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1,2-Naphthoquinone disrupts the function of cAMP response element-binding protein through covalent modification

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Abstract

1,2-Naphthoquinone (1,2-NQ) is an atmospheric contaminant with electrophilic properties that allow it to react readily with protein thiol groups such as those found on the cAMP response element-binding protein (CREB), a transcription factor with conserved cysteine residues that regulate DNA binding. In the present study, we explored the possibility that the interaction of 1,2-NQ with CREB will affect its activity, resulting in down-regulation of gene expression. With bovine aortic endothelial cells (BAECs) and a cell-free system, 1,2-NQ was found to covalently bind to CREB, and inhibit its DNA binding activity under conditions that were blocked by dithiothreitol. CRE-dependent luciferase activity and the down-regulation of Bcl-2 expression were suppressed by exposure of BAECs to 1,2-NQ. This phenomenon was not seen with the hydrocarbon, naphthalene, which lacks any electrophilic properties. The results indicate that CREB is a molecular target for 1,2-NQ which through irreversible binding, inhibits the function of this transcription factor.
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Quinones are ubiquitously distributed in nature and represent a class of toxicological intermediates which can create a variety of hazardous effects *in vivo*, including acute cytotoxicity, immunotoxicity, and carcinogenesis [1]. We reported previously that 1,2-naphthoquinone (1,2-NQ) is found in diesel exhaust particles and atmospheric $PM_{2.5}$ [2]. The α,β-unsaturated carbonyl system present in 1,2-NQ, provides a highly reactive electrophilic site that reacts readily with thiolate anions in proteins to yield a thioether adduct. This reaction also occurs with endogenous compounds as shown by the addition of the electrophilic 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) to cysteine residues on its target molecules to modulate the transcriptional activities of NF-κB, AP-1, PPARγ [3] and estrogen receptor-α [4]. These cysteine thiols are more reactive in proteins with basic amino acids in close proximity because the latter reduce the thiol pK_a. Proteins with such thiols are subject to arylation by 1,2-NQ and presumably alter functions, such as catalytic activity, transcription, and signal transduction capacity of the affected protein.

The cAMP response element-binding protein (CREB) is a transcription factor activated by multiple signal transduction pathways in response to external stimuli, such as those from neurotransmitters, hormones, growth factors, cytokines, and stress [5]. Several studies have suggested that CREB plays an important role in inflammation [6], cardiac function [7], progression of tumor [8,9], and memory [10]. The functional state of CREB is regulated by phosphorylation levels of the serine residue at 133 (Ser133), which promotes its association with the transcription co-activator protein, CREB-binding protein (CBP) and p300 [11]. CREB regulates the activity

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of several genes such as the anti-apoptotic gene for B-cell lymphoma-2 (Bcl-2) and cell cycle genes such as those for cyclin D1 [12].

CREB has a basic leucine-zipper (bZIP) domain that governs the binding efficiency of CREB to its cognate promoter element CRE, a consensus palindromic sequence, TGACGTCA [13]. Interestingly, bZIP transcription factors contain a highly conserved cysteine at regions which have nearby basic amino acids in their DNA binding domains [14,15]. These cysteine residues have a crucial role in DNA binding ability. The activity of a bZIP transcription factor, AP-1, was reported to be inhibited by 15d-PGJ$_2$ through irreversible modification of c-Jun at a cysteine residue located in the DNA binding domain, cysteine 269 [16]. It was also shown that *N*-ethylmalemide, an alkylating agent, is capable of inhibiting the bZIP transcription factor, Nrf2, in its regulation of ARE-mediated NQO1 gene expression [17]. Based these findings, we hypothesized that 1,2-NQ could bind to a CREB, resulting in suppression of the DNA binding activity, transcription activity and thus altered expression of CREB-regulated proteins.

Materials and methods

Cell culture. Bovine aortic endothelial cells (BAECs) were obtained from the Cell Systems (Kirkland, WA). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 using Dulbecco's modified Eagle's medium (DMEM, Wako, Osaka, Japan) containing 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells from 4 to 10 passage were used for experiments.

