Reactive oxygen species induce apoptosis in selenite-treated colorectal cancer cells via PKD1/AKT signaling-mediated mitochondrial translocation of Bad

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ABSTRACT

Mounting evidence shows that supranutritional selenite-induced Reactive Oxygen Species (ROS) could trigger colorectal cancer (CRC) cells apoptosis; however, the molecular mechanism underlying this effect remains unclear. To explore the detailed mechanism how ROS induced apoptosis in selenite-treated CRC cells, we investigated the role of AKT/Bad signaling-mediated mitochondrial cell death in selenite-treated CRC cells and xenograft tumours. First, we found that selenite exposure inhibited Bad phosphorylation, leading to mitochondrial translocation of Bad, which further triggers HCT116 and SW480 CRC cells apoptosis. Furthermore, we identified that AKT-mediated phosphorylation shown to repress Bad function by causing it dissociate from mitochondria and bind to 14-3-3 proteins in the cytoplasm. Additionally, we discovered that selenite-induced ROS cause AKT dephosphorylation by inhibiting the kinase activity of protein kinase D1 (PKD1) in CRC cells. We also corroborated our findings *in vivo* by performing immunohistochemistry experiments. Overall, these observations demonstrate that ROS could induce apoptosis through promoting the mitochondrial translocation of Bad by inhibiting PKD1/AKT signaling in selenite-treated CRC cells *in vitro* and *in vivo*.

Keywords: selenite; apoptosis; mitochondria; PKD1; Bad

1. Introduction

The essential trace element selenium (Se) has shown chemotherapeutic efficacy against cancers [1, 2]. Evidence shows sodium selenite could induce apoptosis in various cancer cell lines [3, 4]. Our previous work demonstrated that sodium selenite triggers apoptosis of colorectal cancer (CRC) cells through mitochondrial pathways [5-7]. Oxidative stress is involved in apoptosis in various systems and our previous studies demonstrated that ROS play an important role in mitochondrial apoptotic pathways in cancer cells [6, 8-10]. Our work also demonstrated that a supranutritional dose of sodium selenite specifically killed CRC cells through ROS-mediated apoptosis [6]. However, the detailed mechanism of ROS-mediated apoptosis in selenite-treated CRC cells remains poorly understood. In this study, we further investigated the mechanism underlying ROS-mediated mitochondrial apoptosis in sodium selenite-treated CRC cells.

Evidences presented that sodium selenite inhibited the PI3K/AKT survival pathway in a reactive oxygen species (ROS)-dependent pathway [6]. AKT, a survival kinase, could inhibit the apoptotic process by phosphorylating a Bcl-2 family member Bad [11]. The phosphorylation of Bad leads Bad binding to 14-3-3 protein, which prevents the subsequent association of Bad with Bcl-2 or Bcl-xL and further inhibits mitochondrial apoptosis [12, 13]. However evidences suggest a role for PKD1 in proliferation by activating survival pathway in several cancer cell lines such as ERK, Hsp27, NFκB, little is known about the downstream effectors regulated by PKD1 and how altered expression of PKD1 could affect cell proliferation [14, 15]. We also found PKD1 might participate in the AKT-mediated mitochondrial apoptosis in CRC cells, but the detailed mechanism remains unclear. Therefore, exploration of the mechanisms of PKD1-mediated apoptosis is of great significance for clinical applications of selenium.

In the current study, we corroborated that sodium selenite conferred its proapoptotic effect through inducing the mitochondrial translocation of Bad in CRC cells. We also confirmed that sodium selenite inhibited the kinase activity of PKD1, which further inhibited AKT/Bad

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pathway and the interaction between Bad and 14-3-3 protein. Furthermore, sodium selenite inhibited the PKD1/AKT survival axis in a ROS-dependent pathway. *In vivo* experiments also corroborated our findings *in vitro*.

2. Materials and methods

2.1. Cell lines and culture

HCT116 and SW480 were cultured in DMEM (Invitrogen, Paisley, Scotland) containing 10% advanced foetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 units/ml penicillin and 100 mg/ml streptomycin at 37°C in a 5% CO_2 humidified environment.

2.2. Reagents and antibodies

Sodium selenite was purchased from Sigma-Aldrich (St. Louis, MO, USA). Manganese (III) tetrakis-(1-methyl-4-pyridyl)-porphyrins (MnTMPyP), CID755673 and phorbol-12-myristate-13-acetate (PMA) were purchased from Merck Calbiochem (San Diego, CA, USA). LY294002 was purchased from Promega (Madison, WI, USA). The Pgex-4T1-hBad vector was obtained from Addgene (www.addgene.org), and the pEGFP-HA-PKD1 KD and pEGFP-HA-PKD1 WT vectors were a kind gift from Dr. Angelika Hausser. DAPI were purchased from Beyotime (Haimen, Jiangsu, China). Mito-Tracker was obtained from Thermo Fisher Scientific (Rockford, IL, USA).

Antibodies recognizing cleaved caspase-9, cleaved PARP, p-PKD1 (Ser744), p-PKD1 (Ser916), PKD1, p-AKT, AKT, p-Bad and Bad were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against B23, 14-3-3 and VDAC were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against b-actin was purchased from Sigma-Aldrich.

2.3. Western blot assay

The total proteins were separated as described previously and then quantified by pixel densitometry and expressed as means ± SD (n=3) [6]. Specifically, the nuclear and cytoplasmic fractions were prepared using the Cytoplasmic Extraction and NE-PER Nuclear Reagents and the mitochondria were fractionated using the Mitochondria Isolation Kit for Mammalian Cells (Thermo Fisher Scientific, Rockford,

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IL, USA).

2.4. Transient transfection analysis

Approximately 4×10^5 cells were plated into a six-well plate 24 h before transfection experiments. Cells were transfected with the plasmids using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) and the cells were treated as indicated 24 h later.

2.5. Small interfering RNAs

AKT1 siRNA (5'-AAGGAGGGUUGGCUGCACAAA-3'); Bad siRNA (5'-AAGAAGGGACTTCCTCGCCG-3') was chemically synthesised by GenePharm (Shanghai, China). PKD1 siRNA (h) and 14-3-3 siRNA (h) were purchased from Santa Cruz Biotechnology. Cells cultured in six-well plates were transfected with 100 pM siRNA by lipofectamine 2000 and then subjected to further treatment as demanded.

