

Protein kinase A activation by β -Lapachone is associated with apoptotic cell death in NQO1-overexpressing breast cancer cells

SAHIB ZADA¹, JIN SEOK HWANG¹, MAHMOUD AHMED¹, TRANG HUYEN LAI¹,
TRANG MINH PHAM¹, DONG-HEE KIM² and DEOK RYONG KIM¹

Departments of ¹Biochemistry and Convergence Medical Sciences, ²Orthopaedic Surgery, Institute of Health Sciences,
Gyeongsang National University School of Medicine, Jinju, South Gyeongsang 527-27, Republic of Korea

Received March 6, 2019; Accepted June 28, 2019

DOI: 10.3892/or.2019.7243

Abstract. One million females are diagnosed worldwide every year with breast cancer, and the mortality rate of these patients remains high. Several treatments, including surgery, are available for breast cancer. β -Lapachone (β -Lap), a natural quinone compound, has been developed for cancer treatment due to its strong cytotoxic effect through its action on NAD(P)H:quinone oxidoreductase 1 (NQO1)-dependent activity. However, the mechanism in regards to how β -Lap induces cytotoxicity in breast cancer cells is still elusive. In the present study, we showed that β -Lap induced apoptotic cell death via activation of protein kinase A (PKA) in NQO1-overexpressing MDA-MB-231 human breast cancer cells. This PKA-dependent cell death was observed solely in NQO1-overexpressing 231 cells via the high production of reactive oxygen species (ROS). Cell survival of antioxidant [N-acetylcysteine (NAC)]-treated NQO1-overexpressing 231 cells was significantly recovered, and NQO1-negative 231 cells did not respond to β -Lap. Antiapoptotic proteins such as Bcl2 and Bcl-xL were decreased, while proapoptotic proteins, including cytochrome c, activation of caspase-3, and cleavage of PARP were increased after β -Lap treatment of NQO1-overexpressing 231 cells. Furthermore, PKA activators, forskolin or dibutyryl-cAMP, an analog of cAMP, aggravated the β -Lap-induced apoptotic cell

death by decreasing antiapoptotic proteins and further activating proapoptotic proteins in NQO1-positive 231 cells. Treatment with a PKA inhibitor, H89, significantly increased cell viability even in NQO1-overexpressing cells treated with β -Lap. These data showed that β -Lap activated PKA via ROS accumulation, subsequently leading to apoptotic cell death in NQO1-positive breast cancer cells.

Introduction

Cancer, a multi-step disease, is characterized by increased cell division, dysregulation of cell growth and resistance to cell death. Increases in oxygen consumption rates in cancer cells leads to hypoxia and consequently stimulates angiogenesis. In particular, breast cancer is both a genetic disease as well as a multiple factor-associated disease (1-4). It is the foremost cause of cancer-related death corresponding to 23% of all cancer incidents in women, and worldwide, one million women are diagnosed with breast cancer and about half of them succumb to the disease every year (5,6). More than 8% of women present with invasive breast cancer that can be associated with a high death rate, and unfortunately, this percentage is expected to rise to 26% in 2020 (7,8). For this reason, it is necessary to develop new strategies for the treatment of breast cancer. However, it is difficult to discover new strategies for breast cancer due to its high potential of metastasis (9).

Apoptosis, programmed cell death, plays a crucial role in the development and maintenance of cellular homeostasis. However, dysregulation of this process has been implicated in various diseases including cancer (10,11). Cancer cells employ many strategies to resist apoptotic cell death and to combat the host immune system, and many anticancer therapies have been targeted to the activation of cell survival signals (12). Protein kinase A (PKA), composed of two regulatory and two catalytic subunits, is activated upon stimulation by various extracellular or intracellular signals. Cyclic AMP (cAMP), a key intracellular mediator, binds to the PKA regulatory subunits, and the consequent dissociation of catalytic subunits results in PKA activation. PKA activity is specifically blocked by the H-89 inhibitor, derived from H-8 N-[2-(methylamino)ethyl]-5-isoquinoline-sulfonamide (13,14). Besides its general role in antiapoptotic cell death, PKA is also involved in the enhancement of apoptosis (15-17). In particular, in certain cell death mechanisms, PKA directly induces apoptosis

Correspondence to: Professor Deok Ryong Kim, Department of Biochemistry and Convergence Medical Sciences, Gyeongsang National University School of Medicine, Jinju, South Gyeongsang 527-27, Republic of Korea
E-mail: drkim@gnu.ac.kr

Abbreviations: β -Lap, β -Lapachone; NQO1, NAD(P)H:quinone oxidoreductase 1; PKA, protein kinase A; ROS, reactive oxygen species; NAC, N-acetylcysteine; cAMP, cyclic AMP; RPMI, Roswell Park Memorial Institute 1640 medium; FBS, fetal bovine serum; DCFDA, 2',7'-dichlorofluorescein diacetate; CCK-8, Cell Counting Kit-8; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Key words: apoptosis, PKA, β -lapachone, ROS, NQO1, breast cancer cells

even in the absence of cAMP (18). The role of cAMP-dependent signals has also been investigated in regards to both cell death and survival (19,20). Moreover, various protein regulators in the cAMP signaling pathway have been proposed as possible therapeutic targets to stimulate apoptosis for the treatment of certain cancers (15-17). PKA-dependent apoptosis is mediated via either phosphorylation of targeted proteins involved in cell death or by activation of an intrinsic mitochondrial cell death pathway, in many cancers (17,21-26). Furthermore, cAMP can also sensitize cells to the proapoptotic action of agents, such as DNA damaging agents, via a non-cAMP pathway (27).

β -Lapachone (β -Lap), a quinone compound derived from the Lapacho tree (*Tabebuia impetiginosa*), has been shown to be highly effective in treating various types of cancer in experimental models, including liver cancer and melanoma (28-33). β -Lap reacts with a cellular enzyme, NAD(P)H:quinone oxidoreductase 1 (NQO1), which is overexpressed in many cancers (34-38). In particular, 84.7% of breast cancer tissues showed positive expression of NQO1 while only 30.8% of adjacent non-tumor tissues showed NQO1 expression (39). The reaction of β -Lap with NQO1 activates a futile cycle by consuming NADPH and generating ROS (33-36). ROS from the redox cycle of β -Lap also contribute to cellular toxicity in cancer cells (34,35). This demonstrates the importance of NQO1 in the anticancer action of β -Lap, especially in cancer cells highly expressing NQO1 (38-41). The differential role of NQO1-mediating redox activation by β -Lap, from the mitochondria to produce ROS, suggests this approach could be useful as a potential anticancer treatment (41,42). However, the mechanism underlying how β -Lap induces apoptosis is still unknown.

In the present study, we showed that production of ROS in NQO1-overexpressing breast cancer cells with highly activated PKA led to apoptotic cell death. In contrast, inhibition of PKA activity caused decreased apoptosis. We suggest that β -Lap may be a potential treatment strategy for NQO1-positive cancer cells as β -Lap-dependent PKA activation is specific to cancer cells, and is different from other cAMP-PKA activation pathway treatments that have harmful effects on normal cells.

Materials and methods

Reagents. Roswell Park Memorial Institute (RPMI)-1640 medium and fetal bovine serum (FBS) were purchased from Gibco Life Technologies/Thermo Fisher Scientific, Inc. (Waltham, MA, USA). β -Lapachone, NAC (*N*-acetylcysteine), DCFDA (2',7'-dichlorofluorescein diacetate), forskolin, dibutyryl-cAMP (cAMP-analog) and H89 were obtained from Sigma-Aldrich/Merck KGaA. The CCK-8 (Cell Counting Kit-8) was purchased from Dojindo (Tokyo, Japan). Primary antibodies against caspase-3 (cat. no. 9662; dilution 1:1,000), cleaved caspase-3 (cat. no. 9661; dilution 1:1,000), PARP (cat. no. 9542; dilution 1:1,000), and cleaved PARP (cat. no. 95411; dilution 1:1,000) were from Cell Signaling Technology (Beverly, MA, USA), and Bcl-2 (cat. no. 7382; dilution 1:1,000), Bcl-xL (cat. no. 56021; dilution 1:1,000), cytochrome *c* (cat. no. 13560; dilution 1:1,000), Bak (cat. no. 832; dilution 1:1,000), p-PKA α / β / γ T198 (cat. no. 32968; dilution 1:1,000) and PKA α (cat. no. 903; dilution 1:1,000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). β -actin was obtained from Sigma-Aldrich/Merck KGaA. Secondary antibodies

against rabbit, mouse and goat were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA).

