

Celecoxib induces p53-PUMA pathway for apoptosis in human colorectal cancer cells

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ABSTRACT

Celecoxib, a clinical non-steroidal anti-inflammatory drug, displays anticarcinogenic and chemopreventive activities in human colorectal cancers, although the mechanisms of apoptosis by celecoxib are poorly understood. The existence of functional p53 but not securin in colorectal cancer cells was higher on the induction of cytotoxicity than the p53-mutational colorectal cancer cells following celecoxib treatment. The p53-wild type HCT116 cells were more susceptible to increase ~25% cell death than the p53-null HCT116 cells after treatment with 100 μ M celecoxib for 24 h. Transfection with a small interfering RNA of p53 reduced the celecoxib-induced cytotoxicity in the RKO (p53-wild type) colorectal cancer cells. Celecoxib (80–100 μ M for 24 h) significantly increased total p53 proteins and the phosphorylated p53 proteins at serine-15, -20, -46, and -392 in RKO cells. However, the phospho-p53 (serine-15, -20, and -392) proteins were presented on the nuclei of cells but the phospho-p53 (serine-46) protein was located on the cytoplasm of apoptotic cells following treatment with celecoxib. Interestingly, the p53 up-regulated modulator of apoptosis (PUMA) protein, which located on the mitochondria, was induced by celecoxib in the p53-functional colorectal cancer cells but not in the p53-mutational cells. Together, this study provides the first time that celecoxib induces the various phosphorylated sites of p53 and activates p53-PUMA pathway, which potentiates the apoptosis induction in human colorectal cancer cells.

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1. Introduction

Colorectal cancer is one of most common cancers around the world [1]. Development of the novel anticancer drugs or strategies for colorectal cancer therapy is highly

desired. Celecoxib, a non-steroidal anti-inflammatory drug (NSAID), which has been approved as adjuvant treatment in patients with familial adenomatous polyposis, exhibits anticarcinogenic and chemopreventive activities in human colorectal cancer [2–4]. It has been shown that celecoxib displayed anticancer ability by inducing apoptosis in tumor cells [5–8]. However, the mechanisms of celecoxib-induced apoptosis in human colorectal cancer cells are still unclear.

Anticancer agents display the anticancer activities by inducing the various signal proteins for apoptosis in cancer cells [9]. p53 is a key tumor suppressor for controlling apoptosis in cancer cells [10–12]. The diverse phosphorylation sites of p53 have been shown to play a variety of roles in the responses of cellular stresses [13–15]. The p53 up-regulated modulator of apoptosis (PUMA) was recently identified a p53 downstream protein for promoting apoptosis [16]. PUMA encodes a BH3-only protein member of

Abbreviations: NSAID, non-steroidal anti-inflammatory drug; PUMA, p53 up-regulated modulator of apoptosis; PI, propidium iodide; ERK, extracellular signal-regulated kinase; MTT, 3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyl tetrazolium bromide; FITC, fluorescein isothiocyanate; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

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the BCL-2 family that is a key mediator to trigger apoptosis via a p53-dependent pathway [16]. Moreover, PUMA can sensitize cancer cells to chemotherapeutic agents [17,18]. Accordingly, the activation of p53-PUMA pathway by anticancer drugs may contribute to cancer therapy.

In this study, celecoxib induced the phosphorylation of p53 at various sites in human colorectal cancer cells. The existence of functional p53 in colorectal cancer cells was higher on the induction of apoptosis and PUMA protein expression following exposure to celecoxib. We provide that the phosphorylated sites of p53 and p53-PUMA pathway play an important role on the regulation of apoptosis in the celecoxib-treated colorectal cancer cells.

2. Materials and methods

2.1. Reagents and antibodies

Celecoxib was purchased from Toronto Research Chemical Inc. (North York, Canada). BODIPY FL phalloidin (B-607) and Mitotracker orange (M-7510) were purchased from Invitrogen (Carlsbad, CA). 3,3'-dihexiloxadadicarbocyanine [DiOC6(3)] was purchased from Calbiochem (San Diego, CA). Hoechst 33258, propidium iodide (PI), and 3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-extracellular signal-regulated kinase-2 (ERK-2) (C-14), anti-PUMA (N-19), anti-COX-2 (sc-19999), and the FITC (fluorescein isothiocyanate)-labeled goat anti-mouse IgG (sc-2010) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The Cy5-labeled goat anti-rabbit IgG was purchased from Amersham Pharmacia Biotech (Little Chalfont Buckinghamshire, UK). Anti-p53 (AHO0132) was purchased from BioSource International, Inc. (Camarillo, CA). Anti-phospho-p53 (serine-15, -20, -46, and -392) antibodies, control siRNA, and p53 siRNA were purchased from Cell Signaling Technology, Inc. (Beverly, MA). The monoclonal anti-securin (ab-3305) was purchased from Abcam (Cambridgeshire, UK).

2.2. Cell lines and cell culture

RKO colorectal carcinoma cells can express the functional p53 proteins [19]. SW480 was a colorectal carcinoma cell line that contained double mutations at codon 273 (G to A mutation) and codon 309 (C to T mutation) in the p53 gene [20]. The wild type p53 and securin, securin-null, and p53-null HCT116 colorectal carcinoma cell lines were kindly provided by Dr. B. Vogelstein of Johns Hopkins University (Baltimore, MD). RKO and SW480 cells were maintained in DMEM medium (Invitrogen). The different genotypes of HCT116 cells were cultured in McCoy's 5A medium (Sigma Chemical). The complete medium was supplemented with 10% fetal bovine serum (FBS). These cells were maintained at 37°C and 5% CO₂ in a humidified incubator (310/Thermo, Forma Scientific, Inc., Marietta, OH).

2.3. Cytotoxicity assay

Briefly, the cells were plated in 96-well plates at a density of 1×10^4 cells/well for 12–16 h. Following celecoxib

treatment, the cells were washed with phosphate-buffered saline (PBS) and were replaced fresh medium for cultured 2 days. Thereafter, the medium was replaced and the cells were incubated with 0.5 mg/ml of MTT in complete medium for 4 h. The surviving cells converted MTT to formazan that generates a blue-purple color when dissolved in dimethyl sulfoxide. The intensity of formazan was measured at 565 nm using a plate reader (Molecular Dynamics, OPTImax). The relative percentage of cell viability was calculated by dividing the absorbance of treated cells by that of the control in each experiment.

