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Research Article

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Reserpine Inhibit the JB6 P+ Cell Transformation Through Epigenetic Reactivation of Nrf2-Mediated Anti-oxidative Stress Pathway

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Abstract. Nuclear factor erythroid-2 related factor 2 (Nrf2) is a crucial transcription factor that regulates the expression of defensive antioxidants and detoxification enzymes in cells. In a previous study, we showed that expression of the Nrf2 gene is regulated by an epigenetic modification. *Rauvolfia verticillata*, a traditional Chinese herbal medicine widely used in China, possesses anticancer and antioxidant effects. In this study, we investigated how Nrf2 is epigenetically regulated by reserpine, the main active component in *R. verticillata*, in mouse skin epidermal JB6 P+ cells. Reserpine induced ARE (antioxidant response element)-luciferase activity in HepG2-C8 cells. Accordingly, in JB6 P+ cells, it upregulated the mRNA and protein levels of Nrf2 and its downstream target genes heme oxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase 1 (NQO1), while it only increased the protein level of UDP-glucuronosyltransferase 1A1 (UGT1A1). Furthermore, reserpine decreased the TPA (12-*O*-tetradecanoylphorbol-13-acetate)-induced colony formation of JB6 cells in a dose-dependent manner. DNA sequencing and methylated DNA immunoprecipitation further demonstrated the demethylation effect of reserpine on the first 15 CpGs of the Nrf2 promoter in JB6 P+ cells. Reserpine also reduced the mRNA and protein expression of DNMT1 (DNA methyltransferase 1), DNMT3a (DNA methyltransferases 3a), and DNMT3b (DNA methyltransferases 3b). Moreover, reserpine induced Nrf2 expression via an epigenetic pathway in skin epidermal JB6 P+ cells, enhancing the protective antioxidant activity and decreasing TPA-induced cell transformation. These results suggest that reserpine exhibits a cancer preventive effect by reactivating Nrf2 and inducing the expression of target genes involved in cellular protection, potentially providing new insight into the chemoprevention of skin cancer using reserpine.

KEYWORDS: epigenetics; JB6 P+; Nrf2; reserpine; skin cancer.

INTRODUCTION

Skin cancer is one of the most commonly diagnosed cancers, accounting for at least 40% of cases globally, particularly among fair-skinned people (1–3). The pathogenesis of skin cancer might be associated with many factors, such as exposure to ultraviolet radiation, chemical carcinogens, and inflammation. Among these factors, it has been reported that more than 90% of cases are induced by exposure to ultraviolet radiation (UVR) from the sun (4–7). UVR (between 200 and 400 nm) increases free radical production in human skin, causing DNA damage in skin cells and resulting in skin cancer (8–12). Free radicals produce oxidative stress, an important factor associated with many diseases and aging (13). Oxidative stress and inflammation are closely related, and once one process occurs in the body, the other will generally follow. The idea that oxidative stress leads to cancer has been confirmed in many studies (14,15). Nrf2 is a basic helix-loop-helix leucine zipper transcription factor that plays a key role in reducing cellular oxidative stress through regulation of the defense system (16,17). Nuclear translocation of Nrf2 activates the expression of anti-oxidative stress/detoxifying enzymes such as heme oxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase 1

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(NQO-1), and UDP-glucuronosyl transferase (UGT) by binding to the antioxidant response element (ARE) in the promoter region of target genes. In addition, we recently demonstrated that Nrf2 is downregulated during 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced neoplastic transformation of mouse skin epidermis JB6 P+ cells (18). The deregulation of the antioxidant defense system has received increased attention because this complication promotes susceptibility and neoplastic progression (18–21).

Previous studies have reported that carcinogenesis can be modulated by epigenetic alterations, such as DNA methylation, of tumor suppressor genes (22,23). DNA methylation represents an early molecular event preceding the observation of actual neoplastic lesions on the epidermis (24). In addition to genetic changes, accumulating evidence suggests that carcinogenesis is associated with aberrant epigenetic alterations, defined as gene expression that can be regulated without alteration of DNA sequences, in tumor suppressor genes or oncogenes (25,26). The regulation of DNA methylation by DNA methyltransferases (DNMTs) maintains cellular DNA stability and integrity and is the one of the major epigenetic mechanisms regulating the transcriptional activity of genes. DNMT inhibitors such as 5-azadeoxycytidine (5-aza) have been introduced as cancer therapeutics (27,28). However, the severe toxic effects and lack of gene specificity limit the application of these drugs. However, phytochemicals with DNA methylation-modulating properties are promising alternatives for cancer chemoprevention, as these compounds have minor side effects (29,30). In this study, we examined the anticancer effect of reserpine on a JB6 P+ cell transformation model and the epigenetic reactivation of the Nrf2 signaling pathway.

Rauvolfia verticillata (Lour.) Baill. (Luo Fu Mu in Chinese), which belongs to the family Apocynaceae, has been commonly used as a traditional Chinese medicine (TCM) for centuries to treat hypertension, snake bites, inflammation, and pruritus, among other diseases (31–33). *R. verticillata* is primarily distributed in the Yunnan and Guangxi provinces of China, India, and other tropical regions worldwide. A major group of compounds in *R. verticillata*, indole alkaloids, has been identified to include reserpine, yohimbine, and ajmalicine. Among these components, reserpine is the major active ingredient officially used as a quality control marker in the Chinese Pharmacopoeia (Fig. 1). To expand the clinical application of *R. verticillata*, we examined the potent effects of this compound against skin diseases. In China, as early as the 1950s, *R. verticillata* extract was used to effectively cure skin diseases that cause various degrees of itchiness and rash, with few side effects. However, few reports exist about the therapeutic mechanism underlying *R. verticillata* action. Therefore, we proposed that the production of free radicals in human skin induces skin disease, and the main components in *R. verticillata* extract exert an antioxidant response to the free radicals produced. Al-Qirim *et al.* (34) reported that *R. verticillata* extract protects mouse cardiomyocytes from damage caused by elevated levels of oxidative free radicals. In another study, Li *et al.* (35) showed that a water-soluble alkaloid extract from *R. verticillata* demonstrated strong antioxidant activity through scavenging 1,1-Diphenyl-2-picrylhydrazyl radical 2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydarazyl (DPPH) *in vitro*.