Cell culture. Two breast cancer cell lines, MDA-MB-231 over-expressing NQO1 (231-NQO1^{+/+}) and MDA-MB-231 lacking NQO1 (231-NQO1^{-/-}), were described previously (43) and were provided by Dr David Boothman (UT Southwestern Medical Center, Dallas, TX, USA). These cells were cultured in RPMI medium supplemented with 5% (v/v) FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a 5% CO₂ humidified atmosphere.

Cell viability. Cell viability was determined by the CCK-8 kit. In brief, both 231-NQO1^{+/+} and 231-NQO1^{-/-} cells (5x10³ cells/well) were treated with β -Lap in the presence or absence of inhibitors or activators for 2 h in 96-well plates, and after removal of medium, were further incubated in fresh RPMI medium with 5% FBS for 4 h. CCK-8 reagent (10 μ l) was added into each well and the cells were further incubated for 4 h at 37°C in a 5% CO₂ humidified atmosphere. Absorbance was measured at 485 nm using a microplate reader (Hidex 1 FN/Chameleon; Turku, Finland).

Determination of intracellular ROS. Intracellular ROS were determined by a DCFDA cellular ROS detection assay kit (cat. no. ab113851; Abcam, Burlingame, CA, USA). Briefly, both 231-NQO1^{+/+} and 231-NQO1^{-/-} cells (5x10³ cells/well) were seeded into 96-well plates containing RPMI medium (200 μ l) supplemented with 5% FBS. After incubation for 24 h at 37°C, cells were treated with different concentrations (0, 2, 3 and 4 μ M) of β -Lap for 2 h, under the same conditions. After subsequent incubation for 4 h, 30 μ M DCFDA dissolved in DMSO/PBS was added to each well, followed by incubation for 30 min under light-free conditions. The plates were read using a GloMax[®] detection system (Model #E 8032; Promega, Sunnyvale, CA, USA) at 485/535 nm.

Western blot analysis. Cells were collected and washed twice with ice-cold 1X PBS. Total proteins were extracted with cell lysis buffer (cat. no. 87788; Pierce; Thermo Fisher Scientific, Inc.) containing protease and phosphatase inhibitor cocktails (Halt[™] Proteases & Phosphatase Single-Use Inhibitor Cocktail (100X; Thermo Scientific, Inc.), and the protein concentration was determined using the Pierce protein assay kit (Thermo Scientific, Inc.). Total protein lysates (30 μ g) were separated on a 10% SDS-PAGE gel, and the target proteins were specifically detected by western blotting using the indicated antibodies by incubating with primary antibodies at 4°C overnight and subsequently with secondary antibodies at room temperature for 1 h. Proteins were visualized with ECL Detection Reagent (Thermo Scientific, Inc.), and quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Protein level was corrected by β -actin normalization as the control value.

Statistical analysis. Each experiment was conducted independently at least three times, and values are expressed as the mean value \pm standard deviation (SD). The difference between two groups was assessed by a two-tailed Student's t-test. One-way analysis of variance (ANOVA) was used to compare the means of three groups or more, and each comparison of these groups

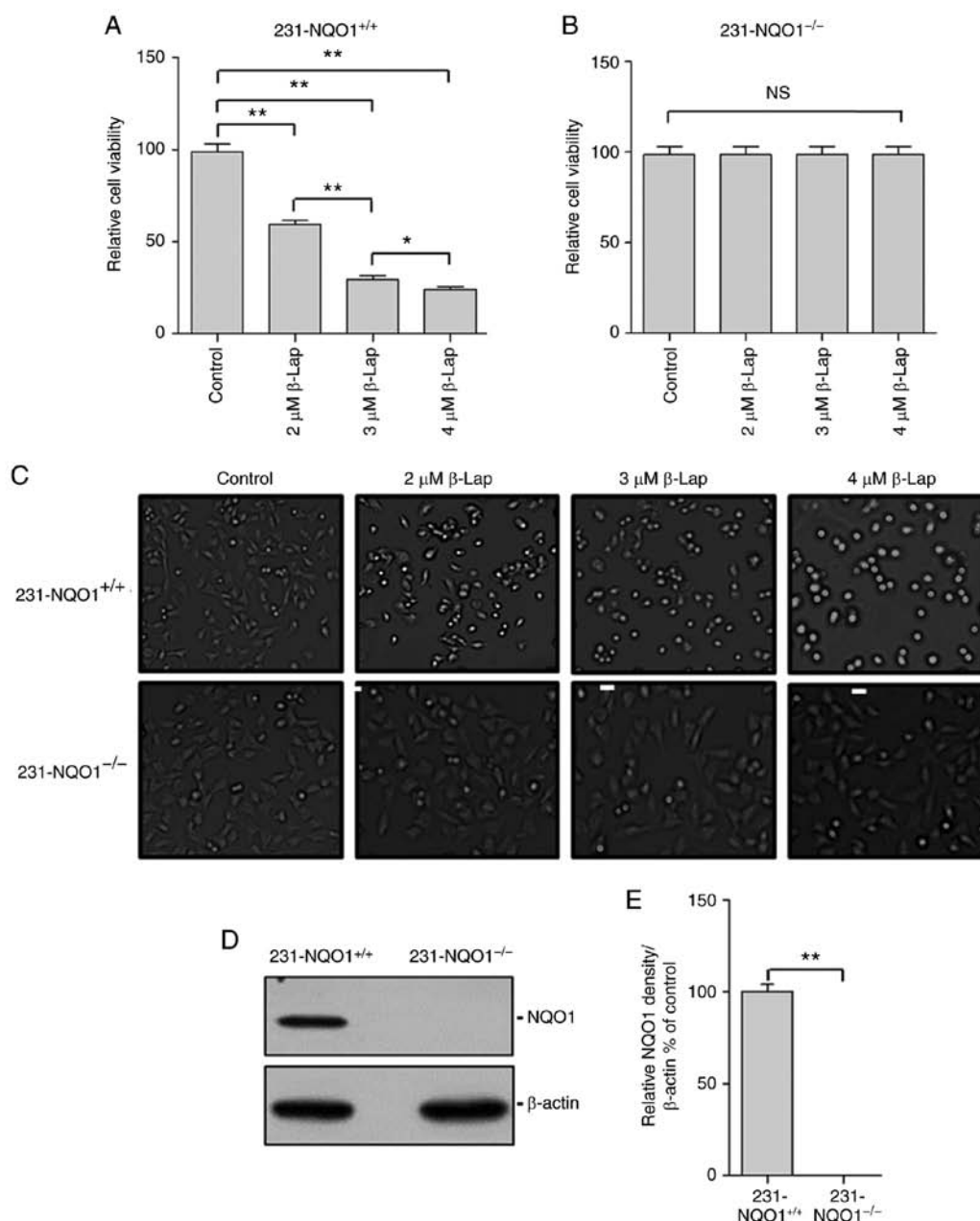


Figure 1. β -Lapachone (β -Lap) decreases the cell viability of NQO1-overexpressing human breast cancer cells in a dose-dependent manner. (A and B) Determination of cell viability. The 231-NQO1^{+/+} (A) and 231-NQO1^{-/-} (B) cells were treated with β -Lap (0, 2, 3 and 4 μ M) for 2 h. After further incubation in fresh RPMI medium with 5% FBS for 4 h, cell viability was determined by CCK-8 assay. (C) Cell morphological change. The 231-NQO1^{+/+} (upper panels) and 231-NQO1^{-/-} (lower panels) cells were treated with β -Lap at different concentrations as indicated. After incubation for 4 h, images were taken for cell morphology (x100 magnification) using bright-field microscopy. Scale bar, 10 μ m. (D and E) Determination of NQO1 expression. The 231-NQO1^{+/+} and 231-NQO1^{-/-} cells were subjected to western blot analysis using anti-NQO1 antibody (D) and results were quantified (E) NQO1, NAD(P)H:quinone oxidoreductase; 231-NQO1^{+/+}, NQO1-overexpressing MDA-MB-231 cells; 231-NQO1^{-/-}, MDA-MB-231 cells lacking NQO1. Data represent the mean (\pm SD) of three independent experiments (* P <0.05, ** P <0.01; NS, not significant).

was followed by multiple comparison Tukey's tests. Probability values of * P <0.05 and ** P <0.01 were considered significant (as indicated by the relevant symbols in the figure).