2.6. Co-immunoprecipitation

Cell lysates were prepared in RIPA buffer after having been washed by ice-cold PBS. 200 μg aliquots of lysates were immunoprecipitated by the appropriate antibodies and then captured by 25 ml protein A+G agarose beads (Santa Cruz). After being washed and elutioned, the immunoprecipitates were boiled with western blot sample buffer for 10 min for western blot assays.

2.7. Immunofluorescence

Cells were grown on coverslips for 24 h before treatment with 10 μM selenite. Cells were treated with Mito-Tracker for 2h and then were fixed in Immunol staining fix solution for 10 min. After washing with PBS, the samples were blocked with Immunol staining blocking buffer. Then, the slides were incubated with the indicated primary antibodies overnight at 4°C. Thereafter, cells were incubated with FITC- or Cy3-labeled secondary antibodies, followed by counterstaining with DAPI solution. Images were acquired on an Olympus laser scanning confocal FV1000 microscope and analyzed using Olympus Fluoview software.

2.8. Apoptosis assay

Apoptosis was determined using Annexin V/PI double staining kit (Merck Calbiochem). Briefly, cells were harvested

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and washed twice with PBS buffer, and then cells were stained with Annexin V and PI in 1×binding buffer. The stained cells were analyzed with a flow cytometer. All of the experiments were repeated thrice independently, and the data are represented as the means ± SD (n=3). *P<0.05.

2.9. Quantification of ROS production

First, the cells were seeded in six-well plates and grown to approximately 50% confluency. Then, the cells were incubated with 5 μM dichlorofluorescein diacetate (DCFH-DA) (Beyotime, Haimen, China) at 37°C for 30 min. After washing with serum-free medium, the cells were treated with 10 μM sodium selenite or PBS solution. The DCFH-DA fluorescence distribution was recorded hourly by a fluorospectrophotometer up to 24 h, and the data are represented as the mean±SD (n=3). (DCF: excitation wavelength 488 nm and emission wavelength 535 nm.).

2.10. Immunohistochemical staining

The colorectal xenograft model was established as previously described[5]. Portions of the tissue samples from the xenograft tumour model were homogenized and subjected to Western blotting and immunohistochemical analysis.

2.11. 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. A P-value of P<0.05 was considered significant.

3. Results

3.1. Bad mediated sodium selenite-induced CRC cells apoptosis

Bcl-2 antagonist of cell death (Bad) is a Bcl-2 homology domain 3 (BH3)-only proapoptotic Bcl-2 family member and our previous work showed that Bad is involved in supranutritional dose of selenite (10 μM) induced CRC cells apoptosis [16, 17]. To elucidate the underlining molecular mechanisms, we investigated the effect of selenite on Bad in CRC cells. Treated with a siRNA targeting Bad and the cells were exposed to selenite for 24 h. The western blot results showed that the knockdown of

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Bad further repressed PARP and caspase-9 cleavage (Fig. 1A). Apoptosis detection demonstrated that the selenite-induced apoptosis was suppressed after the knockdown of Bad (Fig. 1B). On the contrary, overexpression of Bad enhanced selenite-induced activation of PARP and caspase-9 was reduced (Fig. 1C). The analysis of apoptosis of these cells demonstrated that overexpression of Bad enhanced apoptosis in selenite-treated CRC cells (Fig. 1D).

3.2. Sodium selenite induced Bad dephosphorylation and translocated to mitochondria

Bad is a proapoptotic protein activated by dephosphorylation which triggers apoptosis by facilitating the release of mitochondrial cytochrome *c* into the cytoplasm [12, 18]. To explore the mechanism of Bad mediated apoptosis in selenite-treated CRC cells, we performed a series of experiments to detect the phosphorylation and translocation of Bad in selenite-treated CRC cells. As shown in Fig. 2A, in the presence of 10 μM selenite, Bad phosphorylation decreased in both cell lines, whereas Bad increased. To explore whether Bad participated in mitochondria-mediated apoptosis in CRC cells, we separated cytoplasmic and mitochondrial fractions from selenite-treated CRC cells, immunoblotted for Bad and found that selenite treatment induced the translocation of Bad from the cytoplasm to the mitochondria (Fig. 2B). Moreover, the immunostaining result showed in Fig. 2C also confirmed that selenite induced the colocalization of Bad with the mitochondria.

3.3. Selenite disrupted the association between Bad and 14-3-3 protein

The survival kinase such as Akt/protein kinase B, protein kinase A (PKA), and p90 ribosomal S6 kinase (RSK) mediated phosphorylation of Bad has been shown to repress Bad function by causing it to dissociate from mitochondria and bind to 14-3-3 proteins in the cytoplasm [18-20]. To explore the mechanism of Bad mediated CRC cells apoptosis, we performed a co-immunoprecipitation experiment. As shown in Fig. 3A, selenite treatment significantly decreased the association between p-Bad and 14-3-3 in CRC cells. This result suggested that the reduction in Bad phosphorylation leads to

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the selenite-induced disruption of the association between p-Bad and 14-3-3 in CRC cells. To verify the role of 14-3-3 protein in translocation of Bad and selenite-induced apoptosis, we performed several experiments. First, we found that the knockdown of 14-3-3 by siRNA transfection increased the co-localization of Bad with the mitochondria as well as caspase-9 and PARP cleavage (Fig. 3B). The analysis of the apoptotic rate demonstrated that 14-3-3 inhibition increased the apoptosis rate of selenite-treated CRC cells (Fig. 3C). Furthermore, our immunofluorescence results (Fig. 3D) supported that selenite promoted Bad translocation to mitochondria. Taken together, these results indicate that selenite induced dephosphorylation and mitochondrial translocation of Bad, which further caused CRC cells apoptosis.

3.4. Selenite induced Bad dephosphorylation by depressing AKT

Having discovered that selenite treatment induced Bad dephosphorylation and dissociation from 14-3-3 protein, we aimed to figure out the mechanism of Bad dephosphorylation in selenite-treated CRC cells. Large amount of evidence has indicated that AKT-mediated phosphorylation has been shown to repress Bad function by causing it to bind to 14-3-3 proteins and our previous work has proved that selenite induced CRC cell apoptosis by inhibiting AKT signaling [16, 21]. Thus, we conducted experiments to investigate the relationship between Bad and AKT in selenite-treated CRC cell. On one hand, as revealed in Fig. 4A and B, inhibiting AKT in selenite-treated CRC cells with either AKT siRNA or the inhibitor LY294002 decreased the p-AKT level. Inhibiting AKT further suppressed the phosphorylation of Bad. Conversely, when we activated AKT, using constitutively activated AKT constructs, we found that AKT activating increased phosphorylation of AKT and Bad (Fig. 4C). Furthermore, co-immunoprecipitation and mitochondrial fractions detective experiment showed that inhibiting the AKT further induced Bad dissociation from 14-3-3 protein and accumulation in mitochondria (Fig. 4D). Moreover, the immunostaining result shown in Fig. 4E also corroborated the finding that the inhibition of AKT enhanced the colocalization of Bad with the

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mitochondria.