2.4. Apoptotic morphology analysis

To examine the effect of celecoxib on apoptosis, RKO or SW480 cells were cultured on coverslip in a 60-mm Petri dish for 12–16 h before treatment. At the end of treatment, the cells were carefully and gently washed with isotonic PBS (pH 7.4), and then fixed with 4% paraformaldehyde solution in PBS for 1 h at 37°C. The actin filament (F-actin) was stained with 20 U/ml BODIPY FL phalloidin for 30 min at 37°C. The nuclei were stained with 2.5 µg/ml Hoechst 33258 for 30 min at room temperature. The cell morphology of apoptosis was confirmed by the observation of nuclear fragmentation, cell membrane blebbing, and F-actin cytoskeleton disruption under a fluorescence microscope.

2.5. The analysis of sub-G₁ fraction by flow cytometry

The sub-G₁ fractions were determined by flow cytometer. Briefly, RKO or SW480 cells were plated at a density of 1×10^6 cells per 60-mm Petri dish in complete medium for 16–20 h. At the end of treatment, the cells were collected and fixed with ice-cold 70% ethanol overnight at –20°C. The cell pellets were treated with 4 µg/ml PI solution containing 1% Triton X-100 and 100 µg/ml RNase for 30 min. To avoid cell aggregation, the cell solutions were filtrated through nylon membrane (Becton-Dickinson, San Jose, CA). The percentage of sub-G₁ fractions was quantified by using CellQuest software (Becton-Dickinson).

2.6. Immunofluorescence staining and confocal microscopy

To view the protein localization and expression of p53 and PUMA after celecoxib treatment, the cells were subjected to immunofluorescence staining and confocal microscopy as described [21]. After fixation with 4% paraformaldehyde solution, the cells were washed three times with PBS, and non-specific binding sites were blocked in PBS containing 10% FBS and 0.3% Triton X-100 for 1 h. Thereafter, the cells were separately incubated with rabbit anti-phospho-p53 (1:100), mouse anti-p53 (1:50) and goat anti-PUMA (1:250) antibodies in PBS containing 10% FBS overnight at 4°C, and washed three times with 0.3% Triton X-100 in PBS. Then the cells were incubated with goat anti-rabbit Cy5 (1:100), goat anti-mouse FITC (1:50), or donkey anti-goat FITC (1:50) in PBS for 2.5 h at 37°C. F-actin and nuclei were stained with BODIPY FL phalloidin and Hoechst 33258, respectively. The samples

were examined under a laser scanning confocal microscope (Leica, Wetzlar, Germany).

2.7. Mitochondrial hyperpolarization

Mitochondrial function was evaluated by the cells stained with the mitochondrial sensitive probe DiOC6(3). Lipophilic cation DiOC6(3) accumulated in the mitochondrial matrix driven by the electrochemical gradient [22]. RKO cells were cultured in 60-mm Petri dish at a density of 1×10^6 cells for 12–16 h. After treatment with or without celecoxib, the cells were washed with ice-cold PBS. The cells were trypsinized and collected by centrifugation. The cell pellets were fixed in 70% ethanol at -20°C for 2 h. Then cell pellets were incubated with 500 nM DiOC6(3) at 37°C for 30 min (in the dark). Finally, the collected cell pellets were resuspended in 0.5 ml ice-cold PBS and analyzed by flow cytometer (FACScan, Becton Dickinson, San Jose, CA).

2.8. Western blot

The total cellular protein extracts were prepared as described [21]. Briefly, proteins were separated on 10–12% sodium dodecyl sulfate-polyacrylamide gels, and electrophoretic transfer of proteins onto polyvinylidene difluoride membranes. The membranes were sequentially hybridized with primary antibody and followed with a horseradish peroxidase-conjugated secondary antibody. The protein bands were visualized on the X-ray film using the enhanced chemiluminescence detection system (PerkinElmer Life and Analytical Sciences, Boston, MA). Western analyses of various phospho-p53, total p53, PUMA, and ERK-2 were performed using specific antibodies. To verify equal protein loading and transfer, ERK-2 was used as the protein loading control. A gel digitizing software, Un-Scan-It gel (Ver. 5.1, Silk Scientific, Inc.), was used to analyze the intensity of bands on X-ray film by semi-quantification.

2.9. Transfection

The cells (5×10^5 cells/35-mm Petri dish) were transfected with control siRNA or p53 siRNA by using Lipofectamine™ 2000 (Invitrogen) in serum-free DMEM medium according to the manufacturer's recommendations.

2.10. Statistical analysis

Each experiment was repeated at least three times. Data were analyzed by one-way or two-way analysis of variance (ANOVA), and further post hoc tests using the statistic software of GraphPad Prism 4 (GraphPad software, Inc. San Diego, CA). A p value of <0.05 was considered as statistically significant in each experiment. Differences between control and celecoxib-treated samples were compared by one-way ANOVA with post hoc Tukey's tests. Differences between two different genotype cell lines from control and celecoxib treatments were compared by two-way ANOVA with Bonferroni post-tests.

3. Results

3.1. The existence of functional p53 but not securin in colorectal cancer cells increases cytotoxicity after treatment with celecoxib

A variety of human colorectal cancer cell lines including RKO, SW480, HCT116 (securin-wild type), and HCT116 (securin-null) expressed the high levels of COX-2 proteins (Fig. 1A). The protein levels of p53 presented in these cell lines; however, SW480 cells expressed the mutant p53 (contained a G to A mutation in codon 273 and a C to T mutation in codon 309). The securin proteins existed in RKO, SW480, and HCT116 (securin-wild type) cells but did not express in the securin-null HCT116 cells (Fig. 1A). ERK-2