Results

Cell death by β -Lap in MDA-MB-231 breast cancer cells is dependent on NQO1 and increases in intracellular ROS. β -Lap, an anticancer drug that reacts against many cancers, selectively targets only those cells highly expressing NQO1 proteins, and β -Lap-induced cytotoxicity in these cancer cells

is dependent on the accumulation of ROS produced from the futile cycle reactions of β -Lap and NQO1 (31-40). To further investigate the mechanistic details of β -Lap-induced cell death in breast cancer cells, two syngeneic breast cancer cell lines, MDA-MB-231 overexpressing NQO1 (231-NQO1^{+/+}) and MDA-MB-231 lacking NQO1 (231-NQO1^{-/-}), were treated with different concentrations of β -Lap (0, 2, 3 and 4 μ M) for 2 h. The cell viability of 231-NQO1^{+/+} cells was significantly decreased in a β -Lap dose-dependent manner, while 231-NQO1^{-/-} cells maintained their viability regardless of β -Lap concentration (Fig. 1A and B) and microscopic

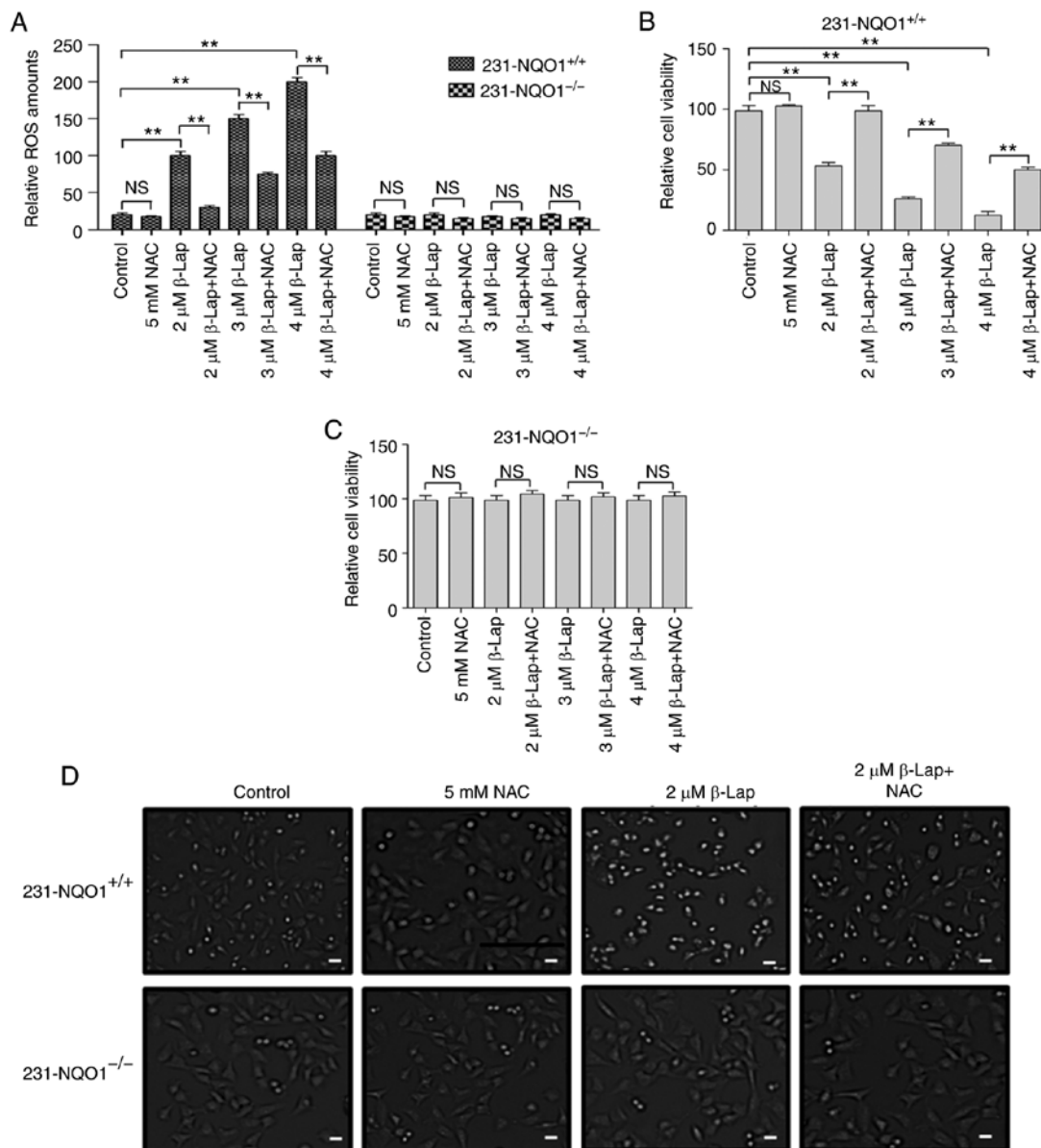


Figure 2. Decrease in β -Lapachone (β -Lap)-induced cell viability is dependent on production of ROS in NQO1-positive cells. (A) Determination of ROS. The 231-NQO1^{+/+} (left histogram) and 231-NQO1^{-/-} (right histogram) cells were treated with β -Lap (0, 2, 3 and 4 μ M) in the presence or absence of 5 mM *N*-acetylcysteine (NAC) for 2 h. After 4 h incubation in fresh RPMI medium with 5% FBS, intracellular ROS levels were determined. (B-D) Cell viability. The 231-NQO1^{+/+} and 231-NQO1^{-/-} cells were treated with β -Lap with/without 5 mM NAC for 2 h. Cell viability of 231-NQO1^{+/+} (B) and 231-NQO1^{-/-} (C) cells were determined as described previously. Data represent the mean (\pm SD) of three independent experiments (^{*}P<0.01; NS, not significant). (D) Images show the cell morphology of 231-NQO1^{+/+} (upper panels) and 231-NQO1^{-/-} (lower panels) cells as captured under a bright-field microscope. Scale bar, 10 μ m. NQO1, NAD(P)H:quinone oxidoreductase; 1231-NQO1^{+/+}, NQO1-overexpressing MDA-MB-231 cells; 231-NQO1^{-/-}, MDA-MB-231 cells lacking NQO1.

phenotype (Fig. 1C). According to the western blot analysis, 231-NQO1^{+/+} cells showed a high level expression of NQO1, while there was no expression of NQO1 in the 231-NQO1^{-/-} cells (Fig. 1D and E).

We further investigated whether a decrease in cell viability of 231-NQO1^{+/+} cells treated with β -Lap is due to the accumulation of cellular ROS. When 231-NQO1^{+/+} cells were treated with β -Lap (0, 2, 3 and 4 μ M), intracellular ROS levels were gradually increased in a dose-dependent manner, and were specifically inhibited by treatment with the ROS scavenger [5 mM NAC (*N*-acetyl cysteine)] (Fig. 2A). However, 231-NQO1^{-/-} cells showed no accumulation of ROS with β -Lap treatment (Fig. 2A). Cell viability and morphological changes under light microscopy were similarly observed in

both 231-NQO1^{+/+} and 231-NQO1^{-/-} cells treated with β -Lap in the presence of NAC, as shown by cell viability results (Fig. 2B-D). These data suggested that β -Lap specifically induced cell death in 231-NQO1^{+/+} breast cancer cells through stimulation of cellular ROS production.