3.5. Selenite-induced inhibition of PKD1 activation is required for AKT dephosphorylation

Our previous work has proved that sodium selenite induced CRC cells apoptosis via PKD1 through phosphorylation of CREB in selenite-treated CRC cells [5]. To determine whether selenite-induced PKD1 dephosphorylation could exert any impact on AKT/Bad signalling in CRC cells, we performed a series of experiments. First, we validated the interaction between PKD1 and AKT by co-immunoprecipitation and found selenite treatment greatly decreased the association between p-PKD1 and p-AKT in CRC cells (Fig. 5A). Furthermore, 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 inhibited phosphorylation of AKT and Bad (Fig. 5B, C, D). Conversely, both PKD1 WT over-expression and PMA (PKD1 activator) treatment significantly increased the p-AKT and p-Bad levels, compared with selenite treatment alone (Fig. 5C, E).

Next, to verify whether PKD1 regulate the association between 14-3-3 protein and Bad, we performed a co-immunoprecipitation experiment. As shown in Fig. 5F, selenite treatment greatly induced Bad released from 14-3-3 protein. Moreover, western blot results of mitochondrial and cytoplasmic fractions also corroborated the finding that selenite induced mitochondrial translocation of Bad by inhibiting PKD1 activity (Fig. 5G).

3.6. Selenite-induced ROS is indispensable for PKD1/AKT/Bad signaling pathway-mediated apoptosis in CRC cells

Numerous works have demonstrated that ROS play an important role in the induction of apoptosis in cancer cells [10, 22-24]. Our previous work demonstrated that sodium selenite treatment increased the level of ROS in CRC cells [6, 25]. Thus, we investigated whether ROS were involved in PKD1/AKT/Bad-mediated apoptosis in selenite-treated CRC cells. First, we measured the ROS level in selenite-treated HCT116 and SW480 cells and found that selenite elicited significant

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increase of ROS in both cell lines in a time-dependent manner (Fig. 6A).

To explore the possible link between ROS and the PKD1/AKT/Bad pathway, we depleted ROS in selenite-treated cells with the ROS scavenger MnTMPyP and found that depletion of ROS almost completely inhibited selenite-induced apoptosis and caused a decrease in cleaved PARP and caspase-9. Furthermore, selenite-induced inhibition of this signaling pathway was reversed by ROS depletion, which suggests a vital role for ROS in selenite-induced PKD1/AKT/Bad-mediated apoptosis in CRC cells. Notably, cells treated with H_2O_2 were used as a positive control, and the results from these experiments provided further evidence that ROS resulted in induction of apoptosis and PKD1/AKT/Bad axis inhibition by activating p38 MAPK (Fig. 6B).

3.7. The PKD1/AKT/Bad signaling pathway is regulated by selenite *in vivo*

Having defined the role of PKD1/AKT/Bad signaling in selenite-induced apoptosis in CRC cells, we further tested whether selenite could regulate this signaling *in vivo*. We first performed western blot analysis of tissues from both control and selenite-treated samples, and the results showed that selenite could inhibit the phosphorylation of PKD1/AKT/Bad (Fig. 6C) [5, 6]. Additionally, we examined the patterns of critical molecules in this signaling pathway with in a series of immunohistochemistry experiments, including p-PKD1, PKD1, p-AKT, AKT, p-Bad, Bad, and 14-3-3, and discovered that each of these proteins displayed a similar pattern as that detected in tumour cell lines (Fig. 6D) [5, 6].

4. Discussion

Accumulating evidence has underscored that selenite has therapeutic effects on a wide range of cancer cells. Our previous study has shown that selenite could induce mitochondrial cell death in selenite-treated CRC cells, whereas the detailed molecular mechanism is not completely understood[6]. The current study explored the mechanism of selenite-induced mitochondrial cell death in CRC cells. First, selenite induced ROS production and inhibited the phosphorylation of PKD1. This inhibition of PKD1 activation caused dephosphorylation of the survival kinase

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AKT. As AKT has high affinity for the phosphorylation of the proapoptotic protein Bad, the inhibition of AKT led to Bad dephosphorylation and dissociation from 14-3-3 proteins to mitochondria, which further induced apoptosis. This study explored the mechanisms by which selenite induced mitochondrial apoptosis in CRC cells.

Mitochondria are often termed the 'powerhouse' of the cell and are involved in regulating apoptosis in various cancer cells. The mitochondrial apoptosis signaling pathway is operated by the Bcl-2-family members, which are generally divided into three categories, pro-apoptotic executioners, anti-apoptotic proteins and pro-apoptotic BH3-only proteins [11, 16, 26]. Anti-apoptotic family members prevent apoptosis by preventing BH3-only proteins interaction with Bax/Bak. The sensitizers BH3-only proteins induce Bax/Bak oligomerization indirectly by displacing activator BH3-only proteins [11, 26]. Our previous work has shown that selenite could repress the expression of Bcl-2 by inhibiting PKD1/CREB pathway in CRC cells [5]. In that study, we observed selenite influenced the location of Bad (One of the sensitizers BH3-only proteins), which prompted us selenite might induce apoptosis by other mechanism. In this study, immunoblotting and immunostaining result (Fig. 2B/C) demonstrated that selenite-treatment promoted Bad translocation to mitochondrial, which provides more details of the mitochondrial apoptosis. Those evidences showed the vital role of Bcl-2-family members in mitochondrial apoptosis and selenite could induce CRC cell death by multiple mechanisms.

The 14-3-3 proteins are a family of phosphor – Ser / phosphor Thr – binding molecules which promote cell survival by sequestering and inactivating proapoptotic proteins. Binding of 14-3-3 proteins sequesters the target protein and the release of 14-3-3 proteins then allows the target to relocate [27]. This relocation is often due to the exposure of an intrinsic subcellular targeting sequence masked by the 14-3-3 protein dimer. Bad is localized mostly in the cytoplasm but redistributes to the mitochondria in response to survival factor deprivation [28]. In our study, co-immunoprecipitation experiment showed selenite treatment greatly

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decreased the association between p-Bad and 14-3-3 (Fig. 3A). Knockdown of 14-3-3 increased the co-localization of Bad with the mitochondria, which further verifies the prosurvival function of 14-3-3 protein (Fig. 3B). Furthermore, the phosphorylation or expression inhibition of PKD1 and AKT had no effect on 14-3-3, on the contrary, it regulated the phosphorylation of Bad and further influenced its binding to 14-3-3 protein.