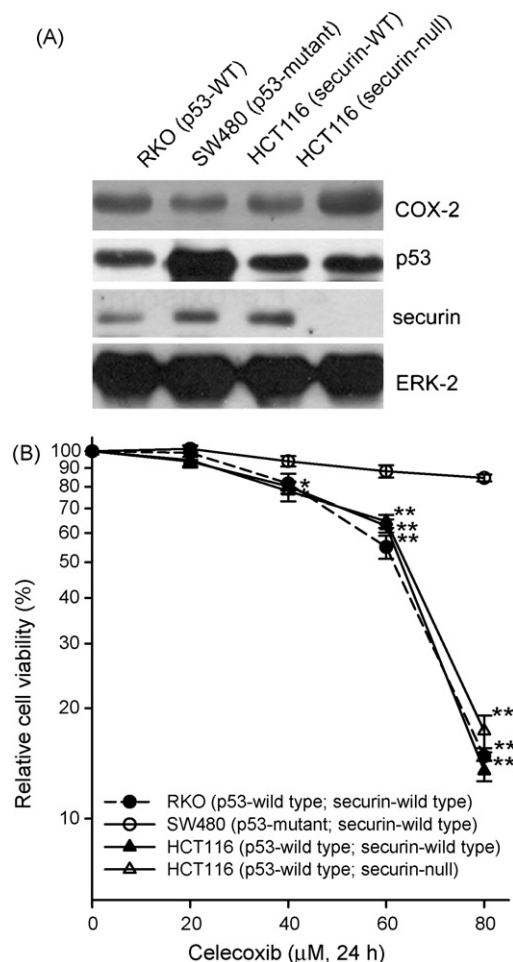


Fig. 1. The effect of celecoxib on the cell viability in a variety of human colorectal cancer cell lines. (A) The total protein extracts from various colorectal cancer cell lines were subjected to Western blot analysis using specific anti-COX-2, anti-p53, anti-securin, and anti-ERK-2 antibodies. Representative Western blot data were shown from one of three separate experiments with similar findings. (B) The cells were treated with 0–80 μM celecoxib for 24 h. After drug treatment, the cells were washed with PBS, and incubated for 2 days before they were measured by MTT assay. Results were obtained from 6 to 12 experiments and the bar represents the mean \pm S.E. * $p < 0.05$ and ** $p < 0.01$, indicate significant difference between control and celecoxib treated samples in various colorectal cancer cell lines.

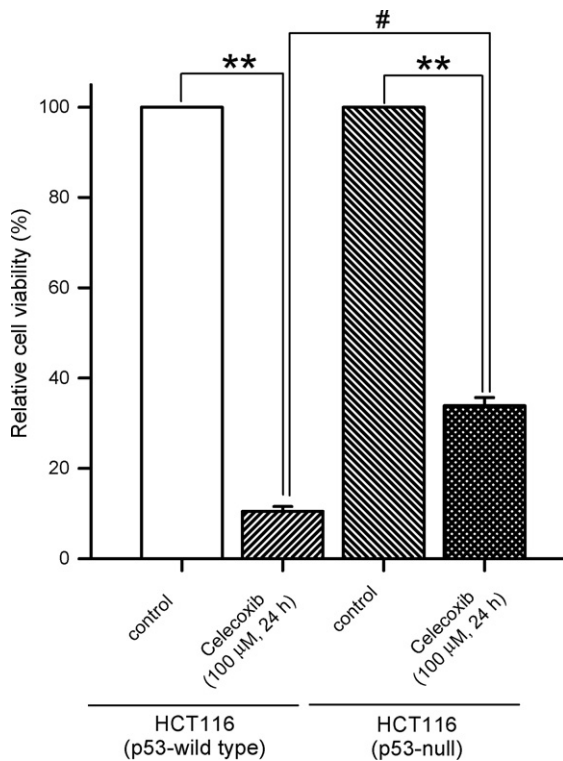


Fig. 2. The comparison of cytotoxicity between the p53-wild type and p53-null HCT116 colorectal cancer cells following celecoxib treatment. The cells were treated with or without 100 μM celecoxib for 24 h. After treatment, the cell viability was measured by MTT assay. Results were obtained from 6 to 7 experiments and the bar represents the mean ± S.E. ** $p < 0.01$, indicates between the control and celecoxib treated samples in the p53-wild type or p53-null HCT116 cells. # $p < 0.05$, indicates between the p53-wild type and p53-null HCT116 cells by the control and celecoxib treated samples.

was used as a loading control protein. The roles of p53 and securin on the regulation of cytotoxicity in human colorectal cancer cells were investigated by exposure to celecoxib. Celecoxib (40–80 μM, 24 h) significantly induced cancer cell death in the p53-wild type cell lines including RKO, securin-wild type and securin-null HCT116 but not SW480 cells (Fig. 1B). Moreover, the securin-wild type HCT116 cells were similar to the securin-null HCT116 cells on the cytotoxicity following treatment with celecoxib (Fig. 1B). To further determine the role of p53 on the celecoxib-induced colorectal cancer cell death. We have further compared the p53-wild type and p53-null HCT116 cells on the celecoxib-induced cytotoxicity. As shown in Fig. 2, both the p53-wild type and p53-null cells were reduced the cell viability following celecoxib treatment. However, the p53-wild type cells were more susceptible to increase ~25% cell death than the p53-null cells after treatment with 100 μM celecoxib for 24 h (Fig. 2).

3.2. Celecoxib increases the total p53 protein expression and p53 phosphorylated proteins

Treatment with 40–100 μM celecoxib for 24 h increased total p53 proteins and the phosphorylated proteins of

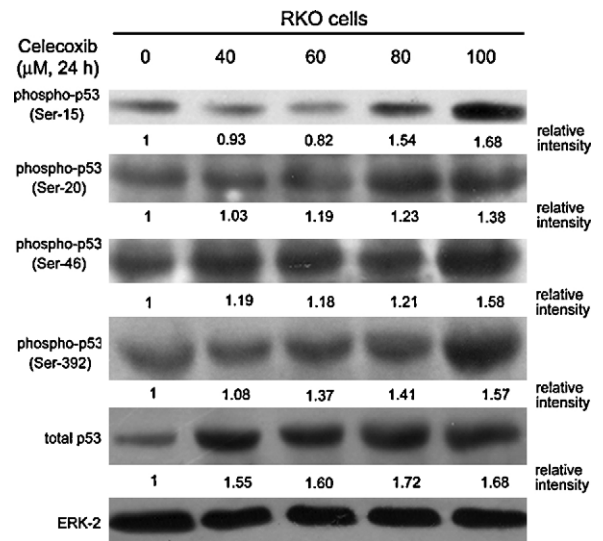


Fig. 3. The effect of celecoxib on the protein levels of total p53 and various phospho-p53 in RKO cells. The cells were treated with 0–100 μM celecoxib for 24 h. The total protein extracts were subjected to Western blot analysis using specific anti-p53 and anti-phospho-p53 (serine-15, -20, -46, or -392) antibodies. The relative protein intensities under each blot were the average of three-independent experiments.