β -Lap treatment in 231-NQO1^{+/+} cells induces apoptotic cell death via activation of PKA. According to many previous studies, β -Lap can induce apoptosis in a variety of cancer cells (31-38). In order to examine the mechanisms underlying β -Lap-mediated cell death, we compared the expression levels of antiapoptotic or apoptotic proteins in both 231-NQO1^{+/+} and 231-NQO1^{-/-} cells upon treatment with different concentrations of β -Lap (0, 2, 3 and 4 μ M). As expected, while the expression

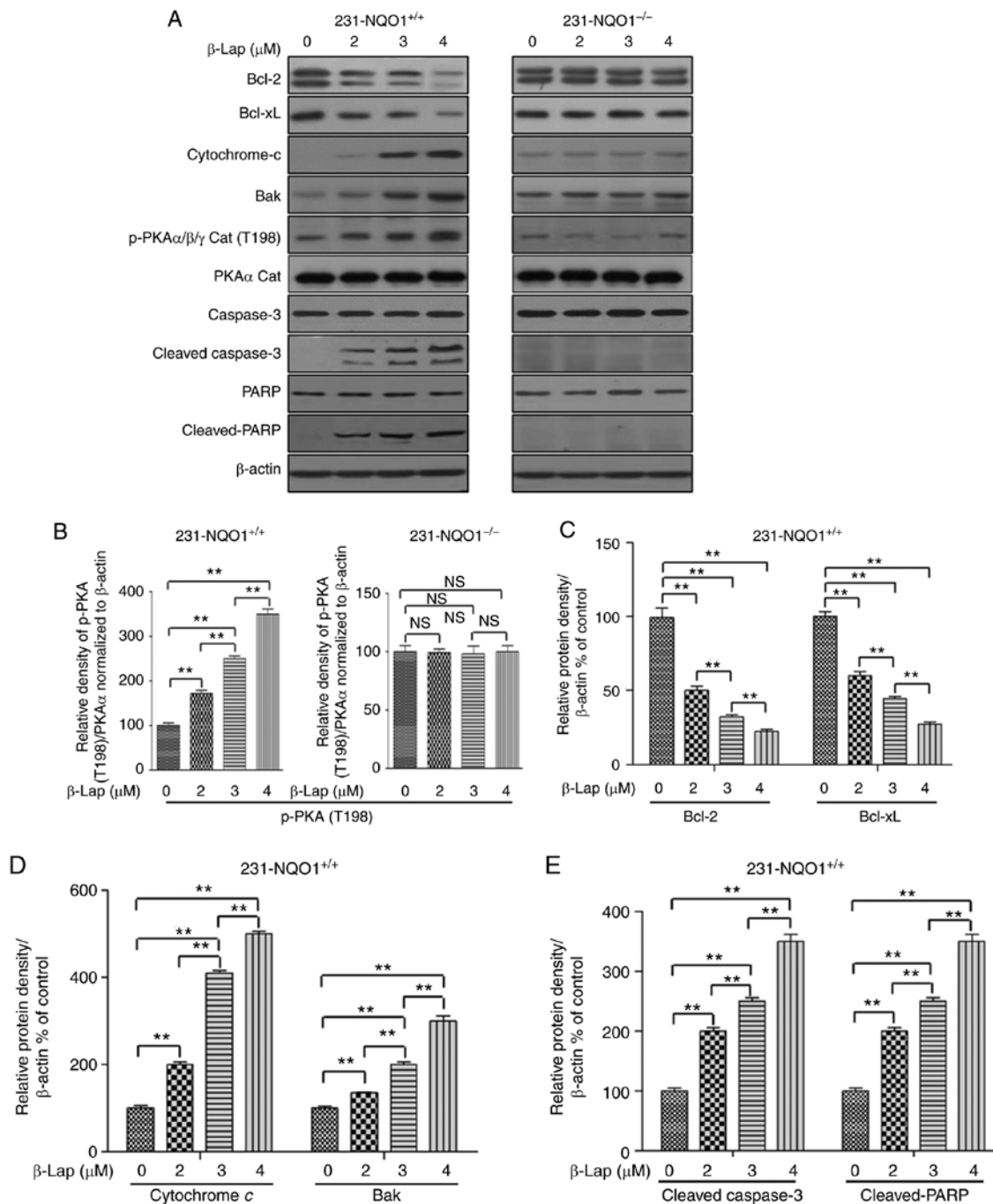


Figure 3. PKA is highly activated during β -Lapachone (β -Lap)-induced apoptosis. (A) Western blots. The 231-NQO1^{+/+} (left panel) and 231-NQO1^{-/-} (right panel) cells were treated with β -Lap (0, 2, 3 and 4 μ M) for 2 h and further incubated in fresh medium with 5% FBS for 4 h. After cell lysis, total cell extracts (30 μ g) were separated on 8 or 10% SDS-PAGE and analyzed by western blotting using primary antibodies against proteins (Bcl-2, Bcl-xL, cytochrome *c*, Bak, p-PKA α /β/γ cat, PKA cat, caspase-3, cleaved caspase-3, PARP and cleaved PARP). β -actin was used as a loading control. (B-E) Quantification of protein expression and activation. The relative amount of all proteins are shown in western blot analyses and were quantified by NIH ImageJ software and represented as a graph, respectively. The relative amount of p-PKA α /β/γ to PKA cat was determined first and subsequently normalized to β -actin. Data represent the mean (\pm SD) of three independent experiments (** P <0.01; NS, not significant). NQO1, NAD(P)H:quinone oxidoreductase; PKA, protein kinase A; Bcl-2, B-cell lymphoma-2; Bcl-xL, B-cell lymphoma-extra large; PARP, Poly(ADP-ribose) polymerase; 1231-NQO1^{+/+}, NQO1-overexpressing MDA-MB-231 cells; 231-NQO1^{-/-}, MDA-MB-231 cells lacking NQO1.

levels of antiapoptotic Bcl-2, and Bcl-xL proteins were gradually decreased with increased concentrations of β -Lap in 231-NQO1^{+/+} cells, the expression levels in NQO1-deficient cells (231-NQO1^{-/-}) were unchanged regardless of the level of β -Lap used (Fig. 3A and C). In contrast, proapoptotic Bak and cytochrome *c* were significantly increased in the 231-NQO1^{+/+} cells in a β -Lap dose-dependent manner, but this was not

observed in 231-NQO1^{-/-} cells (Fig. 3A and D). In addition, caspase-3 activation (cleaved caspase-3) and PARP cleavage was significantly stimulated when 231-NQO1^{+/+} cells were incubated with different concentrations of β -Lap (Fig. 3A and E). Importantly, we observed a significant increase in PKA activation (phosphorylated-PKA α /β/γ at T198) in β -Lap-treated 231-NQO1^{+/+} cells, but not in 231-NQO1^{-/-} cells (Fig. 3A and B).

