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Elevated pressure, a novel cancer therapeutic tool for sensitizing cisplatin-mediated apoptosis in A549

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ABSTRACT

Intensive cancer therapy strategies have thus far focused on sensitizing cancer cells to anticancer drug-mediated apoptosis to overcome drug resistance, and this strategy has led to more effective cancer therapeutics. Cisplatin (cis-diamminedichloroplatinum(II), CDDP) is an effective anticancer drug used to treat many types of cancer, including non-small cell lung carcinoma (NSCLC), and can be used in combination with various chemicals to enhance cancer cell apoptosis. Here, we introduce the use of elevated pressure (EP) in combination with CDDP for cancer treatment and explore the effects of EP on CDDP-mediated apoptosis in NSCLC cells. Our findings demonstrate that preconditioning NSCLC cells with EP sensitizes cells for CDDP-induced apoptosis. Enhanced apoptosis was dependent on p53 and HO-1 expression, and was associated with increased DNA damage and down-regulation of genes involved in nucleotide excision repair. The transcriptional levels of transporter proteins indicated that the mechanism by which EP-induced CDDP sensitization was intracellular drug accumulation. The protein levels of some antioxidants, such as hemeoxygenase-1 (HO-1), glutathione (GSH) and glutathione peroxidase (Gpx), were decreased in A549 cells exposed to EP via the down-regulation of the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf-2). Furthermore, normal human fibroblasts were resistant to EP treatment, with no elevated DNA damage or apoptosis. Collectively, these data show that administration of EP is a potential adjuvant tool for CDDP-based chemosensitivity of lung cancer cells that may reduce drug resistance.

1. Introduction

The platinum drug cisplatin (cis-diamminedichloroplatinum(II), CDDP) is an important chemotherapeutic drug with anticancer activity and is widely used for the treatment of various types of solid tumors, including non-small cell lung carcinomas (NSCLC) [1]. Many studies have elucidated the mechanism of action of CDDP [2–4], and the widely-accepted primary mechanism of CDDP involves cytotoxic effects due to the formation of DNA intra-strand adduct [5]. However, some therapeutic limitations remain, such as inherent and acquired drug resistance. Combining available therapeutic tools offers a powerful new approach to overcome drug resistance and to sensitize cancer cells to anticancer therapy.

Tumor microenvironmental factors are known to cause alterations in the cellular status of cancer cells, which may alter the sensitivity of these cells to CDDP-mediated apoptosis [6,7]. Recent cancer therapeutic strategies have considered the tumor microenvironment, as the growth and behavior of tumors is strongly affected by factors in the microenvironment, such as hypoxia and vascularization [8,9]. ROS generation and oxygenation of the hypoxic tumor microenvironment may be effective forms of cancer therapy. Furthermore, treatment with ROS generators during chemotherapy and radiotherapy improves the response of many solid tumors [10–12]. However, the effectiveness of ROS generators is controversial, since ROS have also been reported to promote angiogenesis and carcinogenesis as well as to stimulate tumor formation [13–16].

Mechanical stresses have also emerged as important factors affecting the cancer cell microenvironment. Elevated pressure (EP), the application of which involves an extrinsic mechanical force applied to cells or whole tissues, inhibits cellular growth in *in vitro* models of venous hypertension [17] and senescence [18–20] through several molecular mechanisms. Recent studies from our laboratory demonstrated that EP (2ATA) enhanced TNF-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis through up-regulation of death receptor 5 and activation of caspase-8 in various cancer cell lines, including a NSCLC line, which was the first to demonstrate potentiating apoptosis in cancer cells by combining TRAIL and EP [21].

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The results from our present study show that pretreatment with EP potentiates cancer cell apoptosis when cells are then treated with CDDP. The mechanism of this sensitizing effect is likely through EP inhibiting cancer cell CDDP resistance, including a reduction in nuclear excision repair genes and the antioxidant HO-1.

2. Materials and methods

2.1. Cell lines and culture conditions

The source of all cell lines for the present study was ATCC (Manassas, VA). The non-small cell lung carcinoma cell lines A549, H460, H1299, and H157 were grown in RPMI 1640 medium. WI-38 cells, a lung fetal normal fibroblast line, were maintained in Dulbecco's modified Eagle's medium. Elevating pressure to 2ATA has been described previously [18]. Cells were pretreated with EP for 2 days prior to incubation with CDDP.