The interaction of Bad with Bcl-2 or Bcl-XL depends on the dephosphorylation and translocation of Bad to the mitochondria, which may cause consequent release of cytochrome c, which further induces apoptosis [20, 28, 29]. In our study, we focused on the mechanism of the dephosphorylation of Bad in selenite-treated CRC cells. Numerous reports suggest that some protein kinase such as PKA, AKT, RSK are involved in Bad [18, 30, 31]. To investigate the mechanism of Bad phosphorylation in CRC cells, co-immunoprecipitation and immunostaining results showed AKT directly phosphorates Bad in CRC cells. Previous work has indicated that activated PKD1 phosphorylates proteins and participated in the prolife of CRC cells. The work of Siu shows PKD-mediated phosphorylation of Ser^{92} upon proper folding of Syx N terminus induces binding of 14-3-3 protein [19]. To confirm the details of Bad phosphorylation, we performed experiments to detect the relationship between PKD1 and AKT/Bad. The summary of the work demonstrated that PKD1 regulates the phosphorylation of Bad indirectly by activating AKT.

ROS are involved in selenite-induced apoptosis in various cancer cell lines [10, 22, 32]. We detected intracellular ROS in selenite-treated CRC cells and observed a sharp increase in a time-dependent manner, which is supported by results from Luo [25]. Our previous work indicated that ROS are an upstream regulatory factor of the apoptosis pathway in selenite-treated CRC cells [6, 33]. In this study, we used H_2O_2 as positive control and verified that selenite-induced apoptosis occurs through ROS activation, which was supported by the work of Liu [34]. We also observed that in CRC cells treated with selenite combined with the ROS scavenger MnTMPyP, the ROS-mediated increase in apoptosis was reversed. These results

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confirmed that ROS are an upstream regulatory factor of selenite-induced apoptosis via the PKD1/AKT/Bad pathway. We found that ROS induced Bad dephosphorylation; furthermore, previous work in our lab indicated that ROS regulated the expression of Bax and Bim [6, 17]. Together, these results suggest that the mitochondrial pathway plays a role in selenite-induced apoptosis in CRC cells.

Overall, our study elucidated the involvement of the PKD1/AKT signaling pathway in the anti-cancer effect of selenite in CRC cells. Selenite could induce Bad dissociation from 14-3-3 protein and translocate to mitochondria through inhibiting the PKD1/AKT survival axis in a ROS-dependent manner, which further activated the mitochondrial apoptosis pathway. These data provide a theoretical basis for the clinical application of sodium selenium drugs in malignant cancer.

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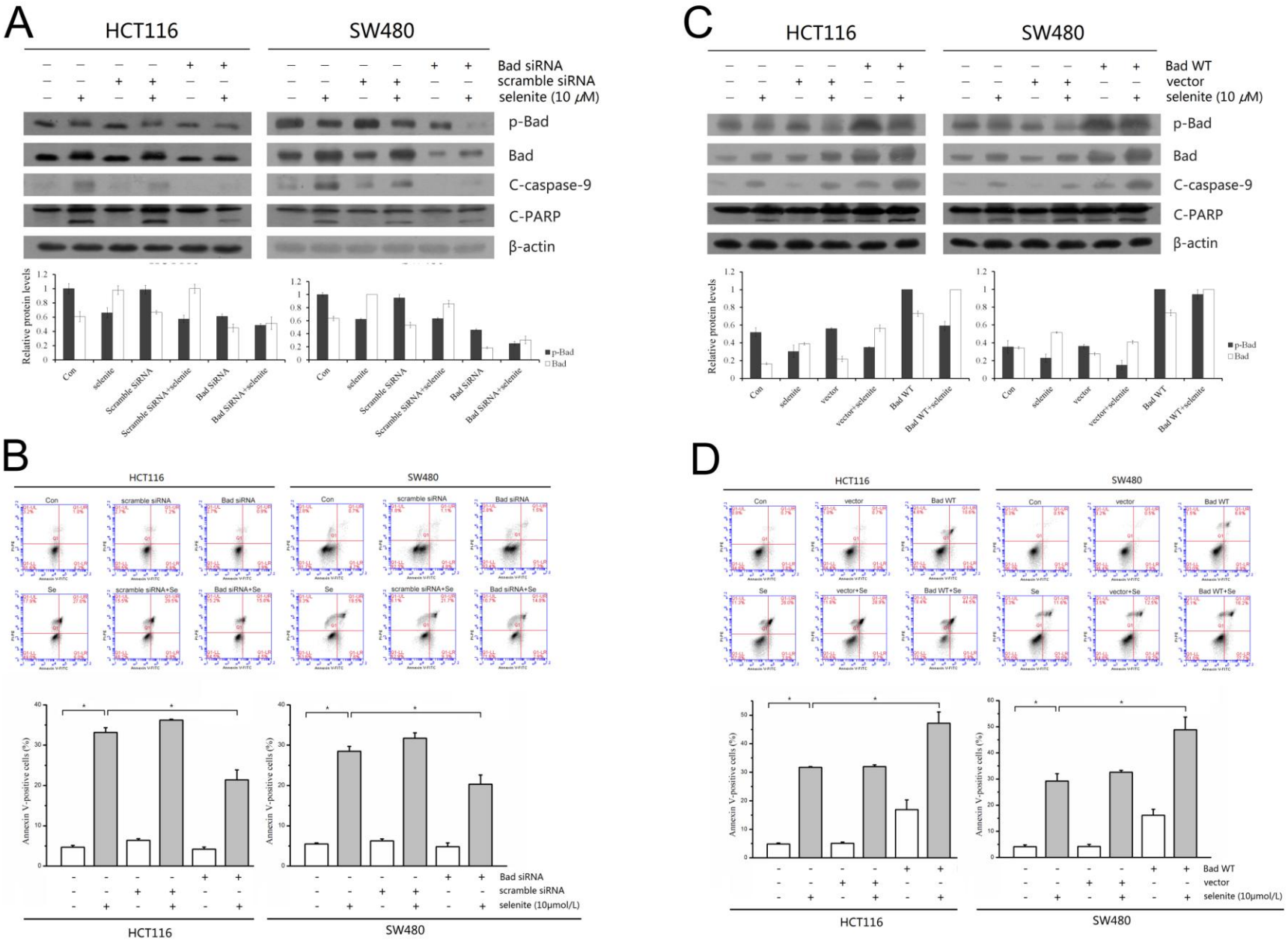