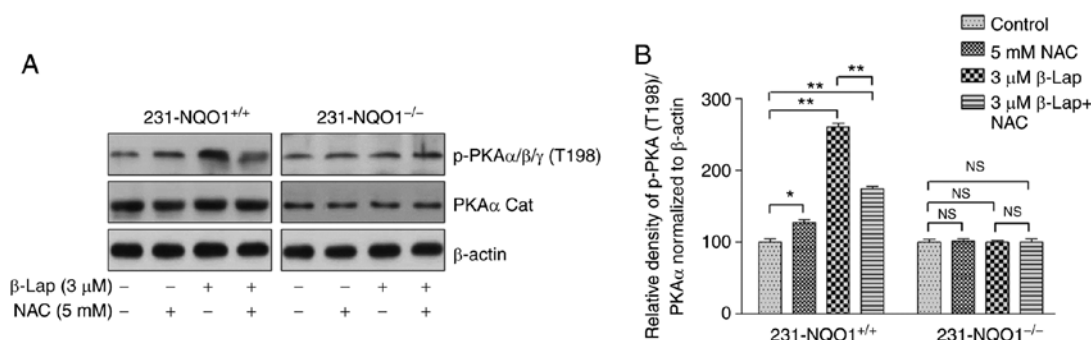


Figure 4. PKA activation is ROS-dependent. (A) The 231-NQO1^{+/+} (left panel) and 231-NQO1^{-/-} (right panel) cells were treated with β -Lapachone (β -Lap) (3 μ M) in the presence or absence of 5 mM N-acetylcysteine (NAC) for 2 h and further incubated in fresh medium with 5% FBS for 4 h. After cell lysis, total cell extracts (30 μ g) were separated on a 10% SDS-PAGE and analyzed by western blotting using the indicated antibodies. β -actin was used as a loading control. (B) The relative amount of all proteins were quantified by NIH ImageJ software and represented as a graph. The relative amount of p-PKA α / β / γ to PKA cat was determined first and subsequently normalized to β -actin. Data represent the mean (\pm SD) of three independent experiments (* P <0.05, ** P <0.01; NS, not significant). ROS, reactive oxygen species; PKA, protein kinase A; NQO1, NAD(P)H:quinone oxidoreductase; 231-NQO1^{+/+}, NQO1-overexpressing MDA-MB-231 cells; 231-NQO1^{-/-}, MDA-MB-231 cells lacking NQO1.

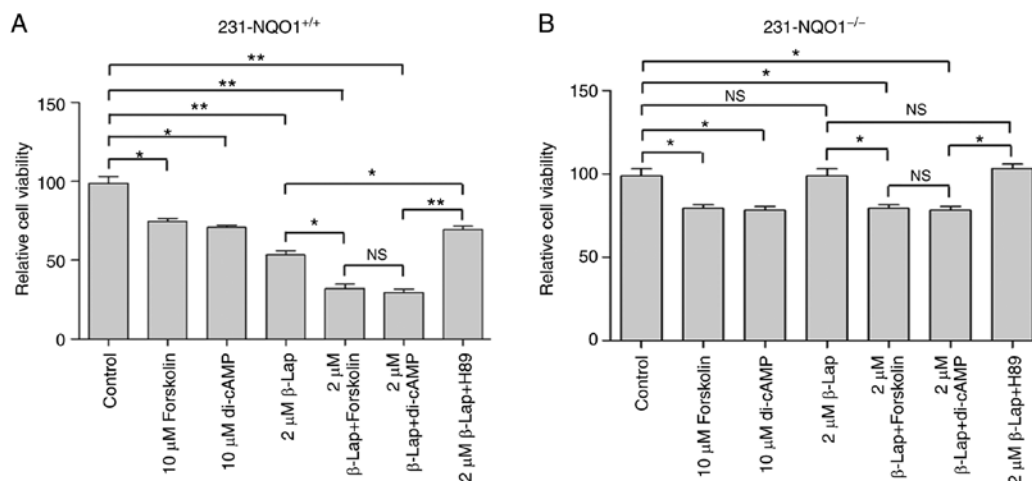


Figure 5. PKA activators aggravate cell death induced by β -Lapachone (β -Lap). (A) The 231-NQO1^{+/+} and (B) 231-NQO1^{-/-} cells were treated with 2 μ M β -Lap in combination with PKA activators [10 μ M dibutyryl-cAMP (di-cAMP), 10 μ M forskolin] or PKA inhibitor (5 μ M H89) for 2 h, and additionally incubated in fresh medium + 5% FBS containing PKA activators or inhibitor for 4 h. Cell viability was determined by CCK-8 assays. Data represent the mean (\pm SD) of three independent experiments (* P <0.05, ** P <0.01; NS, not significant). PKA, protein kinase A; NQO1, NAD(P)H:quinone oxidoreductase; 231-NQO1^{+/+}, NQO1-overexpressing MDA-MB-231 cells; 231-NQO1^{-/-}, MDA-MB-231 cells lacking NQO1.

In addition, the pattern of PKA activation was comparable with induction of proapoptotic events, such as caspase-3 activation and PARP cleavage, as shown in Fig. 3. Moreover, this PKA activation was ROS-dependent. Indeed, the phosphorylation of PKA α / β / γ at T198 was significantly inhibited by 5 mM NAC in β -Lap-treated 231-NQO1^{+/+} cells, but not in 231-NQO1^{-/-} cells (Fig. 4), indicating that PKA activation is closely associated with apoptotic cell death induced by β -Lap-dependent ROS in 231-NQO1^{+/+} cells.

PKA activation deteriorates the β -Lap-induced cell death. As shown above, PKA significantly activated β -Lap-induced cell death in MDA-MB-231 breast cancer cells overexpressing NQO1 (231-NQO1^{+/+}). Additionally, in some studies, PKA has been suggested to be a potential apoptotic cell death activator (15-26). In order to further investigate the possible role of PKA activation during β -Lap-induced cell death, we treated 231-NQO1^{+/+} and 231-NQO1^{-/-} cells with PKA activators (dibutyryl-cAMP, an analog of cAMP, and forskolin) or

PKA inhibitor (H89) and examined the effects on cell viability. The viability of 231-NQO1^{+/+} cells was dramatically decreased when cells were treated with both β -Lap and dibutyryl-cAMP (di-cAMP) or forskolin when compared to the control. In addition, treatment with PKA activators, either di-cAMP or forskolin, caused a significant decrease in cell viability in both 231-NQO1^{+/+} and 231-NQO1^{-/-} cells in the absence of β -Lap when compared to the control (Fig. 5A and B). Furthermore, a decrease in cell viability by β -Lap treatment in 231-NQO1^{+/+} cells was significantly recovered by PKA inhibitor H89 (Fig. 5A), but not completely, suggesting that ROS-mediated cell death might be additionally regulated by some other mechanisms. H89 had a positive influence on the recovery of cell viability, and this was slightly decreased by di-cAMP even in 231-NQO1^{-/-} cells (Fig. 5A and B).

We next examined the molecular levels of cell death-related proteins and PKA activation in both 231-NQO1^{+/+} and 231-NQO1^{-/-} cells treated with β -Lap in the presence of PKA inhibitors or activators. When 231-NQO1^{+/+} cells were treated