p53 at serine-20, -46, and -392 (Fig. 3). The protein phosphorylation of p53 (serine-15) was elevated at the relative higher concentrations of 80–100 μM celecoxib ($p < 0.05$) (Fig. 3). Celecoxib slightly reduced the phospho-p53 (serine-15) proteins at 40–60 μM treatment but not significant difference after statistic analysis ($p > 0.05$). ERK-2 protein was used as an internal control, which was not altered by celecoxib. To further vision the localization of phosphorylated p53 proteins after treatment with celecoxib, the cells were subjected to immunofluorescence staining and confocal microscopy. In these experiments, we therefore selected the concentrations of celecoxib at range 80–100 μM by inducing the high levels of phospho-p53 proteins in colorectal cancer cells. The red fluorescence (Cy5) of phospho-p53 (serine-15) proteins was increased by celecoxib in RKO cells (Fig. 4). The phosphorylated p53 (serine-15) proteins were concentrated and located on the nuclei in the celecoxib-treated cells (Fig. 4, arrows). The phosphorylated p53 proteins at serine-20 and -392 were also located on the nuclei by celecoxib treatment (data not shown). However, the protein phosphorylation of p53 (serine-46) was located in the cytoplasm of the celecoxib-induced apoptotic cells (Fig. 5, red color). Celecoxib induced nuclear fragments (Figs. 5 and 6A, arrows) and the disruption of F-actin cytoskeleton and membrane blebbing (Figs. 5 and 6A, stars).

3.3. The existence of p53 in colorectal cancer cells increases apoptosis by celecoxib treatment

To investigate the role of p53 on apoptotic induction in the celecoxib-exposed cells, the p53-wild type and

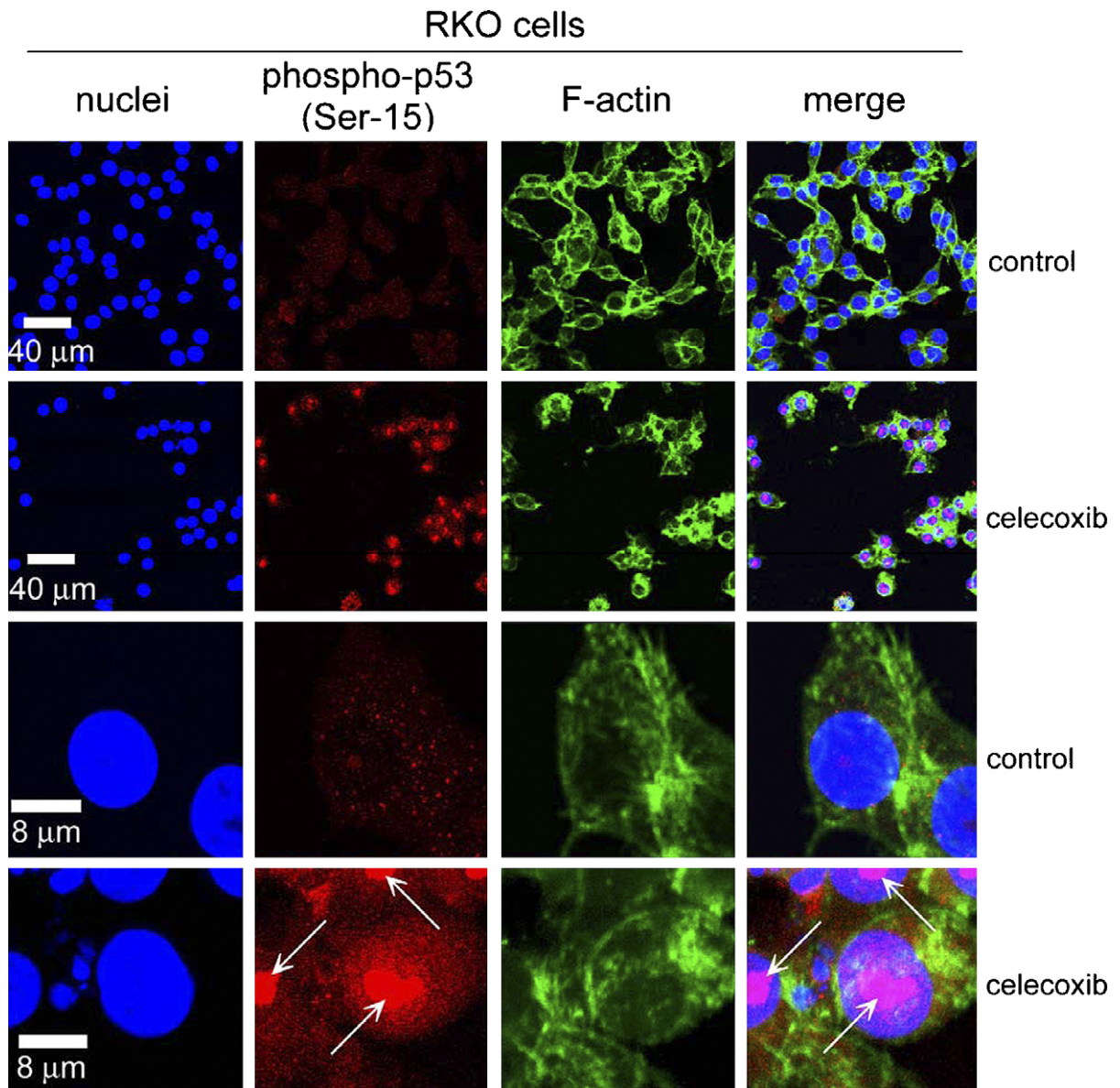


Fig. 4. The expression and localization of phospho-p53 (serine-15) proteins following treatment with celecoxib in RKO cells. The cells were treated with or without 80 μM celecoxib for 24 h. After celecoxib treatment, the cells were incubated with rabbit anti-phospho-p53 (serine-15) antibodies and then incubated with goat anti-rabbit Cy5. F-actin and nuclei were stained with BODIPY FL phalloidin and Hoechst 33258, respectively. The arrows indicate phospho-p53 (serine-15) proteins located in the nuclei.

p53-mutant colorectal cancer cells were compared with apoptosis by treatment with celecoxib. Celecoxib induced the cytoskeleton disruption and apoptotic nuclei in RKO cells (Fig. 6A). Counting the apoptotic cell number, celecoxib (60 μM for 24 h) significantly increased apoptosis in RKO cells; however, the apoptotic cell number was not altered by celecoxib in SW480 cells (Fig. 6B). Moreover, treatment with 40–80 μM celecoxib for 24 h increased the sub- G_1 fractions via a concentration-dependent manner in RKO cells but not in SW480 cells by flow cytometry analysis (Fig. 6C). In addition, transfection with a p53 siRNA (50 nM, 24 h) partially restored the celecoxib-induced cell death in RKO cells (Fig. 6D).

