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Original Article

Activation of Akt–cAMP response element-binding protein (CREB) signaling as an adaptive response to an electrophilic metabolite of morphine

Kohei Matsuo[1], Shigeru Yamano[1], Akira Toriba[2], Kimihiko Matsusue[1], Yoshito Kumagai[3] and Yumi Abiko[2]

[1]Faculty of Pharmaceutical Sciences, Fukuoka University, Fukuoka 814-0180, Japan
[2]Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan
[3]Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Fukuoka 812-8582, Japan

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ABSTRACT — Morphinone (MO) is an electrophilic metabolite of morphine. Electrophiles can modify thiol groups of proteins, resulting in the activation of redox signaling pathways and toxicity. We have previously reported that the atmospheric electrophile, 1,4-naphthoquinone, and electrophilic organometallic compound, methylmercury, activate protein kinase B (Akt) signaling through modification of phosphatase and tensin homolog deleted from chromosome 10 (PTEN), which is a negative regulator of Akt. In the present study, we examined whether MO activates Akt signaling. Exposure of HepG2 cells to MO enhanced translocation of Akt to the nucleus in a concentration-dependent manner. MO phosphorylated Akt and its downstream protein, cAMP response element-binding protein (CREB), and upregulated B-cell lymphoma 2 (Bcl-2), an anti-apoptotic protein. An analogue of MO dihydromorphinone that was not electrophilic did not enhance the phosphorylation of Akt and CREB or expression of Bcl-2, suggesting the importance of electrophilicity of MO in activation of the cascade. Pretreatment of the cells with wortmannin suppressed MO-mediated phosphorylation of Akt and CREB and expression of Bcl-2, and enhanced MO-induced cytotoxicity, indicating that MO activates Akt–CREB–Bcl-2 signaling in HepG2 cells. This signaling pathway might be capable of modulating MO-mediated toxicity in cells.

Key words: Electrophile, Protein kinase B (Akt), cAMP response element-binding protein (CREB), Morphine, Morphinone, Redox signaling

INTRODUCTION

Electrophiles, which are produced through phase I metabolic activation of xenobiotics *in vivo*, easily bind to nucleophilic endogenous molecules such as proteins and DNA, leading to cellular responses and toxicity. Electrophilic modification of proteins causes functional changes in the proteins, resulting in activation of redox signaling pathways (Zheng *et al*., 2020). The narcotic analgesic, morphine, is metabolized to morphinone (MO) by aldo-keto reductase 1C subfamily of isozymes (Yamano *et al*., 1985; Endo *et al*., 2013). MO is electrophilic because of its α,β-unsaturated carbonyl groups; consequently, MO binds to thiol groups to yield MO–glutathione and MO–protein adducts (Kumagai *et al*., 1990; Nagamatsu *et al*., 1982). Morphine is used at high doses to reduce chronic pain in many cases (Bercovitch and Adunsky, 2004); the produced MO is thought to cause electrophilic stress

Correspondence: Yumi Abiko (E-mail: yumi.abiko.11@nagasaki-u.ac.jp)
Yoshito Kumagai (E-mail: kumagai.yoshito.864@m.kyushu-u.ac.jp)

under these conditions. However, the underlying mechanisms of cellular responses and toxicity of MO have not been studied. Our recent studies have revealed that MO exposure activates the Kelch-like ECH-associated protein 1 (Keap1)–nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway and heat shock protein (HSP) 90–heat shock factor 1 (HSF1) in human hepatocellular carcinoma HepG2 cells (Matsuo *et al*., 2023b; Matsuo *et al*., 2023a). These results indicate that MO can activate several redox signaling pathways.

Protein phosphorylation and dephosphorylation regulate signal transmission, which is essential for the modulation of cellular homeostasis (Karin and Hunter, 1995). The protein kinase B (Akt)–cAMP response element-binding protein (CREB) signaling pathway contributes to cell survival and protects cells from apoptosis (Du and Montminy, 1998). Phosphorylation of Akt is negatively regulated by phosphatase and tensin homolog deleted from chromosome 10 (PTEN) through *S*-modification of cysteine residues of PTEN by endogenous electrophiles, such as 4-hydroxynonenal and Δ12-prostaglandin J_2, leading to the activation of Akt (Shearn *et al*., 2011; Fang *et al*., 2013; Covey *et al*., 2010; Stambolic *et al*., 1998). We have previously reported that 1,4-naphthoquinone (1,4-NQ) and methylmercury (MeHg), exogenous electrophilic quinone and heavy metal, respectively, activate the PTEN–Akt–CREB pathway through *S*-modification of cysteine residues of PTEN and expression of its downstream signaling proteins, such as B-cell lymphoma 2 (Bcl-2) (Abiko *et al*., 2017b; Unoki *et al*., 2016). These findings suggest that MO activates the PTEN–Akt pathway. Therefore, in the present study, we aimed to clarify the MO-mediated activation of the PTEN–Akt pathway in HepG2 cells.