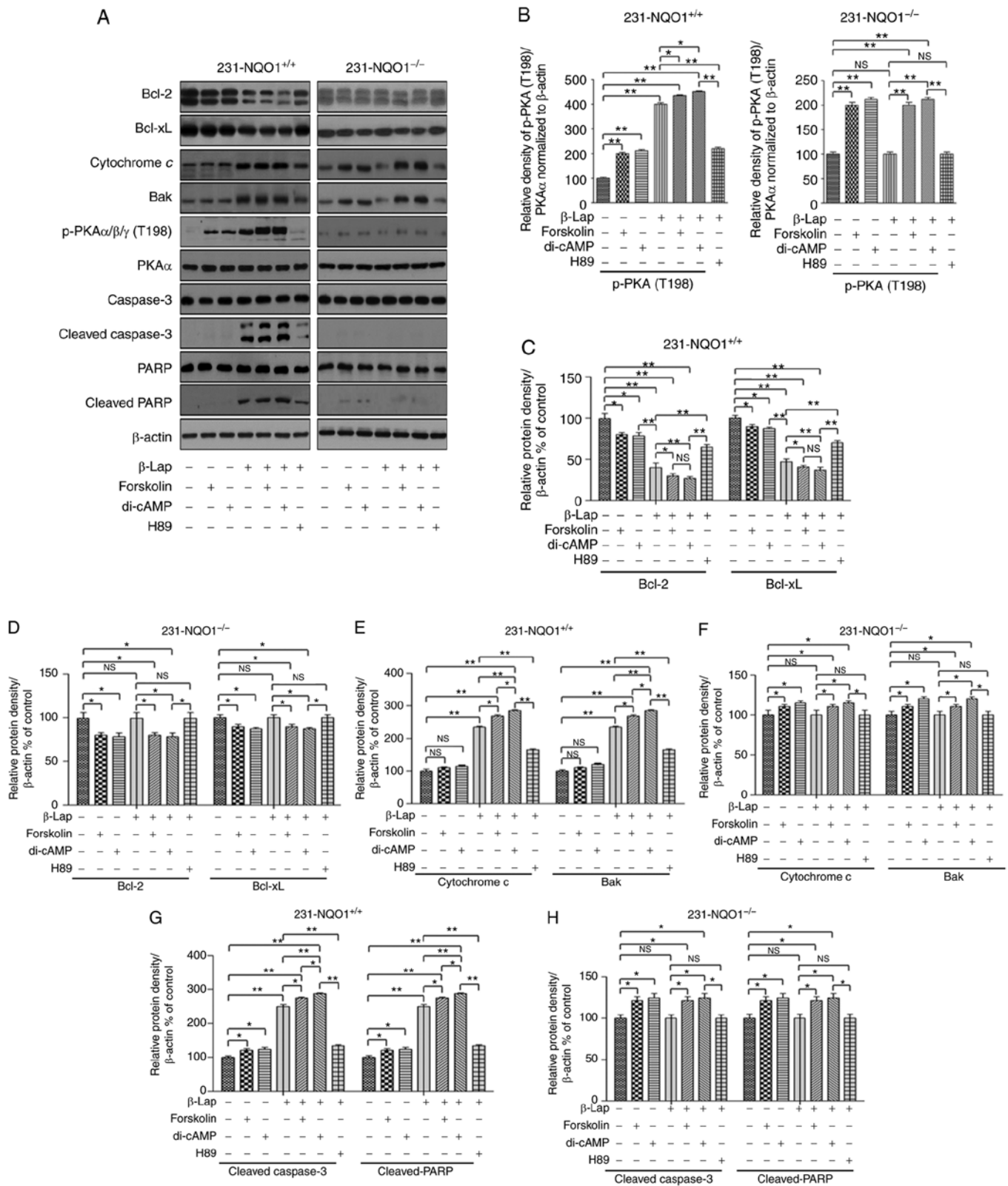


Figure 6. Treatment with PKA activators further increases β -Lapachone (β -Lap)-induced apoptotic cell death through activation of the PKA signaling pathway. (A) Western blots. The 231-NQO1^{+/+} (left panel) and 231-NQO1^{-/-} (right panel) cells were treated with 2 μ M β -Lap in combination with PKA activators (10 μ M dibutyryl-cAMP (di-cAMP), 10 μ M Forskolin) or PKA inhibitor (5 μ M H89) for 2 h, and additionally incubated in fresh medium + 5% FBS containing PKA activators or inhibitor for 4 h. Cells were lysed, and total cell extracts (30 μ g) were separated by 8 or 10% SDS-PAGE and analyzed by western blotting using primary antibodies to Bcl-2, Bcl-xL, cytochrome c, Bak, p-PKA α / β / γ cat, PKA cat, caspase-3, cleaved caspase-3, PARP, and cleaved PARP. β -actin was used as a loading control. (B-H) The relative amounts of all proteins shown in western blot analyses were quantified by NIH ImageJ software and represented as a graph: p-PKA (B) in 231-NQO1^{+/+} (left) and 231-NQO1^{-/-} (right) cells, Bcl2/Bcl-xL in 231-NQO1^{+/+} (C) and 231-NQO1^{-/-} (D) cells, cytochrome c/Bak in 231-NQO1^{+/+} (E) and 231-NQO1^{-/-} (F) cells, and cleaved caspase 3/cleaved PARP in 231-NQO1^{+/+} (G) and 231-NQO1^{-/-} (H) cells, respectively. The relative amount of p-PKA α / β / γ to PKA cat was determined first and subsequently normalized to β -actin. Data represent the mean (\pm SD) of three independent experiments (* P <0.05, ** P <0.01; NS, not significant). NQO1, NAD(P) H:quinone oxidoreductase; PKA, protein kinase A; Bcl-2, B-cell lymphoma-2; Bcl-xL, B-cell lymphoma-extra large; PARP, Poly(ADP-ribose) polymerase; 231-NQO1^{+/+}, NQO1-overexpressing MDA-MB-231 cells; 231-NQO1^{-/-}, MDA-MB-231 cells lacking NQO1.

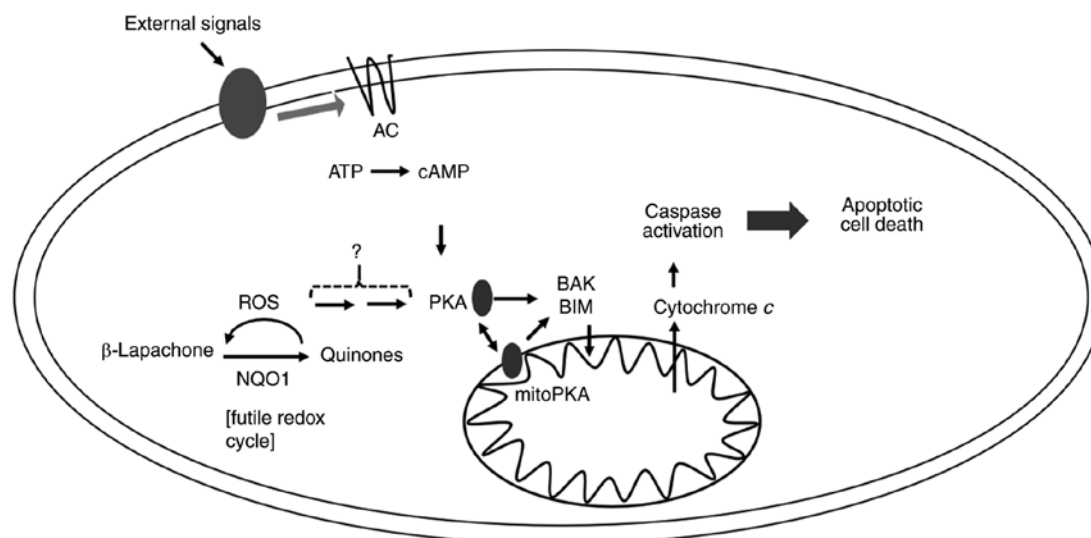


Figure 7. Illustration of the activation of the PKA signaling pathway in β -Lapachone (β -Lap)-induced apoptotic cell death. External signals activate adenylate cyclase (AC) through its specific receptor to produce cAMP, leading to the subsequent activation of PKA. Additionally, ROS produced the β -Lap-induced futile redox cycle to activate cytosolic PKA and mitochondrial PKA through unknown mechanisms (indicated by?). The activated PKA is associated with apoptotic cell death via caspase activation. PKA, protein kinase A; cAMP, cyclic AMP; ROS, reactive oxygen species; NQO1, NAD(P)H:quinone oxidoreductase.

with either di-cAMP or forskolin in combination with β -Lap, we observed a significant decrease in antiapoptotic proteins, such as Bcl-2 and Bcl-xL (Fig. 6A and C), and also a large increase in proapoptotic cytochrome *c*, Bak expression, caspase-3 activation, and PARP cleavage (Fig. 6A, E and G), with a substantial activation of PKA (p-PKA $\alpha/\beta/\gamma$ at T198) (Fig. 6A and B), compared to treatment with PKA activators alone, either di-cAMP or forskolin without β -Lap. Furthermore, we also observed a slight increase in proapoptotic proteins and a decrease in antiapoptotic proteins in 231-NQO1^{-/-} cells treated with either cAMP or forskolin, regardless of β -Lap treatment (Fig. 6A, D, F and H). On the contrary, antiapoptotic proteins and proapoptotic events were significantly increased and decreased, respectively, in 231-NQO1^{+/+} cells treated with β -Lap in the presence of H89, a PKA inhibitor (Fig. 6). Furthermore, combination of β -Lap and H89 in NQO1-negative cells (231-NQO1^{-/-}) caused a slight increase in antiapoptotic proteins and a substantial decrease in cytochrome *c* and Bak expression (Fig. 6).