3.4. The existence of wild type p53 in colorectal cancer cells elevates the PUMA protein expression following celecoxib treatment

We have examined the effect of celecoxib on the PUMA protein expression in human colorectal cancer cells. Celecoxib increased PUMA protein expression in RKO cells but not SW480 cells (Fig. 7A). The semi-quantified data from Western blot showed that celecoxib (40–100 μM for 24 h) significantly increased the protein levels of PUMA via a concentration-dependent manner in RKO cells (Fig. 7B). To investigate the expression and location of PUMA proteins relative to mitochondrial damage, the cells

RKO cells

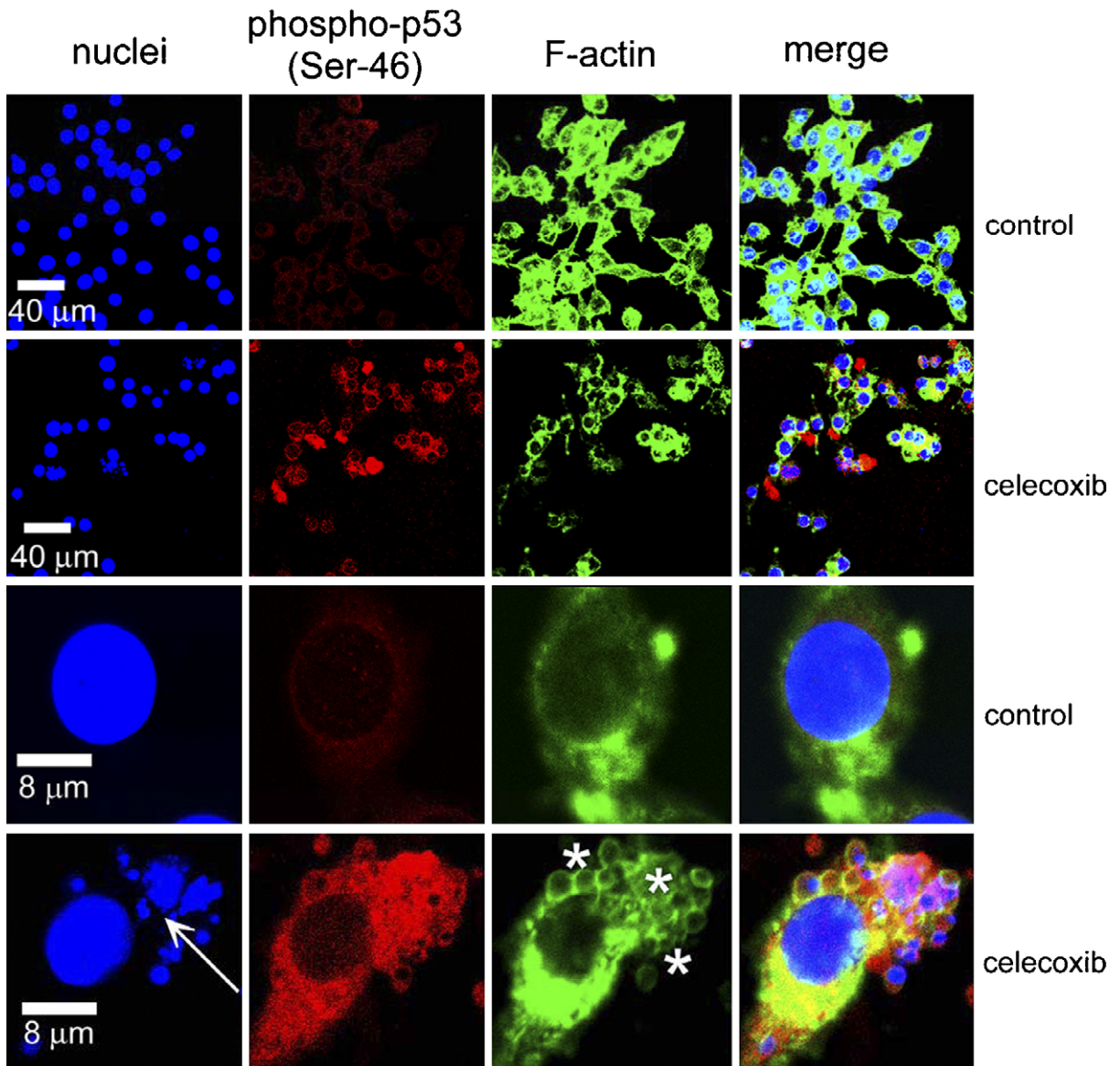


Fig. 5. The expression and localization of phospho-p53 (serine-46) proteins following treatment with celecoxib. RKO cells were treated with or without 100 μ M celecoxib for 24 h. After celecoxib treatment, the cells were incubated with rabbit anti-phospho-p53 (serine-46) antibodies and then incubated with goat anti-rabbit Cy5. F-actin and nuclei were stained with BODIPY FL phalloidin and Hoechst 33258, respectively. The arrow indicates the location of nuclear fragments. The stars indicate the location of the disrupted cytoskeleton of F-actin and the membrane blebbing. (For interpretation of the references to color in the citation of figure, the reader is referred to the web version of the article.)

were subjected to immunofluorescence and mitochondrial staining. Celecoxib increased the green fluorescence intensities of FITC exhibited by PUMA proteins in RKO cells (Fig. 8A). The fluorescence intensities of Mitotracker orange (a mitochondria-sensitive probe) were elevated in the celecoxib-treated cells. The yellow color of the merged pictures indicated the co-localization of PUMA proteins and mitochondria (Fig. 8A, arrows). Similar to Mitotracker orange, celecoxib also increased the fluorescence intensity of DiOC6(3) in RKO cells (Fig. 8B). The quantified data showed that treatment with celecoxib (40–100 μ M for

24 h) significantly increased the levels of DiOC6(3) intensities in a concentration-dependent manner (Fig. 8B).