Therefore, we hypothesized that reserpine (the most abundant and main active compound in *R. verticillata* extract) might protect skin cells from ROS (reactive oxygen species) injury by activating the Nrf2 pathway via epigenetic modulation. In this study, we examined the underlying epigenetic changes caused by reserpine that protect cells from TPA-induced carcinogenesis by restoring Nrf2 expression through DNA methylation in a preneoplastic epidermal JB6 P+ cell line.

MATERIALS AND METHODS

Materials and Chemicals

Reserpine was extracted from *Rauvolfia verticillata* (Lour) Baill. (identification data are shown in the Supplementary Materials). Dimethyl sulfoxide (DMSO), 5-aza (5-azadeoxycytidine, a DNMT inhibitor, has been used as a potential chemotherapeutic agent for cancer), TPA, trichostatin A (TSA, (27,28), bacteriological agar, and Eagle's basal medium (BME) were purchased from Sigma (CO., CA). JB6 P+ cells were purchased from the American Type Culture Collection. Minimum essential media (MEM), fetal bovine serum (FBS), and trypsin-EDTA solution were purchased from Gibco Laboratories (Grand Island, NY). The primary antibodies anti-Nrf2, anti-HO-1, anti-NQO-1, anti-UGT1A1, and anti-β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-DNMT primary antibodies (DNMT1, DNMT3a, and DNMT3b) were obtained from IMGENEX (San Diego, CA).

Cell Culture and Treatment

The human hepatocellular HepG2-C8 cell line was previously established by stable transfection with an ARE-luciferase construct (36). The cells were cultured and maintained in DMEM supplemented with 10% (*V*/*V*) FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. JB6 P+ cells were maintained in MEM containing 5% (*V*/*V*) FBS in a humidified incubator with 5% CO_2 at 37°C. DMSO was used as a vehicle in all of the experiments at a concentration of 0.1%. After incubation for 24 h, the cells were treated with various concentrations of reserpine or 5-aza (250 nmol/L) in MEM containing 1% FBS. For the combination treatment of 5-aza and TSA, TSA (50 nmol/L) was added to the medium on the sixth treatment day. The treated cells were harvested on day 7 for additional assays.

Cell Viability Assay

JB6 P+ cells were seeded in 96-well plates containing MEM at a density of 1×10^4 cells/mL (100 μL/well) for 1, 3, and 5 days, and HepG2-C8 cells were seeded in plates containing DMEM. After incubation for 24 h, the cells were treated with either DMSO or various concentrations of reserpine. For JB6 P+ cells, the medium was changed every 2 days for the 3-day and 5-day treatments. Cell viability was assessed using a CellTiter 96 Aqueous One Solution Cell Proliferation (MTS) assay kit (Promega, Madison, WI) according to the manufacturer's instructions. The absorbance of the formazan product was read at 490 nm, and the cell viability was calculated and compared with the DMSO control group.

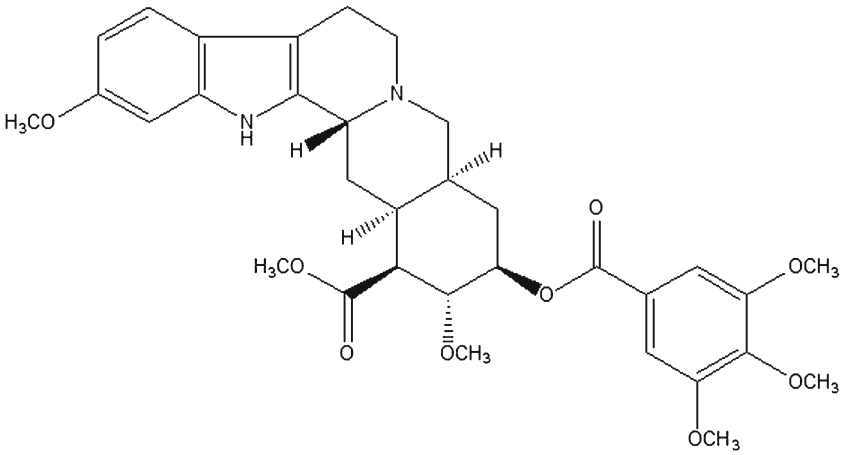

Fig. 1. Chemical structure of reserpine

Luciferase Reporter Activity Assay

The effects of reserpine on Nrf2-ARE activation were examined using HepG2-C8 cells stably expressing the ARE-luciferase construct. HepG2-ARE-C8 cells (1.0×10^5 cells/well) were seeded into 12-well plates in 1 mL of medium containing 10% FBS, incubated for 24 h and were subsequently treated with various concentrations of compounds. ARE-luciferase activity was determined using a luciferase assay kit according to the manufacturer's instructions (Promega, Madison, WI). The reporter lysis buffer was used to lyse the cells, and 10 μL of cell lysate and 50 μL of luciferase solution were combined to analyze luciferase activity using a Sirius luminometer (Berthold Detection System Gmbh, Pforzheim, Germany). We used a bicinchoninic acid (BCA) protein assay (Pierce Biotech, Rockford, IL, USA) to normalize the luciferase activity to protein concentrations. The data were obtained from three independent experiments and expressed as the inducible fold change compared with the DMSO control group.