Plasmid construction. Human CREB was amplified from total RNA extracted HepG2 cells by reverse transcription-polymerase chain reaction (RT-PCR). The total RNA was isolated by Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan). Three microgram of total RNA was reverse-transcribed to first strand cDNA for 1 h at 42 °C with PrimeScript™ Reverse Transcriptase (Takara, Shiga, Japan). Amplification of these cDNA by PCR was performing with PrimeSTAR™ HS DNA Polymerase (Takara, Shiga, Japan) using sense primer: 5′-TCGAATTCATGACCA TGGAATCTGGAGCCGAGAACC-3′, anti-sense primer: 5′- GCTCTA GATTAATCTGATTTGTGGCAGTAAAGGTCC-3′ (Underlined letters indicate the translation initiation codon and termination codon, respectively). Amplified cDNA was cloned into TOPO TA Cloning (Invitrogen, CA). The sequenced CREB cDNA was cloned into modified pCR™3 (pCRIIFL) to overexpress FLAG–CREB fusion protein in mammalian cells (hCREB/pCRIIFL).

In vitro translation and transcription of CREB protein. This procedure utilized the TNT® Coupled Reticulocyte Lysate System from Promega (Madison, WI) according to the manufacturer's instructions.

Immunoprecipitation. BAECs (4×10^6) were seeded in 10 cm dishes. Transfection was performed with HilyMax according to the manufacturer's protocol. Transfected BAECs were treated with or without 1,2-NQ (dissolved in DMSO) for 15 min. Treated cells were washed with D-PBS and then harvested with 1 ml of D-PBS. After centrifugation, cells were lysed with RIPA buffer containing protease-inhibitor for 20 min on ice. Cells were immunoprecipitated with anti-FLAG antibody (Sigma, MO) and Protein A-Sepharose™ CL-4B (Amersham Biosciences, UK) at 4 °C overnight. Pellets were subjected to Western blot analysis.

Western blot analysis. Samples for each analysis were separated by 10% (for CREB and FLAG) or 12% (for Bcl-2) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Gels were transferred to an immune-blot PVDF membrane, and then placed in blocking solution (TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) and 5% nonfat milk) for 1 h. Blots were incubated overnight with anti-1,2-NQ polyclonal antibody, anti-FLAG antibody conjugated with HRP (Sigma, MO), anti-CREB polyclonal antibody (Cell Signaling, MA), or anti-Bcl-2 monoclonal antibody (Upstate Biotechnology, NY), washed with TBST and incubated with HRP-conjugated secondary antibody. Polyclonal antibody against 1,2-NQ was prepared according to the methods of Zheng and Hammock [18]. Bound IgG was visualized using an ECL™ Western Blotting Detection Reagents (Amersham Bioscience, UK) according to the manufacturer's protocol. Band intensities were quantified using the NIH-image system (http://rsb.info.nih.gov/nih-image/).

Electrophoresis mobility shift assay (EMSA). Nuclear extracts were extracted from BAECs with the NE-PER® Nuclear Extraction Reagent (Pierce, IL). CREB concensus oligonucleotide (Promega, WI) was end-labeled using [γ-^{32}P]ATP, ~6000 Ci/mmol (GE Healthcare Bio-Sciences, NJ) and T4 polynucleotide kinase (Promega, WI). Nuclear protein extract (5 μg) was incubated for 30 min at room temperature in binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol (DTT), 50 mM NaCl, 10 mM Tris–HCl, and 50 μg/ml poly(dI–dC)). Then, appropriate cpm radiolabeled consensus oligonucleotide was added to each sample and incubated at room temperature for an additional 30 min. Protein–DNA complexes were subsequently resolved in a 7% native TBE (Tris–Borate–EDTA) gel in 0.5× TBE buffer. For competition experiments, a 50-fold molar excess of cold oligonucleotides were incubated in the mixture. Gels were dried and exposed to autoradiographic film at −80 °C. In studies using *in vitro* translated CREB, 5 μl of reaction mixtures were incubated with each concentration of 1,2-NQ (dissolved in DMSO) for 30 min at room temperature. After incubation, EMSA was performed as above-mentioned.

Transfection and luciferase assay. BAECs (2×10^5) were seeded in 12-well plates and transfected with 2.0 μg/well pCRE-Luc *cis*-reporter plasmid (Stratagene, CA) and 2 μl/well HilyMax (Dojindo Laboratories, Tokyo, Japan) according to the manufacturer's protocol. Transfected cells were treated with or without 1,2-NQ (dissolved in DMSO) for 30 min.