2.2. In vitro cytotoxicity and apoptosis assay

Cell viability was measured using the CellTiter 96 Aqueous One Solution Assay (Promega, Madison, WI). The soluble reaction product was quantified spectrophotometrically at 490 nm using an ELISA reader VERSAmax (Molecular Devices, Sunnyvale, CA). Apoptosis was quantified using the Annexin V-FITC apoptosis detection kit (BD Pharmingen, San Diego, CA) according to the manufacturer's protocol. Cell analyses were performed using a FACS Calibur flow

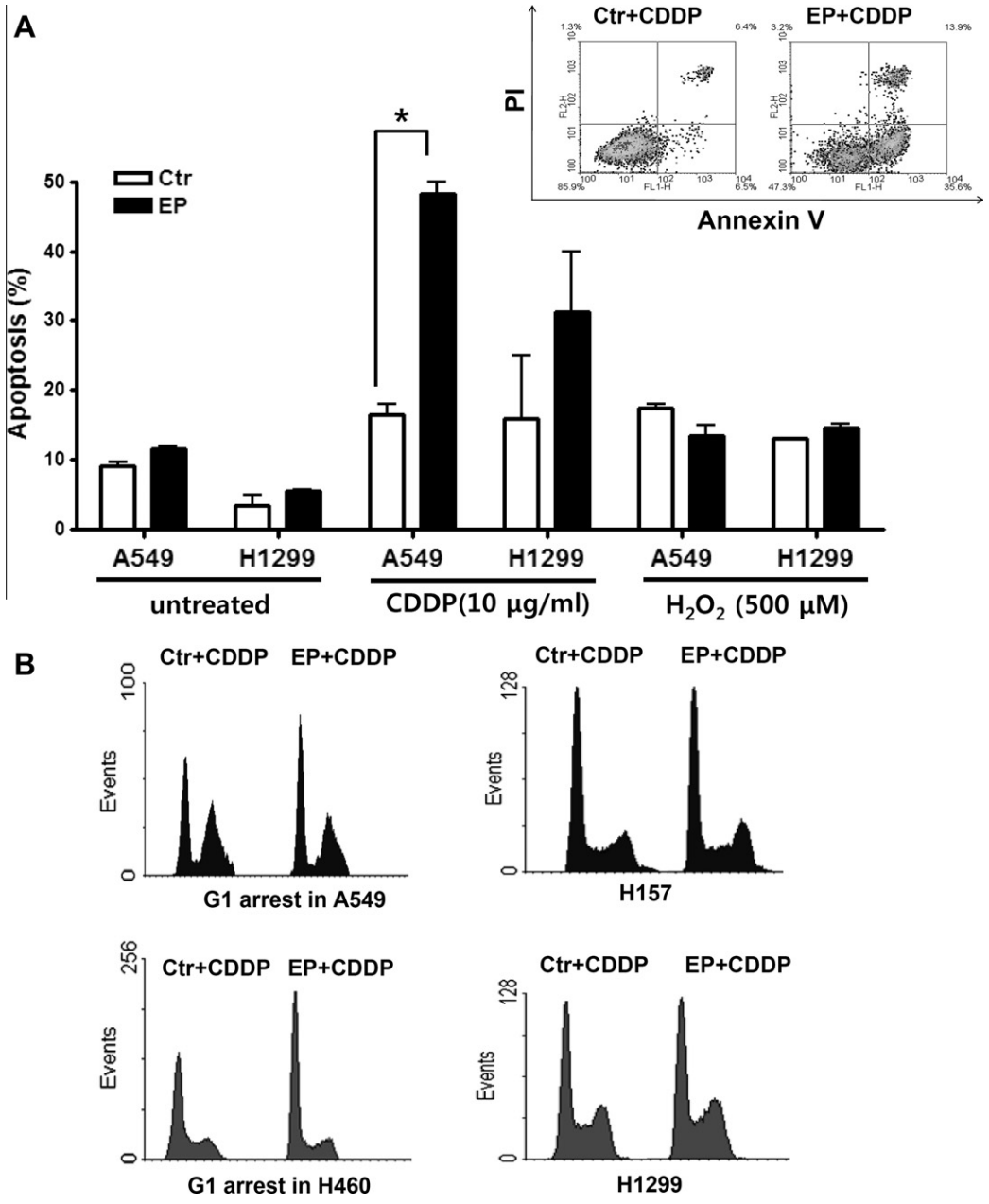

Fig. 1. Pretreatment of NSCLC cancer cells with EP induces CDDP-mediated apoptosis and G1 arrest in a p53-dependent manner. (A) A549 (p53+/+) and H1299 (p53−/−) cells were pretreated with EP for 2 days prior to addition of CDDP or media alone. Rate of apoptosis was determined by Annexin V/PI staining and flow cytometric analysis after exposure of cells to CDDP for 24 h. Hydrogen peroxide was also used to measure apoptosis under corresponding EP and time conditions. The two dot plots are representative for A549 cells exposed to Ctr/CDDP (left) and EP/CDDP (right). *Indicates difference is statistically significant compared to the control group ($p < 0.05$). (B) p53 Wild type (A549 and H460) and p53 mutant type (H1299−/−, H157 mut) cells were pretreated with EP, incubated with CDDP (10 μg/ml) for 6 h, and stained with PI (20 μg/ml) for cell cycle analysis.

cytometer (Becton–Dickinson, Mountain View, CA) and CellQuest software (BD Biosciences).

2.3. Cell cycle analysis