4. Discussion

This is the first report providing that celecoxib induces the various phosphorylated sites of p53 proteins, which located on both of cytoplasm and nuclei in human colorectal cancer cells. Celecoxib induced the phosphorylated p53 proteins (serine-15, -20, and -392) presenting on the nuclei but the phosphorylated p53 (serine-46)

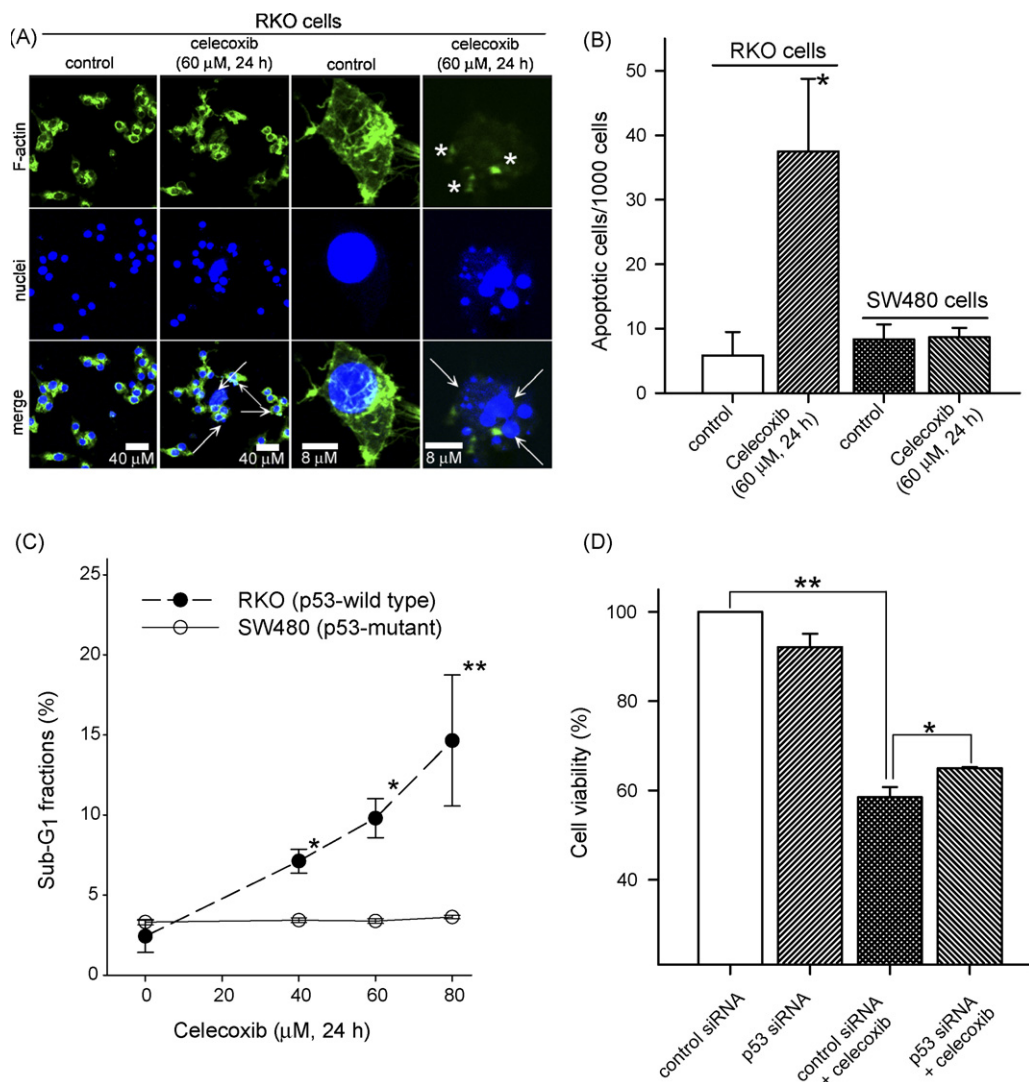


Fig. 6. The effect of celecoxib on apoptosis in colorectal cancer cells. (A) RKO cells were treated with or without 60 μM celecoxib for 24 h. The F-actin and nuclei were stained with BODIPY FL phalloidin and Hoechst 33258, respectively. The arrows indicate the apoptotic nuclei and nuclear fragments. The stars indicate the cytoskeleton disruption of F-actin. (B) The cells were treated with or without 60 μM celecoxib for 24 h. The percentage of apoptosis was scored by the apoptotic cells under a fluorescence microscope. Results were obtained from three experiments and the bar represents the mean ± S.E. * $p < 0.05$ indicates significant difference between control and celecoxib treated samples in RKO cells. (C) After celecoxib treatment, the cells were trypsinized and then subjected to flow cytometry analyses. The populations of sub-G₁ phases were quantified. * $p < 0.05$ and ** $p < 0.01$, indicate significant difference between RKO and SW480 from the same concentrations of celecoxib. D, RKO cells were transfected with 50 nM control siRNA or p53 siRNA for 24 h. After transfection, the cells were treated with or without 60 μM celecoxib for 24 h. The cell viability was measured by MTT assay. * $p < 0.05$ indicates between control siRNA and p53 siRNA transfection in the celecoxib-treated cells. ** $p < 0.01$ indicates significant difference between untreated and celecoxib treated samples by control siRNA transfection. The bar represents the mean ± S.E.

proteins were located on the cytoplasm of apoptotic cells. The diverse phosphorylation sites of p53 have been indicated to play different roles by the cellular stresses [13–15]. The phosphorylation of p53 at serine-15 and -20 sites have been shown to cause the activation and stabilization of p53 proteins [13,23,24]; however, the phosphorylation of p53 at serine-46 mediates the induction of apoptosis [14,25]. The tumor suppressor p53 has been proposed as an important target for cancer treatment [26,27]. Development of anticancer strategies by transcription-dependent and transcription-independent p53 functions is useful for cancer therapy [27]. p53 is a transcription

factor, which presents on nuclei to regulate its downstream gene expression, but the cytoplasmic p53 may be through the transcription-independent proapoptotic effects of p53 on mitochondrial membrane damage for apoptosis [26,27]. We suggest that p53 proteins presenting in the nuclei may regulate its downstream gene expression after treatment with celecoxib; however, p53 locating in the cytoplasm may interact with signal proteins for executing apoptosis. Nevertheless, the precise mechanisms of varying phosphorylated p53 sites induced by celecoxib on the regulation of apoptosis need to be further determined.