Anchorage-Independent Cell Growth Assay

An agar mixture was divided into control (DMSO), TPA, and reserpine (2.5–10 μM) groups. BME containing 0.5% agar with 10% FBS without cells was added to the bottom of 6-well plates (3 mL/well) and maintained at room temperature for 1 h. Subsequently, the JB6 P+ cells (8×10^3/well) were transferred to 1 mL of BME in 0.33% soft agar containing TPA or various concentrations of reserpine layered on top of the agar. The cells were cultured with TPA (20 ng/mL) and other compounds at room temperature for an additional hour and subsequently incubated in a 5% CO_2 incubator at 37°C for 14 days. The cell colonies in soft agar were photographed using a computerized microscope system with the Nikon ACT-1 program (Version 2.20; LEAD Technologies) and counted using ImageJ (Version 1.40 g; NIH).

RNA Isolation and Quantitative Real-Time PCR

JB6 P+ cells were seeded into 10-cm dishes at a density of 1×10^4 cells/mL. The cells were treated with different concentrations of reserpine for 5 days after incubation for 24 h. Total RNA was extracted from the treated cells using an RNeasy Mini kit (Qiagen, Valencia, CA), and a Superscript III First-Strand cDNA Synthesis system (Invitrogen) was used to synthesize first-strand cDNA from total RNA. The mRNA expression of specific genes (β-actin, Nrf2, HO-1, NQO1, UGT1A1, DNMT1, DNMT3a, and DNMT3b) was subsequently determined by quantitative real-time PCR (qPCR) using first-strand cDNA as the template and Power SYBR Green PCR Master Mix (Applied Biosystems). The primer pairs have been previously described (37), and β-actin mRNA expression level was used as an internal loading control.

Whole Lysate Preparation and Western Blotting

After incubation for 24 h, JB6 P+ cells (1×10^5 cells/10-cm dish) were treated with various concentrations of reserpine. Whole cell lysates were prepared from the treated cells using radioimmunoprecipitation assay buffer (Cell Signaling Technology, Danvers, MA) supplemented with a protease inhibitor cocktail (Sigma), and a BCA kit was used to determine protein concentrations. The proteins were separated using 4–15% SDS-polyacrylamide gel electrophoresis (Bio-Rad) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). After blocking with 5% BSA in Tris-buffered saline-0.1% Tween 20 buffer for 1.5 h at room temperature, the membrane was sequentially incubated with specific primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies. The Super Signal enhanced chemiluminescence (ECL) detection and Gel Documentation 2000 system (Bio-Rad) were used to detect and record the antibody-bound proteins on the membrane. The densitometry of the bands was analyzed using ImageJ (Version 1.40 g; National Institutes of Health, NIH).

DNA Isolation and Bisulfite Genomic Sequencing

Genomic DNA was isolated from treated cells using a QIAamp DNA Mini kit (Qiagen). After incubation for 24 h, the cells were treated with reserpine at various concentrations or with 5-aza (250 nM) in combination with TSA (50 nM) in MEM containing 1% FBS for 7 days, and the medium was

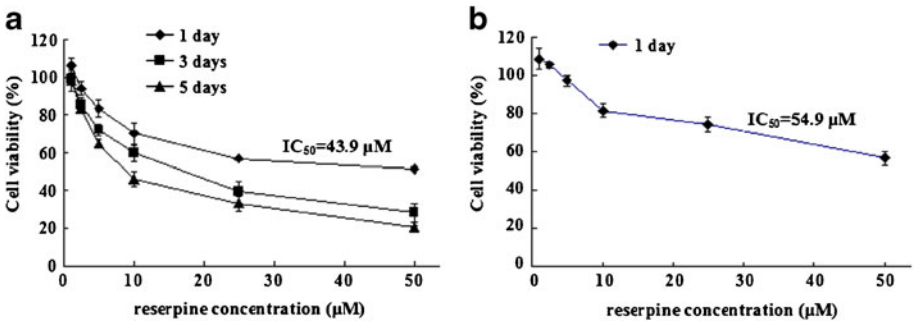

Fig. 2. Cell viability of JB6 P+ and HepG2-C8 cells after treatment by reserpine was determined and calculated using the MTS assay. **a** JB6 P+ cells were treated by reserpine for 1, 3, and 5 days. **b** HepG2-C8 cells were treated by reserpine for 1 day. The IC50 values were calculated using Origin Pro 7.5 software. The data are expressed as the mean ± SD ($n = 3$)

refreshed every 2 days. TSA was added to the medium on day 6, and the cells were harvested on day 7. The bisulfite conversion of genomic DNA was performed using a EZ DNA Methylation Gold kit (Zymo Research Corp.) according to the manufacturer's instructions, as previously described (38). The DNA fragment containing the first 15 CpGs, located between −863 and −1226 in the murine Nrf2 gene with the translation start site defined as position +1, was amplified from the converted DNA with PCR using Platinum Taq DNA polymerase (Invitrogen). The following primer sequences were used: sense, 5′-AGT TAT GAA GTA GTA GTA AAA A-3′ and anti-sense, 5′-ACC CCA AAA AAA TAA ATA AAT C-3′. The PCR products were cloned into the PCR 4 TOPO vector, and ten colonies from each treatment group were randomly selected. The plasmids were prepared using a QIAprep Spin Miniprep kit (Qiagen) and analyzed by sequencing (GeneWiz, South Plainfield, NJ).

Methylation DNA Immunoprecipitation Assay

Methylation DNA immunoprecipitation (MeDIP) analysis was performed using a EpiQuik™ MeDIP Ultra kit according to the manufacturer's instructions as previously described (23,39). The extracted DNA from treated cells was suspended in nuclease-free water and sonicated on ice to generate fragments of approximately 100–800 bp. The fragmented DNA was denatured at 95°C for 5 min and immunoprecipitated overnight at 4°C. The primers 5′-TTT CTA GTT GGA GGT CAC CAC A-3′ (sense) and 5′-CCC AGG GAG ATG GAT GAG T-3′ (anti-sense) were used to probe the DNA sequence containing the 15 CpG sites in murine Nrf2. The enriched MeDIP DNA content was calculated based on calibration using the serial dilution of input DNA, and the relative methylated DNA ratios were calculated based on the control, which was defined as 100% methylated DNA.

