PKD1/CREB and other survival pathways, which are a main focus of our future research.

It has been reported that p38 MAPK acts as a tumour suppressor in various cancer cells, and our previous work indicated that p38 MAPK was involved in selenite-induced apoptosis [28]. Activated p38 MAPK can induce apoptosis by regulating several protein kinases, such as MAPK-activated kinase 2 (MK2) and MSK1 [38]. However, whether p38 MAPK is involved in the regulation of PKD1 signalling in selenite-treated CRC cells remains unknown. Here, we found that p38 MAPK binds to PKD1 and that their interaction was enhanced in selenite-treated CRC cells. Additional experiments showed that the up-regulation of p-p38 MAPK induced apoptosis by inhibiting the PKD1 survival pathway. The study conducted by Ricci [39] demonstrated that p38 MAPK inhibits PKD1, which strongly supports our conclusion.

Previous work in our lab demonstrated that a supranutritional dose of sodium selenite (2 mg/kg/day) had anticancer effects on SW480 cell lines in nude mice [40]. To demonstrate that this supranutritional dose of sodium selenite is appropriate for cancer therapy, we treated mice with two different doses: 2 mg/kg/day or 1 mg/kg/day. The average tumour volume of the 2 mg/kg/day-dose sodium selenite-treatment group was significantly reduced compared with the control and the 1-mg/kg/day-dose sodium selenite-treated group. Moreover, the TUNEL assay results reported by Luo [41] and Wu [20] support our conclusion that a supranutritional dose of sodium selenite induces apoptosis in xenograft tumours. Furthermore, our analyses of the apoptosis-related signalling pathways in tumour tissues were in agreement with our *in vitro* studies.

We discovered that selenite treatment did not have obvious effects on body weight and activity of nude mice, which is consistent with our previous work [40]. Normal mice were treated with selenite (2 mg/kg/day) or PBS, and then liver tissues from both control and selenite-treated mice were analysed by hematoxylin–eosin (HE) staining. Comparing with the liver tissues in control group, there were no marked pathological changes in the liver tissues of selenite-treated group (unpublished data). To verify the potential toxicity of selenite on normal cells, we treated monocytes with selenite (10 μM) and found selenite had no obvious toxicity for monocytes (Supplementary Table S1). Our results also are supported by the work of Nilsonne et al. [42] and Husbeck et al. [43], which showed