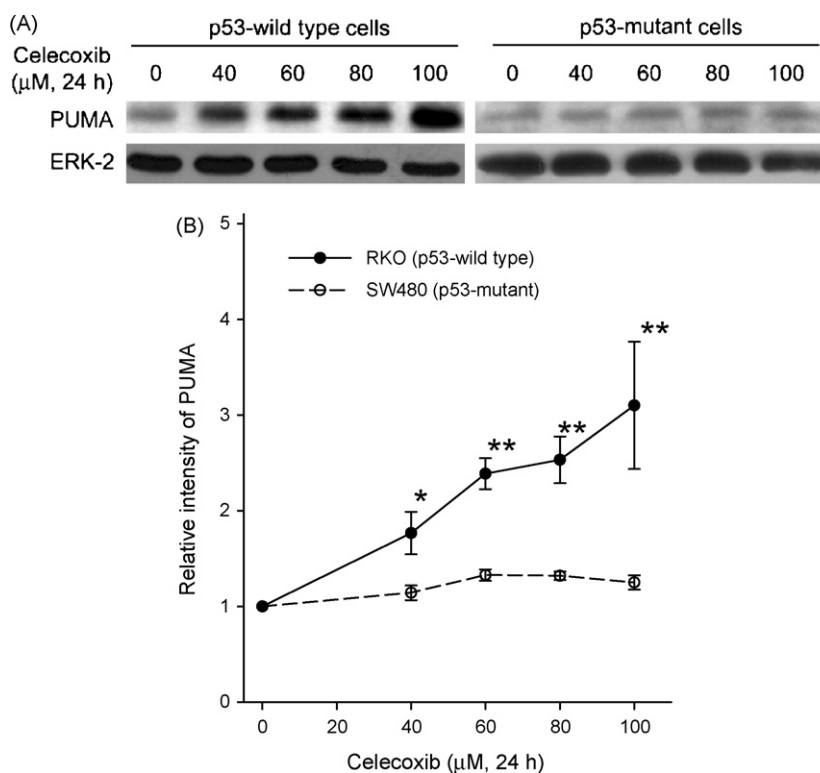


Fig. 7. The effect of celecoxib on PUMA protein expression in the p53-functional and p53-mutational colorectal cancer cells. (A) RKO and SW480 cells were treated with or without celecoxib for 24 h. The total protein extracts were subjected to Western blot analysis using anti-PUMA and anti-ERK-2 antibodies. (B) The relative protein intensities of PUMA were from Western blot by semi-quantification. * $p < 0.05$ and ** $p < 0.01$, indicate significant difference between RKO and SW480 from the same concentrations of celecoxib.

The physiologic events of p53 are mediated through its downstream genes [10–12]. PUMA belongs BCL-2 family and is a key mediator to trigger apoptosis via a p53-dependent pathway [16]. PUMA can sensitize cancer cells to anticancer agents [17,18]. We found that celecoxib induced the PUMA protein expression in the p53-functional cells but not in the p53-mutational cells. Active cytochrome *c* release is associated with early mitochondrial hyperpolarization that relates to caspase activation and apoptosis [28,29]. Celecoxib induced the activation of caspase-3 and caspase-9 that mediated by mitochondrial pathway in cancer cells [6,30]. We found that celecoxib increased the PUMA proteins, which localized on the mitochondria. The fluorescence intensities of Mitotracker orange and DiOC6(3) in colorectal cancer cells were elevated by celecoxib indicating celecoxib-induced mitochondrial hyperpolarization. However, we cannot exclude that celecoxib may induce the loss of mitochondrial membrane potential during late apoptotic stages. The up-regulation of PUMA by celecoxib has been shown to induce apoptosis in human gastric carcinoma cells [31]. The gene knockdown of PUMA by siRNA inhibited the celecoxib-induced apoptosis via a mitochondrial dysfunction pathway [31]. We also found that celecoxib induced the caspase-3 activation for apoptosis in colorectal cancer cells [8]. Accordingly, our findings suggest that the activation of p53-PUMA may be through the mitochondrial pathway for inducing apoptosis in the celecoxib-treated colorectal cancer cells. In addition

to PUMA, for example, Bax is another important downstream of p53 by inducing mitochondrial dysfunction for apoptosis [32]. Thus, the effects of Bax and other p53 downstream proteins on the regulation of celecoxib-induced apoptosis in human colorectal cancer cells need further investigation.

The p53-functional colorectal cancer cells are more vulnerable than p53-mutational cells to the celecoxib-induced cytotoxicity and apoptosis. Blockade of p53 by transfection with a p53 siRNA reduced the celecoxib-induced cell death. Although statistically significant between control siRNA and p53 siRNA transfection in the celecoxib-treated cells, it only restored ~8% survival by treatment with p53 siRNA (Fig. 6D). We have compared the p53-wild type and p53-null colorectal cancer cells on the celecoxib-induced cytotoxicity. Celecoxib induced the cytotoxicity in both p53-wild type and p53-null HCT116 cells. The p53-wild type cells were more susceptible to increase ~25% cell death than the p53-null cells after treatment with celecoxib (Fig. 2). These results indicate that p53 pathway is partially involved in the celecoxib-induced apoptosis in human colorectal cancer cells. Indeed, we have reported that celecoxib induced p38 MAP kinase pathway to induce apoptosis in colorectal cancer cells [8]. Blockage of survivin protein expression by celecoxib caused the caspase-3 activation and apoptosis [8]. Additionally, the 3-phosphoinositide-dependent protein kinase-1/Akt signaling pathway mediates celecoxib-induced apoptosis in