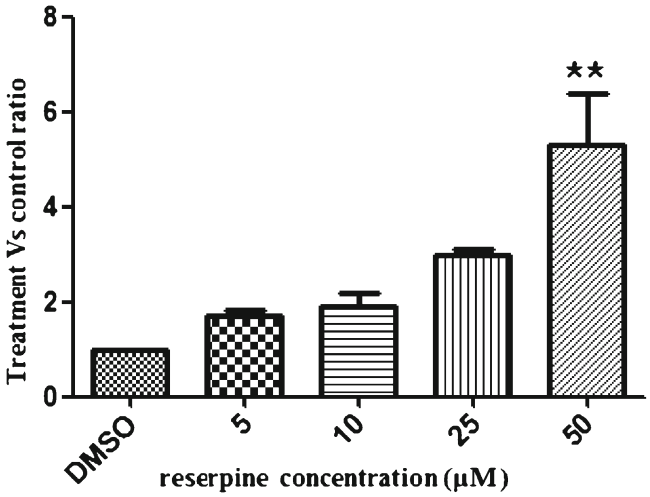

Fig. 3. The induction of ARE-luciferase activity of the treatment of reserpine with concentration from 5–50 μM on HepG2-C8 cells expressed with ARE-luciferase vector. The BCA protein assay was determined to normalize the luciferase activity. The data obtained from three independent experiments expressed the inducible fold change compared with the vehicle control. *Two asterisks* indicate significant difference $p < 0.01$ between the treatment and control group

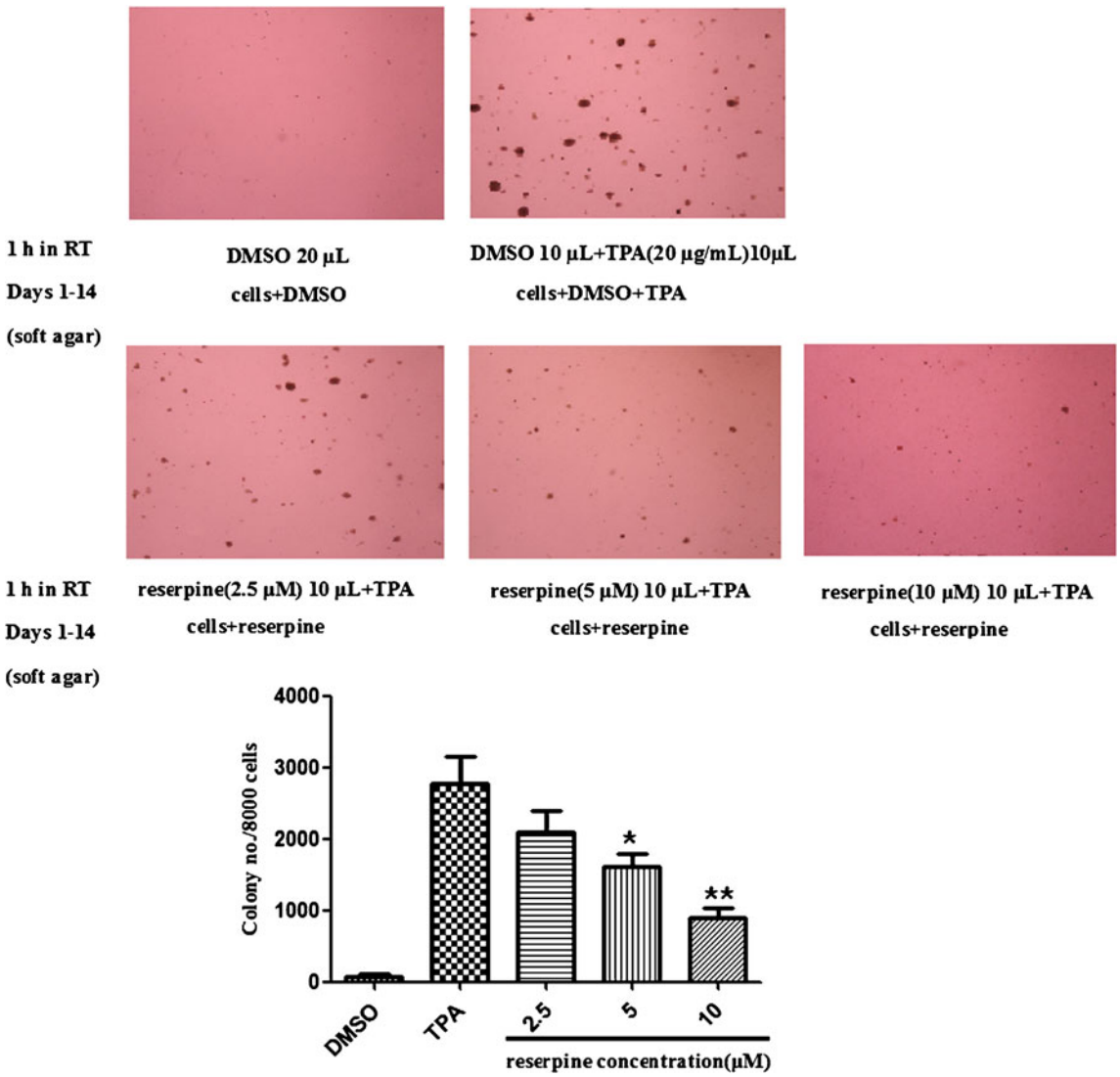

Fig. 4. Inhibitory effects of reserpine on the TPA-induced transformation of JB6 P+ cells. The colonies exhibiting anchorage-independent growth were counted under a microscope using ImageJ software. The data are represented as the average of triplicate results. *One asterisk* and *two asterisks* represent $P < 0.05$ and $P < 0.01$, respectively, which indicate significant differences between the reserpine-treated group and cells treated with TPA alone in soft agar

Statistical Analysis

The data are represented as the mean ± SD of three independent experiments with similar results. The statistical analyses were performed using ANOVA followed by post-hoc test (Dunnett's *t* test). The means were considered significantly different at $P < 0.05$ and $P < 0.01$.