Cells were trypsinized, pelleted by centrifugation, and washed twice with PBS. Harvested cells were fixed with 70% EtOH at 4 °C overnight. Fixed cells were washed to remove EtOH, resuspended in 1 ml of PBS containing RNase (100 μg/ml) and propodium iodide (PI, 10 μg/ml), and incubated for 20 min at room temperature in the dark. Analyses were performed on a FACs Calibur flow cytometer. PI was excited at 488 nm, and fluorescence emissions were collected at 640 nm. Data were analyzed with WinMDI (multiple document interfaces for windows).

2.4. Single-cell gel electrophoresis (SCGE, comet assay)

DNA damage was measured by the alkaline comet assay, as described previously [18]. The slides were incubated with Gel Red and examined using a Komet 5.5 image analysis system (Kinetic Imaging, Liverpool, UK) fitted with an Olympus BX50 fluorescence microscope, for which the parameters of Olive tail moment (OTM) and tail distance (TD) were automatically calculated.

2.5. Western blot

Lysate (15 μg) was fractionated by 12.5% SDS gels and transferred to PVDF membranes (Millipore, Bradford, MA). The membranes were probed with antibodies specific for the proteins of interest, washed with Tween 20 in PBS, and incubated with peroxidase-conjugated secondary antibody. Immunoreactivity was detected using an ECL kit (GE Healthcare, Buckinghamshire, UK), and quantitative data were obtained using molecular imaging software (Kodak, New Haven, CT).

2.6. Immunofluorescence assay (IFA)

A549 cells were plated on cover slips in 24-well cell culture plates. Following treatment, cells were fixed with 3.7% formaldehyde in PBS. Samples were then permeabilized with cold 90% MeOH and blocked for 30 min with 1% bovine serum in PBS. The samples were incubated with anti-γH2AX (Ser-139) primary antibody (Milipore, Billerica, MA) (2 μg/ml) at room temperature for