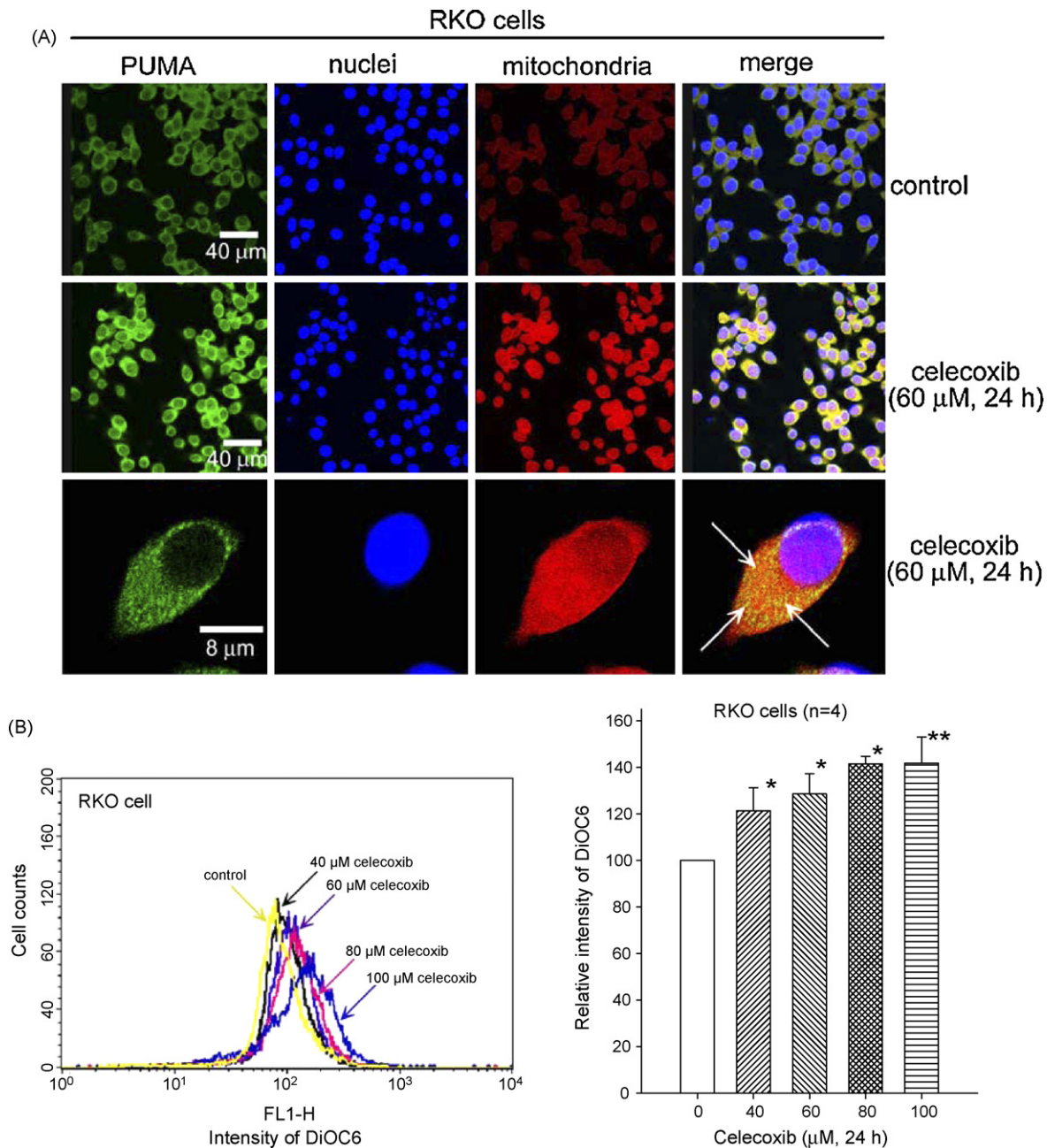


Fig. 8. The effect of celecoxib on the location of PUMA and the hyperpolarization of mitochondria in colorectal cancer cells. (A) RKO cells were treated with or without 60 μM celecoxib for 24 h. The cells were incubated with goat anti-PUMA antibody and subsequently incubated with donkey anti-goat FITC. Mitochondria and nuclei were stained with Mitotracker orange and Hoechst 33258, respectively. The yellow color indicated the localization of PUMA on mitochondria (arrows). (B) After treatment with or without celecoxib, the cells were incubated with 500 nM DiOC6(3) and then analyzed by a flow cytometer. The fluorescence intensities of DiOC6(3) were quantified by a CellQuest software. Results were obtained from 4 experiments and the bar represents the mean ± S.E. * $p < 0.05$ and ** $p < 0.01$, indicate significant difference between control and celecoxib treated samples. (For interpretation of the references to color in the citation of figure, the reader is referred to the web version of the article.)

prostate cancer cells [33]. We suggest that celecoxib can induce different signal transduction pathways for inducing apoptosis. Nonetheless, celecoxib elevated the cell death at similar degree in both of the securin-wild type and securin-null colorectal cancer cells. Therefore, celecoxib induces apoptosis via a securin-independent pathway in colorectal cancer cells.

Celecoxib exhibits high selectivity for the COX-2 enzyme and exerts anticarcinogenic and chemopreventive activities [2–4]. However, Pyrko et al. recently reported that the induction of apoptosis by celecoxib via a COX-independent pathway [34]. In addition, celecoxib mediates cell cycle arrest and apoptosis via COX-2-independent pathways in human cholangiocarcinoma and prostate cancer cells

[33,35]. We found that various genotype of human colorectal cancer cell lines expressed high levels of COX-2 proteins (Fig. 1A). Moreover, the phosphorylated p53 and apoptosis were induced at the relative higher concentrations of celecoxib (80–100 μ M). Therefore, we suggest that the activation of p53 and apoptosis by celecoxib may result from a COX-2-independent pathway in colorectal cancer cells.

In summary, we provide the p53-PUMA pathway that plays an important role for celecoxib-induced apoptosis in human colorectal cancer cells. Understanding the mechanisms by which p53-PUMA and other signal transduction pathways regulate apoptosis following treatment with celecoxib may contribute to the novel therapeutic strategies in colorectal cancers.

Conflict of interest

None declared.

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