Discussion

In the present study, we showed that β -Lap induced apoptotic cell death via the PKA pathway in human breast cancer MDA-MB-231 cells overexpressing NQO1 protein. According to previous studies, β -Lap-induced cell death is mostly dependent on ROS production by intracellular metabolism combined with a futile cycle in a NQO1-dependent manner (34-38). We also observed an increase in intracellular ROS when 231-NQO1^{+/+} cells were treated with β -Lap, in a dose-dependent manner. Additionally, we observed activation of PKA in the β -Lap-induced cell death of 231-NQO1^{+/+} cells, and we also showed that PKA activators further exacerbated β -Lap-induced cell death; PKA inhibitor substantially recovered cell survival even in cases of β -Lap-treated 231-NQO1^{+/+} cells. These results suggested that the PKA activity activated by β -Lap was associated with apoptotic cell death in these breast cancer cell lines.

Although cAMP-induced PKA activation is an essential pathway used to maintain cell viability in normal and cancer cells, PKA activation has also been suggested to induce apoptotic cell death in many cancer cells (15-17). Indeed, activation of cAMP and PKA signaling pathways stimulates apoptosis through phosphorylation of apoptosis-associated proteins such as Bim under stress conditions, such as during DNA damage (25). Most of these cell death mechanisms are associated with an intrinsic mitochondrial pathway (22). Furthermore, PKA-induced cell death in cancer cells is observed in not only a cAMP-dependent but also a cAMP-independent manner (18). In particular, despite the potent anticancer effects of cAMP/PKA activation by cancer drugs, these substances causing PKA activation via increase of intracellular cAMP are not recommended to be used as anticancer drugs because of their high cytotoxicity via the cAMP/PKA/CREB signaling pathway in normal cells. Therefore, the discovery of novel drugs that induce cAMP-independent PKA activation in cancer cells could be a beneficial strategy for future cancer treatment.

According to our data, β -Lap, a quinone compound, highly activated PKA and consequently induced apoptotic cell death in NQO1-positive breast cancer cells but not in NQO1-negative cells, suggesting that β -Lap-mediated ROS production in NQO1-positive cells might be essential for cell death via both PKA activation and other mechanisms. In particular, although direct treatment with PKA activators such as dibutyryl-cAMP or forskolin of 231-NQO1^{-/-} cells substantially activated PKA, it led to less cell death even in the presence or absence of β -Lap, when compared with 231-NQO1^{+/+} cells treated with β -Lap, indicating that PKA activation in β -Lap-treated 231-NQO1^{+/+} cells might not be solely cAMP-dependent; rather, some other cAMP-independent mechanisms may activate PKA, as shown in another study (18).

In addition to cAMP-independent PKA activation, β -Lap might also mediate the coordinative regulation of cytosolic and mitochondrial PKA activation through high production

of ROS in mitochondria. β -Lap-mediated ROS were increased and inhibited by antioxidant NAC in 231-NQO1^{+/+} cells, and coordinatively PKA activation was observed. PKA was more highly activated upon treatment with β -Lap, than by direct treatment with PKA activators. We observed a cumulative upregulation of PKA with treatment of the PKA activator and β -Lap, suggesting that β -Lap-mediated ROS were additionally related to PKA activation involved in cell death (Fig. 7). Indeed, some studies have suggested that increases in intracellular ROS activate the PKA signaling pathway (44-46).

In agreement with previous studies (31-38), this study also showed that β -Lap induced apoptotic cell death by NQO1-mediated ROS in a dose-dependent manner. β -Lap treatment caused a significant decrease in antiapoptotic proteins, such as Bcl-2 and Bcl-xL. In contrast, β -Lap caused a large increase in proapoptotic events including caspase activation, PARP cleavage, and cytochrome *c*. Nevertheless, our study still has some limitations to make a solid conclusion. In this study we only used a single type of breast cancer cell, and we did not examine the effect of β -Lap on normal breast cells with or without NQO1 overexpression, which will be investigated in the future.

As mentioned previously, a combination of different processes may be effective for the treatment of cancer. However, a new strategy targeting apoptosis in cancer cells still remains to be developed. In particular, in this study we suggest a novel mechanism by which β -Lap can induce apoptosis via activation of PKA, and use of β -Lap as a selective treatment in cancer patients.

Acknowledgements

We thank Dr David Boothman (UT Southwestern Medical Center, Dallas, TX, USA) for providing MDA-MB-231 cells overexpressing NQO1 (231-NQO1^{+/+}) and MDA-MB-231 cells lacking NQO1 (231-NQO1^{-/-}).

Funding

This study was supported by the Basic Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education Science and Technology [2018R1D1A1B07043715 (to DRK), 2018R1D1A1B07049185 (to D-HK)] and by the Ministry of Science, ICT, and Future Planning (NRF-2015R1A5A2008833).

Availability of data and materials

The datasets used during the present study are available from corresponding author upon request.

Authors' contributions

SZ and DRK conceived and designed the study. SZ carried out experiments, analyzed the data, and wrote the primary manuscript. JSH, THL, TMP, DHK and MA analyzed the data and edited the manuscript. DHK and DRK edited the manuscript and received the research funding. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or

integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Semenza GL: Molecular mechanisms mediating metastasis of hypoxic breast cancer cells. *Trends Mol Med* 18: 534-543, 2012.
2. Valastyan S and Weinberg RA: Tumor metastasis: Molecular insights and evolving paradigms. *Cell* 147: 275-292, 2011.
3. Lambert AW, Pattabiraman DR and Weinberg RA: Emerging biological principles of metastasis. *Cell* 168: 670-691, 2017.
4. Lerebours F and Lidereau R: Molecular alterations in sporadic breast cancer. *Crit Rev Oncol Hematol* 44: 121-141, 2002.
5. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. *CA Cancer J Clin* 61: 69-90, 2011.
6. Li CI, Beaber EF, Tang MC, Porter PL, Daling JR and Malone KE: Reproductive factors and risk of estrogen receptor positive, triple-negative, and HER2-neu overexpressing breast cancer among women 20-44 years of age. *Breast Cancer Res Treat* 137: 579-587, 2013.
7. Coughlin SS and Ekwueme DU: Breast cancer as a global health concern. *Cancer Epidemiol* 33: 315-318, 2009.
8. Tangjitgamol S, Anderson BO, See HT, Lertbutsanukul C, Sirisabya N, Manchana T, Ilancheran A, Lee KM, Lim SE, Chia YN, *et al*: Management of endometrial cancer in Asia: Consensus statement from the Asian Oncology Summit 2009. *Lancet Oncol* 10: 1119-1127, 2009.
9. Mark HF, Aswad B, Bassily N, Taylor W, Brown S, Sun CL, Samy M, Zolnier K, Wong E, Bland KI and Hsu PH: HER-2/neu gene amplification in stages I-IV breast cancer detected by fluorescent in situ hybridization. *Genet Med* 1: 98-103, 1999.
10. Thompson CB: Apoptosis in the pathogenesis and treatment of disease. *Science* 267: 1456-1462, 1995.
11. Fulda S: Apoptosis pathways and their therapeutic exploitation in pancreatic cancer. *J Cell Mol Med* 13: 1221-1227, 2009.
12. Varfolomeev E and Vucic D: Inhibitor of apoptosis proteins: Fascinating biology leads to attractive tumor therapeutic targets. *Future Oncol* 7: 633-648, 2011.
13. Taskén K and Aandahl EM: Localized effects of cAMP mediated by distinct routes of protein kinase A. *Physiol Rev* 84: 137-167, 2004.
14. Fimia GM and Sassone-Corsi P: Cyclic AMP signalling. *J Cell Sci* 114: 1971-1972, 2001.
15. Cross TG, Scheel-Toellner D, Henriquez NV, Deacon E, Salmon M and Lord JM: Serine/threonine protein kinases and apoptosis. *Exp Cell Res* 256: 34-41, 2000.
16. Lerner A, Kim DH and Lee R: The cAMP signaling pathway as a therapeutic target in lymphoid malignancies. *Leuk Lymphoma* 37: 39-51, 2000.
17. Insel PA, Bourne HR, Coffino P and Tomkins GM: Cyclic AMP-dependent protein kinase: Pivotal role in regulation of enzyme induction and growth. *Science* 190: 896-898, 1975.
18. Hedrick ED, Agarwal E, Leiphrakpam PD, Haferbier KL, Brattain MG and Chowdhury S: Differential PKA activation and AKAP association determines cell fate in cancer cells. *J Mol Signal* 8: 10, 2013.
19. Almeida MQ and Stratakis CA: How does cAMP/protein kinase A signaling lead to tumors in the adrenal cortex and other tissues? *Mol Cell Endocrinol* 336: 162-168, 2011.
20. Chowdhury S, Howell GM, Rajput A, Teggart CA, Brattain LE, Weber HR, Chowdhury A and Brattain MG: Identification of a novel TGF β /PKA signaling transduceome in mediating control of cell survival and metastasis in colon cancer. *PLoS One* 6: e19335, 2011.