RESULTS

Cytotoxicity of Reserpine in JB6 P+ and HepG2-C8 Cells

The viability of JB6 P+ cells after treatment with reserpine for 1, 3, and 5 days and HepG2-C8 cells for 1 day was analyzed using an MTS assay to determine the cytotoxic effect of reserpine. The results are shown in Fig. 2. IC50 values of 43.9 and 54.9 µM were obtained after 1 day of treatment in JB6 P+ and HepG2-C8 cells, respectively. We selected a reserpine concentration (2.5–10 µM) no greater than the IC50 value, ensuring viability greater than 70% for subsequent studies of the epigenetic modification of the Nrf2 promoter and avoiding substantial toxicity.

Reserpine Induces ARE-Luciferase Reporter Activity

The luciferase activity in cells transfected with the ARE-luciferase reporter vector in the treatment groups compared with the control group is shown in Fig. 3. Reserpine induced luciferase activity in a dose-dependent manner at concentrations ranging from 5 to 50 µM, and no significant induction was observed at concentrations lower than 5 µM.

Reserpine Inhibits TPA-Induced JB6+ Cell Transformation

JB6 P+ cells were incubated with TPA with or without reserpine in soft agar for 14 days to induce transformation. The effects of reserpine treatment on the TPA-induced anchorage-independent growth of JB6 P+ cells are shown in Fig. 4. Reserpine treatment at concentrations of 5 and 10 μM significantly decreased the number of JB6 P+ colonies compared with the TPA-treated control group ($p < 0.05$ and $p < 0.01$, respectively), although no significant difference was observed between the 2.5-μM reserpine treatment group and the control group. These results indicated that reserpine might exert chemopreventive effects against TPA-induced carcinogenesis in JB6 P+ cells.

Reserpine Upregulates the mRNA and Protein Expression of Nrf2 Target Enzymes in JB6 P+ Cells

The expression of enzymes regulated by Nrf2 in JB6 P+ cells treated with reserpine for 5 days was measured using qPCR, and the reserpine treatment groups exhibited increased mRNA expression of Nrf2, HO-1, NQO1, and decreased mRNA expression of UGT1A1 in a concentration-dependent manner (Fig. 5a), although the effect on UGT1A1 was not statistically significant. Western blotting experiments were further used to evaluate the protein levels of Nrf2, HO-1, NQO1, and UGT1A1 in JB6 P+ cells treated with reserpine (Fig. 5b) and revealed that reserpine (2.5–10 μM) also increased the protein expression of Nrf2, HO-1, and NQO1. However, UGT1A1 expression was only slightly increased in a concentration-dependent manner in JB6 P+ cells (Fig. 5c). These experimental results suggested that the increased expression of Nrf2 in JB6 P+ cells indicates the potential of reserpine to increase Nrf2-mediated mRNA and protein expression of antioxidant and detoxifying enzymes.

Reserpine Inhibits the mRNA and Protein Expression of Epigenetic Modification Enzymes in JB6 P+ Cells

Epigenetic modification enzymes such as DNMTs, which silence gene expression, are a major target of cancer prevention and therapeutic strategies. Thus, the effect of reserpine on DNMT1, DNMT3a, and DNMT3b was examined to investigate possible epigenetic mechanisms. Reserpine at concentrations of 2.5–10 μM decreased the mRNA expression of DNMT1, DNMT3a, and DNMT3b in a concentration-dependent manner in JB6 P+ cells after 7 days of treatment (Fig. 6a). Reserpine at 10 μM generated a significant difference for DNMT3a expression ($p < 0.05$). As shown in Fig. 6b, the protein expression of DNMT1, DNMT3a, and DNMT3b was decreased in JB6 P+ cells after reserpine treatment. DNMT3a and DNMT3b expression was lower after reserpine (10 μM) treatment compared with the control group, although the effect was not statistically significant. Reserpine significantly decreased DNMT1 protein expression in a concentration-dependent manner, especially in the concentration of 5 and 10 μM ($p < 0.01$). According to the results of the mRNA and protein expression analyses, Nrf2 plays a key role in the inhibitory effects of reserpine on TPA-induced JB6 P+ cell transformation by regulating the expression of anti-oxidative stress enzymes.