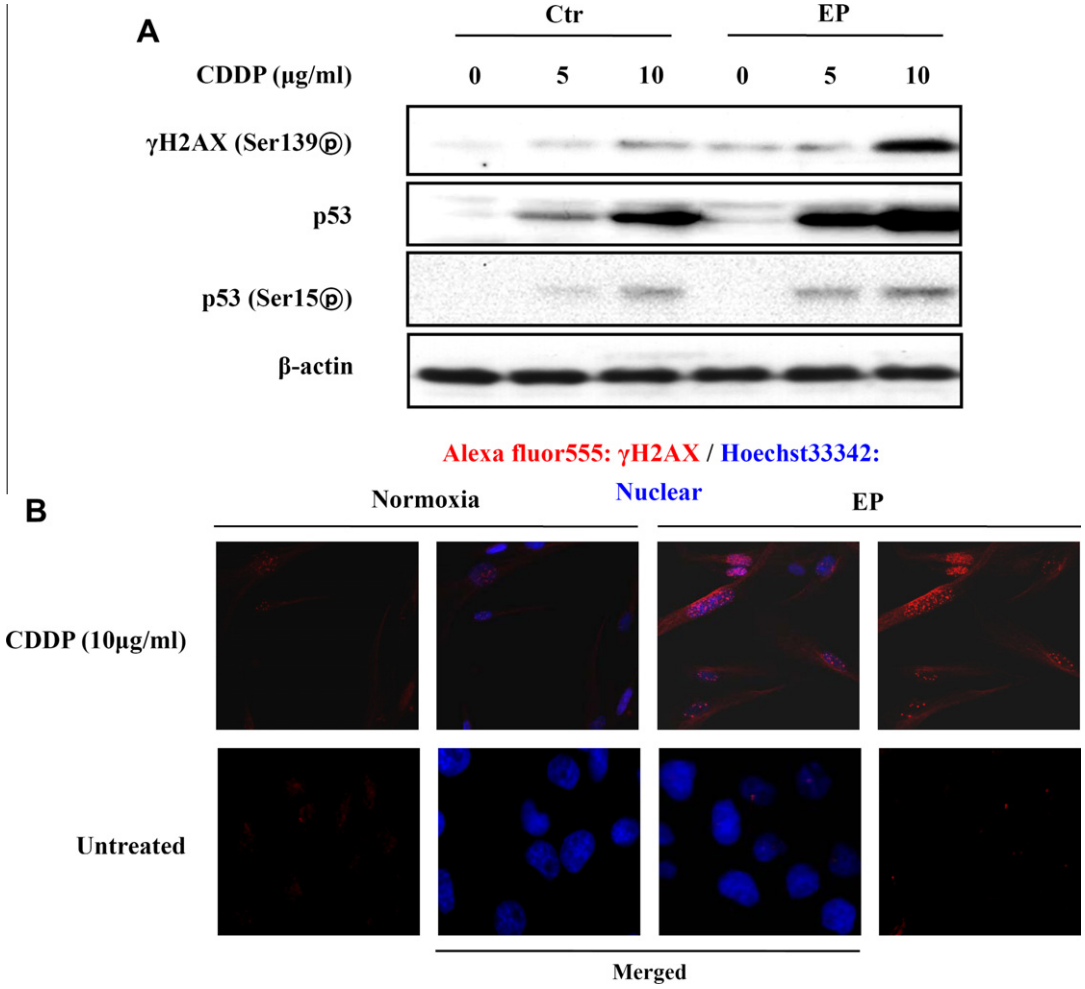

Fig. 2. EP pretreatment induces high levels of γH2AX and p53 activation in A549 cells. (A) A549 cells were pretreated with EP for 2 days and then treated with CDDP (5, 10 μg/ml) for 6 h. γH2AX was detected by Western blot analysis with anti-phospho-H2AX (ser 139) from whole cell lysates. Total and activated p53 were also analyzed by Western blotting. (B) Immunofluorescence micrographs of A549 cells showing γH2AX (red) and Hoechst 33342 (blue). Representative fluorescent micrographs are shown for A549 cells pretreated with EP for 2 days, followed by CDDP treatment (10 μg/ml for 6 h) as in (A). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1 h, followed by incubation with anti-mouse-IgG cross-adsorbed with Alexa 555 (5 μg/ml) and Hoechst 33342 dye (7 μg/ml) as a secondary antibody and dead cell marker, respectively, for 1 h at room temperature in the dark. Samples were mounted in mounting solution, and imaged by fluorescence microscopy (Observer D1, Carl Zeiss, Dusseldorf, Germany) at a magnification of 200×.

2.7. Reverse transcriptase PCR

Total RNA was isolated from cells using RNAiso Plus reagent (TaKaRa, Shiga, Japan) according to the manufacturer's instruction. cDNA was synthesized from total RNA using amfiRivert cDNA Synthesis Master Mix (GenDEPOT, Barker, TX), and the PCR reaction contained EmeraldAmp PCR Master Mix (TaKaRa, Shiga, Japan), for which the specific primers are listed in Supplementary Table 1.

2.8. Statistical analyses

All data are expressed as mean ± SE of three independent experiments. Student's *t*-test and two-way ANOVA were applied to evaluate statistical significance. $p < 0.05$ or $p < 0.01$ indicates statistical significance, which is represented by asterisk(s).

3. Results

3.1. EP induces CDDP-mediated apoptosis and G1 arrest in NSCLC cell lines

To determine whether EP influences CDDP-induced cancer cell apoptosis, we administered these potential therapeutic agents to the NSCLC cancer cell lines A549 (p53 wild type) and H1299 (p53 null). Cells were pretreated with EP for 2 days and then treated with CDDP (10 μg/ml, treated group) or PBS (untreated group) for 24 h. Pretreatment of cells with EP alone did not induce apoptotic cell death (Fig. 1A, untreated). Cells treated with CDDP underwent enhanced apoptosis when pretreated with EP, which is shown in dot plot images generated by FACS analysis of cells stained with Annexin V ($p < 0.01$). Although we detected a slightly increased rate of apoptosis in H1299 cells pretreated with EP versus cells not treated with EP, the difference was not statistically significant, indicating that the CDDP-induced apoptosis following EP-pretreatment is dependent upon p53 (CDDP, 10 μg/ml;