21. Zambon AC, Zhang L, Minovitsky S, Kanter JR, Prabhakar S, Salomonis N, Vranizan K, Dubchak I, Conklin BR and Insel PA: Gene expression patterns define key transcriptional events in cell-cycle regulation by cAMP and protein kinase A. *Proc Natl Acad Sci USA* 102: 8561-8566, 2005.
22. Zhang L, Zambon AC, Vranizan K, Pothula K, Conklin BR and Insel PA: Gene expression signatures of cAMP/Protein Kinase A (PKA)-promoted, Mitochondrial-dependent Apoptosis: Comparative analysis of wild-type and camp-deathless s49 lymphoma cells. *J Biol Chem* 283: 4304-4313, 2008.
23. Coffino P, Bourne HR and Tomkins GM: Mechanism of lymphoma cell death induced by cyclic AMP. *Am J Pathol* 81: 199-204, 1975.
24. Yan L, Herrmann V, Hofer JK and Insel PA: beta-Adrenergic receptor/cAMP-mediated signaling and apoptosis of S49 lymphoma cells. *Am J Physiol Cell Physiol* 279: C1665-C1674, 2000.
25. Zhang L and Insel PA: The pro-apoptotic protein Bim is a convergence point for cAMP/protein kinase A-and glucocorticoid-promoted apoptosis of lymphoid cells. *J Biol Chem* 279: 20858-20865, 2004.
26. Naviglio S, Di Gesto D, Romano M, Sorrentino A, Illiano F, Sorvillo L, Abbruzzese A, Marra M, Caraglia M, Chiosi E, *et al*: Leptin enhances growth inhibition by cAMP elevating agents through apoptosis of MDA-MB-231 breast cancer cells. *Cancer Biol Ther* 8: 1183-1190, 2009.
27. Ugland H, Boquest AC, Naderi S, Collas P and Blomhoff HK: cAMP-mediated induction of cyclin E sensitizes growth-arrested adipose stem cells to DNA damage-induced apoptosis. *Mol Biol Cell* 19: 5082-5092, 2008.
28. Blanco E, Bey EA, Khemtong C, Yang SG, Setti-Guthi J, Chen H, Kessinger CW, Carnevale KA, Bornmann WG, Boothman DA and Gao J: Beta-lapachone micellar nanotherapeutics for non-small cell lung cancer therapy. *Cancer Res* 70: 3896-3904, 2010.
29. Li CJ, Li YZ, Pinto AV and Pardee AB: Potent inhibition of tumor survival in vivo by beta-lapachone plus taxol: Combining drugs imposes different artificial checkpoints. *Proc Natl Acad Sci USA* 96: 13369-13374, 1999.
30. Dong Y, Chin SF, Blanco E, Bey EA, Kabbani W, Xie XJ, Bornmann WG, Boothman DA and Gao J: Intratumoral delivery of beta-lapachone via polymer implants for prostate cancer therapy. *Clin cancer Res* 15: 131-139, 2009.
31. Pardee AB, Li YZ and Li CJ: Cancer therapy with beta-lapachone. *Curr Cancer Drug Targets* 2: 2002, 227-242.
32. Reinicke KE, Bey EA, Bentle MS, Pink JJ, Ingalls ST, Hoppel CL, Misico RI, Arzac GM, Burton G, Bornmann WG, *et al*: Development of beta-lapachone prodrugs for therapy against human cancer cells with elevated NAD(P)H:Quinone oxidoreductase 1 levels. *Clin Cancer Res* 11: 3055-3064, 2005.
33. Trachootham D, Alexandre J and Huang P: Targeting cancer cells by ROS-mediated mechanisms: A radical therapeutic approach? *Nat Rev Drug Discov* 8: 579-591, 2009.
34. Pink JJ, Planchon SM, Tagliarino C, Varnes ME, Siegel D and Boothman DA: NAD(P)H:Quinone oxidoreductase activity is the principal determinant of beta-lapachone cytotoxicity. *J Biol Chem* 275: 5416-5424, 2000.
35. Siegel D, Yan C and Ross D: NAD(P)H:Quinone oxidoreductase 1 (NQO1) in the sensitivity and resistance to antitumor quinones. *Biochem Pharmacol* 83: 1033-1040, 2012.
36. Park EJ, Choi KS and Kwon TK: β -Lapachone-induced reactive oxygen species (ROS) generation mediates autophagic cell death in glioma U87 MG cells. *Chem Biol Interact* 189: 37-44, 2011.
37. Choi EK, Terai K, Ji IM, Kook YH, Park KH, Oh ET, Griffin RJ, Lim BU, Kim JS, Lee DS, *et al*: Upregulation of NAD(P)H:Quinone oxidoreductase by radiation potentiates the effect of bioreductive beta-lapachone on cancer cells. *Neoplasia* 9: 634-642, 2007.
38. Garate M, Wani AA and Li G: The NAD(P)H:Quinone oxidoreductase 1 induces cell cycle progression and proliferation of melanoma U87 MG cells. *Free Radic Biol Med* 48: 1601-1609, 2010.
39. Yang Y, Zhang Y, Wu Q, Cui X, Lin Z, Liu S and Chen L: Clinical implications of high NQO1 expression in breast cancers. *J Exp Clin Cancer Res* 33: 14, 2014.
40. Lewis AM, Ough M, Hinkhouse MM, Tsao MS, Oberley LW and Cullen JJ: Targeting NAD(P)H:quinone oxidoreductase (NQO1) in pancreatic cancer. *Mol Carcinog* 43: 215-224, 2005.
41. Lyn-Cook BD, Yan-Sanders Y, Moore S, Taylor S, Word B and Hammons GJ: Increased levels of NAD(P)H:Quinone oxidoreductase 1 (NQO1) in pancreatic tissues from smokers and pancreatic adenocarcinomas: A potential biomarker of early damage in the pancreas. *Cell Biol Toxicol* 22: 73-80, 2006.
42. Li JZ, Ke Y, Misra HP, Trush MA, Li YR, Zhu H and Jia Z: Mechanistic studies of cancer cell mitochondria- and NQO1-mediated redox activation of beta-lapachone, a potentially novel anticancer agent. *Toxicol Appl Pharmacol* 281: 285-293, 2014.
43. Cao L, Li LS, Spruell C, Xiao L, Chakrabarti G, Bey EA, Reinicke KE, Srougi MC, Moore Z, Dong Y, *et al*: Tumor-selective, futile redox cycle-induced bystander effects elicited by NQO1 bioactivatable radiosensitizing drugs in triple-negative breast cancers. *Antioxid Redox Signal* 21: 237-250, 2014.
44. Liu Y, Yang F, Li S, Dai J and Deng H: Glutaredoxin deletion shortens chronological life span in *Saccharomyces cerevisiae* via ROS-Mediated Ras/PKA activation. *J Proteome Res* 17: 2318-2327, 2018.
45. Li Z, Ji G and Neugebauer V: Mitochondrial reactive oxygen species are activated by mGluR5 through IP3 and activate ERK and PKA to increase excitability of amygdala neurons and pain behavior. *J Neurosci* 31: 1114-1127, 2011.
46. Bovo E, Lipsius SL and Zima AV: Reactive oxygen species contribute to the development of arrhythmogenic Ca^{2+} waves during β -adrenergic receptor stimulation in rabbit cardiomyocytes. *J Physiol* 590: 3291-3304, 2012.