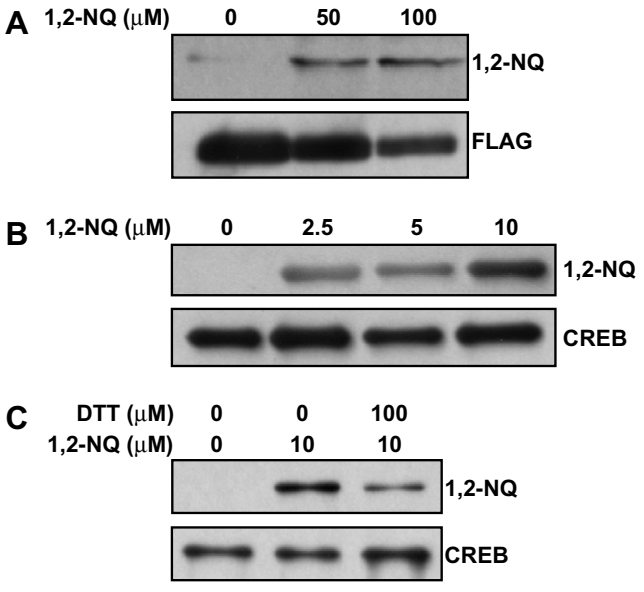

Fig. 1. Covalent binding of 1,2-NQ to CREB. (A) BAECs were transfected with hCREB/pCRIIFL for 24 h. Transfected cells were treated with vehicle, 50 or 100 μM of 1,2-NQ for 15 min. Cells were immunoprecipitated with anti-FLAG antibody, then Western blot analyses were performed with anti-FLAG or anti-1,2-NQ antibody. (B) Recombinant human CREB protein was incubated with vehicle and 2.5, 5 or 10 μM of 1,2-NQ for 30 min. Western blot analyses were performed with anti-1,2-NQ or anti-CREB antibody. (C) Recombinant human CREB was incubated with vehicle or 10 μM of 1,2-NQ in the absence or presence of DTT (100 μM) for 30 min. After incubation, Western blot analysis was performed with anti-1,2-NQ or anti-CREB antibody.

After washing with Dulbecco's phosphate buffered saline (D-PBS, Invitrogen, CA), cells were lysated in 100 μl of lysis buffer provided in the Promega Luciferase Assay System and centrifuged to remove the cell debris. The luciferase activity was measured in the relevant light units using a Luminometer TD-20/20 (Promega, WI). To normalize the transfection efficiency, the luciferase activity was expressed as a ratio of relative light units to the β-galactosidase obtained from the same cell lysates.

Statistical analysis. Data were obtained from three separate experiments. Each value represents the mean ± SD. Statistical significance was assessed by the Student's *t*-test for unpaired values, and differences between treatment groups were considered statistically significant at $P < 0.05$ (two-sided).

Results

1,2-NQ forms a covalent adduct with CREB

To test the hypothesis that CREB might be a molecular target for 1,2-NQ through covalent modification, we first immunoprecipitated with anti-FLAG and subsequently conducted a Western blot analysis with specific antibody against 1,2-NQ. As shown in Fig. 1A, exposure of hCREB/pCRIIFL-transfected BAECs to 1,2-NQ (50 and 100 μM) resulted in the irreversible binding of 1,2-NQ to CREB. Similar results were also observed in a concentration-dependent manner with recombinant CREB exposed to 1,2-NQ (Fig. 1B). Pretreatment with DTT (100 μM) blocked the formation of CREB-1,2-NQ adduct (Fig. 1C), suggesting that 1,2-NQ is covalently bound to CREB, presumably by Michael addition to thiol groups.

1,2-NQ inhibits CREB DNA-binding activity

To investigate whether 1,2-NQ affects the DNA binding function of CREB, we performed EMSA experiments. Treatment of BAECs with 2.5 and 5 μM of 1,2-NQ strongly inhibited the ability to bind DNA (Fig. 2A) in a dose-dependent manner (Fig. 2B). This effect was also