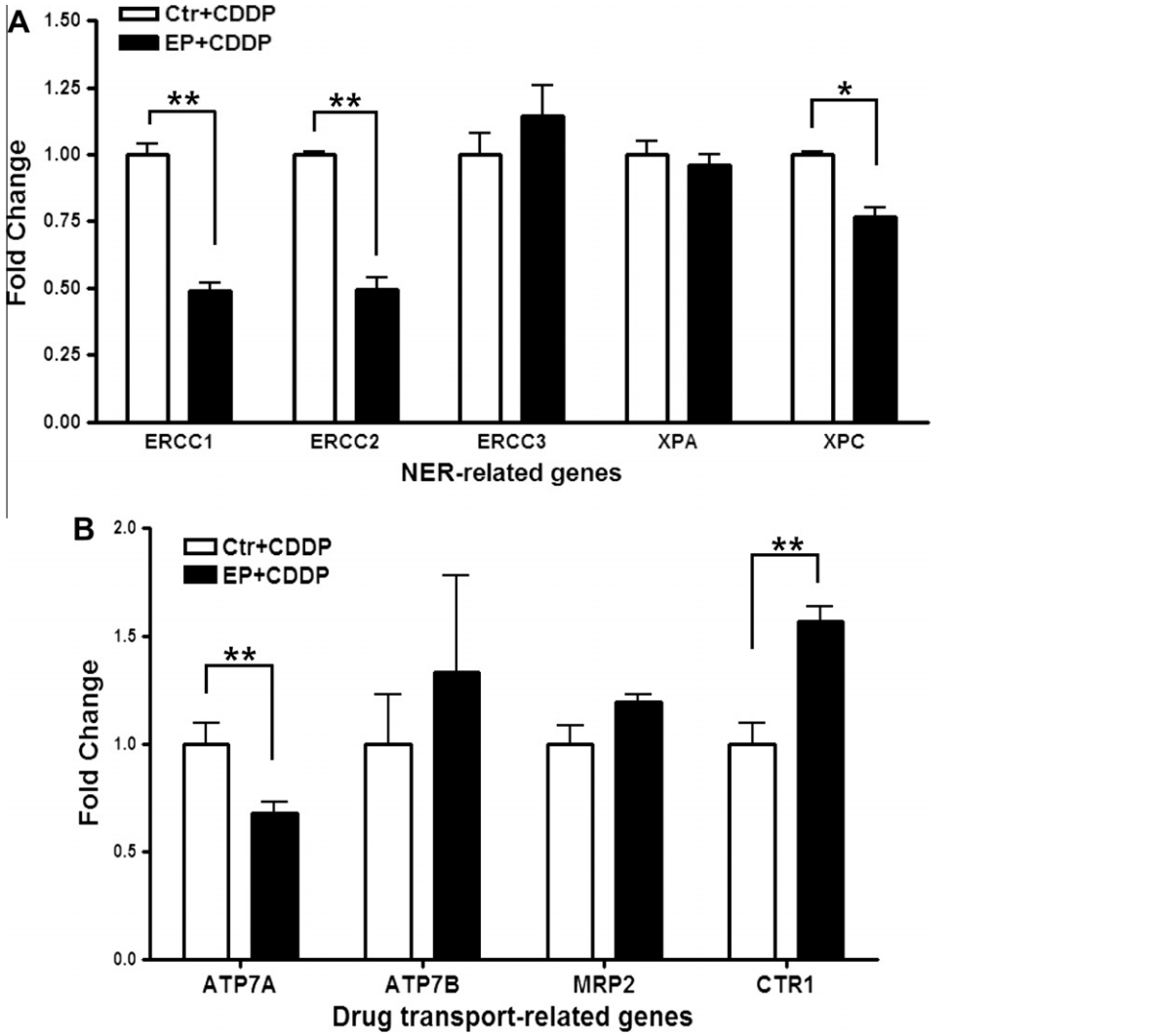

Fig. 3. Changes in transcriptional levels of NER- and drug transporter-related genes. (A) Relative transcriptional levels of NER genes (ERCC1, ERCC2, ERCC3, XPA and XPC), (B) efflux genes (copper-transporting P-type ATPases, ATP7A and ATP7B) and influx genes (copper influx transporters, CRT1 and multidrug resistance gene, MRP2) were determined by RT-PCR. All cells were treated with CDDP for 12 h with or without EP pretreatment. Fold changes were normalized by level of each gene from control. * and ** indicate statistically significant differences ($p < 0.05$ and $p < 0.01$, respectively), as compared to the control group.