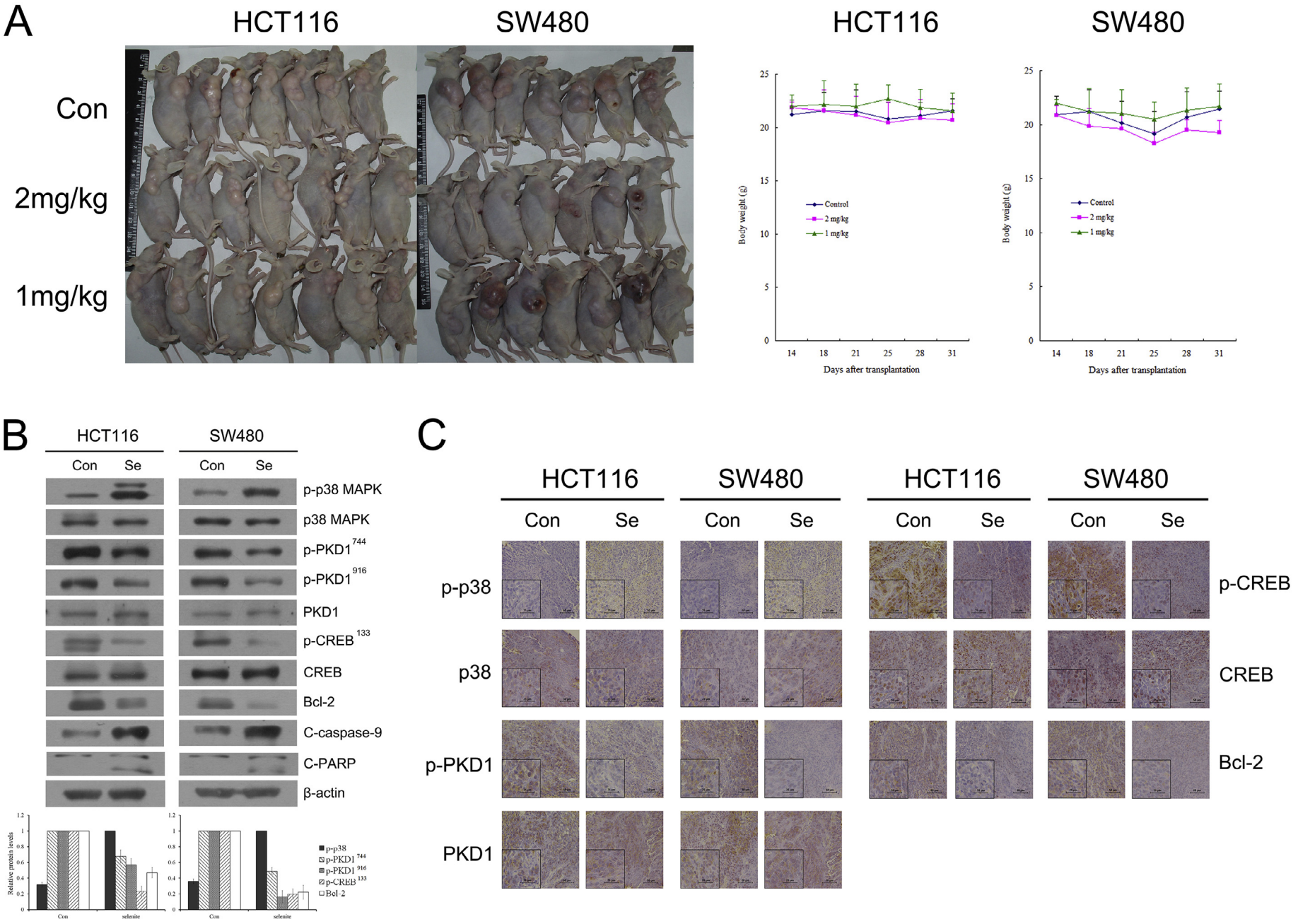

Fig. 5. The p38 MAPK/PKD1/CREB axis is altered by selenite *in vivo*. (A) Selenite treatment of tumour-bearing mice markedly attenuated tumour growth. (B) Western blot analysis of proteins extracted from tumour tissues using antibodies against the molecules indicated and quantified by pixel densitometry. (C) Selenite-mediated regulation of the p38 MAPK/PKD1/CREB signalling pathway *in vivo*. Tumour tissues from a colon xenograft animal model were analysed by immunohistochemistry with antibodies specific for p38 MAPK, p38, p-PKD1, PKD1, p-CREB, CREB and Bcl-2. Bar: 50 μm.

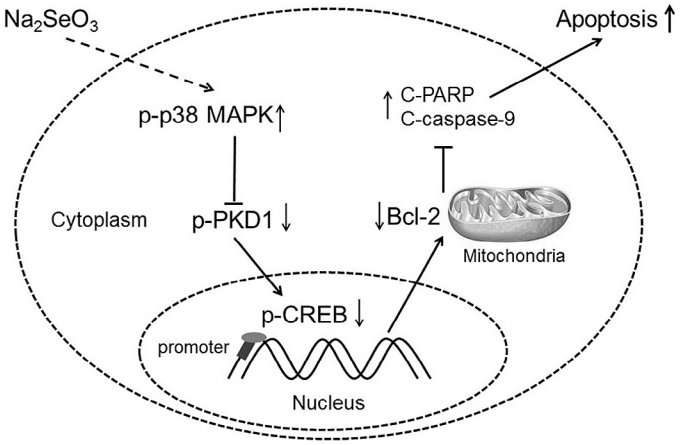

Fig. 6. A schematic illustration delineating the role of the p38 MAPK/PKD1/CREB/Bcl-2 pathway in selenite-induced apoptosis of CRC cells. Selenite triggered the up-regulation of p-p38 MAPK and subsequently inhibited PKD1 phosphorylation. The inhibitory effect on PKD1 was associated with the suppression of CREB transcriptional activity, which led to decreased expression of its target gene Bcl-2. Together, these signalling cascades ultimately contributed to the selenite-induced apoptosis in CRC cells.

selenite selectively killed the cancer cells while has little effect on normal cells. Olm's [44] work showed cancer-specific cytotoxicity of selenite may be due to specific high uptake and accumulation in malignant cells. To be concluded, selenite could induce cancer cell apoptosis with less toxicity *in vivo* and had potential clinical applications.

Supplemental Table S1. Selenite had no obvious toxicity on monocytes. Monocytes were isolated from umbilical cord blood of a normal person. After treatment of selenite for various lengths of time, live cells were counted. Result showed there is no significant difference between control group and selenite treated group ($p > 0.05$).

In summary, our study demonstrated that selenite induces apoptosis in CRC cells through the p38 MAPK-regulated PKD1/CREB/Bcl-2 signalling pathway. These findings provide a theoretical basis for the use of sodium selenite in clinical applications as a treatment for malignant tumours.

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Conflict of interest statement

We declare that there are no conflicts of interest.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2014.08.009.

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