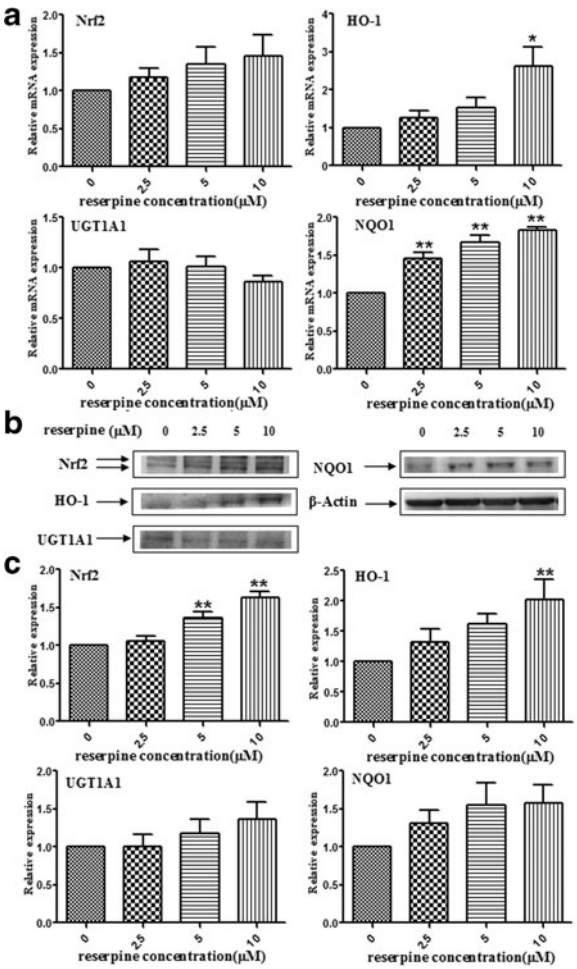

Fig. 5. Effect of reserpine (2.5–10 μM) on Nrf2 mRNA and protein expression of Nrf2 target genes (HO-1, NQO1, and UGT1A1) in JB6 P+ cells were determined using real-time qPCR and Western blot methods. The graphical data are presented as the mean ± SD from three independent experiments. *One asterisk* and *two asterisks* represent $P < 0.05$ and $P < 0.01$, respectively, which indicate significant difference in each treatment compared with the DMSO control group. **a** Reserpine increased the mRNA levels of Nrf2 and its downstream enzymes; **b** Western blot images of Nrf2 and its downstream genes; **c** Reserpine increased the protein expression of Nrf2 and its downstream genes. The relative protein expression levels were quantified based on the signal intensity of the corresponding bands from three independent experiments and normalized using β-actin for the total cellular protein level. The images were analyzed using ImageJ software

Reserpine Decreases the Proportion of Methylated CpG Sites in the Nrf2 Promoter in JB6 P+ Cells

The Nrf2 promoter region encoding 15 CpGs was converted and amplified, and the methylation status of the CpGs was examined to determine whether the CpG sites were demethylated after a 5-day treatment with bisulfite genomic sequencing (BSG), using 0.1% DMSO and the combination of 5-aza/TSA (250/50 nM) as controls. The results showed that JB6 P+ cells treated with DMSO were highly methylated (85.16%; Fig. 7). Treatment with reserpine at 10 μM significantly decreased ($P < 0.01$) the methylation status of these CpG sites, showing 65.11% methylated CpG.

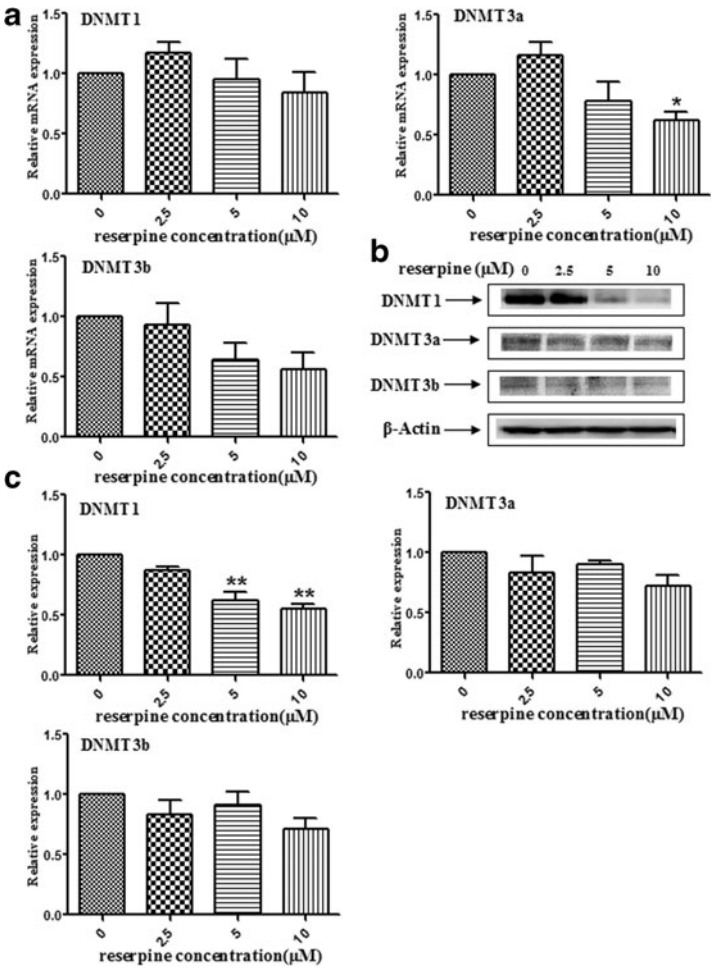

Fig. 6. Effect of reserpine (2.5–10 μM) on DNMT (DNMT1, DNMT3a, and DNMT3b) mRNA and protein expression in JB6 P+ cells. The expression of DNMTs genes mRNA and proteins were detected by real-time PCR and Western blotting, respectively. **a** Reserpine decreased the mRNA level of DNMT1, DNMT3a, and DNMT3b; **b** Western blot images of DNMTs including DNMT1, DNMT3a, and DNMT3b; **c** Reserpine significantly inhibit the protein levels of DNMTs; The graphical data are represented as the mean ± SD from three independent experiments, $*p < 0.05$ and $**p < 0.01$, respectively, indicate significant differences compared treatment with the control groups

The 5-aza/TSA group also showed significant demethylation ($p < 0.01$, 63.73% methylated CpG) compared with DMSO-treated cells. These results suggest that reserpine leads to demethylation of the Nrf2 promoter in JB6 P+ cells.

Reserpine Significantly Decreases the Binding of Anti-methyl Cytosine Antibody to the 15 CpG Sites in the Nrf2 Promoter in JB6 P+ Cells

We performed MeDIP to confirm the above findings. The methylated DNA fragments were enriched by immunoprecipitation using the anti-methylcytosine (anti-MeCyt) antibody, which specifically binds to methylated cytosines. The enriched methylated DNA was used as a template for qPCR analysis to amplify the Nrf2 promoter region (Fig. 8), and the relative amount of MeDIP DNA was calculated using a standard curve of delta CT values obtained through the serial dilution of inputs. The qPCR results showed that reserpine (5 and 10 μM) and 5-aza/TSA significantly reduced the total amount of MeCyt enrichment at the 15 CpG sites in the Nrf2 promoter compared with DMSO (control group) ($p < 0.01$) and that the relative methylated DNA ratio was less than 60%. Even the low concentration of reserpine (2.5 μM) significantly reduced the amount of MeCyt enrichment at the 15 CpG sites compared with the control group ($p < 0.01$), and the relative methylated DNA ratio was 80.3%. Thus, these results suggest that reserpine reverses the methylation level of specific CpG sites in the Nrf2 promoter in JB6 P+ cells.