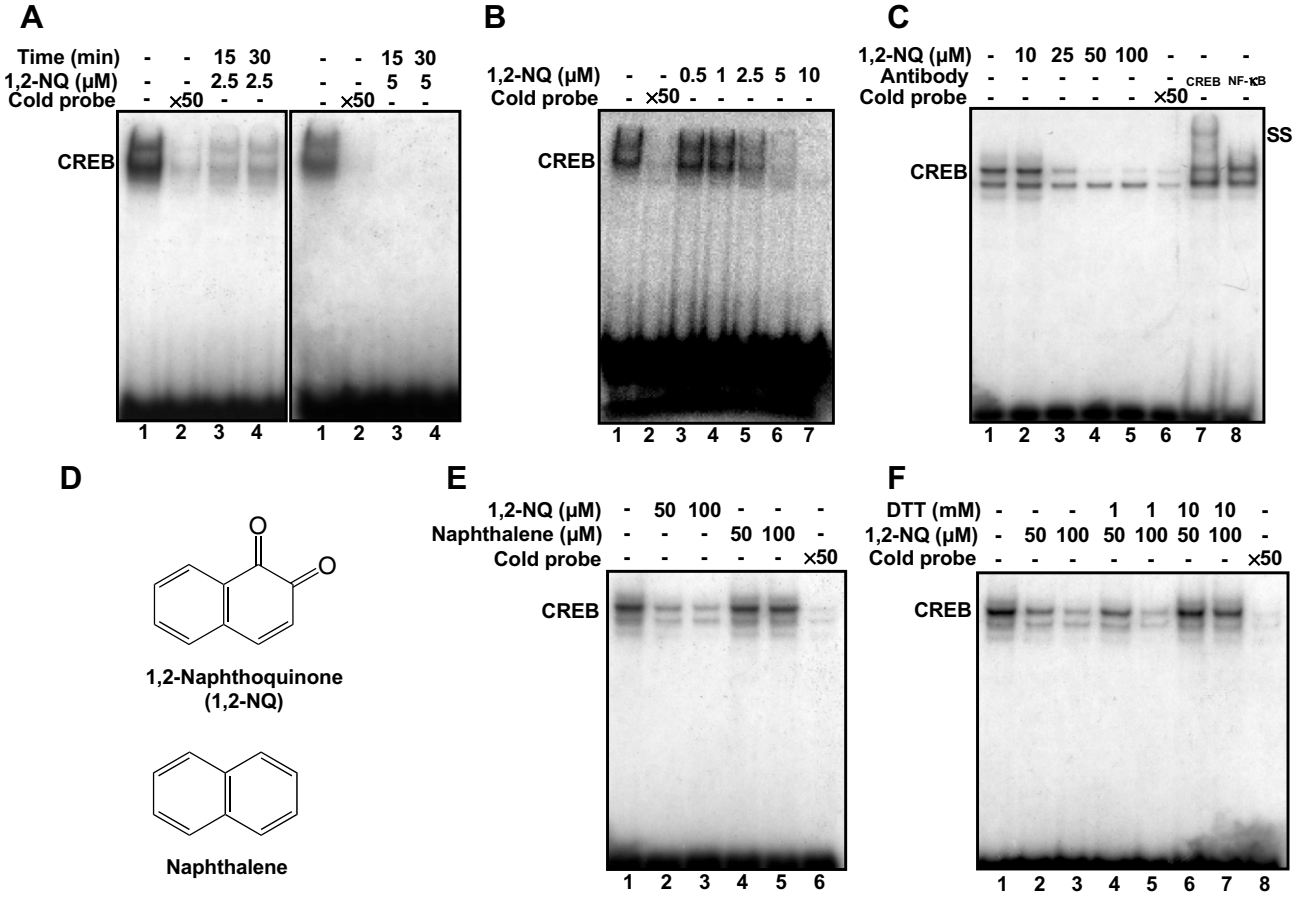

Fig. 2. 1,2-NQ inhibits CREB DNA binding activity. Nuclear extracts were obtained from BAECs and EMSA performed with 5 μg samples (A,B). (A) *Left*, cells were treated with vehicle (lane 1) or 2.5 μM of 1,2-NQ for 15 min (lane 3) or 30 min (lane 4). *Right*, cells were treated with vehicle (lane 1) or 5 μM of 1,2-NQ for 15 min (lane 3) or 30 min (lane 4). (B) Cells were treated with vehicle (lane 1) or the indicated concentrations of 1,2-NQ (lanes 3–10) for 30 min. (C) *In vitro* translated CREB was obtained using TNT® Coupled Reticulocyte Lysate System. The reaction mixtures were incubated with vehicle (lane 1) or the indicated concentrations of 1,2-NQ (lanes 2–5). Anti-CREB and NF-κB antibodies were used for the supershift assay (lanes 7 and 8). (D) Structures of 1,2-NQ and naphthalene. (E) Reaction mixtures were incubated with vehicle (lane 1), indicated concentrations of 1,2-NQ (lanes 2 and 3) or naphthalene (lanes 4 and 5). (F) Reaction mixtures were incubated with vehicle (lane 1), the indicated concentrations of 1,2-NQ in the absence (lanes 2 and 3) or presence of DTT (1 mM: lanes 4 and 5; 10 mM: lanes 6 and 7). DNA–CREB complexes were confirmed in the presence of 50-fold excess unradiolabeled oligonucleotides (A and B, lane 2; C and D, lane 6; F, lane 8).

observed in AP-1, another member of the bZIP family (data not shown). DNA binding, using CREB produced by *in vitro* transcription/translation system, supported the reduction in DNA binding for CREB caused by 1,2-NQ (Fig. 2C). As shown in lanes 7 and 8 in Fig. 2C, the