MATERIALS AND METHODS

Materials

MO and dihydromorphinone (DHMO) were prepared following the procedures described by Rapoport *et al*. (Rapoport *et al*., 1957; Rapoport *et al*., 1950) Anti-Akt, anti-histone deacetylase 1, anti-phosphorylated Akt (Ser473), anti-phosphorylated CREB (Ser33), anti-Bcl-2 antibodies, and horseradish peroxidase (HRP)-conjugated anti-rabbit and HRP-conjugated anti-mouse antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Protease inhibitor cocktail, anti-β actin antibody and avidin agarose were sourced from Sigma-Aldrich (St. Louis, MO, USA). Anti-glyceraldehyde-3-phosphate dehydrogenase antibody and phosphatase inhibitor cocktail were obtained from Santa Cruz Biotechnology (Dallas, TX, USA) and Nacalai Tesque (Kyoto, Kyoto, Japan), respectively. Dulbecco's modified Eagle's medium (DMEM), penicillin–streptomycin, and bicinchoninic acid assay kit were purchased from Thermo Fisher Scientific (Waltham, MA, USA). All other reagents used were of the highest grade available.

Cell culture

HepG2 cells (RIKEN Cell Bank, Ibaraki, Japan) were cultured in DMEM containing 10% fetal bovine serum (FBS; Biowest, Nuaillé, France) and 1% penicillin–streptomycin at 37°C under 5% CO_2. The cells were seeded on 35 mm culture dishes (8×10^5 cells/dish) and incubated for 24 hr. The medium was then replaced by serum-free DMEM, and the cells were incubated overnight. Next, the cells were exposed to MO or DHMO for the indicated time periods and washed thrice with ice-cold phosphate-buffered saline (PBS).

Western blotting

The cells were collected in a lysis buffer (50 mM Tris-HCl [pH 7.5], 6 M urea, 1% Triton X-100, 5 mM EDTA, and 1% protease inhibitor cocktail), and the concentration of proteins in the lysate was determined using the bicinchoninic acid assay, following the manufacturer's protocol. Each sample was mixed with half the volume of loading buffer (10% sodium dodecyl sulfate [SDS], 62.5 mM Tris-HCl [pH 6.8], 20% glycerol, 5 mM 2-mercaptoethanol, and 0.015% bromophenol blue) and heated for 5 min at 95°C. Equivalent amounts of the proteins were separated using SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Millipore-Sigma, St Louis, MO, USA). The membrane was blocked with 5% skimmed milk for 60 min at room temperature and washed thrice with Tris-buffered saline containing Tween 20. Next, the membrane was incubated with primary antibodies, followed by HRP-conjugated secondary antibodies. Immunoreactive protein bands on the membrane were detected using Amersham ECL Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK), and the band intensities were measured using ImageJ software (Schneider *et al*., 2012).

Nuclear and cytoplasmic extraction

After washing with PBS, the cells were incubated with trypsin and collected in DMEM containing 10% FBS. After centrifugation at 1,000 rpm for 10 min, the cell pellet was re-suspended in PBS and then centrifuged again at 4°C. The obtained pellet was gently re-suspended and incubated in 300 μL of suspension buffer (10 mM