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Figure 1. Sodium selenite induced CRC cells apoptosis through activating Bad. (A) Bad knockdown decreased the sodium selenite-induced apoptosis protein cleavage. The cells were transfected with a siRNA targeting Bad and then treated with 10 μM selenite for 24 h. Next, the Bad and apoptosis markers levels were detected by western blot. (B) Bad knockdown reversed the selenite-induced apoptosis. The cells were treated as described in (A), and the rate of apoptotic cells was then determined by flow cytometry. The data are represented as the means ± SD (n=3). *P<0.05. (C) The overexpression of Bad increased the selenite-induced cleavage of apoptosis makers. After transfection with Bad plasmids, the cells were cultured for 24 h and treated with 10 μM selenite for 24 h. The apoptotic markers and Bad levels were detected by western blot. (D) The overexpression of Bad enhanced the selenite-induced apoptosis. The cells were treated as described in (C), and the apoptotic cells were then detected by flow cytometry. The data are represented as the means ± SD (n=3). *P<0.05.

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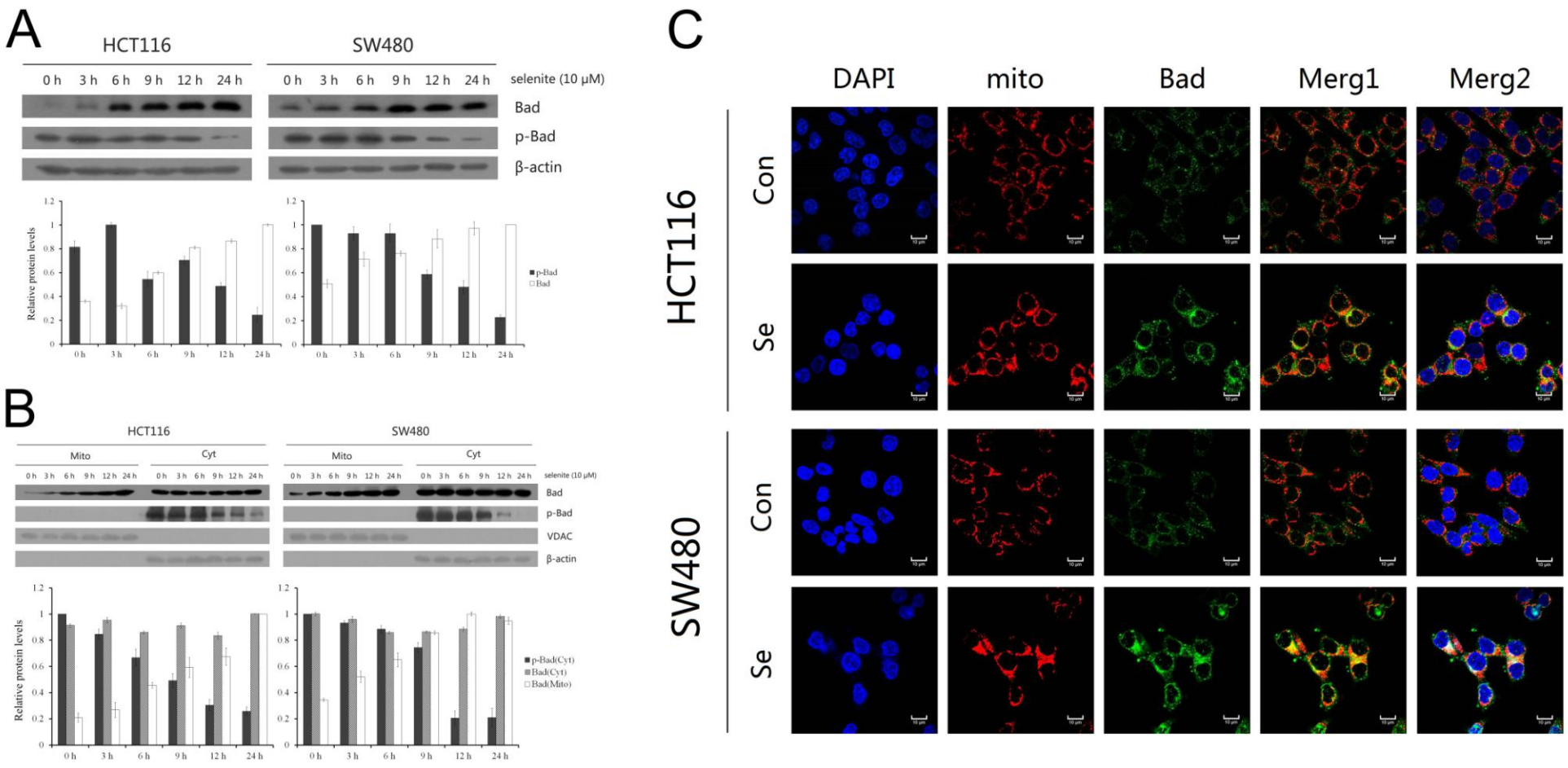

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Figure 2. Sodium selenite induced Bad dephosphorylation and translocation to mitochondria. (A) Sodium selenite induced Bad dephosphorylation in CRC cells. SW480 and HCT116cells were treated with 10 μM selenite for the times points indicated. Bad and p-Bad were detected by Western blot. (B) Selenite induced the mitochondrial translocation of Bad. Mitochondrial and cytoplasmic fractions were prepared from selenite-treated and control cells, Bad and p-Bad were detected by Western blot. VDAC and β-actin were used as loading controls for the mitochondrial and cytoplasmic fractions, respectively. (C) The effect of selenite on Bad distribution in cells was detected by immunostaining. Cells were treated with selenite for 24h, and Mitotracker solution was added to stain the mitochondria. Cells were then stained with Bad primary antibody and a FITC-conjugated secondary antibody (green). Nuclei were stained with DAPI solution. The images were generated with a confocal microscope. The scale bar represents 10 μm.