super-shift experiment with anti-CREB antibody, or anti-NF-κB antibody as a negative-control, demonstrated that this binding was identified as CREB-CRE. Unlike 1,2-NQ, naphthalene (Fig. 2D) without α,β-unsaturated carbonyl groups, had no effect on the DNA binding activity of CREB (Fig. 2E). Furthermore, incubation of CREB to DTT (10 μM) prior to 1,2-NQ addition markedly suppressed the inhibition of DNA binding of CREB (Fig. 2F).

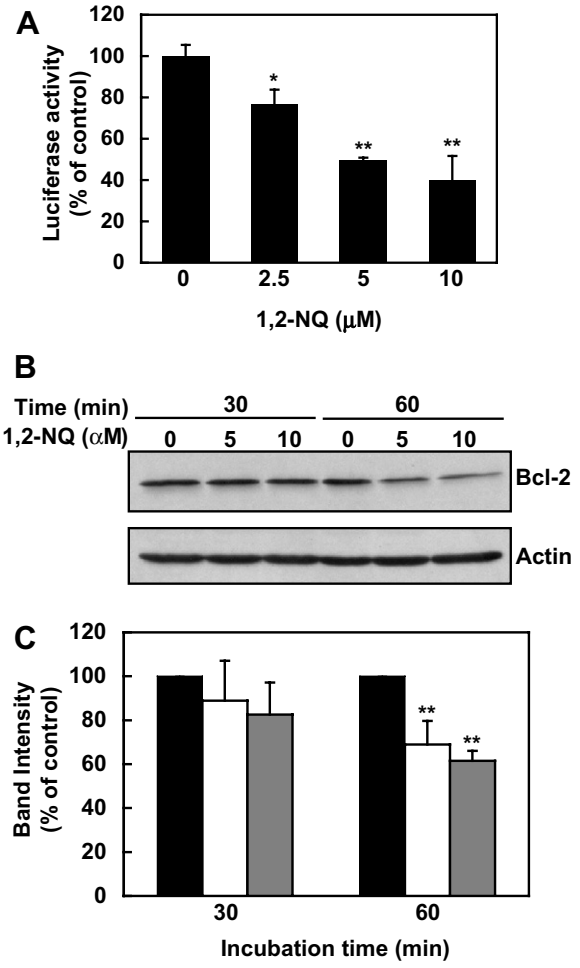

Fig. 3. 1,2-NQ inhibits CRE-dependent luciferase activity and down-regulates Bcl-2 protein expression. (A) BAECs were transfected with pCRE-Luc and pSV-β-gal for 24 h. Transfected cells were treated with the indicated concentration of 1,2-NQ for 30 min. To normalize the transfection efficiency, luciferase activity was expressed as a ratio to β-galactosidase, obtained from the same cell lysates. The % of control for luciferase activity was calculated and further normalized to vehicle ($^{*}P < 0.05$, $^{**}P < 0.01$ vs vehicle). (B) BAECs were treated with the indicated concentrations of 1,2-NQ for 30 or 60 min. After incubation, cell lysates (20 μg total protein) were subjected to Western blot analysis with anti-Bcl-2 antibody. (C) Statistical evaluation was conducted using three experiments for Bcl-2 (black bar; vehicle, white bar; 5 μM, gray bar; 10 μM). Bcl-2 band intensity was quantified with NIH-image system and normalized to the actin band intensity. The % of control for expression of Bcl-2 protein was calculated and further normalized to vehicle ($^{**}P < 0.01$ vs vehicle at same incubation time).