DISCUSSION

Oxidative stress caused by endogenous and exogenous ROS accelerates the development of carcinogenesis, resulting

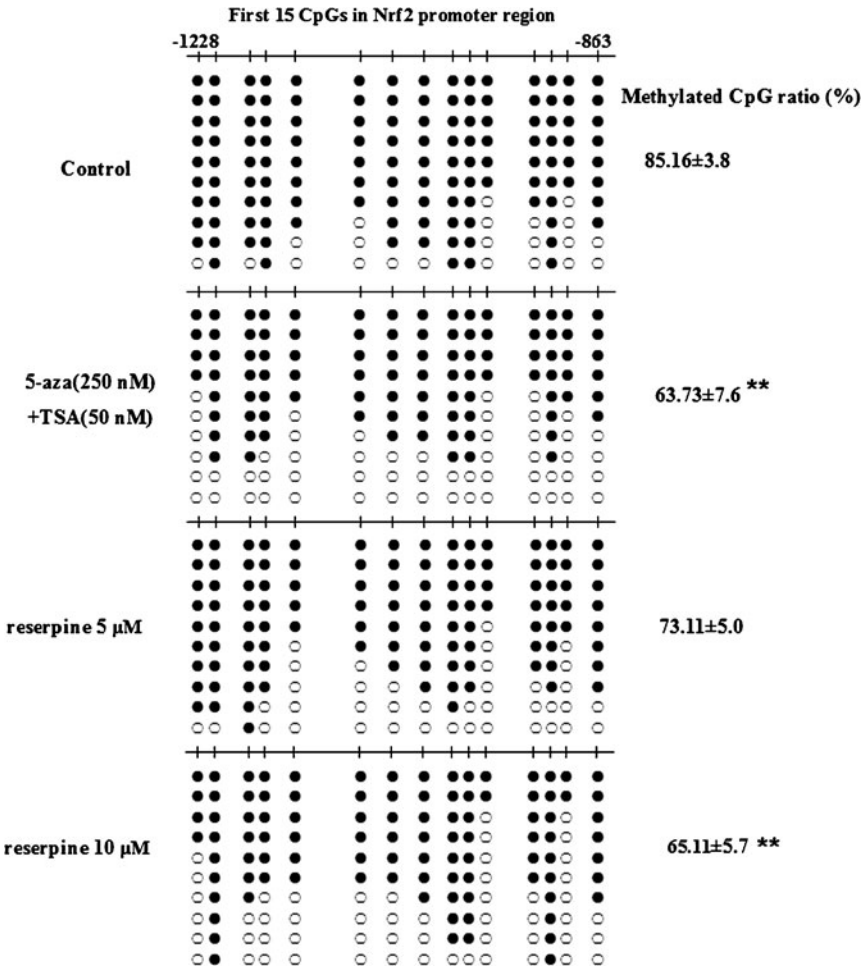

Fig. 7. The methylation level at the 15 CpG sites (located between −1226 and −863) in the Nrf2 promoter was determined using bisulfite genomic sequencing (BGS). DNA samples were extracted from reserpine-treated JB6 P+ cells (1×10^5/10-cm dish) after 5 days of treatment. Reserpine of 2.5 to 10 μM and combination of 5-aza (250 nM)/TSA (50 nM) groups were compared with the DMSO control group to show the significant differences (**$p < 0.01$) in methylation level. *Black dots* indicate methylated CpGs; *open circles* indicate non-methylated CpGs. The 15 CpG sites were the murine Nrf2 gene with the translational start site defined as +1. The values of methylated CpG ratio are the mean ± SD of at least ten clones from three independent experiments

in genetic mutation and neoplastic transformation (40,41). Skin carcinogenesis is often triggered by exogenous ROS inducers such as air pollution, UV irradiation, and xenobiotics (42); however, the cellular endogenous defense system can be enhanced with factors such as antioxidants to protect skin cells from ROS-induced injury (43,44). Previous studies have revealed that many chemical or dietary factors can function as potential cancer chemopreventive agents, reflecting the induction effects of these compounds on phase II detoxifying and antioxidant enzymes, including HO-1, NQO1, and glutathione-S-transferases (45,46). In addition, the tumor promoter TPA induces lipid peroxidation in ICR mouse skin (47), and TPA also induces ROS overproduction and neoplastic transformation in mouse skin JB6 cells (48). In this study, the inhibitory effect of reserpine on TPA-stimulated neoplastic transformation in a mouse epidermal JB6 P+ cell line was investigated to understand the chemopreventive potential of reserpine against skin tumorigenesis. The results suggest that reserpine suppressed the TPA-induced anchorage-independent growth of JB6 P+ cells in soft agar (Fig. 4) by upregulating HO-1, NQO1, and UGT1A1 (Fig. 5).