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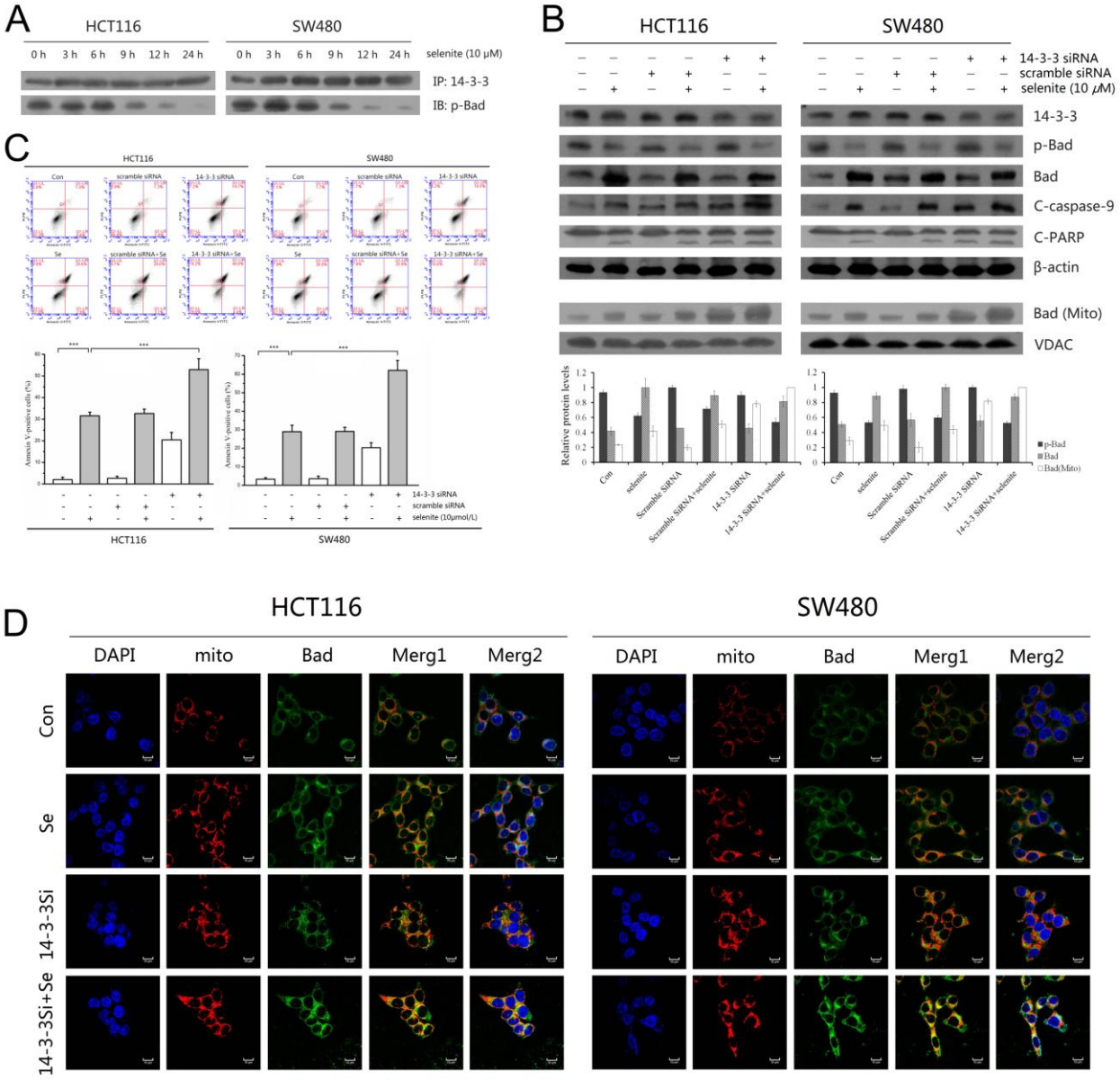

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Figure 3. Selenite induced Bad translocation to mitochondria. (A) Selenite induced Bad released from 14-3-3 protein. The cells were exposed to 10 μM selenite for 24 h, and co-immunoprecipitation was used to detect the interactions between 14-3-3 and Bad. (B) 14-3-3 knockdown enhanced the apoptosis and Bad translocation. The cells were transfected with a siRNA targeting 14-3-3 and then treated with 10 μM selenite for 24 h. Next, the 14-3-3, Bad and apoptosis markers levels were detected by western blot; co-immunoprecipitation was used to detect the interactions between 14-3-3 and Bad. (C) The cells were treated as described in (B), and the rate of apoptosis was then determined by flow cytometry. The data are represented as the means ± SD (n=3). $^{*}P<0.05$. (D) The effect of selenite on Bad distribution after 14-3-3 been knockdown in cells was detected by immunostaining. Cells were transfected with a siRNA targeting 14-3-3 and then treated with 10 μM selenite for 24 h. Bad was immunostained with FITC, mitochondria were stained with MitoTracker and nuclei were stained with DAPI solution. The scale bar represents 10 μm.