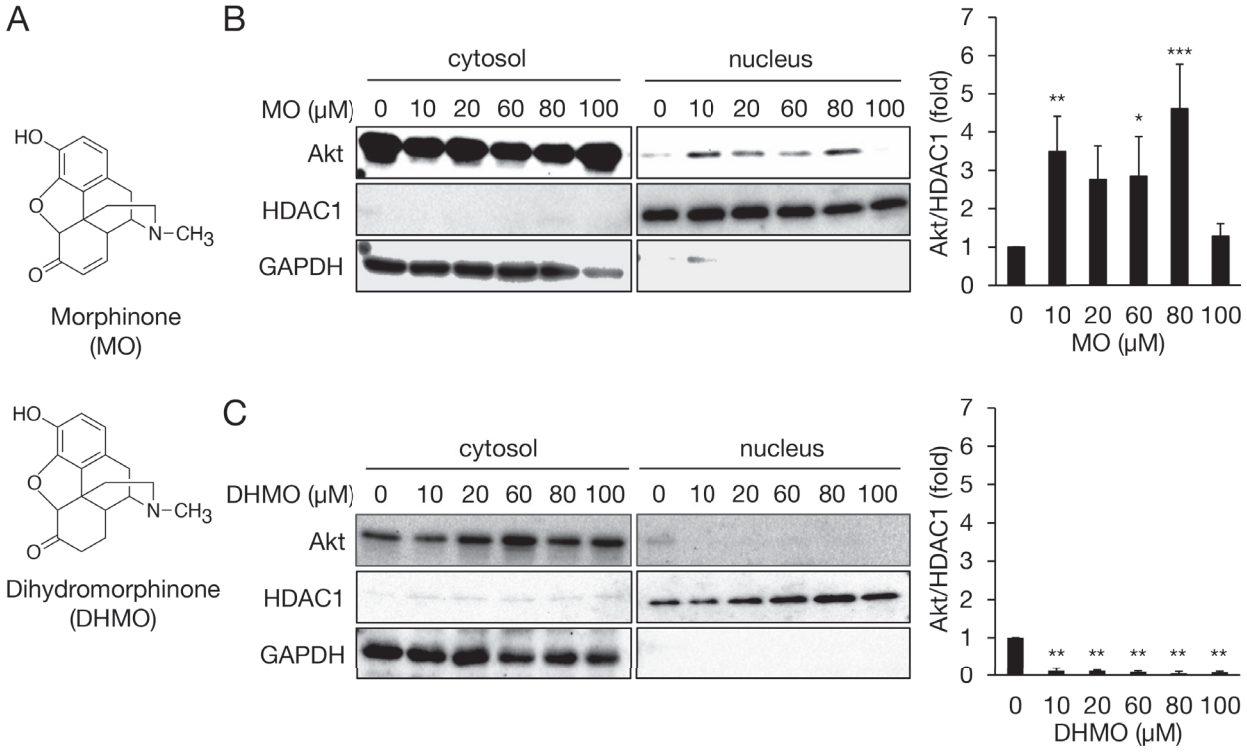

Fig. 1. Nuclear localization of protein kinase B (Akt) during exposure of HepG2 cells to morphine. (A) Structure of morphinone (MO) and dihydromorphinone (DHMO). HepG2 cells were exposed to MO (B) and DHMO (C) for 1 hr. Proteins in the cytosol and nuclear fractions were analyzed using western blotting with antibodies against Akt, histone deacetylase 1 (HDAC1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Protein bands were quantified using ImageJ software; fold changes in expression levels are shown in the graph. Each value is the mean ± SE of three determinations. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ *vs*. untreated cells.

HEPES-KOH [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 100 μM digitonin, and 1% protease inhibitor cocktail) at 4°C for 10 min, then centrifuged at 15,000 rpm for 10 min at 4°C. Cytoplasmic and nuclear fractions were collected from the supernatant and pellet, respectively.

Cell viability

The cells were plated in 96-well plates and treated with 5 μM wortmannin for 30 min following exposure to MO for 6 hr. Cell counting kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan) was then performed according to the manufacturer's instructions. Briefly, 10 μL of CCK-8 working solution per 100 μL of medium was added into the microplates and the cells were incubated for 1 hr; absorbance was measured at 450 nm.