Fig. 1A.). To determine whether this increased rate of apoptosis was CDDP-specific or CDDP-nonspecific, we also measured apoptosis after treating cells with hydrogen peroxide following pretreatment with EP. However, the rate of apoptosis was not significantly different between cells pretreated or not pretreated with EP (Fig. 1A; H_2O_2 500 μM).

We then subjected p53-intact and p53-deficient cancer cells to cell cycle analysis and found that G1 arrest was significantly induced in the p53 wild type cell lines A549 and H460 treated with CDDP (Fig. 1B, left), but there was no significant cell cycle arrest in p53 mutant cells following CDDP-treatment (H1299 and H157; Fig. 1B, right). These data indicate that pretreatment of cells with EP potentiates CDDP-mediated apoptosis by a p53-dependent mechanism, resulting in G1 arrest. We then sought to determine the mechanism by which EP-pretreated, CDDP-treated A549 cells underwent apoptosis and whether this mechanism is related to overcoming CDDP resistance.

3.2. Increased DNA damage response after EP pretreatment

DNA double-strand breaks induce histone H2AX phosphorylation (γH2AX), which is one event in a complex DNA damage-induced signaling cascade [22]. Because of the strong association of initial or residual γH2AX with lethal cell damage, γH2AX, which occurs on Ser 139, may be a useful marker of cellular sensitivity for a variety of DNA damaging agents [23]. To determine if EP pretreatment increases the amount of γH2AX induced by CDDP, A549 cells were pretreated with EP for 2 days. CDDP was then administered for 6 h, followed by western blot (Fig. 2A) and immunofluorescence assay (Fig. 2B). Elevated γH2AX and an increased number of γH2AX-positive cells were detected in A549 cells pretreated with EP. Furthermore, total and activated forms of p53 were significantly induced in EP-conditioned cells. These data indicate that cells pretreated with EP were sensitized to CDDP-mediated DNA damage, causing γH2AX, p53 activation, and apoptosis.