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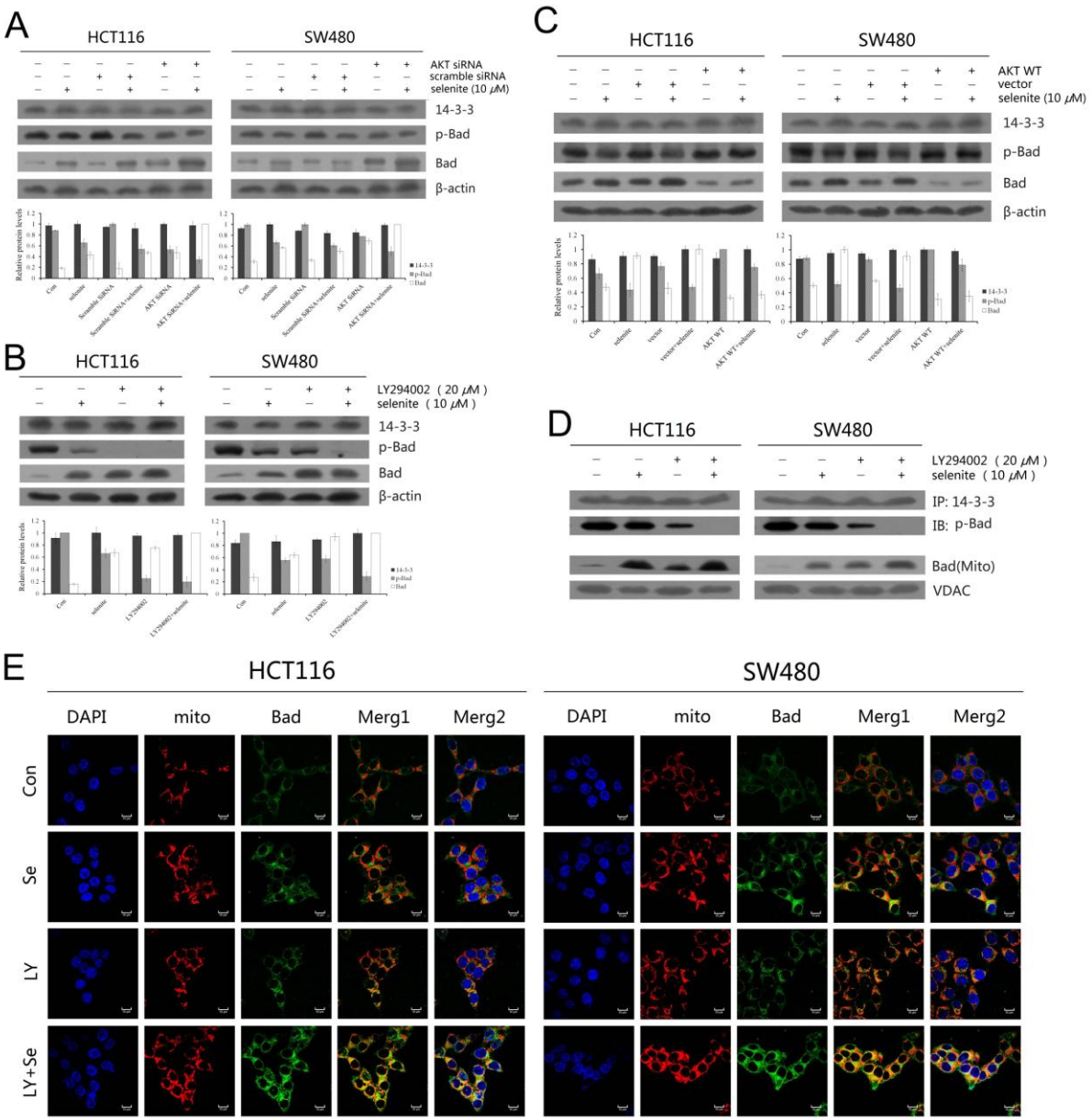

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Figure 4. Selenite inhibited AKT further induced Bad dephosphorylated and translocated to mitochondria. (A, C) AKT induced Bad phosphorylation. The cells were transfected with a siRNA targeting AKT or AKT vector and then treated with 10 μM selenite. Next, the 14-3-3, p-Bad, Bad levels were detected by western blot. (B) The cells were pretreated with 20 μM LY294002 for 2 h. After the cells were treated with 10 μM selenite for 24 h, the 14-3-3, p-Bad, Bad levels were detected by western blot and quantified by pixel densitometry. (D) Treated as scribbled in (B), co-immunoprecipitation was used to detect the interactions between 14-3-3 and p-Bad. The mitochondrial fractions were prepared and Bad were detected by western blot. (E) Treated as scribbled in (B) Bad was immunostained with FITC, mitochondria were stained with MitoTracker and nuclei were stained with DAPI solution. The scale bar represents 10 μm.

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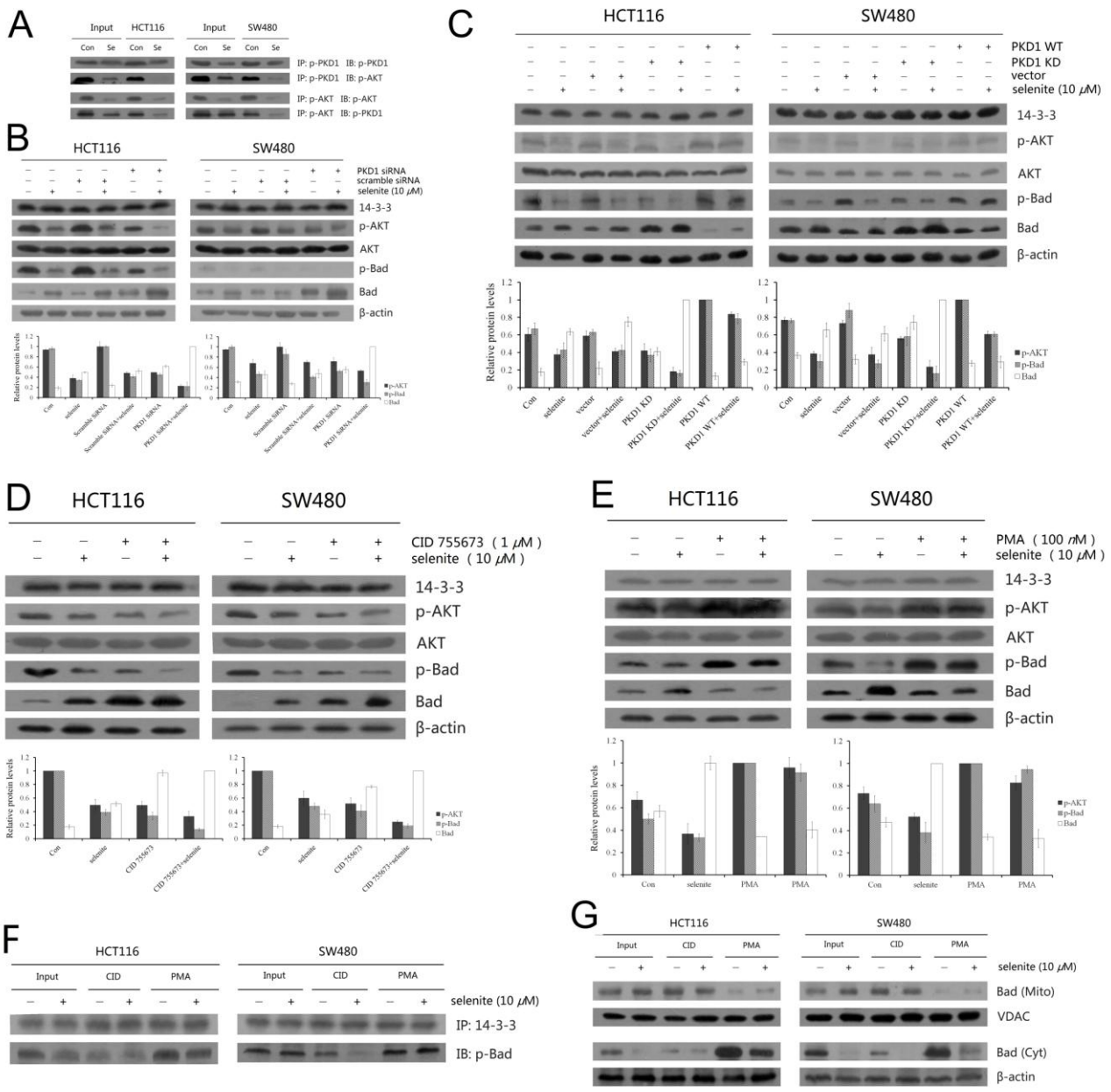