1,2-NQ diminishes CRE-dependent gene transcription and expression of CREB-regulated protein (Bcl-2)

As shown in Fig. 3A, there was a concentration-dependent suppression of transcription activity of CREB following exposure of BAECs to 1,2-NQ. Under these conditions, expression of CREB-regulated gene product, Bcl-2 was significantly down-regulated by 1,2-NQ exposure (Fig. 3B and C).

Discussion

The present study indicates that covalent interaction of 1,2-NQ with CREB causes suppression of the DNA binding activity and substantially down-regulates CREB-regulated protein expression in BAECs. With hCREB/pCRIIFL-transfected BAECs and recombinant CREB, we found that 1,2-NQ causes irreversible modification of CREB (Fig. 1A and B). Under these conditions, DNA binding activity of CREB, as evaluated by EMSA, was drastically inhibited by 1,2-NQ, but not naphthalene (Fig. 2A–E). These results suggest that covalent attachment of 1,2-NQ to CREB participates in the decreased DNA binding activity. Consistent with this notion, irreversible binding of 1,2-NQ to CREB and inhibition of the DNA binding activity of CREB during 1,2-NQ exposure were effectively blocked by treatment with DTT (Fig. 2F), suggesting that a 1,2-NQ forms covalent bonds with CREB through reactive thiol groups.

CREB has a bZIP domain at its C-terminal and binds to CRE as a homodimer or heterodimer with members of the CREB/ATF family. Transcription factors containing bZIP domain, have highly conserved cysteine residues in their DNA binding domains [15]. In this context, 15d-PGJ_2, as a Michael accepter, directly inhibits the DNA binding activity via modification of cysteine 269, which is located in the c-Jun DNA binding domain [16]. Human CREB has two cysteine residues in this domain, and chemical reduction of these thiols by DTT enhances DNA binding activity [19]. Overall, we speculate that modification of reactive thiols in DNA binding domain of CREB through oxidation and, now, arylation, plays a critical role in DNA binding activity of the transcription factor. There was an approximately 10-fold difference in 1,2-NQ concentration required to show an inhibitory effect in cells (Fig. 2A and B) and *in vitro* translated CREB (Fig. 2C, E, and F). This discrepancy may be due to an interaction of 1,2-NQ with the reticulocytes used in the study, since 1,2-NQ has a high affinity for hemoglobin [20,21].

In normal conditions, CREB is phosphorylated at Ser 133 by several upstream-protein kinases thereby promoting the transcription [22–28]. In a preliminary experiment, we found little inhibitory effect of 1,2-NQ on CREB phosphorylation during exposure of BAECs to 1,2-NQ (data

not shown), suggesting that decreased transactivation of CREB caused by 1,2-NQ is not due to blockage of its phosphorylation.

When DNA binding activity was suppressed by 1,2-NQ, CRE-dependent luciferase activity and CREB-regulated gene expression of proteins such as Bcl-2 were also suppressed (Fig. 3A–C). Bcl-2 exerts a survival function in response to a wide range of apoptotic stimuli through the inhibition of mitochondrial cytochrome *c* release [29]. Watanabe et al. [30] reported that cilostazol, potent inhibitor of type III phosphodiesterase, significantly enhances CREB phosphorylation, resulting in the up-regulation of Bcl-2. This increase in Bcl-2 was associated with improved learning memory and a decrease in the number of apoptotic cells in a rat model of chronic cerebral hyperfusion-induced white matter lesions. These results suggest that electrophiles may disturb learning, memory, and enhance apoptotic signaling through Bcl-2 down-regulation. CREB is able to mediate the cellular signals of numerous physiological stimuli which change a broad array of cellular responses. In summary, 1,2-NQ is an electrophile that affects CREB-mediated biological responses through interaction with protein thiols in mammalian cells. Since the functions of transcription factors such as NF-κB, AP-1, PPARγ [3], and estrogen receptor-α [4] are also regulated by cysteine residues, alterations in these transcriptional activities caused by 1,2-NQ is likely and remain to be elucidated.

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