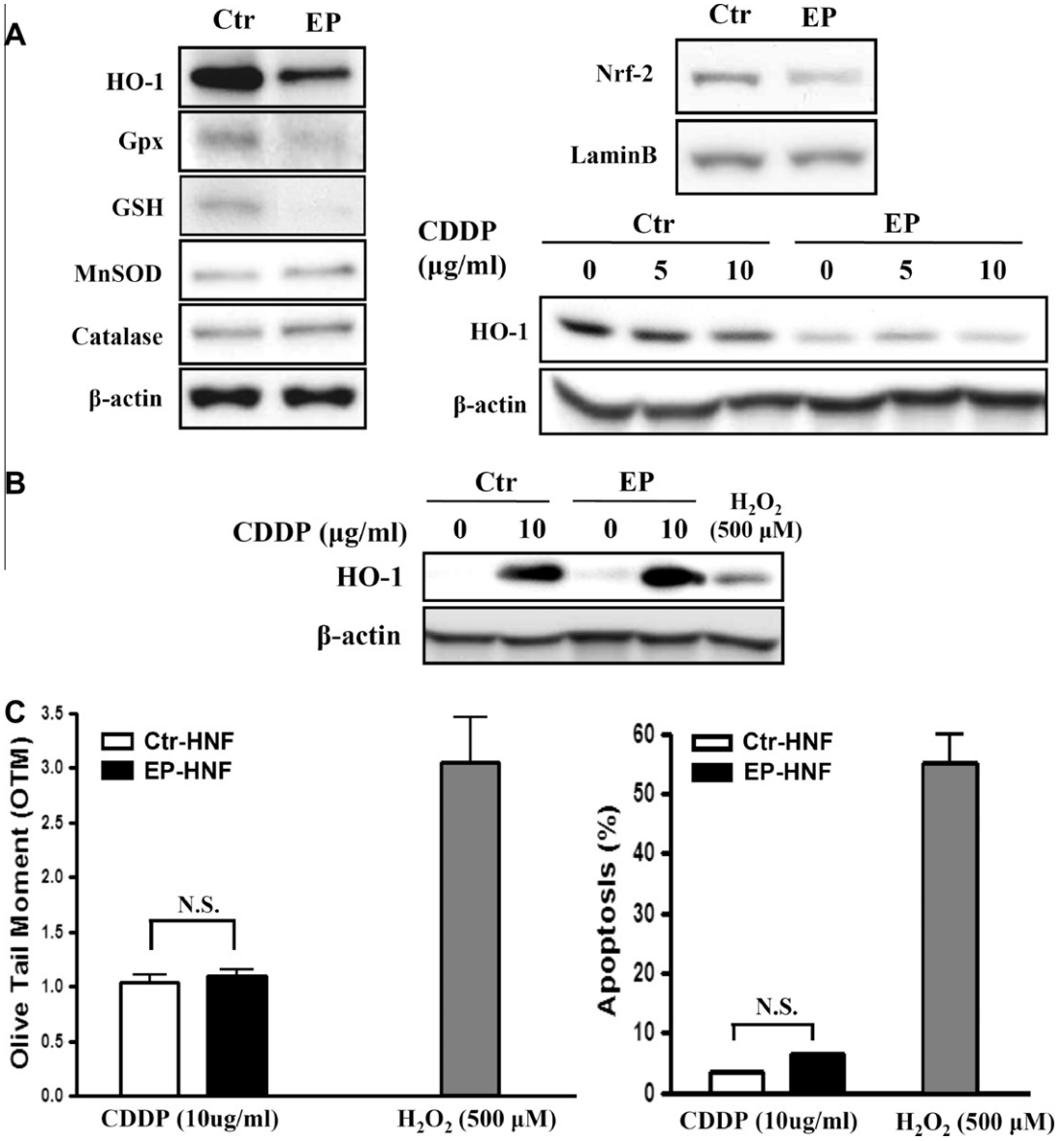

Fig. 4. Suppression of antioxidants of A549 cells and resistance in human normal fibroblasts (HNFs) after EP treatment. (A) Western blot of whole A549 cell lysates. A549 cells were pretreated with EP for 2 days, and levels of HO-1, Gpx, GSH, MnSOD, and catalase were determined, with β-actin as loading control. Nrf-2 was analyzed in the nuclear fraction with lamin B detected for loading control and nuclear envelope marker. Cells treated with CDDP for 6 h were also used for detecting HO-1 by Western blotting. (B) Western blot of HNF cell lysate for phosphorylated-p53 under identical EP-pretreatment and CDDP-treatment conditions. (C) Alkaline comet assay and Annexin V/PI staining assay results, revealing DNA damage and apoptosis, respectively, in HNFs. All experiments were performed with a positive control (treatment of H_2O_2). N.S.: "not significant".

3.3. Transcriptional level of DNA repair-related genes and drug transporters

The repair of CDDP-induced DNA damage is primarily performed by nucleotide excision repair (NER) [24]. The sensitivity of tumor cells to CDDP is inversely correlated with cellular NER capability [25,26]. Many copper transporters, including import transporter (hCtr1) and export transporters (ATP7A, ATP7B, and MRP2), are involved in CDDP transport and drug resistance [27]. To determine the transcriptional level of several genes involved in NER and drug transportation, A549 cells were preconditioned with EP for 2 days and exposed to CDDP for 12 h. Then gene expression was determined by RT-PCR. Among NER-related genes, ERCC1, ERCC2, and XPC were significantly down-regulated in A549 cells pretreated with EP (Fig. 3A). Additionally, EP pretreatment resulted in decreased expression of the drug exporter ATP7A and increased expression of the drug importer CTR1 (Fig. 3B). These data indicate that apoptosis of A549 cells is sensitized by EP pretreatment due to increasing intracellular CDDP accumulation and reducing NER activity.

3.4. Suppression of HO-1 and its transcription factor, Nrf-2

Our previous study showed that intracellular ROS production was elevated in cells treated with EP [18]. The expression of several antioxidants, including HO-1, glutathione peroxidase (Gpx), glutathione (GSH), MnSOD, catalase, and a detoxification gene mediated by electrophiles or oxidants such as Nrf-2, was screened in A549 cells treated with EP. Although catalase and MnSOD expression was not affected by EP, HO-1, Gpx, and GSH expression were all significantly down-regulated when cells were pretreated with EP (Fig. 4A, left). Additionally, the level of Nrf-2 in the nuclear fraction was reduced, as determined by Western blot (Fig. 4A, upper right). Although HO-1 was down-regulated during administration of CDDP to A549 cells after pretreatment with EP (Fig. 4, lower right), the expression of this molecule was undetectable in H1299 cells (Fig. S1). These data indicate that HO-1 may be important in the sensitizing of A549 cells to CDDP-mediated apoptosis of A549 cells by pretreatment with EP.