In this study, we found that reserpine inhibited the transformation of JB6 P+ cells at the concentration of 2.5–10 μM. Reserpine has been used as antihypertensive drug for many years. The doses range from 0.03 to 0.3 mg every day for adults (49). A dose range from 0.1 to 0.25 mg typically can effectively deplete catecholamines from peripheral sympathetic nerve endings. As well, reserpine has been reported to be tumorigenic and teratogenic at low doses (50). Nevertheless, in the context of skin cancer, the drug exposure used in our study could be achieved locally by topical administration of reserpine. In addition, our results indicate that the IC50 is around 44 uM in HepG2 cells, which is much higher than the concentration resulting in the epigenetic regulation on Nrf2-mediated anti-oxidative pathway. Therefore, the

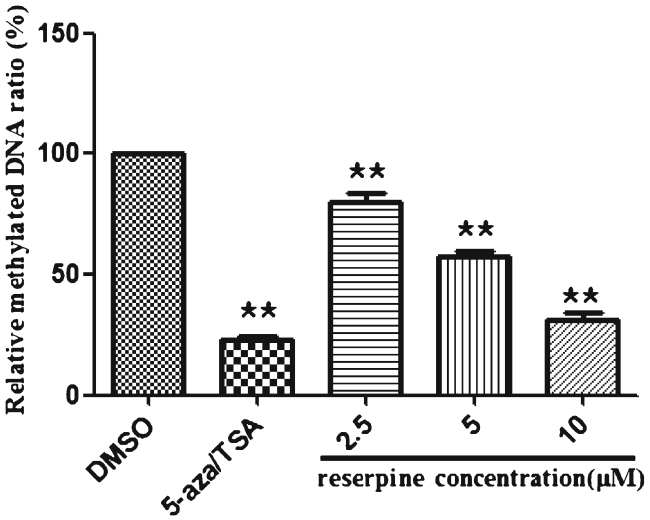

Fig. 8. Methylated DNA immunoprecipitation experiment was performed using the Epigentek kit. Then we used qPCR to analyze the immunoprecipitated DNA and inputs using primers covering the 15 CpG sites in the Nrf2 promoter. Reserpine significantly decreases the binding of anti-methyl cytosine antibody to the 15 CpGs sites in the Nrf2 promoter in JB6+ cells compared with the DMSO control group, which was defined as 100% of methylated DNA. The values are expressed as the mean ± SD of three separate experiments. *Two asterisk sign* indicated significant differences ($p < 0.01$) in the relative methylated DNA ratio

concentration we selected could be effective and avoid unexpected cell damage as a chemopreventive strategy.

Nrf2-deficient mice are susceptible to carcinogen-induced tumorigenesis (51,52). Nrf2 induces the expression of anti-oxidative stress/detoxifying enzymes, such as HO-1, NQO-1, UGT1A1, and GST (53–55), which has been partially associated with cancer chemoprevention (56,57). Nrf2 is regulated by the adaptor protein Keap1 (Kelch-like ECH-associating protein 1), which bridges Nrf2 and Cul3 in the cytoplasm, resulting in the ubiquitination of Nrf2 (58). Once the interaction of Keap1 and Nrf2 is disrupted and cytoplasmic and nuclear Nrf2 accumulates, Nrf2 binds to the ARE in the promoter region of some phase II enzyme genes and triggers the expression of Nrf2 downstream enzymes (59–61). Therefore, many dietary phytochemicals with chemopreventive potential have been reported to enhance the activities of phase II enzymes through the Nrf2-ARE pathway (20). Interestingly, the results of this study showed reserpine-induced ARE-luciferase activity in HepG2-C8 cells (Fig. 3), suggesting that reserpine also induced the Nrf2-ARE pathway in JB6 P+ cells, upregulating HO-1, NQO1, and UGT1A1 (Fig. 5).

In a recent study, we demonstrated that the expression of Nrf2 is regulated by epigenetic changes in both the prostate tissue of transgenic adenocarcinoma of the mouse prostate (TRAMP) mice and in tumorigenic TRAMP C1 cells (38). We also reported that Nrf2 expression is enhanced in TRAMP C1 cells by epigenetic regulation after treatment with phytochemicals, including curcumin (39), a γ-tocopherol-rich mixture of tocopherols (62), Z-ligustilide (37), sulforaphane (63), and 3,3′-diindolylmethane (64). The chemopreventive effect of phytochemicals such as sulforaphane (18), apigenin (65), and tanshinone IIA (66) against TPA-induced skin tumorigenesis by activating the critical Nrf2-mediated pathway through epigenetic modification has also been demonstrated in JB6 P+ cells. These results suggest that the silenced Nrf2 gene can be activated by DNA demethylation using chemopreventive compounds as epigenetic modifiers. In this study, the induction of Nrf2 by reserpine via DNA demethylation was demonstrated in JB6 P+ cells (Figs. 7 and 8), likely contributing to the prevention of TPA-induced neoplastic transformation (Fig. 4). As shown in Fig. 8, with increased concentration of reserpine (2.5, 5, and 10 μM), the relative methylated DNA ratio decreases significantly. Corresponding with previous report from our group, the positive control 5aza/TSA can reduce the total amount of MeCyt enrichment of the 15 CpG sites in the Nrf2 promoter, which is associated with the enhanced transcription activity of Nrf2 (18). Our current results suggest that reserpine can reverse the methylation status of these specific CpG sites in the Nrf2 promoter in JB6 cells. Additionally, the suppression of DNA methylation enzymes such as DNMTs, which silence gene expression, is a major strategy for cancer prevention and therapy (67). We also showed that the mRNA and protein levels of DNMT1, DNMT3a, and DNMT3b were decreased in JB6 P+ cells after treatment with reserpine in this study (Fig. 6).

In conclusion, the potential of reserpine as a cancer preventive has not previously been reported (68,69); thus, this study is the first to demonstrate that reserpine promotes cellular antioxidant activity, particularly through the Nrf2 pathway, contributing to the prevention of the neoplastic growth of JB6 P+ cells induced by TPA. Interestingly, we also showed that reserpine might alter DNA demethylation and epigenetically enhance Nrf2 expression, suggesting that the preventive potential of reserpine against skin carcinogenesis is mediated through a novel molecular mechanism.

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Analysis and interpretation of data (*e.g.*, statistical analysis, biostatistics, and computational analysis): B. Hong, Yuqing Yang, and Yue Guo.

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COMPLIANCE WITH ETHICAL STANDARDS

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Conflict of Interest The authors declare that they have no competing interests.

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