Data analysis

Statistical analysis was performed using one-way ANOVA followed by Sidak's or Dunnett's multiple-comparison test using GraphPad Prism version 8.4.3 (GraphPad Software, San Diego, CA, USA); $P < 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

Akt is reported to translocate from the cytosol to the nucleus as a result of phosphorylation (Meier *et al*., 1997; Nguyen *et al*., 2003). First, we detected nuclear translocation of Akt during exposure of HepG2 cells to MO. Exposure to MO for 1 hr significantly enhanced the nuclear translocation of Akt (Fig. 1A). In contrast, DHMO which is a metabolite of morphine that is not electrophilic, did not translocate Akt to the nucleus (Fig. 1B). This result indicates that electrophilicity of MO is the key to Akt

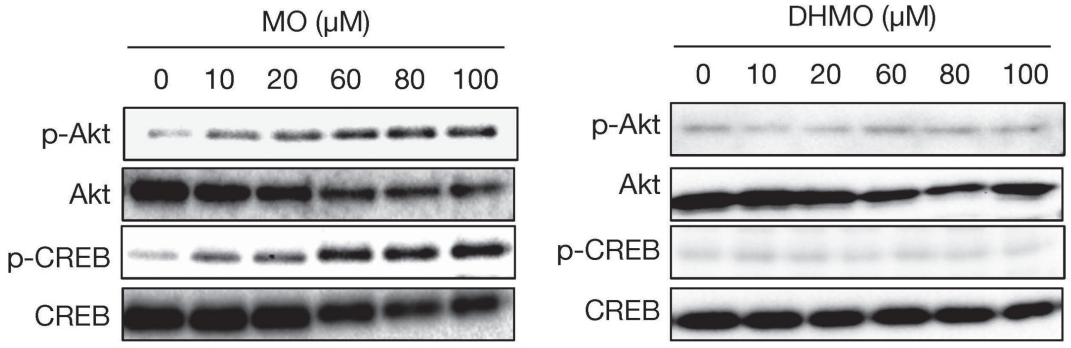

Fig. 2. MO-mediated activation of Akt–cAMP response element-binding protein (CREB) signaling in HepG2 cells. HepG2 cells were exposed to 0 to 100 μM MO or DHMO for 6 hr. Whole cell lysates were analyzed by western blotting using the indicated antibodies. Representative data are shown.

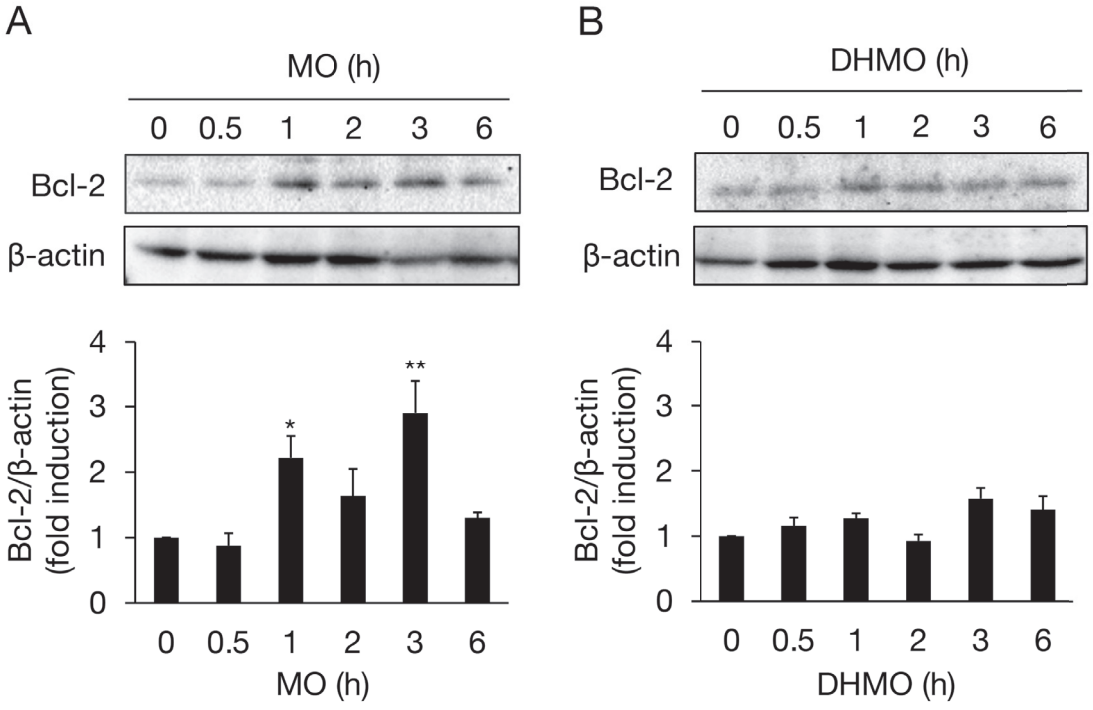

Fig. 3. Up-regulation of B-cell lymphoma 2 (Bcl-2) through exposure to MO in HepG2 cells. HepG2 cells were exposed to 30 μM MO (A) or 30 μM DHMO (B) for 0 to 6 hr. Whole cell lysates were analyzed by western blotting using the indicated antibodies. Protein bands were quantified using ImageJ software; fold changes in expression levels are shown in the graph. Each value represents the mean ± SE of three measurements. *$P < 0.01$, **$P < 0.001$ *vs*. 0 hr.