3.5. Human normal fibroblasts (HNFs) remain resistant against EP

We found no differential expression of HO-1 in normal lung fibroblast cells in response to EP pretreatment, even though HO-1 expression was highly inducible in A549 cells in response to CDDP treatment, with and without EP-pretreatment (Fig. 4B). To determine whether HNFs are susceptible to EP-induced apoptosis, DNA damage and apoptosis were measured by single-cell gel electrophoresis and Annexin V/PI staining, respectively. EP did not induce DNA strand breaks (Fig. 4C, right) or apoptosis (Fig. 4C, left). These results show that HNFs remain resistant to EP treatment, providing the possibility that EP conditioning is a selective sensitizing tool for CDDP cancer therapy.

4. Discussion

Strategies for sensitizing cancer therapy should include methods for overcoming drug resistance. Drug resistance, and specifically CDDP resistance, is associated with multifactorial mechanisms, including inactivation by glutathione, metallothionein, or other sulphur-containing molecules [28–30], and reduced intracellular drug accumulation by changing the profile of drug-influx or -efflux molecules [31–32]. In addition, increased DNA damage repair mechanisms such as NER [3,33,34] and tolerance or failure of apoptotic pathways [35] also result in cancer cell resistance to CDDP. DNA damage triggered by CDDP results in the recruitment of p53, which is crucial for the inhibition of cell proliferation by inducing cell cycle arrest or apoptosis [36] and for inducing DNA repair by interacting with NER machinery [37]. Sensitivity to CDDP positively correlated with the status of a functional p53 pathway in a study involving 60 human cancer cell lines from the National Cancer Institute [30]. However, other studies revealed that there is no correlation or possibly even a negative correlation between p53 status and the effectiveness of CDDP [38].

In this study, we introduced EP as a mechanical stressor to precondition cancer cells prior to CDDP treatment and evaluated the effect of EP on CDDP-mediated apoptosis in NSCLC cell lines. Although 2ATA of pressure is not a natural condition *in vivo*, our results indicate that EP has potential clinical applications as a sensitizing adjuvant tool. Recently, we reported the sensitizing effect of EP for TRAIL-induced apoptosis in various cancer cell types, including NSCLC cells, which resulted in induced DR5 expression and DISC formation in H460 cells [21]. However, A549 cells failed to overcome TRAIL resistance when EP and TRAIL were combined, suggesting that EP can efficiently facilitate TRAIL-mediated apoptosis, but not overcome resistance in all cancer cells.

Interestingly, data from the present study show that A549 cells were sensitive to CDDP-mediated apoptosis after pretreatment with EP. These results led us to evaluate the effect of EP pretreatment on several mechanisms related to CDDP resistance, including DNA damage, NER apparatus activation, and expression of drug transporters and antioxidants.

Our results indicate that preconditioning cancer cells with EP facilitates CDDP-mediated apoptosis in A549 cells that have a functional p53. Apoptosis is mediated by an increased DNA damage response, G1 arrest, and reduced transcription of NER-related genes such as ERCC1, ERCC2, and XPC. EP-pretreated cells down-regulated expression of ATP7A and up-regulated expression of CTR-1, which are involved in efflux and influx of drug molecules respectively, suggesting a mechanism of intracellular CDDP accumulation. Other previous studies have shown that mRNA levels of the major components of NER machinery were highly correlated with CDDP resistance *in vitro* and in clinical cases [25,26]. Drug transporters, including multidrug resistance-associated proteins, are crucial for the CDDP accumulation in many types of cancer cells [39,40]. Interestingly, HO-1, GSH and Gpx which are Nrf-2-dependent targets and act as defense molecules significantly down-regulated in response to EP-pretreatment in this study are promising targets for various methods of chemotherapy, including CDDP treatment [41–43]. Therefore, the potentiating effect of EP on the CDDP-mediated cancer cell apoptosis may be explained by the diverse effects executed by EP/CDDP treatment to reduce resistance.

Collectively, these data show that EP administration in combination with the anticancer drug CDDP induces cellular changes favorable to cancer eradication by overcoming cancer cell drug resistance. However, the pretreatment of HNF with EP demonstrates that EP pretreatment results in neither genotoxicity nor cytotoxicity, suggesting EP as a selective sensitizing tool for cancer therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.07.047.

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