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Figure 5. Selenite inhibited AKT phosphorylation via PKD1. (A) Selenite inhibited the interaction between PKD1 and AKT. The cells were exposed to 10 μM selenite and co-immunoprecipitation was used to detect the interactions between PKD1 and AKT. (B, C) PKD1 induced AKT phosphorylation. The cells were transfected with a siRNA targeting PKD1 or PKD1 vector and then treated with 10 μM selenite. Next, the 14-3-3, AKT, Bad levels were detected by western blot and quantified by pixel densitometry. (D, E) The cells were pre-treated with 1 μM CID 755673 or 100 nM PMA for 2 h. The cells were then treated with selenite (10 μM) for 24 h, and the AKT, 14-3-3, Bad levels were detected by western blot. (F, G) The cells were treated as (D, E). Co-immunoprecipitation was used to detect the interactions between 14-3-3 and p-Bad. The mitochondrial fractions were prepared and Bad were detected by western blot. The mitochondrial and cytoplasmic fractions were prepared from cells treated as (D, E), and Bad was detected by western blot. β-actin and VDAC were used as loading controls for the cytoplasmic and mitochondrial fractions, respectively.

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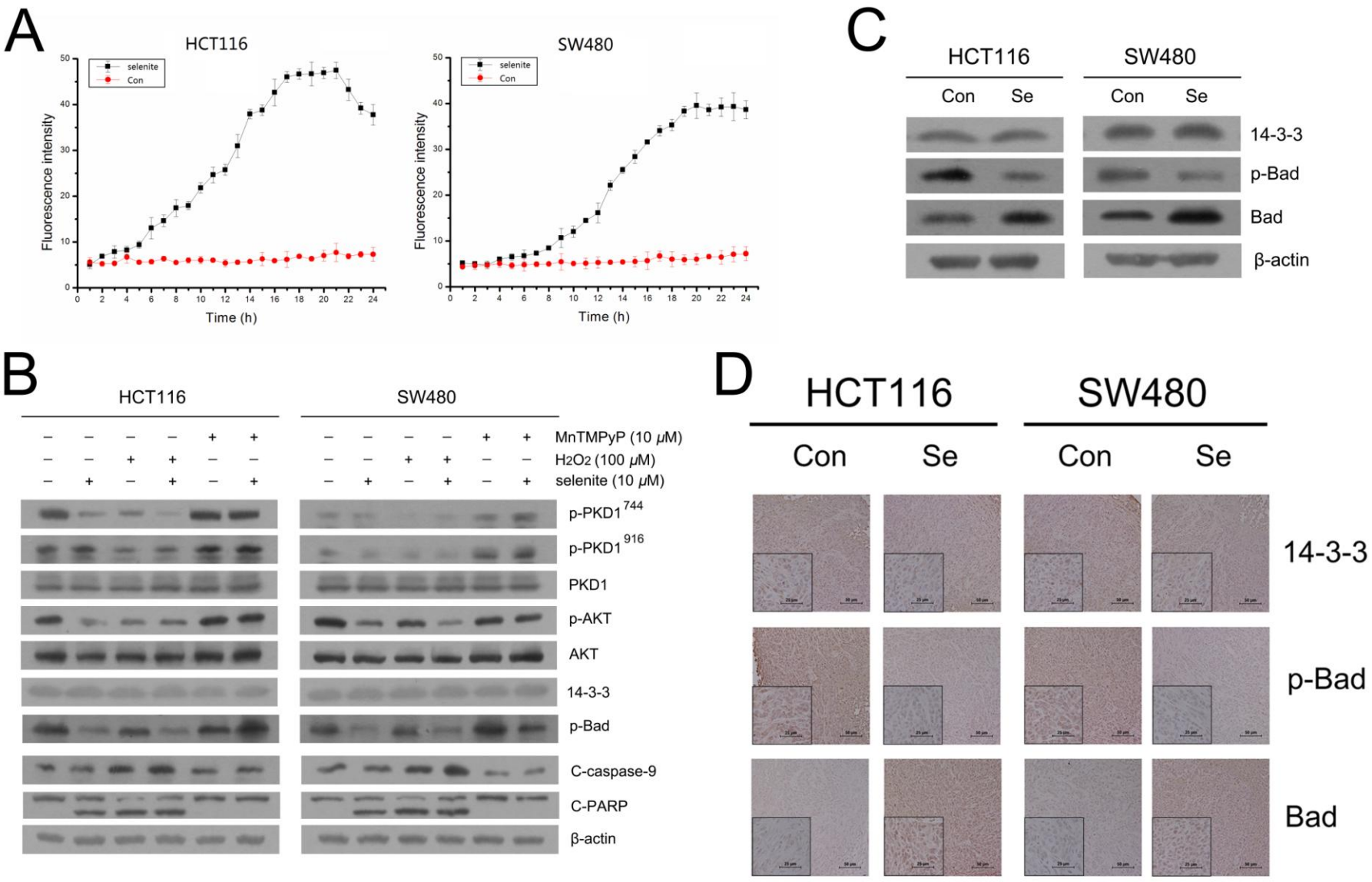

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Figure 6. (A) Selenite triggers ROS increase in CRC cells. CRC cells were treated with 10 μM sodium selenite. Intracellular superoxide or hydrogen peroxide was detected with DHE or DCF-based measurements, respectively. Data are presented as the mean±SD (n=3). (B) Selenite-induced ROS plays a role in the PKD1/AKT/Bad signaling pathway in CRC cells. Cells treated with 100 μM H_2O_2 were used as a positive control. Cells were treated with 10 μM MnTMPyP and 10 μM selenite in combination, and then apoptosis markers and the PKD1/AKT/Bad axis were detected by Western blot. (C) The PKD1/AKT/Bad axis is altered by selenite *in vivo*. Western blot analysis of proteins extracted from tumour tissues. (D) Tumour tissues from a colon xenograft animal model were analyzed by immunohistochemistry with antibodies against PKD1/AKT/Bad axis related molecules. Bar: 50 μm.

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