activation in cells. Notably, higher concentrations of MO (100 μM) did not enhance the translocation of Akt. This may be due to disruption of Akt activity through extensive modification of cellular proteins by MO. Supporting this, our previous study indicated that a higher concentration of MeHg modified not only PTEN, but also Akt and CREB, thereby inhibiting the Akt–CREB signaling pathway (Unoki *et al*., 2016). Next, we assessed the phosphorylation of Akt and its downstream protein, CREB, by MO. As shown in Fig. 2, concentration-dependent phos-

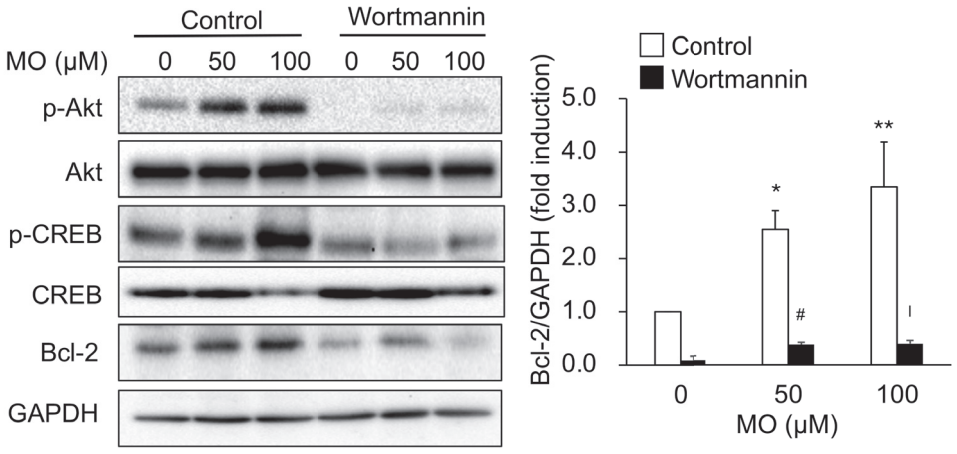

Fig. 4. Effect of a phosphatidylinositol-3 kinase inhibitor on the Akt–CREB–Bcl-2 signaling pathway in HepG2 cells. HepG2 cells were pretreated with 5 μM of wortmannin for 30 min, followed by exposure to MO for 6 hr. Whole cell lysates were analyzed by western blotting using the indicated antibodies. Protein bands were quantified using ImageJ software; fold changes in expression levels are shown in the graph. Each value represents the mean ± SE of three measurements. *$P < 0.01$, **$P < 0.001$ *vs*. control MO (0 μM). #$P < 0.001$ *vs*. control MO (50 μM). †$P < 0.001$ *vs*. control MO (100 μM).

phorylation of Akt and CREB by MO but not by DHMO, was observed. Because Bcl-2 is a protein that is present downstream in the PTEN–Akt–CREB pathway, MO may upregulate Bcl-2 expression. As expected, MO but not DHMO, significantly induced Bcl-2 expression, which reached a plateau at 3 hr and then declined at 6 hr (Fig. 3). To determine whether the MO-mediated phosphorylation of CREB and induction of Bcl-2 expression were Akt-dependent, we pretreated HepG2 cells with wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase. As shown in Fig. 4, wortmannin markedly inhibited the MO-mediated phosphorylation of Akt and CREB and suppressed the expression of Bcl-2. This indicates that MO activated the Akt–CREB–Bcl-2 pathway and wortmannin treatment decreased cell viability after exposure to MO (Fig. 5), thus suggesting that this pathway contributes to MO-mediated cell death.

Akt is dephosphorylated by PTEN, which has highly reactive cysteine residues such as Cys71, Cys83, and Cys124 (Lee *et al*., 2002; Lee *et al*., 1999; Numajiri *et al*., 2011). We have previously demonstrated that an exogenous electrophilic quinone, 1,4-NQ, activates Akt–CREB signaling through the modification of Cys71 and Cys83 of PTEN (Abiko *et al*., 2017b). In the present study, although we did not identify the modification site of PTEN by MO, our observations suggest that

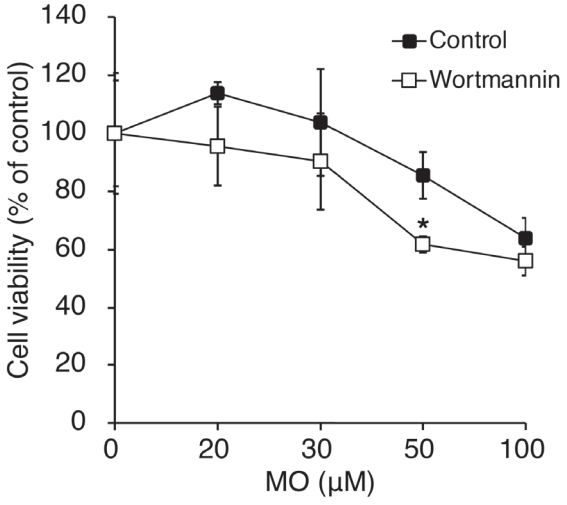

Fig. 5. Protective role of Akt signaling in MO-induced cell death in HepG2 cells. HepG2 cells were pretreated with 5 μM of wortmannin for 30 min, followed by exposure to MO for 6 hr. Cell viability was analyzed using the CCK-8 assay. Each value represents the mean ± SE of three measurements. *$P < 0.001$ *vs*. control.

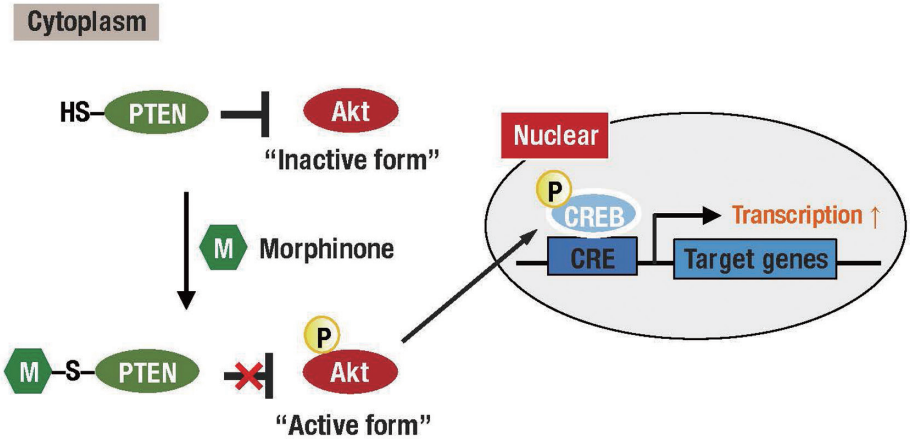

Fig. 6. A prospective regulation mechanism of Akt–CREB signaling pathway by MO. Akt, protein kinase B; CRE, cAMP response element; CREB, CRE-binding protein; MO, morphinone; PTEN, phosphatase and tensin homolog deleted from chromosome 10.

MO modifies such reactive cysteines in PTEN to activate Akt (Fig. 6).

Several of our previous studies indicated that MO targets sensor proteins such as HSP90 and Keap1, leading to the activation of HSF1 and Nrf2, respectively (Matsuo *et al*., 2023b; Matsuo *et al*., 2023a). HSP90–HSF1 signaling and the Keap1–Nrf2 pathway are known to exhibit cytoprotective roles against endogenous and exogenous electrophiles (Jacobs and Marnett, 2007; Abiko *et al*., 2017a; Shinkai *et al*., 2017). In the present study, we have demonstrated that MO also activates the Akt–CREB–Bcl-2 signaling pathway. Since Akt signaling is associated with cell survival and Bcl-2 is an anti-apoptotic protein (Boise *et al*., 1993; Kandel and Hay, 1999), we speculate that the cells may be protected from cytotoxic effects caused by the electrophilic metabolite of morphine, morphinone, through the activation of such redox signaling pathways (Nagamatsu *et al*., 1986).

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Conflict of interest---- The authors declare that there is no conflict of interest.

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